Journal of Molecular and Cellular Cardiology 56 (2013) 72–80

Contents lists available at SciVerse ScienceDirect

Journal of Molecular and Cellular Cardiology

journal homepage: www.elsevier.com/locate/yjmcc

Original article

Thrombomodulin, a novel molecule regulating inorganic phosphate-induced vascular smooth muscle cell calcification

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ARTICLE INFO

Article history:
Received 11 August 2012
Received in revised form 23 November 2012
Accepted 14 December 2012
Available online 26 December 2012

Keywords:
Thrombomodulin
Calcification
Apoptosis
EGF receptor
ERK

ABSTRACT

Hyperphosphatemia has emerged as a cardiovascular risk factor that stimulates calcification in vessels. We explored molecules that were induced by inorganic phosphate (Pi) at an early stage in vascular smooth muscle cells (VSMC). In the present study, we examined the role of thrombomodulin (TM) in Pi-induced VSMC calcification based on the results of DNA microarray analysis. Both mRNA and protein expression of TM were markedly augmented in Pi-induced calcification. Conversely, knockdown of TM by siRNA significantly inhibited calcification, in addition to Pi-induced apoptosis which plays critical roles in VSMC calcification. We further found that TM suppressed both of mRNA and protein expression of growth arrest-specific gene 6 (Gas6), a key molecule regulating apoptosis. Recombinant extracellular epidermal growth factor (EGF)-repeat domain of TM exaggerated calcification and this effect was abrogated by a neutralizing antibody for EGF receptor, suggesting that the cleaved and secreted form of TM may activate EGF receptor. We also found that downregulation of Gas6 by TM/EGF receptor axis was mediated by ERK in VSMC calcification. In the aorta of adenine-fed rat, a typical medial calcification model with hyperphosphatemia, we found that TM expression was increased. Furthermore, in human calcified aorta, increased TM expression was also observed. These results indicate that TM is a novel molecule that promotes apoptosis and vascular calcification by regulation of Gas6, presumably via EGF receptor/ERK axis.

1. Introduction

Vascular calcification is a significant feature of vascular pathology, since this lesion is associated with cardiovascular disease [1–3]. It has been recognized that inorganic phosphate (Pi) is an important inducer of vascular smooth muscle cell (VSMC) calcification, which is morphologically similar to that observed in calcified human heart valves and the aortic media [4]. Pi transport via type III sodium-phosphate cotransporter (Pit-1) is a critical step in the initiation of calcification [5]. However, few studies have explored the target molecules of Pi in calcification of VSMC.

To identify molecules that play an initial and central role in Pi-induced calcification, we employed DNA microarray analysis. Among the identified genes, we focused on thrombomodulin (TM), which was increased 1.4 fold at 24 h after treatment with Pi, because TM has been found to have diverse effects on cellular proliferation [6], cell–cell adhesion [7], and inflammation [8], all of which are important steps in the pathogenesis of atherosclerosis.

TM is a cell membrane-bound glycoprotein that functions as a thrombin cofactor in the activation of protein C. Normally, expression of TM in the arterial wall is limited to the endothelium and is not expressed in the medial smooth muscle. Interestingly, in human atherosclerotic lesions, TM is expressed in VSMC of the media and intima [9,10], suggesting its pathological roles. TM consists of five distinct domains: the NH2-terminal domain, epidermal growth factor (EGF)-like domain, serine/threonine-rich region, transmembrane domain, and cytoplasmic tail [9]. In particular, the EGF-like domain consists of six tandem EGF-like motifs that are homologous to domain III of the human EGF precursor [11]. In VSMC, the EGF-like domain of TM has been reported to exert proatherosclerotic effects [6].

ERK is a well-known downstream signaling molecule of TM [6,12]. It has been reported that TM prolongs thrombin-induced ERK phosphorylation and nuclear retention in vascular endothelial cells [12]. In VSMC, recombinant TM containing the EGF-like domain stimulates ERK phosphorylation, which increases cell proliferation [6]. The ERK

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http://dx.doi.org/10.1016/j.yjmcc.2012.12.013

pathway is also known to play a critical role in osteoblast differentiation and mineralization [13–15]. Furthermore, in calcifying valvular interstitial cell cultures, prolonged elevation of phosphorylated ERK (pERK) was found, and blocking pERK resulted in a marked decrease in nodule number, nodule size, and total calcified area. Although the involvement of ERK activation in the process of VSMC calcification has been shown in previous reports [16,17], the upstream and downstream signaling of ERK is unclear.

Regarding the molecular mechanisms of vascular calcification, we have recently reported that downregulation of the growth arrest-specific gene 6 (Gas6)-mediated survival pathway plays a pivotal role in Pi-induced apoptosis and subsequent calcification [18,19]. In the present study, we demonstrated that TM is a novel molecule regulating vascular calcification. Pi induces TM expression and cleaves the extracellular domain of TM, which stimulates the EGFR/ERK axis. Then, ERK inhibits Gas6 expression, leading to cell death and the development of calcification.

2. Materials and methods

2.1. Cell culture and materials

Human aortic smooth muscle cells (HASMC) were purchased from Clonetics Corp. (San Diego, CA) HASMC were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% 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. MEK inhibitors (PD98059 and U0126) and a matrix matalloproteinase (MMP) inhibitor (GM6001) were purchased from Calbiochem (La Jolla, CA, USA). Lavendustin A was from TOCRIS (Ellisville, MO, USA), and EGF receptor (EGFR) neutralizing antibody was from Millipore (Temecula, CA, USA). Recombinant human TM peptide containing 6 EGF-like domains (rhTME1-6) was prepared as described previously [20].

2.2. Calcifying medium and quantification of calcification

HASMC were maintained in growth medium (DMEM containing 15% FBS and 100 U/mL of penicillin and 100 mg/mL of streptomycin; final P_i concentration = 1.4 mmol/L). Pi(−) means the concentration of Pi (1.4 mmol/L) in growth medium without additional Pi stimulation. To induce VSMC calcification, we added Pi, a mixed solution of 1 mol/L Na_2HPO_4 and 1 mol/L NaH_2PO_4 whose pH was adjusted to 7.4 to serum-supplemented DMEM to a final concentration of 2.6 mmol/L (Pi(+)). Ca deposition was evaluated by the o-cresolphthalein complexone method (C-Test; WAKO). von Kossa staining was performed as follows. After treatment with 5% silver nitrate (Wako) solution for 1 h, the samples were exposed to strong light to visualize calcium deposits, and then 5% sodium thiosulfate solution was added.

2.3. High-density oligonucleotide microarray analysis

Total RNA was prepared using an RNeasy RNA extraction kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. One microgram of RNA extracted from VSMC with or without 2.6 mmol/L Pi treatment for 24 h was amplified up to approximately 100 Ag cRNA and hybridized to a high-density oligonucleotide microarray (GeneChip Rat Genome U34A; Affymetrix, Santa Clara, CA, USA) as described previously [21].

2.4. Small interfering RNA

Four small interfering RNAs (siRNA) were designed to target human TM (Dharmacon). The sequences of TM siRNAs were 5′-GGACGUGG AUGACUGCAUA-3′, 5′-GUCAUUUCCUUGC UACUGA-3′, 5′-GCACUCAA UGCUCAAUG-3′, and 5′-GCAUUCGGGCUUGCUCAUA-3′. Those of Pit-1 were 5′-CCAUGGUGGCAAUGACGUA-3′, 5′-GAAUGUGAACUUCGGGCAA-3′, 5′-CCAAGAAGCGAAUUCGAAU-3′, and 5′-CCUAAUGGUUUGCGAGCU U-3′. Those of Gas6 siRNAs were 5′-GUGACGAGGGCUUUGCGUA-3′ and 5′-GGAGAAGGCUUGCCGAGAU-3′. Regarding the experiments using siRNAs, we used all four siRNAs targeting TM or Pit-1, and two siRNAs targeting Gas6 at the same time. Non-targeting control siRNA was synthesized using standard templates (Dharmacon). The efficiency of siRNA was validated by immunoblotting and real-time PCR of the cell lysates and total RNA at 6 days. To evaluate the effect of TM on VSMC calcification, siRNAs were transfected using transfection reagent (Upstate Biotechnology) when HASMC had reached 70~80% confluence, and were then transfected each time the medium was changed every 2 days. On day 6, apoptosis and Ca deposition were measured.

2.5. Aortic calcification in renal failure rats

Renal failure was induced in rats by a 0.75% adenine-containing diet as previously described [22]. All procedures and animal care were in accordance with the Guide for the Care and Use of Laboratory Animals of the University of Tokyo. Twelve-week-old male Wistar rats (Nippon Clea Inc., Japan) were pair-fed standard CE-2 chow (containing 1.2% calcium and 0.6% phosphorus; Nippon Clea Inc.) in the control group or CE-2 chow containing 0.75% adenine (Sigma) in the renal failure group for 4 weeks. Then, the diet was returned to normal chow for an additional 4 weeks. After induction of renal failure for 8 weeks in total, the rats were sacrificed to collect samples. After perfusion with saline at a constant, nonpulsatile pressure of 100 mm Hg, the aorta was immediately embedded in OCT compound frozen section and sequentially cut into cross-sections with 5-μm thickness from each part of the aorta. To detect calcification in the aortic wall, each cross-section was subjected to von Kossa staining to demonstrate mineralization.

2.6. Calcified aortic specimens of human

Human aortic specimens were obtained from four autopsy cases (3 cases from patients who have atherosclerosis and chronic kidney disease (CKD); 82- and 84-year-old men and 90-year old woman and 1 case from patient without atherosclerosis and CKD; 33-year-old man). This protocol was approved by the Institutional Review Board of Tokyo Metropolitan Geriatric Hospital.

2.7. Statistical analysis

All values are presented as mean ± SEM. Statistical comparisons were conducted by ANOVA, followed by Fisher's test. A value of $p<0.05$ was considered statistically significant.

An expanded Methods section is available in the online Supplemental Methods.

3. Results

3.1. Expression and localization of TM in Pi-induced VSMC calcification

To confirm the results obtained by DNA microarray analysis (Table S1), we examined the expression of TM in the process of Pi-induced calcification. Both mRNA and protein expression of TM were markedly increased by treatment with Pi for 6 days in a time-dependent manner (Figs. 1A, B). The increased expression of TM was also observed by immunofluorescent staining at 6 days (Fig. 1C). We found that this increase was limited in the cytoplasmic fractions, not in the nuclear fraction of Pi-treated HASMC by immunoblotting (Fig. S1A). Furthermore, Pi-induced TM upregulation was blocked by Pit-1 siRNA, suggesting that the increase in TM expression by Pi was mediated by Pit-1, type III sodium-phosphate cotransporter (Fig. 1D). We also examined whether TM transcription activity was increased by Pi. When we