Journal of Inorganic Biochemistry 186 (2018) 1–9

Contents lists available at ScienceDirect

Journal of Inorganic Biochemistry

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

Collagen promotes matrix vesicle-mediated mineralization by vascular smooth muscle cells

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ARTICLE INFO

Keywords:
Annexins
Atherosclerosis
Matrix vesicles
Tissue-nonspecific alkaline phosphatase
Vascular calcification
Vascular smooth muscle cells

ABSTRACT

Vascular calcification (VC) is a hallmark of atherosclerotic plaques. Calcification of advanced plaques shares common features with endochondral ossification of long bones and appears to be protective. On the other hand, microcalcification of early plaques, which is poorly understood, is thought to be harmful. Tissue-nonspecific alkaline phosphatase (TNAP) and collagen are the two proteins necessary for physiological mineralization. Here, we demonstrate the presence of membrane-bound TNAP, detected by immunofluorescence, that seems to form clusters on the plasma membrane of vascular smooth muscle cells (VSMCs) cultured in mineralizing conditions. We observed that TNAP activity and mineralization were increased when VSMCs were cultured in the presence of ascorbic acid (AA) and β-glycerophosphate (β-GP). Increased TNAP activity was observed in whole cell lysates, total membrane fractions and, more particularly, in matrix vesicles (MVs). We have shown that TNAP-enriched MVs released from VSMCs subjected to collagenase contained more apatite-like mineral than the less TNAP-rich/TNAP-enriched vesicles isolated without collagenase treatment. These results suggest a role for collagen in promoting calcification induced by TNAP in atherosclerotic plaques.

1. Introduction

It has been known for decades that advanced atherosclerotic plaques undergo endochondral-like ossification resulting in formation of macrocalcifications, which in advance lesions strongly resemble bone tissue. Ossified plaques consist of carbonated apatite associated with collagen, very similar in organization and composition to the crystals formed by cartilage growth plate [1]. More recently, microcalcifications, measuring < 10 μm in diameter, have been observed within the fibrous caps of early lesions, before the appearance of chondrocyte or osteoblast phenotypes in vascular smooth muscle cells (VSMCs) within the plaque [2]. An interesting study revealed that nearly all fibrous caps contain microcalcifications, and that those with a diameter above 5 μm are particularly harmful [3]. Moreover, based on *in silico* analysis, it was proposed that microcalcifications are particularly harmful when located in a thin fibrous cap [4]. Finally, the culprit segments of vessels of acute myocardial infarction patients generally contain such microcalcifications, whereas those of stable angina pectoris patients contain macrocalcifications [5].

Deciphering when and where microcalcification begins in atherosclerotic plaques is therefore crucial. Two proteins appear to be necessary for normal mineralization: a fibrillar collagen and TNAP [6]. VSMCs normally secrete type I collagen. Tissue-nonspecific alkaline phosphatase (TNAP) overexpression in VSMCs *in vitro* and *in vivo* triggers vascular calcification [7–9]. We therefore aimed at determining the molecular mechanisms of VSMC mediated mineralization, with a particular focus on collagen and TNAP.

TNAP is an enzyme playing a key role during physiological mineralization mediated by chondrocytes and osteoblasts that deposit calcium phosphate crystals in the extracellular matrix (ECM) [10–12]. TNAP regulates mineralization by hydrolyzing a constitutive mineralization inhibitor - inorganic pyrophosphate (PP_i) [13]. PP_i is a small molecule that binds to the arising calcium phosphate crystals and prevents further incorporation of phosphate [14]. Early stages of mineralization take place in nanostructures called matrix vesicles (MVs) that are released by mineralizing cells and serve as nucleation sites for apatite synthesis [15].

Besides TNAP, other proteins may play an important role in calcification. For instance, annexins, the calcium- and phospholipid-binding proteins, are engaged in calcium homeostasis of mineralizing cells and in the influx of Ca^{2+} to MVs [16]. The large variety of annexins present in MVs and their ability to bind to different sides of biological

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https://doi.org/10.1016/j.jinorgbio.2018.05.007
Received 1 February 2018; Received in revised form 20 April 2018; Accepted 16 May 2018
Available online 18 May 2018
0162-0134/

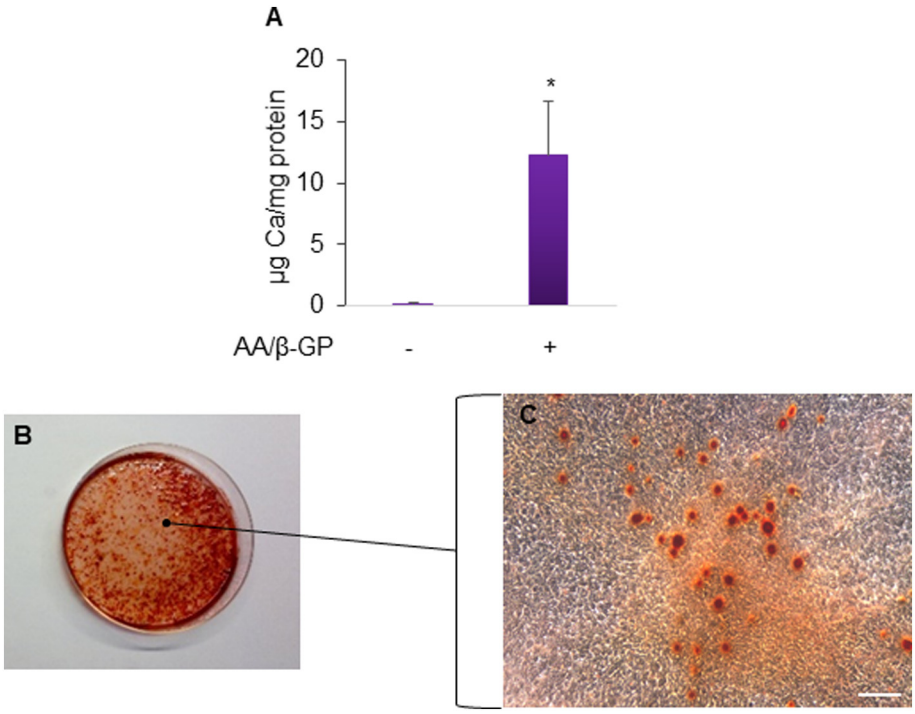

Fig. 1. Mineralization of MOVAS. A) Total calcium concentration measured by calcium colorimetric assay in MOVAS cells after 21 days of osteogenic stimulation in the presence of 50 µgmL^{-1} AA and 10 mM β-GP, $n = 4 \pm$ SEM. B) Calcium deposits produced by MOVAS stained with Alizarin Red S after 21 days of stimulation for mineralization in the presence of 50 µgmL^{-1} AA and 10 mM β-GP, C) microscopic view of calcium deposits produced by trans-differentiated MOVAS taken by Zeiss Axiovert light microscope at 100× magnification, scale bar - 100 µm. * indicates a statistical difference with $p < 0.05$.

membranes suggest that they serve different functions during the mineralization process [17]. Initiation of mineralization during endochondral ossification is a multistep process that correlates with specific interactions of Annexin A5 (AnxA5) and collagens [18]. In addition, lack of AnxA6 in MVs, which initiate the mineralization process in growth plate cartilage, resulted in reduced TNAP activity and Ca^{2+} and P_i content and in an inability to form apatite-like crystals *in vitro* [19].

Sortilin is a protein that has been recently characterized as a key regulator of vascular cell calcification due to its capacity to load TNAP into extracellular vesicles [20,21]. Interestingly, mice deficient in the gene encoding this protein, *Sort1*, have decreased arterial calcification while normal bone formation is not affected.

There are several reports indicating that MVs are necessary for the progression of smooth muscle cell calcification [22–25]. Here we further delineate the localization of TNAP activity in VSMCs and in MVs and determine the requirement of collagen for the ability of MVs to calcify.

2. Materials and methods

2.1. Cell culture and treatment

Murine MOVAS VSMC cell line was purchased from ATCC (Molsheim, France). The cells were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 gL^{-1} glucose and supplemented with 10% fetal bovine serum (FBS), 100 UmL^{-1} penicillin, 100 µg/mL streptomycin, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) and 2 mM L-glutamine at 37 °C in a humidified atmosphere containing 5% CO_2. MOVAS cells were seeded at a density of 4000 cells cm^{-2}. At confluence, trans-differentiation into mineralization-competent cells was induced by cell treatment with 50 µgmL^{-1} ascorbic acid (AA, Sigma) and 10 mM β-glycerophosphate (β-GP, Sigma).

2.2. Immunofluorescence

To analyze TNAP, AnxA2, AnxA6 and cholesterol localization in MOVAS, the cells were seeded at low concentration (1000 cells cm^{-2}) on coverslips coated with 30 µg/mL Collagen Type I from rat tail (Sigma) and cultured in the presence of AA and β-GP for 1–4 days to initiate trans-differentiation. The cells were washed with PBS and fixed with 3% paraformaldehyde for 20 min at room temperature (RT). After washing with PBS, the cells were incubated with 50 mM NH_4Cl in PBS for 10 min at RT. In the case of AnxA2 and AnxA6, permeabilization step was included using 0.1% Triton X-100 in PBS for 5 min on ice. Afterwards, the cells were washed with TBS and subjected to blocking stage (5% FBS in TBS) for 1 h. The samples were incubated for 1.5 h at RT with rabbit anti-TNAP, mouse anti-AnxA2 or mouse anti-AnxA6 primary antibody (Abcam) prepared in 0.5% FBS in TBS supplemented with 0.05% Tween (TBST). Then, the cells were washed several times with TBST and incubated for 1 h at RT with anti-mouse Alexa Fluor 488 or anti-rabbit Alexa Fluor 594 secondary antibody prepared in 0.5% FBS in TBST. For visualization of cholesterol, the cells were incubated with filipin (Sigma) at the concentration of 25 µgmL^{-1}. After extensive washing several times with TBST and once in TBS, coverslips were mounted with Mowiol 4–88 (Calbiochem), supplemented with 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma) on microscope slides. Images were taken by Zeiss AxioObserver Z.1 fluorescence microscope at the 630× magnification with appropriate fluorescent filter.

2.3. Isolation of total membranes and MVs

Membrane fractions were isolated according to Briolay and collaborators [26]. Briefly, MOVAS cells (25×10^6 cells) were disrupted in a Potter homogenizer (40 strokes) on ice in a TNE buffer consisting of 25 mM Tris-HCl, 150 mM NaCl and 5 mM EDTA (pH 7.5), supplemented with 10 µgmL^{-1} of protease inhibitor cocktail (Sigma). The homogenate was then centrifuged at 900*g* for 10 min at 4 °C. The supernatant was collected and then centrifuged at 100,000*g* for 45 min at 4 °C in an Optima L-100 XP ultracentrifuge (Beckman Coulter). After ultracentrifugation, the pellet containing crude membrane fraction (plasma membrane and cytoplasmic vesicular structures) was resuspended in 200 µL of TNE buffer.

Isolation of VSMC-derived vesicles was performed according to the procedures described earlier [22,27]. The vesicles isolated from culture