cAMP Stimulates Osteoblast-like Differentiation of Calcifying Vascular Cells

POTENTIAL SIGNALING PATHWAY FOR VASCULAR CALCIFICATION*

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The role of the cAMP signaling pathway in vascular calcification was investigated using calcifying vascular cells (CVC) derived from primary aortic medial cell cultures. We previously showed that CVC have fibroblastic morphology and express several osteoblastic differentiation markers. After confluence, they aggregate into cellular condensations, which later mature into nodules where mineralization is localized. Here, we investigated the effects of cAMP on CVC differentiation because it plays a role in both osteoblastic differentiation and vascular disease. Dibutyryl-cAMP or forskolin treatment of CVC for 3 days induced osteoblast-like "cuboidal" morphology, inhibited proliferation, and enhanced alkaline phosphatase activity, all early markers of osteoblastic differentiation. Isobutylmethylxanthine and cholera toxin had the same effects. Treatment of CVC with pertussis toxin, however, did not induce the morphological change or increase alkaline phosphatase activity, although it inhibited CVC proliferation to a similar extent. CAMP also increased type I procollagen production and gene expression of matrix γ-carboxyglutamic acid protein, recently shown to play a role in in vivo vascular calcification. CAMP inhibited the expression of osteopontin but did not affect the expression of osteocalcin and core binding factor. Prolonged CAMP treatment enhanced matrix calcium-mineral incorporation but inhibited the condensations resulting in diffuse mineralization throughout the monolayer of cells. Treatment of CVC with a protein kinase A-specific inhibitor, KT5720, inhibited alkaline phosphatase activity and mineralization during spontaneous CVC differentiation. These results suggest that the cAMP pathway promotes in vitro vascular calcification by enhancing osteoblast-like differentiation of CVC.

Arterial calcification is a common and clinically significant complication associated with atherosclerosis (1, 2). Hoeg and colleagues showed that calcific atherosclerosis is significant in patients with homozygous familial hypercholesterolemia (3). Previously, we found expression of bone morphogenetic protein (BMP-2), a potent bone differentiation factor that drives endochondral bone formation (4) in human calcified plaque (5).

Previously we isolated subpopulations of cells from the bovine artery wall that aggregate into mesenchymal condensations that later mature into mineralized multicellular nodules (6). Although nodules occasionally form in primary smooth muscle cells culture, these calcifying vascular cell (CVC) cultures differ from primary smooth muscle cell cultures in an approximately 10-fold enrichment for nodule formation as well as the expression of molecular markers such as osteopontin, type I collagen, and the epitope for monoclonal antibody 3G5 (6). CVC retain their phenotype through multiple passages, and they exhibit several osteoblastic markers including type I collagen (Coll I), alkaline phosphatase, osteopontin, and osteocalcin (6). Certain agents present in atherosclerotic arteries, such as 25-hydroxycholesterol, transforming growth factor β-1, and lipid oxidation products, such as minimally oxidized low density lipoprotein and 8-isoprostaglandin E2, promote CVC differentiation (6, 7).

Other cloned subpopulations of artery wall cells do not form nodules even in prolonged culture conditions, suggesting that CVC represent a specific subpopulation (6). There are intriguing similarities between CVC and the mesenchymal stem cells present in adult nonhematopoietic tissue (8–10) that are capable of differentiating into osteoblasts, chondroblasts, adipocytes, and myoblasts. Such cells may account for pathologic calcification in other mesenchymal tissues.

The cAMP signaling pathway plays a role in both osteoblast differentiation and vascular disease. In osteoblasts, parathyroid hormone modulates differentiation via the cAMP-mediated pathway (11, 12). CAMP functional response elements have been reported in promoters of osteoblast-associated genes (13–15). In vascular smooth muscle cells, stimulation of CAMP inhibits proliferation, relaxation, and migration (16–18). In addition, the cAMP pathway is involved in activation of endothelial cells by oxidized lipoproteins (19). Levels of cAMP are also significantly increased in atherosclerotic lesions and aortas of animals on a high cholesterol diet (20, 21).

During osteoblast development, a series of events occurs as cells undergo differentiation (22). Proliferation declines before the onset of differentiation, and various osteoblastic marker genes, involved in extracellular matrix development and mineralization, are expressed in waves: Coll I is expressed maximally during proliferation and declines progressively, whereas alkaline phosphatase and matrix GLA protein (MGP) expression start low and peak during the matrix development/maturity stage, and osteopontin and osteocalcin expression increase and reach a maximum during the matrix mineralization.

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1 The abbreviations used are: CVC, calcifying vascular cell(s); Coll I, type I collagen; GLA, γ-carboxyglutamic acid; MGP, matrix GLA protein; RT, reverse transcription; PCR, polymerase chain reaction; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Cbfa-1, core binding factor 1; db-cAMP, dibutyryl cAMP.

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Because the cAMP pathway plays a role in both osteoblast differentiation and vascular disease, we investigated its regulatory function in CVC differentiation. In this report, we show that the cAMP pathway stimulates the osteoblast-like differentiation of CVC by inducing morphological change, inhibiting proliferation, enhancing osteoblastic markers (alkaline phosphatase, matrix GLA protein, and type I procollagen), and increasing matrix calcium incorporation yet inhibiting CVC condensation resulting in a diffuse pattern of mineralization.

EXPERIMENTAL PROCEDURES

Materials—[3H]Thymidine, 4\(^{Ca}\)Cl\(_2\) and [\(^{32}\)P]dCTP were from Amersham Corp. Dibutyryl cAMP, forskolin, cholera toxin, isobutylmethylxanthine, pertussis toxin, and a protein kinase A-specific inhibitor, KT5720, were from Calbiochem (San Diego, CA). The β-glycophosphate was purchased from Sigma. Human osteopontin (24) and human type α(I) collagen cDNA (25) probes for Northern analysis were from American Tissue Culture Collection, and human 28 S rRNA probe was purchased from CLONTECH (Palo Alto, CA). Type I procollagen polyclonal antibody for Western analysis was from Chemicon International Inc. (Temecula, CA). CVC. CVC seeded on 60-mm dishes and vehicle alone, dibutyryl cAMP (1 mM), or forskolin (25 μM) were extracted. For the cAMP treatments, CVC were grown in duplicate in 24-well plates at subconfluence for 2 days at 37 °C.

RESULTS

Spontaneous Differentiation of CVC

Morphology—During spontaneous differentiation, CVC displayed distinct morphological transitions. In post-confluent cultures (5–7 days after plating), cells aggregated into ridge-like structures closely resembling embryonic condensations (arrows, Fig. 1B). From 9–11 days after plating, these condensations formed multicellular nodules (arrow, Fig. 1C), which became increasingly darkened after 14–16 days in culture due to mineralization, identified by strongly positive von Kossa staining (arrow, Fig. 1D).

Expression of Osteoblastic Differentiation Markers—Previously, we reported that CVC exhibit several osteoblastic differentiation markers (6). Here, we determined the time course of their expression during the stages described above (Fig. 1, A–D). Total RNA was isolated from duplicate dishes at stages of subconfluence (1 day after plating), confluence (3 days after plating), condensation (6 days after plating), nodules (10 days after plating), and calcification (14 days after plating). Type I procollagen, osteopontin, and 28 S rRNA (used as an internal control) expression were determined by RT-PCR with specific primers designed for each gene (Fig. 2A).

Autoradiographs shown in Fig. 2A were scanned, and data were plotted as the percentage of maximum expression over the number of days in culture that correspond to the stages shown in Fig. 1 (A–D) and subconfluence (1 day post-plating; not shown). Coll I, alkaline phosphatase, matrix GLA protein, and osteocalcin expression increased as CVC underwent distinct morphological transitions, whereas osteopontin expression declined progressively (Fig. 2, A and B). Cbfa-1 was expressed constitutively during CVC differentiation (Fig. 2, A and B).

To determine whether the expression of these differentiation markers during the later stages of CVC differentiation was limited to the cells...
perturbation of CVC d at 70–90% confluence with 1 mM db-cAMP or 25 μM forskolin at 70–90% confluency and incubated for 2 days. [3H]Thymidine was added to the medium during the last 24 h, and cellular proliferation was assessed (prior to condensation and nodule formation). The results showed that increased cAMP inhibited CVC proliferation (Fig. 3). Because it has been shown in osteoblasts that the decline of proliferation is functionally coupled to the onset of differentiation (22), we investigated whether cAMP stimulation also initiated expression of osteoblastic differentiation markers.

Osteoblastic Differentiation Markers—Alkaline phosphatase activity, a well recognized early marker of osteoblastic differentiation (30), increased during spontaneous CVC differentiation (7). Therefore, we first measured its activity in response to cAMP stimulation. CVC at 70–90% confluence were treated with various concentrations of db-cAMP or forskolin and incubated for 3 days. Alkaline phosphatase activity was dose-dependently induced (Fig. 4, A and B, respectively). In addition, cholera toxin (500 ng/ml) and isobutylmethylxanthine (200 μM), other agents known to increase intracellular cAMP levels, also had similar effects on alkaline phosphatase activity (Fig. 4C).

Because the results showed that cAMP induced early markers of osteoblast-like differentiation in CVC, we next determined its effects on later markers described above. Duplicate dishes of CVC were treated at 70–90% confluence with 1 mM db-cAMP or

Table I

| Osteoblastic differentiation markers | Expression localized to nodules |
|-------------------------------------|---------------------------------|
| Coll I                              | 54                              |
| Alkaline phosphatase                | 51                              |
| Matrix GLA protein                  | 75                              |
| Osteopontin                         | 58                              |
| Osteocalcin                         | 52                              |
| Chd-1                               | 49                              |

Effect of cAMP on Osteoblast-like Differentiation of CVC

Morphology—Short term treatment of CVC with 1 mM db-cAMP in a single administration at 70–90% confluence and incubated for 2 days induced a morphological change from an elongated to a “cuboidal” shape (Fig. 1, E versus A, control), which is an indication of preosteoblast differentiation into osteoblastic cells (27–29). The same morphological change was observed when CVC were treated with forskolin (25 μM), isobutylmethylxanthine (200 μM), or cholera toxin (500 ng/ml) (data not shown).

Prolonged treatment of CVC with db-cAMP has marked effects on later differentiation stages of CVC, including condensation, nodule formation, and mineralization. When CVC were treated with 1 mM db-cAMP (at 70–90% confluence and fed every 3–4 days with fresh medium containing 1 mM db-cAMP), there was inhibition of condensation (Fig. 1: control (B) versus treated cells (F) after 6 days in culture) and subsequent nodule formation (control (C) versus treated cells (G) after 10 days in culture). However, von Kossa staining for mineralization showed that calcification occurred in both treated and control cells after 15 days in culture (control (D) versus treated cells (H)). In control cells, calcification was confined within nodules, whereas in treated cells, calcification was diffuse throughout the monolayer with some patches of increased density, despite the absence of nodules.

Proliferation—Cells were treated with 1 mM db-cAMP or 25 μM forskolin at 70–90% confluency and incubated for 2 days. [3H]Thymidine was added to the medium during the last 24 h, and cellular proliferation was assessed (prior to condensation and nodule formation). The results showed that increased cAMP inhibited CVC proliferation (Fig. 3). Because it has been shown in osteoblasts that the decline of proliferation is functionally coupled to the onset of differentiation (22), we investigated whether cAMP stimulation also initiated expression of osteoblastic differentiation markers.

Distribution of osteoblastic differentiation markers between nodules versus intervening monolayer cells

Nodules were separated from monolayer cells by filtration, and expression levels were determined from both RNA derived from the nodules and that from the monolayer cells. The expression found in nodules is expressed as a percentage of the total.

Within the nodules and not in the intervening monolayer cells, nodules were separated from the intervening cells by suspension and filtration, and total RNA from both sets were extracted. Results showed that all differentiation markers except MGP were expressed at similar levels in both the monolayer and the cells forming nodules (Table I). MGP was expressed at 3-fold higher levels in the nodules than in the monolayer cells.
FIG. 2. Expression of osteoblastic differentiation markers during spontaneous CVC differentiation. A, total RNA at the same time points shown in Fig. 1 were isolated, and gene expression of Coll I, osteopontin, and 28 S rRNA were analyzed by Northern analysis; alkaline phosphatase, matrix GLA protein, Cbfa-1, and GAPDH expression were analyzed by RT-PCR. For the subconfluence stage, RNA was isolated 1 day post-plating. d, day(s); subconf, subconfluence; conf, confluence; cond, condensation; nod, nodules; calc, calcification. B, the densitometric data of scanned autoradiographs, normalized for 28 S rRNA (for osteopontin and Coll I) or GAPDH (for alkaline phosphatase, matrix GLA protein, osteocalcin, and Cbfa-1), was plotted as the percentage of maximum expression (average of duplicate samples) over the number of days in culture that represent each differentiation stage.
25 μM forskolin and incubated for 3 days, and total RNA was analyzed by Northern analysis or RT-PCR as described above. Treatment with either agent caused increased gene expression of alkaline phosphatase (11- and 8-fold, respectively) and matrix GLA protein (4- and 2-fold, respectively) but a decrease in osteopontin expression (Fig. 5A). cAMP had no effect on the expression of osteocalcin and Cbfa-1 (data not shown). Type I collagen production was enhanced 2-fold as shown by Western analysis when cAMP was stimulated by 25 μM forskolin (Fig. 5B).

Mineralization—To quantify the amount of mineralization in both control and treated cells, radiolabeled calcium incorporation was measured. CVC were treated with 1 mM db-cAMP at 70–90% confluence and fed every 3–4 days with fresh medium containing 1 mM db-cAMP or control media. After 7 days in culture, 4 mM CaCl₂ and 5 mM β-glycerophosphate were added to the media to enhance mineralization. After an additional 6–7 days in culture, cells were washed twice and changed to media containing 5 mM β-glycerophosphate, labeled calcium (⁴⁵CaCl₂), and either 1 mM db-cAMP or control medium and incubated for an additional 48 h. In validation studies, incorporated labeled calcium has been shown to represent primarily matrix-bound calcium, because similar results were obtained in cultures permeabilized with Triton X-100, which removes ionic calcium (7). The results showed that calcium incorporation was enhanced approximately 4-fold with db-cAMP treatment (Fig. 6).

Relationship between Differentiation and Proliferation—Because osteoblastic differentiation has been considered functionally coupled to inhibition of proliferation, we assessed whether inhibition of CVC proliferation is sufficient to promote osteoblast-like differentiation. CVC were treated with pertussis toxin, which has been shown to inhibit smooth muscle cell proliferation (at 0.001–100 ng/ml) without affecting cell viability and the level of intracellular cAMP (31). The results showed that pertussis toxin inhibited CVC proliferation (87% at 20 ng/ml; 89% at 100 ng/ml) without inducing cuboidal morphology or significantly increasing alkaline phosphatase activity (2-fold increase at both concentrations of pertussis toxin), suggesting that the inhibition of proliferation is not sufficient to promote osteoblast-like differentiation of CVC.

To further determine whether blocking the cAMP pathway decreases osteoblast-like differentiation, CVC were treated at 70–90% confluence with 10 μM KT5720, previously used as a protein kinase A-specific inhibitor (32, 33). Results showed that the increase in alkaline phosphatase activity during spontaneous CVC differentiation was blocked (>90% inhibition; data not shown). In addition, Ca⁴⁺ incorporation assay showed that mineralization was also inhibited (>80% inhibition; data not shown), suggesting that the cAMP pathway has a direct effect on osteoblast-like differentiation of CVC independent of its effect on proliferation.

To assess whether cAMP induces osteoblast-like differentiation in non-CVC, a subpopulation of primary smooth muscle cells that do not form nodules, two clones were treated with forskolin. The results showed that proliferation was inhibited approximately 70% without inducing a significant increase in alkaline phosphatase activity: at 3 days, the same dose of
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**DISCUSSION**

These findings indicate that short term cAMP treatment (≤3 days) triggered the onset of osteoblast-like differentiation of CVC in several aspects: 1) induction of a morphological change, which is characteristic of preosteoblasts differentiating into osteoblasts (27–29); 2) inhibition of CVC proliferation, which is considered a requirement for the initiation of differentiation (22); 3) acceleration of the induction of osteoblastic differentiation markers including alkaline phosphatase, type I procollagen, and matrix GLA protein, which were also found to increase during spontaneous CVC differentiation; and 4) acceleration of the decline in osteopontin expression, which also occurs in spontaneous CVC differentiation.

The results further showed that long term cAMP treatment altered the mineralization pattern in CVC. During spontaneous differentiation, CVC aggregate to form condensations that mature into mineralized nodules. Prolonged treatment of CVC with db-cAMP inhibited condensation and subsequent nodule formation. Therefore, in cAMP-treated cells, enhanced production of alkaline phosphatase, extracellular matrix components, and increased matrix calcium incorporation were no longer confined to the nodules, as in the case with control cells, resulting in a diffuse pattern of mineralization throughout the monolayer. This diffuse pattern was not perfectly homogeneous, having some patchy areas of increased density, despite absence of nodules. This pattern has intriguing similarities to in vitro mineralization of bone cell lines (7).

The mechanism by which cAMP blocks condensation is not known but may be related to elimination of a chemotactic gradient (34) and/or altered expression of adhesion molecules such as hyaluronan, clusterin, or N-CAM, which have been suggested to regulate aggregation (35, 36). The inhibition of condensation by cAMP may also occur through effects on the proliferation signal that precedes aggregation in many epithelial-mesenchymal interactions and probably provides the critical density or quorum of cells required for condensation (35).

Previously, we showed the similarities between CVC and osteoblastic cells. Our present data reveal that the time course of expression of osteoblastic markers in CVC differs from that previously shown for bone cells by Stein, Lian, and co-workers (22). The most evident differences are in osteopontin and collagen I expression. In CVC, osteopontin expression declines progressively, whereas in osteoblastic cultures, its expression increases progressively, peaking during the late stage (matrix maturation stage) of osteoblast-like differentiation. In contrast, in CVC, type I procollagen expression increases progressively peaking during the late stage, but in osteoblastic cultures, its expression declines progressively during differentiation (22). We have hypothesized that reciprocal responses of vascular and bone cells to lipid exposure may have a role in the simultaneous occurrence of vascular calcification and osteoporosis in humans (7) and in essential fatty acid-deficient mice (37).

The role of some of the osteoblastic differentiation markers in mineralization is still unclear. Much evidence points to control of formation and maturation of extracellular matrix, providing an environment that facilitates mineral deposition (22, 38). Our data indicate that the expression of one of these proteins, osteopontin, decreases with spontaneous CVC differentiation and in response to cAMP stimulation, whereas it increases in atherosclerotic calcification (39, 40). The increased expression of osteopontin in human atherosclerotic plaques, however, is largely attributable to other cell types, particularly the macrophage-derived foam cells (39, 41), which synthesize osteopontin as an early inflammatory response to tissue injury (42) and use osteopontin also as an opsonin for adhesion to and phagocytosis of calcified particulate matter (43). In areas of plaque composed of predominantly smooth muscle cells, osteopontin expression was not detected (39).

Another osteoblastic marker, matrix GLA protein, increases during CVC differentiation and in response to cAMP stimulation. This is consistent with previous reports of increased MGP expression predominantly by vascular smooth muscle cells in atherosclerotic lesions (39). These results may initially appear paradoxical in light of the recent report from Luo and colleagues demonstrating extensive vascular calcification and ossification in MGP null mouse (44). One might expect a decrease in MGP in association with in vitro vascular calcification. How-

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**FIG. 5. Effect of cAMP on expression of osteoblastic differentiation markers.** A, RNA isolated from CVC treated with db-cAMP (1 mM) or forskolin (25 μM) for 3 days were analyzed by either Northern analysis (osteopontin and 18 S rRNA) or RT-PCR (alkaline phosphatase, matrix GLA protein, and GAPDH). B, whole cell lysate from CVC treated with forskolin (25 μM) for 3 days were analyzed by Western. Type I procollagen probed with anti-bovine polyclonal antibody was detected as two bands representing α1(I) and α2(I) chains. cont, control.

**FIG. 6. Effect of prolonged cAMP stimulation on CVC mineralization.** Calcium mineral deposition was assayed by 45Ca incorporation in CVCs treated with repeated doses of db-cAMP over a 15-day period as described in the text.
ever, there are other examples such as leukocytosis, when a stimulus induces its own inhibitory factor. That is, absence of white cells in immunodeficient mice allows extensive infection; yet, in human infection, white cells are increased rather than absent. Likewise, absence of MGP in the knock-out mice allows extensive vascular calcification; yet, in human vascular calcification, MGP expression is increased rather than absent. Thus, MGP may be up-regulated in response to vascular calcification, perhaps to limit its extent.

In conclusion, these results support the hypothesis that cAMP modulates in vitro vascular calcification. The findings in atherosclerotic calcification are consistent with the findings in CVC, both in spontaneous and cAMP-induced differentiation, supporting the in vivo relevance of this model.

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