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A novel organ culture model of aorta for vascular calcification

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ABSTRACT

Vascular calcification is a characteristic feature of aging, atherosclerosis, diabetes mellitus, and end-stage renal disease. The use of organ culture provides complementary information that may bridge the gap between traditional cell culture and animal models, and establishes easily controlled experimental conditions. Therefore, we investigated whether organ culture of the aorta could be used as a model of vascular calcification, applying it to animal models of other conditions.

Thoracic aortas were dissected from C57BL/6 mice and cultured. To induce vascular calcification, stimulation with inorganic phosphate (Pi) was performed. Morphometric assessment of medial calcium deposition was quantitatively performed, and the amount of dissolved calcium was measured. Pi-stimulation induced calcium deposition in medial layers in a time- and dose-dependent manner. To investigate the phenotypic change of vascular smooth muscle cells (VSMC), the expression of Runx2, osterix, osteocalcin, and ALP activity were determined. Finally, to investigate the influence of Pi-stimulation on the cultured aorta in other models, aortas from streptozotocin (STZ)-induced diabetic mice, aged mice, and *Sirt1* knockout (+/−) mice were dissected. These cultures showed a greater tendency for aortic calcification by Pi-stimulation than did control cultures.

These results indicate that organ culture of the aorta from mice reflects the state of calcification and suggests that this model will be useful to explore the molecular mechanisms of vascular calcification and the pathology of senescence.

1. Introduction

Vascular calcification is a risk factor for cardiovascular events and has a high prevalence among the elderly, and atherosclerosis, diabetes mellitus, and chronic kidney disease (CKD) patients [1]. Clinical and experimental studies have shown that phosphate overload plays a central role in the pathogenesis of vascular calcification in CKD [2]. Emerging evidence indicates that vascular calcification is a regulated process that resembles embryonic endochondral osteogenesis, involving osteoblastic differentiation of vascular smooth muscle cells (VSMC) [3]. However, the molecular mechanism underlying this pathogenic process is still obscure. To clarify the mechanism of vascular calcification, the development of animal models that exhibit extensive and robust vascular calcification is an important issue for research in vascular biology [4]. To this end, some types of animal models with vascular calcification have been used [5]. For example, the adenine-fed model is one of the most frequently used rat models for its relatively easy induction [6]. Adenine-fed rats develop a series of renal failure-related phenotypes including arterial medial calcification. However, the original adenine (0.75%) model has many confounding factors in researching vascular calcification, such as high blood pressure, lipid disorder, and relatively low prevalence, leading to severe, rapid malnutrition and high fatality in 4–6 weeks [7]. In contrast, calcification of VSMC is simply and easily induced by stimulation with inorganic phosphate (Pi) *in vitro*. In a monoculture of VSMC, the initial experimental condition can also be easily arranged for each detection time or dose-concentration and the detection accuracy can be improved. However, morphologic variation has been seen at passage numbers of over 7–8, and variations in the gene phenotype of VSMC occur at passage numbers beyond 12–13. Moreover, a single cell culture cannot examine the influence

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of the interaction between cells. Therefore, we hypothesized that organ culture of the aorta would provide complementary information that may bridge the gap between traditional animal models and cell culture, and provide a feasible culture technique to evaluate vascular calcification in a form that is almost an *in vivo* condition. Here, we tried to culture whole thoracic aortas dissected from mice and estimate the status of calcification under Pi-stimulation. In addition, we evaluated aorta cultures of not only wild type mice but also other types of mice such as streptozotocin (STZ)-induced diabetic mice, aged mice, and mammalian sirtuin 1 (*Sirt1*) knockout (KO) (+/−) mice.

2. Materials and methods

2.1. Animal experiments

The animal experiments were approved by our institutional review board. Male young and aged wild-type C57BL/6 mice aged 10 weeks, 12–18 weeks, and 40–52 weeks were supplied by Charles River Laboratories Inc. *Sirt1*-heterozygous KO mice (provided by Dr F.W. Alt), designated *Sirt1*(+/−), were generated in a previous study [8]. We made mice diabetic (C57BL/6) by two intraperitoneal injections (day 0 and 5) of STZ (60 mg/kg, SIGMA-ALDRICH, Missouri, USA). Tail blood glucose level was assayed 3 days after the second injection using glucose test strips (Johnson and Johnson, New Jersey, US), and all mice showed glucose levels above 250 mg/dl. Male mice were all housed and maintained in a room at 22 ± 2 °C with an automatic light cycle (12 h light/dark) and relative humidity of 40–60%.

2.2. Organ culture of aorta

Thoracic aortas were dissected from mice and cultured in DMEM supplemented with 15% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 4500 mg/ml glucose at 37 °C in a humidified atmosphere with 5% CO_2. The culture medium was changed every 2 days. To induce calcification, Pi ($Na_2H_2PO_4$/NaH_2PO_4, pH 7.4) was added to supplemented DMEM to final concentrations of 1.4, 1.8, 2.2, 2.6, and 3.0 mmol/L. We defined 1.4 mM as the normal concentration of Pi and 2.6 mM as a high concentration of Pi. After the indicated incubation periods (6, 12 h, 1, 2, 3, 4, 5, 6, 10 days), samples were taken and analyzed. Sirtinol (Calbiochem, San Diego, CA) and phosphonoformic acid (PFA) were added to supplemented DMEM at final concentrations of 100 μM and 100 nM, respectively. To remove endothelial cells, a 5 cm thread (Daiso-Sangyo Inc., Hiroshima, Japan) was passed once through the lumen to excoriate the endothelium.

2.3. Evaluation of calcification

Dissolved calcium was measured by OCPC (*o*-cresolphthalein complexone) method (C-Test, WAKO, Tokyo, Japan) [9]. Briefly, this OCPC method is based on calcium reacting with o-cresolphthalein complexone in an alkaline solution. They form an intense violet colored complex with maximal absorbance at 577 nm. To remove interference by magnesium and iron, 8-hydroxyquinoline is added. The area of calcification in cultures was determined by von Kossa staining. Aortas were washed with deionized water and fixed with 10% buffered formalin and embedded in paraffin. Each sample were sliced at 5 μm thickness, and de-paraffinised before staining. Samples were incubated with 5% silver nitrate under UV light for 1 h, and then washed with 5% sodium thiosulfate for 5 min. Digital photographs of the stained culture plates were taken using a microscope. Alizarin Red S staining was also performed to clarify the calcium deposition. Staining was performed with Alizarin Red Solution (pH to 4.1–4.3 with 10% ammonium hydroxide) for 5 min, and the reaction was observed microscopically. For the detection of alkaline phosphatase (ALP) activity, p-Nitrophenyl-phosphate (pNPP) is used as the substrate in Enzyme Immunoassays (EIA) assays (WAKO, Osaka, Japan). All procedures were carried out at room temperature.

2.4. Immunoblot analysis

Aortas were washed with ice-cold PBS(−) twice and homogenized in RIPA buffer containing 10 mM Tris–HCl (pH 7.5), 50 mM NaF, 0.2 mM Na_3VO_4, 1 mM ethylene-glycol-tetraacetic acid (EGTA), 1 mM ethylene-diamine-tetraacetic acid (EDTA), 1% NP-40; 1% sodium deoxycholate, 10 μg/ml leupeptin, 10 μg/ml aproptonin, 1 mM PMSF; and 20 nM okadaic acid. Samples were kept on ice and gently mixed every 5 min for 20 min, and then centrifuged at 12,000 rpm for 20 min at 4 °C. Supernatants were collected and protein concentrations were quantified using a Protein Assay Kit (Thermo Scientific). Protein was denatured by boiling at 100 °C for 5 min in Laemmli buffer. Each sample containing equal amounts of protein was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to a polyvinylidene fluoride (PVDF) membrane. After 1 h blocking at room temperature using 5% skimmed milk, the membrane was incubated overnight with primary antibody (runt-related transcription factor 2 (Runx2), PiT-1, osteocalcin, SIRT1 (Santa Cruz Biotechnology, Dallas, USA), osterix (Abcam plc, MA, USA), β-tubulin (SIGMA-ALDRICH, Missouri, USA)) in Tris-buffered saline solution/Tween (TBST) containing 5% skimmed milk at 4 °C. After incubation, the membrane was washed three times in TBST and incubated with secondary antibody for 1 h at room temperature. After three washes in TBST, the membrane was developed using a chemiluminescence assay system (Millipore) and exposed to Kodak film.

2.5. Data analysis

Values are shown as mean ± SD in the text and figures. Differences between the groups were analyzed using one-way or two-way analysis of variance. Probability values less than 0.05 were considered significant.

3. Results

3.1. Pi-stimulation induced calcification of aortic culture

To investigate whether Pi-stimulation induces vascular calcification in cultures of the aorta, thoracic aortas were dissected from C57BL/6 wild type mice (N = 6) and cultured for 10 days in medium containing a high concentration of Pi (2.6 mM). Morphometric assessment of medial calcium deposition (Supplementary Figure 1A) was quantitatively performed by von-Kossa/Alizarin Red S staining, and the amount of dissolved calcium was measured by OCPC method (N = 6). Pi-stimulation increased the von-Kossa/Alizarin Red S-stained area for 10 days in the aorta, with saturation at 6 days (Fig. 1A and B). The amount of dissolved calcium in the aorta was increased by Pi-stimulation for 10 days, with saturation at 6 days as well (Fig. 1C). Next, we estimated the degree of calcification at various Pi concentrations (1.4–3.0 mM). The normal serum concentration of Pi in wild type mice is 1.4 mM. Vascular calcification induced by Pi-stimulation was observed in a dose-dependent manner (Fig. 1D, E, and F). These results indicate that vascular calcification is induced by Pi-stimulation in organ culture of the aorta in a time- and dose-dependent manner. To confirm osteoblastic differentiation of VSMC, expression of Runx2, a transcription factor for osteoblast differentiation, was

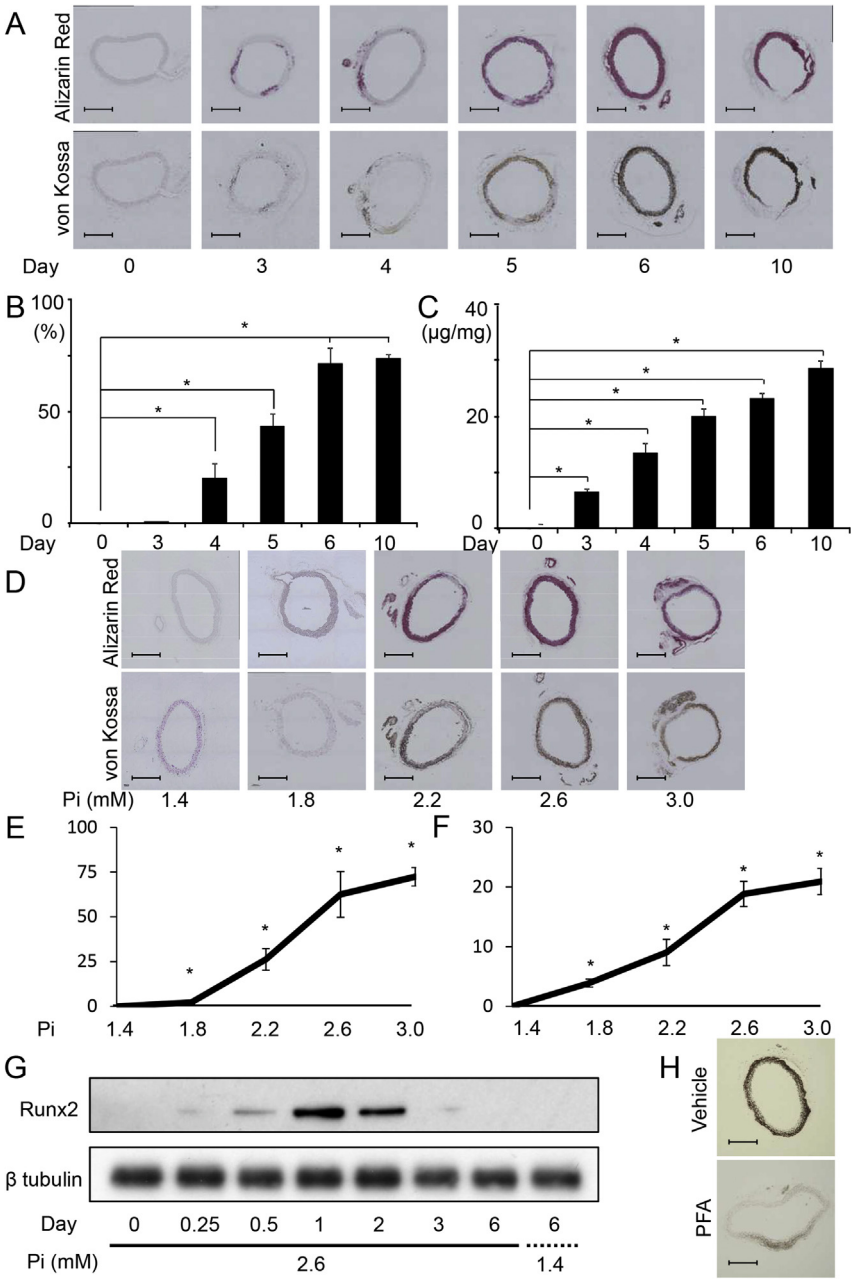

Fig. 1. A. Area of calcification in cultured aortas incubated with medium containing a final Pi concentration of 2.6 mM for 10 days was determined by Alizarin Red S (upper)/von-Kossa (lower) staining. (scale bar: 300 μm) B. von-Kossa-stained area (%) in aortas cultured for 10 days compared with 0 day. C. Dissolved calcium in aortas cultured for 10 days was measured by OCPC method. D, E. Area of calcification in cultured aortas incubated with medium containing Pi (1.4, 1.8, 2.2, 2.6, 3.0 mM) was determined at 6 days by Alizarin Red S (upper)/von-Kossa (lower) staining. (scale bar: 300 μm) F. Dissolved calcium in cultured aortas incubated with medium containing Pi (1.4, 1.8, 2.2, 2.6, 3.0 mM) was measured at 6 days by OCPC method. G. Runx2 expression was increased for 1 day and decreased after 2 days by Pi-stimulation (2.6 mM). H. Treatment with PFA (100 nM) ameliorated vascular calcification as assessed by von-Kossa staining. (scale bar: 300 μm) ($^{*}p < 0.05$, N = 6.)

determined. Runx2 expression was increased by Pi-stimulation (2.6 mM) until 2 days (Fig. 1G). In addition, we observed that osterix, osteocalcin expression and ALP activity were increased by Pi-stimulation (2.6 mM) (Supplementary Figure 1B). This result indicates that osteoblast differentiation occurred at an early stage of Pi-stimulation, and degradation of Runx2 protein occurred when vascular calcification developed.

Pit1, a cellular phosphate transporter, plays an important role in Pi-induced calcification. Therefore, we investigated whether Pit1 is related to vascular calcification. Pit1 was blocked by treatment with PFA, which also decreased vascular calcification (Fig. 1H).

3.2. Cultured aorta of STZ-diabetic mice showed accelerated vascular calcification induced by Pi-stimulation

To apply organ culture of the aorta to mice with other backgrounds, we used STZ-diabetic mice, because patients with diabetic mellitus frequently have vascular calcification in the thoracic aorta. When cultured aortas were treated with Pi (2.6 mM), STZ-diabetic mice showed a greater increase in the von-Kossa-stained area compared with non-diabetic control mice (N = 6) (Fig. 2A and B). The amount of dissolved calcium was also more markedly increased in the cultured aorta of STZ-diabetic mice than in control at 3 days

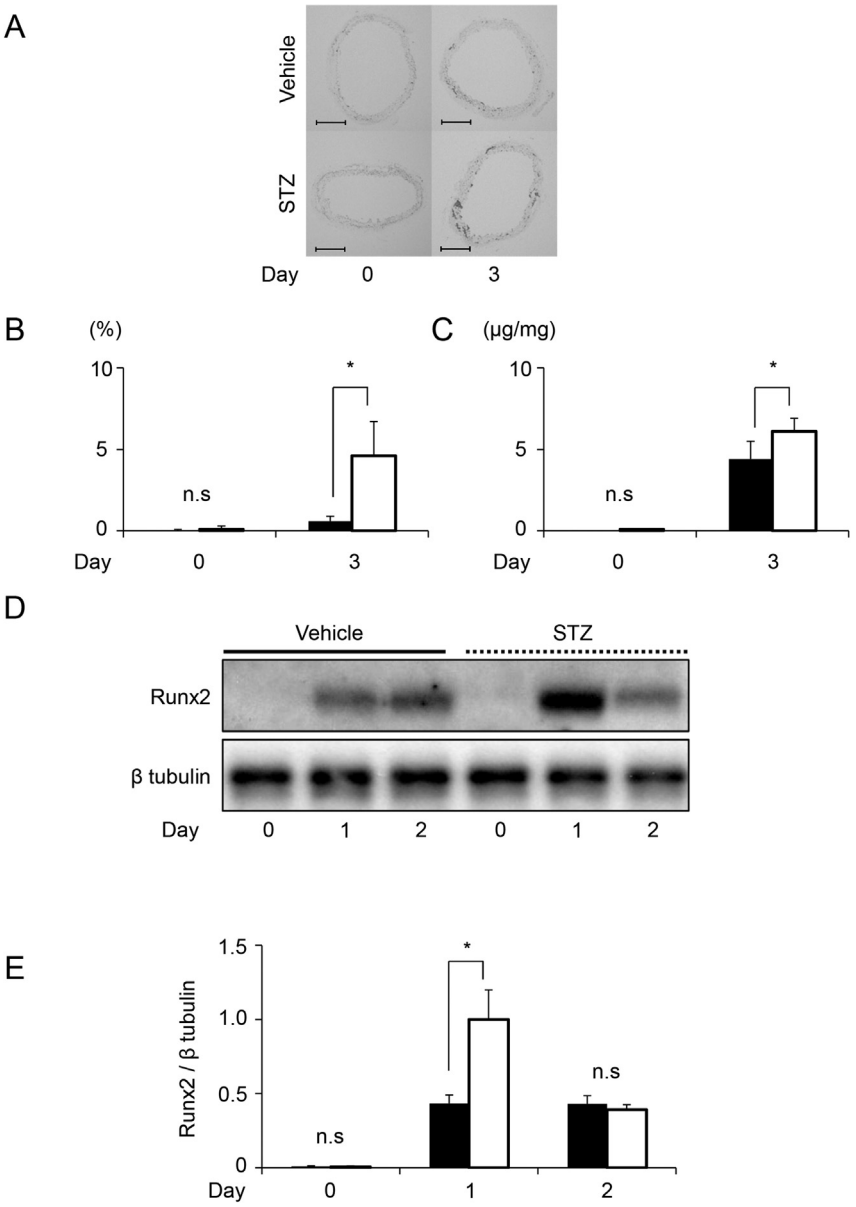

Fig. 2. A, B. Area of calcification in cultured aortas of STZ-diabetic mice incubated with medium containing final concentration of Pi of 2.6 mM for 3 days was determined by von-Kossa staining. (scale bar: 300 μm) C. Dissolved calcium in aortas of STZ-diabetic mice cultured for 3 days was measured by OCPC method. D, E. Runx2 expression in aortas of STZ-diabetic mice compared with control mice. (Black columns: control mice, White columns: STZ-diabetic mice.) ($^{*}p < 0.05$, N = 6, n.s.: not significant.)

(N = 6) (Fig. 2C). Runx2 expression in the aorta of STZ-diabetic mice was increased at an earlier time than in control (Fig. 2D). Moreover, Runx2 expression in the aorta of STZ-diabetic mice was decreased at 2 days and changed to a similar level to control (Fig. 2E). These results indicate that the cultured aorta of STZ-diabetic mice showed accelerated vascular calcification.

3.3. Cultured aorta of aged mice showed accelerated medial calcification induced by Pi-stimulation

Because the aging process is associated with a tendency for vascular calcification, we compared the cultured aorta of aged mice (40–52 weeks) with that of young mice (12–18 weeks). The cultured aorta of aged mice, but not young mice, already showed von-Kossa-stained areas before Pi-stimulation (N = 6) (Fig. 3A and B). In addition, the cultured aorta of aged mice showed accelerated von-Kossa staining until 6 days after the start of Pi-stimulation (2.6 mM) compared with young mice (N = 6) (Fig. 3C). Dissolved calcium was also detected in the cultured aorta of aged mice before Pi-stimulation, and was accelerated by Pi-stimulation (2.6 mM) in aged mice compared with young mice (N = 6) (Fig. 3D). Runx2 expression increased at an earlier time (0–12 h) in the cultured aorta of aged mice than in young mice (Fig. 3E and F). Moreover, Pit1 expression did not differ between young and aged aorta (Fig. 3G and H). These results indicate that the cultured aorta of aged mice shows accelerated vascular calcification compared with that of young mice, and suggest that organ culture of the aorta could reflect the aging process.

3.4. Cultured aorta of Sirt1 KO mice showed accelerated medial calcification induced by Pi-stimulation

As mentioned above, we observed that the cultured aorta of aged mice has a tendency to undergo vascular calcification.

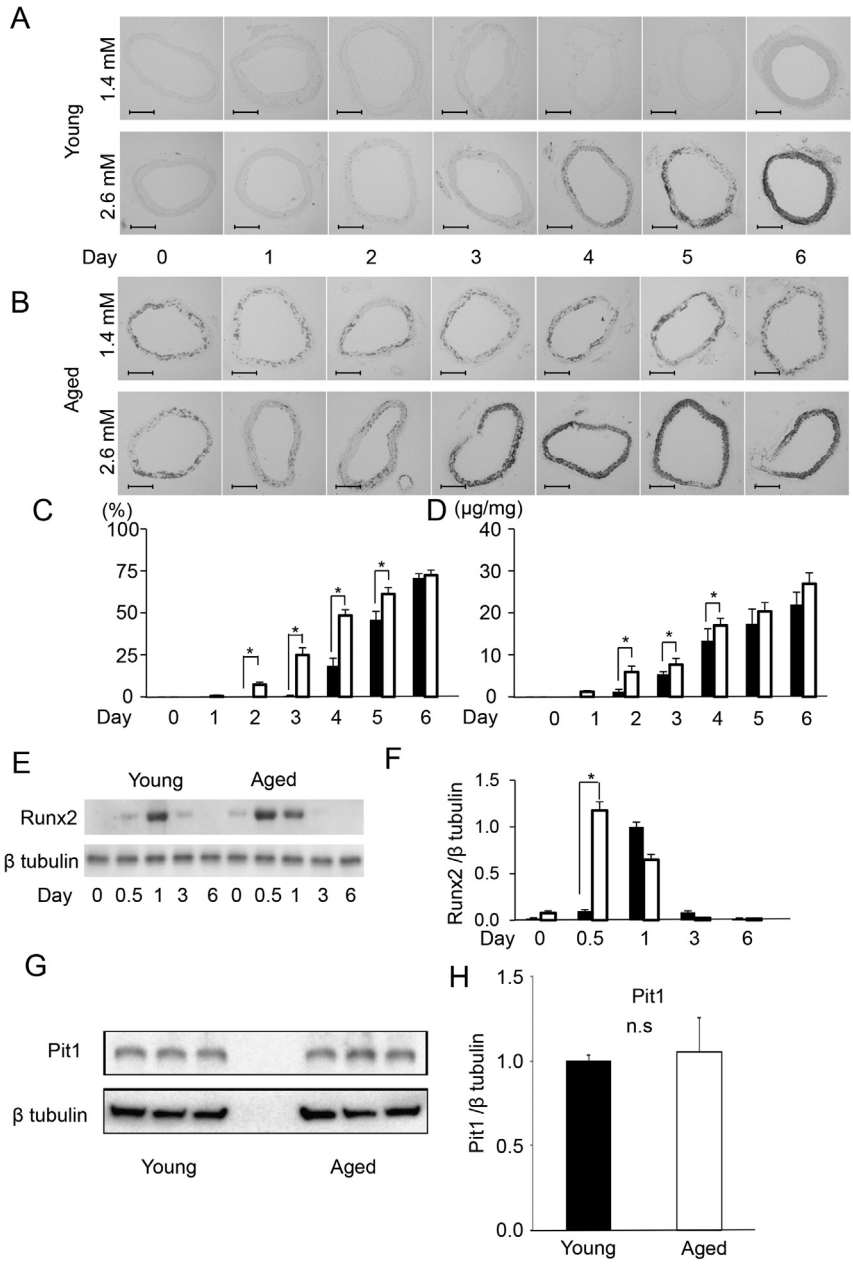

Fig. 3. A, B. Area of calcification in cultured aortas of young (12–18 weeks) (A) and aged (40–52 weeks) (B) mice incubated with medium containing final concentrations of Pi of 1.4 and 2.6 mM for 6 days was determined by von-Kossa staining. (scale bar: 300 μm) C. von-Kossa-stained area (%) in aortas of young and aged mice cultured for 6 days with 2.6 mM Pi. D. Dissolved calcium in aortas of young and aged mice cultured for 6 days was measured by OCPC method. E, F. Runx2 expression in aortas of aged mice compared with young mice. G, H. Pit1 expression in young and aged cultured aorta. (Black columns: young mice, White columns: aged mice.) ($^*p < 0.05$, N = 6, n.s.: not significant.)

Therefore, we next examined other aged phenotype mice with a genetic defect. SIRT1 is an NAD^+-dependent protein deacetylase that has functions such as regulated longevity, stress resistance, and metabolic signaling. It is reported that *Sirt1* (+/−) KO mice are more prone to develop organ damage by various stressors [10]. Therefore, we treated the cultured aorta of these mice with Pi (2.6 mM). The cultured aorta of *Sirt1* (+/−) KO mice showed a greater increase in von-Kossa-stained area than that in control mice at 3 days (N = 6) (Fig. 4A and B). The amount of dissolved calcium was also more markedly increased by Pi-stimulation (2.6 mM) in the cultured aorta of *Sirt1* KO mice than in control at 3 days (N = 6) (Fig. 4C). Pi-stimulation (2.6 mM) also increased the expression of Runx2 in the cultured aorta of *Sirt1* KO mice compared to control mice for 2 days (N = 6) (Fig. 4D and E). Next, the aorta of wild-type mice was treated with a SIRT1 chemical inhibitor, sirtinol. The cultured aorta treated with sirtinol showed a larger von-Kossa-stained area than that in non-treated aorta at 3 days (N = 6) (Fig. 4F and G). The amount of dissolved calcium was also increased by Pi-stimulation (2.6 mM) in the cultured aorta of sirtinol-treated aorta compared to that of non-treated aorta at 3 days (N = 6) (Fig. 4H). These results indicate that the cultured aorta of *Sirt1*-deficient mice showed accelerated vascular calcification, and raise the possibility that SIRT1 may play a critical role in vascular calcification.

4. Discussion

In this study, we found that Pi stimulation induced calcification

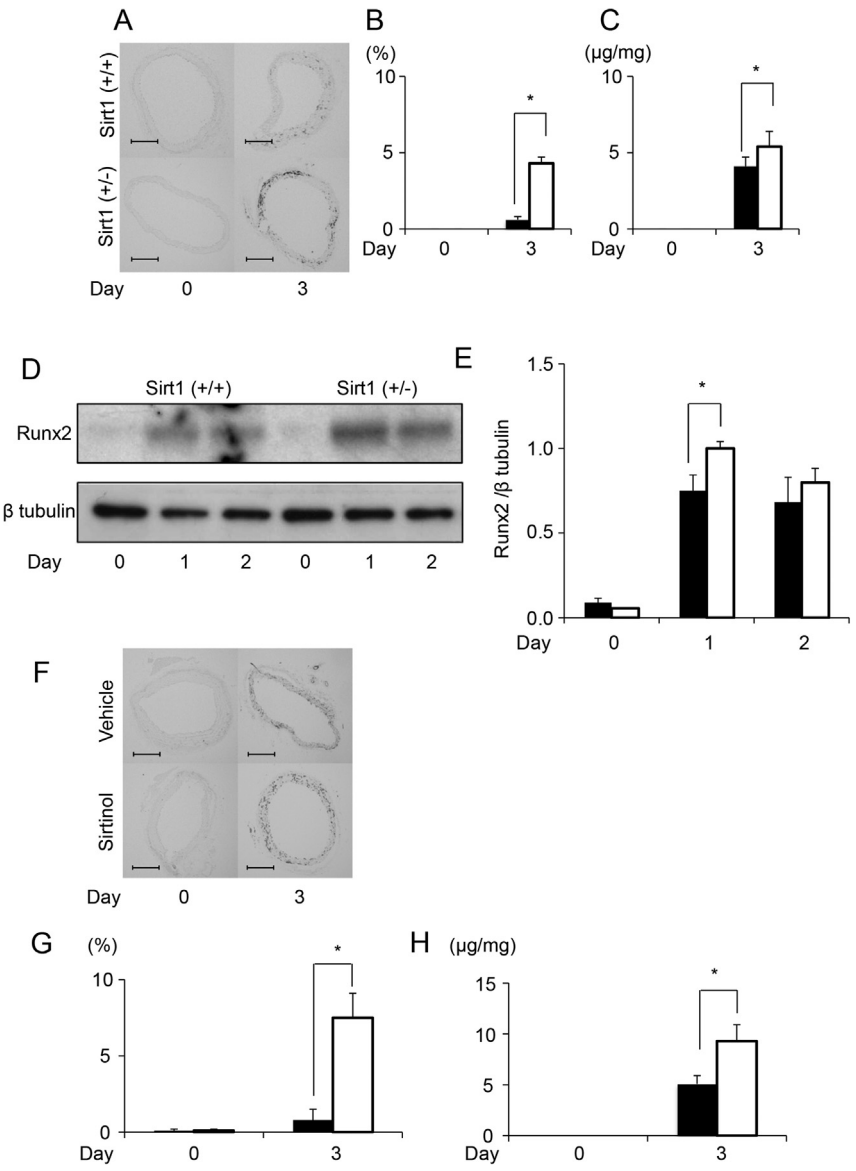

Fig. 4. A, B. Area of calcification in cultured aorta of *Sirt1* KO (+/−) mice incubated with medium containing final concentration of Pi of 2.6 mM for 3 days was determined by von-Kossa staining. (scale bar: 300 μm) C. Dissolved calcium in aorta of *Sirt1* KO (+/−) mice cultured for 3 days was measured by OCPC method. D, E. Runx2 expression in aorta of *Sirt1* KO (+/−) mice compared with control mice. (Black columns: control mice, White columns: *Sirt1* KO (+/−) mice.) F, G. Area of calcification in cultured aorta of sirtinol (100 μM)-treated mice incubated with medium containing final concentration of Pi of 2.6 mM for 3 days was determined by von-Kossa staining. (scale bar: 300 μm) H. Dissolved calcium in aorta of sirtinol-treated mice cultured for 3 days was measured by OCPC method. ($^{*}p < 0.05$, N = 6.).

of cultured aortas, and this organ culture of the aorta was useful for analysis of vascular calcification using mice with diabetes mellitus, and development of the aging process. The experimental procedures for aortic culture are very simple and easy, and it takes less time to evaluate vascular calcification precisely while maintaining the whole structure of the blood vessel. Moreover, we observed that aortic rings of the dorsal and abdominal regions were also calcified by Pi stimulation, similarly to whole aortic cultures (data not shown). In addition, this calcification of different regions was also observed in rats, but not mice (Supplementary Figure 1C). This is useful for minimizing the number of animals used.

In this study, there is a possibility that vascular endothelial cells may have had an influence on vascular calcification. Therefore, endothelial cells were removed using a thread, and the degree of medial calcification was estimated after Pi-stimulation. We found that medial calcification was induced in the cultured aorta with or without endothelial cells, and there was no significant difference between them (data not shown). It is thought that one reason for this is that using a thread to remove endothelial cells might have adversely affected culture of the aorta, and that this process could have made access of Pi to the deep portions of the tissue easier, thereby increasing its accumulation and subsequent von Kossa-positive staining. Therefore, the interaction between endothelial cells and VSMC could not be detected. Further studies without the influence of a removal method are needed.

In this study, we decided to use a Pi concentration of 2.6 mmol/L mainly because at this concentration it was easy to detect vascular calcification compared with control. In wild type mice, the normal serum concentration of Pi is 1.4 mM. Therefore, the concentration of Pi of 2.6–3.0 mmol/L we used is a high concentration *in vivo*. In a human retrospective study, these concentrations were shown to be significantly associated with the outcome of fatal or nonfatal

myocardial infarction. In addition, higher levels of serum Pi were also associated with increased risk of new heart failure, myocardial infarction, and the composite of coronary death or nonfatal myocardial infarction [11].

Following exposure to an excessive extracellular phosphate concentration, VSMC undergo reprogramming into osteogenic cells [12]. Such vascular osteo-induction is characterized by osteogenic transcription factors, and is triggered by increased Pi concentration. A key role in this process is assigned to cellular Pi transporters, most notably the type III sodium-dependent Pi transporter, Pit1 [13]. To investigate whether Pit1 is related to vascular calcification under excessive Pi-stimulation (2.6 mM) in these cultured aortas, Pit1 was blocked by PFA. Treatment with PFA ameliorated vascular calcification (Fig. 1H). These results indicate that excessive Pi-stimulation induced medial calcification through Pit1 in the cultured aorta. However, there is a possibility that other mechanisms related to cell survival such as Gas6/Axl signaling also contribute to calcification in this model, and future study is needed.

In clinical practice, vascular calcification is frequently observed in diabetic patients [14]. In fact, the cultured aorta of STZ-diabetic mice showed accelerated medial calcification induced by Pi-stimulation in this study. However, it was not observed in the cultured aorta of STZ-diabetic mice without Pi-stimulation. In other words, hyperglycemia cannot induce vascular calcification in this model. Consistent with this, it was reported that treatment with STZ only did not show vascular calcification in the rat, and adenine feeding was required to induce it [15]. These results indicate that organ culture of the aorta of STZ-diabetic mice shows a tendency for vascular calcification, but Pi-stimulation is necessary to trigger it.

Similarly to diabetic mice, the cultured aorta of aged mice showed accelerated vascular calcification induced by Pi-stimulation. In this study, the cultured aorta of aged mice, but not young mice, already showed calcification without Pi-stimulation (Fig. 3B). Blood tests showed no differences in renal function (BUN, Cre) and serum Pi concentration between young and aged mice (Supplementary Figure 2A). Moreover, the expression of Pit1 did not differ between young and aged aorta (Fig. 3G and H). In general, vascular calcification is associated with aging, and this pathological process can develop as a consequence of atherosclerosis, diabetes, or CKD [16]. That is, these conditions are among the main causes of vascular aging [17]. In addition, we recently reported that calcium deposition is more readily induced in senescent SMC compared with young cells through SIRT1/p21 signaling *in vitro* [18]. These results suggest that this organ culture model may represent calcification processes in aging, and these conditions have a tendency for calcification. Medial calcification is seen in diabetes and with aging in the presence of normal serum Pi concentrations [19], indicating that hyperphosphatemia is not required for medial calcification. Previous studies suggest that vascular calcification is modulated by several inhibitory factors within the vessel wall [20,21]. Several proteins such as matrix Gla protein or osteopontin may have been involved in our young sample.

In *S. cerevisiae*, the *Sir2* (silent information regulator-2) family of genes governs budding exhaustion and replicative life span [22]. *Sir2* has been identified as an NAD^+-dependent histone deacetylase and is responsible for maintenance of chromatin silencing and genome stability. *Sirt1*, the closest homolog of *Sir2*, is related to the aging process under caloric restriction and regulates the cell cycle, senescence, apoptosis and metabolism [23]. In our study, vascular calcification in the cultured aorta of *Sirt1* KO (+/−) mice was accelerated by Pi-stimulation. It is reported that *Sirt1* KO (−/−) mice died early within a few weeks after birth, whereas heterozygous (+/−) mice had normal body weight, fat content, lean body mass, and a normal mean life span relative to their WT littermates [24], and did not show any remarkable difference in a series of histologic and gene expression analyses. However, when treated with various stressors such as a high fat diet, these mice were more prone to develop organ damage [10]. Our study showed that excessive Pi-stimulation induced calcification in *Sirt1* KO (+/−) mice and sirtinol-treated aortas, providing conclusive evidence that SIRT1 deletion has a tendency to favor vascular calcification. Our previous study showed that Pi-stimulation dose-dependently increased senescent cells, and Pi-induced senescence was associated with downregulation of SIRT1 in cultured VSMC [18]. In addition, activation of SIRT1 by resveratrol significantly reduced senescence-associated calcification of VSMC. In accordance with this, downregulation of SIRT1 and Runx2 was observed under excessive Pi-stimulation in the cultured aorta of wild type-mice (Supplementary Figure 2B and C). In addition, a previous study showed that activation of SIRT1 by resveratrol directly deacetylated Runx2 and led to osteogenesis in mesenchymal stem cells [25]. Considering our results in VSMC, further investigation exploring the interaction of SIRT1 and Runx2 under hyperphosphatemia is needed, but these results suggest that the SIRT1/Runx2 pathway may play an important role in the link between atherosclerosis and osteoporosis in elderly patients, and may provide a new therapeutic strategy for these patients.

In conclusion, the aging process is an important factor in the induction of calcification, and we showed that excessive Pi transportation itself into SMC in some pathological conditions significantly induced vascular calcification even at a young age. This novel culture model can be applied as a reflection of aging, such as in diabetic mice which show a tendency for calcification in the presence of Pi stimulation, and we found that this culture model may be attributable to the aging process itself. We believe that this model will be useful to explore the molecular mechanisms of vascular calcification and senescent pathology.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2015.11.005.

Conflict of interest

The authors declare no conflict of interest.

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