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Tissue-Engineered Grafts Matured in the Right Ventricular Outflow Tract

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Autologous smooth muscle cell (SMC)-seeded biodegradable scaffolds could be a suitable material to repair some pediatric right ventricular outflow tract (RVOT) cardiac anomalies. Adult syngenic Lewis rat SMCs (2×10^6) were seeded onto a new biodegradable copolymer sponge made of ε-caprolactone-*co*-L-lactide reinforced with poly-L-lactide fabric (PCLA). Two weeks after seeding, the patch was used to repair a surgically created RVOT defect in an adult rat. At 8 weeks after implantation the spongy copolymer component was biodegraded, and SM tissue and extracellular matrices containing elastin fibers were present in the scaffolds. By 22 weeks more fibroblasts and collagen were present ($p < 0.05$). The number of capillaries in the grafts also increased ($p < 0.001$) between 8 and 22 weeks. The fibrous poly-L-lactide component of the PCLA scaffold remained. The 22-week grafts maintained their thickness and surface area in the RVOT. The SMCs prior to implantation were in a synthetic phenotype and developed in vivo into a more contractile phenotype. By 8 weeks the patches were endothelialized on their endocardial surfaces. Future work to increase the SM tissue and elastin content in the patch will be necessary before implantation into a pediatric large-animal model is tested.

Key words: Congenital heart defects; Pediatric cardiac surgery; Myocardium; Tissue engineering; Smooth muscle cells; Phenotype; Biodegradable scaffold; Biomaterial

INTRODUCTION

The optimal cardiac graft for repairing congenital heart defects might be a biodegradable scaffold with the patient's muscle cells preseeded and attached to the scaffold. After implantation the scaffold must facilitate the implanted cells to form muscle tissue in the graft before excessive fibroblast infiltration from the host and fibrous tissue occurs in the scaffold. The biomaterial should biodegrade at a rate compatible with the myocardial repair process so that the patch ultimately becomes a mature muscle tissue supplied by host blood vessels.

There are few reports of scaffolds used to build cell-engineered grafts to repair the myocardium. Eschenhagen and colleagues tested a collagen scaffold to construct a cell-engineered graft (7). Cells were grown in gelatin (15,17), polyglycolic acid (21), and alginate (6,14) scaffolds. Although these biomaterials permitted diffusion of nutrients and metabolic waste necessary for cell growth and enhanced cell growth, they were not sufficiently strong to prevent damage from the contracting myocardium. After testing different biomaterials (20) we believed that PCLA [a copolymer sponge made of 50% ε-caprolactone-*co*-L-lactide and 50% L-lactide reinforced on the outside with a woven poly-L-lactide fabric] promised to be an excellent scaffold (Fig. 1). In vivo the spongy matrix was biodegraded in about 2 months and the outer reinforcing fabric biodegraded between 1 and 2 years (information provided by the manufacturers). Both in vitro and in vivo host fibroblast adhesion and migration and extracellular collagenous matrix formation occurred. Complete endothelialization of the endocardial surface of the PCLA patch develops after implantation. An important property of PCLA scaffold was that the seeded cells have sufficient time to form a tissue in the myocardial milieu and for new blood vessels to perfuse the tissue before the supporting outer fabric was completely biodegraded.

Although fetal cardiomyocytes (14,15,17,21,24), bone marrow stromal cells (8,18,33), and smooth muscle cells (SMCs) (19) have been studied in scaffolds, we studied SMC because they were easily cultured, prevented ven-

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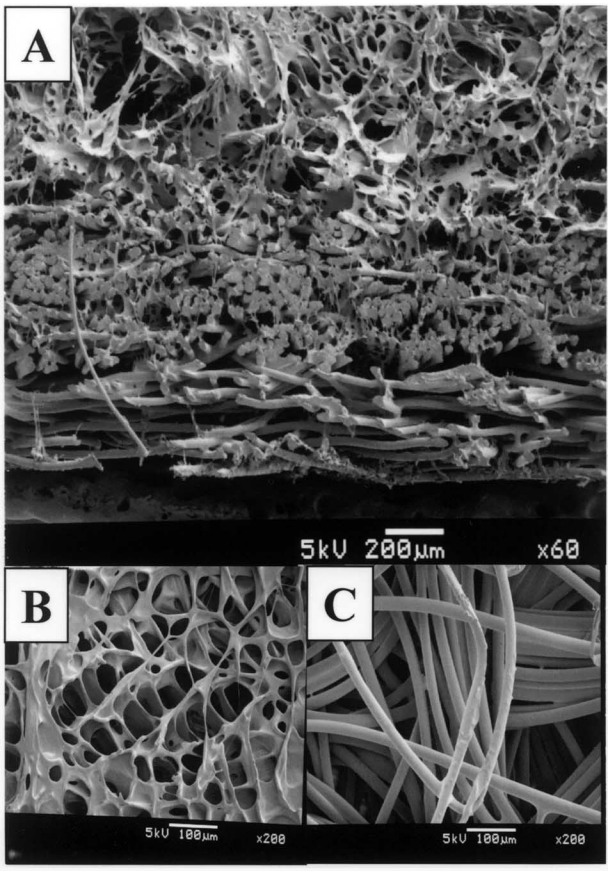

Figure 1. Scanning electron micrographs of PCLA copolymer sponge made of ε-caprolactone-*co*-L-lactide reinforced with an outer poly-L-lactide fabric. (A) Gross appearance of a vertical section of the patch. (B) Spongy component made of 50% ε-caprolactone and 50% L-lactic acid. (C) Fibrous component of poly-L-lactic acid fabric.

tricular remodeling in a myocardial infarct model, induced angiogenesis (16), formed an elastin-rich extracellular matrix (19,30), and were unlikely to cause a myocardial arrhythmia.

In this 5-month study we reported the morphological and histological changes of an implanted cultured autologous SMC-seeded PCLA patch used to repair a surgically created right ventricular outflow tract (RVOT) defect in an adult rat. The SMCs seeded in the patch were in a synthetic phenotype prior to implantation. At 8 weeks after implantation the spongy component of ε-caprolactone-*co*-L-lactide was absorbed and replaced by cells and extracellular matrices. After 22 weeks of implantation, the phenotype of the SMCs in the scaffold changed slowly from a synthetic to a more contractile phenotype. Elastin fibers were colocalized with the SMCs and the elastin sites were included within the SMC distribution. Collagen was observed throughout the patches. No inflammatory reaction indicative of immunorejection was detected. At 22 weeks after implantation the outer fibrous components of poly-L-lactide of the PCLA patch remained.

METHODS

Experimental Animals

All animal procedures were approved by Animal Committee of Toronto General Hospital and carried out in compliance with the *Guide to the Care and Use of Experimental Animals* of the Canadian Council on Animal Care and the *Guide for the Care and Use of Laboratory Animals* prepared by the Institute of Laboratory Animal Resource, National Research Council, and published by the National Academy Press, revised 1996. Adult Lewis rats (Charles River Canada Inc, Quebec, PQ, Canada) are used.

Isolation and Culture of Vascular Smooth Muscle Cells

Rat aortas were harvested and SMCs were isolated as previously described using a protease solution containing 0.4% trypsin, 0.2% collagenase, and 0.02% glucose in phosphate-buffered saline (PBS) (16). The cells were expanded in culture medium (Iscove's modified Dulbecco medium, GIBCO Laboratory, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum, 0.1 mmol/L β-mercaptoethanol, penicillin G (100 U/ml), and streptomycin (100 µg/ml) at 37°C in 5% carbon dioxide and 95% air. When the cells reached confluence, they were detached from the dish with 1 ml of 0.05% trypsin in PBS solution with 0.02% glucose and subcultured.

Cell Seeding Onto the Patch

The SMCs (2×10^6; purity of $89 \pm 6\%$) from a passage 2 culture were resuspended in 30–50 µl of culture medium and seeded onto PCLA patch material ($7 \times 7 \times 1$ mm, Gunze Ltd., Kyoto, Japan). After 60 min of incubation, 20 ml of culture medium was added to the culture dishes. The patches with SMCs were cultured and the culture medium was changed every 2 days.

Right Ventricular Outflow Tract Free Wall Resection and Replacement With Patches

The rats were anesthetized with an intramuscular injection of ketamine hydrochloride (22 mg/kg), followed by an IP injection of sodium pentobarbital (30 mg/kg). The rats were endotracheally intubated and ventilated at a rate of 60 cycles/min with a tidal volume of 3 ml with room air supplemented with oxygen (2 L/min) and 1–2.5% isoflurane. The rat heart was exposed through a median sternotomy to undergo RVOT free wall resection and replacement with patches as we previously described (24). Briefly, a purse-string stitch (5–6 mm in diameter) was placed in the free wall of the RVOT and

tightened through a tourniquet. The bulging part of the RVOT wall was resected. The size and thickness of the PCLA patch at 2 weeks after cell seeding were measured and then sutured along the margin of the purse-string stitch with an over-and-over method with 7-0 polypropylene (Prolene; Ethicon, Inc., Somerville, NJ) to cover the defect in the RVOT. The cell-seeded surface of the patch was oriented to the endocardial side. After completion of suturing, the tourniquet was released and the purse-string stitch removed. The chest incision was closed in layers with running sutures of 3-0 silk. Penlong XL (penicillin G benzathine, 150,000 U/ml, and penicillin G procaine, 150,000 U/ml) was injected intramuscularly (0.3 ml per rat). Buprenorphine hydrochloride (0.01 mg/kg) was given subcutaneously.

Morphological Evaluation of the In Vivo Patches

Eight weeks ($N = 6$) and 22 weeks ($N = 7$) after implantation the rats were euthanized after heparin injection. Each heart was excised and fixed with 10% phosphate-buffered formalin solution for 2 days. The patch area was measured. The patch was then cut in the center and the thickness of the center of the patch was measured. The measurements were compared among the 0-, 8-, and 22-week groups.

Histological Studies

Adult rat aortas and PCLA patches 2 weeks after being seeded with SMCs and at 8 and 22 weeks after implantation ($N = 5$, 6, 6, and 7) were fixed in 10% phosphate-buffered formalin solution for 2 days. The tissues were sectioned by the Department of Pathology, University Health Network, Toronto, Ontario. The slices were processed using JFC solution (Milestone S.r.l., Sorisole, BG, Italy), embedded in paraffin, and sectioned to yield 3-μm-thick specimens. The sections were stained for α-smooth muscle actin (monoclonal antibody for α-actin; 1:3000, Sigma-Aldrich Corp., St. Louis, MO), smooth muscle myosin heavy chain (SM-MHC) (monoclonal antibody for SM-2; 1:400, Yamasa Corp., Tokyo, Japan), nonmuscle myosin heavy chain (monoclonal antibody for SM-emb; 1:3000, Yamasa Corp.) (2,3,13), and for factor VIII (polyclonal antibody 1:2000, DAKO Diagnostics Canada, Inc., Mississauga, ON, Canada). The patch sections were also stained with hematoxylin and eosin as described in the manufacturer's specifications (Sigma-Aldrich Corp.) to evaluate the cellularity and with elastica van Gieson (EVG) to assess the extracellular matrix in the scaffolds (29,32). Within each patch five microscopic fields at 400× magnification were randomly selected and images taken using a digital camera (Coolpix, Nikon, Tokyo, Japan). All the digital images of histological and immunohistochemical samples were analyzed (19,20) using the public domain NIH image program (National Institutes of Health, Springfield, VA) and Adobe Photoshop (Adobe Systems Incorporated, San Jose, CA). Briefly, after tracing the outlines of the target areas and subtracting the backgrounds, the color images were converted to gray images. A fixed threshold of gray color scale determined automatically the number of nuclei staining positively for hematoxylin and expressed as a percentage of the cells or extracellular matrices in each field staining positively for α-actin, SM-2, SM-emb, elastin, and collagen.

The sections stained for factor VIII were used to measure the vascular density within the patch by counting the number of vessels at a magnification of 250×. Ten fields of each section were randomly selected and the vascular density was averaged and expressed as number of blood vessels per field (0.4 mm^2).

Data Analysis

All data were expressed as the mean ± SD. The StatView (SAS Institute Inc., Cary, NC) was used to analyze statistical data. Comparisons between two groups were performed by unpaired or paired *t*-test appropriately. Comparisons of continuous variables among more than two groups were performed by one-way analysis of variance (ANOVA). Scheffe's test was used to specify differences among groups if an *F* ratio of the ANOVA was significant ($p < 0.05$). A value of $p < 0.05$ was considered statistically significant.

RESULTS

Morphological Changes of Cell-Engineered Biomaterial

There were no significant differences in the preoperative patch surface areas that became the 8-week patches (17.5 ± 4.0 mm^2, $n = 6$) and the 22-week patches (17.1 ± 2.2 mm^2, $n = 7$). At explantation the postoperative patch area was greater than preoperatively in both groups ($p < 0.01$), but there was no significant difference in area between the 8-week-old patches (23.1 ± 3.4 mm^2, $n = 6$) and the 22-week-old patches (22.0 ± 3.2 mm^2, $n = 7$). The cell-seeded patches did not thin significantly between 8 weeks (0.84 ± 0.19 mm, $n = 6$) and 22 weeks (0.85 ± 0.28 mm, $n = 7$) after the implantation. Although the SMCs were scattered throughout the scaffold, the cells were located mainly in the layer that was seeded and became the endocardial surface with surgical implantation. At 4 weeks the endocardial surface of the graft was covered by endothelial cells that stained positively for factor VIII (results not shown).

Cell Survival, Extracellular Matrix Secretion, and Muscle Tissue Formation in the Scaffolds

Eight weeks after implantation, although the fibrous skeleton (poly-L-lactide) remained, the spongy copoly-

mer scaffolds [poly(ε-caprolactone-*co*-L-lactide)] were degraded and replaced by cellular components and extracellular matrices of collagen and elastin. Twenty-two weeks after implantation, the fibrous skeleton still remained and the interstices were tightly occupied with cells, predominantly smooth muscle cells and fibroblasts, and extracellular matrix (Fig. 2). The number of cells in the 22-week patches (8356 ± 1723 nuclei/mm^2) was greater ($p < 0.05$) than in the 8-week patches (6065 ± 1528 nuclei/mm^2). The SM tissue identified by α-actin expression was seen throughout the cell-seeded patches at both 8 and 22 weeks. Because there was no significant difference in the percentage of cells staining positively for α-actin at 8 weeks ($20 \pm 7\%$) and at 22 weeks ($27 \pm 8\%$), the increase in cell number at 22 weeks was mainly due to an increase in fibroblasts. The amount of collagen in the scaffold was greater ($p < 0.05$) at 22 weeks ($42 \pm 10\%$) than at 8 weeks ($27 \pm 10\%$). Elastin fibers were observed predominantly in the subendocardial layer of the patch and near the border zone between the patch and host myocardium in the cell-seeded patches at 8 and 22 weeks after implantation (Fig. 3). There was no statistical difference in the amount of elastin between the groups (8 weeks: $22 \pm 10\%$; 22 weeks: $16 \pm 9\%$). The elastin fibers were found within the distribution of SM tissue. The number of factor VIII-stained capillaries in the grafts increased ($p < 0.001$) between 8 weeks (8 ± 2 vessels/0.4 mm^2) and 22 weeks (19 ± 2 vessels/0.4 mm^2) (Fig. 4).

Phenotypic Modulation of Seeded SMCs for Graft Maturation

In the tunica media of adult Lewis rat aortic tissue the amount of SM-2 expressed was $26 \pm 12\%$ ($n = 5$) and the SM-emb expression rate was $4 \pm 3\%$ ($n = 5$). Culturing the SMCs in the scaffold for 2 weeks resulted in the expression of α-actin ($35 \pm 9\%$) and SM-emb ($25 \pm 9\%$) but very little SM-2 ($4 \pm 2\%$). The cells in the grafts implanted for 8 and 22 weeks expressed α-actin, mature smooth muscle myosin heavy chain isoform (SM-2), as well as nonmuscle myosin heavy chain (SM-emb). Between 8 and 22 weeks the amount of SM-2 expression did not change ($16 \pm 7\%$ at 8 weeks; $21 \pm 4\%$ at 22 weeks, $p = 0.15$), but the amount of SM-emb decreased from 8 to 22 weeks ($21 \pm 6\%$ at 8 weeks; $14 \pm 5\%$ at 22 weeks, $p < 0.05$) (Fig. 5).

DISCUSSION

The RVOT is a myocyte transitional zone between the cardiomyocytes in the right ventricle and the SMC in

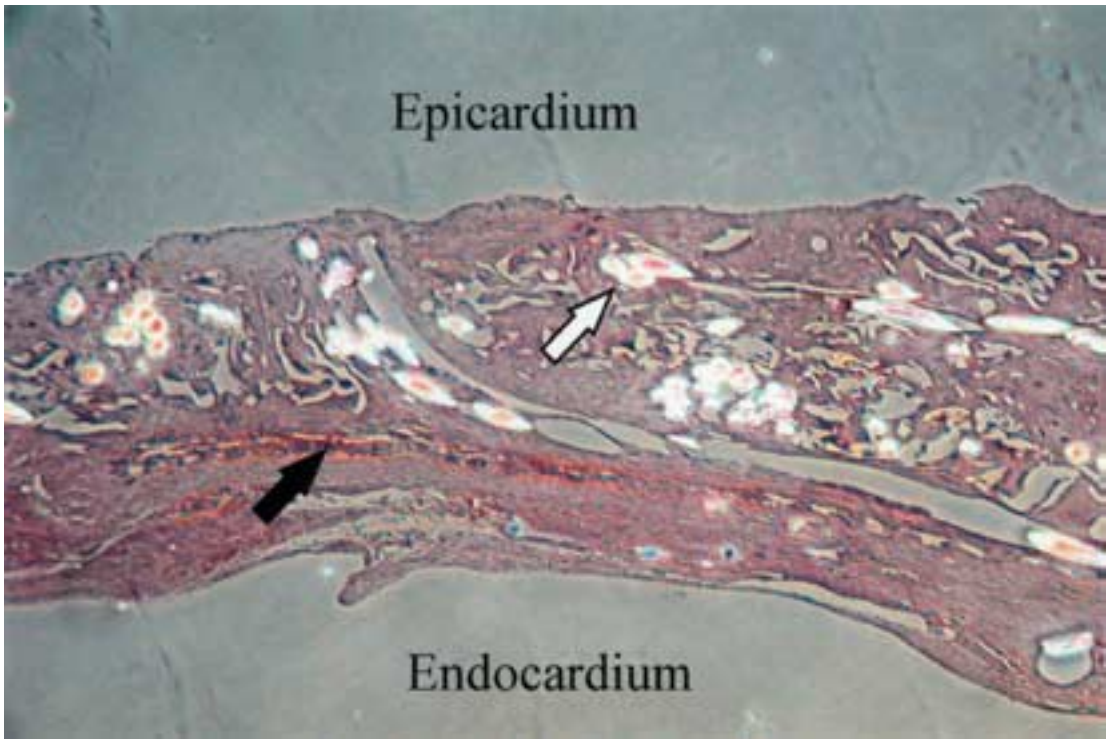

Figure 2. Photomicrograph of a cross section of a 22-week SMC-seeded PCLA patch stained with hematoxylin and eosin (40×). The white arrow points at the unstained translucent polymer scaffold and the black arrow points at smooth muscle tissue. The endocardial surface is lined with endothelial cells.

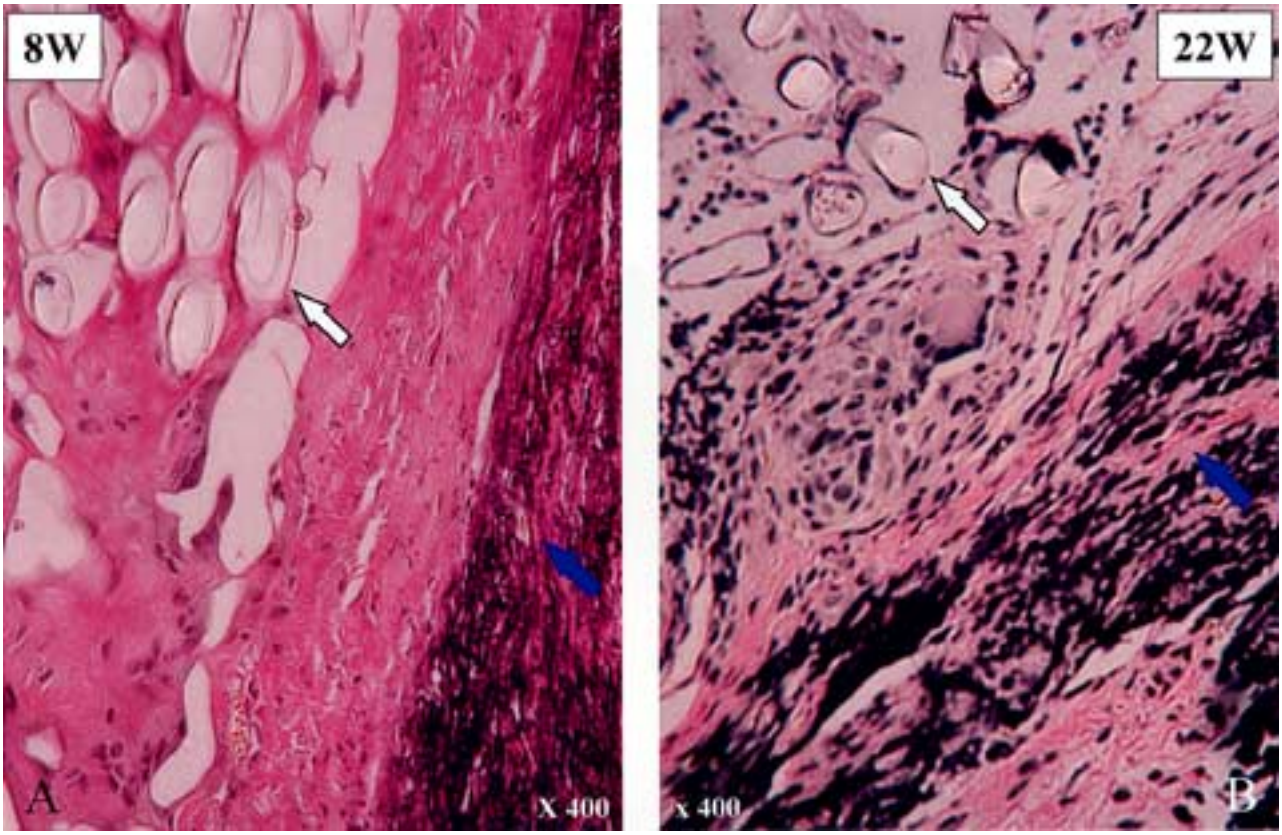

Figure 3. Photomicrographs of 8- and 22-week smooth muscle cell-seeded PCLA patches stained with van Gieson stain for elastin. The white arrows point at unstained translucent polymer and the blue arrows at smooth muscle cells. The elastin stained black in color and the collagen stained pink in color.

the pulmonary artery. We elected to repair an autologous RVOT defect with a SMC biodegradable patch because it would maintain the transitional zone and should grow with the child without necessitating reoperation for RVOT stenosis that can occur when nonbiodegradable patches or conduits are used. SMCs are readily harvestable from a patient's saphenous vein and are easily cultured. If the transplanted smooth muscle cells secreted an extracellular matrix rich in elastin that absorbed wall stress (potential energy) during isovolumic contraction, the elastic recoil could become a contractile force enhancing the ejection of blood into the lungs when the pulmonary valve opened. The elastic muscle graft could also attenuate the increase in wall stress within the adjacent myocardium during the cardiac cycle. Because SMCs respond to external stresses by hypertrophy, hyperplasia, and migration in vivo, these properties might enhance tissue formation after the RVOT reconstruction. Because SMCs require neuronal stimulation to contract and do not form gap junctions with the host cardiomyocytes, SMC transplants are unlikely to cause myocardial arrhythmias. The major disadvantage of using SMC rather than fetal cardiomyocytes or marrow stromal stem cells, which can build gap junctions and differentiate into cardiomyocytes (5), is that synchronous beating of the SMC-seeded patch would not be expected.

To optimize this technology several biodegradable materials and different cell types were being evaluated (6,7,14,15,17,20,21,24–27,30). The optimal cardiac graft would require gradual degradation of the biomaterial so that the seeded autologous cells have time to develop into a tissue with its own blood supply. It was important that immunorejection of the scaffold and the seeded cells did not occur. Graft failure in the myocardium would be catastrophic for the recipient. Syngenic Lewis rats were used as donors and recipients in the study to simulate clinical autotransplantation (5). The animals were so inbred that immunorejection of the SMCs in the scaffold transplants should be minimal.

Phenotypes of SMCs included contractile and immature synthetic states (31). Most SMCs in vivo were in a contractile phenotype, performed a contractile function, and proliferated only in response to stress. A transition from a contractile to a synthetic phenotype occurred

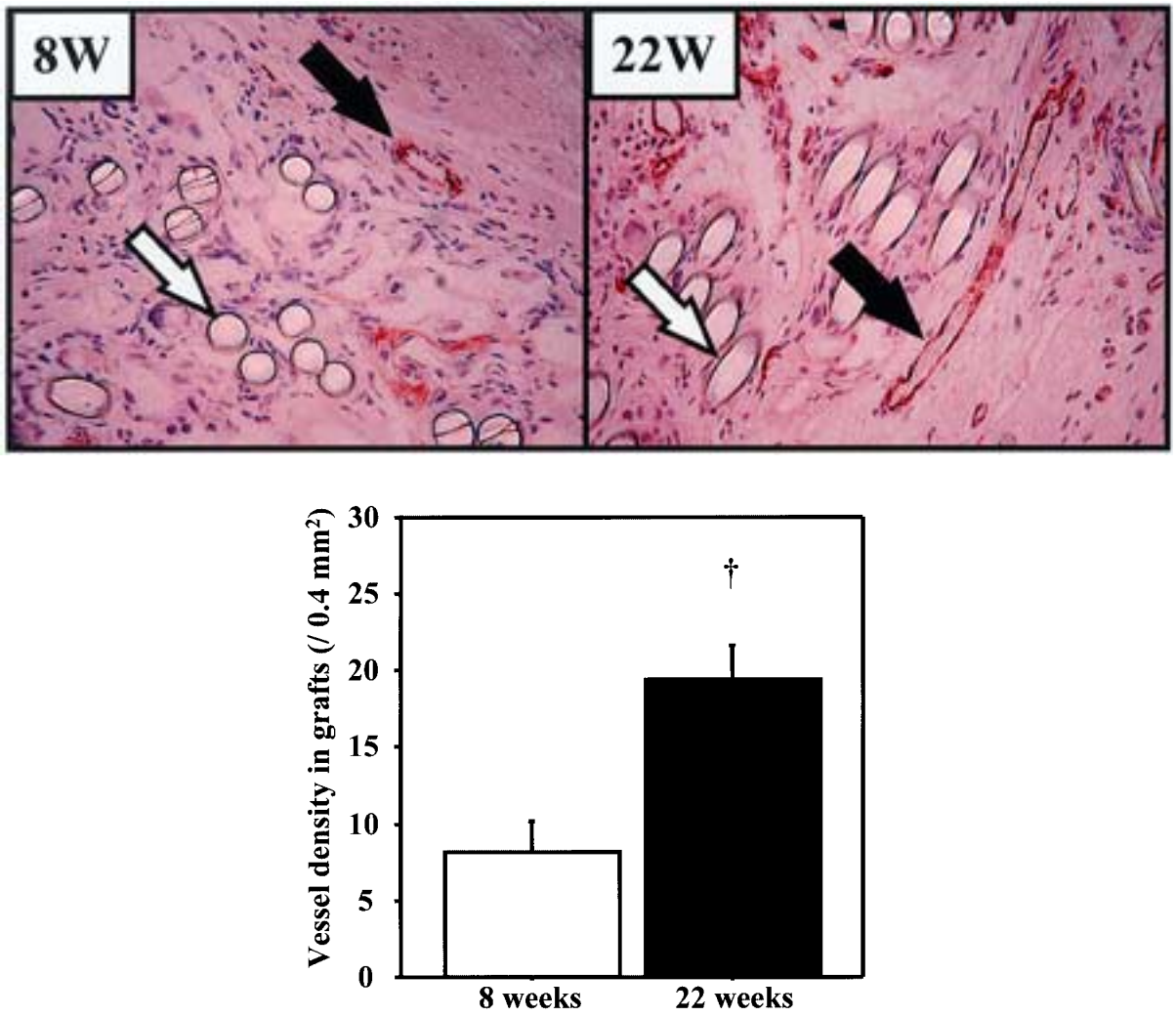

Figure 4. (Top) Photomicrographs of smooth muscle cell-seeded patches stained for factor VIII to identify the endothelial cells of blood vessels (200×). The PCLA scaffold did not stain (white arrows). The endothelial cells of the blood vessels (black arrows) stained a brown in color. (Bottom) The blood vessel density of the 22-week patch was increased (†$p < 0.0001$) compared with the 8-week patch.

when smooth muscle cells were established in culture (28). If the cells did not differentiate into a mature state in vivo, the cells might continue to proliferate and not become mature muscle. The SMCs cultured in the PCLA biomaterial expressed nonmuscle myosin heavy chain (SM-emb) but little contractile smooth muscle myosin heavy chain (SM-2). This indicated that the in vitro SMCs were in a synthetic phenotype that should favor cell proliferation and migration in the graft. In contrast, the SMCs in the graft expressed in vivo both SM-emb and SM-2. Although the SMCs in the 22-week grafts contained less SM-emb expression than that observed in the 8-week grafts, the SM-emb phenotype was still threefold more than that found in vivo in the tunica media of adult aortic tissue. The significant increase in proportion of SM-2 molecules in the SMCs after graft implantation compared with preimplantation indicated that the SMC phenotype changes in the graft from a synthetic to a contractile state during this period. The SM-emb expression in some of the SMCs in the graft at 8 and 22 weeks might indicate that some cells were proliferating in response to the ventricular pressure, or because the graft maturation process was continuing slowly. The SMCs in the grafts at 22 weeks had more SM-2 and less SM-emb, which was consistent with maturation and elastin expression during the period. The maturation of SMC from a synthetic to a contractile state was associated with elastin secretion. The maturation of the graft was also supported by the tissue formation within the graft. The grafts implanted for 22 weeks had

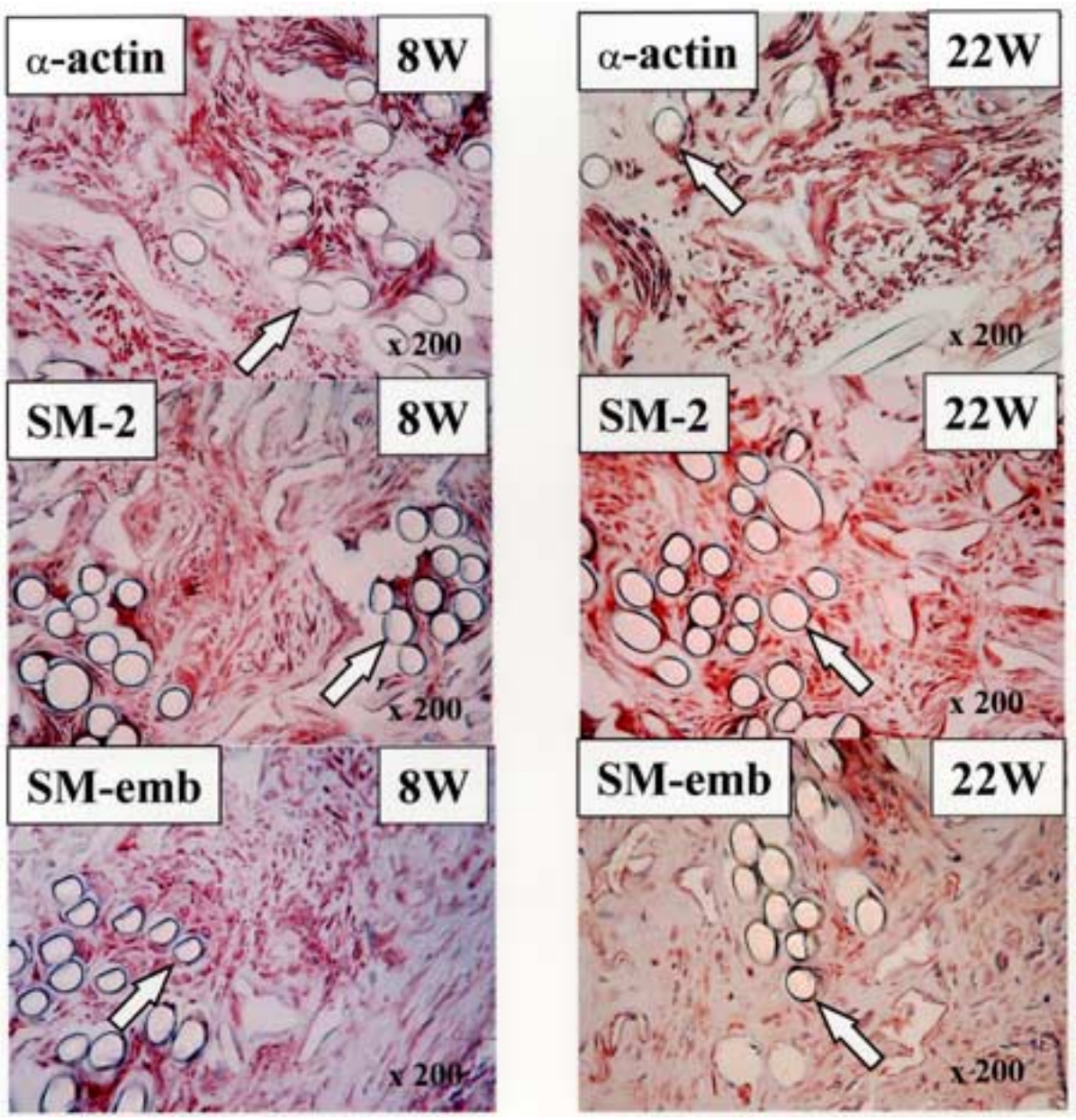

Figure 5. Photomicrographs of smooth muscle cell-seeded PCLA scaffolds at 8 and 22 weeks after implantation into the right ventricular outflow tract stained for α-actin, smooth muscle myosin heavy chain 2 (SM-2), and nonmuscle myosin chain (SM-emb). White arrows point at the translucent unstained PCLA polymer scaffolds. The brown color of the immunohistochemistry stains for α-actin, SM-2, and SM-emb indicates the presence of smooth muscle cells. The decrease ($p < 0.05$) in SM-emb in the 22-week scaffold compared with the 8-week scaffold reflects a change from a synthetic to a more contractile phenotype.

more blood vessels and fibroblasts than at 8 weeks. These results were consistent with the electron microscopic findings of Hirai and Matsuda, which showed a contractile phenotype of some cultured SMC seeded into a venous graft that was tested in vivo for 6 months (9). The extracellular matrix components of the graft might also modulate cellular phenotype. SMCs have been shown to proliferate on a type I collagen matrix more rapidly than on an elastin matrix because collagen promoted transition of the cells into the synthetic phenotype but elastin maintained the cells in the contractile phenotype (31). Kim et al. (12) compared cell growth and patterns of extracellular matrix gene expression on two types of synthetic polymer scaffolds and type I collagen scaffolds. The growth rates of SMC on the synthetic polymer scaffolds were significantly higher than on type

I collagen sponges. The rate of elastin production by SMCs on a synthetic polymer scaffold was also faster than that on type I collagen scaffold. Modification of the cellular environment by changing matrices, such as collagen, elastin, and even synthetic polymeric scaffolds, might alter SMC phenotypic modulation (10,23). The current study showed that the PCLA scaffold maintained the grafted SMCs in a synthetic phenotype that was consistent with SMC proliferation in vitro. As the biomaterial degraded in vivo, the SMCs underwent phenotypic transition from a synthetic to a contractile state and secreted elastin and collagen.

Unseeded PCLA patches used to repair a similar RVOT defect were infiltrated only with host fibroblasts that formed a collagenous extracellular matrix (20). To try to avoid the overcolonization of the patch with fibroblasts the patches were seeded with cultured SMCs and then cultured for 2 weeks prior to implantation to increase their numbers and distribution within the scaffold. Although the SMCs were cultured in the graft for 2 weeks prior to graft implantation, host fibroblast infiltration into the graft was significant. Seeding the scaffold with more SMCs and culturing effectively in vitro, such as the use of bioreactor (1,4,11,21), might yield a more muscular and elastic patch that could decrease fibroblast infiltration of the patch. If the PCLA spongy matrix physically limited in vitro cellular migration throughout the matrix, the matrix would need to be resynthesized to increase its porosity. The PCLA spongy matrices, made of ε-caprolactone-*co*-L-lactic acid, degraded during the 2 months after implantation while the fibrous matrices made of poly-L-lactic acid persisted and remained intact even after 5 months. Although the SMC phenotype in the in vivo grafts changed and tissue formation progressed and replaced the biodegraded biomaterial, the size and thickness of implanted grafts did not change between 8 and 22 weeks after transplantation. The reason that the PCLA patch sustained its physical dimension for 5 months could be attributed to the mechanical property of the fibrous poly-L-lactic acid matrices that would resist the pressure of the right ventricle. The fibrous poly-L-lactic acid components persisted for as long as 1 to 2 years (as suggested by the manufacturer). A previous study demonstrated that polymers blended with higher poly-L-lactic acid proportions exhibited higher elastic moduli, ultimate tensile strength, and lower degradation rates (22).

In conclusion, the synthetic SMCs increased in number in the PCLA scaffold during culturing and the scaffold maintained the SMCs in the synthetic phenotype. After the SMC-seeded patches were used to repair the defect of RVOT, some of the SMCs changed their phenotype from a synthetic to contractile type, survived, and formed smooth muscle tissue and elastin by 2 months. The grafts continued to mature, increased in cellularity, and developed capillaries by 5 months. The SMC-seeded biodegradable scaffold might be a bioengineered graft that could be used to repair some right ventricular outflow tract anomalies in congenital heart diseases.

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