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

Bo-Kyung Son, Koichi Kozaki, Katsuya Iijima, Masato Eto, Taro Kojima, Hidetaka Ota, Yuka Senda, Koji Maemura, Toru Nakano, Masahiro Akishita, Yasuyoshi Ouchi

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 (HASMTC), 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 HASMTC 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 HASMTC 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. It has been recognized that statins exhibit various protective effects against atherosclerosis, including modification of endothelial function, decreased inflammation, and inhibition of vascular smooth muscle cell (VSMC) proliferation and migration, 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 and coronary artery calcification. Statins also inhibited calcification of atherosclerotic plaques in experimental hyperlipidemic animals. On the other hand, some recent clinical trials were not able to find such an inhibitory effect. 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, 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. Recently, it has been shown that similar structures to matrix vesicles, derived from apoptotic VSMC, have been identified in human calcified arteries. 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. 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.

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Gas6 is a secreted protein that harbors a γ-carboxyglutamic acid–rich domain and 4 epidermal growth factor–like repeats.\textsuperscript{19} Gas6-Axl interaction has been shown to be implicated in the regulation of multiple cellular functions, including growth, survival, adhesion, and chemotaxis.\textsuperscript{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 fluavastatin 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.\textsuperscript{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\textsubscript{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\textsubscript{2}HPO\textsubscript{4} and NaH\textsubscript{2}PO\textsubscript{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\textsubscript{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 \textsuperscript{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 for 6 days. Ca deposition was measured by \textsuperscript{35}S-cresolphthalein complexone method and normalized by cell protein content. 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 of 18S.

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**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).
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'-ACCTCGTGCAGCAT-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 for 6 days. Ca content was measured and normalized by cell protein content. All values are presented as mean±SEM (n=6). *\( P<0.05 \) vs 2.6 mmol/L Pi by Fisher’s test. **\( P<0.01 \) vs 2.6 mmol/L Pi, ZVAD.fmk(-) by Fisher’s test. Experiments were performed with at least 3 different cell populations.

**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 calcification...
During calcification, Pi increased the rate of apoptotic cell death detected by terminal deoxynucleotidyl 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.\(^2\) 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 \(\mu\)mol/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...
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 and a prospective study have shown beneficial effects, whereas recent prospective studies were unable to show such effects. 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, 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+}). Statins have been shown to inhibit VSMC proliferation and reduce the acute increase of [Ca^{2+}], in a mevalonate and isoprenoid pathway–independent manner. On the other hand, [Ca^{2+}], is reported to be 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 in calcification was not affected by an inhibitor of Rho kinase (Y-27632), an important modulator of the mevalonate and isoprenoid pathway-taking 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, farneslylpyrophosphate, 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, although the precise mechanism has not been shown. The pleiotropism of statins is of continuing interest.
An antiapoptotic effect of statins has been shown in various cell types. 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. Simvastatin (1 μmol/L) promoted endothelial cell survival. However, in contrast to the results of the present and other studies, a proapoptotic effect of statins has also been reported in VSMC, endothelial cells, and cardiac myocytes. 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. 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. Proudfoot et al also showed that in vascular calcification, several PS-exposing cells are observed within and on the periphery of the nodules. PS exposure by apoptotic bodies generates a potential Ca-binding site and membrane surface suitable for hydroxyapatite deposition. 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. 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 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.
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References


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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**Materials and Methods**

**Determination of Apoptosis**

**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 minutes, and subsequently incubated with terminal deoxyribonucleotidyl transferase in the presence of digoxigenin-conjugated dUTP for 1 hour at 37°C. The reaction was terminated by incubating the samples in stopping buffer for 30 minutes. After 3 rinses with PBS, a fluorescein-labeled anti-digoxigenin antibody was applied for 30 minutes, and the samples were rinsed 4 times with PBS. The samples were then stained, mounted with DAPI/antifade, and examined by fluorescence microscopy.

**Detection of DNA Fragmentation by ELISA**

Cytoplasmic histone-associated DNA fragments were detected with a cell-death detection ELISAplus 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 lysate was used for the assay. Following addition of substrate, colorimetric change was determined by the absorbance value measured at 405 nm.

**Sodium-dependent Phosphate Cotransporter (NPC) Activity Assay**

NPC activity was determined at 37°C in Earle’s buffered salt solution (EBSS) with the radiolabeled
substrate $H_3^{32}$PO$_4$. EBSS is composed of 143 mmol/L sodium or choline, 5.36 mmol/L potassium, 0.8 mmol/L magnesium, 1.8 mmol/L calcium and 125 mmol/L chloride.$^1$ Transport was initiated by adding 1 mL EBSS with $H_3^{32}$PO$_4$. After incubation with 2.6 mmol/L Pi and statins, the reaction was stopped by three rinses with ice-cold EBSS. Radioactivity of a 100 µL aliquot was counted by a standard liquid scintillation technique.

**Generation of Promoter Reporter Construct and Luciferase Activity Assay**

The 1925-bp Gas6 promoter (-1827/+99) corresponding to the Gas6 promoter sequences was generated by PCR from human genomic DNA with the appropriate sets of primers (available upon request). These inserts were cloned into a pGL3 basic vector (Promega) by standard molecular biological techniques. The construct was verified by sequencing. HASMC were transiently transfected in 12-well plates with 0.8 µg plasmid DNA and lipofectamine 2000 (Invitrogen) according to the procedure recommended by the manufacturer. Cells were treated with atorvastatin and Pi at 4 hours after transfection, incubated for an additional 44 hours, and assayed for luciferase activity according to the manufacturer’s protocol.

**Immunoblotting and Immunoprecipitation**

Cell lysates were collected in both short-term (1-12 hours) and long-term (1-10 days) conditions. The effect of atorvastatin on the expression of Gas6 and Axl was examined at 12 hours (short-term
condition) and 6 days (long-term condition). Conditioned medium at 12 hours was concentrated 40-fold by centrifugation in a concentrator fitted with a YM-30 membrane (Amicon). Concentrated medium and the 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 Inc.). After incubation with horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia), blots were visualized by enhanced chemiluminescence and autoradiography (ECL Plus, Amersham Pharmacia). For analysis of Gas6 expression in the medium on day 10 (long-term condition), conditioned medium was immunoprecipitated with anti-Gas6 polyclonal antibody or control IgG, then mixed with 60 µL of 50% v/v protein A/G plus agarose beads (Santa Cruz Biotechnology Inc.) overnight at 4°C. Immunocomplexes were washed in lysis buffer, and resuspended in 30 µL of 3 × electrophoresis sample buffer. Immunoblotting was performed as described above with anti-Gas6 polyclonal antibody. Experiments were performed with at least three different cell populations.

Reference

Online Figure Legends

**Online Figure I.** Pi promotes HASMC apoptosis in long-term condition. HASMC were cultured with the indicated concentrations of Pi for 10 days. A quantitative index of apoptosis determined by an ELISA specific for histone-associated DNA fragments was presented as the relative value to that 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. Experiments were performed with at least three different cell populations.

**Online Figure II.** Anti-apoptotic effect of atorvastatin in long-term condition. HASMC were treated with the indicated concentrations of atorvastatin in the presence of 2.6 mmol/L Pi for 6 days. Apoptosis index relative to 2.6 mmol/L Pi, statin (-) was measured. All values are presented as mean±SEM (n=3). *p<0.05 by Fisher’s test. A representative example of three independent experiments is shown.

**Online Figure III.** Downregulation of Gas6 and Axl expression by Pi in long-term condition. HASMC were cultured in the presence of 2.6 mmol/L Pi for the indicated period. Cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6, Axl or β-tubulin. Experiments were performed with at least three different cell populations.
Online Figure IV. Inhibitory effect of atorvastatin on Gas6 expression in long-term condition. HASMC were treated with atorvastatin (0.1 µmol/L) in the presence of 2.6 mmol/L Pi. On day 6, cell lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies to Gas6. A representative example of three independent experiments is shown.

Online Figure V. Effect of atorvastatin on Gas6 promoter activity. HASMC were transfected with the Gas6 promoter-luciferase construct. Four hours after transfection, atorvastatin (0.1 µmol/L) and 2.6 mmol/L Pi were added and the cells were incubated for an additional 48 hours. Luciferase activity was normalized to that of untreated cells (vehicle). All values are presented as mean±SEM (n=6).

Online Figure VI. Effect of atorvastatin on Pi-induced osteoblastic differentiation. HASMC were incubated with 2.6 mmol/L Pi in the presence of atorvastatin (0.1 µmol/L) for 6 days. Messenger RNA was isolated and Northern blot analysis for MGP was performed. The panel shows a representative blot.

Online Figure VII. Effect of rho protein on Pi-induced calcification and inhibition of calcification by atorvastatin. A, HASMC were treated with the indicated concentrations of a rho-pathway inhibitor, Y27632, in the presence of 2.6 mmol/L Pi. On day 6, Ca deposition was measured by o-cresolphthalein complexone method, and normalized by cell protein content. All
values are presented as mean±SEM (n=6). B, HASMC were treated with Y27632 (10 µmol/L) and atorvastatin (0.1 µmol/L) in the presence of 2.6 mmol/L Pi for 6 days. All values are presented as mean±SEM (n=6).
Online Figure I

![Bar chart showing apoptosis relative to control over 3, 6, and 10 days for different Pi concentrations (1.4 mmol/L, 2.0 mmol/L, 2.6 mmol/L, and 3.2 mmol/L). The chart indicates a significant increase in apoptosis with increasing Pi concentration, especially after 10 days.]
Online Figure II

Apoptosis relative to control

Pi 2.6 mmol/L

Atorvastatin (µmol/L)

-  +  +  +

0 0.01 0.1

* *
Online Figure III

<table>
<thead>
<tr>
<th>Pre</th>
<th>1</th>
<th>3</th>
<th>6</th>
<th>10 (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi 2.6 mmol/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

- **Gas6**
- **Axl**
- **β-tubulin**
Online Figure IV

<table>
<thead>
<tr>
<th></th>
<th>−</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pi 2.6mmol/L</td>
<td>−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atorvastatin</td>
<td>−</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

Gas6
Online Figure V

![Bar graph showing relative luciferase activity](image)

- Pi 2.6 mmol/L
  - –
  - +
  - –
  - +

- Atorvastatin
  - –
  - –
  - +
  - +
Online Figure VI

<table>
<thead>
<tr>
<th></th>
<th>Pi 2.6 mmol/L</th>
<th>+</th>
<th>+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atorvastatin</td>
<td>-</td>
<td>-</td>
<td>+ (6 days)</td>
</tr>
</tbody>
</table>

MGP mRNA
Online Figure VII

A

B

Ca deposition (µg/mg protein)

Ca deposition (µg/mg protein)

Y27632 (µmol/L)

Pi 2.6 mmol/L

Atorvastatin

Y27632

+ + + +

- + + –

- – + –

0.001 0.1 10