Role of Glutaraldehyde in Calcification of Porcine Aortic Valve Fibroblasts

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Glutaraldehyde-treated porcine aortic valve xenografts frequently fail due to calcification. Calcification in the prostheses begins intracellularly. In a previous study, various types of cell injury to canine valvular fibroblasts, including glutaraldehyde treatment, led to calcification. An influx of extracellular Ca^{2+} into the phosphate-rich cytosol was theorized to be the mechanism of calcification. To test the Ca^{2+} influx theory, cytosolic Ca^{2+} and P_i concentrations were assessed in glutaraldehyde-treated porcine aortic valve fibroblasts, and their relationship to a subsequent calcification was studied. Glutaraldehyde caused an immediate and sustained massive cytosolic Ca^{2+} increase that was dose dependent and a several-fold increase in P_i . Calcification of cells followed within a week. The earliest calcification was observed in blebs formed on glutaraldehyde-treated cells. Live control cells or cells fixed with glutaraldehyde in Ca^{2+} -free solution did not calcify under the same conditions. Concomitant increases in Ca^{2+} and P_i in glutaraldehyde-treated cells appear to underlie the mechanism of calcification, and the presence of extracellular Ca^{2+} during glutaraldehyde fixation promotes calcification. (*Am J Pathol* 1999, 154:843–852)

Calcification in human aortic valves (AVs) occurs commonly. It begins at approximately the age of 20 years, increases progressively with age, and gives rise to calcific aortic stenosis in 2.9% of the elderly population.^{1,2} Calcification is enhanced in damaged valves such as those with congenital anomalies or rheumatic valvulitis. Because of the shortage of human replacement prosthetic heart valves, aortic valves with calcific stenosis have been replaced with glutaraldehyde (GA)-fixed valvular prostheses (GFVPs) from animals. GA fixation eliminates antigenicity and provides GFVPs with tensile strength and pliability. Unfortunately, one-third of GFVPs in elderly recipients fail within 10 years mainly due to calcification.^{3,4} Calcification of GFVPs in children, like

other pathological calcifications in general, progresses more rapidly, resulting in their failure in 2 to 5 years.⁵ Forty-two percent of GFVP failures occur in patients younger than 40 years of age.⁶ The mechanism of calcification in GFVPs is incompletely understood. There are currently four mechanistic theories that have been proposed to explain the cause of GFVP calcification: 1) glutaraldehyde fixation, 2) organic matrix composition, 3) mechanical stress, and 4) cell injury theories.

There has been a view that GA molecules retained in GFVPs may cause calcification. GFVPs are known to calcify in rat subcutis, whereas fresh valves provoke inflammation but do not calcify.⁷ The amount of GA in bovine pericardium rat implants has also been shown to have a quantitative relationship with calcific deposits.⁸ However, there have been other reports that question the role of GA in calcification. Fifty percent of GA leaches out of GFVPs in rat subcutis over a period of weeks, and in failed human GFVP xenografts, acid hydrolyzable GA is known to decrease with the graft's duration.^{9,10} Paradoxically, fixation of porcine aortae in a higher concentration of GA diminishes calcification in rat subcutis.¹¹

The role of the extracellular matrix in calcification has also been extensively studied. Collagen, as in other calcifying tissues, has been implicated as a nucleator of apatite in GFVPs.¹² Osteocalcin and osteopontin have been isolated from calcified tissues, including GFVPs, suggesting they may play a role in calcification.^{7,13} However, a study with isolated matrix demonstrated that osteopontin and osteocalcin did not nucleate apatite *in vitro*.¹⁴ Other noncollagenous proteins, especially phosphoproteins, have been implicated in GFVP calcification.¹⁵

In orthotopically grafted GFVPs, calcific deposits have been found selectively in the areas of mechanical stress.¹⁶ Insudation of the plasma contents into the stressed areas may have a role in calcification.¹⁷ Alternatively, relentless oscillations of the valves may let calcified particles migrate and accumulate in the stressed areas.

Calcification in xenografted GFVPs has been shown to begin intracellularly,¹⁸ and several types of cell injury,

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including GA treatment, result in canine aortic valve (AV) fibroblast calcification.¹⁹ Furthermore, removal of cells from GFVPs with detergents and lipid solvents has been shown to prevent calcification.^{20–22} Nevertheless, the cell's role in GFVP calcification has not been studied in detail. Cell injury has long been known to cause an influx of Ca^{2+} into the cell.²³ Recently, there has been increasing evidence supporting that cell injury also increases cytosolic phosphate ($[\text{P}_i]_i$).^{24–26} Concomitant increases in intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) and $[\text{P}_i]_i$ have been theorized to be the underlying mechanism of calcification.²⁷ In the present study, we have tested the hypothesis that calcification in GFVPs results from GA-induced increases in $[\text{Ca}^{2+}]_i$ and $[\text{P}_i]_i$ during the fixation process. Porcine AV fibroblasts were grown in culture and subsequently fixed with GA while measuring cellular and media Ca^{2+} and P_i changes immediately after fixation and over a period of 7 days. GA was found to cause a massive increase in $[\text{Ca}^{2+}]_i$ and several-fold increases in $[\text{P}_i]_i$ followed by calcification of the cells.

Materials and Methods

The acetoxymethyl esters of Fura-2, Indo-1, Fluo-3, Calcium Green-1, and Oregon Green 488 BAPTA-1 (fura-2/AM, indo-1/AM, fluo-3/AM, CaGr-1/AM, and OrGr-1/AM) were purchased from Molecular Probes (Eugene, OR), and GA (50%) was from E. F. Fullam (Schenectady, NY). Fetal bovine serum was obtained from GIBCO (Grand Island, NY). Tissue culture media and analytic grade chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

Porcine aortic valves were obtained from a local slaughterhouse within 30 minutes of sacrifice. Valves for cell culture were transported in ice-chilled RPMI 1640 with 20% fetal bovine serum, 1.25 $\mu\text{g}/\text{ml}$ amphotericin B, and 50 $\mu\text{g}/\text{ml}$ gentamicin sulfate. The valves were treated with 15% collagenase for 15 minutes, endothelial cells were removed with a scalpel, and the remaining tissue was rinsed twice. The tissue was minced with scalpels, explanted, and subcultured in minimal essential medium supplemented with 10% fetal bovine serum, 100 IU of penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin under 5% CO_2 at 37°C and 90% humidity.

Measurement of $[\text{Ca}^{2+}]_i$

Dual-excitation ratiometry of fura-2-loaded cells was performed with a model RM-D Deltascan photometry system (Photon Technology International, Brunswick, NJ). Dual-emission ratiometry of indo-1-loaded cells and single-wavelength fluorescence image analysis of $[\text{Ca}^{2+}]_i$ were performed with a Bio-Rad MRC-1000 confocal microscope (Bio-Rad Microscience, Herts, UK) with a Diaphot inverted microscope (Nikon, Garden City, NY) with krypton-argon and ultraviolet argon ion laser attachments

(Coherent Enterprise, Salt Lake City, UT). LaserSharp 1024 Time Course software (Bio-Rad) and a 40 $\times$ Fluor oil emulsion objective lens with NA of 1.3 (Nikon) were used.

Semiconfluent cells on 25-mm round coverslips were mounted on an Attofluor cell chamber (Molecular Probes) and were loaded with 5 $\mu\text{mol}/\text{L}$ indo-1/AM in phenol-red-free Hanks' balanced salt solution with 25 mmol/L HEPES, and the total calcium was adjusted to 2.0 mmol/L with CaCl_2 , pH 7.4 (HBSS2.0), for 30 minutes at 37°C. The cells were allowed to de-esterify for an additional 30 minutes. Alternatively, the cells were loaded with fura-2/AM for 20 minutes and de-esterified for 20 minutes at 37°C or loaded with 5 $\mu\text{mol}/\text{L}$ fluo-3, 5 $\mu\text{mol}/\text{L}$ CaGr-1/AM, or 5 $\mu\text{mol}/\text{L}$ OrGr-1/AM for 30 minutes at room temperature and de-esterified for 30 minutes. The de-esterification step was omitted for fluo-3. Ratiometry of indo-1-loaded cells was performed at an excitation wavelength (WL_{EX}) of 351 nm and emission wavelengths (WL_{EM}) of 460 and 405 nm, and with fura-2, at WL_{EX} of 340 and 380 nm and WL_{EM} of 510 nm. Single-wavelength $[\text{Ca}^{2+}]_i$ measurements were performed at WL_{EX} of 488 nm and WL_{EM} of 531 nm for CaGr-1 and 523 nm for fluo-3 and OrGr-1.

$[\text{Ca}^{2+}]_i$ in CaGr-1-loaded cells was measured either in HBSS2.0 or in HBSS with the serum level (2.5 mmol/L) of calcium (HBSS2.5). $[\text{Ca}^{2+}]_i$ in selected samples was measured in Ca^{2+} -free HBSS (HBSS-0) or in HBSS2.0 with 10 mmol/L EGTA to determine possible intracellular redistribution of Ca^{2+} caused by GA. To convert fluorescence intensity (FI) to nanomoles per liter, $[\text{Ca}^{2+}]_i$ measurement with CaGr-1 was calibrated using a calibration kit (Bio-Rad, 170-3141) according to the formula $[\text{Ca}^{2+}]_i = K_d[F - F_{\text{min}}]/[F_{\text{max}} - F]$, where K_d is the dissociation constant of CaGr-1, F is the fluorescence of the indicator at the experimental calcium level, and F_{min} is the fluorescence in the absence of extracellular Ca^{2+} ($[\text{Ca}^{2+}]_o$); F_{max} was determined by treating CaGr-1-loaded cells with 1.66 $\mu\text{mol}/\text{L}$ Ca^{2+} ionophore 4-Br-A23187.²⁸ The LaserSharp software has a built-in calibration program. To simulate GFVP preparation, $[\text{Ca}^{2+}]_i$ in cells treated with 0.2% to 0.6% GA was measured at room temperature. Optimal loading of the dyes and the topography of GA-induced changes in $[\text{Ca}^{2+}]_i$ were assessed with fluorescence images. Cells with mitochondrial loading of the dyes and signs of injury, ie, blebs, were excluded from the study. To illustrate the distribution of $[\text{Ca}^{2+}]_i$ increase in GA-treated cells, 12-band pseudocolors ranging from blue to white were assigned to 0 to 255 gray scales of captured images with the LaserSharp program.

$[\text{P}_i]_i$ Measurement

A total of 5×10^5 cells suspended by trypsinization in triplicate were fixed with 0.6% GA in HBSS-0 or HBSS2.5 at room temperature, rinsed three times with 25 mmol/L HEPES in 0.9% saline solution, pH 7.4, by centrifugation, and pelleted in microfuge tubes. The pellets were ground with a motorized pestle, dissolved in 0.25 ml of 0.6 mol/L

perchloric acid, neutralized with 1.25 ml of 3 mol/L KOH, and centrifuged at $16,000 \times g$ for 5 minutes. The supernatants were stored at -80°C for up to a week. P_i in the supernatant was measured by an ammonium molybdate color reaction at 720 nm by the method of Ohnishi et al.²⁹ The extraction procedures were carried out on ice in a cold room.

Calcification in Vitro

Confluent cells in 25-cm² flasks in triplicate were treated with 0.6% GA in HBSS2.5 for 24 hours at room temperature. After rinsing away the GA, cells were incubated in 10 ml of HBSS2.5 without GA for 7 days at 37°C . Live cells incubated in minimal essential medium with 2.5 mmol/L total calcium (MEM2.5) in a CO₂ incubator or cells fixed with 0.6% GA in HBSS-0 and incubated in HBSS2.5 served as controls. Samples were harvested on days 1, 2, 3, and 7. Free Ca^{2+} in the supernatant was measured with an ion-selective electrode (Radiometer, Cleveland, OH). P_i was measured by an ammonium molybdate color reaction at 720 nm.¹³ The cells were subsequently fixed in 10% formalin in phosphate-buffered saline for 3 minutes and stained with 0.03% calcein for 1 minute. Calcein is a fluorochrome that has been used to fluoresce calcium phosphate deposits in mineralizing cell cultures,³⁰ and fluorescence densitometry of calcein-stained samples has been shown to be a suitable technique for quantification of calcific deposits in fibroblasts.¹⁹ Fluorescence densitometry was performed on 10 tandem areas, using a $10\times$ objective lens at WL_{EX} of 488 nm and WL_{EM} of 520 nm. To capture detached cells and blebs, the supernatant was filtered through a polycarbonate membrane filter with pore size of 0.2 μm using a syringe. The surface area of the filter was corrected for 25 cm², and the FI was added to that of attached cells. Polycarbonate membrane is neither autofluorescent nor stainable with calcein.

Electron Microscopy

Cells suspended by trypsinization were fixed with 0.6% GA and incubated in HBSS2.5 in airtight plastic tubes on a rotary drum at 37°C for a week. Cell pellets were embedded in agar, cut into 1-mm³ cubes and fixed in 4% GA in cacodylate buffer, post-fixed in OsO₄ in s-collidine buffer, dehydrated in ethanol and propylene oxide, and embedded in Epon. Thin sections were stained with uranyl acetate and lead citrate and examined with a Philips 200 microscope. Selected area electron diffraction was performed at 80 kV. The diffraction pattern was measured according to $d = D/K$, where d is interplanar spacing, D is diameter of the powder pattern, and K is the camera constant; K is determined by diffraction of ThCl sputtered grids at each diffraction of the sample.³¹ Crystals were identified with the Inorganic Powder Diffraction Files, Joint Committee on Powder Diffraction Standards (Philadelphia, PA).

Figure 1. Photometric data of $[\text{Ca}^{2+}]_i$ response to 0.2% to 0.6% GA treatment in CaGr-1-loaded porcine AV fibroblasts. GA caused an immediate increase in $[\text{Ca}^{2+}]_i$ followed by progressive increases in $[\text{Ca}^{2+}]_i$ in a dose-dependent manner.

Statistical Analysis

Statistical analysis was performed using the SigmaStat software (SPSS, Chicago, IL).

Results

In fura-2- and indo-1-loaded fibroblasts, GA treatment caused an immediate increase in the fluorescence ratio that was soon followed by a decrease in FI (data not shown). In contrast, when 0.6% GA was added to 1 $\mu\text{mol/L}$ potassium salt of fura-2 in cell-free 6 mmol/L HEPES buffer containing 2 mmol/L CaCl_2 , pH 7.4, and the ratiometry was repeated, GA completely abolished fura-2's fluorescence within minutes. The quenching of fluorescence was less pronounced in cells loaded with fluo-3 or OrGr-1. No such fluorescence depression was noted in CaGr-1-loaded cells, although the spike in FI still occurred. The spike was produced even when CaGr-1-loaded cells were in HBSS-0 or in HBSS2.0 with 10 mmol/L EGTA. Because GA appeared to have quenching effects on fura-2, indo-1, OrGr-1, and fluo-3 and because CaGr-1 fluorescence did not appear to be affected by GA, CaGr-1 was chosen for $[\text{Ca}^{2+}]_i$ measurements.

When CaGr-1 was used, GA caused a progressive rise in FI for over 1 hour and in a dose-dependent manner. FI of cells treated with 0.6% GA approached the F_{max} obtained from the cells treated with Ca^{2+} ionophore (1.66 $\mu\text{mol/L}$ 4-Br-A23187). Throughout the experiment, the intensity of cell-free background changed little or none. CaGr-1 FI was converted to $[\text{Ca}^{2+}]_i$ using the aforementioned BioRad kit and fluorescence equation. Photometric data for CaGr-1-loaded cells are shown in Figure 1. $[\text{Ca}^{2+}]_i$ tended to be higher when measured in HBSS2.5 than in HBSS2.0. Otherwise, the $[\text{Ca}^{2+}]_i$ response to GA was similar in both solutions (data not shown). Pseudo-colored images demonstrated the increase of $[\text{Ca}^{2+}]_i$, especially in the perinuclear area (Figure 2).

GA also caused several-fold increases in $[\text{P}_i]_i$ within hours. It remained elevated for 24 hours and returned to

Figure 2. A montage of pseudocolored images of a chain of CaGr-1-loaded fibroblasts treated with 0.6% GA demonstrating increases in $[Ca^{2+}]_i$. Images were captured at time zero and every 12 minutes. $[Ca^{2+}]_i$ increase is seen mainly in perinuclear areas. Pseudocolors ranging from blue to white were assigned to 0 to 255 gray scales.

the ground status in a week. The increase in $[P_i]_i$ was greater in cells fixed with 0.6% GA in HBSS2.5 than those fixed in HBSS-0 (Figure 3). A slight decrease in Ca^{2+} and an increase in P_i in the supernatant during GA fixation for over 24 hours was noted.

Cells fixed with 0.6% GA and incubated in HBSS2.5 progressively depleted Ca^{2+} and P_i from the supernatant, whereas cells fixed in HBSS-0 did not (Figure 4). After an initial decline on day 1, the slopes of ion deple-

Figure 3. $[P_i]_i$ in cells treated with 0.6% GA in HBSS2.5 (solid bars) and in HBSS-0 (gray bars). GA caused an increase in $[P_i]_i$ within hours especially in cells fixed in the presence of $[Ca^{2+}]_o$. $[P_i]_i$ remained elevated for 24 hours and returned to the ground status in a week. Error bars indicate SEM.

tions leveled off for a few days followed by a precipitous decline in day 7. No ion depletion from the supernatant of the unfixed live control cells was noted for a week. In the supernatant of cells fixed in HBSS-0, the precipitous decline did not occur at day 7 and ion concentrations remained level.

Calcein has been shown to have a greater sensitivity for calcium detection than tetracycline or alizarin red-S.¹⁹ When stained with calcein, the control cells without GA treatment displayed a higher FI in nuclei than the cytoplasm. The higher nuclear FI was also seen in routinely cultured cells without incubation in MEM2.5. Cells fixed with 0.6% GA in HBSS2.5 began to form blebs with increased calcein FI during the fixation period. Blebs frequently detached from the cells and floated in the supernatant. Compared with other types of cell injury,²⁷ the size and the number of blebs formed by GA-treated cells were smaller. Most blebs were smaller than the corresponding cellular nuclei. The number of calcified blebs increased mildly during the incubation in HBSS2.5 followed by calcification of the entire cells by day 7. What appeared to be mitochondria frequently emitted more intense fluorescence in the earlier stages of the incubation. Nuclei were usually spared from calcification in heavily calcified cells (Figure 5). Calcified cells also tended to detach from the flask surface. A comparison of media Ca^{2+} depletion with cellular calcein FI revealed an inverse linear relationship with a regression coefficient of 0.79 (Figure 6). Blebs were scanty to absent and calcifi-

Figure 4. A: Depletions of Ca^{2+} from HBSS2.5 by cells fixed with 0.6% GA in HBSS2.5 (●) and in HBSS-0 (○). HBSS2.5 Ca^{2+} leveled off for a few days after an initial drop. A precipitous decline of Ca^{2+} occurred with cells previously fixed in HBSS2.5, whereas Ca^{2+} remained level with cells previously fixed in HBSS-0. B: A progressive depletion of P_i from HBSS2.5 was caused by cells fixed with 0.6% GA in HBSS2.5. Cells fixed in HBSS-0 and incubated in HBSS2.5 did not deplete P_i .

cation was not evident in cells fixed in HBSS-0, incubated in HBSS2.5, and stained with calcein.

Electron microscopy of cells fixed in 0.6% GA and incubated in HBSS2.5 frequently revealed blebs. The earliest (least) calcific deposits were seen in blebs, either in apposition to the inner surface of plasma membrane or in what appeared to be swollen mitochondria (Figure 7). Blebs detached from the cells frequently contained large amounts of needle-shaped apatite. In heavily calcified cells, apatite deposited diffusely in the cytoplasm in apposition to the surface of organelles. In occasional cells, only nuclei were selectively calcified. Although a limited number of cells were examined, cells with nuclear calcification did not appear to form calcifying blebs. Cells fixed in HBSS-0 showed only ruffling of the plasma mem-

brane resulting from trypsinization but no blebs or calcific deposits. Electron diffraction of calcified cells yielded the powder pattern consistent with hydroxyapatite.

Discussion

Crystals are formed by the orderly packing of atoms and molecules that are mainly brought together by van der Waals attractive forces.³² The attractive forces are weaker than chemical bonds, and crystal nucleation requires supersaturation of lattice ions. The kinetics of crystal formation differ from that of chemical reactions in that once nucleation takes place after a latent period, the subsequent crystal growth is autocatalytic and occurs rapidly. Crystal nucleation can be entirely dependent on lattice ion concentrations, in which case it is referred to as homogeneous nucleation, or the nucleation may be catalyzed by solid particles, a process known as heterogeneous nucleation.^{33,34} Because of the abundance of solids that exist in cells, Ca^{2+} and P_i crystallization *in vivo* most likely occurs by heterogeneous nucleation. As a consequence, calcific deposits *in vivo* are frequently observed in apposition to membranous surfaces.²⁷ Under normal conditions, the serum Ca^{2+} and P_i concentrations are low enough that their solubility coefficient is not exceeded, and homogeneous nucleation does not occur. The addition of a minute particle of apatite to serum, however, results in a rapid proliferation of apatite crystals until a new Ca^{2+} and P_i equilibrium is reached at a value approximately one-third the normal serum level.³⁵ Thus, calcific deposits *in vivo* appear to exist in a disequilibrium with serum Ca^{2+} and P_i . Apatite nucleation is prevented by plasmalemmal partitioning of the high $[\text{Ca}^{2+}]_o$ away from the high $[\text{P}_i]_i$. Live tissues also apparently have a capacity to curb the spread of calcification probably by secreting inhibitors for apatite growth; hence, osteocytes survive in densely calcified bone.

In our studies, CaGr-1 was used to assess the influx of Ca^{2+} into AV fibroblasts during fixation with GA. $[\text{Ca}^{2+}]_i$ in resting valvular fibroblasts ranges between 100 and

Figure 5. A: Calcein fluorescence micrographs of live control cells incubated in MEM2.5 for a week. Nuclei are brighter than the cytoplasm. B: Calcein-stained cells fixed in 0.6% GA in HBSS2.5 for 24 hours and incubated in HBSS2.5 for a week. Calcified cells and blebs (arrow) emitted strong fluorescence. Nuclei of heavily calcified cells are usually spared from calcification (arrowhead). The pale, noncalcified cells in the background are due to automated camera exposure time. Magnification, $\times 300$.

Figure 6. A: Calcein fluorescence of cells treated with 0.6% GA and incubated in HBSS2.5 showed an inverse relationship with Ca^{2+} depletion. B: Regression analysis yielded a coefficient of 0.79. Elimination of the outlier on day 1 increased the coefficient to 0.99.

150 nmol/L, whereas $[\text{Ca}^{2+}]_o$ is approximately 1.5 mmol/L. Thus, there is a steep electrochemical gradient of Ca^{2+} across the plasmalemma. GA fixation led to dose-dependent, progressive increases in $[\text{Ca}^{2+}]_i$ over a period of 1 hour (see Figures 1 and 2). After an initial immediate increase in $[\text{Ca}^{2+}]_i$, there was a relatively slow and sustained increase when compared with the transient surges usually associated with signal transduction. This increase eventually reached a value comparable to that obtained when unfixed porcine AV fibroblasts were treated with Ca^{2+} ionophore 4-Br-A23187 and subsequently allowed to equilibrate with $[\text{Ca}^{2+}]_o$. These observations suggest that GA initially permeabilizes the plasmalemma to Ca^{2+} and allows leakage into the cell. Intracellular Ca^{2+} overload exerts many deleterious effects to the cell and is considered to be the underlying mechanism of cell death, both apoptosis and necrosis.^{23,36} Physiological increases of $[\text{Ca}^{2+}]_i$ stimulate mitochondrial ATP synthesis, but a massive increase in mitochondrial Ca^{2+} collapses the proton-motive electric potential of the mitochondrial inner membrane and arrests ATP synthesis.³⁷ In acute cell injury, mitochondria themselves frequently calcify.

The mechanism of GA fixation is complex and incompletely understood. GA is believed to cross-link proteins mainly by piperidine ring formation via a Schiff base reaction.³⁸ Despite its extensive use for fixation of tissues for electron microscopy, relatively little is known about the detailed effects of GA on cell metabolism. As it diffuses into the cell, GA is likely to inactivate many enzymes. The progressive increase of $[\text{Ca}^{2+}]_i$ in GA-treated cells suggests that Ca^{2+} - Mg^{2+} ATPase of the plasma membrane no longer functions. GA has been shown to bind specif-

ically to the ATP-binding sites of sarcoplasmic reticulum Ca^{2+} pumps and to abolish the pump function.³⁸ A similar inactivation of the plasma membrane Ca^{2+} - Mg^{2+} ATPase seems likely. The cessation of ATP synthesis by overloaded $[\text{Ca}^{2+}]_i$ and a generalized devitalization of the cell by GA are likely to contribute further to the abolition of the plasma membrane Ca^{2+} pump activity. On devitalization of the cell, $[\text{Ca}^{2+}]_i$ will equilibrate with $[\text{Ca}^{2+}]_o$. Inhibition of plasma membrane Ca^{2+} pumps has been shown to induce calcification of canine AV fibroblasts.¹⁹

In contrast to Ca^{2+} , P_i is mainly intracellular. It exists mostly in organic forms. The $[\text{P}_i]_i$ in resting cells is estimated to range between 0.5 and 2 mmol/L by ^{31}P nuclear magnetic resonance (NMR).³⁹ NMR tends to underestimate $[\text{P}_i]_i$, detecting only one-third of the values obtained by chemical analysis.²⁴ $[\text{P}_i]_i$ is closely linked to hydrolysis of ATP, and in metabolically active cells, eg, rat skeletal muscle, it can be as high as 8 mmol/L.⁴⁰ With the recent advent of NMR, it became apparent that $[\text{P}_i]_i$ increases during cell injury, usually in association with a reversal of the ATP/ P_i ratio.^{24–26} In our GA-treated AV fibroblasts, GA led to a several-fold increase in $[\text{P}_i]_i$ over a period of hours that remained elevated for at least 24 hours (see Figure 3). In addition, the presence of Ca^{2+} in GA solution was associated with a greater increase in the $[\text{P}_i]_i$ than when GA solution contained no Ca^{2+} . Overloaded $[\text{Ca}^{2+}]_i$ has been shown to activate cell enzymes, ie, phospholipases and endonucleases.³⁶ The additional increase of $[\text{P}_i]_i$ in GA-treated cells can be attributed to the activation of hydrolytic enzymes for organic phosphates by Ca^{2+} and the arrest of ATP synthesis in Ca^{2+} -overloaded mitochondria. The occurrence of calcification in

Figure 7. Electron micrographs of suspended cells fixed in 0.6% GA for 24 hours and incubated in HBSS2.5 for a week. A: A cell with calcified blebs. No calcific deposits are seen in the cytoplasm. Magnification, $\times 7500$. B: A closer view of the tip of a bleb indicated by the arrowhead in A. Needle-shaped apatite crystals abut against the inner surface of the membrane. Magnification, $\times 32,500$. C: A calcific deposit in what appears to be a swollen mitochondrion in a bleb. Magnification, $\times 8300$. Mitochondria outside of the bleb show matrix condensation but no apatite. Radiating needles are seen in a closer view of the deposit (inset, $\times 34,000$). D: A heavily calcified cell with diffuse apatite deposition in the cytoplasm. The nucleus did not calcify. Magnification, $\times 7500$. E: A higher magnification of the perinuclear area in D. Needle-shaped apatite crystals are apparent. Magnification, $\times 32,500$. F: Electron diffraction of a calcified cell yielded the powder pattern consistent with hydroxyapatite. G: In occasional cells, the deposits were selectively seen in nuclei. No calcified blebs but a ruffled plasma membrane due to trypsinization were present. Magnification, $\times 8500$. H: A closer view of an area in G demonstrates needle-shaped apatite in the nucleus. Magnification, $\times 25,000$.

mitochondria in GA-treated cells concurs with such Ca^{2+} increases in mitochondria. Alkaline phosphatase has been implicated to have a role in calcification, especially of matrix vesicles.⁴¹ The enzyme may hydrolyze phosphoesters and thereby contribute to the increase in $[\text{P}_i]_i$ in GA-treated cells. The enzyme has been shown to persist in cells of GFVPs.^{42–44} The gradual decline of $[\text{P}_i]_i$ in a week after a several-fold increase in $[\text{P}_i]_i$ in hours in GA-treated cells is evidently due to its leakage into the supernatant. P_i in the supernatant increases in association with the decrease in $[\text{P}_i]_i$ (results not shown). In our study, the $[\text{Ca}^{2+}]_i \times [\text{P}_i]_i$ products in GA-treated cells exceed by several times that of serum, reaching values sufficient for heterogeneous nucleation of apatite. Valvular fibroblasts not treated with GA and maintained under otherwise similar conditions did not calcify. The occurrence of calcification in GA-treated cells but not in live control cells under similar conditions attests that the influx of $[\text{Ca}^{2+}]_o$ into the P_i -rich cytosol is most likely the mechanism of calcification. The absence of calcification in cells fixed in HBSS-0 in comparison with cells fixed in HBSS2.5 is consistent with a necessary Ca^{2+} influx during the early stage of GA fixation, presumably when the $[\text{Ca}^{2+}]_i \cdot [\text{P}_i]_i$ products are at the peak, leading to subsequent calcification of the cells. GA-treated cells apparently remain permeable to Ca^{2+} as evidenced by the progressive depletion of Ca^{2+} from HBSS2.5 (see Figure 4A). In addition to the progressive Ca^{2+} depletion from HBSS2.5, the P_i content of the buffer also diminished with time, suggesting intracellular nucleation of Ca^{2+} and P_i was occurring in GA-treated fibroblasts.

After an initial relatively rapid depletion rate, HBSS2.5 ion depletions lagged for a few days, then progressed rapidly once again. These observations suggest that the intracellular apatite mass grows to a critical size and levels off before additional, more rapid extraction occurs from the buffer. There was also observed a relative delay in HBSS2.5 P_i depletion, when compared with Ca^{2+} . This delay was most likely attributable to a higher $[\text{P}_i]_i$ than $[\text{Ca}^{2+}]_i$ found initially in cells. Cells fixed in Ca^{2+} -free media did not deplete P_i (see Figure 4B). Calcein fluorescence densitometry studies of cells and blebs revealed that the earliest calcification preceded the aforementioned lag phase and occurred in blebs and mitochondria. This was followed by intense FI in the fibroblast soma (see Figure 5B). HBSS2.5 Ca^{2+} depletion significantly and inversely correlated with cellular calcein FI, further indicating that intracellular nucleation occurred concomitantly with media ion depletion (see Figure 6). Interestingly, the ion depletion lag phase is not observed in other types of cell injury such as anoxia or repeated freezing and thawing,¹⁹ presumably because membranes are damaged. Membranes are well preserved after GA treatment (see Figure 7). The mechanism of P_i entry into GA-treated cells was not addressed in the present study, but in functioning cells, P_i is co-transported along with Na^+ .³⁹ In this study, HBSS2.5 is tailored for the short-term experiments of cellular calcification, and the $[\text{Ca}^{2+}]$ was higher, whereas $[\text{P}_i]$ was lower than in serum. This medium, however, allowed the identification of calcified cells within a week.

We also observed that the nuclei of control valvular fibroblasts exhibited a stronger calcein fluorescence than the cytosol. The nuclear concentration of Ca^{2+} is higher or lower than the cytosol depending on the type and the physiological status of the cells and is believed to have a role in cell proliferation. The nuclear membrane, which is continuous with the endoplasmic reticulum membrane has its own Ca^{2+} transport mechanisms, including inward Ca^{2+} pumps and inositol 1,4,5-trisphosphate receptors.⁴⁵ The observation of nuclear calcification indicates that nuclear Ca^{2+} and P_i can reach sufficiently high levels for apatite nucleation. Nuclear calcification has been described in calcification of GFVPs *in vivo*.¹⁸

In addition to nuclear calcification, calcification may be promoted in injured cells through blebbing. Blebbing has long been known to occur in cell injury.⁴⁶ Blebs have been shown to be associated with Ca^{2+} overload in injured cells and to contain higher concentrations of Ca^{2+} than the remaining cytosol.^{47,48} Cells apparently have a capacity to condense overloaded Ca^{2+} into blebs. Blebbing may be a cellular defense mechanism to protect against Ca^{2+} overload. Isolated blebs formed by senescent canine AV fibroblasts have been shown to readily calcify in MEM2.5,¹⁹ and calcification in membranous cell debris has been observed in GFVPs removed from human hearts.⁴⁹ In a variety of human calcinosis, including aging aortic valves, bleb-like membranous vesicles are the predominant foci of calcification regardless of the type of tissues involved.²⁷ It has been proposed that matrix vesicles are blebs formed by apoptotic cells. For example, hypertrophic chondrocytes of the epiphysis, the major producer of matrix vesicles, undergo apoptosis.^{see 27}

Our *in vitro* data are consistent with the hypothesis that blebbing may contribute to calcification in GFVPs. Calcein fluorescence of fibroblasts fixed in HBSS2.5 containing GA frequently revealed cellular blebbing (Figure 5), and the earliest calcific deposits were observed in these blebs. Electron microscopy showed these deposits were usually found either in close apposition to the inner surface of the plasma membrane or in swollen mitochondria. Cells and blebs that became detached often contained large amounts of needle-shaped crystals that were confirmed by electron diffraction to be hydroxyapatite (Figure 7). By the 7th day after GA exposure, calcification of entire cells was observed. In heavily calcified cells, apatite was deposited throughout the cytoplasm but usually in apposition to organelle surfaces. Blebs were scanty to absent, and calcification was not evident in cells fixed in HBSS-0 containing GA.

Thus, our studies of porcine AV fibroblasts suggest that the GA fixation process itself may be an important contributor to the calcification that occurs following after with GFVP. The data suggest that GA fixation leads to an early, sustained influx of Ca^{2+} into the valvular cell followed by apatite nucleation. In addition to the fixation process, others have provided evidence that tissue injury before GA fixation may affect the subsequent calcification of GFVPs. A delay in GA fixation of bovine pericardium and porcine aorta intensifies calcification of GFVPs in rat subcutis.^{11,50} Furthermore, anoxic exposure at

room temperature before GA fixation resulted in a heavy calcification of GFVPs, whereas the exposure at 4°C significantly reduced calcification in rat subcutis.⁵¹ It has been theorized that GA preserves certain structures that promote calcification during the early stage of fixation, thus resulting in delayed calcification.⁵² The major difficulty of the Ca^{2+} influx theory is linking GA-induced cell calcification, or any short-term calcification, with the slow progression of calcification of GFVPs that occurs *in vivo*. Calcification in GFVPs xenografted in human hearts progresses over years^{4,5} and, in aging human aortic valves, over decades.¹

Several explanations can be offered for the delayed calcification *in vivo*. First, a variety of calcification inhibitors have been identified in tissue fluid.⁵³ Fibroblasts inhibit calcification of bone cells cultured in the same dish.⁵⁴ Cryopreserved AV allografts with viable cells survive twice as long as GFVPs in human hearts.⁵⁵ Thus, calcification of GFVPs *in vivo* appears to be delayed by cellular inhibitors. Second, GFVPs are usually fixed in a Ca^{2+} -free solution. Therefore, GFVP extracellular Ca^{2+} is likely diluted by the fixative. This could lead to less Ca^{2+} influx into valvular cells during fixation and delayed calcification. Third, nucleated apatite during GA fixation of GFVPs is likely to be dissolved during the storage in Ca^{2+} -free solutions. It has been shown that when calcified rat aortae were decalcified with EDTA, they calcify more readily than the aorta without previous calcification (unpublished data). Once tissues have been calcified in GA, they appear to retain a template for heterogeneous nucleation of apatite. If such a template exists in GFVPs, it could predispose the valves to delayed calcification after grafting. Fourth, cellular membranes may cause delayed calcification. Liposomes made of phosphatidylserine and red cell ghosts readily calcify in serum, suggesting the phospholipid bilayer may serve as a nidus for heterogeneous nucleation.⁵⁶ This may be one of the reasons that removal of cells from GFVPs prevents calcification.¹⁹⁻²¹ In addition, a complex and poorly understood interaction exists between plasma membrane and the extracellular matrix.⁵⁷ The extracellular matrix may play the role of diffusion barrier for both fixative and ions and thus delay calcification. At the same time, the matrix may predispose valvular grafts to this delayed calcification. For example, collagen is known to stimulate calcification in matrix vesicles isolated from epiphyseal growth plates.⁵⁸

Along with tetracycline, calcein has been used for the study of new bone deposition. In view of its relative inertness, acetoxymethyl ester of calcein has also been used as a vital dye. Although calcein has a strong affinity for Ca^{2+} , it also reacts with a variety of divalent metals. However, as opposed to Ca^{2+} and Mg^{2+} , binding of other metal ions quenches the fluorescence of calcein.⁵⁹ This property of the dye has allowed the measurement of labile iron pools in certain cells.⁶⁰ Calcific deposits in GFVPs are usually quantified by atomic absorption spectrophotometry of charred samples. The method is destructive and ignores topography of the deposits. Findings in this study further support that calcein can be useful for monitoring the progress of calcification, espe-

cially in cell cultures. Plasma membrane is impermeable to calcein. Fixation of the cells, eg, with formalin, is usually required for staining of cells with calcein. GA-treated cells obviates the need for the fixation. A small amount of calcein (0.15 $\mu\text{mol/L}$) added to HBSS2.5 allows a continuous monitoring of the progress of calcification in the same cells (results not shown).

In conclusion, cultured porcine AV fibroblasts appear to be a useful model for studies of the Ca^{2+} influx theory of GFVP calcification. GA treatment of cultured fibroblasts led to increases in $[\text{Ca}^{2+}]$, and $[\text{P}_i]$, and intracellular heterogeneous nucleation of apatite. Early cellular blebbing appeared to play a significant role in the calcification process.

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