

Mechanical stimulation and mitogen-activated protein kinase signaling independently regulate osteogenic differentiation and mineralization by calcifying vascular cells

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Accepted 9 January 2004

Abstract

Ectopic calcification of vascular tissue is associated with several cardiovascular pathologies and likely involves active regulation by vascular smooth muscle cells and osteoblast-like vascular cells. This process often occurs in sites with altered mechanical environments, suggesting a role for mechanical stimuli in calcification. In this study, we investigated the effect of mechanical stimulation on the proliferation, osteogenic differentiation, calcification, and mitogen-activated protein kinase (MAPK) signaling in calcifying vascular cells (CVCs), a subpopulation of aortic smooth muscle cells putatively involved in vascular calcification. Application of equibiaxial cyclic strain (7%, 0.25 Hz) to CVCs had no effect on cell proliferation, but accelerated alkaline phosphatase expression and significantly increased mineralization by 3.1-fold over unstrained cells. Fluid motion in the absence of strain also enhanced mineralization, but to a lesser degree. Because MAPK pathways mediate mechanically regulated osteoblast differentiation, we tested whether similar signaling was involved in mineralization by CVCs. In static cultures, pharmacological inhibition of the extracellular signal-regulated kinase (ERK1/2), p38 MAPK, and c-Jun N-terminal kinase pathways significantly attenuated mineral production by as much as –94%, compared with uninhibited CVCs. Strikingly, although mechanical stimulation activated each of the MAPK pathways, inhibition of these pathways had no effect on the mechanically induced enhancement of alkaline phosphatase activity or mineralization. These novel data indicate that mechanical signals regulate calcification by CVCs, and although MAPK signaling is critical to CVC osteogenic differentiation and mineralization, it is not involved directly in transduction of mechanical signals to regulate these processes under the conditions utilized in this study.

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Keywords: Vascular calcification; Vascular smooth muscle cells; Mitogen-activated protein kinase; Osteogenesis; Biomechanics

1. Introduction

Ectopic calcification in the cardiovascular system (e.g., in arteries, cardiac tissue, and heart valves) contributes to atherosclerotic lesions (Rumberger et al., 1995), increased risk of cardiovascular (Iribarren et al., 2000; Wong et al., 2000) and cerebrovascular (Vliedgenhart et al., 2002) events, increased risk of

dissection after angioplasty (Fitzgerald et al., 1992), and failure of native (Braunwald, 1997) and bioprosthetic (Vyavahare et al., 1997) heart valves. Vascular and valvular calcification, previously believed to be non-specific responses to tissue injury, are now recognized as highly regulated processes (Giachelli, 2001; Mohler et al., 2001; Wallin et al., 2001), similar in many ways to bone formation (Bostrom et al., 1993; Mohler et al., 2001). Smooth muscle cells (SMCs) contribute significantly to the active regulation of vascular calcification (Campbell and Campbell, 2000; Giachelli, 2001; Shanahan et al., 2000; Shioi et al., 2000). In healthy tissue, normal SMCs express proteins (e.g., matrix gla protein) that actively

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inhibit calcification (Luo et al., 1997; Shanahan et al., 2000). In response to pro-calcific regulatory factors, however, SMCs can transform to an osteoblast-like phenotype (Jono et al., 2000; Shioi et al., 1995; Steitz et al., 2001). In vitro, these transformed SMCs express bone-associated genes and proteins, and are able to produce mineralized nodules. These findings from cell culture studies are supported by observations of active osteoblast-like cells in calcific lesions in heart valves (Mohler et al., 2001) and bone-associated proteins in atherosclerotic plaques (Shanahan et al., 1994). Additionally, subpopulations of calcifying vascular cells (CVCs) that exhibit osteoblast characteristics have been isolated from the aortic media (Watson et al., 1994) and the aortic valve interstitium (Mohler et al., 1999). These cells respond to factors associated with vascular and valvular calcification, resulting in increased mineralization in vitro (Mohler et al., 1999; Parhami et al., 2001; Tintut et al., 2000; Watson et al., 1994). Thus, regulation of calcification is dependent on the activities of both normal SMCs and, when present, osteoblast-like CVCs. How these specific cell types respond to signals from a milieu of factors that induce or inhibit calcification likely dictates whether calcification is initiated, and if so, how it progresses.

Notably, vascular and valvular calcification are often prevalent in regions with significant or altered mechanical environments, such as in native heart valves (Thubrikar et al., 1986), bioprosthetic valves (Vyavahare et al., 1997), and vascular lesions (Caro et al., 1969), suggesting a role for mechanical stimuli in calcification. In bone cells and their mesenchymal precursors, mechanical strain increases matrix mineralization via activation of the extracellular-regulated protein kinase (ERK1/2) signaling pathway (Simmons et al., 2003; Wang et al., 2002; Ziros et al., 2002), a sub-family of the mitogen-activated protein kinases (MAPKs). The effect of physical stimuli on vascular calcification is less clear. We recently showed that cyclic mechanical strain inhibits the phenotypic conversion of vascular smooth muscle cells to a pattern of osteoblast-like gene expression (Nikolovski et al., 2003). However, the effect of physical stimulation on the osteoblast-like vascular cells that are present in lesions and likely involved in advancing calcification is not known. Determining the effect of physical stimuli on the function of this subpopulation in particular is important to understanding the role of mechanical signals in the initiation and progression of vascular calcification.

Thus, our objective in this study was to determine the effect of mechanical stimulation on calcifying vascular cells, a subpopulation of smooth muscle cells putatively involved in vascular calcification. We hypothesized that mechanical signals regulate osteogenic differentiation and calcification by CVCs. We also tested the secondary hypothesis that, similar to bone cells and their

mesenchymal progenitors, mineralization by CVCs in response to mechanical signals is regulated by activation of MAPK pathways.

2. Methods

2.1. Cell culture

Bovine calcifying vascular cells (generously provided by Linda L. Demer, University of California, Los Angeles) were used as a model of osteoblast-like vascular cells putatively involved in calcification. These cells are isolated and cloned by limiting dilution and single cell harvesting from cultures of aortic SMCs in which multicellular nodules appear spontaneously (Watson et al., 1994). The resulting clonal lines were previously identified as CVCs by positive staining with monoclonal antibody 3G5 and by their ability to aggregate into mesenchymal condensations and form calcified nodules in vitro (Watson et al., 1994). The cells were expanded in high glucose Dulbecco's Modified Eagle Medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen), 140 mM sodium bicarbonate (Sigma-Aldrich, St. Louis, MO), 25 mM HEPES (Sigma), 100 U/ml penicillin, and 0.1 mg/ml streptomycin (both from Invitrogen). Serum from the same lot was used within a single experiment for both control and experimental cell populations. Cells from passages 14–17 were used for experiments. For all experiments, the CVCs were cultured in their standard medium supplemented with 10 mM β -glycerophosphate (Sigma) to provide a pro-calcific environment.

2.2. Mechanical stimulation of cultured cells

Cells were plated on six-well plates with flexible silicone rubber bases (Flexcell, Hillsborough, NC). The flexible bases were pre-coated with $1 \mu\text{g}/\text{cm}^2$ type I collagen by adsorption of the collagen in a carbonate/bicarbonate buffer (15 mM Na_2CO_3 and 35 mM NaHCO_3 in PBS, pH 9.4) for 24 h at 4°C . Cells were seeded at a density of 3×10^4 cells/ cm^2 with seeding confined to a 5cm^2 area in the center of the wells using Teflon o-rings as physical barriers. After 24 h, the o-rings were removed, new medium was added, and the cells were immediately subjected to mechanical stimulation. In most experiments, a modified Flexercell system (FX-2000, Flexcell) was used to apply a 7% equibiaxial cyclic strain to the cells continuously at 0.25 Hz (square-wave; 2 sec strain, 2 sec relaxation) for the duration of the experiment, with media changes every other day. With the Flexercell system, deformation of the cell substrate not only mechanically strains the cells, but also results in motion of the cell culture medium, which itself can be a potent physical stimulus. To test whether motion of the

culture media alone in the absence of substrate deformation had an effect on cell function, a set of experiments were performed in which Flexcell plates were prepared as described, placed on an orbital shaker in an incubator, and rotated at 0.25 Hz for the duration of the experiment, with media changes every other day. In all cases, static control plates were maintained identically except for the application of strain or fluid motion.

2.3. DNA content assay

The total amount of DNA in each well was determined as a measure of cell proliferation. DNA content was also used to normalize alkaline phosphatase activity to account for differences due simply to cell number. Cell nuclei were disrupted by addition of a lysis buffer (0.025 M trisHCl, 0.4 M NaCl, 0.5% SDS, pH = 7.4), followed by sonication and centrifugation. Samples of the supernatant and standard solutions of calf thymus DNA were measured in parallel using the Hoescht 33258 assay (Cesarone et al., 1979) to determine DNA content.

2.4. Alkaline phosphatase assay

Alkaline phosphatase (ALP) activity was measured as a marker of early osteogenic differentiation. ALP activity was determined quantitatively from cell lysates obtained by washing the cell layers twice with PBS and then harvesting using a passive lysis buffer (Promega, Madison, WI). The lysate was sonicated and centrifuged at 4°C and the supernatant was assayed for ALP activity by incubating with 50 mM *p*-nitrophenyl phosphate in an assay buffer (100 mM glycine, 1 mM MgCl₂, pH 10.5) at 37°C for 15 min. The absorbance was measured at 405 nm and converted to *p*-nitrophenol (*p*NP) concentration based on standard solutions prepared in parallel. Alkaline phosphatase activity was normalized to DNA content determined from the same lysates, as described previously.

2.5. Matrix mineralization assays

Mineral deposition was assessed qualitatively by the von Kossa method after 10 days of straining. Prior to staining, cell layers were washed twice in PBS and then fixed with 70% ethanol for 30 min at 4°C. Fixed cell layers were incubated with 5% (w/v) silver nitrate solution for 30 min under bright light. After incubation, the cell layers were washed in dH₂O and rinsed twice with 5% (w/v) thiosulphate to remove unreacted silver. Samples for quantitative assessment of matrix mineralization were obtained by rinsing the cell layers twice with calcium-free PBS and solubilizing the accumulated matrix-deposited calcium in 0.6 N HCl at 4°C for ~18 h (Wada et al., 1999). After centrifugation, the supernatant

was assayed for calcium content using the *o*-cresolphthalein reaction (Sigma Kit #587), with the absorbance read at 575 nm. Calcium concentration was determined based on standard solutions prepared in parallel.

2.6. Immunoblotting analysis

Using immunoblotting analysis, we first determined whether physical stimulation activated MAPK pathways in CVCs. Cells were seeded as described previously at a density of 3×10^4 cells/cm² and allowed to adhere for 24 h in serum-containing media. To synchronize the cells and reduce basal MAPK activity, the cells were serum-starved for 4 h prior to and during the application of strain, which lasted up to 60 min. Immediately after straining with the Flexercell, cell layers were washed once with ice cold PBS and incubated on ice in cell lysis buffer (Cell Signaling Technology, Beverly, MA). The DNA content in sonicated lysates was measured using the Hoescht 33258 assay, and the results confirmed that there were equal numbers of cells in each sample. Lysate supernatants were retained for selective immunoprecipitation and chemiluminescent detection of ERK1/2, p38 MAPK (p38), or c-Jun N-terminal kinase (JNK) activity using MAPK Activity Kits (Cell Signaling Technology) according to the manufacturer's protocols.

2.7. MAPK inhibition studies

The role of individual MAPK pathways in alkaline phosphatase expression and matrix mineralization by CVCs was assessed by blocking the pathways using specific inhibitors. The ERK1/2 pathway was blocked with U0126 (Cell Signaling), which inhibits MEK1/2, an upstream molecule of the ERK phosphorylation cascade (Favata et al., 1998). The p38 MAPK was specifically inhibited with SB203580 (Calbiochem, San Diego, CA) (Cuenda et al., 1995) and JNK was inhibited with SP600125 (Calbiochem) (Bennett et al., 2001). Preliminary experiments showed that the optimal concentrations for inhibition of MEK1/2, p38, and JNK without cytotoxicity were 10 μM U0126, 15 μM SB203580, and 20 μM SP600125, respectively. To assess the effects of the inhibitors on MAPK activity, inhibitors were added to the serum-free cell culture media one hour prior to application of the mechanical stimulus, the CVCs were then strained for 15 min, and the lysates were collected for immunoblot analysis, as described above. To determine the effects of the inhibitors on alkaline phosphatase expression and mineralization by CVCs, experiments were performed for 5 or 15 days as described previously, with the MAPK inhibitors added with each replacement of media. In contrast to the immunoblotting experiments in which serum-free media were used to reduce basal MAPK activity, fully supplemented media were used in these experiments.

3. Results

3.1. Effect of mechanical stimulation on proliferation, osteogenic expression and mineralization by CVCs

The DNA content was measured in static cultures and in cultures strained with the Flexercell as an indicator of cell proliferation. Both static (control) and strained CVCs proliferated with time in culture, but there was no difference in DNA content between the two conditions (Fig. 1), indicating this mechanical stimulus did not affect the net proliferation of CVCs.

To determine the effect of mechanical stimulation on CVC osteogenic differentiation, alkaline phosphatase activity, an early marker of osteogenic expression, and matrix mineralization were measured in static and strained cultures. Both control and strained CVCs expressed increasing levels of alkaline phosphatase with time in culture, consistent with their osteoblast-like differentiation behavior (Fig. 2). Mechanical stimulation resulted in increased levels of alkaline phosphatase activity at earlier time points ($P < 0.05$; Fig. 2). As is typical of CVCs, the cells aggregated into mesenchymal condensations (Fig. 3A and B). Mechanical stimulation also resulted in more mineralized nodules being formed (Fig. 3C and D), with a 3.1 fold increase in matrix-deposited calcium after 15 days in culture ($P < 0.001$; Fig. 3E). Together these data indicate that mechanical signals stimulate osteogenic differentiation of CVCs.

3.2. Effect of mechanical stimulation on MAPK activation in CVCs

Since we observed that CVC osteogenesis was mechanoresponsive and mechanical stimuli are known

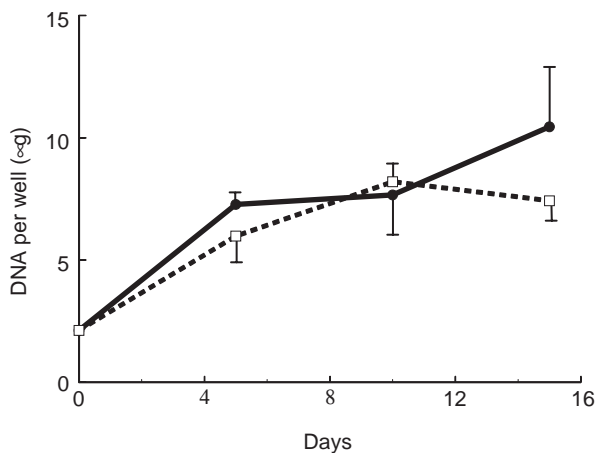


Fig. 1. Mechanical stimulation does not affect proliferation of CVCs. The total amount of DNA increased with time in culture, but there was no difference in amount of DNA between the unstrained cells (•, solid line) and strained cells (□, dashed line). Data represent means \pm SD of pooled data from two experiments in which there were three samples for each condition.

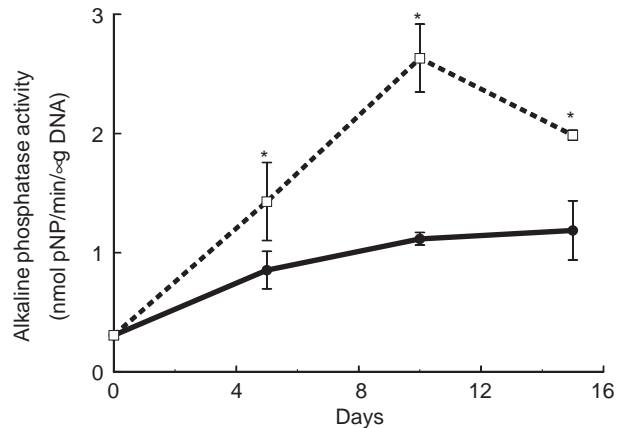


Fig. 2. Mechanical stimulation accelerates alkaline phosphatase expression by CVCs. Alkaline phosphatase expression by unstrained cells (•, solid line) increased with time in culture, but mechanical stimulation accelerated this process (□, dashed line). Significantly higher levels of alkaline phosphatase activity were noted throughout the 15 day experiment in mechanically simulated versus static control cells. Data represent means \pm SD of a typical experiment with $n = 3$ samples for each condition. Two additional independent experiments showed similar results. * $P < 0.05$ vs. unstrained control by ANOVA and post-hoc Tukey pairwise comparisons.

to enhance osteogenic differentiation of bone cells through activation of MAPK pathways, we tested whether the mechanical stimulus applied with the Flexercell similarly activated the ERK1/2, p38, and JNK pathways in CVCs. All three pathways were activated by mechanical stimulation, with prolonged activation for up to 30 min in the case of ERK1/2 (Fig. 4A) and p38 (Fig. 4B), and for at least 60 min in the case of JNK (Fig. 4C).

3.3. Effect of MAPK inhibition on osteogenic differentiation of unstrained CVCs

We next determined the role of MAPK signaling in the osteogenic differentiation of CVCs in the absence of mechanical stimuli by blocking individual MAPK pathways pharmacologically and measuring the effect on alkaline phosphatase activity after 5 days and on mineralization after 15 days in static culture. Inhibition of ERK1/2, p38, and JNK activation by 10 μ M U0126, 15 μ M SB203580, and 20 μ M SP600125, respectively, was confirmed with immunoblotting (data not shown).

When ERK1/2 activity was blocked by U0126 in unstrained cells, we observed an increase in alkaline phosphatase activity at day 5 relative to uninhibited cells (2.3-fold; not statistically significant (NS)) (Fig. 5A), but a significant decrease in mineral production at day 15 (-89% ; $P < 0.05$) (Fig. 5B). The pattern of mineralization with ERK1/2 inhibited was also altered, with more diffuse matrix mineralization, and fewer and smaller mineralized condensations (Fig. 5C). These data suggest critical but distinct roles for ERK1/2 signaling in early

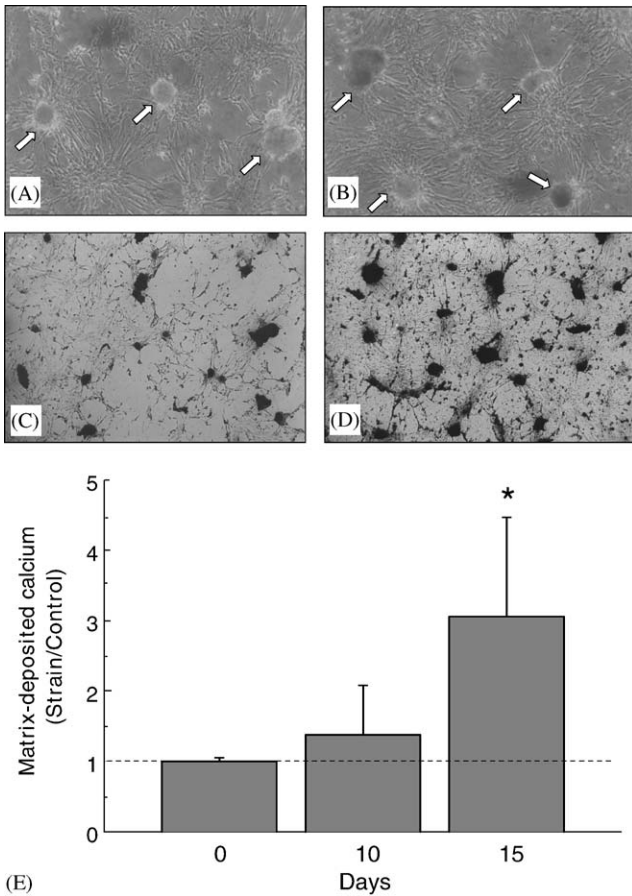


Fig. 3. Mechanical stimulation enhances matrix mineralization by CVCs. Cellular condensations (arrows) typical of CVCs were evident in both the (A) unstrained and (B) strained cultures after 15 days (phase contrast images, original magnification $100\times$). However, von Kossa staining demonstrated fewer mineralized nodules in the (C) unstrained condition than in the (D) strained condition (brightfield images, original magnification $40\times$). (E) Measurement of matrix-deposited calcium by mechanically stimulated cells (normalized to that by static control cells) confirmed that there was significantly more mineral formed by the strained cells after 15 days. Data represent means \pm SD of pooled data from two experiments in which there were three samples for each condition. Two additional independent experiments showed similar results after 15 days of straining. * $P < 0.001$ by t-test.

osteogenic differentiation and later processes leading to mineralization. Inhibition of p38 by SB203580 and of JNK by SP600125 had little effect on alkaline phosphatase activity at day 5 in unstrained cells (Fig. 5A). However, both p38 and JNK pathways were necessary for normal mineralization, as inhibition of these pathways resulted in significantly decreased matrix mineralization, compared with uninhibited cells (-94% for p38; -88% for JNK; both $P < 0.05$) (Fig. 5B). Histochemical staining showed no evidence that blocking these pathways altered the pattern of condensation or mineralization; instead, there were simply fewer nodules with the p38 and JNK pathways inhibited (data not shown). Although the p38-inhibited cells were able to

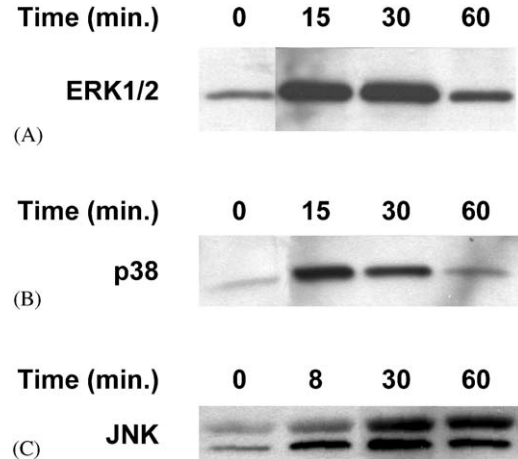


Fig. 4. ERK1/2, p38, and JNK are activated in CVCs by mechanical stimulation. CVCs were strained and, at the times indicated, cells lysates were collected. The activity of (A) ERK1/2 (phospho-Elk-1), (B) p38 (phospho-ATF-2), and (C) JNK (phospho-c-Jun) were analyzed by immunoblotting with quantification by densitometry. Strain activated the pathways in a time-dependent manner, with maximum activity of ERK1/2 (2.7-fold over unstrained cells) and p38 (6.0-fold) after 15 min of straining and of JNK (2.7-fold) after 30 min of straining.

produce mineralized nodules, the morphology of the cells surrounding the nodules was distinct from that of normal differentiating CVCs, with abundant intracellular vacuoles (Fig. 5D). The JNK-inhibited cells had normal morphology (data not shown).

3.4. Effect of MAPK inhibition on osteogenic differentiation of mechanically stimulated CVCs

We next examined whether the enhancement of osteogenesis by mechanical stimulation was mediated by MAPK pathways in CVCs. Again these pathways were inhibited pharmacologically (confirmed by immunoblotting) and alkaline phosphatase activity at day 5 and mineralization at day 15 were measured as indicators of osteoblast-like differentiation.

Interestingly, although mechanical stimuli activated MAPK pathways (Fig. 4), inhibition of these pathways had little effect on the mechanically induced enhancement of alkaline phosphatase activity observed in uninhibited cells (Fig. 6A). Without inhibition, we observed a significant increase in alkaline phosphatase activity at day 5 in the stimulated cells, relative to the static control cells (3.6-fold; $P < 0.05$) (Fig. 6A). Similarly, with p38 or JNK inhibited, the mechanical stimulus enhanced alkaline phosphatase activity significantly over the inhibited, static counterpart by 3.7-fold and 3.5-fold for p38 and JNK, respectively ($P < 0.05$) (Fig. 6A). With the ERK1/2 pathway inhibited, the stimulated cells expressed higher alkaline phosphatase levels (Fig. 6A), but the degree of enhancement by the mechanical stimulus was comparable to the other

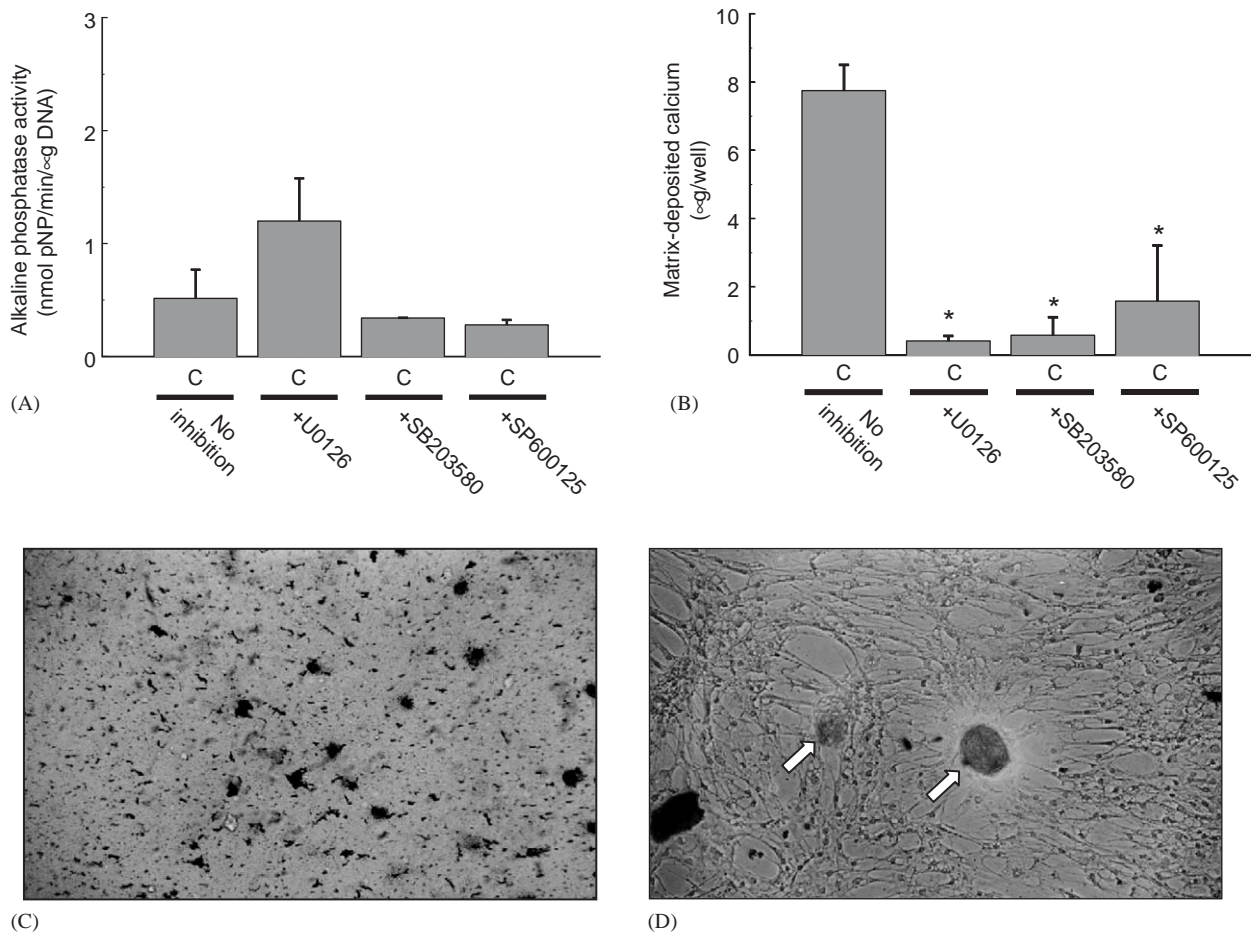


Fig. 5. Alkaline phosphatase and mineralization are differentially regulated by MAPK pathways in unstrained CVCs. CVCs were cultured statically in media with or without MAPK inhibitors for 5 or 15 days. (A) After 5 days, alkaline phosphatase activity by unstrained cells was increased when the ERK1/2 pathway was blocked with 10 μ M U0126. Inhibiting the p38 pathway with 15 μ M SB203580 or the JNK pathway with 20 μ M SP600125 had no effect on alkaline phosphatase expression by unstrained cells. (B) Matrix mineralization by unstrained cells after 15 days was significantly attenuated with inhibition of each MAPK pathway. (C) With the ERK1/2 pathway inhibited, mineralization by unstrained cells (von Kossa stain, brightfield image, original magnification 40 \times) was diffuse rather than confined primarily to nodules (compare with Fig. 3C). (D) With p38 inhibited, the unstrained cells formed mineralized nodules (arrows) (phase contrast image, original magnification 100 \times), but the cellular morphology was abnormal (compare with Fig. 3A). Note the photomicrographs in (C) and (D) were taken with different magnifications and contrast methods. Data in (A) and (B) represent means \pm SD of individual experiments with $n = 3$ samples for each condition. An additional experiment showed similar results. * $P < 0.05$ vs. uninhibited sample by Student t-test.

cases (4.7-fold over unstrained, ERK-inhibited cells; $P < 0.05$).

Similar to mechanically induced alkaline phosphatase expression, mechanically induced mineralization was unaffected by inhibition of each of the ERK1/2, p38, and JNK pathways. As with the static control cells, inhibition of the MAPK pathways attenuated the degree of mineralization by the mechanically stimulated cells relative to stimulated, uninhibited cells (Fig. 6B). However, relative to the inhibited, static counterpart, mechanical stimulation resulted in a significant increase in the amount of matrix-deposited calcium regardless of the pathway inhibited ($P < 0.05$) (Fig. 6B). These data indicate that mechanical stimulation enhanced mineralization independent of MAPK signaling. Interestingly, whereas ERK1/2 inhibition resulted in diffuse

mineralization by static control cells (Fig. 5C), mechanical stimulation seemed to recover some of the normal pattern of mineralization (Fig. 6C).

3.5. Effect of fluid motion on osteogenic expression and mineralization by CVCs

To distinguish between the effects of fluid motion and strain, CVCs were subjected to fluid agitation alone on an orbital shaker, and alkaline phosphatase activity at day 5 and mineralization at day 15 were measured as indicators of osteogenesis. Fluid agitation alone resulted in a significant increase in alkaline phosphatase activity at day 5 compared with static control cells ($P < 0.01$) (Fig. 7A). Mineralization also increased 1.7-fold with application of fluid motion alone relative to static

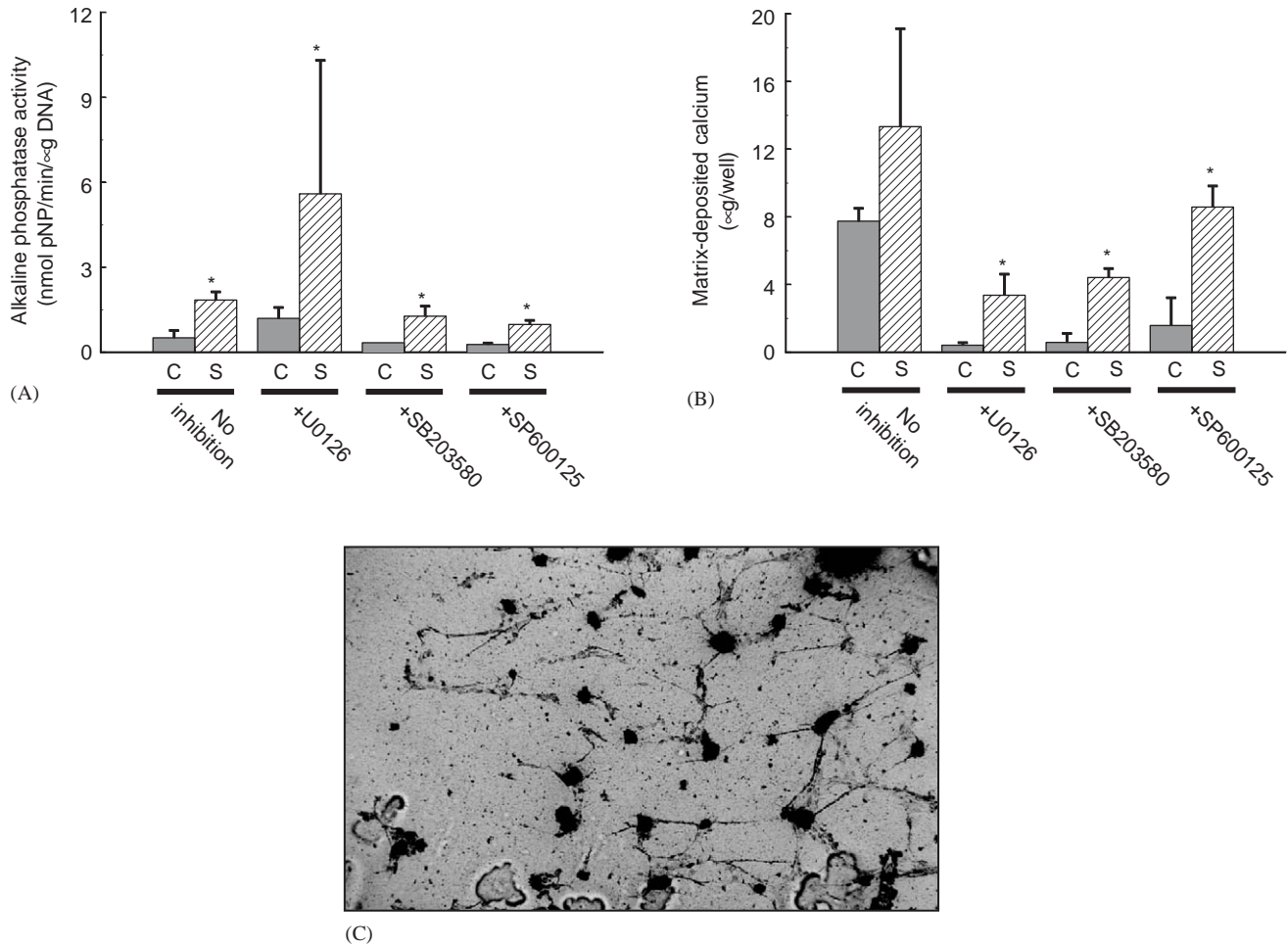


Fig. 6. Enhancement of osteogenic differentiation by mechanical stimulation is not mediated by MAPK pathways. CVCs were cultured in media with or without MAPK inhibitors and with mechanical stimulation (S; hatched bars) or statically (C; shaded bars) for 5 or 15 days. (A) Application of strain increased alkaline phosphatase activity in uninhibited cells after 5 days, relative to static control cells. The enhancement of alkaline phosphatase activity by mechanical stimulation was not affected by inhibition of the ERK1/2 (by U0126), p38 (by SB203580), or JNK (by SP600125) pathways. (B) Similarly, mechanical stimulation increased mineralization relative to static control cells after 15 days, even when the MAPK pathways were inhibited. Notably, the pattern of mineralization by stimulated, ERK1/2-inhibited cells (C) was more nodular (von Kossa stain, brightfield image, original magnification $40\times$) than the diffuse mineralization seen with static, ERK1/2-inhibited cells (compare with Fig. 5C). Data in (A) and (B) represent means \pm SD of individual experiments with $n = 3$ samples for each condition. An additional experiment showed similar results. * $P < 0.05$ vs. unstrained counterpart by t-test.

controls (Fig. 7B), but the difference was not significant and on average less than that observed when the CVCs were subjected to strain (Figs. 3E and 6B).

4. Discussion

The association between vascular calcification and local mechanical stimuli suggests mechanical signals modulate the calcification process. However, the effect of mechanical signals on the specific cell populations putatively involved in the active regulation of vascular calcification is not known. The results of this study demonstrate that mechanical stimulation of calcifying vascular cells accelerated osteogenic differentiation,

resulting in increased mineral production. Although activation of ERK1/2, p38, and JNK signaling pathways was critical to aspects of CVC differentiation and each pathway was activated by mechanical stimulation, the regulation of mineralization by the mechanical stimuli applied here was not mediated by MAPK signaling. These novel results identify mechanical signals and MAPK signaling as independent regulators of mineralization by vascular cells, suggesting roles for each in the etiology of vascular calcification.

Although mechanical factors and vascular calcification are associated, there was little previous evidence supporting a direct effect of mechanical signals on calcification by vascular cells. The results presented here are the first to demonstrate that mechanical stimuli

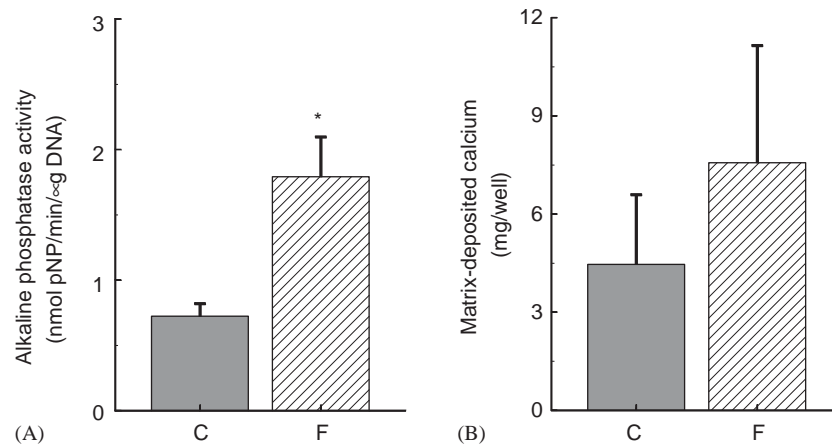


Fig. 7. Fluid motion in the absence of substrate deformation enhances osteogenic differentiation. CVCs were agitated on an orbital shaker to generate fluid motion (F; hatched bars) or maintained as static cultures (C; shaded bars) for 5 or 15 days. (A) Fluid motion increased alkaline phosphatase activity after 5 days, relative to static control cells. (B) Fluid motion also increased mineralization relative to static control cells after 15 days, although not significantly. Data represent means \pm SD with $n = 3$ samples for each condition. * $P < 0.01$ vs. static control by t-test.

regulate osteogenic differentiation, condensation, and mineralization by a calcifying subpopulation of vascular cells. Notably, this response differs from that seen with vascular SMCs. Rat aortic SMCs cultured in collagen sponges and subjected to chronic cyclic strain have lower expression of bone-associated genes and mineralize less than SMCs cultured under static conditions (Nikolovski et al., 2003). While differences in the mechanical stimulus, culture system, and cell species limit the implications of these observations, the data suggest that mechanical stimuli might have distinct effects on specific subpopulations of cells within the vascular wall. Accordingly, there might be distinct roles for each cell type in the etiology of vascular calcification. For instance, in normal vascular smooth muscle tissue, mechanical signals might protect against mineralization, acting to maintain the normal SMC phenotype. However, in circumstances in which pro-calcific regulators initiate transformation from a SMC phenotype to an osteoblast-like phenotype, appropriate mechanical signals may act in concert with other regulators to stimulate osteogenic differentiation and mineralization by the osteoblast-like vascular cells specifically, thereby contributing to the progression of calcified lesion formation. Therefore, similar to atherosclerosis, vascular calcification may be a multi-factorial disease in which mechanical signals act on specific vascular cell populations in concert with other stimuli to prevent or promote disease initiation and progression. Clearly further studies, including in vivo experiments, are necessary to test these hypotheses.

In the absence of mechanical stimulation, inhibition of each of the MAPK signaling pathways affected expression of the osteogenic markers. A possible mechanism by which inhibited MAPK signaling might affect mineralization is to increase apoptosis, which is an initiating factor for mineralization of CVC cultures

(Proudfoot et al., 2000). Our data do not support this mechanism, however. For instance, the ERK1/2 pathway typically promotes cell survival (Howe et al., 2002) and its inhibition would therefore be expected to increase apoptosis and mineralization. While we did observe a small decrease in net proliferation with this pathway inhibited (data not shown), this was accompanied by a decrease in mineralization and an increase in alkaline phosphatase expression, suggesting delayed osteogenic differentiation of the cells. Inhibition of the p38 and JNK pathways had no effect on cell proliferation, again supporting a direct effect on osteogenic conversion of the CVCs rather than an apoptosis-mediated calcification mechanism.

Similar to bone cells (Gebken et al., 1999; Granet et al., 2001, 2002; Kletsas et al., 2002; Pommerenke et al., 2002; Schmidt et al., 1998; You et al., 2001; Ziros et al., 2002) and SMCs (Li et al., 1999; Li and Xu, 2000), the strain stimulus activated MAPK pathways in CVCs. In contrast to bone cells, however, the regulation of osteogenic differentiation and mineralization by strain was not mediated by MAPK signaling in CVCs, as pharmacological inhibition of MAPK pathways did not abolish mechanically enhanced osteogenesis. While MAPK signaling is one pathway for transduction of mechanical signals, mechanoreceptors regulate cell function through several mechanisms that are independent of MAPKs (Davies, 1995; Li and Xu, 2000; Ruwhof and van der Laarse, 2000). For example, the cAMP signaling pathway, which mediates osteogenic differentiation of CVCs by tumor necrosis factor- α (Tintut et al., 2000) and other activating factors (Tintut et al., 1998), is known to be mechanoresponsive in vascular SMCs (Mills et al., 1997). Furthermore, the responses of SMCs and CVCs to mechanical stimulation are likely dependent on the specific nature of the mechanical stimulus, as in bone

cells (You et al., 2000). In this study, we used the Flexercell system to apply a mechanical stimulus via cyclic deformation of the cell substrate at a magnitude comparable to that in the medial layer of blood vessels (Bella et al., 1999). A secondary stimulus generated by this level of substrate deformation is reactive motion of the cell culture medium (Brown et al., 1998), making it difficult to distinguish the effects of strain and fluid motion on cell function with the Flexercell system. Differentiating these effects is important because fluid flow has the potential to regulate cell function by generating membrane shear stresses and by enhancing local mixing and chemotransport (Donahue et al., 2003). In bone cells, fluid flow generated by mechanical loading appears to be a more potent regulator of metabolism than mechanical strain at physiological levels (Smalt et al., 1997; You et al., 2000). We addressed this issue by subjecting CVCs to fluid agitation in the absence of strain. Our data indicate that CVC osteogenesis is responsive to fluid motion alone. While we cannot determine definitively whether these effects are due to shear stresses or enhanced chemotransport, the differential effects of MAPK inhibition with and without mechanical stimulation suggest that enhanced chemotransport is not solely responsible for the osteogenic response. Furthermore, our data suggest that both strain and fluid flow regulate CVC osteogenesis, as the effects on mineralization due to fluid motion alone were attenuated from those observed when the CVCs were stimulated with the Flexercell system. Critical to a better understanding of the (patho)physiological relevance of these mechanical signals is more thorough characterization of the cellular-level mechanical stimuli experienced in situ by SMCs and CVCs in normal and diseased vascular tissue.

In conclusion, we have shown that mechanical stimulation regulates calcification by CVCs. Although not involved in transduction of the mechanical stimulus, signaling via the ERK1/2, p38, and JNK pathways regulates CVC osteogenesis and mineralization, suggesting mechanisms by which pro- and anti-calcific regulators might act. Together these data support the premise that vascular calcification is a cell-regulated process, involving a multitude of regulatory pathways and factors, including mechanical stimuli. Further investigation into the mechanisms by which specific mechanical signals regulate vascular calcification may reveal novel regulatory pathways and potential targets for therapeutic intervention.

Acknowledgements

We gratefully acknowledge the assistance of Dr. Rajaram Gopalakrishnan (University of Minnesota) and the support of the NIH (R01 DE13033), a Natural

Sciences and Engineering Research Council of Canada Postdoctoral Fellowship to C. Simmons, and an American Heart Association Fellowship to J. Nikolovski.

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