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Effects of lectins on calcification by vesicles isolated from aortas of cholesterol-fed rabbits

Howard H.T. Hsu [a,*], Ossama Tawfik [a], Francis Sun [b]

[a] *Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, Kansas City, KS 66160-7410, USA*
[b] *Department of Animal Care Facility, University of Kansas Medical Center, Kansas City, KS 66160-7410, USA*

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Abstract

Advanced vascular calcification in atherosclerosis weakens arterial walls, thereby imposing a serious rupturing effect. However, the mechanism of dystrophic calcification remains unknown. Although accumulating morphological and biochemical evidence reveals a role for calcifiable vesicles in plaque calcification, the mechanism of vesicle-mediated calcification has not been fully explored. To study whether vesicles' membrane components, such as carbohydrates, may have a role in vesicle-mediated calcification, the effect of sugar-binding lectins on calcification was investigated. Atherosclerosis was developed by feeding rabbits with a diet supplemented with 0.5% cholesterol and 2% peanut oil for 4 months. Calcifiable vesicles were then isolated from thoracic aortas by collagenase digestion. The histological examination of aortas with hematoxylin counter-staining indicated abnormal formation of large plaques enriched with macrophage-derived foam cells. Fourier transform spectroscopy revealed mild calcification in aortas indicating that advanced stages of heavy calcification have yet to be reached. However, vesicles isolated from the aortas were capable of calcification in the presence of physiological levels of Ca^{2+}, Pi, and ATP. Thus, at this stage of atherosclerosis, aortas may start to produce calcifiable vesicles, but at a level insufficient for substantial formation of mineral in aortas. The assessments by FT-IR analysis and Alizarin red staining indicated that concanavalin A (Con A) substantially increased mineral formation by isolated vesicles. Con A also exerted a marked stimulatory effect on ^{45}Ca and ^{32}Pi deposition in a dose-dependent fashion with a half-maximal effect at 6–10 μg/ml. Either α-methylmannoside or α-methylglucoside, but not mannitol, at 10 mM abolished the stimulation. Con A stimulation was abolished after Con A was removed from calcifying media, suggesting that covalent binding may not be involved in the effect. Galactosides appear to also be implicated in ^{45}Ca and ^{32}Pi deposition since *Abrus precartorius* agglutinin, which specifically binds galactosides, enhanced the deposition. Neither wheat-germ agglutinin that binds *N*-acetylglucoside nor *N*-acetylgalactoside-specific *Helix pomatia* agglutinin was effective, suggesting that the acetylated forms of carbohydrate moieties are either absent in vesicles or may not be involved in calcification. None of these lectins exerted an effect on ATPase. Thus, the effects of lectins appeared to be mediated through interactions with carbohydrate moieties of calcifiable vesicles. Whether stimulation of vesicle-calcification by lectins is of pathological significance in atherosclerotic calcification requires further investigation.

Keywords: Calcification; ATP; Calcifiable vesicle; Lectins

1. Introduction

Advanced arterial wall calcification associated with atherosclerosis can weaken the aorta and thereby

Abbreviations: Con A, concanavalin A; FT-IR, Fourier transform infrared spectroscopy

* Corresponding author. Fax: +1-913-588-7073; E-mail: hhsu@kumc.com

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imposes a serious rupturing effect on its wall [1–3]. The mechanism of dystrophic calcification, however, remains unknown and is likely to involve a number of interacting factors in aorta walls. These factors are also commonly found in bone including osteopontin, osteocalcin, bone morphogenetic proteins, collagens, lipids, and subcellular organelles (for a review see [4]). The precise role of these putative factors in calcification, nevertheless, remains unclear. Investigations derived from morphological evidence [5,6] and a recent in vitro study of calcification by vesicles isolated from atherosclerotic human aortas [7] suggest that mineral-associated vesicles may have a role in vascular calcification. The mechanisms of how these subcellular organelles can initiate calcification, however, have remained unclear. In addition to the enzyme requirement, whether membrane components, such as membrane proteins, lipids, or carbohydrates also have a role requires further studies. Since an enzyme marker study [7] indicated that calcifiable vesicles are likely derived from plasma membranes, cell surface-associated carbohydrates may be present in the vesicles and therefore may exert an effect on vesicle-mediated calcification. To determine whether carbohydrates, if present inside or on the surface of vesicles, play a role in calcification, we hereby report the effect of various carbohydrates-specific binding lectins on vesicle-mediated calcification.

2. Materials and methods

2.1. Induction of atherosclerosis by high cholesterol diet

Four-month-old rabbits were fed with a daily diet supplemented with 0.5% cholesterol and 2% peanut oil (supplied by Harland Teklad) for 4 months.

2.2. Isolation of calcifiable vesicles from aortas

To isolate calcifiable vesicles, the method of Hsu and Camacho for the isolation of vesicles from human aortas was used with a slight modification [7]. Segments of ascending thoracic aortas (about 3 in. long) were removed, the attached fats removed, and immediately submerged in phosphate-buffered saline at 4°C. The tissues were minced into fine pieces and digested for 3 h at 37°C in a crude collagenase solution (15 ml/g of tissue) containing 0.1% of crude collagenase (Boehringer Mannheim, Type B), 0.25 M sucrose, 0.12 M NaCl, 0.01 M KCl, 100 U/ml of penicillin, 1 mg/ml of streptomycin, and 0.02 M TES buffer (*N*-tris[hydroxmethyl]-methyl-2-aminoethanesulfonic acid), pH 7.45. The digests were centrifuged at $800\times g$ to spin down cells and cell debris. The supernatants were then centrifuged at $30\,000\times g$ for 10 min to spin down mitochondria and microsomes. The resultant supernatants were centrifuged at $300\,000\times g$ for 20 min. The pellets were resuspended in TBS (10 mM Tris-buffered saline, pH 7.6)–0.25 M sucrose and centrifuged. The resultant precipitates were then resuspended in 1 ml TBS–0.25 M sucrose to yield a protein concentration of about 0.3 mg/ml.

2.3. Calcium and Pi deposition

The term 'Ca and Pi deposition' is used to describe the process by which Ca or Pi was deposited either as undefined forms of calcium phosphate, or as ions that were taken up by or bound to calcifiable vesicles. To measure Ca or Pi deposition, the method of Hsu and Camacho [7] was used with a slight modification. Unless otherwise stated, the freshly prepared standard calcifying medium (100 μl) consisted of 50 mM Tris, pH 7.65 (37°C), 85 mM NaCl, 15 mM KCl, 1 mM $MgCl_2$, 30 mM $NaHCO_3$, 1.45 mM $CaCl_2$, 2.3 mM Pi, and ± 1 mM ATP. For controls, ATP was omitted. $^{45}Ca^{2+}$ and ^{32}Pi (1×10^6 cpm) were used as tracers. The reaction was initiated by addition of aliquots of calcifiable vesicles (final protein concentration: 15 μg protein/ml) to calcifying media and then incubated for 5 h at 37°C in a water-vapor saturated incubation chamber, which was used to minimize vaporization of the reaction mixture during incubation. At the end of incubation, the reaction mixture was filtered through 0.1 μm pore-size Durapore membranes (Millipore) attached to a Millipore vacuum trap device. The membrane filters were washed twice each with 1 ml of Tris-buffered saline, pH 7.6 (TBS) and then transferred to vials containing scintillation fluids for radioactivity counting. The non-specific $^{45}Ca^{2+}$ or ^{32}Pi binding is defined as the radioactivity non-specifically bound to

the filters under the identical conditions in the absence of calcifiable vesicles ($0.6 \pm 0.2\%$ of the total radioactivity). These non-specific counts were then subtracted from the radioactivity in the presence of calcifiable vesicles with or without ATP under various experimental conditions. Ca or Pi deposition is expressed as nmol Ca or Pi/μg vesicle proteins/5 h and is calculated by the following formula. [cpm with calcifiable vesicles minus non-specific binding/total cpm], multiplied by the concentration of $CaCl_2$ or Pi (nmol/ml), and then divided by the concentration of calcifiable vesicle proteins (μg/ml). Transmission electron microscopy and the ATPase activity measurement indicate that no significant amounts of vesicles and mineral passed through filters although the size-ranges of mineral and vesicles can be smaller than the pore size of the filters (not shown).

2.4. Alizarin red staining for mineral

For histochemical staining, a small segment of aortas was fixed with 2% formalin overnight at 4°C, blocked, 3 μm thin sectioned, dehydrated in 95% ethanol, and then stained with 1% Alizarin red. To stain mineral extracted from the aortas, the residues remaining after collagenase digestion, which was used for vesicle preparations, were further treated with 50 μg/ml proteinase K and 0.5% SDS for 1 h to maximize the removal of proteins and lipids. The mineral was precipitated at $800 \times g$ and then stained with 1% Alizarin red.

To stain mineral deposited by isolated vesicles, calcifying media containing mineral and vesicles after 5-h incubation were spun down at $300\,000 \times g$ and the precipitate was resuspended in 2 ml deionized and distilled water at pH 7.6. The suspension was centrifuged at $300\,000 \times g$. The mineral-containing precipitate was resuspended in 50 μl of neutral water, spotted onto a 3.5-cm culture dish, dried overnight, and stained with Alizarin red.

2.5. Scanning electron microscopy

Calcifiable vesicles in 100 μl TBS were centrifuged and then collected on 0.45-μm nucleopore filters using a vacuum trap. The filters were then fixed, dehydrated, and then processed by a scanning electron microscopy procedure. For examination of mineral deposited by isolated vesicles, the following steps were taken. After a 5-h exposure of vesicles to calcifying media with or without 1 mM ATP, calcified vesicles and mineral deposits were collected in Beckman polyallomer tubes by centrifugation at $270\,000 \times g$ for 30 min. The resulting pellets were washed twice with TBS by centrifugation and then resupended in 50-μl water (pH 7.6). The suspensions were then spotted directly onto the sample holders and air-dried. The dried residues were examined and photographed in a Hitachi scanning electron microscope.

2.6. Fourier transform infrared spectroscopy (FT-IR)

Aliquots of calcifiable vesicles ($\sim$25 μg protein/aliquot) were exposed to calcifying medium in the presence and absence of 1 mM ATP for 5 h and then the reaction mixtures were centrifuged at $300\,000 \times g$ for 20 min. The vesicle precipitates were washed twice with saline at pH 7.6 and resuspended in 100-μl solution containing 10 mg KBr to assure total recovery of the sample. The precipitate/KBr mixtures were freeze-lyophilized. The lyophilized powders were placed onto micro-sample holders prefilled with KBr and analyzed on a Mattson Satellite FT-IR spectrometer (Mattson Instruments, Madison, WI) equipped with a diffuse reflectance accessory. Typically, 64 scans were acquired at 4 cm^{-1} resolution under N_2 purge. WinFirst software was used for data analysis. Protein, lipid and phosphate species (from the mineral phase) absorb at infrared frequencies characteristic of that particular molecule and thus can be used to identify the components present in a sample. Spectra of non-mineralized vesicles were subtracted from the mineralized spectra to isolate the mineral phase. To analyze mineral phases in the tissue, aortas were minced and then treated with the proteinase K and SDS mixture. The mixture was then centrifuged at $300\,000 \times g$ to precipitate mineral for FT-IR analysis.

2.7. ATPase assay

Various ATPases were assayed by combining the methods of Hsu and Anderson [8] and Cunningham et al. [9]. For the basic Mg-ATPase, the reaction mixture (100 μl) contained 25 mM MOPS

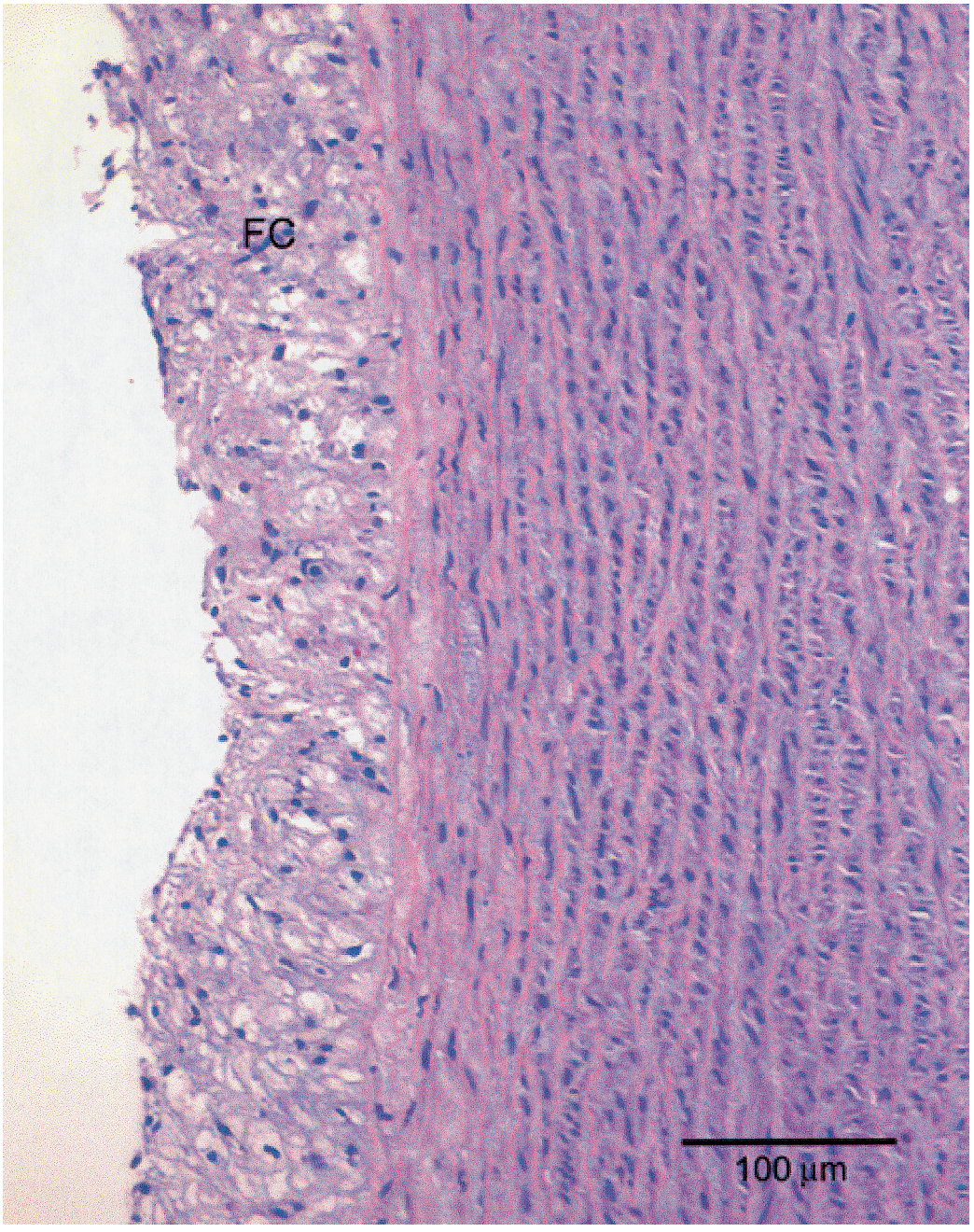

Fig. 1. Histology of an aorta isolated from a high cholesterol-fed rabbit. The media area appears normal whereas the intima expanded with numerous macrophage-derived foam cells (FC) and extracellular lipid deposits in the atheromatous area, a finding that is totally absent from control animals. (hematoxylin and eosin stain).

(pH 7.3), 1 mM $MgCl_2$, and 1 mM ATP (1×10^6 cpm [γ-^{32}P]ATP). An aliquot of vesicles (final protein concentration: 20 µg/ml) was added to initiate the reaction. For the blanks, vesicles were not added. The reaction mixture was incubated for 30 min at 37°C and terminated by addition of 250 µl of 0.2 M silicotungstic acid. The ^{32}Pi yielded from ATP hydrolysis was then extracted into the isobutanol-toluene phase according to the method of Martin and Doty [10] and the organic extract was transferred to a vial containing scintillation fluid for radioactivity counting. One unit (U) is expressed as that amount of

ATPase required to produce 1 μmol ^{32}Pi/min from [γ-^{32}P]ATP. Ca-ATPase was measured as the increase in basal activity upon the addition of 0.2–1 mM $CaCl_2$ and 4 μM calcium ionophore (A23187). Na,K-ATPase was measured as the increase in basal activity upon the addition of 110 mM NaCl, 10 mM KCl, 1 mM valinomycin, and 1 mM monensin.

2.8. Alkaline phosphatase assay

The enzyme was assayed by measuring the release of *p*-nitrophenol from *p*-nitrophenol-phosphate at ambient temperature. The assay mixture (0.3 ml) contained 0.3 M 2-amino-2-methyl-1-propanol, pH 10.2, 4 mM *p*-nitrophenol-phosphate, and aliquots of diluted vesicle preparations. The rate of *p*-nitrophenol formation was determined spectrophotometrically at 410 nm.

2.9. NTP pyrophosphohydrolase assay

The activity is expressed as the hydrolysis of *p*-nitrophenyl thymidine-5-phosphate at pH 10.2 to nitrophenol, which was determined by spectral changes at 410 nm [11,12].

2.10. Protein and phosphate assays

Inorganic orthophosphate was determined by the method of Martin and Doty [10]. The protein concentration was measured by the method of Lowry et al. [13] using bovine serum albumin as standard.

3. Results

3.1. Induction of atherosclerosis in rabbits by a high lipid diet

Rabbits fed with 0.5% cholesterol and 2% peanut oil for 4 months developed severe atherosclerosis as revealed by the formation of large atheromatous plaques populated with numerous macrophage-derived foam cells (Fig. 1). Fourier transform-infrared spectroscopy (FT-IR) of the aorta extracts indicates the presence of a small, but significant, absorbance peak corresponding to mineral phosphate phases (Fig. 2, wavenumber 1169 cm^{-1}). This characteristic peak was absent in normal aorta extracts (not shown).

3.2. Calcifiability of isolated vesicles

Calcifiable vesicles were isolated from rabbit thoracic aortas according to the method of Hsu and Camacho for the isolation of calcifiable vesicles from atherosclerotic human aortas [7]. Similar to human aorta vesicles, the rabbit vesicles appeared heterogeneous in shape and size in scanning microscope (Fig. 3a). However, some filament-like structures were also present and seemed to be closely associated with some vesicles, despite a further purification by

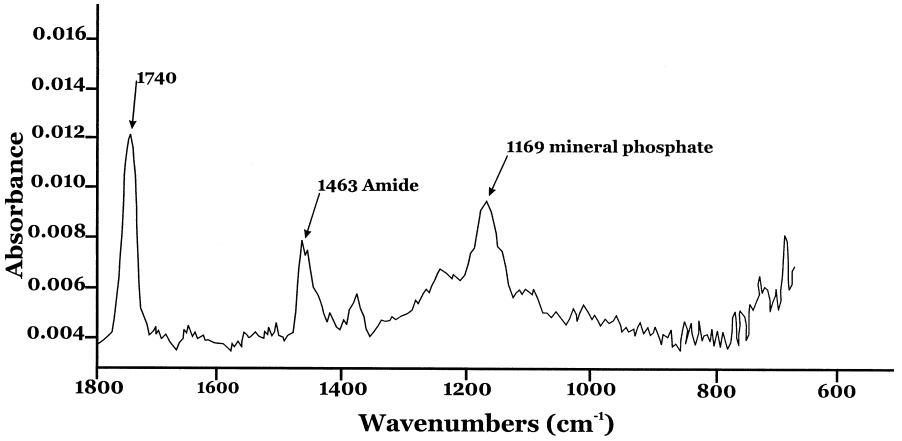

Fig. 2. Fourier transform infrared (FT-IR) spectrum of an aorta extract obtained from a high cholesterol-fed rabbit. The preparation of the aorta extract is described in detail in Section 2. A small peak corresponding to mineral phosphate is located around the wavenumber of 1169 cm^{-1}, which is not present in the extract from the control aorta.

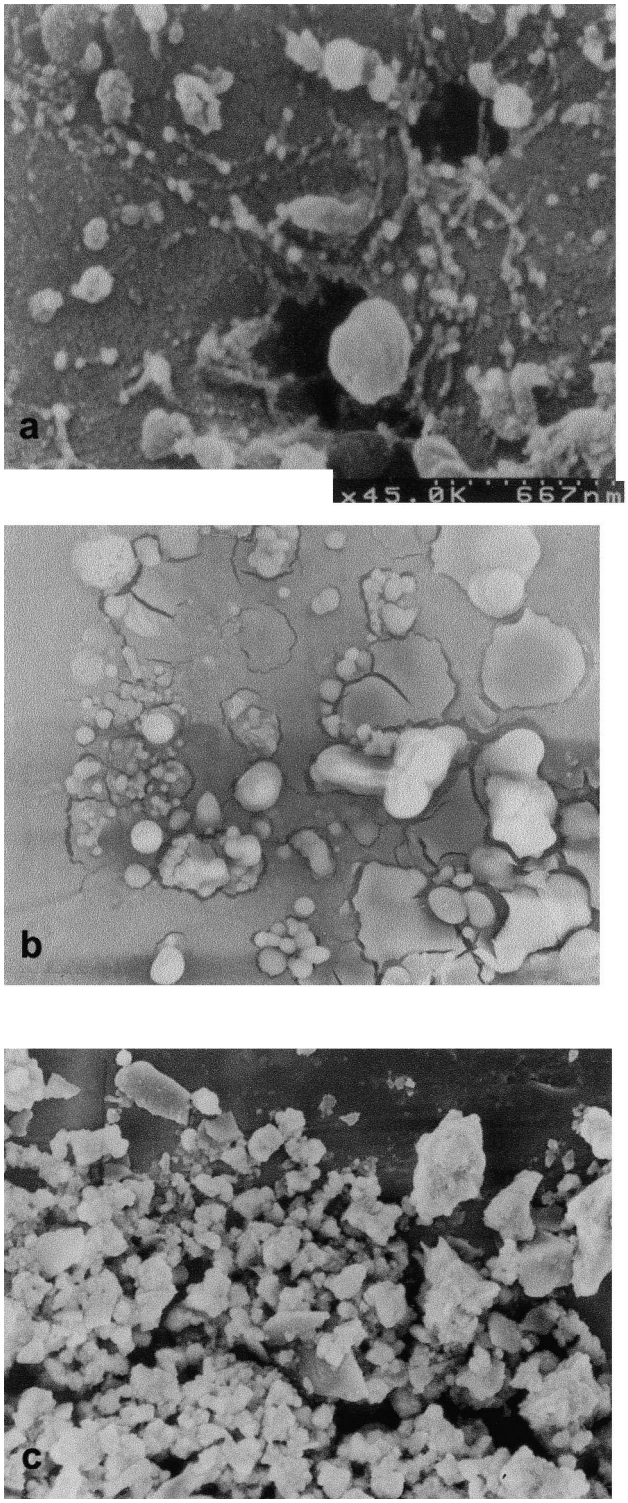

Fig. 3. Scanning electron microscopy of calcifiable vesicles and mineral deposits. The details of scanning electron microscopy are described in Section 2. (a) Vesicles retained on the filter appear to be heterogeneous in size and shape. Some vesicles seem to be closely associated with some filament-like substances (magnification: ×42 750). Two empty filter pores (0.45 μm) can be seen. (b) Mineral deposited by vesicles (magnification: ×950). (c) Synthetic hydroxyapatite (magnification: ×950). Only the scale bar is given in (a) as a reference to (b) and (c), which are 45-fold less magnified.
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sucrose-gradient centrifugation or by further treatment with crude collagenase. To visualize mineral induced by calcifiable vesicles, the precipitates containing vesicles and mineral were centrifuged at 250 000×*g*. The precipitates were washed twice with aliquots of water (pH 7.6) by centrifugation, resuspended in 50 μl water, and spotted directly onto aluminum sample holders of a scanning electron microscope, and air-dried. After exposing calcifiable vesicles to calcifying media for 5 h, mineral deposits with various sizes, shapes, and surface smoothness were formed (Fig. 3b). Some small, smooth, and round deposits appeared to be growing into larger mineral. Synthetic hydroxyapatite particles were included for comparison (Fig. 3c). Hydroxyapatite deposits appear to have more rough and irregular surfaces as compared to the vesicle-induced mineral.

The vesicles were capable of depositing 4.2 ± 1.5 nmol $^{45}CaCl_2$ per μg protein (mean ± S.D., four samples) after 5-h incubation in the presence of calcifying media containing physiological levels of Ca^{2+}, Pi, and 1 mM ATP. We also included normal aortas as the control to consider the possibility that some plasmalemmal fragments or vesicles may form by cell degradation as a result of prolonged proteolytic digestion by crude collagenase during vesicle preparation. There was a 88 ± 8% less yield of control vesicles proteins per mg of aortas from normally fed rabbits than that from high cholesterol-fed rabbits ($P < 0.05$). On the basis of the specific Ca deposition activity and protein yields of the vesicles, the total $^{45}CaCl_2$ deposition of the control vesicles per mg tissue was found to decrease by the same magnitude. Since $^{45}CaCl_2$ deposition or uptake may not fully reflect mineral formation per se, we further used Alizarin red stain for mineral to confirm

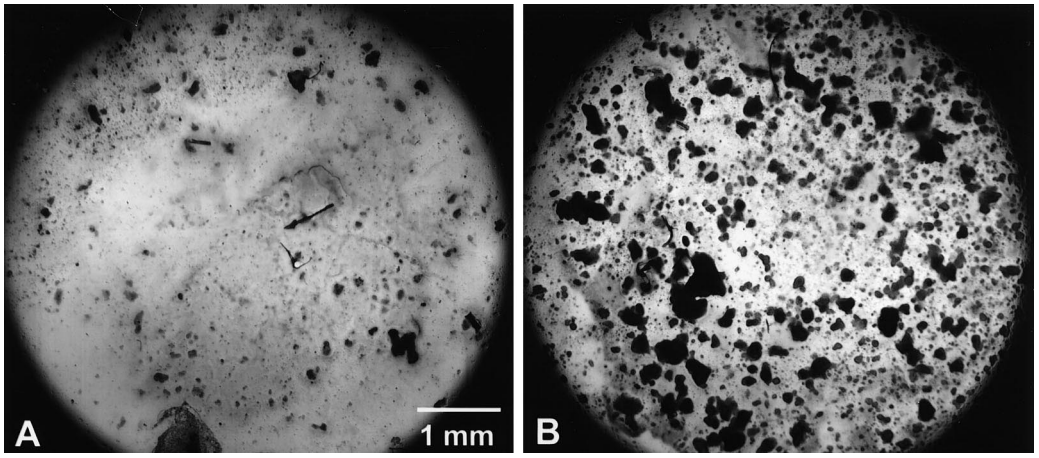

Fig. 4. Comparison of mineral formation induced by vesicles isolated from control aorta and aortas of high-lipid-fed rabbits. The extent of Alizarin red staining was used as a qualitative indication of the amount of mineral deposited by calcifiable vesicles. See Section 2 for details. (A) Normal vesicles. (B) Vesicles from aortas of high-lipid-fed rabbits.

$^{45}CaCl_2$ data. Alizarin red staining (Fig. 4) observations demonstrated that vesicles from cholesterol-fed rabbits tended to have higher calcifying activity than those obtained with normal aortas.

3.3. *Effect of concanavalin A on calcification by isolated calcifiable vesicles*

In an attempt to assess the role of ATPase in ATP-initiated calcification by vesicles, the effect of an ecto-ATPase activator, concanavalin A (Con A), on calcification was studied. Comparison of FT-IR spectral peaks obtained from untreated and Con A-treated vesicles indicated that, while amide peak levels overlapped each other, those peak intensities corresponding to mineral phosphates (1050–650 cm^{-1}) were substantially higher with Con A-treated vesicles than those with untreated vesicles (Fig. 5). The difference in the calcifiability was further demonstrated by Alizarin red staining (Fig. 6). However, ATPase activity was not altered by Con A (data not shown). The stimulation of Ca^{2+} and Pi deposition was dose-dependent with a half-maximal stimulation at 6–10 μg/ml of Con A (Fig. 7).

To determine whether binding of a putative carbohydrate to Con A was responsible for the stimulation, the effect of α-methylmannoside and α-methylglucoside, which bind specifically to Con A [14], on stimulation was studied. The addition of these carbohydrate derivatives at 10 mM completely abolished the stimulation (Fig. 8, $P < 0.01$). Non-specific carbohydrates that do not bind to Con A, such as galactoside, *N*-acetylglucosamine, mannitol, or galac-

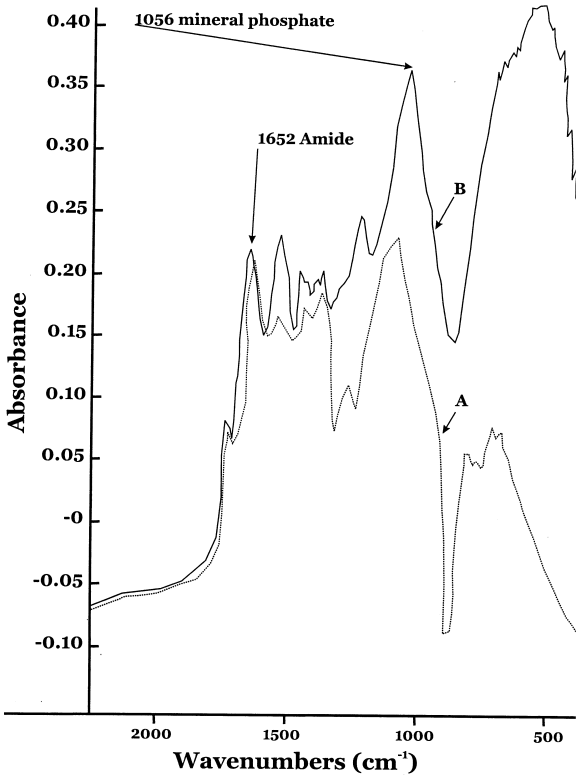

Fig. 5. FT-IR of mineral deposits by untreated vesicles and Con A-treated vesicles. (A) Untreated vesicles exposed to calcifying media without Con A. (B) vesicles exposed to calcifying media in the presence of 12 μg/ml Con A. See Section 2 for details.

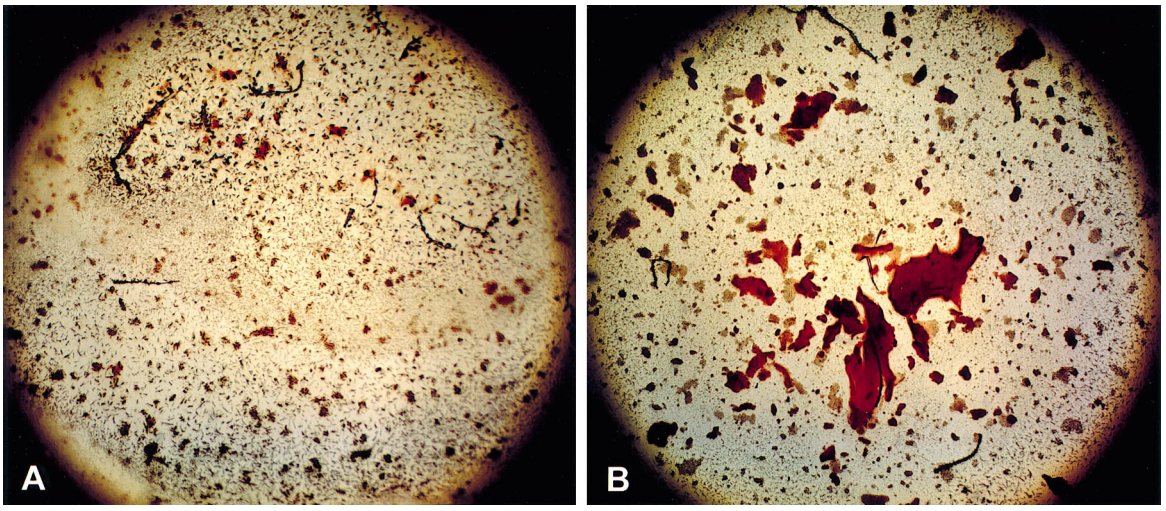

Fig. 6. Effect of Con A on mineral formation induced by calcifiable vesicles. The extent of Alizarin red staining was used as a qualitative indication of the amount of mineral deposited by calcifiable vesicles. See Section 2 for details. In order to visualize the stimulatory effect of Con A, the concentrations of calcifiable vesicles in calcifying media were reduced to 7 μg/ml to minimize mineral staining. (A) Control. (B) Treatment with 12 μg/ml Con A.

tosamine (data not shown), did not affect Con A stimulation ($P > 0.05$).

To evaluate whether Con A stimulation is reversible, the following steps were taken. Calcifiable vesicles were initially incubated with 12 μg/ml Con A for 30 min. Con A was then removed by centrifugation. The treated calcifiable vesicles were tested to see if the stimulated level of calcification remained unchanged. As shown in Fig. 9, the stimulated level of ATP-dependent Ca deposition returned to normal levels after Con A was removed (Fig. 9A vs. C, $P > 0.05$). When Con A was added again to calcifying media the stimulation for both TBS- and Con A-treated vesicles was restored (Fig. 9C vs. D; $P < 0.01$).

To determine whether galactosides and their derivatives may also be involved in the mechanisms of Ca and Pi deposition, the effect of *Abrus precartorius* lectin, which specifically binds galactosides [15], was investigated (Fig. 8). *A. precartorius* lectin markedly enhanced calcification and the stimulation was abolished by 10 mM α-methyl galactoside ($P < 0.01$).

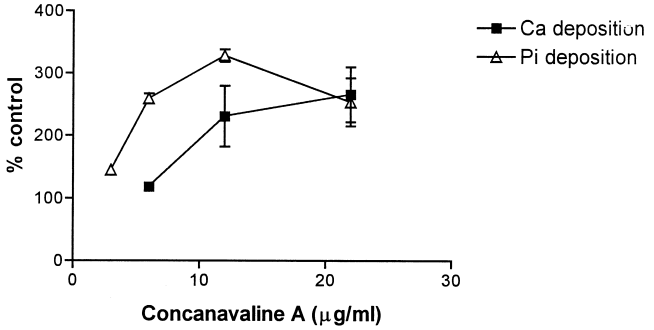

Fig. 7. Effect of Con A concentrations on Ca and Pi deposition by calcifiable vesicles. The details of Ca and Pi deposition are described in Section 2. Values are expressed as means ± S.D. calculated from duplicate data of two replicate experiments. Note that the ratio of Ca to Pi, which was often used to characterize mineral, cannot be calculated from the graph since the vesicle preparation for Ca deposition experiments was different from that for Pi deposition. Furthermore, Ca and Pi deposition not only involves mineralization, but also ion uptake by vesicles.

Neither *N*-acetylglucoside-binding wheat germ agglutinin [16] nor *N*-acetylgalactoside-specific *Helix pomatia* [17] affected calcification ($P > 0.05$).

3.4. Effects of lectins on enzyme activities

Since Con A is known to activate ecto-Mg-ATPase, it is relevant to determine whether the stimulatory effect of Con A or other lectins on calcification was mediated through the activation on ATPase activity. Of all lectins tested, including Con A, wheat-germ agglutinin, *Abrus* lectin, none had significant effects on calcification related enzymes including ATPase, NTP-pyrophosphohydrolase, and alkaline phosphatase activities (data not shown)

4. Discussion

Advanced calcification in severe atherosclerosis constitutes a critical situation whereby patients risk a life-threatening rupture of the aorta. An earlier report with human atherosclerotic aortas indicated [7] that isolated vesicles can induce calcification when the vesicles were exposed to calcifying media containing physiological concentrations of Ca^{2+} and Pi and 1 mM ATP. In an attempt to investigate whether hyperlipidemia, which is known to cause atherosclerosis, can also lead to aorta calcification through the activation or production of calcifiable vesicles, we fed the rabbits with a high lipid diet

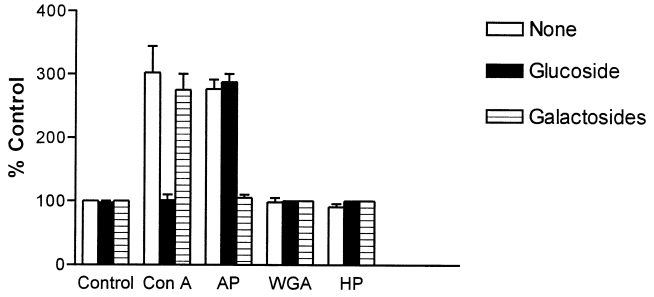

Fig. 8. The effects of various lectins on Ca deposition. The effects of lectins are expressed as a percentage of the control values (4.5 ± 0.8 nmol/μg protein). Values are expressed as means ± S.D. calculated from duplicate data of two replicate experiments. Con A, concanavalin A; AP, *Abrus precartorius* lectin; WGA, wheat germ agglutinin; HP, *Helix pomatia* lectin. The effects of various carbohydrate derivatives at 10 mM on lectin-stimulated calcification were also included in the study.

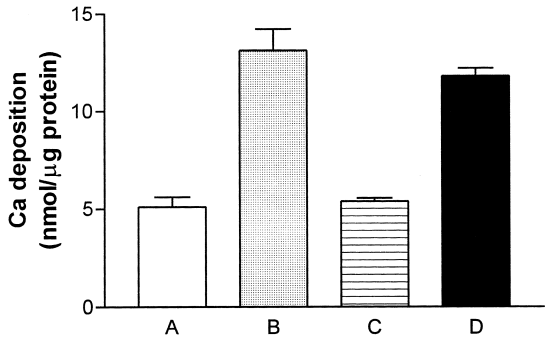

Fig. 9. The reversibility of the stimulation by Con A of ATP-initiated Ca deposition. Calcifiable vesicles were incubated with TBS or 12 μg/ml Con A at 4°C for 30 min and followed by centrifugation at 270 000 × *g* for 30 min to remove Con A. The resulting precipitates were washed twice by TBS and followed by suspension in aliquots of TBS. The washed pellets were then assayed for Ca depositing activity in the presence or absence of Con A. Values are expressed as means ± S.D. calculated from duplicates data of two replicate experiments. The letters under each bar represent: A, TBS-pretreated vesicles; B, TBS-pretreated vesicles exposed to Con A; C, Con A-pretreated-vesicles; D, Con A-pretreated vesicles re-exposed to Con A.

supplemented with 0.5% cholesterol and 2% peanut oil. After 4 months of feeding, atheromas with abundant macrophage-derived foam cells developed in experimental animals. At this stage of development, however, advanced calcification still did not occur. A further attempt to maintain rabbits with longer exposure to a high-lipid diet or with higher levels of lipid in the diets met with difficulty, since rabbits developed severe jaundice and had to be killed. However, the presence of minor calcification in the aortas of cholesterol-fed rabbits suggests that the aortas were not in final stages of atherosclerosis. Furthermore, the presence of vesicles with higher calcifiability than control vesicles also may indicate that calcifiable vesicles may start to accumulate at early stages of atherosclerosis, but at a level insufficient for heavy calcification.

Although ATP can initiate vesicle-mediated calcification, there is no firm evidence for the role of ATPase. Since Con A has been shown to activate ecto-ATPase in a variety of tissues [9], it was tempting to assume that Con A-stimulation of calcification was mediated through the enhancement of ATPase activity. The present study indicates that Con A activated neither vesicle's ATPase nor other calcification related-enzymes including alkaline phosphatase [18,19] and NTP-pyrophosphohydrolase [12,20,21].

Thus, these observations suggest that lectin stimulation may be due to interactions between sugar moieties of vesicles and lectins. This contention was supported by the observations that glucosides that inhibit the binding of Con A to cell surface carbohydrates reversed the stimulation of calcification. We also observed that galactoside-specific *A. precartorius* lectin could stimulate calcification and that the stimulation can be reversed by galactoside derivatives. These observations also indicate that glucosides, mannosides, and galactosides may be present in plasma membrane-derived vesicles and that these carbohydrates may regulate vesicle-mediated calcification.

Since the molecular weight of Con A monomer is about 30 000, it can be estimated that 6 μg/ml of Con A ($K_{0.5}$) would contain about 2 nmol of Con A. Assuming that each mole of Con A bound one mole of glucosides, it is likely that 15 μg of vesicle protein per ml of calcifying media may bind 2 nmol of glucosides. On the basis of these assumptions, it can be estimated that about >0.26 nmol glucosides/μg protein are present in vesicles. A further chemical analysis to identify each individual carbohydrate constituent in or on calcifiable vesicles would be required to confirm the above estimation.

Interestingly, neither *N*-acetylglucoside-binding wheat-germ agglutinin nor *N*-acetylgalactoside-specific *H. pomatia* was effective, suggesting that the acetylated forms of carbohydrate moieties may not be involved in calcification. Alternatively, it might suggest that the acetyl derivatives were absent in vesicles and, therefore, the lectins were unable to interact with vesicles.

To provide direct evidence for the binding of the lectins to specific vesicle-associated carbohydrates as the cause of stimulation, a detailed binding kinetic approach would be necessary. However, such an approach will be difficult because of the reversibility of the binding as suggested by the stimulation reversibility test (Fig. 9). Furthermore, direct chemical analysis to determine carbohydrate constituents responsible for stimulation of calcification would also require a large quantity of vesicle preparations and sophisticated techniques for isolation and identification of specific carbohydrates, thus warranting a separate project.

Although calcifiable vesicles have been isolated from a variety of calcifying tissues, the detailed mechanisms of vesicle-mediated calcification have yet to be elucidated (for a review see [22]). It appears that membrane components, in addition to the enzymes that are involved in calcification, also play an essential role. For example, calcifiability of matrix vesicles isolated from rachitic rat epiphyseal cartilage can be stimulated by a neutral or anionic detergent, but inhibited by a cationic detergent suggesting that hydrophobic and ionic microenvironments of vesicle membranes may play an important role in vesicle-mediated calcification [23]. The marked stimulation by carbohydrate-binding lectins further suggests a role for carbohydrates in vesicle-mediated calcification. Whether additional vesicle-associated components are involved in calcification requires further investigation.

Although calcifiability of isolated vesicles reported here seems to suggest their role in arterial calcification, whether many of agents to which the vesicles were exposed to in vivo remains to be established. Although levels Ca and Pi, and ATP were within physiological ranges as discussed previously [7], the exact concentrations of these ions surrounding calcifiable vesicles have yet to be studied. There is a good possibility that these ions could be higher than normal serum levels because of leakage of the ions into extracellular or intracellular compartments from within or without cells as the disease progresses, such as necrosis or apoptosis. It is also plausible that available Ca can be limited by its binding to proteins. However, whether the binding indeed inhibits, enhances or exerts no effect on calcification remains to be established. Further, the extent of calcification is dependent on $Ca \times P$ ion products. Thus, the reduction in Ca levels can be easily compensated by Pi supplied by ATP, which is the best source of Pi because of its Pi content and abundance in cells (in the range of mM). A further study is needed to address these possibilities before the role of calcifiable vesicles in atherosclerotic calcification can be ascertained.

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