Macrophages Highly Express Carbonic Anhydrase 2 and Play a Significant Role in Demineralization of the Ectopic Calcification

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Vascular calcification is an important risk factor for cardiovascular disease, and is closely associated with all-cause mortality. Recently, it has been revealed that vascular calcification is not a passive precipitation of circulating minerals, but is a process actively regulated through machinery similar to bone formation. During the bone remodeling, osteoclasts execute the bone resorption by releasing hydrogen ions to dissolve minerals; however, molecular mechanisms underlying decalcification of ectopically calcified lesions remain largely unknown. Here, we identified a significant role of macrophages in decalcifying the ectopic calcification. Since carbonic anhydrase-2 (CA2) is critically involved in synthesizing hydrogen ions, we investigated its expression in various cells, and found that macrophages highly express CA2. We established a cell free assay system in which ectopic calcification is quantitatively analyzed in vitro, and using this assay system, we revealed that macrophages efficiently decalcify the ectopic calcification. Interestingly, M1 polarized macrophages showed reduced CA2 expression, whereas treatment with inflammatory cytokines and vasoactive peptides decreased CA2 expression in macrophages. Of note, treatment with angiotensin II significantly reduced the decalcification capacity in macrophages in association with reduced CA2 expression. Furthermore, overexpression of CA2 enhanced decalcification capacity in C2C12 myoblast cells. Together, we unveiled a potential role of macrophages in decalcifying the ectopic calcification, and identified that CA2 is critically involved in the cellular decalcification capacity. Activating cellular CA2 has a therapeutic potential in the treatment of ectopic calcification, especially in regressing vascular calcification.

INTRODUCTION

Ectopic calcification in blood vessel walls is frequently observed in elderly population and in patients with diabetes or chronic kidney disease (1-3). Vascular calcification is a significant risk factor for cardiovascular disease (4, 5); however treatment and/or prevention of vascular calcification are limited in the clinical setting and effective pharmacotherapeutic treatment needs to be developed. Vascular calcification had been thought to be a passive precipitation of excessive minerals in blood for long, but recent many data strongly suggest that mechanism similar to bone formation actively regulates the ectopic calcification in vessel walls (6). Molecules that play a key role in the bone calcification have been identified in blood vessels, particularly at the sites of calcified lesions (7, 8). Matrix GLA protein (MGP) is a potent inhibitor for mineral precipitation and genetic loss of MGP induces severe vascular calcification (9). Of note, restoration of MGP expression in vascular smooth muscle cells (VSMCs) rescued the arterial calcification phenotype in MGP-deficient mice, whereas raising the serum levels of MGP failed to do so (10). These data indicate that MGP locally expressed in blood vessels plays an essential role in the prevention of vascular calcification.

Osteoblasts that play a key role in bone mineralization are differentiated from mesenchymal precursor cells largely through bone morphogenetic protein 2 (BMP2)-mediated activation of runt-related transcription factor 2 (RUNX2) (11, 12). Osteoblasts produce type I collagens in which calcium phosphate mineral crystals precipitate. Osteoblasts also produce alkaline phosphatase (ALP) that hydrolyzes inorganic pyrophosphate, leading to acceleration of mineral precipitation (13). We previously reported that senescent VSMCs acquired osteoblast-like features with enhanced expression of RUNX2, ALP and type I collagen, and exhibited exacerbated VSMC mineralization (14). We also identified that dedifferentiated VSMCs produced BMP2 and accelerated osteoblastic differentiation of mesenchymal precursor cells. Targeted activation of BMP2 in VSMCs enhanced atherosclerotic plaque calcification in apoE-deficient mice, indicating a crucial role of paracrine osteogenic signals exerted by VSMCs in the vascular calcification (15). In addition, macrophages further

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enhanced BMP2 expression in VSMCs, suggesting a role of macrophages in the atherosclerotic plaque calcification in coordination with VSMCs (16).

In contrast to the progress in understanding molecular mechanisms underlying vascular calcification, little is known about the regression or decalcification of ectopically precipitated minerals. During bone remodeling, osteoclasts dissolve minerals by creating acidic environment in the resorption lacuna (17). In this process, carbon anhydrase 2 (CA2) and vacuolar H^+-ATPase play essential roles for H^+ production and proton transportation, respectively (18, 19). Although a potential role of osteoclast-like cells in the regression of vascular calcification has been suggested, their population is very rare and it is unlikely that osteoclast-like cells provide the primary mechanism in the decalcification of ectopically precipitated minerals (20, 21). Here we established a cell free assay system for the analysis of ectopic calcification, and identified a potential role of macrophages in the decalcification process in association with their high CA2 expression. CA2 expression was negatively regulated by M1-polarization or treatment with inflammatory cytokines, endothelin-1 and angiotensin II in macrophages. Of note, CA2 expression levels are well correlated with the cellular decalcification capacity, and therefore macrophage CA2 is an attractive therapeutic target in the treatment of vascular calcification.

MATERIAL AND METHODS

Materials

Fibronectin-coated culture plates were obtained from Corning. Type I collagen-coated plates were purchased from IWAKI. Alizarin red S solution was obtained from Cosmo Bio. Antibody for FLAG and lipopolysaccharide (LPS) were purchased from Sigma. Recombinant endothelin-1 and angiotensin II were purchased from Peptide Institute. Recombinant IFN-γ, TNF-α and IL-1β were obtained from R&D Systems.

Decalcification assay

The calcification medium was prepared by adding 0.4 ml 1.8 M $CaCl_2$ and 0.4 ml 0.9 M NaH_2PO_4 into 39.2 ml Dulbecco's Modified Eagle Medium (DMEM) containing HEPES (Gibco #21063029) supplemented with 15% FBS (final concentration is; 18 mM $CaCl_2$ and 9 mM NaH_2PO_4). Type I collagen-coated 12-well plates were incubated with 500 μl calcification medium for 72 h in the CO_2 incubator. Subsequently, culture plates were washed with PBS for 3 times, and 5 x 10^5 cells were plated in phosphate free DMEM (Gibco #11971025) supplemented with 10% FBS. After 24 h, culture medium was changed with phosphate free DMEM supplemented with 3% FBS, and further incubated for 72 h. Before measurements of remaining calcium precipitates, cells were scraped off using cell scraper followed by washing with distilled water for 3 times. Negative control wells that do not contain cells were also scraped using cell scraper as a sham-procedure. Remaining minerals were dissolved in 500 μl 0.6 M HCl, and calcium concentration was measured using QuantiChrom Calcium Assay Kit (BioAssay Systems). In some experiments, C2C12 cells were transfected with expression construct for GFP or CA2 tagged with FLAG using lipofectamine 3000 (Thermo) 1 day prior to plating on the assay plates. For M1-polarization, RAW cells were treated with 10 ng/ml IFN-γ and 10 ng/ml LPS for 24 h.

Quantitative RT-PCR

Total RNA was extracted using TRIzol (Thermo) followed by purification with NucleoSpin RNA purification kit (TAKARA). cDNA was synthesized from ~1 μg of total RNA using PrimeScript RT reagent Kit with gDNA Eraser (TAKARA). PCR reactions were prepared using FastStart SYBR Green Master (Roche Applied Science) followed by the real time PCR analysis using LightCycler96 (Roche Applied Science). The mRNA levels of target genes relative to 18S were analyzed.

Statistical analysis

All data are presented as mean ± S.E. Differences between groups were analyzed by Student's t-test. Comparisons between 3 groups were assessed for significance by one-way ANOVA with post hoc analysis of Fisher's PLSD . $P < 0.05$ was considered statistically significant.

RESULTS

Establishing a cell free assay system for analysis of ectopic calcification

A major disadvantage in the research for decalcification is that there is no established cell free assay system with which ectopic calcification is quantitatively analyzed. We therefore tried to establish such assay systems. First, we optimized the calcification medium enriched with calcium and phosphates, and found that DMEM containing HEPES supplemented with 15% FBS can maintain 18 mM $CaCl_2$ and 9 mM NaH_2PO_4 without generating mineral crystals. We then prepared cell culture plates that are coated with various extracellular matrix,

and incubated with the calcification medium to induce ectopic mineral precipitation. After 3 days of incubation with the calcification medium, we analyzed the calcium precipitations using alizarin red S-staining as well as by direct measurements of precipitated calcium. Alizarin red S-staining demonstrated significant calcification in type I collagen-coated plates, while modest mineralized nodules were detected in fibronectin-coated plates (Fig. 1A). In contrast, gelatin-coated and non-coated plates showed no alizarin res S-positive calcium nodules (Fig. 1A). Direct measurements of precipitated calcium revealed that type I collagen holds the highest amount of calcium precipitates, while gelatin- and fibronectin-coated plates showed modest calcium precipitations (Fig. 1B). Together, we established cell free assay tools for analysis of the ectopic calcification, which is easily prepared using type I collagen-coated culture plates and the calcification medium we optimized.
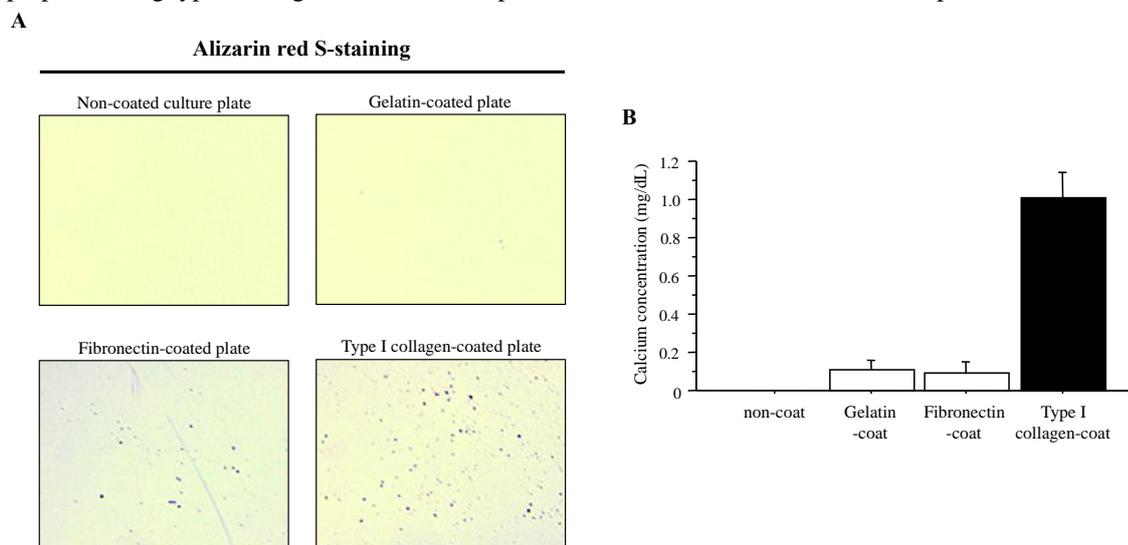
A

Figure 1. Establishment of cell free assay system for the analysis of ectopic calcification.
(A) Alizarin red S-staining of calcium nodules in the culture plates coated with various extracellular matrix. (B) Quantitative analysis for calcium precipitation. Data are presented as mean ± SE, n = 6 each.

Macrophages highly express CA2 and effectively decalcify the ectopically precipitated minerals

Since CA2 and vacuolar H^+-ATPase play essential roles to generate acidic microenvironment for dissolution of calcium and phosphate mineral crystals, we analyzed their expression levels in various types of cells. Accordingly, we identified that macrophage RAW cells highly expressed CA2, while H^+-ATPase expression was comparable among the cells examined (Fig. 2A and 2B). These data suggest a potential role of macrophages in the decalcification of ectopically precipitated minerals. We then examined whether macrophages decalcify the ectopic calcification in comparison with C2C12 cells that expressed CA2 at low levels by using the ectopic calcification assay plates described above. Following the preparation of the calcification plates, we plated RAW or C2C12 cells in phosphate-free DMEM. The calcification plates without cells were treated in the same way as a control group. After 4 days of incubation, cells were removed using cell scraper and remaining calcium precipitation was quantified. Control plates without cells were also treated with cell scraper in the same way. Interestingly, the calcification plates treated with RAW cells exhibited a substantial reduction in calcium
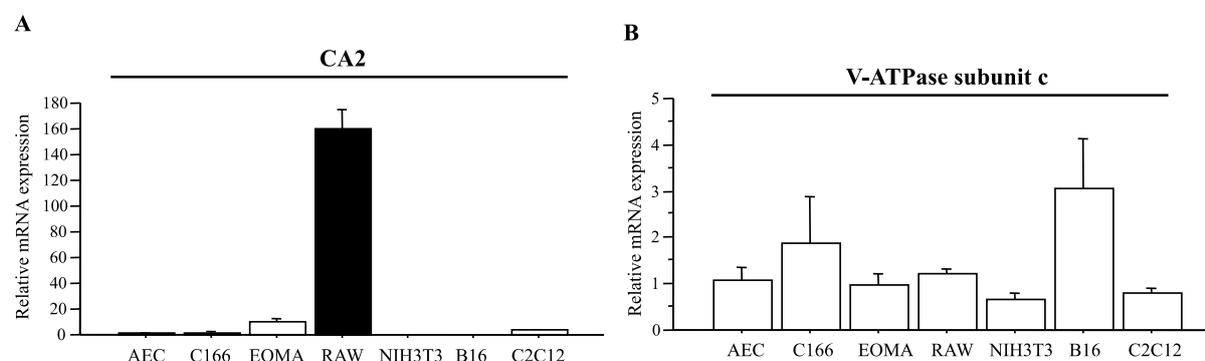

Figure 2. Expression of CA2 and V-ATPase in various types of cells. (A and B) Quantitative analysis for CA2 (A) or vacuolar H^+-ATPase subunit c (B) mRNA expression in various cells. AEC, mouse aortic endothelial cells; C166, mouse endothelial cell line; EOMA, mouse endothelial cell line; RAW, mouse macrophage cell line; NIH3T3, mouse fibroblast cell line; B16, mouse melanoma cell line; C2C12, mouse myoblast cell line. Data are presented as mean ± SE, n = 3 each.

precipitates, while the plates treated with C2C12 cells showed modest calcification reduction comparing to the control plates without cells (Fig. 3A). These results strongly suggest that macrophages effectively decalcify the ectopically precipitated minerals. We then investigated whether CA2 is involved in the cellular decalcification capacity. Overexpression of CA2 significantly enhanced the decalcification of ectopically precipitated minerals by C2C12 cells, indicating a crucial role of CA2 in determining the cellular decalcification capacity (Fig. 3B and 3C).
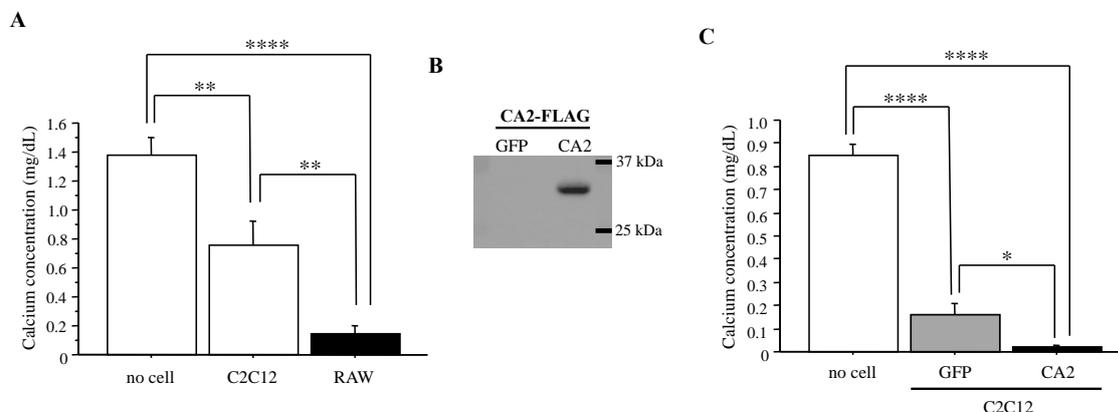

Figure 3. Macrophages effectively decalcify the ectopic calcification. (A) Quantification of remaining calcium precipitates in the calcification plates treated with no cell, C2C12 cells, or RAW cells. Data are presented as mean ± SE, n = 11 for the plates with no cell; n = 10 each for the plates with cells. **P < 0.01 and ****P < 0.0001. (B) Immunoblotting for CA2 tagged with FLAG in C2C12 cells transfected with either GFP or CA2 expression construct. (C) Quantification of remaining calcium precipitates in the calcification plates treated with no cell or C2C12 cells transfected with either GFP or CA2 expression construct. Data are presented as mean ± SE, n = 6 each. *P < 0.05 and ****P < 0.0001.

Various stimuli regulate CA2 expression and decalcification capacity in macrophages
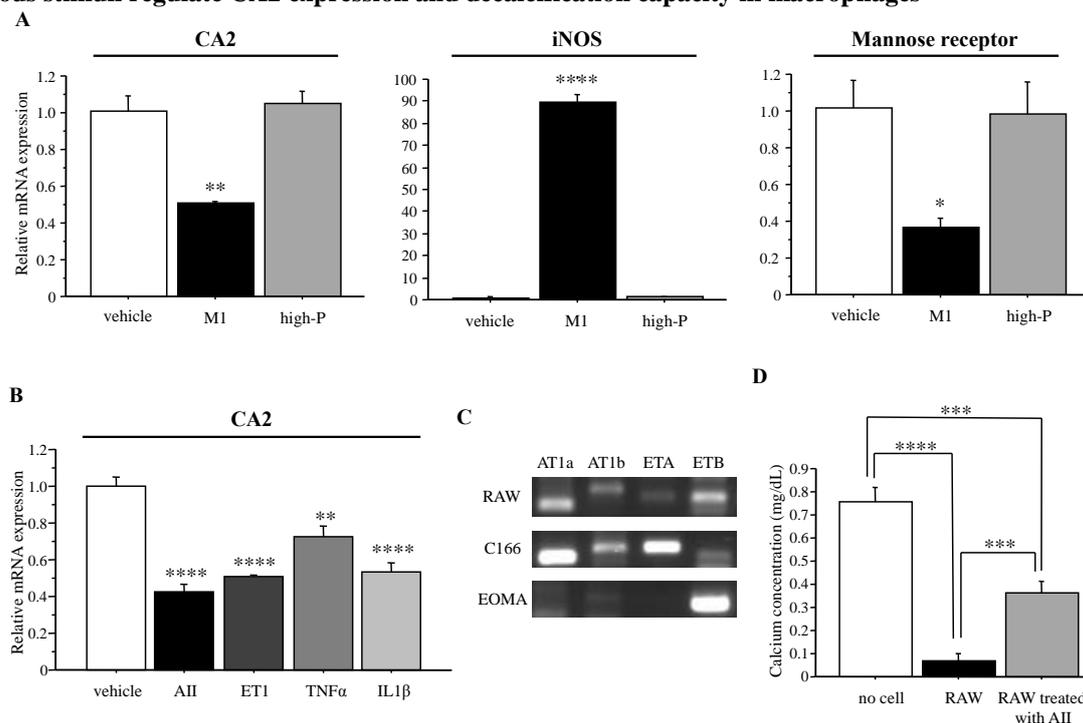

Figure 4. CA2 expression is regulated by various stimuli in macrophages.
(A) Quantitative analysis of CA2, iNOS (M1 marker), and mannose receptor (M2 marker) in RAW cells treated with M1-polarizing stimuli (M1) or 2.5 mM phosphate (high-P). Data are presented as mean ± SE, n = 3 each. *P < 0.05, **P < 0.01 and ****P < 0.0001 versus vehicle-treated cells. (B) Quantitative analysis of CA2 in RAW cells treated with 100 nM angiotensin II (AII), 10 nM endothelin-1 (ET1), 50 ng/ml TNF-α, or 50 ng/ml IL-1β for 24 h. Data are presented as mean ± SE, n = 3 each. **P < 0.01 and ****P < 0.0001 versus vehicle-treated cells. (C) RT-PCR for angiotensin II receptor type 1a (AT1a) and type 1b (AT1b), and endothelin receptor type A (ETA) and type B (ETB) in RAW, C166, and EOMA cells. (D) Quantification of remaining calcium precipitates in the calcification plates treated with no cell or RAW cells in the presence or absence of 100 nM angiotensin II. Data are presented as mean ± SE, n = 4 each. ***P < 0.001 and ****P < 0.0001.

We then analyzed the expressional regulation of CA2 in macrophages. When polarized toward pro-inflammatory M1 phenotype, CA2 expression was significantly reduced in RAW cells (Fig. 4A). In contrast, exposure to high phosphate condition did not affect either CA2 expression or M1/M2 phenotype in RAW cells (Fig. 4A). Treatment with vasoactive peptides such as endothelin-1 and angiotensin II or inflammatory cytokines such as TNF-α and IL-1β also reduced CA2 expression in RAW cells (Fig. 4B). We confirmed the expression of receptors for angiotensin II and endothelin in RAW cells (Fig. 4C). Of note, treatment with angiotensin II significantly impaired the decalcification capacity in RAW cells in consistent with the reduced CA2 expression, although a direct link between the reduced CA2 expression and the impaired decalcification capacity needs to be elucidated (Fig. 4D). These results strongly suggest that macrophage decalcification capacity is modified by various stimuli potentially through the alteration of CA2 expression.

DISCUSSION

In the present study, we successfully established a cell free assay system for the analysis of ectopic calcification. Ectopic calcification is easily and consistently produced, and we revealed that decalcification capacity of cells can be quantitatively analyzed using this assay system. Therefore, this assay system is useful in the analysis of cellular decalcification capacity and its underlying mechanisms. However, detailed characterization of precipitated minerals needs to be investigated and compared to physiological ectopic calcification in the future to further validate this assay system as an appropriate tool for the analysis of ectopic calcification.

Ectopic calcification, represented by vascular calcification is often observed in the clinical setting. Since vascular calcification is a significant risk for cardiovascular disease, regressing the calcification is a promising approach in the prevention of cardiovascular events. It has been reported that 15% of symptomatic patients with coronary artery disease showed regression of coronary calcification, while progression of calcification was detected in 51% of patients in a year, assessed by electron-beam computed tomography (22). Furthermore, treatment with endothelin-1 antagonist has been reported to regress the established vascular calcification in rats (23, 24). These reports suggest that regression of vascular calcification occurs potentially through an actively regulated mechanism.

We found that macrophages highly expressed CA2 and effectively decalcify the ectopically precipitated minerals. CA2 has been reported to be essential for bone resorption and osteoclast differentiation. Interestingly, mice carrying a CA2 null allele showed medial calcification in small arteries in various organs (25). Furthermore, ectopic calcification in subcutaneously implanted bovine pericardium was deteriorated in CA2-deficient mice (26). These data sufficiently indicate that CA2 plays a crucial role in the formation of ectopic calcification although it remains unclear whether CA2 modulates ectopic calcification by altering cellular decalcification capacity. CA2 expression was reduced by inflammatory cytokines, vasoactive peptides such as endothelin-1 and angiotensin II, and M1-polarization in macrophages. Of note, these stimuli are also involved in the progression of cardiovascular disease. Given that decalcification capacity is closely associated with CA2 expression levels, regression of vascular calcification might be inhibited during the progression of cardiovascular disease.

In conclusion, we established a cell free assay system for the analysis of ectopic calcification, and identified that macrophages effectively demineralize the ectopic calcification in association with high CA2 expression. Our assay system would expedite the research in clarifying the mechanism underlying the decalcification of ectopically precipitated minerals, and our data highlighted CA2 as an attractive pharmacotherapeutic target for the treatment of vascular calcification.

ACKNOWLEDGMENTS

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