RAPID

PUBLICATION

Vitamin K-dependent Calcium Binding Proteins in Aortic Valve Calcification

ROBERT J. LEVY, JOHN A. ZENKER, and JANE B. LIAN, *Departments of Cardiology and Orthopaedic Surgery, The Children's Hospital Medical Center, and Departments of Pediatrics and Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02115*

ABSTRACT The pathogenesis of valvar calcification, which complicates the course of cardiac valve disease and also affects tissue valve prostheses, is incompletely understood. The present work explores the possible role of the vitamin K-dependent, calcium-binding amino acid, γ-carboxyglutamic acid (Gla) in valve mineralization. Gla is normally found in the vitamin K-dependent clotting factor proteins, and is also present in unique calcium binding proteins in bone, kidney, and lung. Unique Gla-containing proteins have also been isolated from pathologic calcifications including calcium containing renal stones and calcified atherosclerotic plaque. Calcified valves including specimens with calcific aortic stenosis, calcified porcine xenograft valves, and a calcified aortic homograft valve were analyzed for Gla content, complete amino acid analysis, and tissue calcium and phosphorus levels. Normal porcine valves contained protein-bound Gla (2.0–10.6 Gla/10^4 amino acids): no Gla was present in normal valve leaflets. Furthermore, Gla levels paralleled tissue calcium content in the calcified valves. In addition, complete amino acid analysis indicated a decline in valvar collagen content plus increased acidic proteins in conjunction with valvar calcification and the presence of Gla-containing proteins. These results suggest that calcific valvar disease may result in part from vitamin K-dependent processes.

This work was presented in part at the American Heart Association Scientific Sessions, November 1978, Dallas, Tex.

Received for publication 16 October 1979 and in revised form 13 November 1979.

INTRODUCTION

Pathologic mineralization frequently complicates the course of cardiac valvar disease. (1, 2) Furthermore, calcific degeneration of tissue valve prostheses has also emerged as an important clinical problem (3–5). The etiology of valvar calcification is poorly understood. Congenitally stenotic and bicuspid aortic valves are unusually susceptible to the development of calcific stenosis (1, 2). Aortic valve homografts, when used as prostheses, undergo rapid calcification over a 1–2-yr period (5), and this complication has resulted in the abandonment of this type of prosthesis. In addition, porcine xenograft calcification has been recently noted as a significant complication (3, 4) and this problem has been noted to occur more frequently in children (4).

γ-carboxyglutamic acid (Gla),[1] a highly specialized calcium-binding amino acid, was first discovered in the vitamin K-dependent blood coagulation factors (6, 7). Recently, Gla has been demonstrated to occur in other unique proteins of bone (8), kidney (9), and lung (10), and in proteins isolated from various pathologic calcifications including calcium containing renal stones (11, 12) and calcified atherosclerotic plaque (11, 13). Gla biosynthesis in liver (7), bone (14), kidney (9), and lung (10) has been demonstrated to occur as a vitamin K-dependent posttranslational, enzymatic carboxylation of specific glutamic acid residues (Fig. 1). Gla protein function has been studied in prothrombin

[1] *Abbreviation used in this paper*: Gla, γ-carboxyglutamic acid.

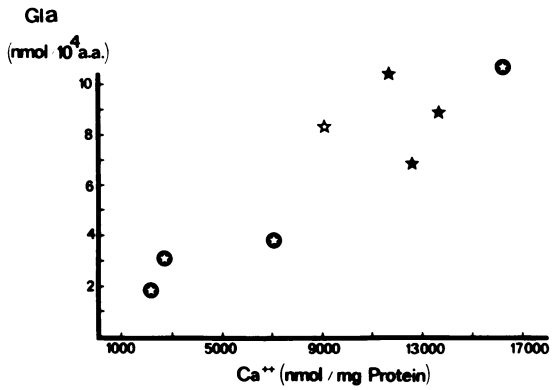

FIGURE 1 Gla and calcium content of calcified aortic valves. Gla is expressed as nanomoles per 10^4 nanomoles of total amino acids and calcium as nanomoles per milligram protein. ★: porcine xenografts; ☆: aortic homografts; ✪: calcific aortic stenosis; a.a., amino acids.

in which Gla residues occur at calcium and phospholipid binding sites (7). In the presence of bound calcium and phospholipid prothrombin can undergo a conformational change promoting its appropriate enzymatic cleavage by factor Xa in the clotting cascade to form thrombin. Vitamin K deficiency or antagonism (produced by warfarin and related anticoagulant drugs) results in deficiency of calcium binding sites resulting from inhibition of Gla synthesis and anticoagulation.

In the present work, data is presented that demonstrates the presence of Gla-containing proteins in calcific cardiac valve disease, indicating a possible relationship of vitamin K-dependent processes to the pathogenesis of these disorders.

METHODS

Valve specimens were prepared as follows: human aortic valves, porcine aortic xenograft valves (implanted 3–4 yr), and a human aortic homograft valve (implanted 2 yr) were obtained fresh at cardiac surgery for valvar stenosis; both grossly calcified and noncalcified specimens were studied. None of the patients operated upon had received warfarin therapy. Normal human aortic valves were obtained at autopsy. Normal porcine valves were obtained fresh at slaughter, and commercially prepared, nonimplanted glutaraldehyde-preserved valves were also studied as control tissue. The valves were rinsed with copious amounts of normal saline, and then freeze-dried. The dried valves were then milled to a coarse powder under liquid nitrogen. Alkaline hydrolysis of valve tissue (performed in order to prevent decarboxylation of Gla to glutamic acid) was carried out with 2 N KOH incubations at 110°C for 24 h as previously published (8). Acid hydrolysis was performed with 6 N HCl hydrolysis of powdered valve tissue for 24 h. Acid hydrolysates were then flash evaporated to remove concentrated acid, and diluted in 0.01 M HCl. Aliquots of the acid hydrolysates were diluted in 0.5% La^{+++} for calcium determination by atomic absorption (13). In addition, aliquots of the acid hydrolysates were analyzed for phosphorus content (15). Amino acid analysis of the alkaline hydrolysates to detect and quantitate Gla was performed on a Beckman-Spinco 121M (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) automated amino acid analyzer according to methodology developed in our laboratory (16). The lower limit of Gla detection with this system is 10 pM, and variation in ninhydrin peak areas among triplicate samples is <2% (16). The presence of Gla was confirmed by 6 N HCl decarboxylation of the putative Gla peak with resultant formation of glutamic acid as previously described (17). Finally, complete acid analysis (from which tissue protein content was also computed) was performed with the same amino acid analysis system on acid hydrolysates according to established procedures (16). Complete amino acid analysis precision is comparable to that described above for Gla.

RESULTS

Gla and mineral analysis. Gla was present in all calcified valve specimens (2.0–10.6 residues Gla/10^4 amino acid residues) and was undetectable in noncalcified tissues (Table I). Furthermore, as shown in Table I, human tissue specimens with calcific aortic stenosis demonstrated markedly elevated Gla levels, whereas noncalcified valves with congenital aortic stenosis had no detectable Gla. Also of interest is the finding of high Gla levels in a calcified aortic valve homograft. As presented in Table I, normal aortic valves had no detectable protein-bound Gla. Gla was also present in calcified porcine xenograft implants (Table I) and was not demonstrable in normal porcine aortic valves (nonfixed) and in nonimplanted glutaraldehyde-preserved valves (Table I). As Fig. 1 illustrates, valvar calcium content of the calcified valves was directly related to the tissue Gla levels.

Complete amino acid analysis. As shown (Table I), calcified valves demonstrated a number of important differences in amino acid composition compared with both normal and noncalcified stenotic valves. Both porcine and human valves showed increased levels of the acidic amino acids, aspartic acid and glutamic acid. Calcified porcine valves exhibited markedly lower hydroxyproline content compared with normal and glutaraldehyde-preserved porcine valves, indicating reduced collagen (18). Calcified human valves however, had only slightly lower hydroxyproline content. Glycine content paralleled the hydroxyproline content as would be expected because collagen is also rich in glycine (18). Hydroxylysine (an amino acid uniquely involved in collagen crosslink formation) was present at relatively lower levels in some valves with calcification. The low hydroxylysine levels in glutaraldehyde-preserved valves are a result of the glutaraldehyde fixation (19).

DISCUSSION

This study represents a demonstration of the presence of Gla-containing proteins in calcific cardiac valve disease. Our finding of high levels of Gla-containing proteins in calcified valves supports the view that Gla-containing proteins and vitamin K-dependent processes

TABLE I
Gla, Mineral, and Amino Acid Analysis in Aortic Valves

Diagnosis	No. of valves	Patient age	Gla	Glutamic acid	Aspartic acid	Hydroxy-lysine	Glycine	Hydroxy-proline	Calcium	Phos-phorus
		yr		*residues/10³ amino acids*					*nmol/mg protein*	
Normal human	2	15, 77	0.0	72–86	62–72	2.4–8.8	219–307	44–92	36.0–164.2	75.5–109.7
Noncalcified congenital aortic stenosis	3	12–18	0.0	78–80	63–64	5.5–8.1	264–280	89–96	68.2–335.0	44.2–303.5
Calcific aortic stenosis	4	52–77	0.2–1.06	82–112	65–93	1.3–7.6	148–258	42–88	2,080–16,197	1,563–12,073
Calcified aortic valve homograft	1	7	0.83	83	72	2.0	213	66	9,180	8,127
Normal porcine	1	—	0.0	82	65	10.0	277	94	146.2	37.5
Glutaraldehyde preserved xenograft (nonimplanted)	1	—	0.0	82	66	1.2	279	99	108.0	61.7
Noncalcified xenograft	1	77	0.0	106	95	10.0	181	66	133.2	198.6
Calcified xenograft	3	12–16	0.68–1.04	111–116	88–114	1.0–2.1	121–191	14–27	11,770–13,735	11,129–11,554

may play a role in the pathogenesis of calcific valve disease, both in congenitally deformed valves and in tissue prostheses. Also supportive of this hypothesis is our finding that Gla levels in the mineralized valves rose relative to tissue calcium content, indicating possible coordinate synthesis of Gla-containing proteins along with mineral incorporation. Complete amino acid composition studies of the mineralized valves showed that compared with normal, the Gla-containing proteins occur in these tissues as part of an overall change in protein composition, which includes relatively diminished collagen content and increased acidic amino acids, indicating increased acidic proteins.

The demonstration of the highly specific, calcium-binding amino acid Gla in calcified valves, suggests that Gla-containing proteins in the mineralizing valve leaflets may bear many functional similarities to other extrahepatic Gla-containing calcium-binding proteins. Osteocalcin, the Gla-containing protein found in normal bone (8) was the first example of an extrahepatic vitamin K-dependent protein (14). Previous work on bone development has shown that the highest Gla levels (and hence, highest osteocalcin levels) occur in the most mineralized regions of bone (18), a finding similar to the relationship in calcified valves shown in the present work (Fig. 1). Furthermore, Gla-containing proteins have also been noted to be present in other pathologic calcifications (11, 12) and unique Gla-containing proteins have, in fact, been isolated from calcium containing renal stones (12), and calcified atherosclerotic plaque (13). In calcified plaque our group has found that Gla levels paralleled calcium content and pathologic severity (13), as is the case in the present work concerning calcified cardiac valves. Atherocalcin (13), the Gla-containing protein we have purified from calcified plaque, has been partially characterized and is a very acidic protein, according to amino acid composition and charge, with an approximate molecular weight of 80,000 and 12 Gla residues/molecule. Its amino acid composition and isoelectric point differ markedly from other known Gla-containing proteins. The relationship of atherocalcin to the Gla-containing proteins in calcific valve disease remains to be established. If atherocalcin is the principal Gla-containing protein in calcified valves, then based upon the present data with respect to Gla content, atherocalcin would constitute 0.8 to 4% of the total protein in calcified valves.

The precise developmental role of the Gla-containing proteins in valvar calcification remains to be elucidated. It may be that Gla-containing proteins found in calcified valves are synthesized elsewhere, and are adsorbed by the mineralizing valve tissue. This, however, seems unlikely for the following reason: the relationship of Gla to calcium content in calcified valves shown in Fig. 1 suggests an ordered synthesis of these proteins *in situ* rather than adsorption. Furthermore, our data show that the typical valvar calcium

levels in the calcified leaflets are several orders of magnitude greater than the molar amounts of detectable Gla. This suggests that the Gla-containing proteins present in calcified values may function as calcification initiators, rather than macromolecules simply adsorbed to the mineralizing matrix. In the case of porcine xenograft and homograft valves, *in situ* biosynthesis could only result from host cell invasion and, in fact, increasing cellular invasion of valvar grafts with time has been reported (20).

Furthermore, the present results suggest that the biosynthesis of Gla-containing proteins found in calcified cardiac valve tissue is vitamin K-dependent, as has been shown for liver (7), bone (14), kidney (9), and lung (10). In the case of the vitamin K-dependent clotting factors, vitamin K-antagonism with warfarin or related drugs results in an inhibition of Gla synthesis with loss of calcium binding and anticoagulation (7). If Gla-containing proteins are necessary for the mineralization of cardiac valves, then vitamin K-antagonism with (perhaps) warfarin or analogous drugs could delay or diminish the pathologic process. Also, modification of tissue prostheses with either warfarin analog pretreatment or actual material incorporation of these compounds could favorably affect calcific degeneration.

ACKNOWLEDGMENTS

The authors wish to thank Dr. Paul Gallop, Dr. William Bernhard, and Dr. Judith Levy for their advice and helpful discussions. We would also like to thank Dr. Joseph Corson of the Peter Bent Brigham Hospital, Boston and Dr. Aldo Castaneda of our institution for providing pathologic specimens. We also thank Ms. Margaret Wall and Ms. Terry McCarthy for the preparation of the manuscript.

This work was supported by National Institutes of Health grants: HL05606, HL24463, and DE0461.

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