MOVAS-1 cell line: A new *in vitro* model of vascular calcification

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Abstract. Vascular calcification has severe clinical consequences in a number of diseases, including diabetes, atherosclerosis and end-stage renal disease. The *in vitro* calcification of primary mouse, human and bovine vascular smooth muscle cells (VSMCs) is commonly employed to examine the mechanisms of vascular calcification. However, to date, no published studies have utilised a murine cell line to investigate this process. In the present study, we aimed to determine whether the mouse VSMC line MOVAS-1 can calcify *in vitro*. We established that the calcification of MOVAS-1 cells can be induced in the presence of calcifying medium (containing β-glycerophosphate and ascorbic acid), as detected by Alizarin Red and von Kossa staining, and quantification of calcium deposition and alkaline phosphatase activity. We also showed that the time course of MOVAS-1 calcification is comparable to that of the primary murine aortic VSMCs, establishing the MOVAS-1 cells as a feasible and relevant model. Significant increases in the mRNA expression profile of key genes associated with vascular calcification (*Ocn, Akp2* and *PiT-1*) were observed in MOVAS-1 cells cultured under calcifying conditions, with similar changes in expression in murine aortic VSMCs. Furthermore, a significant reduction in calcification was observed in MOVAS-1 cells following treatment with levamisole and etidronate, known inhibitors of calcification. In conclusion, we demonstrated that the MOVAS-1 line is a reliable, convenient and economical system in which to investigate vascular calcification *in vitro*, and will make a useful contribution to increasing our understanding of this pathological process.

Introduction

Vascular calcification has severe clinical consequences in a number of diseases, including diabetes, atherosclerosis and end-stage renal disease (1-3). The process of vascular calcification shares many similarities with that of bone formation, which occurs through osteoblast and chondrocyte extracellular matrix calcification (1,2). Vascular smooth muscle cells (VSMCs), the predominant cell type involved in vascular calcification, can undergo phenotypic transition to calcifying osteoblastic and chondrocytic cells in a calcified environment (4-8). Drivers of VSMC chondro-osseous differentiation and calcification include mineral imbalance; bone morphogenic proteins (BMPs); certain oxidised pro-atherogenic lipids and loss of negative regulators of calcification (2,4,5,7-16). The phosphate transporter, *PiT-1,* is the predominant sodium-dependent phosphate co-transporter expressed in VSMCs. Increased *PiT-1* expression leads to elevated intracellular phosphate, which induces the osteogenic conversion of VSMCs (17). Tissue non-specific alkaline phosphatase (ALP), a key regulator of bone calcification, also plays an important role in vascular calcification through the generation of phosphate, and by reducing levels of the calcification inhibitor pyrophosphate in blood vessels (18,19).

The *in vitro* calcification of primary mouse (20,21), human (22,23) and bovine (4,24-26) VSMCs are commonly utilised models of vascular calcification. Cells derived from transgenic mouse models with vascular calcification phenotypes are also frequently studied (7,18,27). However, to date, no published studies have utilised a murine cell line to investigate vascular calcification.

The mouse VSMC line MOVAS-1 was initially described by Afroze *et al* (28). This cell line exhibits a smooth muscle cell-specific phenotype, and has been employed to investigate the VSMC cell cycle (28); neuroendocrine peptide expression profiles (29); vascular circadian rhythms (30) and signal transduction responses to cytokines (31).

This cell line offers an alternative to primary cells derived directly from animals, reducing experimental variation and allowing stable transformation of VSMCS. The present study has therefore evaluated the MOVAS-1 cell line as an *in vitro* model of vascular calcification.

Materials and methods

MOVAS-1 cell culture. MOVAS-1 cells were a kind gift from Professor M. Hussein, Univeristy of Toronto, Canada. Cells were maintained in DMEM (Invitrogen, Paisley, UK) supplemented with 10% FCS (Invitrogen) and 1% gentamycin (Invitrogen). Cells were seeded in multi-well plates (Costar, High Wycombe, Bucks, UK) at a density of 1.0×10^4 cells/cm^2. At confluence, the medium was supplemented with 2.5 mM

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β-glycerophosphate (βGP) (Sigma, Poole, Dorset, UK) and 50 μg/ml ascorbic acid (Sigma) for 21 days to induce calcification. Cells were exposed to 1-100 μg/ml etidronate (Sigma) or to 0.01-1 mM levamisole (Sigma). Incubation was performed at 37˚C in a humidified atmosphere of 95% air/5% CO_2 and the medium was changed every second/third day.

Primary murine VSMC isolation. Primary VSMCs were isolated from aortas dissected from C57Bl6 mice at 5 weeks of age. The adventitia was removed and the aorta cut open to expose the endothelial layer (7). Tissues from eight animals were pooled for digestion with 1 mg/ml trypsin to remove remaining adventitia and endothelium, followed by incubation overnight at 37˚C in a humidified atmosphere of 95% air/5% CO_2 in growth medium consisting of α-MEM supplemented with 10% FCS and 1% gentamycin. Tissues were then digested in 425 U/ml collagenase type II for 5 h. Before experimentation, isolated VSMCs were expanded in growth medium for two passages in T25 tissue-culture flasks (Greiner Bio-One GmbH, Frickenhausen, Baden-Württemberg, Germany) coated with 0.25 μg/cm^2 murine laminin (Sigma) to promote maintenance of the contractile differentiation state (32).

Primary murine VSMC culture. VSMCs were seeded at a density of 1.5x10^4 cells/cm^2. At confluence, growth medium supplemented with 2.5 mM βGP and 50 μg/ml ascorbic acid for 21 days to induce calcification. Cells were incubated was at 37˚C in a humidified atmosphere of 95% air/5% CO_2 and the medium was changed every second/third day.

Detection of calcification. Calcium deposition was evaluated by staining the cell-matrix monolayer with Alizarin Red (33,34). Cells were washed twice with phosphate buffered saline, fixed in 4% paraformaldehyde for 5 min at 4˚C, stained with 2% Alizarin Red (pH 4.2) for 5 min at room temperature and rinsed with distilled water. Alizarin Red-stained cultures were extracted with 10% cetylpyridium chloride for 10 min. The OD was determined at 570 nm using a spectrophotometer (Multiskan Ascent, Thermo Electron Corporation, Vantaa, Finland). To investigate phosphate deposition, von Kossa staining was undertaken (32). Cells were fixed in 4% paraformaldehyde for 5 min at 4˚C, stained with 5% silver nitrate solution for 20 min and fixed in 5% sodium thiosulphate solution for 5 min.

The matrix was decalcified in 0.6 N HCl for 24 h, and free calcium was determined colorimetrically using a commercially available kit (Randox Laboratories Ltd., Crumlin, UK) and corrected for total protein concentration. The protein content of the cells was measured using the Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hertfordshire, UK) based on the Bradford dye binding procedure, and γ-globulin was used as standard (35).

Alkaline phosphatase (ALP) activity. Cell layers were lysed with 0.9% NaCl and 0.2% Triton X-100 and centrifuged at 12,000 x g for 15 min at 4˚C. The supernatant was assayed for protein content and ALP activity. Enzyme activity was determined by measuring the cleavage of 10 mM p-nitrophenyl phosphate (pNPP) at 410 nm using a commercially available kit (Thermo Trace, Melbourne, Australia). Total ALP activity was expressed as nmoles pNPP hydrolysed/min/mg protein (36).

Analysis of SM22α expression using semi-quantitative RT-PCR. RNA was extracted from cells using RNeasy total RNA (Qiagen Ltd., Crawley, UK), according to the manufacturer's instructions. For each sample, total RNA content was assessed by absorbance at 260 nm and purity by A260/A280 ratios. RNA was reverse transcribed and the PCR reaction undertaken as described previously (37,38). For the PCR reaction, primers for *18S* rRNA gene (20 cycles) (Ambion, Huntingdon, UK, sequence not disclosed) and SM22α (35 cycles) (Forward 5'TCC AGT CCA CAA ACG ACC AAG C3', Reverse 5'GAA TTG AGC CAC CTG TTC CAT CTG-3') were used.

Analysis of gene expression using quantitative RT-PCR. RNA was extracted, quantified and reverse transcribed as described above. RT-qPCR was performed using the Stratagene Mx3000P real-time QPCR system (Stratagene, CA, USA). Primers for *PiT-1* (Forward, 5'CAC TCA TGT CCA TCT CAG ACT3'; Reverse, 5'CGT GCC AAA GAA GGT GAA C3'), *Osteocalcin (Ocn)* (Forward, 5'CCG GGA GCA GTG TGA GCT TA3'; Reverse, 5' TAG ATG CGT TTG TAG GCG GTC3'), ALP *(Akp2)* (Forward, 5'GGG ACG AAT CTC AGG GTA CA3'; Reverse 5'AGT AAC TGG GGT CTC TCT CTT T3') and *Gapdh* (sequence not disclosed) were used (Primer Design, Southampton, UK).

Statistical analysis. General Linear Model analysis and the Student's t-test were used to assess the data. All data are expressed as the mean ± SEM. Statistical analysis was performed using Minitab 15. $P<0.05$ was considered to be significant.

Results

Calcification of MOVAS-1 cells. Initial studies were undertaken to determine whether the calcification of MOVAS-1 cells could be induced, when cultured in the presence of calcifying medium (containing βGP and ascorbic acid). At 21 days, Alizarin Red (Fig. 1A) and von Kossa (Fig. 1B) staining confirmed the deposition of calcium and phosphate, respectively. Calcium deposition was quantified following decalcification in 0.6 M HCl, and was significantly increased ($P<0.001$; Fig. 1C). The activity of ALP, a molecule known to play an important role in vascular calcification (18,19), was also significantly elevated ($P<0.001$; Fig. 1D).

Further studies were performed to evaluate the utilisation of MOVAS-1 cells as a model of *in vitro* calcification. The time course of MOVAS-1 calcification was examined, and compared to that of murine aortic VSMCs. Alizarin Red staining in MOVAS-1 cells cultured under calcifying conditions was negligible at 0 days, with significant increases in staining intensity at 7, 14 and 21 days ($P<0.01$; Fig. 2B). Comparable increases in Alizarin Red staining in murine VSMCs were observed ($P<0.01$; Fig. 2A). ALP activity in MOVAS-1 cells was also significantly increased at 7, 14 and 21 days compared to 0 days ($P<0.01$; Fig. 2D). Comparable increases in ALP activity in murine VSMCs were observed ($P<0.01$; Fig. 2C).

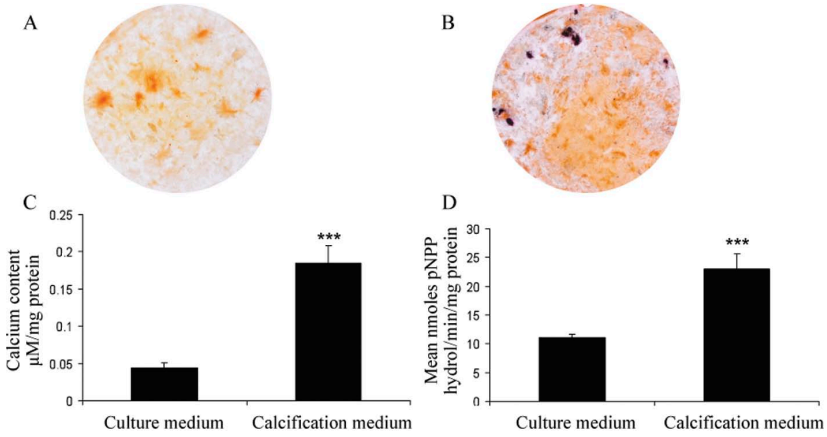

Figure 1. *In vitro* calcification of MOVAS-1 cells. (A) Alizarin Red staining showing calcium deposition and (B) von Kossa staining showing phosphate deposition in MOVAS-1 cells cultured for 21 days under calcifying conditions. (C) Calcium content (μM/mg protein) and (D) alkaline phosphatase activity (mean moles pNPP hydrol/min/mg protein) of MOVAS-1 cells cultured in the presence or absence of calcifying conditions.

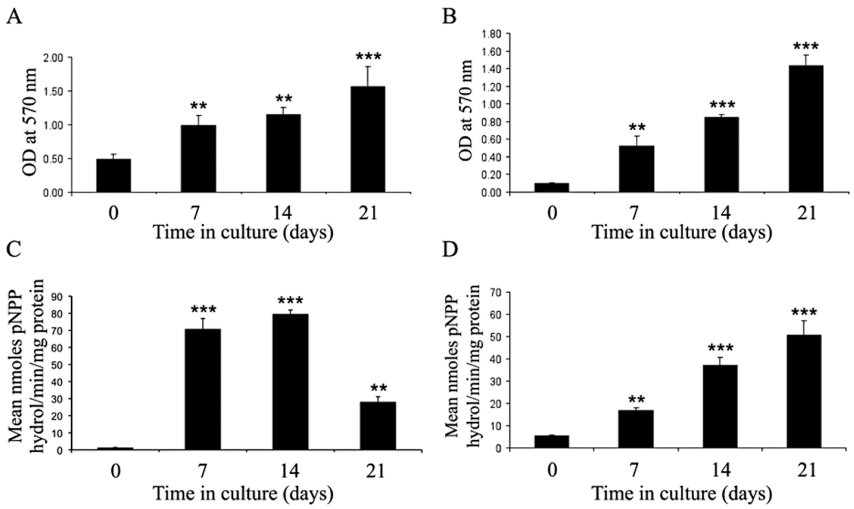

Figure 2. Quantification of Alizarin Red staining showing calcium deposition in (A) murine VSMCs and (B) MOVAS-1 cells cultured for 21 days under calcifying conditions. Quantification of alkaline phosphatase activity (mean moles pNPP hydrol/min/mg protein) in (C) murine VSMCs and (D) MOVAS-1 cells cultured for 21 days under calcifying conditions.

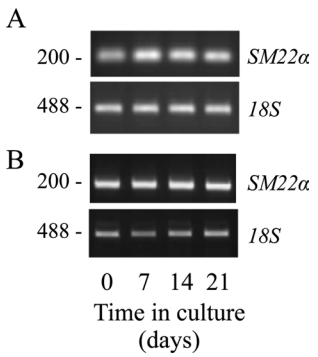

Figure 3. Semi-quantitative RT-PCR analysis of SM22α and 18S expression in (A) murine VSMCs and (B) MOVAS-1 cells cultured for 21 days under calcifying conditions.

Changes in gene expression in calcifying MOVAS-1 cells. In order to verify that MOVAS-1 cells and murine aortic VSMCs maintained a VSMC phenotype throughout the culture period, the expression of the VSMC marker *SM22α* was confirmed by semi-quantitative PCR (Fig. 3). Further gene expression studies were undertaken, to investigate the expression profile of key genes associated with vascular calcification in MOVAS-1 cells, compared to murine aortic VSMCs. Quantitative PCR revealed significant increases ($P<0.05$) in the expression of *Ocn* (Fig. 4B), *Akp2* (Fig. 4D) and *PiT-1* (Fig. 4F) in MOVAS-1 cells cultured under calcifying conditions, with similar changes in the expression in murine aortic VSMCs (Fig. 4A, C and E, respectively). These data suggest that the culture of MOVAS-1 cells in a calcifying medium is an appropriate model with which to study vascular calcification *in vitro.*

Inhibition of MOVAS-1 calcification by etidronate and levamisole. These studies have established that MOVAS-1 cells undergo calcification with associated changes in gene expression comparable to that of murine aortic VSMCs. Further experiments investigated whether this calcification of MOVAS-1 cells could be reduced, using known inhibitors of calcification over a 21-day culture period.

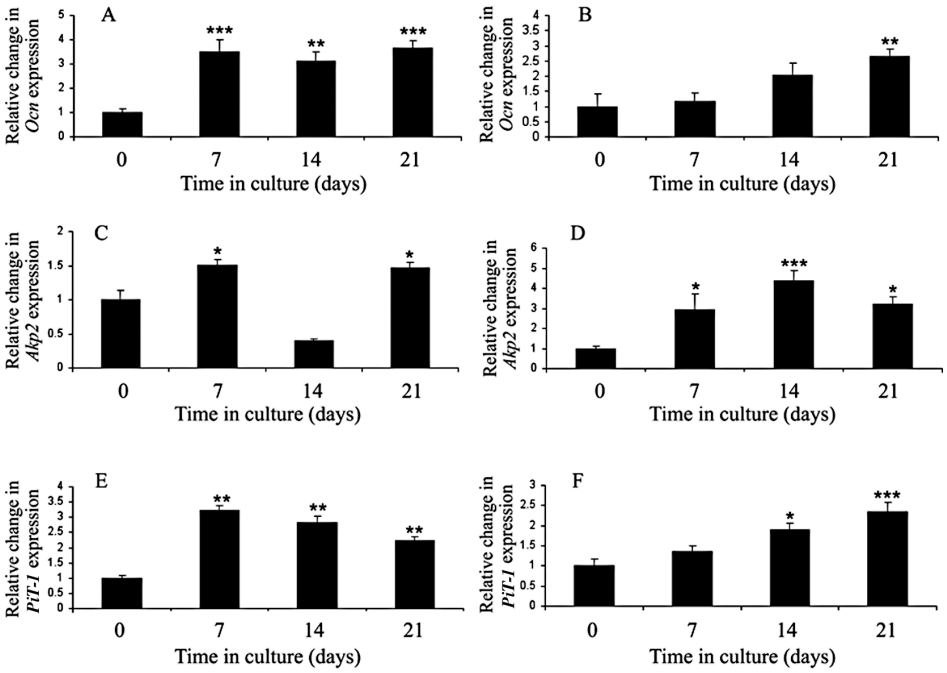

Figure 4. Fold changes in mRNA expression during culture for 21 days under calcifying conditions of *Ocn* in (A) murine VSMCs and (B) MOVAS-1 cells; *Akp2* in (C) murine VSMCs and (D) MOVAS-1 cells and *PiT-1* in (E) murine VSMCs and (F) MOVAS-1 cells.

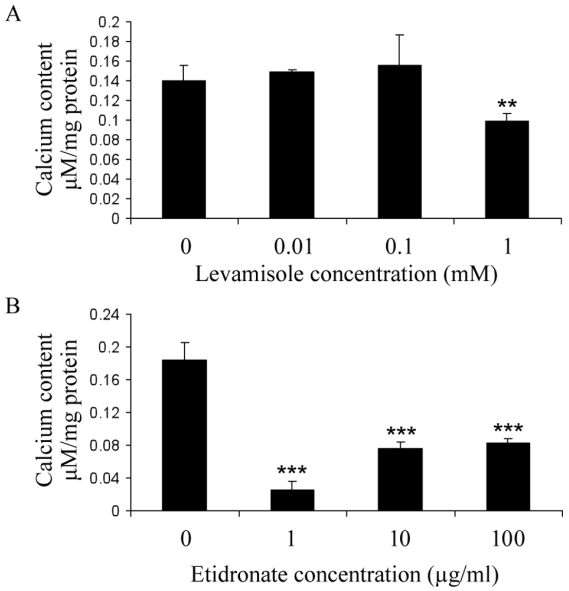

Figure 5. Effect of (A) levamisole (0.01-1 mM) and (B) etidronate (1-100 μg/ml) exposure on MOVAS-1 calcification *in vitro*, following culture for 21 days under calcifying conditions.

The effect of levamisole (0.01-1 mM), a specific inhibitor of ALP (39), on MOVAS-1 calcification was determined. A significant reduction in calcium deposition ($P<0.01$) was observed following exposure to 1 mM levamisole (Fig. 5A). These data confirm the functional significance of ALP in the calcification process.

As the production of hydroxyapatite is involved in vascular calcification, we also examined the effects of the bisphosphonate etidronate (1-100 μg/ml), a known inhibitor of hydroxyapatite formation (40), on MOVAS-1 calcification (Fig. 5B). A significant reduction in calcium deposition ($P<0.01$) was observed following exposure to 1, 10 and 100 μg/ml etidronate. These results demonstrate the formation of hydroxyapatite by MOVAS-1 cells, and confirm the inhibitory effect of etidronate on vascular calcification *in vitro*.

Discussion

The *in vitro* calcification of mouse (20,21), human (22,23) and bovine (4,24-26) vascular smooth muscle cells (VSMCs) are a commonly employed model of vascular calcification. However, primary VSMC cultures exhibit slow growth and cannot be used beyond a limited number of passages due to senescence and phenotypic changes that occur during culture. Furthermore, the use of primary cells is labour-intensive, expensive, and time-consuming (28). Moreover, the utilisation of an immortalized mouse VSMC line in the field of vascular calcification would allow complementary *in vitro* studies of mouse models in which transgenic manipulations and their effects are restricted to VSMCs *in vivo* (41,42). For these reasons, we characterized the *in vitro* calcification of the mouse VSMC cell line, MOVAS-1.

Since the establishment of the MOVAS-1 cell line (28), these cells have been utilised in vascular studies that have investigated the cell cycle (28), neuroendocrine peptide expression profiles (29), circadian rhythms (30) and signal transduction responses (31). However, to date, no published studies have employed the MOVAS-1 cell line to investigate vascular calcification.

In the present study, we showed that the calcification of MOVAS-1 cells can be induced in the presence of calcifying medium containing βGP and ascorbic acid. This was demonstrated through standard staining and enzyme activity assays frequently employed to assess vascular calcification *in vitro* (7,18,32).

We also showed that the time course of MOVAS-1 calcification is comparable to that of primary murine aortic VSMCs, establishing that MOVAS-1 cells are a feasible and relevant model. Furthermore, calcified MOVAS-1 cells showed increased expression of a number of recognized markers of vascular calcification including PiT-1, ALP and Ocn. The phosphate transporter PiT-1 is the predominant sodium-dependent phosphate co-transporter expressed in VSMCs. Increased PiT-1 expression leads to elevated intracellular phosphate and induces the osteogenic conversion of VSMCs (17). Conversely, down-regulation of *PiT-1* gene expression by RNA knockdown has been shown to reduce phosphate uptake by VSMCs and inhibit phosphate-induced VSMC phenotypic transition and calcification (17). ALP, a key regulator of bone calcification, also plays an important role in vascular calcification through the generation of phosphate, and by decreasing levels of PPi in blood vessels (18,19). *Ocn* is a vitamin K-dependent matrix protein proposed to be involved in the regulation of calcium crystal development (43). Our data therefore suggest that the culture of MOVAS-1 cells in a calcifying medium is an appropriate model with which to study vascular calcification *in vitro*.

Using known molecular inhibitors, we also demonstrated that functional studies can be undertaken in MOVAS-1 cells. Levamisole reversibly but non-competitively inhibits ALP (39), and is a potent inhibitor of the calcification of osteoblasts (bone forming cells) (44,45) and VSMCs (19,46). The bisphosphonate etidronate, an inhibitor of hydroxyapatite formation, has also been reported to inhibit calcification of VSMCs and osteoblasts. The reduction in calcification observed in MOVAS-1 cells following treatment with levamisole and etidronate confirms that this cell line is highly applicable to vascular calcification research.

The severe clinical implications of vascular calcification are widely recognized. However, the underlying mechanisms have yet to be fully elucidated and effective therapeutic strategies that may prevent and potentially reverse vascular calcification are not currently available. The MOVAS-1 cell line is a reliable, convenient and economical system in which to investigate vascular calcification *in vitro*, and will make a useful contribution to increasing our understanding of this pathological process.

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