Functional Growth in Tissue-Engineered Living, Vascular Grafts
Follow-Up at 100 Weeks in a Large Animal Model

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Background—Living autologous vascular grafts with the capacity for regeneration and growth may overcome the limitations of contemporary artificial prostheses. Particularly in congenital cardiovascular surgery, there is an unmet medical need for growing replacement materials. Here we investigate growth capacity of tissue-engineered living pulmonary arteries in a growing lamb model.

Methods and Results—Vascular grafts fabricated from biodegradable scaffolds (ID 18±1 mm) were sequentially seeded with vascular cells. The seeded constructs were grown in vitro for 21days using biomimetic conditions. Thereafter, these tissue-engineered vascular grafts (TEVGs) were surgically implanted as main pulmonary artery replacements in 14 lambs using cardiopulmonary bypass and followed up for ≤100 weeks. The animals more than doubled their body weight during the 2-year period. The TEVG showed good functional performance demonstrated by regular echocardiography at 20, 50, 80, and 100 weeks and computed tomography–angiography. In particular, there was no evidence of thrombus, calcification, stenosis, suture dehiscence, or aneurysm. There was a significant increase in diameter by 30% and length by 45%. Histology showed tissue formation reminiscent of native artery. Biochemical analysis revealed cellularity and proteoglycans and increased collagen contents in all of the groups, analogous to those of native vessels. The mechanical profiles of the TEVG showed stronger but less elastic tissue properties than native pulmonary arteries.

Conclusions—This study provides evidence of growth in living, functional pulmonary arteries engineered from vascular cells in a full growth animal model. (*Circulation*. 2006;114[suppl I]:I-159–I-166.)

Key Words: tissue engineering ■ growth ■ pulmonary artery ■ cells ■ scaffolds

Many congenital defects necessitate the use of vascular grafts, for example, in surgical reconstruction of the right ventricle to pulmonary artery continuity or the aorta. Children who undergo these types of operations with either prosthetic or homograft materials often require multiple reinterventions related to conduit failure.[1] All of the clinically available vascular replacements are nonliving, foreign materials with limited long-term function. They lack the potential for growth and remodeling and are associated with an increased risk of thromboembolism and infection. Stimulated by these shortcomings, the search for ideal replacement materials is still ongoing. The essential characteristics of such materials were described by Dwight E. Harken in the 1950s, including durability, absence of

thrombogenicity, resistance to infections, lack of immunogenicity, and the potential for growth; in principle describing the fundamental characteristics of natural, autologous tissues.[2] In vitro creation of living, autologous replacement materials by methods of tissue engineering addresses the critical need for growing substitutes in congenital cardiovascular surgery. The ultimate goal of tissue engineering is to construct tissues with characteristics of the healthy native tissues from their cellular components.

In the past 10 years, research has demonstrated the principle feasibility of the autologous tissue engineering concept for cardiovascular applications in heart valves[3–5] and blood vessels.[6] Tissue-engineered large diameter vascular grafts have been successfully used in low[7] and systemic pressure

applications[8] in sheep, and technology transfer to human cells has been shown.[4,9] Shin'oka et al[10] pioneered clinical applications and recently reported the first clinical data on vascular autografts engineered with human bone marrow cells.[11,12]

Although representing milestones in this field and showing feasibility in principle, the fundamental aspect of growth capacity of engineered cardiovascular tissues has not yet been demonstrated. Because systematic evidence of growth represents a prerequisite to entering larger clinical trials in congenital cardiovascular surgery, this preclinical study investigates function and growth in tissue-engineered living main pulmonary arteries over a period of 100 weeks in lamb, covering the full growth of this animal model.

Methods

We had full access to the data and take full responsibility for its integrity. We have all read and agree to the article as written.

Cell Isolation and Cultivation

The general approach to cell isolation, culture, and seeding has been described in detail previously.[3] All of the procedures were conducted according to Swiss regulations on animal welfare and permission granted by the ethical committee (Permission No. 71/2002, 91/2004). To obtain high cell numbers, vascular-derived cells were obtained from venous and arterial sources.[13] Briefly, 2-to 3-cm segments of carotid artery and jugular vein were harvested from Swiss White Alpine lambs (14.8 ± 4.6 kg). Endothelial cells were obtained using a collagenase instillation technique, with an incubation of 15 minutes at 37°C and 5% CO_2 in Endothelial Cell Basal Medium-2 (Cambrex) containing 0.2% collagenase type A (Roche Diagnostics). After isolation, they were cultured on tissue culture flasks (Nunc) with Endothelial Cell Basal Medium–2 medium supplemented with 10% lamb serum (Gibco). To obtain myofibroblasts, the remaining de-endothelialized vessel segments were minced and cultured in DMEM or advanced DMEM (Gibco) supplemented with 10% lamb serum, 2 mmol/L glutamax (Gibco) and 50 μg/mL gentamicin (Gibco). After myofibroblast migration onto the dishes (after 5 to 7 days), the cells were serially passaged and expanded in a humidified incubator at 37°C and 5% CO_2. Sufficient cell numbers for cell seeding were obtained in pure culture after 10 to 14 days.

Bioabsorbable Pulmonary Artery Scaffolds

Nonwoven polyglycolic-acid (PGA) mesh (thickness, 1.0 mm; specific gravity, 70 to 90 mg/cm³, Cellon SA) was coated with a thin layer of poly-4-hydroxybutyrate ([P4HB] molecular weight, 1×10^6, PHA 4400, Tepha Inc) by dipping into a 1% P4HB wt/vol tetrahydrofuran solution (Sigma). From the PGA/P4HB composite scaffold material, arterial scaffolds were fabricated by using a heat-application welding technique. An additional 10% P4HB coating was applied to the outer surface of the conduits (ID, 18 mm). The constructs were then cold gas sterilized with ethylene oxide.

Cell Seeding and In Vitro (Bioreactor) Culture

Myofibroblasts (4.5 to 5.5×10^6 per cm²) were seeded in 3 steps at 24-hour intervals onto the arterial scaffolds and cultured under static culture conditions with either DMEM or A-DMEM. After the third seeding step, 260 mg/L of ascorbic acid 2-phosphate (Sigma) was added to the culture medium for 2 days. Thereafter, the constructs were seeded with endothelial cells (1×10^6 per cm²) phenotypically confirmed by the presence of von Willebrand factor. After another 3 days of static culture, the constructs were transferred into a pulse duplicator system[3] and grown under gradually increasing nutrient medium flow conditions (50 to 550 mL/min, 1Hz) for 14 days. The medium was changed every 3 to 4 days.

Animal Implants

The tissue-engineered vascular grafts (TEVGs) were implanted into lambs (n=14, 28.4 ± 5.4 kg). The heart was exposed by a left anterolateral thoracotomy through the third intercostal space. Cardiopulmonary bypass was performed via right femoral artery and right atrial cannulation. After exposure and mobilization of the main pulmonary artery, ≈2 cm of native pulmonary artery was excised. TEVGs were implanted in all of the lambs. The distal and proximal anastomoses were completed with 5/0 polypropylene nonabsorbable monofilament interrupted suture. Off-bypass hemostasis was achieved, and heparin was reversed with protamine (300 IU/kg), followed by closure of the chest. Postoperative echo was performed. Maximum follow-up time was 100 weeks, representing the full biological growth cycle of this animal model (assessed by the state of bone maturation)

Echocardiography

2D Doppler transesophageal echocardiography was carried out under general anesthesia using a Siemens Acuson Sequoia C256 (Siemens) machine. The length and diameters of the TEVG were measured postoperatively and at 20, 50, 80, and 100 weeks. The length was defined as the shortest distance between the proximal and distal nonabsorbable suture of the grafts.

Computed Tomography–Angiography

Computed tomography (CT)–angiography scans were performed under general anesthesia on a 64-slice scanner with a 0.37-second rotation time (Somatom Sensation 64, Siemens). A bolus of iodixanol (270 mgI/mL, Amersham Health) was injected into a forearm vein at a flow rate of 4 mL/s, using 2 mL of contrast agent per kilogram. Slices with a thickness of 0.75 mm (increment 0.5 mm) and a medium soft-tissue reconstruction kernel (B30f) were used for evaluation. Measurements of the ID and cross-sectional areas of the tissue-engineered conduits at the midportion of the tissue engineered artery (TEA) were then taken using a commercially available vessel analysis program.

Microstructure

A representative longitudinal sample of each TE conduit construct was fixed in 10% phosphate buffered formalin (pH 7.0) and embedded in paraffin. Each cross-section included native tissue proximal, as well as distal to the entire length of the TEA. Routine histological staining was by hematoxylin and eosin, Masson trichrome, and Movat pentachrome. Immunohistochemical stainings were performed using the Ventana Benchmark automated staining system (Ventana Medical Systems) with Ventana reagents for the entire process. Primary antibodies against the following antigens were applied: von Willebrand factor (polyclonal rabbit anti-von Willebrand factor antibodies; all from DakoCytomation), α-smooth muscle actin ([α-SMA] monoclonal mouse anti-αSMA, clone 1A4, Sigma) and desmin (monoclonal mouse anti-desmin, clone D33). Primary antibodies were detected with the Ventana iVIEW diaminobenzidine detection kit, resulting in a brown reaction product. Counterstaining was performed with hematoxylin.

Quantitative Tissue Analysis

Collagen

As an indicator for collagen formation, hydroxyproline content of lyophilized samples was determined as described by Huszar et al.[14] As a standard, known amounts of trans-4-hydroxy-L-proline (Sigma) were used.

Sulfated glycosaminoglycans (GAGs) were determined colorimetrically using papain digested samples and 1,9-di-methyl-methylene blue stain.[15] As a standard, chondroitin sulfate from shark cartilage was used (Sigma).

Cell Number

The number of cells was estimated by measuring the DNA content from the same papain digested samples using Hoechst dye (Bisben-
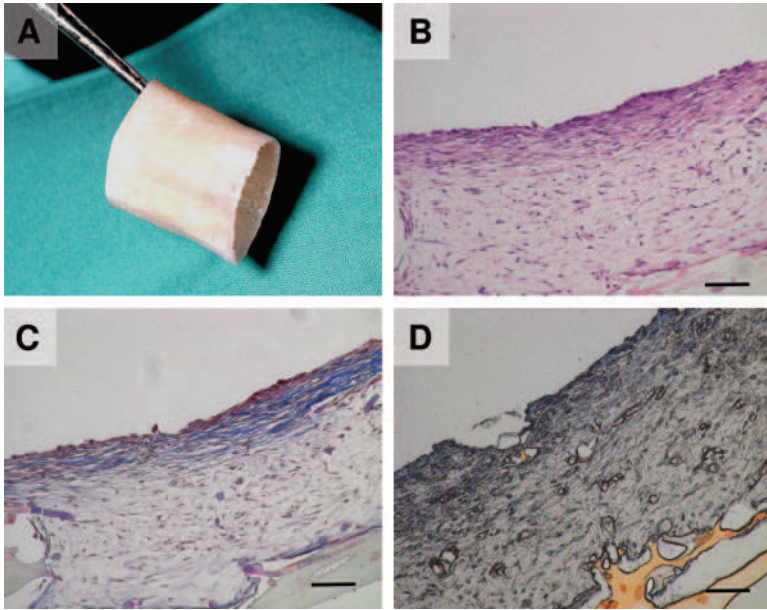
Figure 1. A, the tissue-engineered preimplantation conduit; B through D, histological stainings of pre-implant tissue-engineered conduits in hematoxylin and eosin (B), trichrome Masson (C) and hemalaun Sudan (D). Collagen is stained blue (B), scaffold material is stained orange (C). Bar in B through D represents 200-μm length.

zimide H 33258, Fluka) by fluorometry. Calf thymus DNA (Sigma) was used as a standard. Residual bioresorbable scaffold content in TEA was determined by differential scanning calorimetry using a Mettler-Toledo DSC 822e.

Biomechanical Analysis

Mechanical properties of the tissue-engineered and native pulmonary artery tissues were evaluated using a uniaxial tensile tester (Instron 4411 Instron Corp) equipped with a 100 Newton load cell. Longitudinal strips of 2×0.5 cm were placed within the clamps of the tensile tester and distracted to breaking point. Tensile force and displacement were recorded and transformed into stress–strain curves.

Cell Phenotype

For assessment of changes in cell phenotype of tissue-engineered artery-derived cells after 100 weeks in vivo, pieces (≈ 8 mm^3) of tissue were obtained from both explanted tissue-engineered pulmonary arteries and the corresponding native arteries and cultured as described above. Outgrowing cell phenotypes were characterized by immunohistochemistry using the same antibodies as described above. Furthermore, quantification of antigen expression analysis was performed by Flow cytometry (fluorescence-activated cell sorter) using antibodies against vimentin (clone 3B4), desmin (clone D33), α-SMA (clone 1A4, all from DakoCytomation), and an inside stain kit (Miltenyi Biotech) following the manufacturer's instructions. Primary antibodies were detected with fluorescein isothiocyanate–conjugated goat anti-mouse antibodies (Boehringer Mannheim). Analysis was performed on a Becton Dickinson FACScan. Irrelevant isotype-matched antibodies (IgG1MOPC-21, IgG2a, clone UPC-10, Sigma) served as negative controls.

Statistical Analysis

ANOVA was performed to estimate the means and the significance of the categorical factors using SYSTAT version 10 (SPSS, Inc). Post hoc pairwise mean differences were estimated using the Bonferroni test. A P value <0.05 was considered as significant.

Results

Fourteen animals were initially included in the study. One animal is still alive and will be followed up over 5 years. A second animal was excluded because of postoperative kinking of the TEA after surgical malpositioning. The maximum observa-

tion period of 100 weeks was chosen to monitor the full biological growth within this animal model. Twelve animals were analyzed and sacrificed at 20 (n =3), 50 (n =2), 80 (n =3), and 100 (n =4) weeks postimplantation. A macroscopic image of a tissue-engineered artery before implantation and the corresponding histology is shown in Figure 1.

Echocardiography

Echocardiography performed after surgery and at 20, 50, 80, and 100 weeks demonstrated functional engineered main pulmonary arteries in proximal and distal continuity with the adjacent native arterial tissues. There was no evidence of thrombus, calcification, stenosis, or aneurysm formation at all time points. At 80 and 100 weeks, the wall structure of the tissue-engineered artery was indistinguishable from the native arterial wall (Figure 2). There was a significant increase in the postoperative length as early as 20 weeks postimplantation of 23% and a additional significant increase in length between 20 and 100 weeks of 18%. Altogether this represents a 45% increase in tissue-engineered artery length over a 100-week period.

CT Angiography

Figure 3 shows representative CT–angiograms at 20, 50, 80, and 100 weeks postimplantation. Mean IDs showed a significant increase of 30% between 20 and 100 weeks. No calcification was noted in any tissue-engineered pulmonary artery. A summary of the observed increases in length (assessed by echocardiography, panel A) and diameter (CT angiography, panel B) is provided in Figure 4.

Morphology

The tissue-engineered pulmonary arteries were explanted "in toto" with careful dissection from the surrounding tissues. Figure 5 shows a representative explanted specimen of each group. There was no gross evidence of stenosis, aneurysm, suture dehiscence, or residual scaffold material at all of the time points. In addition, there was a smooth luminal layer in
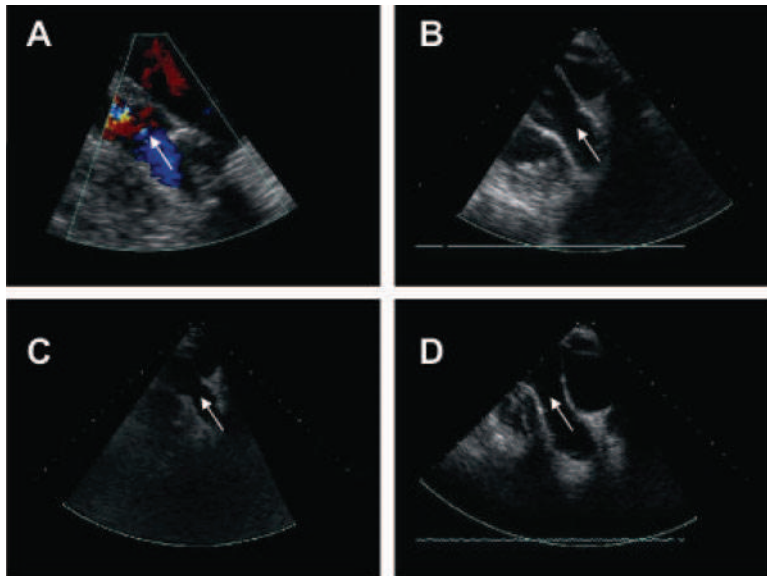
Figure 2. Longitudinal axis follow-up echos of a 100-week animal at time points immediately postoperative (A) showing flow through the tissue-engineered artery and no evidence of stenosis. B through D, tissue-engineered artery at 40, 84, and 102 weeks. White arrows, direction of flow in the tissue-engineered artery.

all of the explanted TEVGs with no evidence of thrombus or calcification. One of the explanted grafts (week 80) showed an area of abnormal induration, presumably because of a production abnormality of the initial scaffold material.

Tissue Microstructure

Tissue-engineered pulmonary arteries at all of the time points from 20 to 100 weeks were well integrated with and had a smooth transition of the luminal surface with that of the native pulmonary artery both proximally and distally. Details are shown in Figure 6. The grafted segments had 3 layers, reminiscent of the structure of natural arteries. The intima of the grafted segments, which structurally resembled a typical intimal healing process and occupied nearly half of the wall thickness, was composed of a closely packed collection of α-SMA cells in an extracellular matrix that stained positively for both collagen and GAGs but was devoid of stainable elastin. This layer was nearly identical in specimens at all of the time intervals. von Willebrand factor positive cells with the morphology of endothelial cells could be seen on the luminal surface. There was a central layer (essentially a media) that consisted of granulation tissue (ie, fibroblasts, collagen, and a rich capillary network) at earlier time points with a focal to diffuse mononuclear inflammation particularly in areas of polymer fibers, which were abundant at 20 weeks and nearly completely resorbed at 100 weeks. At all times, there were foreign body giant cells around residual fibers (marked at 20 weeks and progressively less at longer time
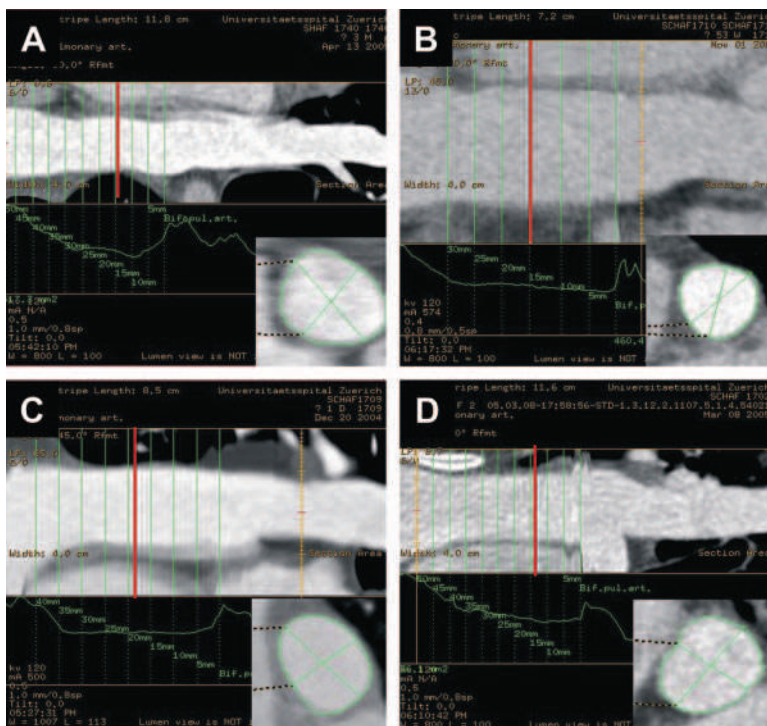

Figure 3. Representative CT–angiographic images of animals at 20 (A), 50 (B), 80 (C), and 100 weeks (D) postimplantation. Inset figures (bottom right corner of A through D) show the tissue-engineered conduits in cross-section. Green vertical lines show in 5-mm increments the entire length of the pulmonary artery from left (proximal) to right (distal, including the PA bifurcation). Red vertical line represent the midpoint of the TEVG.
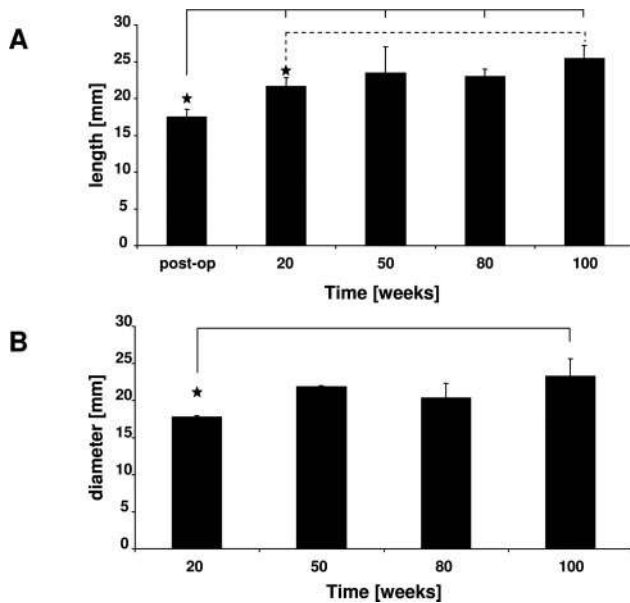
Figure 4. A , average lengths of the tissue-engineered conduits measured from long axis views on echo cardiography in all of the groups. B, average diameters measured on CT–angiography in all of the groups.

durations). The vascularity of the central layer decreased with time. On the outside surface was a loose connective tissue layer with adipose tissue that resembled normal arterial adventitia. There was no evidence of mural thrombus, tissue necrosis, infection, or deep calcification.

Quantitative Tissue Analysis

The DNA assay used to measure cell number and proliferation demonstrated a comparable cellularity between tissue-engineered and native pulmonary arteries at all of the time points in contrast to lower contents before implantation (Figure 7A). As to GAGs, there was no difference between the tissue-engineered samples as compared with the corresponding native tissue (Figure 7B). Collagen formation as measured by hydroxyproline content was significantly in-

creased in all of the tissue-engineered arteries (>36.5 μg/mg) as compared with the corresponding native pulmonary artery tissues ($n=10$; 22.8 μg/mg; Figure 7C). There was increasing collagen formation within the tissue-engineered groups (36.5 to 49.9 μg/mg at 20 to 100 weeks). PGA was completely degraded at as early as 20 weeks of implantation, and P4HB content was $<1\%$ of the wet weight in the 100-week group.

Biomechanical Properties

Compared with the corresponding native pulmonary arteries, the tissue-engineered arteries were, on the one hand, mechanically stronger, and on the other hand, less pliable at all of the time points. This was indicated by the higher tensile strength values of the tissue-engineered arteries reaching statistical significance at 80 weeks and the significantly increased E modulus values at 50, 80, and 100 weeks (Figure 8A and 8B). Representing less elastic tissue properties, strain at break values of native tissues were significantly higher compared with all of the tissue-engineered arteries (Figure 8C).

Cell Phenotype

Tissue-engineered artery-derived cells expressed vimentin and α-SMA but lacked desmin in immunohistochemistry. The same staining pattern was observed in the corresponding native pulmonary artery–derived cells. In addition, flow cytometry revealed no quantitative difference of antigene expression between the tissue-engineered artery-derived cells and their native counterparts (data not shown).

Discussion

There is a substantial need for tissue-engineered, living, autologous replacement materials with the potential for growth in pediatric applications. Approximately 1% of all newborns have congenital heart defects, and many of them require surgical interventions.[1] As of today, there are no clinically available implantation materials with growth capacity. Currently used prosthetic vascular grafts and homografts are nonviable, artificial, or allogeneic materials lacking the capacity of growth, repair, and remodeling.[16] This limits their
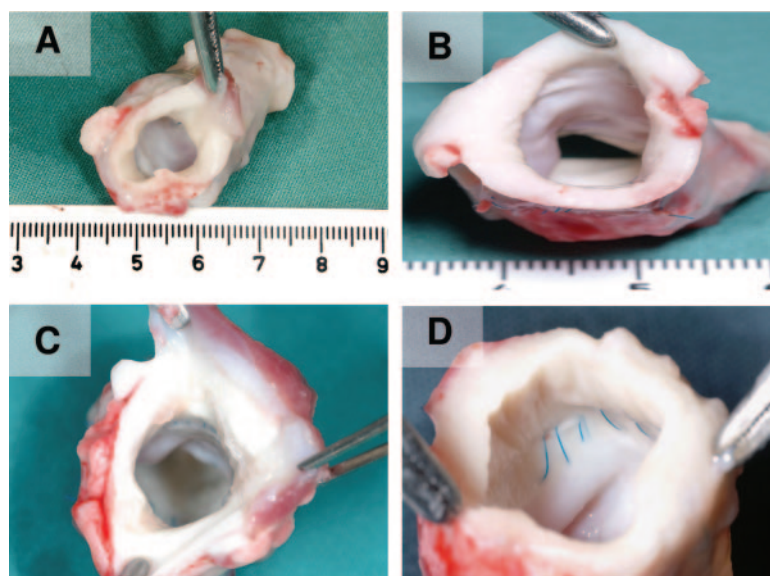

Figure 5. Macroscopic proximal view of explanted artery at 20 (A), 50 (B), 80 (C), and 100 weeks (D) postimplantation.
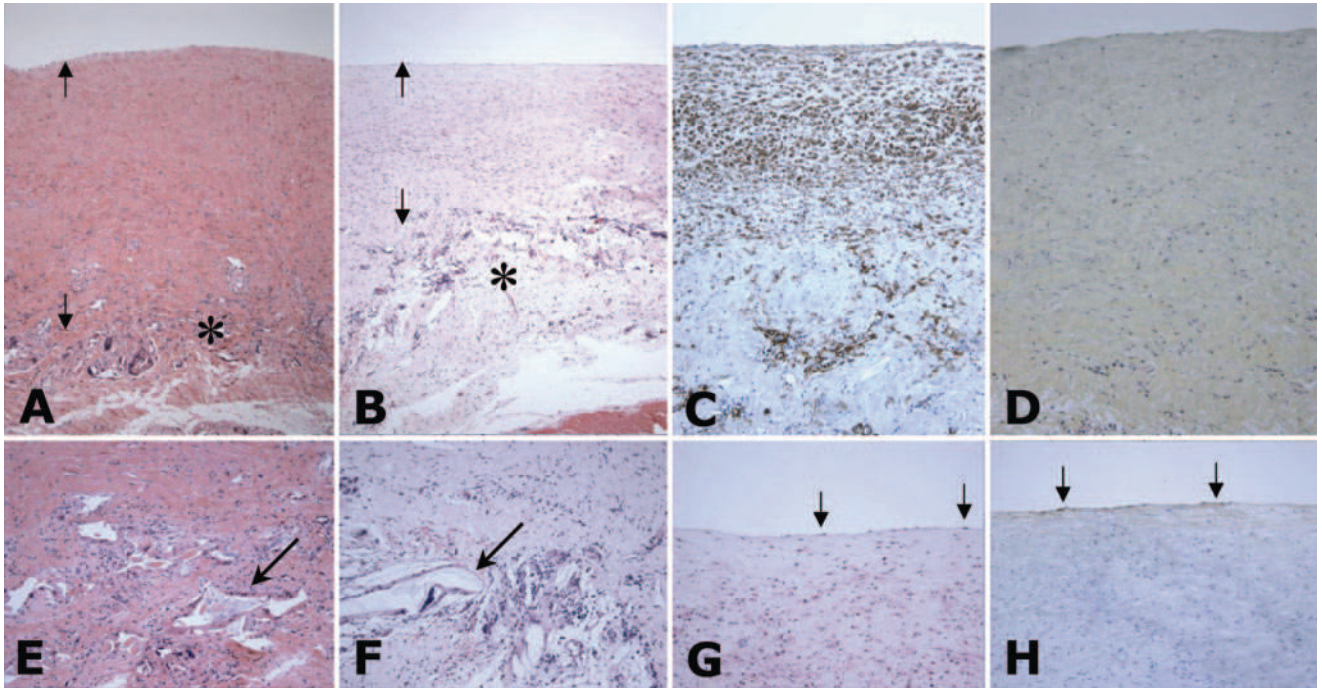
Figure 6. Histology: Tissue-engineered arterial segments at all time points at 20 to 100 weeks had 3 layers, reminiscent of the structure of natural arteries (50 weeks in A and 100 weeks in B). The intima of the grafted segments (between vertical arrows) had a prototypical vascular intimal healing response with closely packed α-SMA–positive cells (C) in an extracellular matrix that stained for both collagen and GAGs but not elastin (D). This layer became progressively condensed and occupied less volume at the longer intervals. There was a central layer (essentially a media; * in A and B) that consisted of granulation tissue (ie, fibroblasts, collagen, and a rich capillary network) and focal to diffuse mononuclear inflammation, particularly in areas of residual polymer fibers, which were abundant at 20 weeks and nearly completely resorbed at 100 weeks. Residual fibers (arrow) were surrounded by marked mononuclear inflammation with foreign body giant cells (shown in E at 50 weeks and F at 100 weeks). Discretely staining von Willebrand factor antigen-positive cells with the morphology of endothelial cells were present on the luminal surface (G and H; arrows denote individual cells). A, B, and E through G stained with hematoxylin and eosin; C, immunoperoxidase for α-SMA; D, stained with Movat pentachrome stain (collagen yellow, glycosoaminoglycans blue-green, and elastin black); H, immunoperoxidase for von Willebrand factor antigen. Magnifications: A and B, $\times100$; C through H, $\times200$.

long-term function, posing the substantial burden of graft failure and related reoperations on this patient population.[1,17]

This clinical demand has stimulated the further search for more optimal therapeutic solutions. In recent years, impressive progress in cell biology and biomedical engineering has led to the birth of tissue engineering.[18] This new experimental approach aims to fabricate living replacements using a concept in which tissue structures are generated in vitro from autologous cells by seeding onto bioabsorbable scaffolds. In the past 10 years, the feasibility of the autologous tissue-engineering concept has been demonstrated for cardiovascular applications in heart valves[3–5] and blood vessels.[6] Engineered vascular grafts have been successfully used in sheep,[7,8] and technology transfer to human cell systems has been shown,[4,9] including first clinical applications.[10–12]

The fundamental question, however, of whether tissue-engineered substitutes have growth capacity has not been systematically investigated yet. Because growth capacity represents a major premise and legitimation of the cardiovascular tissue-engineering concept for congenital use, systematic experimental evidence of growth is a prerequisite for proper clinical trials.

The aim of this study was to provide evidence of growth and develop a more detailed understanding of tissue remodeling mechanisms in vivo in a "model system" representing a relatively simple geometry. Thus, we investigated function and growth in TEVGs used as main pulmonary artery substitutes over 100 weeks in lambs. At this point in time, conventional controls, such as prosthetic vascular grafts or homografts, were not included. This will be necessary within the scope of a full preclinical study to prove the superiority of the new technology with regard to state-of-the-art vascular replacements.

Sheep are the animal model of choice for the evaluation of cardiovascular implants in vivo. The anatomic and hemodynamic conditions are sufficiently comparable to the human situation. Moreover, sheep represent a "worst case model" because of their increased calcium metabolism allowing for the assessment of degenerative processes of cardiovascular implants in a relatively short period of time.[19] Most importantly, the juvenile sheep model enables the monitoring of full growth, which is complete by 2 years of age.

The animals included in this study more than doubled their body weight during 2 years. Concomitantly, there was a significant increase of the mean diameter (30%) and lengths (45%) of the TEVGs in parallel to the native pulmonary arteries indicating adequate growth with the animals. The TEVGs showed good functional performance in all of the groups as demonstrated by regular echocardiography at 20, 50, 80, and 100 weeks and CT angiography. In particular, the
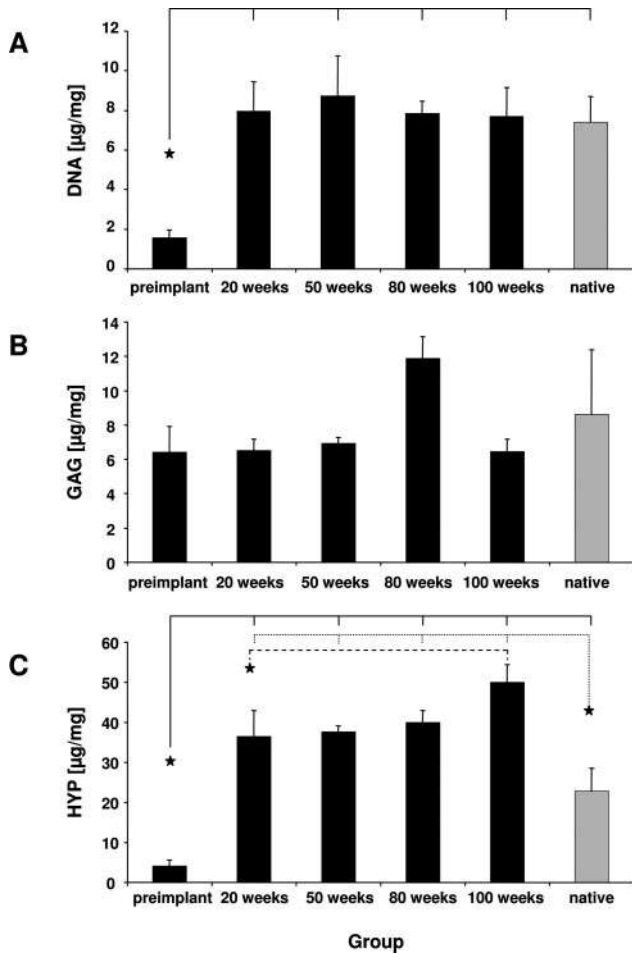
Figure 7. Biochemical analysis of DNA (A), GAG (B), and hydroxyproline (C) for preimplant, 20, 50, 80, and 100 weeks compared with native pulmonary artery.

Figure 8. Biomechanical analysis showing E-modulus (A), tensile strength (B), strain at break (C) at 20, 50, 80, and 100 weeks and native groups.

absence of thrombus, calcification, stenosis, suture dehiscence, or aneurysm formation underlines the functionality of the TEVGs and particularly their capacity to adapt to the changing environment of the growing cardiovascular system. At 80 and 100 weeks, the wall structure of the tissue-engineered arteries was in proximal and distal continuity with and indistinguishable from the corresponding native pulmonary arteries by imaging.

By histology, TEVGs at all of the time points had a 3-layered structure, reminiscent of natural arteries. There was no evidence of superficial mural thrombus, tissue necrosis, infection, or deep calcification. The intima of the grafted segments resembled a prototypical vascular intimal healing response but became progressively condensed at longer intervals. The media initially showed granulation tissue particularly in areas of residual scaffold material resolving over time, and the adventitia consisted of loose connective tissue. These observations are indicative of an in vivo tissue remodeling comparable with healing. The question of to what extent the originally seeded cells still were present and contributed to this in vivo remodeling process will have to be investigated in future studies using long-term cell labeling methods. The biochemical analysis of the engineered tissues revealed native analogous cellularity and proteoglycans but
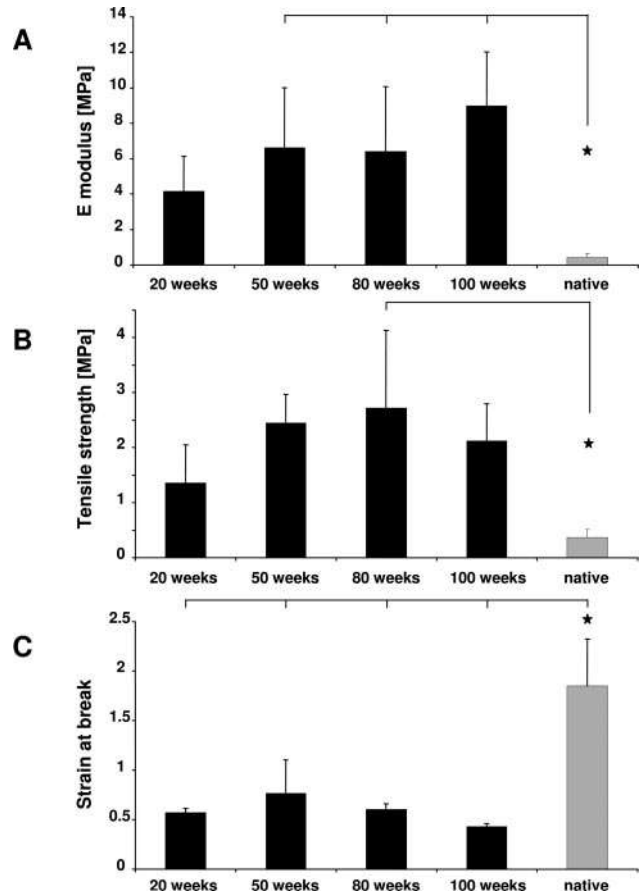
increased collagen contents in all of the postoperative groups. This matrix composition is reflected in the biomechanical properties of the TEVGs showing increased mechanical strength and less pliable tissues.

Although showing morphological approximation to native pulmonary artery tissues over time, the differences even after 2 years suggest ongoing tissue remodeling and maturation with good functional performance and growth. This capacity of tissue remodeling and adaptation to the growing cardiovascular system initiated by in vitro generated engineered cell-seeded vascular grafts may be best characterized as guided tissue regeneration resulting in functional growth.

In summary, this study provides evidence of functional growth in living pulmonary arteries engineered from vascular cells in a full growth animal model. Thus, proof of concept regarding the growth potential of tissue-engineered cardiovascular substitutes is systematically demonstrated in vivo for the first time. These findings support the potential of the tissue-engineering concept for congenital applications and may provide a further experimental basis to justify entering into clinical trials.

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Disclosures

S.P.H. and G.Z. are scientific advisors to Symetis Inc.

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