Review

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New Frontiers in Tissue Engineering: *Applications of Tissue Engineering in Cardiovascular Surgery*

Abstract: **Objective:** Tissue engineering (TE) is an approach by which cells are grown in vitro onto biodegradable polymers to construct tissues for implantation. Preliminary experimental applications of cardiovascular surgery in our laboratory for creating vascular grafts and valve leaflets through TE are presented and discussed.

Material and Methods: Ovine artery and vein segments were harvested, separated into individual cells, expanded in tissue culture and seeded onto synthetic biodegradable tubular scaffolds in 20-day old lambs . After 7 days of in vitro culture, the autologous constructs were used to replace a 2 cm segment of pulmonary artery (N=8) and valve leaflet (N=6). One control animal received an acellular polymer structure in each study design. Animals were sacrificed at intervals of 11 to 24 weeks. Explanted TE conduits were assayed for collagen and calcium content, and a tissue deoxyribonucleic acid assay was used to estimate number of cell nuclei as an index of tissue maturity.

Results: The acellular control constructs developed progressive obstruction and thrombosis. All TE constructs were patent and demonstrated a non-aneurysmall increase in diameter (18.3±1.3 mm, 95.3%of native pulmonary artery). Collagen content was 73.9±8% of adjacent pulmonary artery and showed a gradual increase. Elastic fibers were present in the media layer and deoxyribonucleic acid assay showed a progressive decrease in the number of cell nuclei, suggesting an ongoing tissue remodeling. Calcium content of TE grafts was elevated but no macroscopic calcification was found.

Conclusion: Living vascular TE grafts and leaflets functioned well and demonstrated an increase in diameter, suggesting growth and development of endothelial lining and extracellular matrix. The TE approach may ultimately allow the development of viable autologous structures for clinical use.

Key Words: Cardiovascular suggery, heart valves, prosthesis, biomedical engineering.

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Introduction

Developmental anomalies of cardiovascular structures, including valves and vessels or acquired loss of function of them through disease, are among the most frequent, devastating and costly problems in human health care. Each year there are approximately 1.4 million procedures that require vascular prosthesis, and about 60.000 operations in which valvular prosthesis have to be used in the USA alone (1).

At present, the available prosthetic conduits and homografts lack growth potential and can become obstructed by tissue ingrowth or calcification, leading to the need for multiple conduit replacements (2).

Tissue enginleering (TE) is a new and rapidly expanding field that applies the methods of engineering to the biological sciences to creative viable structures for replacement of diseased or deficient natural structures. Our laboratory has focused on developing techniques for culturing a variety of tissues both in vitro and in vivvo using polymer scaffolds as a cell delivery system with the aim of producing vascular and valcular grafts for repair of cardiovascular damage. Polymer scaffolds are biodegradable, involving compounds that have already been approved for human implantation. Once cells are attached to the three-dimensional biodegradable polymer, the resulting tissue construct is implanted in vivo where cells have the potential for further growth and development. The cellular structure and matrix develop as the polymer degrades, ultimately leaving only engineered tissue without foreign material. The remaining cells and matrix have the potential to organize into functional

tissue that can be used for reconstructive or transplantational surgery.

In this study, we present the recent methodology and results of our preliminary experimental applications for creating vascular grafts and valve leaflets through TE and discuss the implications of use.

Material and Methods

The tecniques of cell isolation, culture, cell sorting, polymer characteristics and cell seeding have previously been described in detail (3).

The aim of this paper is to summarize and discuss further steps of the two individual studies that were carried out in our laboratory and published previously (4, 5). These steps included creating a pulmonary valve leaflet and pulmonary artery (PA) by tissue enginleering and the evaluation of the results of the implantation of these constructs in vivo.

The methodology of both steps was composed of 4 individual steps:

a) Cell seeding b) In vitro cell culture c) Implantation of autologous tissue construct d) Evaluation of the implanted constructs

Cell Seeding and In vitro Cell Culture

2 cm sections of ovine artery for creating the PA leaflet (N=6), and both ovine artery (N=4) and vein (N=4) for creating PA were harvested from 20-day old lambs, minced, and cultured in Dulbecco's Modified Eagle Medium (Gibco BRL-Life Technologies. Grand Island, N.Y.) supplemented with 10% fetal bovine serum (Sigma Chemicals, Tokyo, Japan) and 1% guinea pig serum (29.2 mg/ml l-glutamine, 1000 U/ml penicillin G sodium and 10000 l.g/ml streptomycin/Gibco/media). The media was changed every 7 days, and in vitro cell growth was assessed periodically. The explanted tissue culture was placed in a humidified incubator maintained at 37°C with 5% CO_2 for 6 to 9 weeks. After several days, cell growth from the explants was observed to form mixed cell populations of endothelial cells and fibroblasts. The cultured cells were then labeled with an acetylated low density lipoprotein (LDL) marker (Biomedical Technologies Inc., Stoughton, Mass.), which is selectively taken up by endothelial cells through the scavenger pathway. After a 24-hour incubation period, cells were sorted into LDL (+) endothelial cells and LDL (-) fibroblasts and smooth muscle cells with a fluorescent activated cell sorter. Endothelial cells wereound in 2% to 3% of mixed cell culture in both groups after 2 weeks of in vitro culture.

The tissue scaffold was composed of a polyglactin woven mesh (Ethicon, Inc., Somerville, N.J.) sealed with non-woven PGA mesh (Albany Int Research Co, Mansfield, Mass.) inside the lumen. The scaffold was designed so that it would be biodegraded over 6 to 8 weeks (6).

Ten million mixed culture cells were seeded onto the polymer. Culture media was changed on a daily basis. After seven days, cells became confluent on the polymer.

Implantation of the Constructs

Autologous tissue-engineered conduits and leaflets were implanted into the same lambs from which the cells had been previously harvested (average age: 68.4±15 days, body weight: 18.7±2 kg). In control animals, only polymer scaffold leaflets or conduits were implanted.

Anesthesia was induced with 30 mg/kg of ketamine and maintained with continuous infusion of 0.2 mg/kg/min of propofol. The chest was exposed through left thoracotomy at the third intercostal space. Normothermic femoral arterial and right atrial cardiopulmonary bypass was established. With the heart beating, a longitudinal pulmonary arteriotomy was made in the leaflet replacement group, and the right posterior PA leaflet was excised and replaced with either a tissue-engineered valve leaflet or a plain polymer with 5-0 monofilament sutures. In the PA replacement study, PA was transected and a 2 cm section of main pulmonary artery was resected and replaced with either a tissue-engineered conduit or a plain polymer conduit with 5-0 monofilament sutures.

All animals received human care in compliance with the 'Guide for Care and Use of Laboratory Animals' published by the National Institute of Health (NIH publication No: 85-23, revised 1985).

Evaluation of the Implanted Constructs

After implantation, doppler echocardiography was used periodically to evaluate conduit patency and leaflet function as well as growth potential. All animals underwent angiography immediately before they were killed. The diameters of tissue-engineered conduits were measured angiographic ally and compared with those of adjacent native main PA and expressed as a percentage of native PA.

Animals were sacrificed at 75 to 169 days after implantation (average interval after implanatation: 125±20 days, body weight:38.6±14 kg). After the animals were killed, a portion of the specimen was fixed with 10% formalin for histologic examination with hematoxylin and eosin as well as Miller's elastic stain.

Another portion was immersed in 0.9% saline solution and immediately processed for collagen, calcium and immediately processed for collagen, calcium and deoxyribonucleic acid (DNA) assays. A 4-hydroxyproline assay (7) was used to measure collagen content in tissue-engineered conduits. DNA assay (8) was used to determine cell density in the tissue. The collagen and DNA contents of the constructs were compared with the native PA and leaflets of the same animal and expressed as a percentage of the native PA and leaflets of the same animal and expressed as a percentage of the native artery and leaflets.

The ortho-cresolphthalein complexon (OCPC) method was used for calcium measurements (9). Additional sections of the constructs werelso stained for factor VIII by immunohistochemical technique with a monoclonal anti-factor VIII antibody (Biogenex, San Ramon, Calif.) (10).

The mechanical strength of the leaflets was evaluated in vitro with a Vitrodyne V-1000 mechanical tester (11).

Results

Macroscopic Findings: Persistence of polymer sheet was observed in animals killed up to 11 weeks after implantation. The gross appearance of the engineered leaflets that had been implanted for over 11 weeks resembled the native leaflet (Fig. 1). The gross appearance of the tissue-engineered PA conduits in both Group A (ovine artery origin) and Group V (ovine vein origin) resembled the native PA. The control animal was killed 2 weeks after implantation because of severe dyspnea and pulmonary stenosis. Progressive thrombus formation and contraction of the polymer wall was found.

Echocardiographic and angiographic findings: PA leaflets constructed by tissue engineering seemed to be competent in pulmonary circulation, as was demonstrated by doppler echocardiography. In the control animal, the implanted polymers were completely degraded within 11 weeks.

In the PA conduit replacement step, two weeks after implantation, echocardiography showed patent conduits in all Group A and V animals. Thrombus formation was identified in the control animal as causing PA stenosis. After 10 to 12 weeks, tissue engineered conduits showed no evidence of thrombus formation or calcification in either Group A or V.

On angiography, all conduits showed an increase in diameter compared with that at the time of implantation. However, the percentage diameter remained 95%±1.7% in Group A and 86.8%±2.9% in Group V compared with the native PA, respectively (Fig.2).

Histologic examination: Miller's elastic stain showed evidence of elastic and collagen fibers in the engineered leaflet. Factor VIII stained endothelial cells were found lining the surfaces of the valvular tissue as well as the luminal surface of the tissue engineered conduit (Fig.3).

The specimens from the Group A and Group V showed almost complete disappearance of PGA polymer histologically, and the tissue engineered conduit resembled the native pulmonary vascular cellular
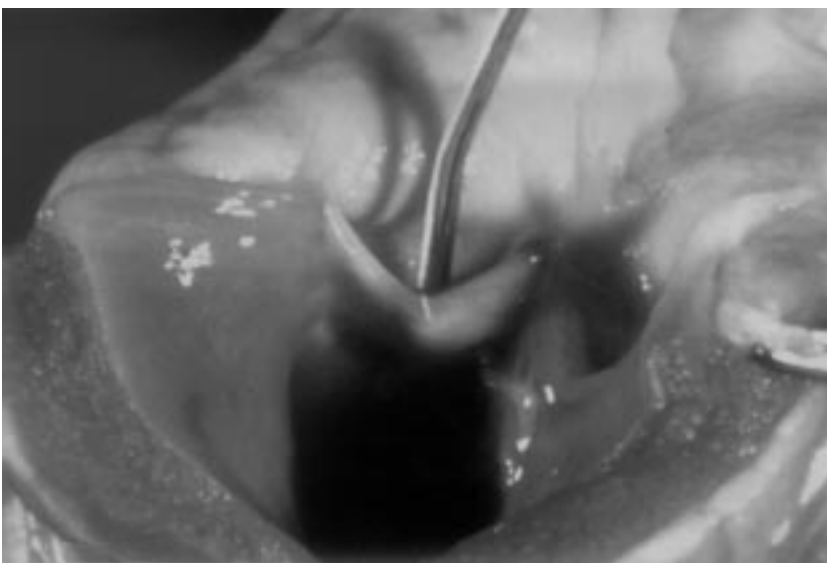

Figure 1. The gross morphology of the tissue engineered leaflet 11 weeks after implantation

architecture. Fig.4 summarizes the comparison of Group A and V with respect to parameters. Fig.4a compares the collagen content of Groups A and V with respect to native PA, showing Group A to be better than Group V. Fig.4b shows the calcium content of both groups; the venous group has relatively higher levels. DNA assay (Fig.4c) is also higher in Group A than in Group V. The diameter of grafts of arterial origin is much better than the venous ones when expressed as a percentage of native (Fig.4d).

Biochemical examination: The 4-hydroxyproline assay demonstrated that the tissue engineered leaflets obtained after 11 weeks of implantation showed a gradual increase in percent collagen content approaching the content of the native PA valve. In the PA replacement s tep, 5-hydroxyproline assay demonstrated that the tissue engineered conduit contained 73.9%±8% collagen compared with native PA.
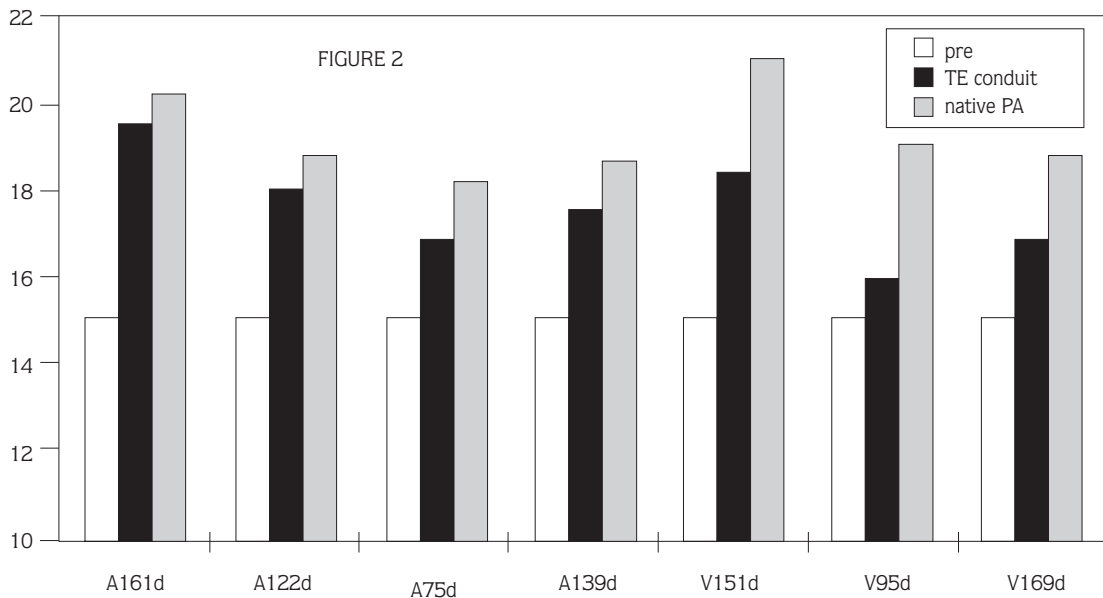

Figure 2.   Changes in diameter of tissue engineered conduits and native PA: Group A (arterial origin) and Group V (venous origin), the numbers represent the days after implantation.
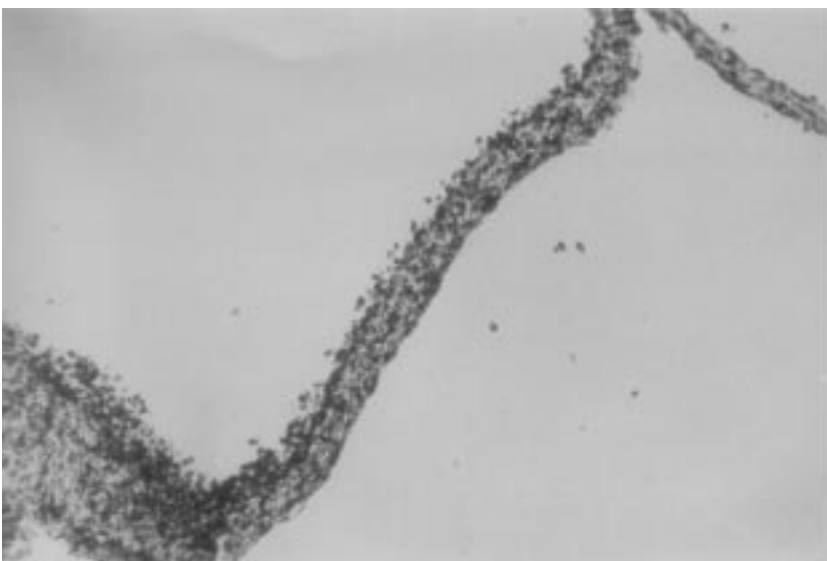

Figure 3.   Immunohistrochemical staining of factor VIII (red staining) shows the lining of endothelial cells on the surface of the tissue engineered leaflet

COLLAGEN CONTENT

CALCIUM CONTENT

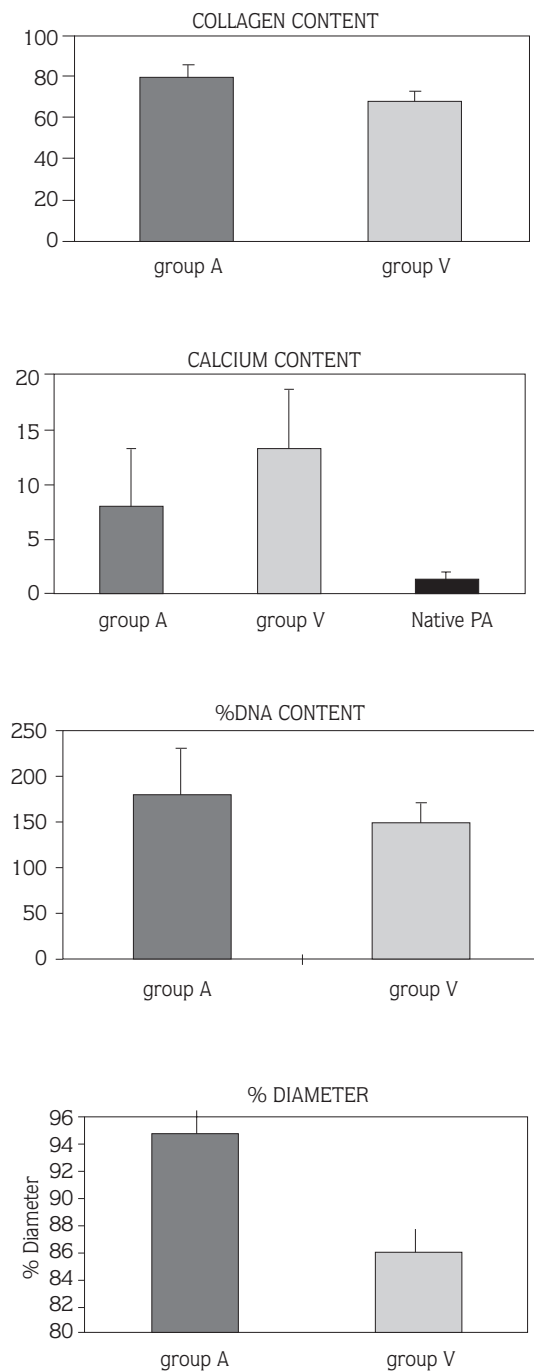## %DNA CONTENT

% DIAMETER

Figure 4. The cmoparison of the tissue engineered conduits between groups of arterial and venous origin
a) Collagen assay is expressed as percentage of native PA
b) Calcium content is expressed as mg per gram of dry tissue
c) DNA assay
d) Diameter of conduit is expressed as percent of native PA

Calcium assay: In tissue engineered constructs, calcium content was higher than native PA (Group A: 7.95 ± 5.09, Group V: 13.2 ± 5.4, native PA: 1.2 ± 0.8 mg/g dry weight), but no macroscopic calcification was found in either PA conduits or leaflets.

DNA assay: DNA cantent (cell nuclei density per gram dry tissue) in the tissue engineered constructs was higher than in native PA tissue when animals were killed (Group A: $180\%\pm50.7\%$, Group V: $148\%\pm22\%$).

Mechanical testing: The maximal tensile strength was measured with the use of a mechanical tester. There was a trend toward an increase in tensile strength over time (Fig.5).

Discussion

The search for an ideal valvular or vascular conduit for use in cardiovascular surgery continues because all the current synthetic or bioprosthetic grafts used clinically are far from ideal. None have growth potential, and therefore durability is limited. All synthetic grafts and valves are also thrombogenic to some extent and usually there is a need for continuous anticoagulation.

These constructs have the disadvantage of poor neointima formation and tissue ingrowth, which promotes calcification (12). Allografts currently seem to be the best conduits. At present, they may be cryopreserved and stored in liquid nitrogen until used. Cryopreservation has been suggested to preserve fibroblast viability, which might theoretically result in growth potential and make them less thrombogenic. Nevertheless, the presence of viable allograft components may enhance immune rejection, leading to calcification and subsequent failure (13). In addition, allograft donor scarcity remains a significant problem that continues to limit its widespread clinical application.

The option of creating living constructs from autologous cells offers many potential advantages, which include elimination of the problems of rejection and donor organ scarcity. These constructs are non-thrombogenic, have growth potential, and require no additional replacement procedures (14).

Tissue engineering is a new and rapidly expanding field in which techniques are being developed for culturing a variety of tissues both in vitro and in vivo using polymer scaffolds to support tissue growth.

Our laboratory has focused an applications of tissue engineering for cardiovascular surgery. Our results have
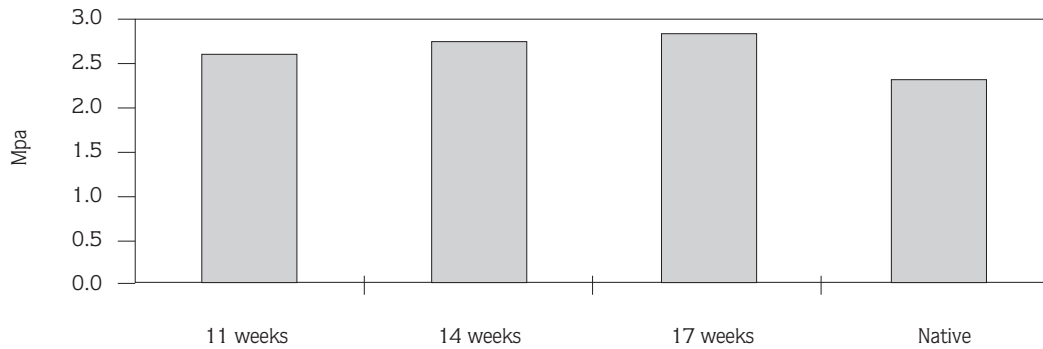
Figure 5. Mechanical testing demonstrated a gradual increase in maximal tensile strength over time.

demonstrated evidence of unctional endothelila cells and viable fibroblasts in implanted constructs.

In the PA leaflet implantation step, we have shown that the cultured cells seeded in vitro onto bioabsorbable polymers could maintain their biologic activities in vivo. With cell tracing, we have demonstrated that the in vitro labeled cells were traced within the matrix even after the complete degradation of the polymer sçaffold (4). However, in the control animal the acellular polymer had disappeared completely by about 11 weeks, suggesting that the presence of implanted cells is critical to the tissue generation process. In addition, the control animals that underwent PA or PA valve leaflet replacement with acelullar polymers had severe thrombus formation, suggesting that cell seeding is also necessary to prevent thrombus formation within the constructs.

It was also demonstrated that most of the cells in the tissue engineered constructs were those resulting from in vitro seeding.

At present, the optimal number of cells necessary to generate an adequate matrix in the engineered constructs remains undetermined. We chose to seed the polymer sheet with $>10^7$ cells and found that a confluent cell-matrix sheath was created within 7 days. In the past, we seeded the fibroblasts onto polymer followed by endothelial cells in a staged fashion. However, we have subsequently learned that simultaneous seeding of mixed fibroblasts and endothelial cells can generate a similar structure. Regarding the smooth muscle cells, we have not observed any significant overproliferation. In our study, that might be related to the low pressure system at the pulmonary circulation. We have observed this problem in small arterial grafts subjected to systemic arterial pressure. We believe that many cell-to-cell interactions and signals would affect this cellular growth

pattern and the formation of intimal hyperplasia. We have not investigated the vascular properties of tissue engineered constructs in this particular study. We inted to research vascular properties in the forthcoming studies in which we try to create a tissue engineered aorta.

It is also unclear whether large arterial wall cells were optimal source of cells for tissue engineered constructs. The PA replacement step shows that venous fibroblasts and endothelial cells have no demonstrable histologic or morphologic differences from arterial wall cells. This may be due to short term follow-up of this study or to the alterations of cellular phenotypes after in vitro expansion. Although the DNA content measurements suggested decreasing cell numbers with time, it was not entirely clear whether or not this represented maturation of the tissue in a true sense.

It has always been very difficult to differentiate dilation from growth. However, there areseveral clues that verify the latter. The diameter of the tissue engineered graft increased in proportion to the native PA. The DNA contents that reflected cell numbers were higher than the native PA at 6 months, while the diameter was increased. In simple dilatation, one would expect an increase in diameter without an increase in cell numbers. Also, the histological level, the collagen content and elastic fibers did not resemble those of dilatation.

This study demonstrates functional endothelial cells, fibroblasts and growth in these tissue engineered constructs. The cells generated collagen, elastin and factor VIII and attained mechanical properties similar to the native PA tissue. These early preliminary results seem promising, although many issues remain to be addressed. The long-term physiologic and growth characteristics of these implanted constructs requireurther research. General limitations still must be overcome before clinical

application of these constructs. They should have additional characteristics such as flexibility, lack of bulkiness, ease of passing sutures and low porosity. A major limitation of the current biodegradable polymer is its tiffness before in vivo implantation. The optimal duration of in vitro seeding reamins to be determined because prolonged incubation will result in the degradation of the bioabsorbable polymer. On the other hand, the composite polymer will remain too stiff for surgical manipulation, if implanted prematurely.

No calcification was observed in any construct. More long-term follow-up is necesarry to determine whether the incresaed DNA content represents a transient active cellular metabolism or the ultimate development of atherosclerotic calcification.

The engineering of autologous cardiovascular structures is still at an early stage of development and there are numerous issues that remain to be investigated at present. However, it is encouraging that the construction of autologous tissue replacements seem to be feasimle. We believe that based on these initial steps, a tissue engineering approach to the development of replacement cardio√ascular structures is worthy of further investigation.

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References

1. Rabago G. A. Worldwide Overview of Valve Usage. Heart Valve Replacement & Future Trends in Cardiac Surgery (Ed. Rabago G) Futura Publishing Company. New York 1987, pp: 156-175.

2. Cannegieter SC, Rosendall FR, Briet E. Thromboembolic and bleeding complications in patients with mechanical heart valve prostheses. Circulation 89: 635-641, 1994.

3. Breuer CK, Shinoka T, Tanel RE, Zund G, Mooney DJ, Ma PX, Miura T, Colan S, Langer R, Mayer JE, Vacanti JP. Tissue engineering lamb heart valve leaflets. Biotech Bioeng 50: 562-7, 1996.

4. Shinoka T, Ma PX, Shum-Tim D, Breuer CK, CusickRA, Zund G, Langer R, Vacanti JP, Mayer JE. Tissue-engineered heart valves- Autologous valve leaflet replacement study in a lamb model. Circulation 94 (Suppl II): II-164-168, 1996.

5. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Isogai N, Langer R, Vacanti JP, Mayer JE. Creation of viable pulmonary artery autografts through tissue engineering. J Thorac Cardiovasc Surg 115: 536-46, 1998.

6. Ma PX, Langer R. Degradation structure and properties of fibrous nonwoven poly/glycolic acid scaffolds for tissue engineering. Polymers in Medicine and Pharmacy (Eds. Mikros AG, Leong KW, Radomsky ML, Tamada JA, Yaszemski MJ) Materials Research Society. Pennsylvania 1995, pp: 99-104.

7. Bergam I, Loxley R. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal Chem 35: 1961-5, 1963.

8. Labarca C, Paigen K. A simple rapid and sensitive DNA assay procedure. Anal Biochem 102: 344-52, 1980.

9. Connerty HV, Briggs AR. Determination of serum calcium by means of orthocresolphtlaein complexon. Am J Clin Pathol 45: 290-6, 1966.

10. Booyse FM, Sedlak BJ, Rafelson M. Culturef arterial endothelial cells: Characterization and growth of bovine aortic cells. Thromb Diath Haemorrh 34: 825-39, 1975.

11. Ma PX, Schloo B, Mooney D, Langer R. Development of biomechanical properties and morphogenesis of in vitro tissue engineered cartilage. J Biomed Mater Res 29: 1587-1595, 1995.

12. Danielson GK, Downing TP, Schaff HV, Puga FJ, Di Donato RM, Ritter DG. Replacement of obstructed extracardiac conduits with autogenous tissue reconstructions. J Thorac Cardiovasc Surg 93: 555-9, 1987.

13. Kirklin JK, Smith D, Novick W, Naftel DC, Kirklin JW, Pacifico A, Nanda NC, Helmcke FR, Bourge RC. Long term function of cryopreserved aortic homografts. J Thorac Cardiovasc Surg 106: 154-166, 1993.

14. Noishiki Y, Tomizawa Y, Yamane Y, Okoshi T, Satoh S, Matsumoto A. Acceleration of neointima formation in vascular prostheses by transplantation of autologous venous tissue fragments. J Thorac Cardiovasc Surg 105: 795-804, 1993.