Elastin Calcification and its Prevention with Aluminum Chloride Pretreatment

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Elastin, an abundant structural protein present in the arterial wall, is prone to calcification in a number of disease processes including porcine bioprosthetic heart valve calcification and atherosclerosis. The mechanisms of elastin calcification are not completely elucidated. In the present work, we demonstrated calcification of purified elastin in rat subdermal implants ($\text{Ca}^{2+} = 89.73 \pm 9.84 \text{ } \mu\text{g}/\text{mg}$ after 21 days *versus* control, unimplanted $\text{Ca}^{2+} = 0.16 \pm 0.04 \text{ } \mu\text{g}/\text{mg}$). X-ray diffraction analysis along with resolution enhanced FTIR spectroscopy demonstrated the mineral phase to be a poorly crystalline hydroxyapatite. We investigated the time course of calcification, the effect of glutaraldehyde crosslinking on calcification, and mechanisms of inhibition of elastin calcification by pretreatment with aluminum chloride (AlCl_3). Glutaraldehyde pretreatment did not affect calcification ($\text{Ca}^{2+} = 89.06 \pm 17.93 \text{ } \mu\text{g}/\text{mg}$ for glutaraldehyde crosslinked elastin *versus* $\text{Ca}^{2+} = 89.73 \pm 9.84 \text{ } \mu\text{g}/\text{mg}$ for uncrosslinked elastin). This may be explained by radioactive (^3H) glutaraldehyde studies showing very low reactivity between glutaraldehyde and elastin. Our results further demonstrated that AlCl_3 pretreatment of elastin led to complete inhibition of elastin calcification using 21-day rat subdermal implants, irrespective of glutaraldehyde crosslinking ($\text{Ca}^{2+} = 0.73\text{--}2.15 \text{ } \mu\text{g}/\text{mg}$ for AlCl_3 pretreated elastin *versus* 89.73 ± 9.84 for untreated elastin). The AlCl_3 pretreatment caused irreversible binding of aluminum ions to elastin, as assessed by atomic emission spectroscopy. Moreover, aluminum ion binding altered the spatial configuration of elastin as shown by circular dichroism (CD), Fourier transform infrared (FTIR), and ^{13}C nuclear magnetic resonance (NMR) spectroscopy studies, suggesting a net structural change including a reduction in the extent of β sheet structures and an increase in coil-turn conformations. Thus, it is concluded that purified elastin calcifies in rat subdermal implants, and that the AlCl_3 -pretreated elastin completely re-

sists calcification due to irreversible aluminum ion binding and subsequent structural alterations caused by AlCl_3 . (*Am J Pathol* 1999, 155:973–982)

Elastin, a structural protein of the extracellular matrix, is a major component of elastic fibers in connective tissue. It is one of the most abundant proteins present in the aorta, arterial walls, and heart valve cusps.¹ The biosynthetic precursor of elastin, tropoelastin, is a linear polypeptide composed of about 700 amino acids and is rich in non-polar amino acids: glycine (>30%) valine, leucine, isoleucine, and alanine. Elastic fiber formation involves lysyl oxidase-mediated formation of characteristic intermolecular crosslinks called desmosine and isodesmosine. These properties make elastin a highly hydrophobic and insoluble protein.² Elastin fibers also contain a microfibrillar component consisting of 10- to 12-nm fibrils, which are located around the periphery of elastin fibers.³ This microfibrillar component is made up of acidic glycoproteins including fibrillin.³

A number of diseases' processes, including atherosclerosis and bioprosthetic heart valves (derived from glutaraldehyde-crosslinked porcine aortic valves) calcification, demonstrate characteristic elastin oriented calcification.^{4–7} Comparable elastin-oriented calcification was shown in rats with subdermally implanted segments of glutaraldehyde-crosslinked rabbit aorta,⁸ with allograft aortic wall in rats,⁹ and in human aortic allografts.^{10,11}

The mechanisms governing aortic wall elastin calcification are incompletely understood and seem to differ from those of bioprosthetic heart valve cusp calcification. For example, glutaraldehyde crosslinking is not a prerequisite for aortic wall calcification.⁹ Previous studies in our laboratory have shown comparable calcification of fresh and glutaraldehyde-fixed aortic root allografts in both a rat subdermal implantation model and in the circulation.⁹ We have shown previously that the ethanol pretreatment of bioprosthetic heart valve completely prevented cusp calcification due to mechanisms based on lipid extraction and collagen structural change; however, the same

Supported by the National American Heart Association SDG (NRV) and NHLBI Grant HL38118 (R.J.L.). Dr. Levy is the recipient of an endowed chair at the Children's Hospital of Philadelphia.

Accepted for publication May 22, 1999.

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pretreatment was only partially effective in preventing aortic wall calcification.^{12,13}

This consistent observation of elastin-oriented calcification in the bioprosthetic heart valve aortic wall as well as in atherosclerosis led us to ask if purified elastin rat subdermal implants undergo calcification. Thus, in the present work, we studied the calcification of purified elastin in a rat subdermal implantation model and its inhibition by aluminum chloride (AlCl_3) pretreatment. The AlCl_3 pretreatment was chosen because previous studies in our laboratory have shown that metal ions such as Fe^{3+} or Al^{3+} completely inhibit bioprosthetic heart valve cusp as well as aortic wall calcification.^{9,14–17} By using pure elastin, its calcification could be studied independently from the effects of other structural components of the aortic wall such as phospholipids, glycosaminoglycans, devitalized cells and cellular debris, intrinsic enzymes, and other extracellular matrix (ECM) proteins. Moreover, the use of isolated purified elastin is also favorable for obtaining insights on the effects of anti-calcification agents in terms of the protein conformation, protein-protein and protein-calcium interactions using biophysical techniques such as circular dichroism (CD) and Fourier transform infrared (FTIR) and nuclear magnetic resonance (NMR) spectroscopies. The goals of the present study were to study the calcification of purified elastin in a rat subdermal implants, to investigate the protein structural changes in elastin due to AlCl_3 pretreatment by CD, FTIR and ^{13}C NMR spectroscopies, and to study the mechanism of AlCl_3 inhibition of elastin calcification in a rat subdermal model.

Materials and Methods

Elastin fibers (5–10 mm) from bovine neck ligament purified by a neutral extraction method,¹⁸ soluble elastin, and alpha elastin were obtained from the Elastin Product Company (Owensville, MO). Absolute 200 proof ethanol (McCormick Distilling Co. Inc., Weston, MO), HEPES (Sigma Chemical Co., St. Louis, MO), aluminum chloride (AR grade, Aldrich, Milwaukee, WI), glutaraldehyde as an 8% electron microscopy grade solution (Polysciences, Warrington, PA) were used as received. Radioactive glutaraldehyde ($1,5\text{-}^3\text{H}$ glutaraldehyde, 250 μCi) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO), Ketamine HCl (Ketaset, Fort Dodge Lab, Fort Dodge, IA) and Xylazine (Rompun, Miles Inc., Shawnee Mission, KS) were used for rat anesthesia.

Amino Acid Analyses of Elastin

The samples (elastin fibers and α elastin, 5–6 mg) were placed in borosilicate glass tubes and 1 ml of 6N ultrapure (ultrapure) HCl was added to each of the tubes. The tubes were then sealed under argon and kept in an oven (110°C) for 20 hours. The tubes were opened and the HCl was evaporated under a constant flow of helium. The residue was dissolved in 1 ml of distilled water and then lyophilized to remove traces of HCl. The lyophilized residue was then redissolved in 0.5 ml of water for amino

acid analyses. For comparison, we also extracted insoluble elastin from fresh porcine aortic wall using 0.1 N NaOH treatment at 100°C .¹⁹ This elastin was then digested per the above protocol for amino acid analysis.

Glutaraldehyde Treatment of Elastin Fibers

The elastin fibers were treated with a 0.6% glutaraldehyde solution (diluted from 8% aqueous solution) buffered with 50 mmol/L HEPES, pH 7.4, then transferred after 24 hours to 0.2% glutaraldehyde solution in the same buffer for 7 days, per the procedure used for bioprosthetic heart valve.¹² The elastin fibers were rinsed free of glutaraldehyde with saline 24 hours before implantation.

Radioactive Glutaraldehyde Treatment of Elastin Fibers

Radioactive (^3H -labeled) glutaraldehyde (specific activity 1–5 Ci/mmol) solution was used under conditions identical to those described above to determine the amount of glutaraldehyde reacted with elastin. Thus, elastin samples were reacted with glutaraldehyde (0.2% ^3H -labeled glutaraldehyde) for 7 days and then washed exhaustively with water to remove unbound glutaraldehyde until the supernatant solution showed no radioactivity. Then the elastin samples (5–10 mg) were lyophilized, weighed, and digested in 1 ml of 0.1 mol/L NaOH at 80°C for 8 hours and 5 ml of scintillation cocktail was added to each vial. The samples were counted for radioactivity using a liquid scintillation counter (LC 3801, Beckman, Irvine, CA).

Aluminum Chloride Pretreatment

Glutaraldehyde-crosslinked and uncrosslinked elastin fibers (50–100 mg) were placed in 100 ml of aqueous 0.1 mol/L AlCl_3 solution (pH 2.0) for 2 hours at 25°C on a shaker bath. After the AlCl_3 pretreatment, samples were washed exhaustively in saline to remove traces of unbound AlCl_3 before implantation. A group of samples were placed in 1.0 mol/L NaCl and samples were taken at 4 hours and at 1, 3, 7, 14, and 21 days for aluminum ion analysis in order to test whether the aluminum ions were tightly associated with elastin. Aluminum levels of the samples were assessed by atomic emission spectroscopy (AES) using Atom Scan 16 (Thermo Jarrell Ash Corp., Franklin, MA) with appropriate standard curves.²⁰

FTIR Spectroscopy

A Nicolet (Model Protege 460, Madison, WI) FTIR spectrometer equipped with a horizontal attenuated total reflectance (ATR) ZnSe cell (SpectraTech Inc., Stamford, CT) was used to obtain ATR-FTIR spectra of elastin fibers. A water spectrum was obtained as a reference, and was then subtracted from the sample by the instrument-controlled computer. The elastin fibers (control and AlCl_3 -pretreated) were equilibrated in double-distilled water and blotted on a filter paper. The hydrated samples were

then placed on an ATR cell and pressed to obtain good surface contact between the cell and the sample. Fifty scans were obtained at a resolution of 4 cm^{-1} . The spectra were then deconvoluted in the region of $1800\text{--}1000\text{ cm}^{-1}$ with the software program (Omnic ESP) provided by Nicolet to resolve the different bands.

Circular Dichroism (CD) Spectroscopy

Soluble alpha-elastin was used for the CD spectroscopy per previously published protocols.²¹ Briefly, 0.25 mg of alpha-elastin was dissolved in 1 ml of trifluoroethanol and 20 μl of aqueous solutions of calcium chloride and aluminum chloride in various concentrations (from 6.25 to 150 mmol/L) were added. After standing for 1 to 2 hours these solutions were used for CD spectral analysis. CD spectra were obtained in 0.01-cm quartz cuvettes from 180 to 260 nm on a Jasco (J-710, Easton, MD) spectrometer.

Nuclear Magnetic Resonance (NMR) Spectroscopy

Soluble elastin, 20 mg (from bovine neck ligament obtained by autoclaving followed by oxalic acid treatment, purchased from the Elastin Product Co.), was placed in 1 ml of D_2O and aluminum chloride (20 mg) was added. The FT ^{13}C NMR (Bruker AM 400, Billerica, MA) spectra were recorded for both the control elastin sample and AlCl_3 -treated sample at a resonance frequency of 400 MHz, with 0.655 seconds acquisition time and $30\text{--}40^\circ$ pulse width for 90,000 scans.

Rat Subdermal Implantation

Male Sprague Dawley rats (21–24 days old, 50–65 g, Charles River Laboratories, Burlington, MA) were anesthetized by intramuscular injections of ketamine HCl and Xylazine (4:3), 0.001 cc/g of body weight. Using a sterile technique, a small incision was made on the backs of rats and two subdermal pouches, one in front and one in back, were created. Two samples of elastin fibers (10–15 mg) were then implanted subdermally in the pouches. The rats (5 per group) were sacrificed by CO_2 asphyxiation at 3, 7, and 21 days, and samples were retrieved. The samples were used for quantitative calcium, phosphorus, and aluminum analyses and representative samples were fixed in Karnovsky's fixative for morphological studies.

Calcium, Phosphorus, and Aluminum Analyses

The elastin samples explanted from rats were washed with an excess of saline followed by distilled water, lyophilized, and weighed. Each sample (15–40 mg) was hydrolyzed in 1 ml of 6 N HCl in a test tube at 70°C for 16 hours. The solution was then completely evaporated at 70°C with a continuous flow of nitrogen in the tube. The residue in the test tube was dissolved in 1 ml of 0.01 N HCl. The calcium contents were measured using atomic

absorption spectroscopy as previously described.²² The same solutions for calcium analyses were also used for quantitative phosphorus analysis by molybdate complexation.²³ The aluminum ion levels were determined by atomic emission spectroscopy.²⁰

Morphological Assessment of Explants

Representative AlCl_3 -pretreated and untreated control elastin samples explanted from the rat subdermal study were fixed in 2.5% glutaraldehyde, 2.0% paraformaldehyde in cacodylate buffer at pH 7.4 (Karnovsky's fixative), dehydrated through graded ethanols, and embedded in glycolmethacrylate (GAM, Polysciences Inc., Warrington, PA). For light microscopic examination, sections cut 3 μm thick were stained with hematoxylin and eosin for overall morphological features and the von Kossa stain for calcium phosphates.

For ultrastructural morphology, representative specimens were fixed as above, postfixed in 2.0% osmium tetroxide, dehydrated ethanol, and propylene oxide, and embedded in Poly/Bed 812 (Polysciences). Sections were cut at 60 nm, stained with lead citrate and uranyl acetate, and examined with a JEOL-100CX transmission electron microscope (JEOL, Cranford, NJ) at an accelerating voltage of 80 kV.

Mineral Phase Analysis of Explanted Elastin

Because the explanted elastin samples have only 10% of mineral and 90% of protein contaminant, we combined several explanted samples together and extracted mineral by hydrazine method.²⁴ This method has been used by bone researchers to extract mineral and this method has been shown not to alter mineral structure.²⁴ We prepared elastin explants for X-ray and FTIR analysis by immediate freezing over dry ice and storage at -80°C until use. Each elastin sample was milled in liquid nitrogen using mortar and pestle. The powdered sample was then extracted with chloroform:methanol (2:1) for 8 hours, followed by washing with ethanol and diethyl ether and lyophilization. The sample was then reacted with anhydrous hydrazine at 4°C for 24 hours with vigorous stirring. After centrifugation, the pellet was suspended and washed with absolute ethanol 4 times to remove hydrazine. The sample was then lyophilized. FTIR of isolated mineral was obtained with Nicolet FTIR spectrometer as a 2 wt% KBr pellet. The FTIR spectra were obtained from $4000\text{ to }400\text{ cm}^{-1}$ at 2 cm^{-1} resolution (100 scans). Resolution-enhanced deconvolution of the spectra was performed with the software provided by Nicolet (Omnic). X-ray dispersive profiles of extracted mineral were collected in the 2θ range of interest, using a Rigaku (Geiger Flex D-max 2B, Danvers, MA) X-ray powder diffractometer equipped with a graphite monochromator and a Cu target. Synthetic hydroxyapatite (Fluka, Buchs, Switzerland) was used as a standard.

Table 1. Amino Acid Analyses for Elastin

Amino acid	Bovine neck ligament elastin (commercial source, prepared by autoclaving ¹⁸)	Porcine aorta elastin, lansing procedure ¹⁹
ASP	6.6	3.5
GLU	16.1	17.1
HYP	13.4	11.4
SER	8.4	7.6
GLY	344.1	348.5
HIS	0.7	0.4
ARG	5.9	4.9
THR	7.5	5.2
ALA	206.7	219.2
PRO	119.9	122.9
TYR	8.7	17.6
VAL	137.5	130.8
MET	2.1	1.0
CYS	0.8	0.2
ILU	25.5	17.8
LEU	62.9	53.3
PHE	30.2	32.9
LYS	2.8	5.5

Values are expressed as residues per thousand residues.

Data Analyses

All data are expressed as mean $\pm$ standard deviation (SD). Statistical analyses of data were performed and probability values (*P*) for significance were calculated using Student's *t*-test. *P* values ≤ 0.01 were considered to be statistically significant.

Results

The present studies demonstrate that purified elastin calcifies in a rat subdermal model independent of glutaraldehyde exposure, and that its calcification can be completely prevented by AlCl_3 pretreatment.

Amino Acid Analyses of Elastin

The purity of the elastin fibers was confirmed with amino acid analyses (Table 1), which showed typical features of purified elastin such as high amounts of alanine and valine residues, the lack of tryptophan, cysteine, methionine, and histidine, and a very low content of polar amino acids such as hydroxyproline, aspartic acid, and glutamic acid (Table 1). The elastin extracted from fresh porcine aorta by refluxing in 0.1 N NaOH for 45 minutes¹⁹ also showed a similar composition (Table 1).

Radioactive Glutaraldehyde Study

When elastin fibers were treated with ^3H -labeled glutaraldehyde, relatively low levels of glutaraldehyde were bound to the elastin (1.71 ± 0.02 nmol/L glutaraldehyde per milligram of dry elastin). For comparison, our previous studies with bioprosthetic heart valve cusps show 117 ± 0.8 nmol/L of glutaraldehyde reacted per milligram of dry tissue.²⁵ Thus, glutaraldehyde pretreatment had

Figure 1. An overlay of ATR-FTIR spectra for control and AlCl_3 -pretreated elastin fibers showing amide I structural alterations caused by AlCl_3 . (A, AlCl_3 pretreated; A1, deconvoluted amide I region; B, control; B1, deconvoluted amide I region). Both spectra were deconvoluted with bandwidth 26.2 cm^{-1} ; sensitivity factor 2.1.

significantly lower reactivity with purified elastin than with porcine aortic cusps.

AlCl_3 Pretreatment Effects on Elastin Structure

FTIR Results

An overlay of ATR-FTIR spectra for uncrosslinked elastin fibers (control and AlCl_3 -pretreated) is shown in Figure 1. There were considerable changes in the carbonyl stretching region (amide I) due to the AlCl_3 pretreatment. For control elastin fibers the deconvolution of amide I region resolved it in five distinct bands, viz., 1681, 1668, 1650, 1631, and 1614 cm^{-1} . After AlCl_3 pretreatment, the band at 1614 cm^{-1} was considerably reduced in intensity and the band at 1650 cm^{-1} was increased in intensity as compared to controls (Figure 1). There were no changes in other regions of the spectra.

CD Spectroscopy

Circular dichroism spectra for α -elastin in trifluoroethanol at a concentration of 0.25 mg/ml showed two negative peaks (205.5 and 221.8 nm) (Figure 2). Circular dichroism spectroscopy for α -elastin in the presence of calcium and aluminum ions indicated significant reduction of the negative peak at 221.8 nm in the CD spectra and a concomitant blue shift (to a lower wavelength) and increase in the 205 nm peak, suggesting structural alter-

Figure 2. An overlay of circular dichroism spectra for α -elastin in trifluoroethanol (0.25 mg elastin/ml) control and in presence of AlCl_3 and CaCl_2 . Elastin showed two negative peaks at 205.5 and 221.8 nm. Both AlCl_3 (dotted line) and CaCl_2 (heavy line) caused significant changes in the spectra at 1.0 mmol/L metal ion concentration.

ations and metal ion binding (Figure 2). The reduction in the negative peak at 221.8 nm was directly proportional to the aluminum ion concentration in the solution (Figure 3). This trend was comparable to that of calcium (Figure 3).

^{13}C NMR Spectroscopy

^{13}C NMR spectra for soluble elastin is shown in Figure 4. Our ^{13}C NMR spectra shows similar peaks that were observed for κ -elastin by others²⁶ (Figure 4). After AlCl_3 treatment, the peak at 43.5 ppm for $\alpha\text{-CH}_2$ of glycine residues is significantly altered (Figure 4). Similarly, sig-

Figure 3. Comparison of changes caused at 221.8 nm in CD spectroscopy by calcium and aluminum ions at different concentrations. The data show a progressive reduction of the negative peak at 221.8 nm related to a conformational change in elastin's structure.

Figure 4. An overlay of ^{13}C NMR spectra for soluble elastin in D_2O showing alterations caused by AlCl_3 (B) as compared to control (A) at 43.7 ppm for $\alpha\text{-CH}_2$ of glycine residues and in the carbonyl carbon region (171–176 ppm shown in inset, B1: AlCl_3 , A1: control), suggesting structural changes in elastin. The changes are indicated by arrows.

nificant changes were seen in the peptide carbonyl region (171–176 ppm; Figure 4, inset).

Elastin Calcification in a Rat Subdermal Model

Progressive calcification of elastin fibers was observed in this model with severe calcification at 21 days (Figure 5). The elastin fibers were calcified irrespective of whether or not they were crosslinked with glutaraldehyde (Figure 5). The phosphorus levels were determined for the samples and molar ratios of Ca:P were determined. The Ca:P molar ratios (around 1.6) obtained suggested the presence of a poorly crystalline hydroxyapatite (Table 2). Morphological analysis of elastin implants confirmed the chemical results and provided insight into the mechanisms of calcification (Figure 6, A–C). Both crosslinked and non-crosslinked elastin calcified heavily. Mild to moderate inflammatory reaction and fibrosis were

Figure 5. Time course of calcification of elastin fibers in a rat subdermal model.

Figure 6. Morphologic aspects of elastin explants. **A–C:** light microscopy; **D** and **E:** electron microscopy. **A:** Non-crosslinked elastin; **B:** crosslinked elastin; **C:** AlCl_3 -treated elastin. Both non-crosslinked and crosslinked elastin have severe mineralization; in contrast, no calcific deposits are present in AlCl_3 -pretreated elastin. **D:** Electron photomicrograph of the explanted crosslinked elastin demonstrating earliest mineralization at the periphery of the individual fibers. **E:** Electron photomicrograph of the AlCl_3 pretreated elastin demonstrating complete absence of even the earliest calcific deposits. **A–C** stained with von Kossa's reagent (calcium phosphates black); original magnification $\times 266$ **D** and **E** stained with lead citrate and uranyl acetate; original magnification, $\times 12,000$. Scale bar, $2\ \mu\text{m}$.

Table 2. 21-Day Rat Subdermal Calcification Data for Elastin Implants

Group ($n = 10$ per group)	Ca ($\mu\text{g}/\text{mg}$)	P ($\mu\text{g}/\text{mg}$)	Ca:P molar ratio	Al levels of elastin before implantation ($\mu\text{g}/\text{mg}$)	Al levels of elastin after implantation ($\mu\text{g}/\text{mg}$)
Unimplanted control elastin fibers	0.16 ± 0.04	0.04 ± 0.02	—	undetectable	undetectable
Uncrosslinked elastin fibers	89.73 ± 9.84	46.98 ± 4.20	1.53	—	—
Glutaraldehyde-crosslinked elastin fibers	89.06 ± 17.93	44.07 ± 8.22	1.62	undetectable	undetectable
Uncrosslinked elastin fibers pretreated with 0.1 mol/L AlCl_3 for 2 hours	0.73 ± 0.19	1.45 ± 0.63	0.40	1.20 ± 0.14	0.88 ± 0.36
Glutaraldehyde-crosslinked elastin fibers pretreated with 0.1 mol/L AlCl_3 for 2 hours	1.66 ± 0.56	5.30 ± 0.69	0.25	1.26 ± 0.45	0.92 ± 0.16
Glutaraldehyde crosslinked elastin fibers pretreated with 0.1 mol/L AlCl_3 for 2 hours and then washed with 1 mol/L NaCl for 4 hours	2.15 ± 0.53	4.61 ± 0.40	0.37	1.71 ± 0.45	1.80 ± 0.17

Figure 7. X-ray diffraction patterns of mineral extracted from 21-day rat subdermal elastin implant. **A:** Mineral from elastin implant. **B:** Synthetic hydroxyl apatite. The spectra demonstrate characteristic diffraction peaks of hydroxyapatite. Radiation source, CuK_{α} .

present at the edges and only occasional mononuclear inflammatory cells were seen permeating the implant between the fibers. The degree of inflammation was equivalent in crosslinked and non-crosslinked elastin implants. Electron microscopy of the explanted crosslinked material revealed that mineralization began at the periphery of the individual fiber bundles and spread within individual fibers, ultimately involving entire bundles. Initial lesions resembled the small spicules of hydroxyapatite and was somewhat characteristic of the nucleation of calcific deposits in bone.

Mineral Phase Analysis by X-ray and FTIR Spectroscopy

Our X-ray analysis of mineral extracted from 21-day elastin implants clearly showed the presence of poorly crystalline hydroxyapatite (Figure 7), similar to results obtained from native bone mineral.²⁷ The resolution-enhanced FTIR spectra of the mineral extracted from elastin

Figure 8. Resolution-enhanced infrared spectra of isolated mineral from 21-day rat subdermal elastin implant showing poorly crystalline hydroxyapatite phases. **Lower**, direct recorded; **upper**, deconvoluted. **A:** $\nu_3\text{PO}_4$ domain (1200–900 cm^{-1}) deconvolution parameters, bandwidth 36 cm^{-1} ; sensitivity factor 2.2. **B:** $\nu_2\text{CO}_3$ domain (900–850 cm^{-1}) deconvolution parameters, bandwidth 10 cm^{-1} ; sensitivity factor 2.0. **C:** $\nu_4\text{PO}_4$ domain (650–500 cm^{-1}) deconvolution parameters, bandwidth 18 cm^{-1} ; sensitivity factor 2.0.

Table 3. Aluminum Levels in Elastin Samples (Uncrosslinked) after 1.0 mol/L NaCl Extraction Protocol

Extraction time	Aluminum levels ($\mu\text{g}/\text{mg}$) $\pm$ SD
Brief rinse	2.24 ± 0.17
4 hours	1.98 ± 0.20
1 day	2.26 ± 0.30
3 days	1.59 ± 0.28
7 days	1.33 ± 0.14
14 days	1.58 ± 0.20
21 days	1.71 ± 0.27

$n = 3$ per time point.

implanted in rats for 21 days provide useful information about the characteristics of the phosphate and carbonate environments (Figure 8).^{28,29} In the ν_1 , ν_3 PO_4 domain of calcium and phosphate phases (1200–900 cm^{-1}), the spectrum is characterized by intense but poorly resolved spectral bands (Figure 7A). A strong band at 1029 with shoulders at 1107 and 1122 cm^{-1} clearly shows poorly crystalline hydroxyapatite mineral phases.²⁸ The deconvolution of the $\nu_2\text{CO}_3$ FTIR domain (900–850 cm^{-1}) shows the same two carbonate bands (872 and 879 cm^{-1}) observed in bone mineral (Figure 7B). The band at 872 represents Type B carbonate (carbonate ions substituted for phosphate ions) and a band at 879 cm^{-1} corresponds to Type A carbonate (carbonate ions occupying monovalent OH sites in the apatitic structure). The shoulder at 866 cm^{-1} , not clearly visible in the spectra as shown but distinctly seen in the expanded spectra (not shown) is due to labile carbonates in non-apatitic environments. Both carbonate and HPO_4 contribute to this weak shoulder. The three main bands at 560, 577, and 601 cm^{-1} in ν_4 PO_4 domain could be assigned to phosphate ions in an apatite configuration and environment (Figure 7C). A strong shoulder at 613 cm^{-1} and a weak shoulder at 622 cm^{-1} could be assigned to a labile non-apatite environment of phosphate ions.²⁸

Prevention of Elastin Calcification by AlCl_3 Pretreatment

Significant levels of aluminum ions were bound to elastin after the AlCl_3 pretreatment of elastin for 2 hours. The levels of aluminum bound were similar for glutaraldehyde-crosslinked and uncrosslinked samples (Table 2). When AlCl_3 pretreated elastin fibers were extracted with 1 mol/L NaCl for up to 21 days to remove unbound or loosely bound aluminum, the levels of aluminum did not significantly decrease (Table 3). Pretreatment of elastin fibers with 0.1 mol/L AlCl_3 for 2 hours followed by 24 hours' incubation in sterile saline before implantation completely prevented elastin calcification in 21-day rat subdermal implants (Table 2). AlCl_3 pretreatment completely prevented calcification of both uncrosslinked and glutaraldehyde-crosslinked elastin (Table 2). The morphological study of the AlCl_3 -pretreated explants using von Kossa staining (which stains calcium phosphates black) and electron microscopic analysis showed complete absence of any calcific deposits (Figure 6, C and

E). The anti-calcification effect of AlCl_3 was still persistent after the fibers were washed in 1 mol/L NaCl solution for 4 hours ($2\times$) after AlCl_3 pretreatment (Table 2).

Discussion

The key findings of the present work are as follows. First, the time course of purified elastin calcification paralleled that of the calcification of aortic wall of bioprosthetic heart valves implanted subdermally in rats,¹³ and therefore may serve as a model to study aortic wall calcification mechanisms. Elastin calcified independent of glutaraldehyde, unlike bioprosthetic aortic cusps. Secondly, the aluminum chloride pretreatment, which was effective in preventing bioprosthetic heart valve calcification,^{14–17} completely inhibited elastin calcification and was associated with a specific alteration in elastin structure.

Calcification of Purified Elastin in a Rat Subdermal Model

Uncrosslinked purified elastin calcified to the same extent as glutaraldehyde-crosslinked elastin. This is in close agreement with previous results with uncrosslinked aortic wall allograft calcification that occurs in the rat subdermal model.⁹ Thus, glutaraldehyde crosslinking is not a prerequisite for elastin-oriented calcification. These results also agree with our radioactive glutaraldehyde study showing less reactivity of elastin with glutaraldehyde than seen with porcine aortic cusps or purified collagen. This was predictable, as our amino acid analysis of elastin showed very few free lysine residues. In the native state, elastin is intermolecularly crosslinked to form isodesmosine and desmosine residues and contains very few free lysine groups to react with glutaraldehyde. Our results showing purified elastin calcification demonstrate that the elastin component of the bioprosthetic heart valve extracellular matrix can also calcify independently. In our previous studies, ethanol pretreatment completely prevented bioprosthetic heart valve cusp calcification (which acts by lipid removal and collagen structural alterations),¹² but the same treatment was only partially effective in preventing aortic wall calcification.¹³ The aortic wall portion of the bioprosthetic heart valve is enriched with elastin and it could be possible that ethanol pretreatment is not able to completely prevent elastin-oriented bioprosthetic heart valve aortic wall calcification.

AlCl_3 Pretreatment Effects on Elastin Structure

Elastin has been postulated to have a mixture of α -helical, β -sheet, and random coil structures.³⁰ Various studies have been reported for elastin structure determination by optical spectroscopies.^{31,32} The carbonyl stretching (amide I, 1700–1600 cm^{-1}) region of the FTIR spectra is highly sensitive to the small changes in protein conformation. A recent paper discusses the amide I region of FT-Raman spectra for elastin, indicating the complex nature of this region.³² After deconvolution of the amide I

band, the elastin's tertiary structure was determined to be a mixture of 10% α -helices, 45% β -sheets and 45% unidentified (turn + coil) conformations.³² Pure insoluble elastin has never been studied by FTIR before our work, partly due to its insolubility. However, recent advances in ATR cells' assembly in FTIR make it feasible to study the conformation of native insoluble elastin fibers. In the present FTIR studies (Figure 1), the amide I band for elastin fibers was deconvoluted into five distinct bands: 1681, 1668, 1650, 1631, and 1614 cm^{-1} . The bands at 1631 and 1614 cm^{-1} can be ascribed to β -sheet structures of elastin, whereas a band at 1650 cm^{-1} can be assigned to random coil, turns, and α -helix parts of elastin structure. It has been postulated based on the FTIR results of tropoelastin that two types of β -sheets coexist in elastin conformation: regular (1630 cm^{-1}) and nonclassical (1614 cm^{-1}).³¹ The AlCl_3 pretreatment caused a significant reduction in the nonclassical β -sheet structures of elastin as assessed by reduction in intensities of the band at 1614 cm^{-1} . Similarly, there was an increase in turn-coil conformations in elastin after AlCl_3 pretreatment, as assessed by an increase in the 1650 cm^{-1} band. Thus, FTIR data suggest that aluminum ions bind to elastin and change its spatial conformation. However, it is warranted that the hydrophobic domains of elastin are highly mobile and that coil-turn or sheet-coil-turn conformational transitions can be mediated by the change in the water of hydration molecules in elastin caused by aluminum ion binding. The other parts of the FTIR spectra, such as amide II (N-H bending deformation and C-N stretching, 1600–1500 cm^{-1}), are less sensitive to the small protein conformational changes and thus, we did not see any appreciable changes in the other regions of the FTIR spectra (Figure 1).

Our experiments with CD and ^{13}C NMR spectroscopies for soluble elastin peptides further supported our FTIR results. The CD spectra of α -elastin in trifluoroethanol showed two negative peaks, 205 nm for unordered turn-coil conformations and 221.8 nm for ordered β structures. In comparing the effects of aluminum ions to those of calcium ions on the elastin structure by CD spectroscopy, we found that the changes in the CD spectra due to aluminum ion additions were comparable to those of calcium, that is, an increase in the intensity and shift to a lower wavelength for a peak at 205 nm (Figure 2), and a decrease in a peak at 221.8 nm, suggesting reductions in ordered β structures, although aluminum ion has only half the effect of calcium ions (Figure 3). These results are in agreement with previous studies by Urry et al showing structural alterations in the elastin due to metal ion binding to α -elastin with a variety of divalent metal ions using CD spectroscopy, although they did not study trivalent metal ions such as aluminum.²¹ In their work, calcium ions showed highest interactions with elastin, followed by Sr^{2+} and Mg^{2+} .²¹ The CD data also indicated that there are at least two conformationally different binding sites on the elastin for metal ions.²¹ The changes caused by calcium ions in the CD spectra of α -elastin were identical for both unblocked and blocked (charged groups such as amino and carboxyl were blocked by forming N-formyl-O-methyl ester derivative) elastin, suggesting

that metal ions interacted with elastin at neutral sites such as peptide carbonyl groups.²¹ Our present CD results are in agreement with the previously published results by Urry et al.

¹³C NMR spectroscopy has been used in the past to study elastin structure.^{26,33} It is well established that elastin molecular structure along with α -helices, β -sheet conformations show β -turns where Gly-Pro residues are adjacent to each other.^{34–36} This pairing of residues greatly favors β -turn formation, where the oxygen of glycine carbonyls is well exposed for co-ordination with cations.³⁴ In our NMR spectra (Figure 4), we observed two different peaks for α -CH₂ of glycine residues, a strong peak at 42.7 ppm and small peak at 43.7 ppm. We assigned a peak at 43.7 ppm for the glycine residues involved in the β -turn conformation. The disappearance of this peak after AlCl₃ pretreatment, together with significant changes in the carbonyl region, strongly suggests that the glycine carbonyls in the β -turn conformation are involved in aluminum ion binding.

Thus, our spectroscopy studies, along with quantitative aluminum ion analysis of elastin, show that aluminum ions irreversibly associate with elastin. This aluminum ion binding could be hypothetically important, as elastin structure would not accommodate calcium after aluminum ion binding or may interact differently with other ECM signaling molecules responsible for the progression of calcification.

AlCl₃ Pretreatment Effects on in Vivo Elastin Calcification

AlCl₃, used either as a pretreatment or with site-specific delivery through polymers, has been shown to be effective in preventing bioprosthetic heart valve calcification in rat subdermal implants as well as in circulatory studies with a rat aortic allograft model.^{9,14–17,37} Previous studies with AlCl₃-pretreated bovine pericardium showed localization of aluminum in phosphorous-rich intracellular loci of devitalized cells.^{16,17} Thus, it was hypothesized that aluminum ions inhibit calcification by preventing the crystallization of calcium phosphates in the devitalized cells (these are thought to be initial sites for bioprosthetic heart valve calcification). The pretreatments of bioprosthetic heart valve cusps with AlCl₃ also inhibited alkaline phosphatase activity, probably due to leaching of Al³⁺ ions, and may have mitigated calcification through this mechanism as well.¹⁷ Further studies with rat aortic allografts have also shown complete prevention of calcification by AlCl₃ pretreatment and it was assumed that it acts by the same mechanisms as mentioned before.^{9,37}

The present study for the first time demonstrates that aluminum ions irreversibly associate with elastin, altering its spatial structures such that it resists calcification. Thus, the irreversible binding of aluminum ions to elastin leading to complete inhibition of elastin calcification is a completely new finding.

Conclusions

The present studies demonstrate that (i) purified elastin undergoes calcification in a rat subdermal model; (ii) glutaraldehyde did not significantly react with elastin and therefore, the glutaraldehyde treatment had no influence on *in vivo* elastin calcification; and AlCl₃ inhibited calcification due to alteration of spatial structures of elastin as noted by CD, FTIR, and NMR spectroscopies and due to irreversible binding of aluminum ions to elastin, as confirmed by quantitative aluminum assay by atomic emission spectroscopy.

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