Surgery for Congenital Heart Disease

Midterm clinical result of tissue-engineered vascular autografts seeded with autologous bone marrow cells

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Objective: Prosthetic and bioprosthetic materials currently in use lack growth potential and therefore must be repeatedly replaced in pediatric patients as they grow. Tissue engineering is a new discipline that offers the potential for creating replacement structures from autologous cells and biodegradable polymer scaffolds. In May 2000, we initiated clinical application of tissue-engineered vascular grafts seeded with cultured cells. However, cell culturing is time-consuming, and xenoserum must be used. To overcome these disadvantages, we began to use bone marrow cells, readily available on the day of surgery, as a cell source. The aim of the study was to assess the safety and feasibility of this technique for creating vascular tissue under low-pressure systems such as pulmonary artery or venous pressure.

Methods: Since September 2001, tissue-engineered grafts seeded with autologous bone marrow cells have been implanted in 42 patients. The patients or their parents were fully informed and had given consent to the procedure. A 5-mL/kg specimen of bone marrow was aspirated with the patient under general anesthesia before the skin incision. The polymer tube serving as a scaffold for the cells was composed of a copolymer of L-lactide and ϵ-caprolactone (50:50). This copolymer is degraded by hydrolysis. The matrix is more than 80% porous, and the diameter of each pore is 20 to 100 μm. Polyglycolic acid woven fabric with a thickness of 0.5 mm was used for reinforcement. Twenty-three tissue-engineered conduits (grafts for extracardiac total cavopulmonary connection) and 19 tissue-engineered patches were used for the repair of congenital heart defects. The patients' ages ranged from 1 to 24 years (median 5.5 years). All patients underwent a catheterization study, computed tomographic scan, or both, for evaluation after the operation. The patients received anticoagulation therapy for 3 to 6 months after surgery.

Results: Mean follow-up after surgery was 490 $\pm$ 276 days (1.3-31.6 months, median 16.7 months). There were no complications such as thrombosis, stenosis, or obstruction of the tissue-engineered autografts. One late death at 3 months after total cavopulmonary connection was noted in patient with hypoplastic left heart syndrome; this was unrelated to the tissue-engineered graft function. There was no evidence of aneurysm formation or calcification on cineangiography or computed tomography. All tube grafts were patent, and the diameter of the tube graft increased with time (110% $\pm$ 7 % of the implanted size).

Conclusion: Biodegradable conduits or patches seeded with autologous bone marrow cells showed normal function (good patency to a maximum follow-up of 32

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months). As living tissues, these vascular structures may have the potential for growth, repair, and remodeling. The tissue-engineering approach may provide an important alternative to the use of prosthetic materials in the field of pediatric cardiovascular surgery. Longer follow-up is necessary to confirm the durability of this approach.

Various vascular grafts are commonly used in the reconstruction of congenital heart defects in children. However, current prosthetic or bioprosthetic materials lack growth potential, and subsequent replacements are therefore required in pediatric patients.[1,2] Tissue engineering is a new discipline that offers the potential to create replacement structures from autologous cells and biodegradable polymer scaffolds.[3] Because tissue engineering constructs contain living cells, they may have the potential to grow, self-repair, and self-remodel. The application of this technique to cardiovascular surgery was pioneered by Drs Mayer and Vacanti in 1993 at the Children's Hospital, Boston.[4]

With the tissue-engineering technique, we demonstrated the feasibility of tissue engineering heart valve leaflet and pulmonary arterial graft in a large animal model in 1995 and 1997, respectively.[5-9] After the successful results of the supplementary examination in a dog inferior vena cava replacement model, the institutional review board in Tokyo Women's Medical University approved the clinical trial of these technique, with the patient's parents' thoroughly informed consent.[10,11] In May 2000, peripheral pulmonary artery was successfully reconstructed in a 4-year-old girl with the patient's own venous cells.[12] After that, 3 patients underwent tissue-engineered graft implantation with cultured autologous venous cells. On the basis of the large animal experiments, we began to use bone marrow cells (BMCs) from the anterior superior spine, readily available on the day of surgery, as a cell source, because cell culturing was time-consuming and xenoserum was required. Our experimental work showed evidence that BMCs contribute to the construction of tissue-engineered vascular autografts in vivo.[13]

Thus far, no clinical investigations have been reported. The institutional review board approved human clinical trials of this new technique. This study presents intermediate-term clinical results of tissue-engineered vascular grafts created by in vitro seeding of autologous bone marrow mononuclear cells on biodegradable polymer scaffolds.

Material and Methods

Biodegradable Scaffolds

A copolymer of lactide acid and ϵ-caprolactone was synthesized by ring-opening polymerization. This copolymer is a polyester with a molar composition of lactide and ϵ-caprolactone at a 50:50 ratio and is degraded by hydrolysis after a few months in vivo. The matrix is more than 80% porous, with a pore diameter of 20 to 100 μm. A woven fabric (thickness 0.5 mm) made with poly-L-lactide acid (PLLA, n = 22) or polyglycolic acid (PGA, n = 20) was used for reinforcement of the porous matrix. The PGA fibers were degraded by hydrolysis in approximately 2 months, and the PLLA fibers were degraded in 2 years. With these polymers, we fabricated a hybrid tubular scaffold that was 12 to 24 mm in diameter, 13 cm in length, and 0.6 to 0.7 mm in thickness. This fabrication was achieved by pouring a solution of the copolymer of lactide acid and ε-caprolactone onto the PLLA or PGA woven fabric sheet, followed by freeze-drying under vacuum (Figure 1). The tensile strength of the PLLA and PGA fiber in warm saline was measured with an Instron tester (model 4302; Instron Corporation, Canton, Mass) at various time points.

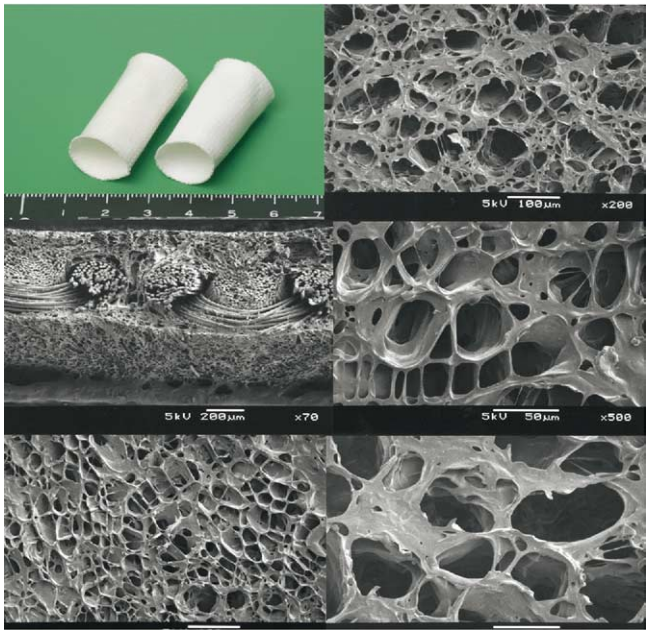

Figure 1. Macroscopic finding of biodegradable scaffolds and scanning electromicroscopic findings of polymer scaffolds. *Upper left,* Macroscopic finding; 18 mm in diameter.Copolymer of L-lactide and ϵ-caprolactone synthesized by ring-opening polymerization, with weight composition ratio of L-lactide and ϵ-caprolactone at 50:50. Polymeric woven scaffold composed of polycaprolactum and polylactic acid reinforced with PGA mesh. *Bars* represent 200 μm *(middle left),* 100 μm *(lower left, upper right),* and 50 μm *(middle right, lower right).*

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Preparation of the Tissue-Engineered Grafts

Cell harvest. As described previously, there were several modifications to isolation of BMCs and seeding on the scaffold. Briefly, recent methods are described here. With the patient under general anesthesia, BMCs (approximately 4-5 mL/kg body weight) were aspirated into a syringe containing heparin (100 units/mL BMCs) from the anterior superior iliac spine of a patient with a puncture needle. Aspirated BMCs were passed through a nylon cell strainer (Becton Dickinson, Franklin Lakes, NJ) to remove fat and bone fractions. BMCs were centrifuged with Histopaque-1077 (Sigma, St Louis, Mo) as described previously.[11]

Seeding onto the scaffold. After several washing and concentration steps, obtained mononuclear BMCs were seeded onto the biodegradable polymer by pipetting. The outer surface of the seeded biodegradable scaffolds was then sprayed with fibrin glue (Tisseel; Baxter, Vienna, Austria). The seeded biodegradable scaffold was then kept in the patient's own serum for approximately 2 to 4 hours at 37°C in 100% humidity and a 5% carbon dioxide atmosphere until use. Approximately 300,000 cells/cm^2 were used for cell seeding.

Quality control of the tissue-engineered grafts. To verify that seeded cells remained in the scaffold, the remnants of the graft before implantation were taken as a sample and examined by scanning micrography with Giemsa staining (Sigma) and immunohistochemical methods. Anti-CD31–conjugated monoclonal antibodies (Sigma) were used to identify the cell type. Cell counting was performed by the method previously described by Otto.[14]

Patients

From May 2000 until March 2004, a total of 42 consecutive patients received tissue-engineered vascular autografts seeded with BMCs (Table E1). Informed consent was obtained directly from patients older than 20 years and from the parents of younger patients. Twenty-three patients had a tube graft as an extracardiac total cavopulmonary connection (TCPC) graft. In the other 19 patients, a sheet-type patch was used. The mean age at operation was 7.3 years (range 1-24 years), and the mean body weight was 21.7 kg (range 7.5-64 kg). Inclusion criteria for patients for this procedure were as follows: elective surgery, age younger than 30 years, patients or familial full understanding of the procedure, and good quality of other organ function. Eighteen patients were male, and 24 were female. Thirty-six patients had undergone one or more previous operations, as described in Table E1. To examine the inflammatory reaction early after surgery, 20 patients who underwent TCPC at the same times (mean age 6.3 ± 3.1 years) with foreign materials served as a control group. White blood cell counts and maximum postoperative C-reactive protein level were compared between groups.

Follow-up

Follow-up visits of the patient were scheduled at 6 to 12 months postoperatively and yearly thereafter. During follow-up visits, transthoracic echocardiography and multislice computed tomography, cineangiography, or magnetic resonance imaging angiogram were performed. Anticoagulation therapy with warfarin sodium and aspirin was continued until 3 to 6 months after the operation. International normalized ratio was kept within 1.5 to 2.0. Thereafter, anticoagulation therapy with aspirin alone was continued for 12 months postoperatively.

Laboratory and Clinical Evaluation

The clinical evaluation was performed to detect whether there was any evidence of immunologic or inflammatory activation, such as leukocytosis or increasing C-reactive protein, during the first week after surgery. At follow-up visits, additional clinical evaluations, such as physical examination, chest roentgenography, and electrocardiography, were performed.

Changes in Transectional Area of Extracardiac TCPC Graft

At 1 year after implantation, computed tomography or magnetic resonance imaging was performed. Because the transectional shapes often appeared elliptic, the changes in maximal transectional area were calculated and compared with the area of the implanted tube. Calculation was performed according to the following formula: % Area change = [(Major axis × Major axis of graft) − (Implanted tube diameter)^2]/(implanted tube diameter)^2 × 100%. Seven of 23 patients who underwent TCPC were included in this calculation.

Results

Biodegradable Scaffolds

The PGA fibers were degraded by hydrolysis in approximately 2 months, the PLLA fibers in 2 years. The remaining tensile strengths of the PLLA fiber were 98.2% at 13 weeks, 88.1% at 26 weeks, 61.3% at 52 weeks, and 23.1% at 78 weeks; those of the PGA fiber were 81.3% at 1 week, 48.8% at 2 weeks, 7.6% at 3 weeks, and 4.6% at 4 weeks.

Quality Control of the Tissue-Engineered Grafts

Mean cell number seeded onto the scaffold was 320,000 ± 29,900 cells/cm^2. Scanning electromicrography showed the presence of seeded cells attached to the polymer surface (Figure E1). Giemsa staining showed a sufficient number of seeded monolayer cells in the inner space of the scaffold wall. Immunohistochemical staining for anti-CD34 and fluorescence-activated cell sorter analysis demonstrated that obtained mononuclear cells contained approximately 3% anti-CD34–positive cells (Figure E2).

Follow-up

Follow-up was 100% complete. Mean follow-up was 16.3 months (range 1.3-31.6 months, median 16.7 months).

Mortality

There were no in-hospital deaths after surgery. One patient with hypoplastic left heart syndrome died 3 months after the TCPC operation because of progressive tricuspid regurgitation and congestive heart failure. This late death was not related to the implanted tissue-engineered graft.

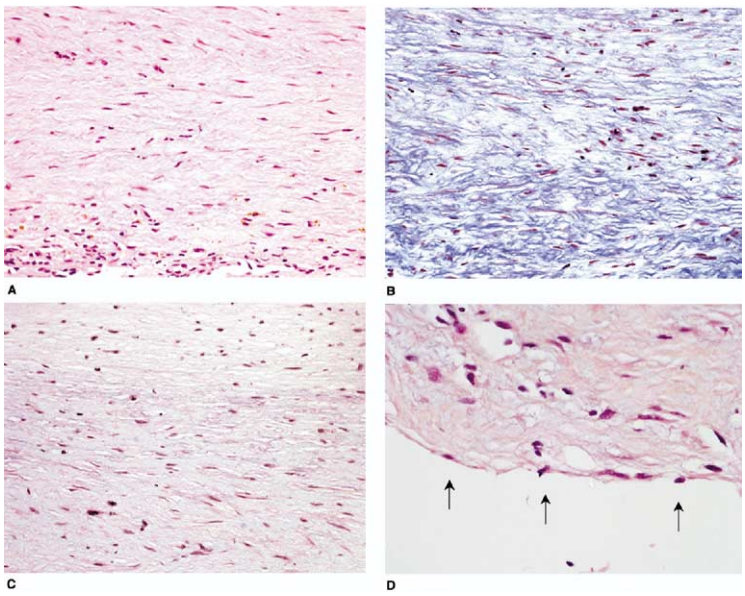

Figure 2. Histopathologic findings on explanted patch in patient with reoperation. *Arrows* indicate endothelium-like cells. A, Hematoxylin-eosin stain, original magnification ×400; B, Masson stain, original magnification ×400; C, Victoria blue, original magnification ×400; D, hematoxylin-eosin stain, original magnification ×800.

Adverse Events and Reintervention

During follow-up, there were no graft-specific lethal complication, such as rupture, thromboembolism, or aneurysm formation. In 1 patient, the tissue-engineered graft was replaced with a polytetrafluoroethylene patch 2 months after the original surgery because of unexpected slower tissue growth on the patch used for lateral tunnel procedure, which had caused massive right-to-left shunting. This patient recovered quickly after this surgical intervention and is currently in New York Heart Association functional class I. The histologic findings of the explanted patch are shown in Figure 2. The surface of the patch was covered with endothelial-appearing monolayer cells. In the medial layer, there were many collagen fibers and no evidence of calcification.

Laboratory and Clinical Evaluation

Fever of unknown origin has not been seen in any of the patients. Maximum elevated temperatures were in the range of 37.4°C ± 0.8°C after surgery. Further temperature measurements till discharge were performed without any increase in temperature. Inflammatory reaction early after operation is shown in Figure E3. There was no statistically significant difference between the tissue-engineering group and a control group randomly chosen for the same period. Transthoracic echocardiography, multislice computed tomography, or magnetic resonance imaging showed calcification to be absent in the tissue-engineered grafts for all investigated patients. Pulmonary angiography showed persistent patency in the peripheral pulmonary artery that was reconstructed with a tissue-engineered patch (Figure 3).

Changes in Area During Follow-up

As shown in Figures 4, 5, and E4, maximal transectional area was calculated and compared with the implanted size in the TCPC group. In patients with azygous connection (in which only hepatic flow goes up through the tissue-engineered graft), the larger grafts implanted tended to decrease in diameter with time. Depending on the flow amount, some grafts decreased in diameter and some increased, which seemed to be some sort of environmental adaptation of the tissue-engineered grafts (Figure 6).

Discussion

A good quality biologic material for use in pediatric heart surgery is not yet available. Allogeneic tissue and prosthetic materials degenerate rapidly relative to the course in older patients.[15,16] Furthermore, these materials are nonviable structures, limiting longer term durability. Currently used prosthetic or bioprosthetic materials, even allogeneic materials, lack growth potential and inevitably require reintervention within 10 to 15 years after the definitive surgery in pediatric patients.[17] Especially in young patients, there is increased calcium turnover, and antimineralization treatments can only decrease this in part.[18,19] A tissue-engineering approach with biodegradable materials and autologous cells might overcome these deficits.

We have previously reported the usefulness in human beings of a tissue-engineered autograft made with cultured cells seeded onto a biodegradable polymer.[12] This innovative challenge was based scientifically on several animal experiments.[5-10] Although these procedures use harvested vessel walls, the process of cell isolation introduces a risk of

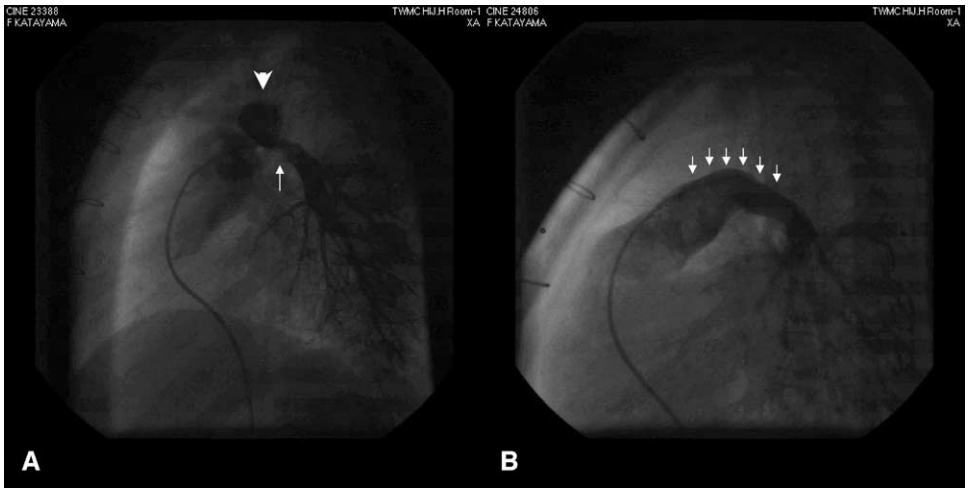

Figure 3. Pulmonary angiograms of patient 31. A, Lateral view of pulmonary angiogram before operation showed peripheral stenosis *(white arrowhead)* with aneurysmal formation *(white arrow)* after balloon angioplasty. B, Same view 6 months after operation. *White arrows* indicate location of tissue-engineered patch.

infection and a potential inability to culture sufficient cells for seeding onto the biodegradable polymer. Culturing cells requires substantial time, which is not available in emergency cases, and the use of serum from other species in the culture medium reduces the merit of the procedure. The benefits of using autologous cells for tissue engineering are that the patients do not need a donor and that there is no fear of rejection. When using BMCs, we were able to obtain sufficient cells on the day of surgery. In consequence, patients did not need extra hospitalization for vein harvesting. In fact, although some patients made a preliminary hospital visit to harvest vein walls, sufficient cells were not obtained by culturing. Furthermore, smaller quantities of culture medium and other reagents are required, leading to improved cost-effectiveness.

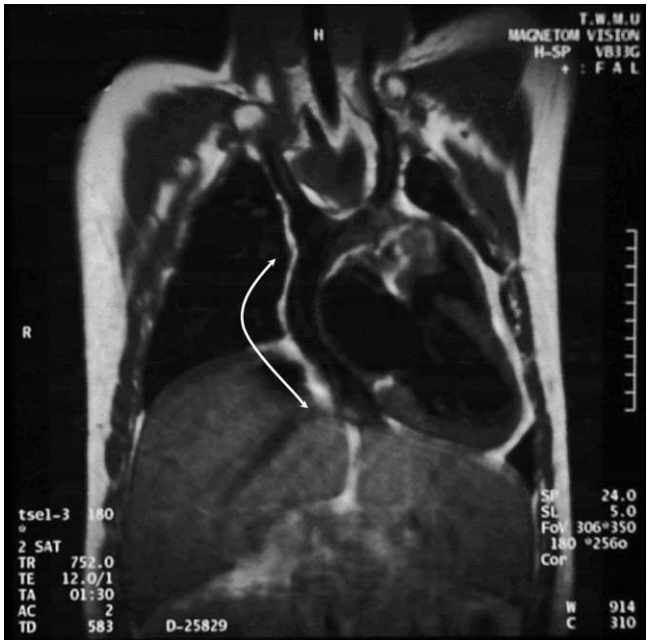

Figure 4. Magnetic resonance image 9 months after implantation of TCPC graft (patient 8). *White arrow* indicates location of tissue-engineered conduit.

From 2001, we changed cell source in tissue engineering for clinical applications because Noishiki and colleagues[20] reported that BMCs implanted onto the surface of an artificial graft led to earlier endothelialization in a large animal model, and it has been reported that some multipotential cells in bone marrow have the potential to differentiate into several cell types in vivo.[21] Furthermore, previous studies have shown the existence of endothelial progenitor cells derived from bone marrow, which contribute to vasculogenesis and angiogenesis,[22-24] and recent studies have demonstrated that endothelial cell lineage cells have potential in endothelialization.[25-27] Recently, we proved the contribution of seeded and circulating BMCs in tissue engineering vascular autografts in a dog model.[13] On the basis of these reports and our experimental results,[9] we were certain that seeding BMCs onto the polymer would result in a valuable tool for constructing tissue-engineered materials.

After the initial three implantations of tissue-engineered grafts seeded with venous cells, we retrospectively analyzed these first implantation data and compared these with those from the new technique. There were no difference in any data at any point (unpublished data).

Anticoagulation therapies have been administered to all patients, because animal experiments showed that it usually took 2 weeks to accomplish the complete endothelialization on the inner surface of the grafts. We decided to use the anticoagulation therapy for 3 months in clinical setting for the first clinical trials. International normalized ratio was kept between 1.5 and 2.0. During the early postoperative period, heparin was continuously

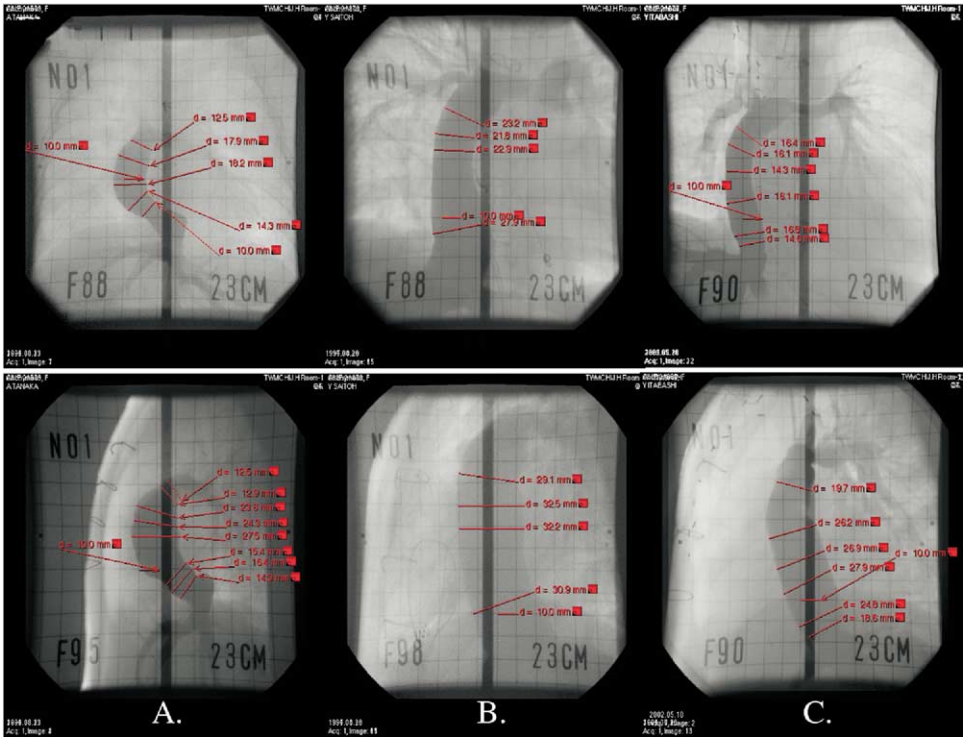

Figure 5. Measurements of major and minor axes of tissue-engineered grafts in patients 2 (A), 4 (B), and 12 (C). *Upper row,* Anteroposterior view; *lower row,* lateral view.

injected, and anticoagulation time was controlled between 180 and 230 seconds. After discharge from the hospital, we gave 5 mg/kg of aspirin and warfarin for 3 months to protect patients from thromboembolic complications. We assumed that autologous tissue would not necessitate anticoagulation and that patients would be free from medication from then on. The duration of warfarin treatment could be shortened in the future; this would improve quality of life, especially for young women who want to become pregnant and need to be free from anticoagulation therapy such as warfarin.

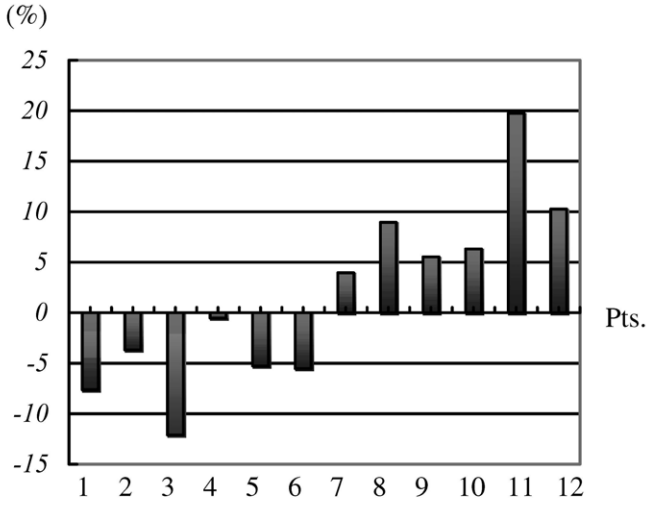

Figure 6. Percentage changes in cross-sectional area of tissue-engineered grafts 1 year after operation.

Regarding the differentiation of BMCs, this method has potential risks, such as bone formation or neoplasm formation. In our 4-year experience, however, there was no calcification or neoplasm formation in 42 patients.

This therapy is in its infancy, and it is true that several problems remain to be resolved at this time. More ideal biodegradable scaffolds, better procedures for cell preparation, and improved culture medium should be investigated in the near future. We do hope that autologous tissue-engineered materials will offer several advantages for children around the world who require cardiac surgery.

Limitations of the Study

This study was not randomized trial or a controlled study. It was also a small study, with a small number of patients. We believe it to be the only safety and feasibility study of tissue-engineered grafts in the clinical setting to date.

Conclusion

In conclusion, tissue-engineered vascular grafts were safe and feasible in pediatric cardiovascular surgery and showed excellent hemodynamic performance during in-

termediate-term follow-up without anticoagulation therapy. It appears that the application of biodegradable scaffold with seeded BMCs might be able to decrease the need for reintervention in growing pediatric patients. Avoidance of anticoagulation therapy will probably improve quality of life. Because our results in both the clinical setting and the experimental model were quite encouraging, we believe that the tissue-engineering approach may play an important role as an alternative method to polytetrafluoroethylene grafts or allografts, especially in the field of pediatric cardiovascular surgery. The potential durability of these tissue-engineered constructs will be determined by longer follow-up.[18,19]

References

1. Kirklin J, Barratt-Boyes B. Ventricular septal defect and pulmonary stenosis or atresia. Philadelphia: Churchill Livingstone; 1993. p. 885-917.
2. Mayer JE Jr. Uses of homograft conduits for right ventricle to pulmonary connections in the neonatal period. *Semin Thorac Cardiovasc Surg.* 1995;7:130-2.
3. Langer R, Vacanti JP. Tissue engineering. *Science.* 1993;260:920-6.
4. Mayer JE Jr, Shin'oka T, Shum-Tim D. Tissue engineering of cardiovascular structures. *Curr Opin Cardiol.* 1997;12:528-32.
5. Breuer CK, Shin'oka T, Tanel RE, Zund G, Mooney DJ, Ma PX, et al. Tissue engineering lamb heart valve leaflets. *Biotechnol Bioeng.* 1996; 50:562-7.
6. Shinoka T, Breuer CK, Tanel RE, Zund G, Miura T, Ma PX, et al. Tissue engineering heart valves: valve leaflet replacement study in a lamb model. *Ann Thorac Surg.* 1995;60(6 Suppl):S513-6.
7. Shinoka T, Ma PX, Shum-Tim D, Breuer CK, Cusick RA, Zund G, et al. Tissue-engineered heart valves. Autologous valve leaflet replacement study in a lamb model. *Circulation.* 1996;94(9 Suppl):II164-8.
8. Shinoka T, Shum-Tim D, Ma PX, Tanel RE, Langer R, Vacanti JP, et al. Tissue-engineered heart valve leaflets: does cell origin affect outcome? *Circulation.* 1997;96(9 Suppl):II102-7.
9. Shin'oka T, Shum-Tim D, Ma PX, Tanel RE, Isogai N, Langer R, et al. Creation of viable pulmonary artery autografts through tissue engineering. *J Thorac Cardiovasc Surg.* 1998;115:536-46.
10. Watanabe M, Shin'oka T, Tohyama S, Hibino N, Konuma T, Matsumura G, et al. Tissue-engineered vascular autograft: inferior vena cava replacement in a dog model. *Tissue Eng.* 2001;7:429-39.
11. Matsumura G, Hibino N, Ikada Y, Kurosawa H, Shin'oka T. Successful application of tissue engineered vascular autografts: clinical experience. *Biomaterials.* 2003;24:2303-8.
12. Shin'oka T, Imai Y, Ikada Y. Transplantation of a tissue-engineered pulmonary artery. *N Engl J Med.* 2001;344:532-3.
13. Matsumura G, Sachiko Miyagawa-Tomita, Shin'oka T, Ikada Y, Kurosawa H. First evidence that bone marrow cells contribute to the construction of tissue-engineered vascular autografts in vivo. *Circulation.* 2003;108:1729-34.
14. Otto RJ. Fluorimetric DNA assay for cell growth estimation. *Anal Biochem.* 1992;207:186-92.
15. Albert JD, Bishop DA, Fullerton DA, Campbell DN, Clarke DR. Conduit reconstruction of the right ventricular outflow tract: lessons learned in a 12-year experience. *J Thorac Cardiovasc Surg.* 1993;106: 228-36.
16. Breymann T, Thies WR, Boethig D, Goerg R, Blanz U, Koerfer R. Bovine valved venous xenografts for RVOT reconstruction: results after 71 implantations. *Eur J Cardiothorac Surg.* 2002;21:703-10.
17. Gott JP, Chih P, Dorsey L, Jay JL, Jett GK, Schoen FJ, et al. Calcification of porcine valves: a successful new method of antimineralization. *Ann Thorac Surg.* 1992;53:207-16.
18. Chen W, Kim JD, Schoen FJ, Levy RJ. Effect of 2-amino oleic acid exposure conditions on the inhibition of calcification of glutaraldehyde cross-linked porcine aortic valves. *J Biomed Mater Res.* 1994;28:1485-95.
19. Weissenstein C, Human P, Bezuidenhout D, Zilla P. Glutaraldehyde detoxification in addition to enhanced amine cross-linked dramatically reduced bioprosthetic tissue calcification in the rat model. *J Heart Valve Dis.* 2000;9:230-40.
20. Noishiki Y, Tomizawa Y, Yamane Y, Matsumoto A. Autocrine angiogenic vascular prosthesis with bone marrow transplantation. *Nat Med.* 1996;2:90-3.
21. McKay R. Stem cells—hype and hope. *Nature.* 2000;406:361-4.
22. Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, et al. Ischemia- and cytokine-induced mobilization of bone marrow–derived endothelial progenitor cells for neovascularization. *Nat Med.* 1999;5: 434-8.
23. Shintani S, Murohara T, Ikeda H, Ueno T, Honma T, Katoh A, et al. Mobilization of endothelial progenitor cells in patients with acute myocardial infarction. *Circulation.* 2001;103:2776-9.
24. Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, et al. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res.* 1999;85:221-8.
25. Shi Q, Rafii S, Wu MH, Wijelath ES, Yu C, Ishida A, et al. Evidence for circulating bone marrow–derived endothelial cells. *Blood.* 1998; 92:362-7.
26. Bhattacharya V, McSweeney PA, Shi Q, Bruno B, Ishida A, Nash R, et al. Enhanced endothelialization and microvessel formation in polyester grafts seeded with CD34(+) bone marrow cells. *Blood.* 2000;95: 581-5.
27. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, et al. Isolation of putative progenitor endothelial cells for angiogenesis. *Science.* 1997;275:964-7.

Discussion

Dr John Mayer, Jr *(Boston, Mass).* First, I congratulate Shin'oka and colleagues from the Tokyo Women's Medical University on their pioneering clinical efforts in applying a tissue-engineering approach to the creation of devices for cardiovascular application. It has been gratifying to me that Dr Shin'oka's work has progressed from the earliest successful feasibility studies that he and Chris Breuer successfully completed in the laboratory several years ago and has now reached the point of a clinical trial.

Dr Shin'oka's use of autologous BMCs to seed biodegradable graft materials has confirmed in human beings what Fraser Sutherland presented at this meeting last year in an ovine pulmonary valved conduit model, that bone marrow mononuclear cells can be a clinically applicable source of cells for tissue-engineered constructs in the cardiovascular system.

However, there are a number of unanswered questions for this emerging field of cardiovascular tissue engineering, and I would like to ask Dr Shin'oka a few questions on his experience thus far. One fundamental question is what happens to the cells that are seeded onto the scaffolds after the scaffolds are implanted? If I understand the article correctly, there are at least 2 scaffolds that may be available for histologic examination after 2 to 3 months in vivo: 1 from the patient with hypoplastic left heart syndrome who died and 1 from the patient who had to have the tissue-engineered baffle replaced after 2 months.

Dr Shin'oka, do you have any histologic or immunohistochemical data on either of these scaffolds, or on any other specimens explanted after a period in vivo? Do you have any preliminary

ideas about what happens to the cells that were implanted within the scaffold?

Dr Shin'oka. First, Dr Mayer, let me express my deep appreciation for your kind instruction when I began the laboratory experiments for tissue engineering in Boston. As you mentioned, many questions remain to be answered in this field, and as a result I have been told by others that a clinical application to my research was premature. However, I have always remembered that you and Dr Vacanti encouraged me by saying "you have to walk before running." Therefore, on the basis of the results of 6 years of research, we have begun clinical application of vascular grafts in a low-pressure system, which we assume is the safest region. However, I agree that our results are just the beginning of a long, long path for successful tissue engineering in the cardiovascular system.

With regard to your first question concerning histologic study of explanted specimens from the patient who died and the patient who underwent reoperation, you are correct in stating that these specimens provided opportunities for histologic examination. Unfortunately, the first patient's family refused an autopsy. In the case of the patient who underwent reoperation, we obtained a specimen during that reoperation, 2 months after implantation (Figure 2).

This specimen demonstrates the partially endothelialized tissue, which contains the collagen fiber in the medial layer. However, this tissue was broken by catheterization and is very fragile in the center of the patch, which shows poor tissue growth. When the scaffold degraded, the current tissue formation was insufficient to provide mechanical support. This is a different situation from that observed in the blood vessels. Because the outer surface of tissue-engineered vascular grafts would be covered with the adhesive tissue, this tissue would reinforce the mechanical properties of the vascular grafts. Because of these results, we stopped using the PGA scaffold as a septum in the bloodstream.

Regarding the fate of the seeded cells, as I have shown in this presentation, we confirmed that many of the seeded BMCs contributed to tissue formation.

Dr Mayer. A second fundamental question is whether it is even necessary to seed these biodegradable polymer constructs before implantation, or whether circulating progenitor cells of bone marrow or other origin could in fact seed a polymer scaffold in vivo after the naked scaffold was implanted. Do you have any experience with implantation of the scaffold without previous seeding with cells? If not, could you speculate on the potential role of circulating progenitor cells for tissue-engineered structures?

Dr Shin'oka. With regard to the second question concerning the contribution of circulating progenitor cells, we did implant many control grafts in the inferior vena cava position without cell seeding. In 20% of these grafts, good quality vessels developed. However, the graft occlusion rate was high. When we administered granulocyte colony-stimulating factor after control graft implantation, the success rate increased to 50%. Further, when we used the cell seeding before implantation, the success rate reached approximately 100%.

Because of these results, we continue to use the cell seeding method before implantation in a clinical setting. The experimental study showed that the seeded cells produced some cytokines, such as vascular endothelial growth factor and angiopoietin 1. We suggest that these cytokines assist in inducing progenitor cell migration from the bloodstream. We speculate and provide evidence that the healing mechanism is due to the cooperation between seeded cells and migrated progenitor cells.

Dr Mayer. And the last fundamental question for cardiovascular tissue engineering is whether the implanted cells on these conduits will continue to proliferate, causing vascular obstruction, or in some way or another will "know" when to stop proliferating.

In your series, 2 of the 19 patients receiving tissue-engineered pulmonary artery patches and 1 patient with a Fontan circulation required subsequent balloon angioplasty, and in the article you have attributed these stenoses to presumed tissue overgrowth. What strategies should we use to prevent such tissue overgrowth, and what role do you think that there might be for the simultaneous seeding of endothelial cells in addition to BMCs in preventing tissue overgrowth?

Dr Shin'oka. In response to the third question, asking when and how seeded cells stop proliferating, as you might have noticed in the clinical setting, the wound healing process is a mysterious function of the human body. When a skin injury is completely healed, the cells stop proliferating. The same mechanics may occur in the tissue-engineering process. Actually, no patients in our series have had neoplasm formation in our 4-year clinical experience.

The reason for the recurrent pulmonary restenosis is related to tissue overgrowth that occurred at the anastomosis site, where the suture material remained. We have no solution for tissue overgrowth at the anastomosis site. In addition, I would like to state that the wound healing process is different from patient to patient, an individual process. Most likely, the process that occurs in the tissue-engineered grafts is similar to this kind of wound healing. Thank you again for your kind questions.

Dr Thorsten Walles *(Hannover, Germany).* Reading your abstract, I have one question. I am surprised by the need for anticoagulants for a period of 6 months in your patients, because a functional tissue-engineered vascular graft should have a functional athrombogenic endothelial lining. Did you use any methods before or after implantation to control endothelial function in your grafts?

Dr Shin'oka. According to the results of the experimental animal model, endothelialization is complete within 2 weeks; anticoagulation treatment for 6 months thus may not be required. In the clinical setting, we desire the best results, but maybe we can shorten the duration of anticoagulation therapy.

Dr Tirone David *(Toronto, Ontario, Canada).* No, the question is regarding the need for anticoagulation. Why did you anticoagulate the patients for 6 months?

Dr Shin'oka. In the experimental animal, endothelialization was complete within 2 weeks. So maybe we don't have to use anticoagulation for 6 months. But in the clinical setting, we want to have better results. So maybe we can shorten the duration of anticoagulation therapy.

Dr Walles. But you don't know if you have endothelial function and an athrombogenic graft at the time of implantation?

Dr Shin'oka. As you pointed out, tissue-engineered grafts do not have the functional endothelial cells at the time of implantation. We therefore used the heparin infusion in acute phase and used anticoagulation therapy in the middle term. Actually, the tissue-engineered graft had obtained functioning endothelial cells

within 2 weeks, which was confirmed by nitric oxide production, in an animal model.

Dr Adrian A. Crucean *(London, UK).* We are currently conducting a similar experiment in Italy. I would like to ask you whether before using these conduits in human beings you have done any animal model or in vitro model regarding the potential of growth for these grafts (designed to be used in the pediatric age group). What is the smallest graft that you used?

Dr Shin'oka. The growth potential was confirmed in the growing animal model. The smallest graft size that we have used in the clinical setting is now 12 mm.

Dr Smruti R. Mohanty *(Bangalore/Karnataka, India).* Can you use BMCs from the sternum?

Dr Shin'oka. We usually aspirate bone marrow from the anterior iliac spine. The sternum is not suitable to obtain a sufficient number of BMCs.

TABLE E1. Characteristics of patients (female/male ratio 24:18)

Patient	Age (y)	Body weight (kg)	Chief diagnosis	Previous operations		Operative procedure with tissue-engineered materials	Scaffold diameter (mm)	Components of biodegradable scaffold
				Radical operations	Palliative operations			
Tissue-engineered vascular autografts								
1	2	11	Asplenia, AVSD(A), small RV		mBTS (×2)	TCPC	16	P(LA/CL) + PLLA
2	1	7.5	Asplenia, SRV, DORV, TAPVC (Ib)		mBTS	TCPC, TAPVC repair	20	P(LA/CL) + PLLA
3	7	18.5	Concordant criss-cross heart, DORV, PAA, MS		oBTS, CS, PA plasty	TCPC	18	P(LA/CL) + PLLA
4	21	44.4	TA (Ib)		oBTS (×2), CS	TCPC	24	P(LA/CL) + PLLA
5	4	14	SRV, DORV, AVVA		PAB, PDA ligation, mBTS, BDG	TCPC	20	P(LA/CL) + PLLA
6	12	36.7	Total sinus defect, ASD, tricuspid valve regurgitation (III)	Septation, TVP (×2)	oBTS, PAB	TCPC	24	P(LA/CL) + PLLA
7	17	46.5	Asplenia, SLV, CAVVR (III)		oBTS, BDG + CAVVP	TCPC	24	P(LA/CL) + PLLA
8	19	47	TA (Ib)		Björk + PDA ligation	TCPC	22	P(LA/CL) + PLLA
9	3	13.5	Polysplenia, SRV		BDG	Hepatic vein rerouting	12	P(LA/CL) + PLLA
10	2	7.5	HLHS, MA, IAA (A)		Norwood, mBTS, BDG	TCPC	16	P(LA/CL) + PLLA
11	2	11	Asplenia, SRV, PAA, nonconfluent PA		mBTS (×2)	TCPC	16	P(LA/CL) + PGA
12	13	23	PPA, ASD (II), sinusoidal communication		oBTS, mBTS	TCPC	20	P(LA/CL) + PLLA
13	14	9.9	SLV, DILV, left AVVA			TCPC	16	P(LA/CL) + PGA
14	2	9.32	DORV, small LV, perimembranous VSD, PS, ASD (II)		mBTS, PAB	TCPC	18	P(LA/CL) + PGA
15	2	11	Polysplenia, cAVSD (A), DORV, PS, CAVVR			Hepatic vein rerouting	12	P(LA/CL) + PGA
16	2	8.67	Asplenia, SRV, CAVV, CA, TAPVC (Ib), CAVVR (I)	TAPVC repair	BDG	TCPC	16	P(LA/CL) + PGA
17	24	51.6	TA (Ia), RA giant thrombosis, AFL, af, congestive liver	Fontan (APC)		TCPC	18	P(LA/CL) + PGA
18	1	8.7	SRV, DIRV, PA, ASD (II)		mBTS	TCPC	16	P(LA/CL) + PGA
19	11	25.5	Asplenia, SRV, PS (inferior and valvular), CA, CAVV			TCPC	18	P(LA/CL) + PGA
20	2	11	Polysplenia, cAVSD, PS, CAVV, CAVVR (I)		TCPS	Hepatic vein rerouting	12	P(LA/CL) + PGA
21	3	10.5	DORV, VSD, small RA, PLSVC, TAPVC (IIb)			TCPC, PVO release	16	P(LA/CL) + PGA
22	4	13	PPA, ASD (II), PDA, sinusoidal communication		mBTS (×2)	TCPC	18	P(LA/CL) + PGA
23	4	14.2	SLV, DILV, PA, ASD, bilateral SVC		mBTS (×2), BDG	TCPC	18	P(LA/CL) + PGA

TABLE E1. Continued

Patient	Age (y)	Body weight (kg)	Chief diagnosis	Previous operations		Operative procedure with tissue-engineered materials	Scaffold diameter (mm)	Components of biodegradable scaffold
				Radical operations	Palliative operations			
Tissue-engineered sheets								
24	6	16.5	TF, PAPVD, valvular PS	ICR		RVOTR		P(LA/CL) + PLLA
25	10	36	D-TGA (I), RV outflow obstruction, branch PS	ASO, PA plasty		PAP		P(LA/CL) + PLLA
26	24	44	TF, PA, conduit stenosis	Rastelli	Waterston	RVOTR		P(LA/CL) + PLLA
27	9	29	SRV, DIRV, DORV, branch PS	Fontan	mBTS	PAP		P(LA/CL) + PLLA
28	7	16.1	TF, branch PS	ICR	mBTS	PAP		P(LA/CL) + PLLA
29	1	13.5	IAA (A), branch and main PA stenosis, SVC occlusion	ICR, arch repair		PAP and SVC Patch		P(LA/CL) + PLLA
30	3	13.8	Polysplenia, SRV, DORV		mBTS	TCPC (LT)		P(LA/CL) + PLLA
31	3	14	Truncus arteriosus communicans (I), conduit stenosis	Rastelli (×2)		PAP		P(LA/CL) + PLLA
32	11	36.5	Congenital aortic regurgitation (III)			RVOTR, Ross		P(LA/CL) + PLLA
33	2	8.5	Asplenia, SRV, DORV			TCPC (LT)		P(LA/CL) + PLLA
34	8	19	DORV, VSD, nonconfluent PA		mBTS (×2), CS + PAP	PAP		P(LA/CL) + PLLA
35	5	14.5	TF, branch PS	ICR	oBTS, mBTS, palliative RVOTR	RVOTR		P(LA/CL) + PGA
36	7	19.5	Polysplenia, L-DORV, PAA, bilateral branch PS	Double-switch operation	mBTS	PAP		P(LA/CL) + PGA
37	10	17.2	Polysplenia, SRV, left AVVA, right AVVR (II)		BDG	TCPC (LT)		P(LA/CL) + PGA
38	17	64	D-TGA (I), supravalvular PS, AR (II)	ASO		RVOTR		P(LA/CL) + PGA
39	3	13.8	TAC (II), conduit stenosis, PAPVR	Rastelli		RVOTR		P(LA/CL) + PLLA
40	9	42.5	BWG, PS	Takeuchi, right PA angioplasty		PAP		P(LA/CL) + PGA
41	8	20.5	TGA (II), CoA, LMT occlusion, left PA stenosis	ASO		PAP		P(LA/CL) + PGA
42	6	19.5	D-TGA (I), SVAS, left PA stenosis	ASO		PAP		P(LA/CL) + PGA

AVSD, Atrioventricular septal defect; *RV,* right ventricle; *mBTS,* modified Blalock-Taussig shunt; *P(LA/CL),* copolymer of L-lactide and ϵ-caprolactone; *SRV,* single right ventricle; *DORV,* double-outlet right ventricle; *TAPVC,* total anomalous pulmonary venous connection; *PAA,* pulmonary artery atresia; *MS,* mitral stenosis; *oBTS,* original Blalock-Taussig shunt; *CS,* central shunt; *PA,* pulmonary artery; *TA,* tricuspid atresia; *AVVA,* atrioventricular valve atresia; *PAB,* pulmonary arterial banding; *PDA,* patent ductus arteriosus; *BDG,* bidirectional Glenn shunt; *ASD,* atrial septal defect; *TVP,* tricuspid valve plasty; *SLV,* single left ventricle; *CAVVR,* common atrioventricular valve regurgitation; *CAVVP,* common atrioventricular valve plasty; *HLHS,* hypoplastic left heart syndrome; *MA,* mitral atresia; *IAA,* interruption of aortic arch; *PPA,* pure pulmonary atresia; *DILV,* double-inlet left ventricle; *LV,* left ventricle; *VSD,* ventricular septal defect; *PS,* pulmonary stenosis; *cAVSD,* complete atrioventricular septal defect; *CAVV,* common atrioventricular valve; *CA,* common atrium; *RA,* right atrium; *AFL,* atrial flutter; *af,* atrial fibrillation; *APC,* atrial premature contraction; *DIRV,* double-inlet right ventricle; *PLSVC,* persistent left superior vena cava; *SVC,* superior vena cava; *TF,* tetralogy of Fallot; *PAPVD,* partial anomalous pulmonary vein drainage; *ICR,* intracardiac rerouting; *RVOTR,* right ventricular outflow tract reconstruction; *D-TGA,* dextrotransposition of the great arteries; *ASO,* arterial switch operation; *PAP,* pulmonary arterial patch angioplasty; *LT,* lateral tunnel method; *AVVR,* atrioventricular valve regurgitation; *AR,* aortic regurgitation; *TAC,* truncus arteriosus; *PAPVR,* partial anomalous pulmonary vein return; *BWG,* Bland-White-Garland syndrome; *CoA,* coarctation; *LMT,* left main trunk of coronary artery; *SVAS,* supravalvular aortic stenosis.

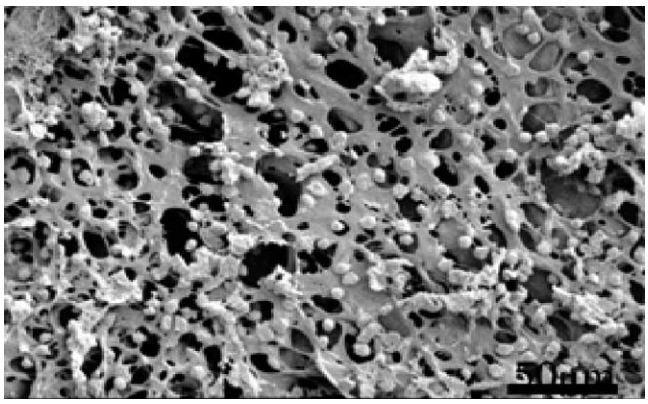

Figure E1. Scanning electromicroscopic findings of seeded BMCs on polymer scaffold. *Bar* represents 50 μm.

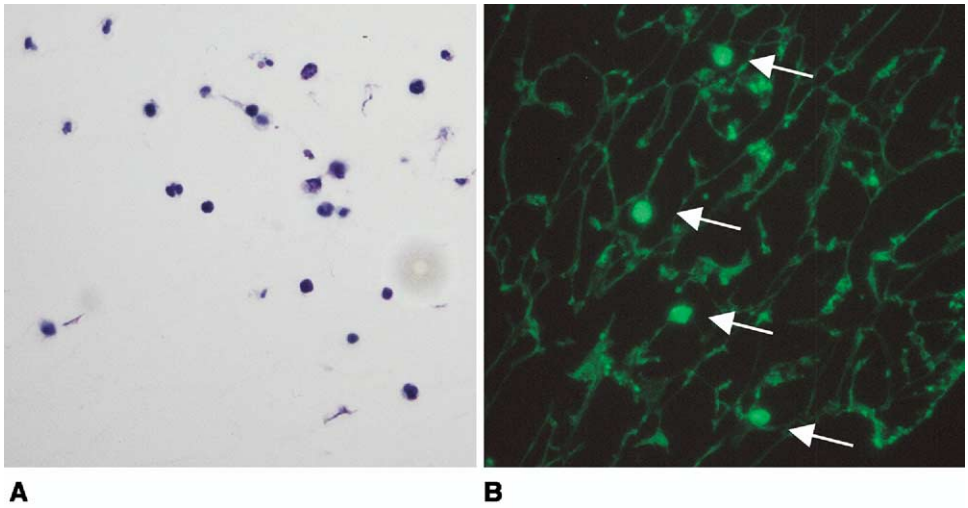

Figure E2. Histologic findings of seeded polymer. A, Mononuclear cells in scaffold (May-Grünwald-Giemsa staining). B, Immunohistochemical staining for CD34 showed positive cells in scaffold *(arrows).*

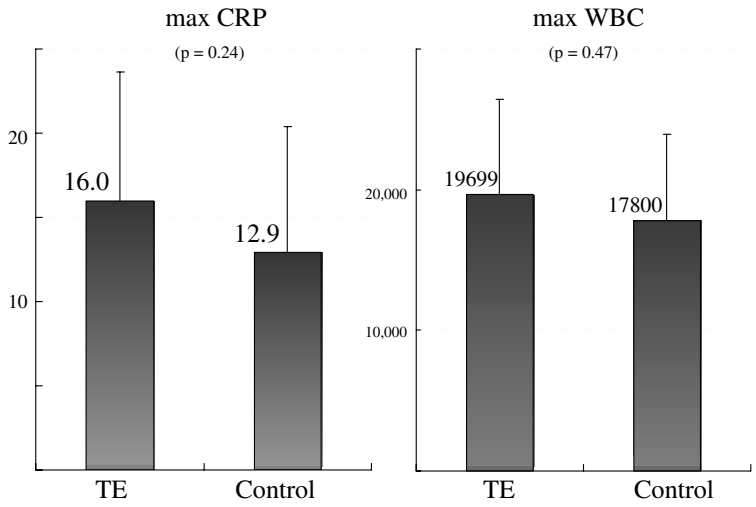

Figure E3. Postoperative maximum values of C-reactive protein *(max CRP)* and white blood cell count *(max WBC)* showed no significant difference between groups. *TE,* Group implanted with tissue-engineered graft.

Figure E4. Three-dimensional computed tomographic image 12 months after implantation of TCPC graft (patient 20). *Red arrow* indicates location of tissue-engineered conduit.