Scanning and Transmission Electron Microscopic Study of Recovered Porcine Aortic Valved Conduits

D. J. Allen  
*Georgia State University*

I. H. Fentie  
*Medical College of Ohio*

J. T. Davis  
*Medical College of Ohio*

Angela Lineen  
*Medical College of Ohio*

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Abstract

Four explanted porcine aortic valved conduits were examined using scanning and transmission electron microscopy. Sources of obstruction such as neointima or "peel" and calcification were observed. In one sample the neointima was found to possess an unusually large expanse of squamous cells partially lining the luminal surface. This lining much resembled a normal endothelium, which is not an expected feature of neointima. Cells, presumably of host origin, were noted upon the leaflet surfaces. They did not seem as well organized as those found on the neointima. Calcification did not seem greatly advanced but was clearly apparent. Certain treatments proposed by others to curtail calcification are discussed and amended herein.

SEM examination of three of these conduits provided good evidence of lining cells on only the inflow surface of the leaflet. The fourth conduit, however, showed cells on both inflow and outflow surfaces. These cells possessed certain characteristics of cells from leaflets of the other three conduits, but questions remain as to the precise identification of all of these lining cells. TEM examination provided cytological evidence of macrophage-like cells lining the inflow surface of a leaflet.

Key Words: Bioprosthesis, heart valve, valved conduit, scanning electron microscopy, transmission electron microscopy, porcine valve, neointima, peel, calcification

Introduction

Valved conduits fashioned from a variety of materials have been applied to the surgical correction of congenital cardiovascular malformations for almost two decades. In 1966, Ross and Sommerville made the first successful anastomosis between the right ventricle and pulmonary artery with a homograft of aortic valve and aorta to correct pulmonary atresia (32). One variation, a homograft aortic valve fitted inside a Dacron tube, was employed by Kouchoukos et al. (25). Another variation was instituted by Horiuchi and associates (21) who prepared both valve and conduit from autologous pericardium. In another example of autologous tissue use, Ionescu and Deac (23) constructed both valve and conduit from fascia lata. Mechanical valves (Bjork-Shiley) have also been inserted in Dacron conduits, as reported by Huse (22) and by Cartmill and colleagues (10). Use of what in the United States seems to be the item of choice presently, a porcine aortic valve fitted in a woven Dacron tube, was first reported by Bowman and co-workers in 1973 (8). Over the past decade many of the porcine valved conduits just described have been recovered from patients for examination. These implants have been found to display characteristics which vary dependent upon intra- or extracardiac location and placement within the pulmonary or systemic circulations. The reports of Miller et al. (29) and Rocchini et al. (31) indicate a lesser tendency for calcification in valves located in extracardiac conduits than with intracardiac valves. The conduits themselves, on the other hand, can develop a neointima, or "peel", first reported by Ciavarella and associates in 1979 (12) which can become obstructive in the lesser circulation. Neointima does form in the left outflow tract but is not reported to attain obstructive dimensions, perhaps, as suggested by Bortolotti and colleagues (7), due to higher flow rates discouraging formation and organization of thrombi in the systemic circulation. In 1981, Agarwal et al. (1) reported on a study of several thick,
obstructive peels. The same group later published a paper on right outflow tract peels which had remained thin and non-obstructive (2). Since the report of Ciaravella et al. (12), mention of peel has become more the rule than the exception in reports of conduit studies.

The investigation reported here presents findings of transmission and scanning electron microscopy, as well as light microscopy, applied to the examination of four conduits recovered from implantation in patients. Apparent re-endothelialization was noted on leaflet surfaces of valves within these conduits. Aspects of calcification found in these valves stimulate questions as to when the process begins, before or after implantation.

Materials and Methods

Each of the explants examined was acquired from a patient undergoing normal surgical replacement of a right outflow tract bioprosthesis. In three of the four cases, replacement was required due to outgrowth of conduits implanted in infancy. In the other instance, fibrosis and calcification led to the removal and replacement of the conduit and bioprosthesis. All of the patients were young males. A summary of the pertinent information is provided in Table 1.

Samples were taken from the valvular cusps and from material (neointima) lining the woven Dacron tubes. As controls, similar tissue samples (with the exception of neointima) were obtained from unimplanted Hancock porcine aorticvalved conduits and from unimplanted Hancock porcine valves intended for intracardiac application.

Specimens intended for light microscopy (LM) or transmission electron microscopy (TEM) were fixed in 0.1 M phosphate buffered 3% glutaraldehyde, pH 7.2 - 7.4, and post-fixed in 2% osmium tetroxide in the same buffer. After washing out post-fixative with buffer, samples were dehydrated through an ascending, graded ethanol series, transferred to propylene oxide and then to a mixture of equal volumes of propylene oxide and Epon-Araldite over night. Tissues were then embedded in an Epon-Araldite mixture and sectioned after curing. Semi-thin sections (1 mm) were stained with methylene blue for LM. Thin sections were stained using uranyl acetate and lead citrate or orcein as described by Nakamura et al. (30) and examined with either a Philips 300 transmission electron microscope or an ultra high resolution Hitachi 11-E transmission electron microscope.

Tissue intended for scanning electron microscopy (SEM) was washed with buffer out of the same glutaraldehyde fixative described above and dehydrated through a graded ethanol series. Samples were then critical point dried using liquid carbon dioxide, mounted on aluminum stubs and coated with gold-palladium in a Tousimis sputter coater previous to examination with a Cambridge Stereoscan S-180 scanning electron microscope.

Selected samples intended for SEM also underwent a post-fixation in osmium tetroxide as described for the TEM samples when other examinations were anticipated for them.

Results

A particularly unusual finding was observed in one conduit. It possessed a neointima that was partially lined by squamous cells as depicted in figure 1. These cells abutted upon surrounding cells completely around their circumference and possessed fine microvilli. Some cells possessed a larger compliment of these microappendages than their neighbors as shown in figure 2. While the cell lining was not continuous, some fields measured over 2.5 x 5 mm of uninterrupted cells as typified by the example of figure 3. This was the only conduit of the group examined here, and to our knowledge, anywhere else, to possess such cells over the surface.

<table>
<thead>
<tr>
<th>I.D.#</th>
<th>Age at Surgery</th>
<th>Time in Position</th>
<th>Sex</th>
<th>Original Diagnosis</th>
<th>Reason Removed</th>
</tr>
</thead>
<tbody>
<tr>
<td>VC4</td>
<td>17.0 yrs.</td>
<td>8.0 yrs.</td>
<td>Male</td>
<td>Transposition of Great Vessels</td>
<td>Calification and Fibrosis</td>
</tr>
<tr>
<td>VC3</td>
<td>6.0 yrs.</td>
<td>4.0 yrs.</td>
<td>Male</td>
<td>Tricuspid Atresia</td>
<td>Outgrown</td>
</tr>
<tr>
<td>VC2</td>
<td>6.5 yrs.</td>
<td>4.5 yrs.</td>
<td>Male</td>
<td>Pulmonary Atresia</td>
<td>Outgrown</td>
</tr>
<tr>
<td>VC1</td>
<td>6.8 yrs.</td>
<td>6.7 yrs.</td>
<td>Male</td>
<td>Persistent Truncus</td>
<td>Outgrown</td>
</tr>
</tbody>
</table>
Extracardiac valved conduits

Fig. 1. This sample, taken from the luminal neointimal surface of VC4, possesses a partial lining which resembles endothelium, an unusual feature for this tissue.

Fig. 2. This cell, from the same sample, possesses a larger quantity of short microvilli when compared with those surrounding it.

Fig. 3. Some scope of the width of tissue lined by these cells is presented in this illustration. These cells present many of the features attributable to endothelium.

Fig. 4. There is perhaps a more expectable view of the luminal surface of the neointima shown in this sample from VC2. There is an obvious array of collagen fibers which have no common direction.

Fig. 5. Some scope of the width of tissue lined by these cells is presented in this illustration. These cells present many of the features attributable to endothelium.

of the neointima. Peel commonly appears quite flat and smooth to gross anatomical or low magnification SEM examination. This is not directly related to the possession of a lining epithelium but rather to a smooth, bare region of connective tissue fibers as shown in figure 4. The collagen fibers shown in this figure were seen to be laid down at various angles and to appear relatively smooth. Figure 5 is a low magnification SEM micrograph of bare woven Dacron which illustrates the interwoven pattern of the corrugated tubing from which conduits are prepared. This segment was taken from an unimplanted valved conduit. Such a tight pattern of interwoven fibers does not permit establishment of firm connections between the conduit and its loosely adherent neointima.

When neointima is examined using TEM methods (fig. 6), connective tissue elements are found which have the appearance of healthy, growing tissue. The fibroblasts
Fig. 5. The corrugate surface of a control conduit is demonstrated here. Note the tightly interwoven Dacron fibers. This tight fiber mesh is thought responsible for the loose manner by which neointima is bound to the conduit lumen, as the luminal tissue cannot easily establish anchoring roots within the mesh.

Fig. 6. A cross-section of neointima taken from VCI is presented here. These cells, presumed fibroblasts, possess easily observed membranes and appear actively involved in collagen production as implied by the considerable array of Golgi apparatus (G) seen in the upper cell. The somewhat "plump" shape in itself is taken to imply activity in such cells.

Fig. 7. The leaflet inflow surface examined by TEM is presented here. Note the dark filaments in lower portion of the photograph (arrowheads). These are thought to be elastic fibers stained with orcein. The three surface cells do not appear to be squamous. These cells seem to be other than endothelium.

Fig. 8. This group of cells, from the valve of VCI again thought to be fibroblasts, show a degree of wear. The collagen fibers appear to be breaking down and open areas may be seen between the cells. Note what may be an area of calcification occurring within the cell (arrowhead).

The cells are surrounded by collagen and some few elastin fibers. The tissue seems to be intact without signs of infiltrate or obvious collagen degeneration.
Extracardiac valved conduits

Examination of valve leaflets using TEM provide some interesting views of the surfaces and deep structures. Figure 7 is a view of two adjacent cells on or near the surface of the leaflet with another cell extending out into the lumen. The borders of these cells clearly are not tightly joined by junctional complexes. From their appearance, such cells appear to be migrating either into or out of the leaflet surface. Some "orcein-positive" stained material with the configuration of elastin fibers was observed within the cusps.

Figure 8 provides a cross-sectional example of the deep structure of the leaflet showing good preservation of cellular elements (fibroblasts). These cells appear to have been actively involved in collagen production at the time of preservation. They possessed many Golgi complexes and large areas of rough endoplasmic reticulum. Dense inclusion granules were seen as well as areas of calcification. The surrounding collagen, however, was not well established. It appeared, rather, to be undergoing modification of some sort. Presumably, from the level of activity of the surrounding cells, new collagen was being synthesized.

Figure 9 shows two cells closely related to the outflow surface of the leaflet. Such cells usually were oval or ovoid in shape and possessed long anchoring microappendages. As in the previous TEM view (fig. 7) of surface cells, no clear junctional complexes were evident. Many lysosomes were seen within these cells which are more indicative of macrophages than endothelial cells.

Other areas of the valve showed signs of breakdown as exemplified in figure 10, a TEM view of a leaflet cross-section. The collagen fibers appeared to be undergoing degeneration. Evidence of lipid infiltration and obvious extracellular calcification were also present.

Figure 11 provided another example of disrepair, in this case including signs of cellular destruction. Large, open areas between the cell and the surrounding structures were seen. No limiting membrane could be followed around the cell.

SEM examination (fig. 12) of three of these conduits provided good evidence of lining cells on only the inflow surface of the leaflet. The fourth conduit, however, showed cells on both inflow and outflow surfaces. These cells possessed certain characteristics of cells from leaflets of the other three conduits, but questions remain as to the precise identification of all of these lining cells. TEM examination provided cytological evidence of macrophage-like cells lining the inflow surface of a leaflet. At higher power (fig. 13), these cells were seen to possess what appeared to be microvilli, however, their borders were often incomplete indicating that they may be a monolayer of macrophages or some form of transitional cell between the two major cell types.

Examination of control tissue provided some interesting comparisons. Leaflets taken from an unimplanted valved conduit fitted with a porcine aortic valve possessed no cells on inflow or outflow surfaces. Leaflets from an unused porcine aortic valve intended for intracardiac use, however, were found to possess cellular remnants as shown in figure 14. This comparison is made to indicate that handling and preparation techniques are imperfect and cell remnants are found in crevasses and also on open areas of the leaflet surface.
Fig. 11. This leaflet cross-section from VCl illustrates a different degree of preservation in the tissue when compared with the cells in figure 8. The collagen also appears to be less well organized with large openings to either side of the cell remnant. No clear membrane can be traced round the cell although membranous structures which probably represent endoplasmic reticulum still are apparent.

Fig. 12. This view of the inflow surface of a leaflet from VCl illustrates the very tenuous nature by which these cells are bound to the leaflets. There are many openings between cells including the wide expanse of open area between the two fields. Note the edge along which many of the cells are being drawn off the surface.

Fig. 13. The cells shown here, from VCl, appear squamous and possess short microvilli. Long microappendages extend between these cells leaving bare areas between them. Note that these cells overlie some erythrocytes (arrowheads) and are not firmly attached to the underlying tissue.

Fig. 14. For the sake of comparison this illustration of an unimplanted control valve is offered. While surface cells, clearly remnants of the donor animal, are present, they appear to be less intact than those previously shown and are found in fewer numbers, often only in crevasses but occasionally on leaflet surfaces. Some treated valves are completely devoid of such cells.
Application of the valved conduit to repair of malformations of the heart and great vessels found with congenital heart disease is particularly significant because, instead of returning a patient's health to a level the patient has never known before. It is unfortunate when such an endeavor is marred, but reports of failures with conduits are not uncommon, often due to obstruction brought about by several mechanisms outlined in the 1981 report of Agarwal and associates (1).

Reasons for early obstruction included the use of undersized conduit, compression of the conduit due to unfortunate placement and obstructions developing in association with the proximal or distal suture lines. Care at surgery to avoid the possible obstructive effects of poor placement or insufficient diameter presumably has resulted in fewer accounts of such difficulties recently. The list of failures (1) goes on to name three mechanisms of late obstruction. In the simplest of these, the patient outgrows a conduit adequately sized at surgery. Calcification, another source of late obstruction, can fix the cusps in a partially stenotic configuration. The last mechanism is the formation of an obstructive neointima or peel. Our examination revealed two such obstructive mechanisms; formation of peel and calcification. As demonstrated in Table 1, patient outgrowth of conduit was the reason for replacement in three of the four samples. The peel from one of these specimens possessed a partial lining of what appeared to be endothelial cells. Dacron grafts in general, with some few exceptions (13,34), were not reported to endothelialize far beyond their attaching suture lines in human applications as indicated by Berger and colleagues (6), DeBakey and associates (13), Sauvage and co-workers (35) and Dilley and Herring (14). The paper of Dilley and Herring described a method of seeding endothelium onto Dacron grafts to produce a surface free of clot forming characteristics. It would appear that in this example, some form of seeding occurred in vivo.

Peel can become obstructive either in relation to its weak binding characteristics with the woven Dacron conduit wall or its capacities for growth. Description of a particularly confounding example was provided by Agarwal et al. (3). After surgery at which a segment of this neointima was left in place, a portion of it broke free, forming a reversed valve cusp opening on systole to produce early post-operative obstruction. Similarly, Ben-Shachar and colleagues (5) reported a case where a segment of neointima came free from the conduit wall and protruded out into the lumen. The reduction in conduit diameter as a result produced obstruction. One of the samples examined here possessed a thin, delicate cusp-like flap which projected out into the lumen of the conduit from a base of similar tissue, presumably composed more of fibrin than other material. Though a particularly delicate structure, it had been present in the patient and must have undergone the rigors of systole for some time. What effect it might have had on cardiac function is a matter of speculation, particularly with respect to the obstructive peel already present. It did resemble what might have become the frame of an extra valve cusp.

In regard to the other mechanism of obstruction, calcification, Geha et al. (20) reported on 17 valved conduits in the right ventricular outflow tract (RVOT). There were also four placed between the right ventricle and the pulmonary artery, for a total of 21 units in the lesser circulation of children. Of these units, two failed due to calcification. With respect to the left ventricular outflow tract (LVOT), in children, Chachoua et al. (11) reported on 21 patients, of which two required reoperation due to early degeneration and calcification. Though the samples were very small, these two reports imply a similar propensity to calcify in both right and left outflow tracts. In 1981, Dunn (15) reported on 77 valved conduits in the RVOT in children, two of which were found to be calcified. Of eight in the LVOT, one was replaced due to calcification. Calcification seems less common in valved conduits than in aortic or mitral positioned porcine valve replacements, apparently independent of the outflow tract (19). The paper of Edwards et al. (16) dealing with the pathology of 37 specimens of obstructed conduits pointed to calcific stenosis as the obstructive mechanism, in whole or in part, for over half of the sample. Calcification, therefore, along with formation of obstructive neointima, certainly was among the major causes of obstruction. A somewhat sinister aspect of these forms of developing obstruction is that often considerable gradients develop asymptomatically (37).

In the present report, some calcification was noted in the remains of cellular organelles and in intercellular regions of connective tissue. It did not seem to have progressed to the degree described in the report of Ferrans et al. (17). For example, calcification directly outlining collagen fibrils was not here apparent. The tissue component most greatly affected was cellular, particularly in association with membranes or within membranous organelles. This agrees with the 1985 report of Schoen and colleagues (36), who found that early calcification was associated with membranes of connective tissue cells. In the experimental animals used by this group and other investigators, calcification appeared a sure and certain fate in short order. This is not the case in humans, even children, and questions concerning the nature or perhaps condition of
the implant, rather than its host are tempting. Is it possible, for example, that some degree of calcification was present in the valve prior to harvest for use in humans? This small amount might serve as a seed crystal for valve calcification, particularly in high pressure placements (9), individuals with active calcium metabolism (33) or in the rapidly beating hearts of children (15). If this were the case, the hypothesis of Levy and co-workers (27) in reference to the work of Lentz and associates with sodium dodecylsulfate (26) is reinforced. Levy et al. suggest that this agent extracts phospholipid membrane components from the leaflet, thus reducing calcification noted to be associated with phospholipids. The extractive activity of SDS or a detergent such as Triton X-100 would extract membranes as suggested, removing a starting point for calcification. Their secondary purpose would be to remove with these membranes any small deposits of calcification we suggest might already be there. Subsequent washes with EDTA or citrate chelation solutions may capture any significant amounts of calcium previously not available due to membranes. Addition of such treatments to tissue harvested for use as valves might serve to reduce the incidence of implanted valve calcification. Along these same lines, another concept offered by Levy and associates (28) involved non-invasive patient tests for levels of proteins containing gamma-carboxyglutamic acid. Increasing amounts of such proteins imply elevated degrees of calcification. Could this test be modified for application some steps earlier to the animal donor, for example, rather than the human graft host? If so, it might prevent use of an animal whose valves already possess deposits of calcium albeit in trace, or larger, amounts.

On one of the control valves examined, some endothelial cell remnants were found both in crevasses and out upon the valve surface. Such cell remnants were not found upon the leaflet surfaces examined from the other control valve. Other authors have described either controls or recovered valves differently with respect to their compliment of endothelial cells originating from the donor animal. Ferrans and associates (18,19) found some endothelial cells on the outflow surfaces of processed, unimplanted valve cusps. Ishihara and colleagues (24) described and pointed out the significance of endothelial cells in implanted porcine bioprosthetic valves. Ashraf and Bloor (4) found endothelium considered native to the donor animal on short-term implanted grafts. These findings, as well as our own, imply variability in the preparation of porcine aortic valves. This variability leaves two possibilities for clarification. First, are the cells found those of the donor animal, or as we presume, those of the host? While there is no way to absolutely disprove this possibility, comparison of the features of the cell remnants of the control with those of the cells of the leaflet explants, the latter being in generally better condition and possessing microvilli while the former appearing more like smooth surfaces ghosts, strongly favors the impression that the leaflet explant surface cells originated more recently than the graft, in other words, from the host. The second point concerns the significance of such cells. Are there enough porcine endothelial cells upon these leaflets to produce a significant immunological threat? In other words, when porcine aortic valves fail, do the ones which fail earliest do so because of immunological effects in response to these cell remnants? If such an immune response is possible, the detergent and other washes proposed above would have a secondary value. The extractive effects such washes produce might serve to remove donor endothelial cell remnants from the leaflet surface, thus reducing a possible immunological factor from the valve implant.

Peel, or neointima, is clearly a tissue of the host and as such, can be expected to thrive in the host's body. It is easy, therefore, to understand the appearance of peel as a healthy, growing tissue when examined microscopically. Leaflets, on the other hand, are porcine tissues and can be expected to be no better than at their time of preservation and perhaps at some lesser state. Whether originating from macrophages or endothelium, the cells which line the cusps are very likely those of the host. In three of the explants, these endothelial cells were located only upon the inflow surface. As peel is not normally expected to provide a surface for endothelial growth, the cells found on the samples might be thought to have arrived with the flow. In the one sample where considerable endothelium was found on the peel surface, the apparent migratory effect here provided cells on both inflow and outflow surfaces of the leaflet. It must be remembered that these cells are not known to be endothelial, and the conduit possessing cells on both leaflet surfaces had cells particularly notable for characteristics resembling to macrophages.

The dissimilar levels of degradation of cellular and collagenous structures seen in TEM might be explained if preparations of cusps for use in patients through low concentration glutaraldehyde treatment left the cells fixed to an inconsistent degree. It might be more reassuring to find all fibroblasts degenerated to the same extent rather than as found here, with some in very bad repair and others apparently very well fixed such that they might have been viable previous to fixation for microscopy.

We believe that, although improvements in bioprosthetic heart valves have been made, there will always be room for more. Perhaps the additional treatments suggested by Levy...
and colleagues (27,28) or the amendments to those treatments included here might serve in such endeavor.

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References


Editor's Note: All of the reviewers' concerns were appropriately addressed by text changes, hence there is no Discussion with Reviewers.