Identification and Characterization of Vascular Calcification–Associated Factor, a Novel Gene Upregulated During Vascular Calcification In Vitro and In Vivo


Objective—Vascular calcification, with its increasing clinical sequelae, presents an important and unresolved dilemma in cardiac and vascular practice. We aimed to identify molecules involved in this process to develop strategies for treatment or prevention.

Methods and Results—Using subtractive hybridization, a novel cDNA, designated vascular calcification–associated factor (VCAF), has been isolated from a bovine retinal pericyte cDNA library generated during the differentiation and mineralization of these cells in vitro. RNA ligase-mediated rapid amplification of cDNA ends was used to compile the 740-bp bovine cDNA sequence. Database searching reveals that VCAF has novel nucleotide/amino acid sequences. RNA analysis confirms that VCAF is upregulated in mineralized pericytes and is present in human calcified arteries but not noncalcified arteries. Protein analysis using a VCAF antibody confirms the presence of an 18-kDa protein in calcified nodules but not in confluent pericytes. Adenoviral antisense VCAF gene delivery reduces VCAF protein levels and accelerates pericyte differentiation compared with controls.

Conclusion—We demonstrate the isolation of a novel gene, VCAF, which is upregulated during vascular calcification in vitro and in vivo. Antisense VCAF gene delivery accelerates pericyte differentiation, implicating a role for VCAF in this clinically significant pathological process. (Arterioscler Thromb Vasc Biol. 2005;25:1851-1857.)

Key Words: atherosclerosis ▪ genes ▪ pericytes ▪ calcification ▪ novel gene

D evelopments in imaging technology show a correlation between the levels of coronary artery calcium and the occurrence of myocardial infarction and stroke.1 Kondos et al2 showed benefits of using measurements of coronary artery calcium for prediction of future cardiovascular events, thereby providing additional prognostic information beyond that obtained from traditional coronary heart disease risk factors. These findings provide the impetus to move beyond observational studies and determine the mechanism underpinning calcium deposition in the vessel wall so that novel therapeutic targets for diagnosis or treatment aimed at modulating vascular calcification can be developed.

In vitro models of calcification have been established that demonstrate that microvascular pericytes,3 smooth muscle cells,4 and calcifying vascular cells,5,6 when grown in culture, form nodules containing the bone mineral hydroxyapatite. The matrix deposited by these vascular cells in vitro resembles that present in calcified vessels in vivo (eg, the bone-related proteins osteopontin [OPN], osteocalcin, matrix Gla protein [MGP], and bone morphogenetic protein-2 are all upregulated).4,5,7

As a step toward identifying genes that are expressed differentially during the osteogenic differentiation of vascular cells, we used subtractive hybridization to screen for cDNA sequences that are either upregulated or downregulated during the process of pericyte differentiation. This study describes the isolation and characterization of a bovine cDNA, originally called clone 15 (now designated vascular calcification–associated factor [VCAF]),8 that is upregulated when bovine pericytes form mineralized nodules in vitro. Furthermore, we report the presence of human VCAF mRNA transcripts in calcified tissues, which have sequence similarity to the bovine cDNA. The predicted molecular weight of 18 kDa has been confirmed by Western blot analysis using an antibody generated against a VCAF peptide. Finally, adenoviral-mediated gene transfer of antisense VCAF cDNA demonstrates downregulation of VCAF protein, and increases the size, rate of formation, and maturation of nodules com-
pared with untreated or control virus-infected cells. These studies are a first step toward characterizing a novel putative calcification regulating protein, the expression of which is upregulated in advanced calcified plaques. Our results support the hypothesis that VCAF may modulate pericyte differentiation toward an osteogenic phenotype.

Methods

Tissue Collection
Approval from the local research ethics committee was granted for the use of human tissue, and procedures were in accordance with institutional guidelines. Blood vessels from 4 different vascular beds were examined: (1) tissue from an aortic aneurysm; (2) peripheral arteries from lower-limb amputations, which included tibial, popliteal, and femoral arteries; (3) carotid tissue from endarterectomy samples; and (4) atheromatous coronary arterial specimens obtained at the time of postmortem examination within 12 hours of death. RNA was harvested from calcified and noncalcified arterial segments, either directly or from sections of the tissue after embedding, to allow correlation of VCAF expression with histological analysis. Vessels were classified as calcified or noncalcified on the basis of von Kossa and Alizarin red staining as described previously.9

Cell Culture and Analysis of Nodule Formation
Pericytes were isolated from adult bovine retinal microvessels, characterized, and maintained as described previously.10 Nodules were counted and areas measured using AnalySIS software (Soft Imaging System GmbH). Data are presented as the total number of nodules in 14 fields of view (14 × 3.6 mm²) per flask (n = 2). RNA and protein were extracted at the times indicated in the experiments.

Adenoviral Infection of Cell Cultures
Cells were plated at a density of 4 × 10⁵ cells/25 cm² flask and infected at 80% confluence using: (1) antisense VCAF virus (Ad/VCAF/AS), generated with the full-length antisense VCAF cDNA; (2) a recombinant adenovirus-encoding green fluorescent protein (Ad/GFP) to assess virus infection efficiency; or (3) PBS alone (mock infected). Culture media was added to the cells, and they were infected at 80% confluence using: (1) antisense VCAF virus (Ad/VCAF/AS), generated with the full-length antisense VCAF cDNA; (2) a recombinant adenovirus-encoding green fluorescent protein (Ad/GFP) to assess virus infection efficiency; or (3) PBS alone (mock infected). Culture media was added to the cells, and they were incubated for up to 6 weeks. The media was changed twice a week, and cells were infected every 7 days with a multiplicity of infection (MOI) of 25. The MOI selected routinely achieves 90% transfection efficiency without any cell toxicity (data not shown). The adenoviral vectors were generated by the Gene Transfer Core of the University of Iowa under the direction of Dr Beverly Davidson.

RNA Isolation, RNA Ligase-Mediated Rapid Amplification of cDNA Ends, and Sequencing
RNA was extracted from cells using RNABee following the manufacturer’s protocol (Biogenesis Ltd). RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) was performed according to manufacturer’s instructions (Ambion) to obtain the full-length cDNA sequence of VCAF. cDNAs were synthesized from 1 μg of total RNA using reverse transcriptase (RT) and gene-specific primers E4 (tggctgtcctagcaggcacttc) and E8 (ttatgggtcctagcaggcacttc) (Figure 1A). Nested polymerase chain reactions (PCRs) were performed on these cDNA templates using the RACE 5’ primer (gtcctgcttctagcaggcacttc) and RT-PCR was conducted using primers as follows: 5’ RACE primer and E2 (tcctagcaggcacttc), E3 (ctctagcaggcacttc) and E4 (gttctagccccaggcacttc) and E8; and E7 and E9 (taagagacatctatatctatagctc) and standard PCR conditions with annealing temperatures of 64°C, 60°C, 64°C, and 58°C, respectively. The amplicons were inserted into the pCR2.1-TOPO vector (Invitrogen) and sequenced using the Big Dye Terminator Cycle Sequencing Ready Reaction sequencing kit and an ABI 377 automated sequencer (Applied Biosystems Inc).

RNA Analysis
RNAs (20 μg) were extracted from cells as above and used for Northern blot analysis. Human tissue RNA was extracted using RiboZyber matrix beads (Hybaid; RYM62111) and RNABee (Biogenesis) and used for RT-PCR (1 μg). Hybridization was performed using UltraHyb (Ambion) at 42°C and a full-length VCAF cDNA probe. Axl, MGP, and 18S ribosomal RNA detection was performed as described previously.11 The autoradiograms were exposed routinely for 48 hours for VCAF, MGP, and OPN, or for 15 hours for the 18S ribosomal probe.

In Situ Hybridization
Sections (7 μm) were cut from human arteries, mounted onto silanated slides, and in situ hybridization was performed as described previously,12 using 35[S]-UTP–labeled single-stranded sense and antisense RNA probes to VCAF and OPN. Autoradiographic exposure times were 2 weeks, and after development, slides were counterstained with hematoxylin and analyzed using an Axioplan microscope attached to Axiovision computer software (Zeiss).

Antibody Generation and Western Blot Analysis
Using the predicted protein sequence, a peptide was designed based on its hydrophilic and antigenic properties, its lack of predicted β-turns and its suitability for conjugation chemistry at the N terminus. This 15mer VCAF peptide, located at the C terminus of the VCAF protein (CVKVGRWKHRLLPTE in the single-letter amino acid code, corresponding to amino acids 152 to 165 of VCAF) was synthesized and conjugated to keyhole limpet hemocyanin (Sigma Genosys). Anti-peptide antibodies were raised in rabbits and used for Western blot analysis. Samples of preimmune serum were used as controls. Whole cell lysates were obtained using Trizol according to manufacturer’s instructions. The proteins (15 μg) were electrophoresed on reducing 15% sodium dodecyl sulfate–polyacrylamide gels.
isolation of VCAF

Subtractive hybridization was performed as described previously,13 and the subtracted cDNAs were used to screen a pericyte cDNA library. This resulted in the isolation of cDNAs that were specifically upregulated in postconfluent pericytes. BLAST search comparisons with sequences from the National Centre for Biotechnology Information revealed that the sequence of one of these clones, which contained a DNA fragment of 420 bp, showed no homology with other sequences in the database. Detailed analysis of this clone, initially called clone 15,8 now designated VCAF, is described in this article.

Sequencing and Primary Structure Analysis of the VCAF Coding Region

A database search of the public-expressed sequence tag set (dbEST) recovered 28 hits with significant alignments extending the sequence of VCAF by 280 bases. RLM-RACE was performed to confirm the computer-generated sequence from the dbEST database and to identify the full 5′ region of the cDNA. cDNA fragments were generated by nested RT-PCR using RNA extracted from mineralized pericytes and different combinations of primers (Figure 1A). Sequencing of the 5′ termini confirmed the 280 bp obtained from the dbEST database and extended the sequence by an additional 40 bp. The compiled 740-bp VCAF cDNA sequence (GenBank No. AY628649) was subjected to an open reading frame (ORF) finder program, which yielded 6 potential ORFs, and the Centre for Biological Sequence Analysis program, which gave a predicted translation initiation site at position 50. The full-length sequence is shown in Figure 1B, with nucleotide numbers on the right and amino acids numbered on the left, beginning with the first methionine of the longest ORF. The use of the ATG triplet, beginning at the longest ORF, is in close agreement with the consensus sequence flanking the translation initiation sites as determined by Kozak,14 with a guanine at position +4 and −6. A predicted 165-aa sequence was obtained (Figure 1B), yielding a predicted protein mass of 18 kDa. Protein database search showed no homology with other proteins. A consensus AATAAA polyadenylation signal15 was identified 28 nucleotides from the end of the 3′ untranslated region (UTR). One domain of VCAF (632 to 636) is AU rich and contains 7 cysteine residues, and whether they potentially undergo γ-carboxylation. A predicted cleavage site is also located between amino acids 17 and 18, although it is thought unlikely to encode a secreted protein because the probability of a signal peptide is predicted as low, according to the Markov model used by the Centre for Biological Sequence Analysis signal peptide prediction server. Furthermore, a bipartite nuclear targeting sequence with a high probability of occurrence17 is detected at residues 58 to 74, which supports the lack of a predicted signal peptide. The importance of these putative domains within the VCAF protein remains to be determined.

Expression of VCAF During the Osteogenic Differentiation of Pericytes

Northern blot analysis of RNA, prepared from confluent pericytes and postconfluent pericyte cultures containing mineralized nodules, was used to examine the pattern of expression of VCAF during pericyte differentiation. Figure 2A confirms that VCAF is upregulated in postconfluent cultures containing mineralized nodules compared with confluent pericytes. Hybridization of the same filter with probes for MGP and Axl, other genes we have identified as being differentially regulated during pericyte mineralization,11,13 confirms the upregulation of MGP and the downregulation of Axl mRNA during the osteogenic differentiation of pericytes. An 18S ribosomal oligonucleotide probe confirms equal loading of RNA.

VCAF Synthesis by Pericytes

To determine the size of the VCAF protein, we raised a polyclonal antibody to a peptide within the predicted VCAF amino acid sequence. Protein was extracted from confluent bovine pericytes and postconfluent cultures containing mineralized nodules. Western blot analysis revealed a band with an apparent molecular mass of 18 kDa, identical to the

and electrotransferred to nitrocellulose membranes (Bio-Rad). The membranes were incubated with the VCAF primary antibody at 22°C for 1 hour (dilution 1:300), and Western blot analysis was performed as described previously11 using a horseradish peroxidase–conjugated swine anti-rabbit secondary antibody (dilution 1:2000).

Results

Figure 2. A, A representative Northern blot using total RNA extracted from confluent pericytes and mineralized nodular pericytes and sequentially probed with VCAF, MGP, and Axl cDNAs and an 18S oligonucleotide. B, Total protein was extracted from confluent bovine retinal pericytes and postconfluent cultures containing large mineralized nodules. Western blots were probed with VCAF antiserum and preimmune serum.

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predicted molecular weight of the VCAF protein in mineral-
ized pericytes but not in confluent pericytes (Figure 2B).
Although higher molecular weight bands are apparent, anti-
body specificity for VCAF was verified by demonstrating the
absence of an 18-kDa band in blots incubated with preim-
une serum (Figure 2B). These data are consistent with the
Northern blot analysis (Figure 2A), confirming a correlation
between the increase in mRNA abundance and VCAF protein
levels as pericytes undergo osteogenic differentiation.

Analysis of VCAF Expression in Human
Atherosclerotic Lesions Using RT-PCR and In
Situ Hybridization
Calcified and noncalcified atherosclerotic tissues were col-
lected, and the extent of calcification in the arterial samples obtained from
our patient group. Some lesions had deep-filled lipid deposits
together with foci of degeneration and calcification. The
representative von Kossa–stained section shown in Figure 3A
exhibits a large calcific deposit and the more granular medial
form of calcification. RNA was extracted from sections, and
first-strand synthesis reaction was performed using a reverse
oligo dT, followed by PCR with primers E3/E4 (Figure 1A).
Successful amplification of a human cDNA fragment of 390
bp was achieved from 1 μg of total RNA extracted from the
calcified (Figure 3B, 1 and 2) but not from the noncalcified arteries (Figure 3B, 3). PCR using actin primers confirmed
the presence of RNA in all samples as shown in the lower gel.
The specificity of the amplicon was confirmed by Southern
blot analysis using a VCAF cDNA probe (data not shown).
The reactions were performed in the absence (−) and
presence (+) of reverse transcriptase (RT), confirming a lack
of contaminating DNA. The 390-bp PCR fragment from the
human calcified arterial segments was isolated, sequenced,
and found to have 96% similarity with the equivalent bovine sequence.

Tissue segments were also fixed for in situ hybridization,
and serial sections were cut from paraffin blocks of all arterial
samples. A representative specimen is documented in Figure
4, showing, in this case, a separation of the base of the lesion
from the media that occurred during processing. Consistent
with reports of OPN expression associated with calcified
lesions,18 we demonstrate high focal expression of OPN
mRNA in groups of cells at the base of a matrix-rich region
of the plaque (Figure 4a and 4d). The OPN sense probe
demonstrates negligible background staining (Figure 4b).
Using an antisense VCAF probe, we demonstrate that VCAF
is also expressed within the atherosclerotic lesions at levels
lower than OPN but in the same location (Figure 4c and 4f).
Negligible background staining was obtained using a VCAF
sense probe (Figure 4e). There were discrete stippling micro-
foci of calcification in the same region where VCAF and
OPN were localized. These results confirm the expression of
VCAF and OPN mRNA within calcified atherosclerotic
lesions.

Inhibition of VCAF Expression and Accelerated
Differentiation of Pericytes Using
Adenoviral-Mediated Gene Transfer of
Antisense VCAF cDNA
To determine the effect of decreasing expression of VCAF in
bovine pericytes, the cells were infected at an MOI of 25
using either VCAF antisense virus (Ad/VCAF/AS) or a
control virus (Ad/GFP) and were cultured for up to 6 weeks.
As shown in Figure 5A, we demonstrated successful down-regulation of VCAF protein expression using this antisense strategy. The loss of VCAF protein in pericytes infected with Ad/VCAF/AS correlates with an increase in the rate of nodule formation, with nodules being formed as early as 2 weeks in culture (Figures 5B and 6), whereas mock- or Ad/GFP-infected cells have fewer and smaller nodules, which took longer to mature (Figures 5B and 6). The large nodules formed in the cells infected with Ad/VCAF/AS showed evidence of maturation (Figure 5B), suggesting that down-regulation of VCAF protein stimulated the deposition of a calcified matrix by pericytes.

**Discussion**

The present study has identified a novel gene (VCAF) that is associated with calcification of bovine pericytes in culture and human calcified atherosclerotic lesions. We screened for genes that are differentially expressed during mineralization of pericytes in culture using subtractive hybridization. Many of the randomly picked clones displayed a restricted expression pattern in mineralized nodules, and the majority were identified as sequences that corresponded to MGP. One clone, identified as clone 15 (now termed VCAF), was found to encode a novel gene and became the focus of this study.

We have shown that VCAF mRNA is modulated during the osteogenic differentiation of pericytes. High expression is detected in postconfluent mineralized nodular pericytes compared with the low or undetectable levels of expression in confluent cells without nodules. Furthermore, using RT-PCR and in situ hybridization, we demonstrate that VCAF mRNA is expressed in human atherosclerotic lesions, in which we detect VCAF transcripts in calcified but not in noncalcified vessels. It is interesting to note that OPN and VCAF mRNA expression is detected at the base of the plaque at sites of early calcification but not in the heavily calcified media. This is consistent with the findings of Shanahan et al, who have also shown that high levels of OPN mRNA expression in the intima is confined to a subset of macrophages. This contrasts with the reported localization of OPN protein at the cell-calcified matrix interface and in the adventitia. Future studies will identify the cell type(s) producing VCAF mRNA in calcified tissues and will determine whether VCAF mRNA and protein colocalize.

Sequence analysis of bovine VCAF along the distal half of the cDNA has revealed 96% similarity at the nucleotide level to the human amplified sequence of VCAF, which was obtained from RT-PCR amplification of RNA isolated from human calcified arterial tissue. This implies a degree of evolutionary conservation, suggesting that VCAF may encode a functionally important protein. Indeed, our data...
establish that VCAF is associated with areas of calcification in vitro and in vivo. According to the apparent molecular weight of VCAF identified by Western blot analysis, the cDNA we have isolated can account for the entire coding region of this novel protein.

Many proteins become functionally active with post-translational modification. The predicted regulatory domains that have been identified within the VCAF protein sequence also suggest possible mechanisms by which the functional role of this molecule may be executed. The predicted amino acid sequence of VCAF contains 6 glutamic acid residues, providing the potential for γ-carboxylation and binding of hydroxyapatite. However, it does not contain a consensus Gla domain, which is thought to be important for substrate recognition by the carboxylase enzyme.19 Also of interest are the number of potential phosphorylation sites in this novel VCAF molecule. OPN, a potent regulator of vascular calcification,20 is a phosphoprotein containing Ser/Thr phosphorylation sites that mediate hydroxyapatite binding.21 It is possible that VCAF acts as a mineral-binding protein through phosphorylation of its serine/threonine residues, representing a potential mechanism by which VCAF could influence calcification in soft tissues. VCAF also possesses an RGD sequence. RGD motifs are known to regulate cell–matrix interactions22 and the activation of procaspases.23 The importance of these domains in determining VCAF function will be established in future studies.

We used a newly generated anti-VCAF antibody to confirm the size of the protein as 18 kDa and to show an upregulation of VCAF in mineralized pericytes. An adenosine expressing antisense VCAF mRNA was used as a gene-silencing technique to determine the function of VCAF in pericyte differentiation. Infection of pericytes with Ad/VCAF/AS downregulated expression of VCAF protein, and this appeared to correlate with accelerated nodule size, formation, and maturation. These findings suggest an involvement of VCAF in pericyte osteogenic differentiation in vitro. Although the precise role of VCAF is unclear at the present time, our data suggest that VCAF may be a negative regulator of differentiation or mineralization. It is tempting to speculate that just as increased MGP expression has been postulated to act as a protective factor against mineralization of vascular tissue,7 VCAF may also be upregulated to limit further calcification in diseased arteries. Because VCAF protein shares no homology with any known protein, we can only speculate as to the mechanism by which this may occur. One possibility is that VCAF protein is a component of a signal transduction pathway that negatively regulates pericyte osteogenic differentiation. Another possibility is that VCAF plays a role in some of the morphological changes that occur when pericytes differentiate into osteoblast-like cells in vitro. It is interesting to note that Garfinkel et al24 suggest that the patterning of cell growth and behavior is predicted by a mathematical model on the basis of molecular morphogens interacting in a reaction diffusion process and highlight the morphogen bone morphogenetic protein-2 and its inhibition by MGP.24 The predicted small size of VCAF (18 kDa) allows it to fall into a category of a protein that could rapidly diffuse and inhibit formation of calcified lesions in a pathological situation. The role of VCAF in inhibiting calcification, the interacting proteins, the signal transduction pathways associated with VCAF function and its tissue distribution will be established in future studies.

In conclusion, we: (1) identified and characterized a novel gene (VCAF) expressed by bovine pericytes; (2) demonstrated elevated abundance of the 720-bp VCAF mRNA transcript during the osteogenic differentiation of pericytes in vitro; (3) confirmed the presence and localization of VCAF mRNA expression in advanced lesions within human blood vessels but its absence in noncalcified arteries using RT-PCR and in situ hybridization; (4) confirmed the size of VCAF protein to be 18 kDa; and (5) using VCAF antisense adenoviral gene delivery, demonstrated downregulation of VCAF protein associated with accelerated maturation of pericytes, indicative of its involvement in the osteogenic differentiation of these cells. These studies represent the first step in characterizing a novel putative calcification regulator protein that is upregulated in bovine mineralized pericytes and may play a role in the osteogenic differentiation of these cells. Our data not only show that VCAF is present in atherosclerotic lesions but suggest that VCAF is an important regulatory protein in vascular calcification. Future studies will establish the mechanism underpinning the role of VCAF in this clinically significant pathology.

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References


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