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CALCIFICATION OF RAT VALVE ALLOGRAFTS

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Abstract

Scanning electron microscopy (SEM) and energy dispersive X-ray microanalysis (EDS) have been used to quantify calcium deposition in bioprosthetic valves. To further characterize the calcification process as it pertains to allograft valve tissue, two models of tissue valve implantation were used. The first model used subcutaneous implantation of glutaraldehyde-preserved allogeneic aortic and pulmonary valve leaflets. The second model used syngeneic or allogeneic fresh aortic valve grafts implanted heterotopically into the abdominal aorta of recipient rats. Reference light microscopy was used to select sections for SEM and EDS. In the subcutaneous model, calcium content in both the pulmonary and aortic valves increased up to three weeks, followed by a plateau. The pulmonary leaflets showed greater calcium content than aortic leaflets. In the heterotopic implantation study, calcification occurred to a significantly greater degree in the allogeneic than in the syngeneic valves. This technique may be useful in analyzing the factors that contribute to deterioration of bioprosthetic and allograft valves.

Introduction

Cardiac valve replacements include substitutes of mechanical or biological origin. Biological substitutes commonly used today are bioprosthetic valves (primarily glutaraldehyde-preserved porcine aortic valves) and human allografts. Unlike mechanical valves, tissue valves do not require anticoagulation, and are therefore preferable for patients in whom long-term coumadin treatment is unsuitable. Despite the increasing use of aortic valve allografts (homografts) in cardiac surgery, there is concern about the long-term durability of these tissues. Sources of this concern include the possible importance of immunologically mediated destruction of the graft, and the potential for graft calcification. Conventional clinical valve replacement using allografts makes no use of tissue typing between donor and recipient. Some investigators have proposed making a concerted attempt to blood group match the valve to the patient (Yankah et al., 1988), although there is no evidence that ABO mismatch diminishes graft longevity (O'Brien et al., 1987).

This report describes our investigation of calcium measurement in heart valve leaflets of the rat using scanning electron microscopy and energy dispersive X-ray microanalysis (EDS). This technique was used to quantify calcium deposition in glutaraldehyde-preserved valves implanted subcutaneously, a model known to result in rapid calcification. It was then used in a model of heterotopic valve implantation in the abdominal aorta, to evaluate calcification in a model more analogous to the clinical methods of allograft implantation.

Materials and methods

Subcutaneous implantation

Aortic and pulmonary valves were harvested from adult, male, Brown Norway rats, 250-300 g body weight. The valves were treated with phosphate-buffered 0.6% glutaraldehyde in 0.9% NaCl for 24 hours. Valve leaflets were dissected, washed in saline, and implanted subcutaneously into the dorsal thorax of recipient rats. After 1 to 5 weeks, the valve leaflets were harvested, fixed, and carbon coated. Additional valves were studied after preservation, but before implantation. All valve recipients were 10-12 week old rats of the Lewis strain. The Lewis and Brown Norway strains are strongly allogeneic, incompatible at both the RT1 (major histocompatibility complex) and non-RT1 loci. Five aortic and five pulmonary valves were studied after implantation for each duration (control, 1, 2, 3, 4, and 5 weeks).

Heteropic transplantation

This procedure has been described previously (El Khatib and Lupinetti, 1990). Inbred male rats (250-300 g body

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weight) underwent general anesthesia with 3.6% chloral hydrate solution (1 ml/100 g body weight) administered by intraperitoneal injection. Under clean conditions, the heart and ascending aorta were excised and rinsed in 0.9% saline. The aortic valve with attached ascending aorta, anterior mitral leaflet, and small portion of left ventricular outflow tract was dissected and placed in chilled, heparinized saline while the recipient animal was prepared. Recipient rats underwent laparotomy and dissection of the abdominal aorta under a 14 X operating microscope. The recipient aorta was occluded proximally and distally and divided. An end-to-end interposition of the allograft was performed using 8-0 polypropylene suture. The anterior leaflet of the aortic valve was incorporated into the suture line to prevent valve competence, which may predispose to thrombosis. Twelve weeks following implantation, the animals were sacrificed and the valve grafts were harvested. All heterotopic valve recipients were 10-12 week old, male Lewis rats, while donors were either Lewis (n=3) or Brown Norway (n=5) rats, also 10-12 week old males.

Scanning electron microscopy and calcium quantification

Following harvest, the valves were rinsed in saline and washed free of salts with distilled water. They were secured to a specimen holder with Tissue-Tek O.C.T. and quick frozen in liquid nitrogen. Sections 8 μm thick were cut using a Reichert-Jung cryostat at -20° C and mounted on carbon stubs. The specimens were freeze-dried at -40° C with an EMScope FD500 for 12 hours, followed by a 40 hour period at room temperature. The tissue was carbon coated. Alternate 8 μm sections were mounted on chrome-alum coated glass slides and stained with Von Kossa's procedure for calcium phosphates and counter-stained with eosin. These served as references for locating specific tissue regions to be analyzed with electron microscopy and energy dispersive spectroscopy. The freeze-dried leaflets were examined with a Hitachi S-570 scanning electron microscope (Mountain View, CA) equipped with a Kevex 8000 energy dispersive spectrometer and quantum detector (San Carlos, CA). X-ray microanalysis was performed on five distinct reduced-screen areas of one leaflet of each valve to detect calcium. The section analyzed was randomly selected, and the five regions measured encompassed all or nearly all of the leaflet. Acquisition was performed at 500 X magnification for 180 seconds, using an accelerating voltage of 15 kV and an emission current averaged at 95μA. The beam angle was 90° and the working distance was 35 mm. For each leaflet, the mean number of calcium counts per region was recorded. Data were pooled for each group of valve leaflets, and the group mean and standard error were calculated.

Results

Results of calcium assays for the subcutaneous implantation of glutaraldehyde-preserved aortic and pulmonary valves are illustrated in Figure 1. Mean calcium counts increased progressively up to three weeks in each group, followed by a plateau beyond three weeks.

Calcium content of syngeneic and allogeneic heterotopically implanted fresh allografts are displayed in Figure 2. Mean calcium counts were 861 ± 260 in the syngeneic group and 6961 ± 2316 in the allogeneic group.

Figure 3 demonstrates a representative aortic valve allograft harvested 12 weeks after implantation. Figure 4 shows a higher power view of the same specimen. Typical of the other valves examined, calcification appeared in a random pattern, with no apparent predominance of any one region of the leaflet.

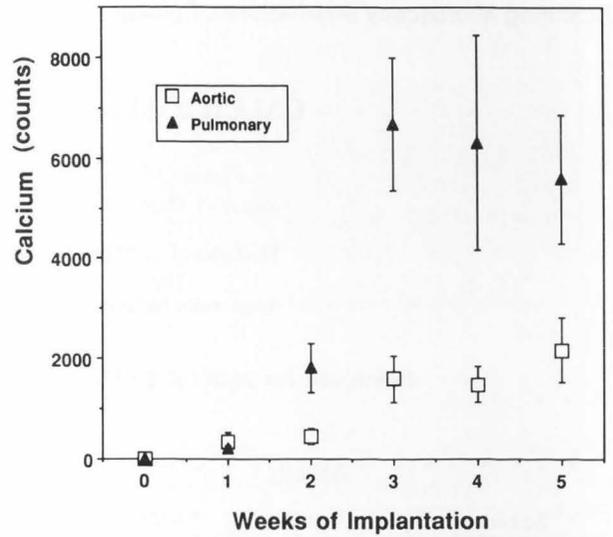


Figure 1. Calcium content in subcutaneously implanted glutaraldehyde-preserved aortic and pulmonary valves (mean values + S.E.M.).

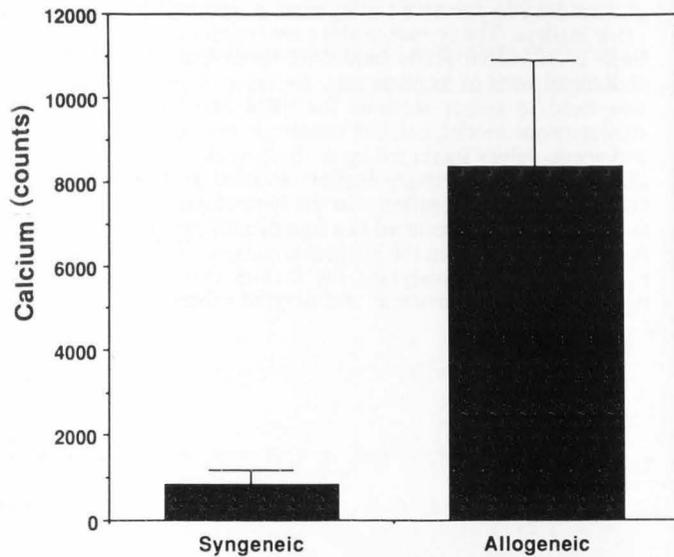


Figure 2. Calcium content in heterotopically implanted syngeneic and allogeneic aortic valves (mean values + S.E.M.).

Discussion

Aortic and pulmonary valve allografts have become used with increasing frequency for treatment of aortic valve disease, pulmonary valve disease, and complex congenital cardiac anomalies. Compared to mechanical prostheses and bioprostheses, allograft valves are associated with fewer infectious and thromboembolic complications, and display excellent hemodynamic characteristics. Chronic degeneration of allografts may occur, however, and may result in valve stenosis and/or insufficiency. Calcification is an important mode of allograft failure.

Bodnar and Ross (1982) found that calcification occurred in 17.5% of explanted allografts. Because 27.5% of grafts

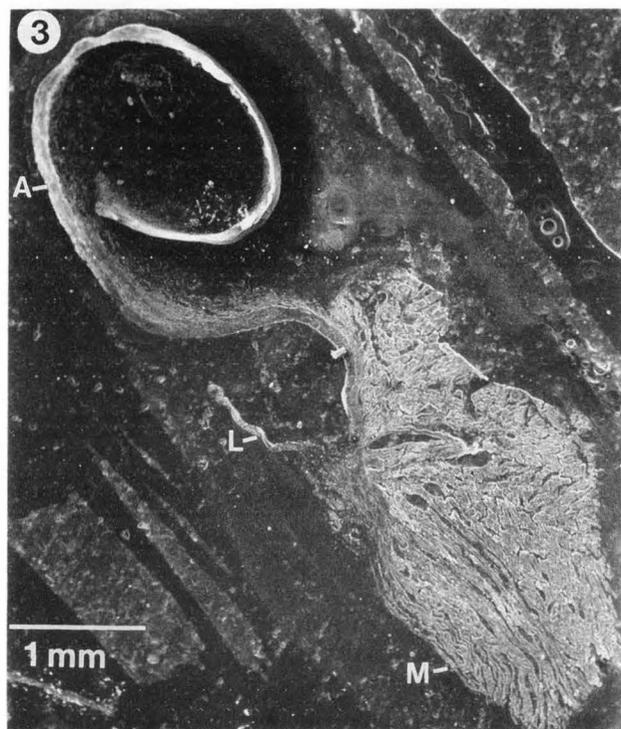


Figure 3. Allogeneic aortic valve leaflet implanted heterotopically for 12 weeks (A--aortic wall; L--valve leaflet; M--myocardium).

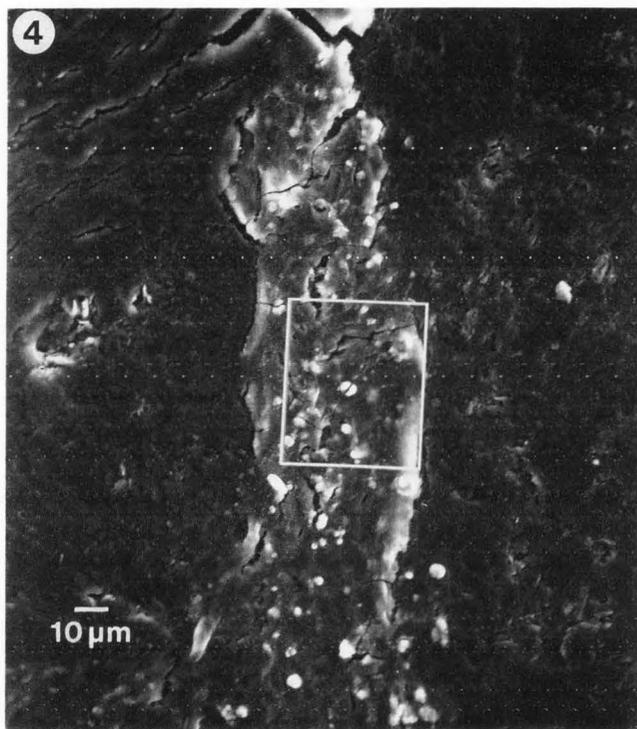


Figure 4. Allogeneic aortic valve leaflet implanted heterotopically for 12 weeks. Rectangle denotes region of measurement using EDS. Calcium (white refractile areas) is randomly distributed within the leaflet.

failed due to technical problems, calcification was second only to tissue fatigue as a cause of primary tissue failure of allografts, ahead of infection, shrinkage, and tissue overgrowth. However, the precise factors that predispose some allografts to calcification, others to fatigue, or others to excellent long-term function are poorly understood. This experimental model, therefore, was used to permit evaluation of immunologic differences between donor and recipient on the magnitude of calcium deposition in the valve leaflets.

The experimental model of heterotopic valve implantation may offer several advantages for studying the pathologic fate of tissue valves. First, the immunogenic potential of the graft may be expressed to a greater degree with intravascular, rather than subcutaneous, insertion. Second, the placement of the valve leaflets in the pulsatile aortic flow may simulate mechanical forces of valve opening and closing that cannot be

exerted on a leaflet outside the arterial stream. Third, this model can be performed in small, inbred species, permitting control of immunologic variables that are less easily standardized in larger animal models.

The subcutaneous implantation model has other advantages, notably the demonstrated comparability of the pattern of calcification seen in this model to that of intracardiac bioprostheses (Schoen et al., 1985). The purpose of performing the subcutaneous studies was to permit some degree of confirmation of the ability of EDS to quantify calcium a model known to provoke tissue calcification. In the subcutaneous implantation phase of this study, it was perhaps not expected that the calcification of the pulmonary valves would exceed that of the aortic leaflets. Our findings are in contradistinction to those of Livi et al. (1987), who found that the pulmonary artery wall contained a much smaller calcium

content and less elastic tissue than the aorta. Although this suggested the possibility that pulmonary grafts may be less prone to calcification than aortic grafts, no confirmatory tests were carried out to support this hypothesis.

In clinical use histocompatibility differences between the donor aortic valve allograft and the recipient are of unknown importance to the longevity of the graft. It has been suggested that valve allografts may be immunologically privileged, either because no microvasculature develops between host and graft, because the cells are rapidly replaced by those of recipient origin, or because the graft does not contain a significant mass of viable tissue. Although allograft valves are employed without immunosuppressive medication, some investigators have recommended that ABO blood group matching between donor and recipient be performed if possible (Yankah et al., 1988).

Previous investigations by this and other laboratories have demonstrated the immunogenic potential of aortic valve allografts both in the fresh state and following cryopreservation (El Khatib and Lupinetti, 1990; Cochran and Kunzelman, 1990). However, whether immunogenicity influences the long-term performance or pathologic fate of the graft remains speculative.

Experimental evidence for the importance of immunologic influences on allograft valve degeneration comes from the work of Gonzalez-Lavin et al. (1988). These investigators found that calcium content of allograft valve leaflets and aortic conduits was greater when the transplant was performed between unrelated dogs than when the transplant was between littermates. Although use of the canine model does not permit the immunologic precision available with highly inbred strains, the prevalence of calcification in failed allografts emphasizes the relevance of this study to clinical practice. Rocchini and associates (1981) have suggested that xenograft valve failure may also be mediated by immunologic reactions.

Energy dispersive X-ray microanalysis (EDS) permits quantification of calcium in small amounts of tissue and in locations that may not be measurable using other methods. The use of EDS to quantitate calcification of bioprosthetic valves was first performed by Nelson et al. (1985) using glutaraldehyde-preserved xenograft valves. That study compared data obtained with EDS to that obtained using biochemical analysis, and found a high correlation between the two measurements. In the present study, initial observations were made using a similar model of glutaraldehyde-preserved valves. It is noteworthy that the time course of calcification observed in the subcutaneously implanted glutaraldehyde-preserved valves in the present study paralleled that observed by Nelson and associates. In that study, as in the present investigation, maximum calcification in subcutaneously implanted tissue valves occurred between three and four weeks after implantation.

Although it would be desirable to correlate EDS measurements of calcium with those obtained by spectroscopy, this does not appear to be practical. First, the mass of tissue in these rat valve leaflets are extremely small. Second, efforts to dissect individual leaflets from the remainder of the specimen with extensive surgical scarring and adhesions is difficult. Finally, measurement of calcium in specimens not thoroughly separated from aortic wall, myocardium, and suture material may yield unrepresentative results. Therefore, this model appears to require EDS measurement to obtain reliable data.

Acknowledgement

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Discussion with Reviewers

F. Bilge: The authors use the term "anterior leaflet of the aortic valve" [referring to the operative technique]. I am not familiar with such terminology. Is it correct or do they mean the anterior leaflet of the mitral valve?

Authors: The anterior leaflet of the aortic valve corresponds to the right coronary ostium. Incorporating this leaflet prevents valve competence which may result in thrombosis. Other leaflets could be used as well for this purpose.

S. R. Khan: How and why were the valve carbon coated?

Authors: Carbon evaporation coating was used to reduce "charging." This is a buildup of electrons in a nonconductive specimen, which may deflect the incident beam, make viewing difficult, and damage the specimen. Although carbon coating will trap some lower energy X-rays, the coating is consistent and should not have affected the data.

G. M. Roomans: Have similar experiments with aldehyde-treated, subcutaneously implanted valves been carried out where donor and recipient were of the same strain? Is there less calcification under those conditions?

Authors: We are aware of no such work having been done. Most of the previous investigations have studied xenograft tissue.

H. Harasaki: What is the rationale that these cross sections can represent the distribution and the intensity of calcification of the aortic valves, which can be quite heterogenic?

Authors: It is acknowledged that the sample subjected to calcium measurement represents only one, randomly selected portion of the entire valve. However, this methodology has been compared to biochemical analysis in larger tissue sections with high correlations between the two methods (Nelson et al., cited above). Because of the very small amounts of tissue in this model, a direct comparison of energy dispersive methods to bulk methods is not practical.

R. J. Levy: If confirmatory x-ray emission counting was used to generate a calcium map, it would be useful to show this as a matching image.

Authors: No calcium maps were generated during this investigation. We agree that it would be a useful adjunct to the quantitative data.

F. J. Schoen: What is the possible explanation for the greater mineralization in the fixed pulmonic valve than in the fixed aortic valve? Are there structural differences between these two valves in the rat; are there differences in the structures of these valves between rat and larger animals?

Authors: Few comparisons have been made between the valves of the rat and those of other animals. Livi et al. (*J Thorac Cardiovasc Surg*; 1987; 93:755-760) found that in humans, pulmonary valve allografts contained less calcium and less elastic tissue than aortic allografts. This study was performed on the arterial walls, and not on the valve leaflets, however. It may be speculated that differences in pulmonary and systemic pressures affect the structure of the valves in such a way as to alter their predisposition to undergo calcification.

F. J. Schoen: In the aortic interposition model, what are the possible explanations for the allogeneic implant calcifying more than the syngeneic implant? Could this be due to an underlying difference in the propensity of these two donor strains to calcify their aortas, rather than an immunological cause?

Authors: It remains a possibility that some strains have a greater propensity to calcification than others. Whether immunological factors play a definitive role in calcification will require further investigation, although our data suggest that such a role may exist. It must be noted that previous studies attempting to answer these questions have focused exclusively on glutaraldehyde-preserved valves. This study, on the other hand, attempts to determine whether differences in calcification occur in untreated, freshly harvested valves.

F. J. Schoen: What was the fate of the aorta wall portion of the abdominal aortic graft with respect to calcification?

Authors: The aortic wall was not studied.