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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