Apoptosis Regulates Human Vascular Calcification In Vitro
Evidence for Initiation of Vascular Calcification by Apoptotic Bodies

Diane Proudfoot, Jeremy N. Skepper, Laszlo Hegyi, Martin R. Bennett, Catherine M. Shanahan, Peter L. Weissberg

Abstract—The mechanisms involved in the initiation of vascular calcification are not known, but matrix vesicles, the nucleation sites for calcium crystal formation in bone, are likely candidates, because similar structures have been found in calcified arteries. The regulation of matrix vesicle production is poorly understood but is thought to be associated with apoptotic cell death. In the present study, we investigated the role of apoptosis in vascular calcification. We report that apoptosis occurs in a human vascular calcification model in which postconfluent vascular smooth muscle cell (VSMC) cultures form nodules spontaneously and calcify after ≈28 days. Apoptosis occurred before the onset of calcification in VSMC nodules and was detected by several methods, including nuclear morphology, the TUNEL technique, and external display of phosphatidyl serine. Inhibition of apoptosis with the caspase inhibitor ZVAD.fmk reduced calcification in nodules by ≈40%, as measured by the cresolphthalein method and alizarin red staining. In addition, when apoptosis was stimulated in nodular cultures with anti-Fas IgM, there was a 10-fold increase in calcification. Furthermore, incubation of VSMC-derived apoptotic bodies with 45Ca demonstrated that, like matrix vesicles, they can concentrate calcium. These observations provide evidence that apoptosis precedes VSMC calcification and that apoptotic bodies derived from VSMCs may act as nucleating structures for calcium crystal formation. (Circ Res. 2000; 87:1055-1062.)

Key Words: calcification ■ vascular smooth muscle ■ apoptosis ■ apoptotic bodies

Intimal calcification is a common and early event in the pathogenesis of atherosclerosis. Calcification also occurs in the media of the vessel wall, particularly in aging, diabetes, and uremia. The presence of calcification in the coronary arteries correlates with an increased risk of myocardial infarction1–3 and predisposes to coronary dissection after angioplasty.4 Vascular calcification is also a potent predictor of future cardiovascular events in asymptomatic patients.5 The calcium deposits exist mainly in the form of calcium apatite, which is the type of mineral normally found in bone.6 Recently, the discovery that bone-associated genes can be expressed in the blood vessel wall strongly suggests that vascular calcification is an actively regulated process.7–9 However, the mechanisms that generate nucleation sites for calcium crystal formation in the vasculature have yet to be determined.

Matrix vesicles are thought to initiate calcification in forming bone and mineralizing cartilage.10 These are membrane-bound vesicles that are produced by budding from chondrocytes, osteoblasts, and odontoblasts and contain the necessary calcium-binding proteins and phosphatases for nucleation of hydroxyapatite. Matrix vesicle-like structures have also been found in calcified arteries and heart valves.11,12 Kockx et al13 showed that in advanced carotid atherosclerotic plaques, these structures were derived from vascular smooth muscle cells (VSMCs) and contained BAX protein, a proapoptotic member of the bcl-2 family, indicating that they may be remnants of apoptotic cells. It is thought that cell death may lead to matrix vesicle generation,10,14 and Hashimoto et al15 recently demonstrated that chondrocyte apoptotic bodies have similarities with matrix vesicles.

We have previously shown that human VSMCs spontaneously form multicellular nodules and deposit calcium crystals after ≈28 days in culture and that, by using electron microscopy, matrix vesicle-like structures can be identified within the nodules.16 In the present study, we have established that apoptosis occurs in VSMC nodules and have tested the hypothesis that apoptosis initiates calcification by inhibiting VSMC calcification with the cell-permeable caspase-inhibitor ZVAD.fmk. We demonstrate that inhibiting apoptosis also inhibits calcification. In addition, stimulation of apoptosis with a combination of anti-Fas IgM and cycloheximide increased nodule calcification. Furthermore, we have shown that apoptotic bodies derived from cultured human VSMCs can concentrate and crystallize calcium. These studies provide experimental evidence for the first time, to our knowl-
edge, to show that apoptosis precedes calcification and that apoptotic bodies are capable of initiating vascular calcification.

Materials and Methods

Cell Culture

VSMCs were prepared from medial explants of human aortic tissue and cultured in 20% FCS in M199 medium (Sigma) and formed multicellular nodules, as described previously.16

Induction of Apoptosis and Apoptotic Body Preparation

Induction of apoptosis in VSMCs was achieved by either addition of anti-Fas IgM (100ng/mL, clone CH11, Upstate Biotechnology) and cycloheximide (10 µg/mL, Sigma) in serum-free M199 to primary human VSMCs for 24 hours or by serum starvation of a human coronary plaque cell line HASMC 66 (human coronary artery smooth muscle cell).17 Apoptotic bodies (ABs) were harvested from these cultures by centrifugation of cell supernatants at 2500 rpm.

Detection of Apoptosis

Nuclear Morphology

Nodules were cultured in 35-mm dishes in M199 containing 20% FCS and monitored live at 37°C. Bisbenzimide 2 µg/mL (final concentration, Hoechst No. 33258, Sigma) was added to the medium, and the nodules were monitored by confocal microscopy (Leica TCS-SP) so that the whole nodule could be imaged. Apoptotic cells were quantified by counting fragmented or condensed nuclei by capturing serial optical sections through the nodules. For this analysis, the glow-over mode was used, because this gave the best contrast between saturated and nonsaturated fluorescence.

TUNEL Labeling

TUNEL labeling was performed on nodule sections pretreated with citric acid, as described previously.18

Phosphatidyl Serine Exposure

Nodules were treated as described above while being monitored by time-lapse confocal microscopy. Annexin V-FITC (4 µL/mL, Clontech) and propidium iodide (500 nmol/L, Clontech) were added to the culture medium and monitored for initiation of annexin V binding.

Detection of Calcification in Multicellular Nodules

VSMCs were grown in 12-well plates fixed in 4% formaldehyde in PBS for 45 minutes at 4°C. The cultures were then washed in distilled water and exposed to alizarin red (2% aqueous, Sigma) for 5 minutes and then washed again with distilled water. Alternatively, the calcified material in each well was extracted from nonfixed cell layers with 0.1 mol/L HCl overnight at room temperature and quantified using cresolphthalein,19,20

45Ca Accumulation into ABs

To measure calcium accumulation in ABs, the method of Hashimoto et al15 was used and modified minimally. The calcifying reaction mixture contained 45Ca (~50 000 cpm) and 40 µg of ABs, and the samples were incubated at 37°C for 24 hours. The samples were then centrifuged at 6500 rpm for 10 minutes, and washed pellets were dissolved in 0.1 mol/L HCl and then placed in HiSafe scintillation fluid. Disodium ATP (100% pure, Roche) and Nonidet P-40 (NP-40, Sigma) were included in some experiments. A synthetic cartilage lymph calcification buffer was also used in some experiments.21

Energy Dispersive X-ray Microanalysis

Adherent ABs mounted on thermonox discs were rinsed in distilled water, quench-frozen in melting propane, and freeze-dried. Elemental content of ABs was performed by energy dispersive x-ray (EDX) analysis, the glow-over mode was used, because this gave the best contrast between saturated and nonsaturated fluorescence.

Figure 1. Human VSMC and nodular cultures. A, VSMC morphology in culture. B, Confluent VSMCs can spontaneously retract and develop into multicellular nodules. C, Section of a day-7 nodule fixed in 4% formaldehyde, embedded in paraffin, and sectioned and stained with H&E. D, Section of an 8-week-old nodule, prepared as in panel C.

Calcium Detection by Confocal Microscopy

ABs were cultured in 35-mm dishes and incubated with calcein-AM (2 µmol/L, Molecular Probes) for 30 minutes. ABs were then washed 3 times in serum-free medium, and green fluorescence was examined using the confocal microscope.

An expanded Materials and Methods section can be found in an online data supplement available at http://www.circresaha.org.

Results

Human VSMC Nodular Cultures

VSMCs cultured from explants of human aortic media formed the distinct hill- and valley-type of morphology, with cells retracting from some areas and grouping into multicellular aggregates or nodules (Figures 1A and 1B). In cross section, nodules contained concentric layers of cells arranged peripherally with many small cells in the middle of the nodule (Figure 1C). As nodules developed in culture, they contained fewer cells, which were mainly located around the periphery of the nodule with central areas of acellularity (Figure 1D). Therefore, with time, cells were lost from the center of the nodules.

Detection of Apoptosis in Nodules

To find out whether apoptosis occurred in VSMC nodules, several different methods were used. Stains including hematoxylin and eosin (H&E) and Hoechst revealed many nuclei with condensed or fragmented nuclei within nodules (Figures 2A through 2C). The TUNEL method was also used, which detects DNA breaks characteristic of apoptosis. Several nuclei stained positively by this method (Figure 2D), indicating that they are likely to have been apoptotic. However, because TUNEL can potentially detect nonapoptotic cells,22,23 we also investigated exposure of phosphatidyl...
serine (PS) by cells in nodules. Several PS-exposing cells were observed within and on the periphery of the nodule, and many cells had already died, as indicated by propidium iodide staining (Figure 2F). In addition, in nodules monitored by time-lapse video microscopy, we observed cells within nodules undergoing surface membrane blebbing, characteristic of apoptosis (data not shown). Collectively, these results confirm that many cells within the nodule undergo apoptosis by day 7 of nodule culture.

We previously observed that calcification was not detected in nodules until day 28 of culture. Because apoptosis was evident before the onset of calcification, we investigated the relationship between apoptosis and calcification by estimating the apoptotic indices in nodules over the 28-day culture. To obtain an accurate measure of the whole nodule apoptotic events, nodules were optically sectioned using the confocal microscope. Apoptotic cells were visualized using Hoechst nuclear morphology, ie, fragmented or condensed nuclei were counted and an index was generated as the percentage of apoptotic cells compared with total nodular cell number. This analysis showed that the apoptotic index did not significantly change between days 7 and 21 but increased at day 28 (Figure 3). Therefore, apoptosis occurs before calcium crystals are deposited, and the higher rates of apoptosis coincide with the onset of calcification.

The apoptotic indices measured in nodules at various time points were relatively high (>20%), which would predict that older nodules would eventually become acellular. However, nodules cultured for 8 weeks still contained viable cells, mainly peripherally (Figure 1D). By observing nodules with time-lapse videomicroscopy, we found that nodules recruit cells from the surrounding monolayer (data not shown). Therefore, although the rate of apoptosis in nodules is relatively high, migration of VSMCs into nodules accounts for the maintenance of cellularity.

**Effect of ZVAD.fmk on Calcification**

To confirm that the caspase inhibitor ZVAD.fmk had an antiapoptotic effect in VSMC nodules, its effects on nodule...
apoptosis were measured using Hoechst, as in Figure 3. ZVAD.fmk (100 μmol/L) decreased the apoptotic index in day-7 nodules, from 39.1±7.5% in the control nodules to 28.0±9.6% in treated nodules (n=6, P=0.03). Examples of optical sections of nodules treated with or without ZVAD.fmk are shown in Figure 4. To test the possibility that ZVAD.fmk affected cell proliferation, Ki-67 staining was performed on nodule sections, but no difference was seen between control and ZVAD.fmk-treated groups (see the online data supplement, available at http://www.circressa.org). Also, we did not observe an effect of ZVAD.fmk on necrotic cell death in VSMCs (data not shown).

After verification of the antiapoptotic effects of ZVAD.fmk on nodule apoptosis, ZVAD.fmk (100 μmol/L) was added throughout the 28-day culture period, and its effects on subsequent calcification were measured. Treatment with ZVAD.fmk reduced the amount of calcification in nodular cultures, as assessed by alizarin red staining (Figures 5A and 5B) as well as calcium content in the nodular cultures (Figure 5C). It is important to note that the presence of ZVAD.fmk had no effect on the total number of nodules. Therefore, inhibition of apoptosis in nodules with ZVAD.fmk reduced the resulting nodule calcification.

Effect of Enhancing Apoptosis in Nodules
The role of apoptosis was investigated additionally by exposure of the nodular cultures to apoptotic stimuli: a combination of anti-Fas IgM and cycloheximide. Treatment of VSMC nodular cultures with anti-Fas IgM and cycloheximide significantly increased the total amount of calcium deposited in cultures by ≈10-fold (Figure 6).

Accumulation of Calcium by VSMC-Derived ABs
To find out whether ABs derived from VSMCs could accumulate calcium in a similar manner to chondrocyte ABs and matrix vesicles, HASMC 66 cells and primary VSMCs were used to generate ABs (Figure 7A). When VSMC-derived ABs were incubated in calcifying medium, they

**Figure 3.** Apoptotic indices in nodules. The apoptotic index was measured by counting apoptotic nuclei stained with Hoechst (condensed or fragmented nuclei) and expressed as percentage of the total cell number per nodule (average of 6 nodules per time point). The apoptotic index did not significantly change between days 7 and 21 of culture but significantly increased at day 28 (compared with indices at days 14 and 21; *P*<0.006). The number of cells per nodule did not change significantly from days 7 to 28 (mean nodule cell number was 36.3±12.4, n=24 nodules).

**Figure 4.** Imaging of cells in nodules treated with or without ZVAD.fmk. These are confocal images showing the section with the greatest cell number in different control (C1 through C3) or ZVAD.fmk-treated nodules (Z1 through Z3). These nodules have been incubated with Hoechst, and the image is in the glow-over mode, as in Figure 2A. Note that ZVAD.fmk-treated nodules appear to contain more cells per nodule than controls.
accumulated $^{45}$Ca from solution (Figure 7B). $^{45}$Ca accumulation was not stimulated by ATP (1 mmol/L), but ABs pretreated with the detergent NP-40 to permeabilize the AB membrane failed to accumulate $^{45}$Ca (Figure 7B). These studies showed that ABs can concentrate $^{45}$Ca by a mechanism that requires an intact AB membrane.

Elemental Analysis of VSMC ABs

To investigate the type of calcium deposited in ABs, ABs were incubated in calcifying medium for 24 hours at 37°C and analyzed for their elemental content by EDX (Figures 8A and 8B). In both preparations there was a large signal for Ca$^{2+}$, confirming that ABs concentrate calcium, but a low signal for phosphate. The backscattered image suggests that the calcium is in a concentrated, crystallized form and, from the elemental profile, is most likely to consist of calcium carbonate. Thus, VSMC-derived ABs are capable of concentrating and crystallizing calcium, which is distributed throughout the AB with occasional voids (Figure 8C).

Discussion

Association of Apoptosis With Calcification

The link between cell death and vascular calcification has been noted in pathological studies. In this study, we provide experimental evidence that implicates a role for apoptosis in the initiation of vascular calcification. The occurrence of apoptosis in our in vitro calcification model was confirmed by several methods, including ultrastructural characteristics, TUNEL, and PS exposure in VSMC nodular cells. At the day-7 stage of nodule development, there was a mixture of apoptotic cells, viable cells, and cells with damaged cell membranes. The propidium iodide–positive cells at this stage may have been the result of apoptosis (ie, secondary necrosis) or primary necrosis within the nodules. Apoptosis occurred early in VSMC nodules, but we have previously shown that calcium crystal deposition is not detected until $\approx$day 28 by von Kossa staining or EDX. At the 28-day time point, the apoptotic index increased, coinciding with detection of calcification. These observations show that apoptosis precedes the onset of calcium crystal formation but that if apoptosis initiates calcification, its effects are delayed in early nodules, which would imply that the calcification process in nodules is regulated. Possible calcification-limiting factors produced by VSMCs in the nodule are mineralization-regulating proteins, such as matrix Gla protein. Another possibility is that if ABs are the initiators of calcification, the cells within the early nodules would be expected to recognize and phagocyte the ABs. Older nodules may contain less-efficient phagocytes, allowing the ABs to stimulate calcium crystal growth. In support of

![Figure 5. Effect of ZVAD.fmk on calcification.](http://circres.ahajournals.org/)

![Figure 6. Effect of anti-Fas IgM and cycloheximide on calcification.](http://circres.ahajournals.org/)
a role for apoptosis in calcification, other studies in cultured cells have shown associations with apoptosis and calcification. Apoptosis occurred in cultures of chick embryonal limb bud mesenchymal cells, which were used as a model of chondrocyte differentiation, and Lynch et al. have shown that apoptosis is an integral part of osteoblast differentiation and calcification in fetal rat calvarial osteoblast cultures.

To test whether apoptosis was actually required for calcification to occur, apoptosis was inhibited in nodules by the caspase inhibitor ZVAD.fmk. Alizarin red staining and calcium quantitation clearly showed that ZVAD.fmk inhibited calcification in VSMC nodules. ZVAD.fmk is a broad-spectrum inhibitor of caspases, and we confirmed its antiapoptotic effects in VSMC nodules. Other studies have shown that the mechanism of action of ZVAD.fmk is to prevent completion of the apoptotic program, which may not involve a delay in the onset of apoptosis. Caspase inhibition can also inhibit the release of ABs from cells. The role of apoptosis in our in vitro calcification model was then additionally examined by stimulating apoptosis in nodules with anti-Fas IgM and cycloheximide. This treatment resulted in a 10-fold stimulation of calcification, which strongly supports the role of apoptosis in calcification.

**Role of VSMC-Derived ABs in Initiating Calcification**

Because a lack of clearance of ABs was a potential mechanism of induction of calcification in VSMC nodules, we were tempted to speculate that ABs derived from VSMCs could initiate calcification in a similar manner to chondrocyte matrix vesicles or ABs. We demonstrated that VSMC-
derived ABs accumulated calcium via a mechanism that involved an intact AB membrane, because when it was permeabilized with NP-40, no calcium accumulation was observed. The induction of calcium accumulation in chondrocyte-derived matrix vesicles and ABs was dependent on the presence of ATP. However, ATP had no effect on VSMC AB calcium uptake. In fact, the role of ATP in calcification is not clear, because in different studies, ATP had stimulatory effects on calcification, was not necessary for matrix vesicle calcification, or inhibited calcification. The lack of dependency on ATP for calcium uptake into VSMC ABs suggests the following: calcium is taken up into VSMC ABs by an ion channel or calcium-binding protein that does not require ATP for its activity; ABs may contain sufficient ATP to accumulate calcium, and by adding exogenous ATP, there is no additive effect; and VSMC ABs may not contain the necessary enzymes for ATP hydrolysis, which are present in matrix vesicles. PS exposure by ABs generates a potential calcium-binding site as well as a membrane surface suitable for hydroxyapatite deposition. However, confocal images of cross sections of ABs loaded with calcine suggested that calcium was concentrated throughout the AB rather than bound at the membrane.

Elemental analysis of the bodies in calcifying solutions revealed that they contained abundant calcium but very low phosphate. The EDX spectra suggested that calcium carbonate may be the form of calcium present in ABs. Calcium carbonate comprises 9% of the total calcium crystals in human atherosclerotic lesions, and carbonate apatite is found in bone as well as ectopic calcification. Calcium carbonate can also act as a precursor to carbonate apatite formation under certain conditions. These observations suggest that VSMC-derived ABs can concentrate and crystallize calcium in a form that is found in vivo. Therefore, VSMC ABs have similarities with chondrocyte-derived matrix vesicles but produce a different type of calcium crystal in vitro. This may be attributable to differences in in vitro culture conditions or perhaps to intrinsic differences in protein expression.

It is interesting to note that not all chondrocyte matrix vesicles calcify, only those at specific sites in the cartilage matrix. Matrix vesicles in vitro will only calcify if they are preincubated in ascorbate- and phosphate-rich medium, which generates matrix vesicles enriched with annexin V (which can act as a Ca2+ channel) and alkaline phosphatase. These observations suggest that matrix vesicles are not all equivalent and that only tissues normally engaged in mineralization produce mineralization-competent vesicles. However, it is also possible that nonmineralizing tissues produce inhibitors of matrix vesicle function to block mineralization. Therefore, one can hypothesize that a lack of production of inhibitors of matrix vesicle calcification may lead to the development of pathological calcification.

Relevance of Apoptosis to Calcification in Disease

Some studies have implied that ABs in atherosclerotic plaques are similar to matrix vesicles and that these may initiate calcification. Atherosclerotic plaques were described as containing lipid-laden VSMCs, which shed calcifying membrane-bound vesicles. In addition, although calcification in atherosclerosis has been detected mainly in association with extracellular structures such as matrix vesicles and extracellular matrix, intracellular calcification has also been observed. These may be calcified cell organelles or calcified structures that have been engulfed by VSMCs.

If the matrix vesicle-like structures in plaques are apoptotic remnants, they should be rapidly cleared by adjacent phagocytic macrophages or VSMCs. In the largely acellular lipid core, phagocytosis may be impaired because of the presence of oxidized lipids that have been shown to compete with ABs for binding to phagocytes. Nonphagocytosed ABs would either undergo secondary necrosis or could proceed to calcify, depending on their local environment. Therefore, the presence of efficient phagocytic cells in atherosclerotic lesions is important for effective scavenging and, thereby, regulation of calcium deposition. This concept was tested experimentally by Kim, who demonstrated that rat aortic segments calcified when placed in Millipore chambers in the peritoneal cavity, but when aortic segments were grafted and contained inflammatory cells, calcification was minimal. Finally, in support of the role of apoptosis in calcification in vivo, mice lacking matrix Gla protein or osteoprotegerin develop medial vascular calcification and both proteins have potential roles in apoptosis.

In summary, we have shown that apoptosis precedes human vascular calcification in vitro and that VSMC-derived ABs can concentrate and crystallize calcium. Therefore, what remains to be tested is whether a lack of phagocytosis of ABs leads to the progression of vascular calcification.

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References


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Materials and Methods

Cell culture

The culture medium used was Medium 199 (M199) (Sigma) buffered with 3.7 g/L NaHCO₃ and 5% CO₂, and supplemented with 100 IU/ml penicillin (Sigma), 100μg/ml streptomycin (Sigma), 250ng/ml amphotericin B (Sigma) and 4 mmol/L of L-glutamine (Sigma). Human VSMCs were obtained from non-atherosclerotic areas of aortas from organ donors of various ages (males and females from 8 to 65 years old). The cells were prepared from medial explants of tissue and were confirmed as smooth muscle cells by positive staining with monoclonal antibodies against αSM-actin and calponin (Sigma). Cells were maintained in M199 containing 20% FCS (heat-inactivated, Sigma) and were used between passages 3 and 11. At least three different isolates from individuals of different ages were used in experiments. In experiments where nodule formation and calcification were studied, VSMCs were plated subconfluently in 12 well plates (Falcon), allowed to grow beyond confluence and when nodule formation initiated, this time-point was termed day 0. In experiments where ZVAD.fmK was added to cultures, ZVAD.fmK was added at day 0. ZVAD.fmK and a control fmK compound, YAAD.fmK were from Bachem (Saffron Walden, Essex, UK) and stored as a stock at -20°C in DMSO.

The human VSMC line 'HASMC 66' was derived from coronary atherosclerotic plaques from a 49 year old male and was SV40 immortalised¹. These cells were maintained in M199 containing 10% FCS.
**Preparation of apoptotic bodies (ABs)**

Induction of apoptosis in VSMCs was achieved by either serum-starvation of HASMC 66 or by addition of anti-Fas IgG (100ng/ml, clone CH11, Upstate Biotechnology,) and cycloheximide (10µg/ml, Sigma) in serum-free M199 to primary human VSMCs for 24 hours. ABs were harvested by tapping the tissue culture flasks and centrifuging the ABs at 2,500 rpm. Pellets of ABs were washed three times with HBSS (without calcium or magnesium) and total protein was estimated by the Lowry method using BioRad reagents. Bovine serum albumin was used as a protein standard (BioRad).

**Detection of apoptosis**

**Nuclear morphology**

In experiments where nuclear morphology was analysed in nodules, 7 day nodules were cultured in 35mm dishes in M199/20% FCS and monitored live at 37\(^0\)C. 2µg/ml bisbenzimide (final concentration, Hoechst no. 33258, Sigma) was added to the medium and the nodules were monitored by capturing serial optical sections by confocal microscopy (Leica TCS-SP-MP) so that the whole nodule could be imaged. Apoptotic cells were quantified by counting fragmented or condensed nuclei throughout a whole nodule. For this analysis, the ‘glow over’ mode was used since this gave the best contrast between saturated and non-saturated fluorescence.
Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP (deoxyuridine-triphosphate)-biotin nick end labelling (TUNEL)

TUNEL labelling was performed as described previously. Briefly, sections of VSMC nodules were incubated with 0.1 U/μl TdT and biotin-dUTP (Roche) for 30 minutes, followed by incubation with streptABComplex/HRP (DAKO) for 20-30 minutes at RT. Binding sites of biotin-dUTP were visualized by incubation with 1 mg/ml diaminobenzidine plus 0.02% (v/v) H₂O₂. The counterstain was Mayer’s haematoxylin. Negative controls were stained identically except the enzyme TdT was omitted.

Phosphatidyl serine (PS) exposure

For PS externalisation studies in nodules, day 7 nodules were cultured in 35mm dishes in M199 containing 20% FCS and kept live at 37°C while being monitored by time-lapse confocal microscopy. Annexin V-FITC (4μl/ml, Clontech) and propidium iodide (500nmol/L, Clontech) were added to the culture medium and monitored for initiation of annexin V binding.

Detection of calcification in multicellular nodules

VSMCs were grown in 12-well plates, washed three times with PBS and fixed in 4% formaldehyde in PBS for 45 minutes at 4°C. The cultures were then washed in distilled water and exposed to the calcium binding dye alizarin red (2% aqueous, Sigma) for 5 minutes, then washed again with distilled water. In addition, we have confirmed by energy dispersive microanalysis that alizarin red binds to calcium as well as hydroxyapatite in nodules (data not shown). Calcification in VSMCs cultured in 12-well plates was quantified by extracting the calcified material in each well with 0.1M HCl overnight at
room temperature and then quantifying the calcium using cresolphthalein purple and CaCl₂ to generate a standard curve. This method has been used for quantitation of vascular and bone calcification in culture. The amount of calcium in each well was corrected for total protein by dissolving the well contents with 0.1M NaOH and 0.1% SDS and performing a protein assay as above. In experiments where apoptosis was induced in nodular cultures with anti Fas IgM and cycloheximide, since this resulted in apoptosis of the monolayer and detachment of the nodules, cells and nodules were collected by centrifugation before calcium was extracted with 0.1M HCl.

\[ {}^{45}\text{Ca accumulation into ABs} \]

To measure calcium accumulation, the method of Hashimoto et al was used and was modified minimally. The calcifying reaction mixture contained \( {}^{45}\text{Ca} \) (approximately 50,000cpm), 2.2mM CaCl₂, 50mM TES, 85mM NaCl, 15mM KCl, 1mM MgCl₂, 1.6 mM KHPO₄, 10mM NaHCO₃, 1% penicillin/streptomycin and, pH7.6. 40μg of ABs were included in each reaction mix and the samples were vortexed and incubated at 37°C for 24 hours. The samples were then centrifuged at 6,500 rpm for 10 minutes, the supernatants collected and the pellets were washed in cold calcifying medium. The washed pellets were dissolved in 0.1M HCl for 1 hour and then placed in HiSafe scintillation fluid for scintillation counting. The amount of \( {}^{45}\text{Ca} \) taken up by the ABs was expressed as % accumulation. Disodium ATP (100% pure, Roche) and Nonidet P-40 (NP-40, Sigma) were included in some experiments.

A synthetic cartilage lymph calcification buffer was also used, described by Kirsch and colleagues. This buffer consisted of 2mM Ca\(^{2+}\) and 1.42 mM PO\(_4\)\(^{2-}\), in addition to 104.5
mM Na⁺, 133.5 mM Cl⁻, 63.5 mM sucrose, 16.5 mM Tris, 12.7 mM K⁺, 5.55 mM D-glucose, 1.83 mM HCO₃⁻, 0.57 mM Mg²⁺, and 0.57 mM SO₄²⁻ pH 7.4.

Energy dispersive microanalysis (EDX)

Adherent ABs mounted on polyester resin sheets were rinsed in distilled water, quench-frozen in melting propane and freeze-dried in an Edwards 360. Elemental content of ABs was performed by EDX in a Philips XL30-FEG system equipped with an Oxford Instrument ISIS, GEM spectrometer. Regions of interest were probed with a reduced raster for 100 seconds live time. Detector efficiency for calcium and phosphorous were determined using isoatomic droplets and normalising the counts for phosphorous against calcium to determine a correction factor for x-ray counts for phosphorous (79.75%).

Calcium detection by confocal microscopy

ABs were cultured in 35mm dishes and incubated with calcein-AM (2μmol/L, Molecular Probes). 30 minutes after the addition of calcein the bodies were washed three times in serum-free medium and green fluorescence was examined using the confocal microscope.

Statistical analysis

Analysis of variance (ANOVA) was used to test for differences within groups of samples. Student’s t-test was used to compare two means. P<0.05 was considered statistically significant.
References for Online Methods Supplement


Online Supplement for MS#1489

Supplement Figure.

This figure shows staining of paraffin-embedded sections for the proliferation marker, Ki-67. This monoclonal antibody (clone MIB-1, DAKO) was used at a dilution of 1:50 and was developed using the ABC immunoperoxidase technique and visualised by DAB. All sections were treated identically (except B) and are counter-stained with haemotoxylin.

A. A positive control for Ki-67 staining; a tubulo-villous adenoma of the colon. Note that many cells are stained positively (brown staining).

B. A negative control for A, where an identical concentration of mouse IgG was used in place of Ki-67 antibody.

C. A section through a day 7 control nodule. Note that no positive brown staining was detected.

D. A section through a day 7 ZVAD.fmk-treated nodule. As for C, no cells were positive for the Ki-67 antigen.