Proteoglycan in Porcine Aortic Tissue after Cryopreservation

This study was to investigate the effects of cryopreservation on proteoglycans of arterial conduit tissue. Proteoglycans from fresh and cryopreserved porcine aorta tissues were extracted with 4 M guanidine hydrochloride (Gdn-HCl) at 4℃ in the presence of protease inhibitors. From the tissue extracts, proteoglycans were isolated by cesium chloride (CsCl) isopycnic centrifugation and fractionated by gel filtration. Quantitative analysis of extracted proteoglycans revealed that the content of proteoglycans from cryopreserved tissue, measured as the amount of uronate and protein per unit weight of wet tissue, was similar to that from fresh tissue (0.44±0.300 versus 0.43±0.007 mg uronate/g wet tissue and 3.14±0.039 versus 2.64±0.015 mg protein/g wet tissue). Gel permeation column chromatography studies suggested that proteoglycans present in three CsCl fractions (I, II, and III) from cryopreserved tissue have approximately the same molecular weights as those from fresh tissue; K_{av}= 0.13 and 0.47 (I), 0.20 (II), and 0.43 (III) from cryopreserved tissue versus K_{av}= 0.13 and 0.50 (I), 0.23 (II), and 0.40 (III) from fresh tissue. These studies indicate that there is no significant alteration in the content and molecular size of proteoglycans in properly cryopreserved aortic tissue. *(JKMS 1997; 12: 398~404)*

Key Words : *Arterial conduit tissue; Cryopreservation; Proteoglycans*

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Received : March 12, 1997
Accepted : June 21, 1997

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INTRODUCTION

Calcific degeneration of cardiovascular implants and diseased cardiovascular tissues is common (1). Calcification of cardiovascular implants often results in clinical failure of the device due to mechanical dysfunction, vascular obstruction, or embolization of calcific deposits. Primary tissue degeneration due to intrinsic cuspal calcification is the most frequent cause of clinical failure of porcine aortic valve bioprostheses (2~4). Bovine pericardial bioprostheses also fail frequently because of calcification (1, 3). Although valved aortic homografts develop calcification much less frequently than bioprosthetic valves, calcification is one of the general causes of homograft failure in the late ultimate results (5, 6).

The pathophysiology of cardiovascular implant calcification is complex and poorly understood, and there is no satisfactory preventive measures or therapies to reverse degenerative calcification. However, calcification occurring normally in skeletal and dental tissues, and pathologically in cardiovascular implants share important features (1, 7, 8). Chief among the common elements in various types of cardiovascular implant calcification is cell-derived components, such as cellular debris and subcellular vesicle-like organelles, which serve as the initial locus of calcification in direct analogy to the matrix vesicles of endochondral skeletal and dental mineralization (9). In addition, extracellular matrix proteoglycans (PGs) are functionally important components of the arterial wall. Little information describing the characteristics of individual proteoglycans and their precise location in the aortic tissue is available. However, arterial proteoglycans have physicochemical properties that are similar to those of hyaline cartilage (10, 11), and recent advances in the study of cartilage mineralization indicate that matrix PGs have an inhibitory effect on cartilage calcification (12~16).

Although the mechanism by which proteoglycans inhibit mineralization is not immediately apparent, proteoglycans might inhibit mineralization by the following mechanisms. First, the polyanionic chains of glycosaminoglycans may help to hold the extended network, repel phosphate anions, and bind calcium. Secondly, proteoglycan aggregates inhibit matrix calcification more effectively than proteoglycan subunits because aggregates physically shield or sequester small mineral clusters within their network of subunits, preventing enlargement of small mineral clusters beyond a critical size that would spread mineralization through the matrix (17). This inhibition occurs because subunits bound to aggregates can not be easily displaced, and they are organized to provide a large, uniformly dense network of negatively charged

glycosaminoglycan chains, and are essentially immobilized in the matrix.

There is very little information describing the effect of cryopreservation on proteoglycans in allograft heart valves. The present study focuses on the matrix components of the arterial conduit tissue since valved aortic homografts develop calcification more rapidly and to a greater extent in the aortic wall than in the valve leaflets. This paper describes the effects of cryopreservation on proteoglycans by the quantitative analysis of proteoglycans and study of the size distribution of proteoglycans present in fresh and cryopreserved tissues.

MATERIALS AND METHODS

Procurement and preparation of tissues

The pig hearts were obtained from a local abattoir (Gwaltney Meat-Packing Company, Smithfield, Virginia, USA) within 20 min of slaughter. Each heart was immediately washed with cold lactated Ringer's solution to remove residual blood and transferred to cold tissue culture media (RPMI 1640, Gibco, USA). Fresh aorta conduit tissues are defined as tissues dissected from the hearts immediately on arrival at the laboratory. Cryopreserved tissues are defined as tissues taken through a standard preimplantation processing for cryopreservation as described by Lange and Hopkins (18). This processing involves dissection, antibiotic sterilization, addition of cryoprotectant (10% dimethylsulfoxide and 10% fetal calf serum in RPMI 1640 tissue culture medium), controlled freezing at rate of -1℃/min, and storage at below -130℃.

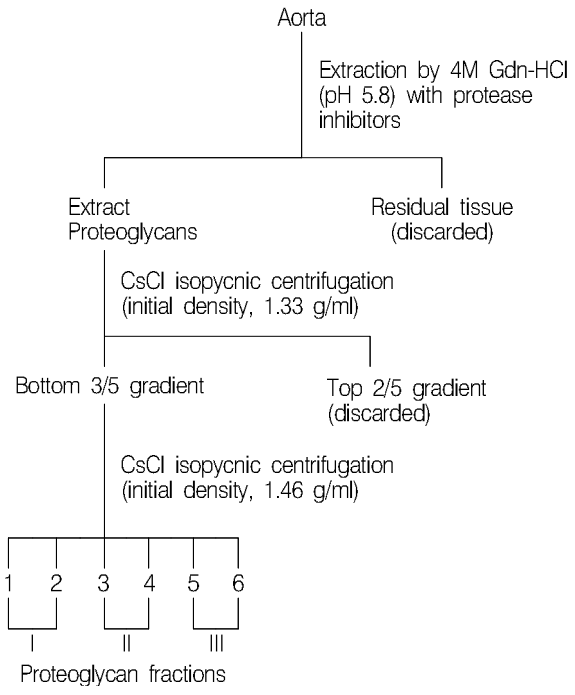

Fig. 1. Experimental scheme of the extraction and isolation of proteoglycans from fresh and cryopreserved porcine aorta tissues under dissociative conditions.

Proteoglycan extraction from conduit tissue

The scheme for the isolation of proteoglycans from conduit tissue is illustrated in Fig. 1. Aortic tissues were rinsed in isotonic saline and minced as finely as possible with dissecting scissors. The finely minced tissues were placed in OakRidge centrifuge tubes, and 5 volumes of cold extraction solution (4 M guanidine-HCl, pH 5.8) with protease inhibitors (0.1 M aminocaproic acid, 0.005 M benzamidine-HCl, 0.01 M EDTA, 0.005 M N-ethylmaleimide, 0.001 M iodoacetamide, and 0.001 M phenylmethylsulfonyl fluoride) were added. Extraction of tissue was performed at 4℃ using a rocker platform. Extracts were centrifuged at 10,000×g for 30 min, and the supernatants were exhaustively dialyzed (Spectra\Por, 3500) against ultrapure water at 0℃.

Cesium chloride (CsCl) centrifugation of proteoglycans

The extracts of aorta were adjusted to a density of 1.33 g/ml by the addition of solid CsCl and centrifuged at 100,000×g for 40 h at 8℃ in an ultracentrifuge (Beckman, Model L8-70). Following centrifugation, the bottom three-fifths of the gradient was obtained and adjusted to a density of 1.46 g/ml by the addition of solid CsCl. These preparations were centrifuged again at 100,000×g for 40 h at 8℃. Six fractions of equal volumes were then collected from each tube, and the density of each fraction was determined. The fractions were exhaustively dialyzed (five times) against ultrapure water at 0℃. Based on uronic acid and protein content profiles, samples were pooled into three aliquots (I, II, and III) and freeze-dried in Freeze Dry/Shell Freeze System (Labconco).

Quantification of proteoglycan concentration

Glycosaminoglycans were isolated from the extract by alkaline treatment as described by Carlson (19). Dialysates were collected and used for the determination of uronic acid content in the proteoglycan fractions by the method of Bitter and Muir (20) using glucuronolactone as a standard. Protein content in the proteoglycan fractions was measured by the procedure of Lowry et al. (21) using bovine serum albumin as a standard.

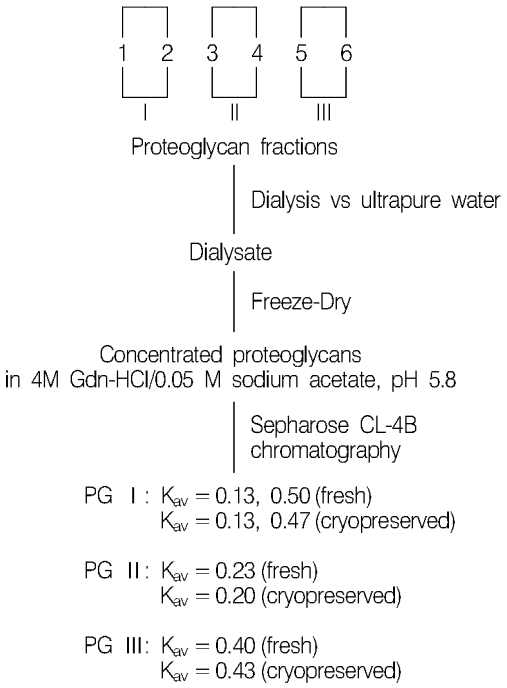

Fig. 2. Scheme used for the characterization of proteoglycans from fresh and cryopreserved porcine aorta conduit tissues.

Gel column chromatography

Gel column chromatography was used for determination of approximate hydrodynamic volumes of purified proteoglycans. Fig. 2 illustrates the procedure used for characterization of proteoglycans by gel permeation chromatography after purification by CsCl isopycnic ultracentrifugation and concentration by freeze-drying. Lyophilized proteoglycans (0.5 mg of uronic acid) were chromatographed on a Sepharose CL-4B (Pharmacia, USA) column (1.5×100 cm) equilibrated with a dissociative buffer (4 M Gdn-HCl/0.05 M sodium acetate, pH 5.8). The column was eluted with the same buffer at a flow rate of 16 ml/h and 3.5 ml fractions were collected. A hollow-fiber system (Fleaker hollow fiber, 88 fibers; Spectrum, USA) using ultrapure water, was used to separate proteoglycans from the dissociative buffer as they were eluted from the column. The column was calibrated using *Escherichia coli* to determine void volume (V_o) by measuring turbidity of the eluent at 600 nm and 5,5'-dithiobis [2-nitrobenzoic acid] to determine total volume (V_t) by measuring the absorbance at 280 nm. Chondroitin sulfate (Sigma Chemical Co.; 0.15 mg in 1 ml of buffer) was used to determine the expected elution volume of glycosaminoglycans that might be released from proteoglycans. The K_{av} values were calculated from the mean elution volume (V_e) of the proteoglycans using the formula : $K_{av}=V_e-V_o / V_t-V_o$.

Statistical evaluation of data

Experiments for the isolation of proteoglycans from separate tissue procurements were repeated more than three times. Data for the contents of uronic acid and protein of proteoglycans were recorded as milligrams/gram wet tissue weight and presented as mean±standard error. Linear regression analyses (Energraphic, 3.0) were used for standard curves. Statistical evaluations of significance were compared by Student's *t* test. The significance level was set at $p<0.05$.

RESULTS

Extraction of proteoglycans

Fresh and cryopreserved porcine aorta tissues were extracted once for 48 h in 4 M Gdn-HCl yielding 0.46 mg uronate/g wet tissue (representing about 47% of total uronate of the tissue) in fresh tissue and 0.47 mg uronate/g wet tissue (representing about 45% of total uronate of the tissue) in cryopreserved tissue (Table 1). More uronate could be extracted by a second 48 h extraction of the tissue, but the extraction was not repeated in order to minimize the presence of degraded proteoglycans in the extracts. Following ultracentrifugation, 92.6±1.7 and 93.5±0.6%, fresh and cryopreserved, of the extracted proteoglycans resided in the bottom three fractions of the CsCl gradient (Table 1).

Quantitative analysis of proteoglycans

Ultracentrifugation profiles of proteoglycans in Gdn-HCl extracts from fresh and cryopreserved tissues are illustrated in Fig. 3. There was no difference in the dissociative CsCl isopycnic centrifugation profiles of proteoglycans between fresh and cryopreserved porcine aorta tissues (Fig. 3). Based on the uronic acid and protein profiles, the ultracentrifugation fractions were pooled into three proteoglycan fractions, I, II, and III, as shown in Fig. 3. In extracts from fresh and cryopreserved tissues, fraction I had the highest uronate concentrations (0.238

Table 1. Uronate recovered in the bottom three-fifths of the gradient after the initial ultracentrifugation

	Total UA[a] (mg/g wet tissue)	UA in bottom three fractions (% of total UA)
Fresh	0.46±0.006	92.6±1.7
Cryopreserved	0.47±0.034	93.5±0.6

[a] UA, uronic acid.
Values represent the mean ± standard error, n=3.

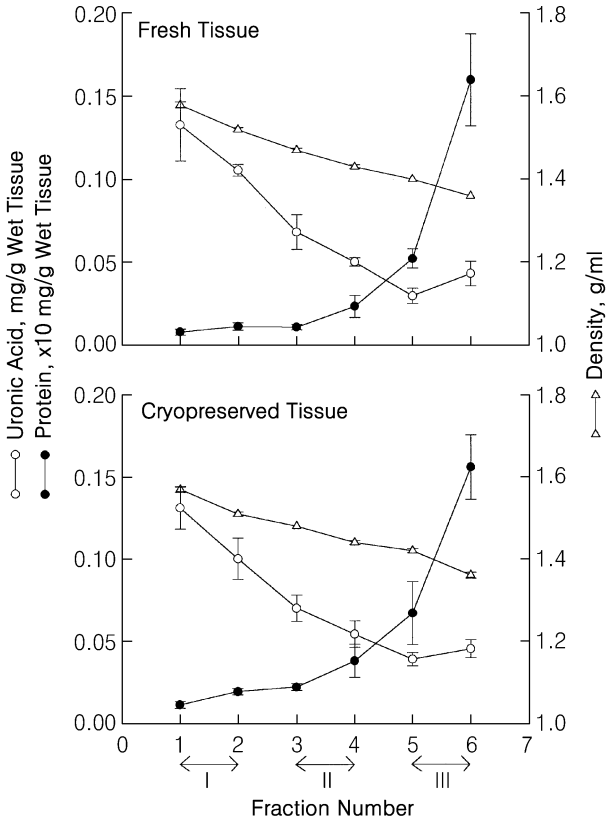

Fig. 3. Ultracentrifugation profiles of proteoglycans from fresh and cryopreserved porcine aorta conduit tissues. Ultracentrifugation was carried out under dissociative conditions (4 M Gdn-HCl) in CsCl at an initial density of 1.46 g/ml. Six fractions were collected starting from the bottom of the centrifuge tube and analyzed for uronic acid and protein. Based on analyses, proteoglycans were pooled into three fractions I, II, and III.

±0.0207 mg uronate/g tissue with fresh tissue and 0.231±0.0208 mg uronate/g tissue with cryopreserved tissue) and fraction III had the lowest (0.072±0.0100 mg uronate/g tissue with fresh tissue and 0.084± 0.0078 mg uronate/g tissue with cryopreserved tissue). Fraction III had the highest protein concentration, consistent with the buoyant density characteristic, of the CsCl gradients. Most of the proteoglycan material (83% of total uronic acid in fresh tissue and 81% of total uronic acid in cryopreserved tissue) sedimented in the bottom I and II fractions (density>1.43 g/ml in fresh tissue and density>1.44 g/ml in cryopreserved tissue). The total uronic acid-positive material ($P>0.05$) and protein contents of proteoglycans ($P>0.05$) extracted from fresh and cryopreserved porcine aorta tissues were not statistically significantly different (Fig. 4).

Gel permeation chromatography studies

Gel permeation column chromatography is one of the most widely applied analytical procedures for identifying and characterizing proteoglycans. Because no appropriate proteoglycans with known molecular weights are available, it was not possible to determine the absolute molecular weights of extracted proteoglycans. Molecular size distributions of extracted proteoglycans are therefore expressed as K_{av} values. The K_{av} value represents the volumetric distribution coefficient for uronic acid-positive and proteinaceous materials eluting from the column. Chondroitin sulfate typically eluted as a single peak with a K_{av} of 0.80 (data not shown).

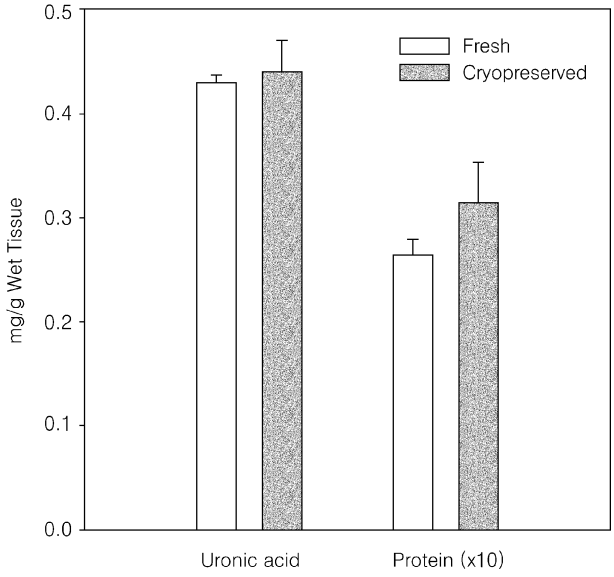

Fig. 4. Total concentrations of uronic acid and protein contents from proteoglycans, after the second ultracentrifugation, in fresh versus cryopreserved aorta conduit tissue. Data shown are mean values with error bars indicating the standard error of the mean (n=3).

In the elution profiles of proteoglycans in fraction I from fresh porcine aorta tissues, the proteoglycans, as the uronic acid-positive material, resolved into two peaks. One hexuronic acid-positive peak occurred near the void volume with a K_{av} of 0.13, and the other peak occurred with a K_{av} of 0.50. The proteinaceous materials absorbing at 280 nm eluted with a K_{av} of 0.93. All hexuronic acid-positive materials were eluted from the column as two peaks without detectable absorption at 280 nm. The proteoglycans in fraction I from the cryopreserved tissue also resolved into two peaks of uronic acid-positive materials (K_{av} values of 0.13 and 0.47) and one peak of proteinaceous materials (K_{av} of 0.90) following gel filtration. Hexuronic acid-positive materials from the proteoglycan fraction I of cryopreserved tissue eluted from the column without detectable absorption at 280 nm.

Hexuronic acid-positive materials present in fraction II from fresh tissue eluted from the column as a broad peak

with a K_{av} value of 0.23, whereas proteinaceous material was resolved into two peaks with K_{av} values of 0.13 and 0.93. The proteoglycans in fraction II from cryopreserved aorta tissue was resolved into one peak of uronic acid-positive materials (K_{av} of 0.20) and two peaks of proteinaceous materials (K_{av} of 0.13 and 0.90).

Proteoglycans in fraction III from fresh tissue showed the most heterogeneity among the fractions in gel filtration profiles of hexuronic acid-positive materials. Hexuronic acid-positive materials in this fraction eluted as a broad peak with a K_{av} value of 0.40. Materials in fraction III from fresh tissue resolved into two groups of proteinaceous materials. One peak of proteinaceous material eluted from the column near the void volume with a K_{av} of 0.10, and the other eluted near the total volume with a K_{av} of 0.83. Fraction III from cryopreserved tissue was also chromatographed on Sepharose CL-4B under dissociative conditions. The uronic acid-positive materials in fraction III eluted as a broad peak with a K_{av} value of 0.43. The materials absorbing at 280 nm eluted near the void volume as a small peak with a K_{av} of 0.10 and with a K_{av} of 0.80 as a larger peak.

Proteoglycans in fractions I, II, and III from both fresh and cryopreserved porcine aorta were equivalent in their respective K_{av} values, indicating no difference in relative hydrodynamic volumes of proteoglycans from fresh versus cryopreserved tissues.

DISCUSSION

The history of using allograft heart valves and greater vessel valve-containing conduits in the repair of complex heart malformations dates back more than 30 years (22). Although the performance of fresh antibiotic sterilized valvular allografts has been superior to that of mechanical or bioprosthetic valves in almost every aspect, severe limitations in the supply of allograft valves has restricted usage. The development of cryopreservation technologies is now permitting long term storage of the allograft heart valve and improving on availability, but issues of long-term durability remain. Allograft valve calcification remains a major concern for continued valve function and no effective means for inhibiting allograft calcification are currently available. The research undertaken in this study was designed to begin an alternative approach to the preparation of allograft valves and conduits that might possess a reduced tendency to calcify following transplantation.

Loss or alteration of proteoglycans accompanies cartilage calcification or transformation of cartilage into bone. Reddi et al. (23) found that the proteoglycans present in plaque at the time of maximum chondrogenesis are primarily in the aggregated form. However, when the hypertrophic cartilage undergoes extensive calcification, there is a decline in the synthesis of proteoglycans and a large proportion of the newly synthesized molecules are of lower molecular weight (23). Lohmander and Hjerpe (15) suggested that the quantitative and qualitative change in the proteoglycans of cartilage during calcification resulted from the concerted actions of released lysosomal hydrolytic enzymes. The presence in cartilage of both protease and hyaluronidase has been demonstrated (24). Chen et al. (25) reported that proteoglycan aggregates inhibit hydroxyapatite growth in a dose-dependent manner, and Boskey et al. (26) demonstrated that the enzyme-mediated alteration in the size of proteoglycans can significantly enhance the amount of minerals formed in an *in vitro* hydroxyapatite formation and growth assay. They suggested that the degraded proteoglycan can function as a hydroxyapatite nucleator or may reflect the easier accessibility of calcium ions to the phosphate ions in solution because of the loss of steric hindrance properties of the proteoglycan. It was also found that there was a parallel increase in alkaline phosphatase activity with increased protease activity in the calcification process of healing fracture callus (27). These observations are consistent with the view that proteoglycans and proteoglycanases are involved in preparing matrix tissue for calcification.

Allograft heart valves are analogous in many ways to mineralizing tissues. They typically contain a population of fibroblast cells with reduced viability at the time of transplantation, and it is generally accepted that donor cells in homograft valve fail to persist much beyond transplantation - a characteristic not much different from chondrocyte degeneration in hypertrophic and mineralizing cartilage (28). Remnants of dead cells mimic the matrix vesicles of endochondral skeletal and dental mineralization in serving as sites of nucleation of calcium to the phosphate-rich phospholipid in cell membranes (9). Dead and dying cells in the homograft would certainly release hydrolytic enzymes that could serve to alter the proteoglycan component (as well as other macromolecular components) of the matrix. Such alteration clearly mimics the changes in proteoglycan content and size associated with cartilage mineralization.

It has generally been assumed that retention of a viable fibroblast cell population in a cryopreserved allograft heart valve improves long-term durability. The basis of this improved durability was thought to be due to a continued synthesis and repair of the valve matrix. As an alternative explanation, it might be suggested that retention of a viable donor fibroblast cell population in cryopreserved allograft valves is important in that a reduction in cell death limits the release of hydrolytic

enzymes into the matrix space. These enzymes would degrade, to varying extents, the collagenous and noncollagenous proteins. It is suggested that degradation, i.e. alteration in quantity and size, of proteoglycans in a transplanted allograft heart valve may be responsible for the initiation of the process of mineralization as has been reported in cartilagenous tissues (bone).

The results described herein suggest that the procedures associated with cryopreservation do not alter the content or size distribution of proteoglycans in porcine conduit tissues. These findings do not, however, alter the basis of the proposed hypothesis. The present studies were conducted with porcine tissues with very short warm-ischemic times, i.e., in cases in which both fresh and cryopreserved valves might expected to retain maximum cellular viability (29, 30). With increasing warm ischemic times, fibroblast cell viability steadily declines and proteoglycan content might be changed. Following transplantation of a cryopreserved allograft valve with some degree of warm ischemic time and cell death, recipient mesenchymal cells may migrate into the conduit portion of the allograft where they may be induced to differentiate into an osteoblast-like cell with increased levels of alkaline phosphatase. Via a process of mineralization similar to that for cartilage (bone formation), i.e. alteration in proteoglycan quantity and size, hydroxyapatite crystals may nucleate on membrane fragments of dead donor cells, and mineralization may proceed along the elastin/collagen fibers until degenerative calcific deposits are formed. That recipient cells are less likely to migrate into leaflet tissues, as opposed to the conduit tissues, may explain the greater tendency of conduit tissue to calcify following transplantation.

Acknowledgements

This study was supported by grant from American Heart Association in USA. The author is grateful to Dr. Lloyd Wolfinbarger, Jr. at Old Dominion University for his continued support.

REFERENCES

1. Schoen FJ, Harasaki H, Kim KM, Anderson HC, Levy RJ. *Biomaterial-associated calcification: Pathology, mechanisms, and strategies for prevention. J Biomed Mater Res Appl Biomater 1988; 22(A1): 11-36.*
2. Gallo I, Nistal F, Artinan E, Fernandez D, Cayon R, Carrion M, Garcia-Marinez V. *The behavior of pericardial versus porcine valve xenografts in the growing sheep model. J Thorac Cardiovasc Surg 1987; 93: 281-90.*
3. Schoen FJ. *Cardiac valve prostheses. Review of clinical status and contemporary biomaterials issues. J Biomed Mater Res Appl Biomater 1987; 21: 91-117.*
4. Valente M, Borotolotti V, Thiene G. *Ultrastructural substrates of dystrophic calcification in porcine bioprosthetic valve failure. Am J Pathol 1985; 119: 12-21.*
5. Maxwell L, Gavin JB, Barratt-Boyes BG. *Differences between heart valve allografts and xenografts in the incidence of dystrophic calcification. Pathology 1989; 21: 5-10.*
6. Miller DC, Shumway NE. *Fresh aortic allografts: Long term results with free-hand aortic valve replacement. J Cardiac Surg 1987; 2: 185-94.*
7. Anderson HC. *Calcific disease: A concept. Arch Pathol Lab Med 1983; 107: 341-8.*
8. Anderson HC. *Biology of disease: Mechanism of mineral formation in bone. Lab Invest 1989; 60: 320-45.*
9. Anderson HC. *Mineralization by matrix vesicles. Scanning Electron Microsc 1984; 2: 953-64.*
10. Gardell S, Baker J, Caterson B, Heinegard D, Roden L. *Link protein and a hyaluronic acid-binding region as components of aorta proteoglycan. Biochem Biophys Res Commun 1980; 95: 1823-31.*
11. Oegema TR Jr, Hascall VC, Eisenstein R. *Characterization of bovine aorta proteoglycan extracted with guanidine hydrochloride in the presence of protease inhibitors. J Biol Chem 1979; 254: 1312-8.*
12. Hirschman A, Dziewiewiatkowski D. *Protein-Polysaccharide loss during endochondral ossification: Immunochemical evidence. Science 1966; 154: 393-5.*
13. Joseph AB. *Proteoglycan structure in calcifying cartilage. Clin Orthop 1983; 172: 207-32.*
14. Larsson S, Ray R, Kuettner K. *Microchemical studies on acid glycosaminoglycans of the epiphyseal zones during endochondral calcification. Calcif Tissue Res 1973; 13: 271-85.*
15. Lohmander S, Hjerpe A. *Proteoglycans of mineralizing rib and epiphyseal cartilage. Biochim Biophys Acta 1975; 404: 93-109.*
16. Mitchell N, Shepard N, Harrd J. *Measurement of proteoglycan in the mineralizing region of the rat growth plate. J Bone Joint Surg 1982; 64A: 32-8.*
17. Cuerro L, Pita J, Howell D. *Inhibition of calcium phosphate mineral growth by proteoglycan aggregate fractions in synthetic lymph. Calcif Tissue Res 1973; 13: 1-10.*
18. Lange PL, Hopkins RA. *Allograft Valve Banking: Techniques and Technology. In: Hopkins RA, ed. Cardiac Reconstructions with Allograft Valves. New York, Springer-Verlag, 1989; 37-63.*
19. Carlson DM. *Structures and immunochemical properties of oligosaccharides isolated from pig submaxillary mucins. J Biol Chem 1968; 243: 616-26.*
20. Bitter T, Muir H. *A modified uronic acid carbazole reaction. Anal Biochem 1962; 4: 330-4.*
21. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. *Protein measurement with the Folin phenol reagent. J Biol Chem 1951; 193: 265-75.*

22. Fontan F, Choussat A, Deville C, Doutremepuich C, Cupilland J, Vosa C. *Aortic valve homografts in the surgical treatment of complex cardiac malformations. J Thorac Cardiovasc Surg 1984 ; 87 : 649-57.*
23. Reddi AH, Gay R, Gay S, Miller EJ. *Transitions in collagen types during matrix induced cartilage, bone, and bone marrow formation. Proc Nat Acad Sci USA 1977 ; 74 : 5589-92.*
24. Woessner JE. *Purification of cathepsin D from cartilage and uterus and its action on the protein-polysaccharide complex of cartilage. J Biol Chem 1973 ; 248 : 1634-42.*
25. Chen CC, Boskey AL, Rosenberg LC. *The inhibitory effect of cartilage proteoglycans on hydroxyapatite growth. Calcif Tissue Int 1984 ; 36 : 285-90.*
26. Boskey AL, Maresca M, Armstrong AL, Ehrlich MG. *Treatment of proteoglycan aggregates with physeal enzymes reduces their ability to inhibit hydroxyapatite proliferation in a gelatin gel. J Orthop Res 1992 ; 10 : 313-9.*
27. Einhorn TA, Hirschman A, Kaplan C, Nashed R, Delin VJ, Warman J. *Neutral protein-degrading enzymes in experimental fracture callus : A preliminary report. J Orthop Res 1989 ; 7 : 792-805.*
28. Thyberg J, Friberg U. *Ultrastructural and acid phosphatase activity of matrix vesicles and cytoplasmic dense bodies in the epiphyseal plate. J Ultrastruct Res 1970 ; 33 : 554-73.*
29. Hu J, Gilmer L, Hopkins RA, Wolfinbarger L Jr. *Effects of antibiotics on cellular viability in porcine heart valve tissue. Cardiovasc Res 1989 ; 23 : 960-4.*
30. Hu J, Gilmer L, Hopkins RA, Wolfinbarger L Jr. *Assessment of cellular viability in cardiovascular tissue as studied with ^{3}H-proline and ^{3}H-inulin. Cardiovasc Rec 1990 ; 24 : 528-31.*