First Evidence That Bone Marrow Cells Contribute to the Construction of Tissue-Engineered Vascular Autografts In Vivo

Goki Matsumura, MD; Sachiko Miyagawa-Tomita, PhD; Toshiharu Shin’oka, MD, PhD; Yoshito Ikada, PhD; Hiromi Kurosawa, MD, PhD

Background—Materials commonly used to repair complex cardiac defects lack growth potential and have other unwanted side effects. We designed and tested a bone marrow cell (BMC)–seeded biodegradable scaffold that avoids these problems.

Methods and Results—To demonstrate the contribution of the BMCs to histogenesis, we labeled them with green fluorescence, seeded them onto scaffolds, and implanted them in the inferior vena cava of dogs. The implanted grafts were analyzed immunohistochemically at 3 hours and subsequently at 2, 4, and 8 weeks after implantation using antibodies against endothelial cell lineage markers, endothelium, and smooth muscle cells. There was no stenosis or obstruction caused by the tissue-engineered vascular autografts (TEVAs) implanted into the dogs. Immunohistochemically, the seeded BMCs expressing endothelial cell lineage markers, such as CD34, CD31, Flk-1, and Tie-2, adhered to the scaffold. This was followed by proliferation and differentiation, resulting in expression of endothelial cells markers, such as CD146, factor VIII, and CD31, and smooth muscle cell markers, such as α-smooth muscle cell actin, SMemb, SM1, and SM2. Vascular endothelial growth factor and angiopoietin-1 were also produced by cells in TEVAs.

Conclusions—These results provide direct evidence that the use of BMCs enables the establishment of TEVAs. These TEVAs are useful for cardiovascular surgery in humans and especially in children, who require biocompatible materials with growth potential, which might reduce the instance of complications caused by incompatible materials and lead to a reduced likelihood of further surgery. (Circulation. 2003;108:r73-r78.)

Key Words: tissue engineering ■ cells ■ polymers ■ surgery

Pediatric cardiovascular surgeons often encounter patients requiring surgical intervention that necessitates use of foreign materials to repair complex lesions. However, the materials that are commonly used lack growth potential, and long-term results have revealed several material-related failures, such as stenosis, thromboembolization, calcium deposition, and risk of infection. To solve these problems—in particular, for children who require implantation of dynamic material with growth potential—we need to develop optimal filling materials with biocompatibility and growth potential.

Previously, we reported the advantages of tissue-engineered vascular autografts (TEVAs) in dog, lamb, and human clinical applications using biodegradable scaffolds. The key benefit from using such scaffolds is that they degrade in vivo, thereby avoiding the long-term presence of foreign materials as the seeded cells proliferate to form new tissue. However, there was a drawback in the procedure in that we had to culture cells in vitro with the use of serum from other species in the culture medium, which entails risks that significantly reduce the merit of the procedure.

Recent studies have demonstrated the existence of bone marrow–derived endothelial progenitor cells that contribute to vasculogenesis and angiogenesis and endothelialization of artificial grafts using bone marrow cells (BMCs). In light of these reports, we hypothesized that BMCs would contribute to histogenesis in TEVAs. To test this hypothesis, we seeded labeled BMCs onto a biodegradable scaffold, which was then implanted to the inferior vena cava (IVC) of dogs. This report describes the characteristics, functions, and roles of seeded BMCs after implantation.

Methods

Biodegradable Scaffolds

A copolymer of lactic acid and ε-caprolactone [P(CL/LA)] was synthesized by ring-opening polymerization. This copolymer is a polyester with a molar composition of lactate and ε-caprolactone at a 50:50 ratio. The matrix is >80% porous, with a pore diameter of 20 to 50 μm. It loses its strength in 16 weeks and is degraded by hydrolysis in vivo after ~24 weeks (not all data are shown). A woven fabric made with poly-ε-lactic acid (PLLA) (thickness, 0.5 mm) that could be degraded by hydrolysis within ~2 years was used for reinforcement of the porous matrix. When a mechanical test using Instron (model 4302; Instrom) was performed, this PLLA fiber showed a remaining tensile strength of 98.2% at 13, 88.1% at 26, 61.3% at 52, and 23.1% at 78 weeks when compared with a control.
fiber (0 day; tensile strength 100%) (not all data are shown). Using these polymers, we fabricated a hybrid tubular scaffold that was 8 mm in diameter, 2 cm in length, and 0.6 mm in thickness. This fabrication was achieved by pouring a solution of P(CL/LA) onto the PLLA woven fabric sheet, followed by freeze-drying in a vacuum.

**Cell Harvest and Seeding Onto the Scaffold**

BMCs (~1 to 3 mL/kg body weight) were aspirated into a syringe containing heparin (1000 U per 10 mL BMCs) from the iliac bone of a dog. Three volumes of red blood lysis solution (Promega) and 0.5 mg of 5- and 6-carboxyfluorescein-diacetate-succinimidyl-ester (CFDA) for tracing were added to the aspirated BMCs. The resulting solution was centrifuged and the supernatant was discarded. Cells were seeded onto the scaffold at a concentration of 758.0±240.0 cells/mm². These cells were pipetted onto the tubular scaffold, which was then incubated in culture medium for 1.5 to 2 hours at 37°C in an atmosphere of 5% CO₂ and 100% humidity.

**Culture Medium**

To preserve the cells seeded onto the scaffolds, we kept them in RPMI-1640 (Sigma) supplemented with 100 mg/L of Tylosin (ICN Biomedicals), 100 U/L of penicillin G, 100 μg/L of streptomycin, 2.5 mg/L of Fungizone (Invitrogen), 1.0 μg/L of recombinant human vascular endothelial growth factor (VEGF) (R&D Systems), 0.5 μg/L of recombinant human hepatocyte growth factor (R&D Systems), and 2.5 μg/L of recombinant human basic fibroblast growth factor (Sigma).

**Implantation of TEVAs**

We used 16 healthy adult beagles (8.7 to 11.2 kg) in this study (NARC Co., Tomisato, Japan). The Animal Care and Use Committee of Tokyo Women’s University Medical School approved the use of the animals. In each case, TEVAs were implanted into the IVC of the dog from which the BMCs were originally obtained, as described above. With the dog sedated with pentobarbital anesthesia (0.1 mg/kg body weight) and atropine sulfate (0.08 mg/kg), a right lateral thoracotomy was performed through the 6th intercostal space. After injection of heparin (0.1 mL/kg), the IVC was clamped and resected, followed by implantation of a TEVA into the remaining IVC. The dogs were observed for 3 hours and subsequently at 2, 4, and 8 weeks, and one dog was observed for 2 years for long-term evaluation. These dogs were monitored without anticoagulants until they were euthanized and dissected.

**Immunohistochemistry and Immunofluorescence Staining**

The harvested graft divided into two parts was either fixed in 4% paraformaldehyde in phosphate-buffered saline and embedded in paraffin or snap-frozen in OCT Compound (TissueTek) and sectioned into 5-μm thickness using a cryostat.

The paraffin sections were stained with hematoxylin and eosin (HE), modified Masson’s trichrome, and Victoria blue–van Gieson. Immunostaining of the paraffin sections was done with antibodies to factor VIII (Dako), anti-α-smooth muscle actin (Dako), and anti-SM1, anti-SM2, and anti-SMemb (Yamasa). The immunoreaction was visualized with 3,3’-diaminobenzidine.

The frozen sections were examined with Alexa-594 conjugated anti-fluorescence/-Oregon Green (1:200; Molecular Probes) to confirm the presence of CFDA in the cells that represented seeded BMCs. The samples were then treated with antibodies (anti–canine-CD34 [1:50; BD Biosciences], anti–human-CD31 [1:20; Dako], anti–human-Fk-1 [1:200; Santa Cruz], and anti–CD146 [1:200; Chemicon]). The samples were then treated with biotinylated anti-mouse, -rabbit, or -goat IgG (1:400; Vector). Finally, the samples were treated with Alexa Flour 594 conjugated streptavidin (1:200; Molecular Probes).

**Reverse Transcription–Polymerase Chain Reaction**

The expression of VEGF was determined by reverse transcription–polymerase chain reaction (RT-PCR). Endothelial cells were stripped and kept in Isogen solution (Nippon Gene). After homogenization in Isogen solution, total RNA was extracted using the acidic guanidium thiocyanate-phenol-chloroform method and reverse-transcribed with a Superscript Preamplification system (BRL) using oligo(dT) primers. PCR amplification was performed using primer sets that amplify 4 splice variants of VEGF (sense, 5’-TTCTGTATCAGTCATCTTTCTGTTGA-3’; antisense, 5’-CGAAGTTGGTGACAGTCATGAGT-3’) and the dog S17 ribosomal subunit (sense, 5’-GAAGGCGGCCCGGGTTGACA-3’; antisense, 5’-GTAGGCCTGATGTCACTG-3’) as previously described. The PCR products were analyzed with an Agilent 2100 Bioanalyzer (Agilent), and S17 mRNA was measured as an internal standard in all RNA samples.

**Results**

**Proliferation of Seeded BMCs in TEVAs**

In this model, CFDA, which appears as green fluorescence, was used to trace the seeded BMCs and to distinguish between seeded BMCs and host endothelial cells, blood cells, or BMCs that might have migrated into the graft. Seeded BMCs expressing green fluorescence were observed at 2, 4, and 8 weeks after implantation, and we confirmed that these cells were seeded BMCs using a fluorescent CFDA antibody (Figure 1). The seeded BMCs adhered to the scaffold and proliferated after implantation, and were at least partly responsible for the histogenesis in the TEVAs.

**Coexistence of Seeded BMCs and Host Cells of Endothelial Lineage in TEVAs**

It is possible that endothelial stem cells present in the aspirated bone marrow and in the circulation would contribute to histogenesis in the scaffold because they have great potential for vasculogenesis and angiogenesis. We examined them for expression of Fk-1 and Tie-2 (markers of endothelial cell receptor tyrosine kinases) and CD31 and CD34 (markers of endothelial cell lineage) to establish the characteristics of the cells at 3 hours and 2 weeks after
implantation. We observed seeded BMCs with green fluorescence expressing Flk-1 (Figure 2A), Tie-2, CD31 and CD34 antigens, and CFDA-negative cells expressing Flk-1 (Figure 2B), Tie-2, CD31, and CD34 (not all data are shown), which are presumed to have originated in the bone marrow and traveled via the circulation to the scaffold, where they adhered and proliferated. In addition, we observed Flk-1–, Tie-2–, and CD31-positive cells with/without CFDA in the specimens at 4 weeks (Figure 3). These results suggest that both seeded BMCs and nonseeded circulating endothelial cells cooperate and contribute to histogenesis in TEVAs.

Growth Factor Production in the TEVAs

As shown above, the presence of cells expressing Flk-1 and Tie-2, which are VEGF and angiopoietin-1 (Ang-1) receptors, respectively, suggests that cells in the TEVAs might synthesize these growth factors. To test this hypothesis, we examined the expression of VEGF and Ang-1 in the TEVAs. We observed a detectable level of VEGF and Ang-1 in the TEVAs at 3 hours and subsequently at 2, 4, and 8 weeks after implantation. VEGF and Ang-1–positive cells were diffuse at 3 hours and 2 weeks and then the cells lined up in the TEVAs gradually. C, Representative data of RT-PCR for VEGF mRNA and s17 in a TEVA at 4 weeks after implantation. PCR products of 405, 534, and 606 bp corresponded to mRNAs encoding VEGF121, VEGF164, and VEGF188, respectively.

Endothelialization of the TEVAs and Seeded BMCs Contribution

There was no stenosis or obstruction in the TEVAs implanted into the dogs. Almost complete endothelialization of the TEVAs was obtained at 4 weeks (Figure 5A). We examined the expression of factor VIII and CD146, which are endothelial cell markers, to confirm the endothelialization of the TEVAs immunohistochemically. Factor VIII (Figure 5B) and CD146 (Figure 7A) were expressed in a monolayer of cells in the TEVAs at 4 and 8 weeks, suggesting that these cells were endothelial cells. Seeded BMCs contributed in part to the endothelialization, as they were seen as yellow cells present in the merged section (Figure 7A).
Expression of Undifferentiated and Differentiated SMCs

Immunostaining with anti-SMemb, anti-SM1, and anti-SM2 antibodies, which are isoforms of myosin heavy chain, and markers of undifferentiated and differentiated SMCs confirmed the presence of SMCs on the scaffold at 2, 4, and 8 weeks after implantation (Figure 6). Coexistence of both mature and partially differentiated SMCs in the TEVAs indicated that the cells were present at varied stages of differentiation, ranging from embryonic-like to mature SMCs expressing SM1 and SM2. Furthermore, expression of CFDA and SM2-positive cells in the specimen indicated that seeded BMCs might differentiated into SM2-positive cells in TEVAs (Figure 7B).

Long-Term Follow-Up of a TEVA

A dog that was implanted with a BMC-seeded TEVA was kept alive for 2 years. The surgical view showed a normal vessel-like appearance of the TEVA (Figure 8A). Cineangiography of the IVC of the dog was performed simultaneously. The TEVA was patent after the surgery without obstruction (Figure 8B).

Histology of the TEVAs showed a flattened cell monolayer lining the surface of the TEVAs. Staining with HE denoted endothelial cells, Masson’s trichrome indicated the production of SMCs and collagen fibers. Victoria blue–van Gieson staining revealed proliferation of elastic fibers in the TEVA. Factor VIII (FVIII) and α-smooth muscle actin (αSMA) were expressed clearly in the TEVA at 2 years after implantation, though less SMemb expression was shown (H). Black arrows indicate SMemb-positive cells (H). Magnification, ×400.
tion of SMCs (red) and collagen fibers (blue), and Victoria blue–van Gieson revealed proliferation of elastic fibers (Figures 8C, 8D, and 8E). Factor VIII and α-smooth muscle actin were expressed clearly in the TEVAs (Figures 8F and 8G). However, SMemb-positive cells that reveal undifferentiated SMC expression were apparently scarce when compared with sections at 2, 4, and 8 weeks after implantation (Figure 8H). These results indicate that a BMC-seeded TEVA would develop with time to construct a new vessel wall.

Discussion
In this study, we demonstrated the feasibility of seeding BMCs onto a biodegradable scaffold to construct TEVAs. We showed positive evidence that the seeded BMCs home in to, adhere to, proliferate in, and differentiate in the scaffold to construct components of a new vessel wall. Furthermore, we observed the presence of endothelial cells, presumably derived from the bone marrow, circulating in the bloodstream to populate the scaffold.9,10 These cells have the potential to differentiate into both SMCs and endothelial cells.17,26 Moreover, our findings suggested the initiation of a process that would lead to the generation of other components of the vessel wall from these cells within a few months in the TEVAs.

The present results suggest that “autograft implantation” using multipotent BMCs is feasible, and we can now extend its use to clinical trials. Because BMCs have the potential to differentiate into many different cell types in vivo,27 they represent a valuable tool in vessel reconstruction. In addition, this technique using BMCs does not require cell culture with serum from other species, which is associated with a risk of (unknown) infections. Compared with the technique that was reported in which cell culture was required,7 this technique using BMCs does not require cell culture with sections at 2, 4, and 8 weeks after implantation. These results indicate that a BMC-seeded TEVA would develop with time to construct a new vessel wall.

Our observation that endothelial cells derived from bone marrow express CD34, CD31, Flk-1, and Tie-2 in TEVAs (not all data are shown) suggests that these cells can differentiate into mature endothelial cells, as shown in previous studies. In the present study, we showed that seeded BMCs and/or circulating endothelial cells proliferated and differentiated in the TEVAs, resulting in the construction of new vessel walls. These cells are therefore extremely important in the formation of TEVAs, together with the biodegradable scaffold.

Macroscopic examination revealed that endothelial coverage was completed by 4 weeks in this model. Recent studies have revealed that endothelial stem cells show a great potential for endothelialization.15,17,26 These studies strongly support our observation. Our recent study compared histogenesis in TEVAs with or without seeding of BMCs onto the scaffolds. The nonseeded TEVAs showed stenosis or atresia in vivo, but the TEVAs that had been seeded with bone marrow–derived cells did not show these negative character-istics and instead generated what appeared to be normal tissue. Therefore, we believe that seeding bone marrow–derived cells onto a biodegradable scaffold is essential for tissue generation within TEVAs.

Previous reports have shown that several growth factors synthesized by endothelial cells, bone marrow–derived cells, and/or pericytes and their receptors play a critical role in vasculogenesis and angiogenesis.28–30 and that endothelial cells secrete VEGF, enhance endothelial cell migration and proliferation,19,20 and promote differentiation,26,31 whereas pericytes and SMCs secrete Ang-1.21 It seems probable that all of these cells play a crucial role in mediating reciprocal interactions between the endothelium and the surrounding matrix.28,32 Interestingly, our results showed the expression of VEGF and Ang-1 in the vicinity of the endothelial cells and their respective receptors in the TEVAs. This fact supports the notion that endothelial cells or cells near the endothelium secrete these factors and thus might have induced endothelial stem cells to migrate into the TEVAs, proliferate, and differentiate into mature cells with para- and/or autocrine signaling to construct new tissues.

Our tissue-engineering technique seems to be able to generate essentially normal vessel walls within a few months. However, there are many questions that remain as to how randomly seeded bone marrow–derived cells proliferate, differentiate, and arrange themselves in an appropriate fashion to constitute a new tissue, and which type of cells contribute to the generation of different types of newly formed tissues. Finding answers to these questions would permit us to obtain even better TEVAs.

In conclusion, our findings provide strong evidence for the validity of our protocol and justify moving forward with clinical trials of tissue engineering to perform autotissue implantation using BMCs. This therapy is an ideal strategy in cardiac surgery that could contribute to the patient’s wellbeing, inasmuch as TEVAs might reduce the instance of complications caused by incompatible materials and lead to a reduced likelihood of further surgery.

Acknowledgments
This work was supported by a Grant-in Aid for Scientific Research (C) (Japan) and an Open Research Grant from the Japan Research Promotion Society for Cardiovascular Diseases. The authors are indebted to Barbara Levene, Prof Margaret L. Kirby (Duke University Medical Center), and Prof J. Patrick Barron (Tokyo Medical University) for their review of this manuscript.

References
6. Shinoka T, Sham-Tim D, Ma PX, et al. Creation of viable pulmonary
1998;115:536–545 [Discussion pp 545–536].
7. Shin’oka T, Imai Y, Ikada Y. Transplantation of a tissue-engineered
mobilization of bone marrow-derived endothelial progenitor cells for
2001;103:2776–2779.
endothelial progenitor cells responsible for postnatal vasculogenesis in
physiological and pathological neovascularization. Circ Res. 1999;85:
221–228.
vascular prosthesis with bone marrow transplantation. Nat Med. 1996;2:
90–93.
growth factor receptors in smooth muscle cells. J Cell Physiol. 2001;188:
359–368.
coronary collateral growth is dependent on vascular endothelial growth
16. Asahara T, Chen D, Takahashi T, et al. Tie2 receptor ligands,
angiopoietin-1 and angiopoietin-2, modulate VEGF-induced postnatal
17. Bhattacharya V, McSweeney PA, Shi Q, et al. Enhanced endotheli-
alization and microvessel formation in polyester grafts seeded with
mononuclear cells into ischemic myocardium enhances collateral per-
fusion and regional function via side supply of angioblasts, angiogenic
growth factor–induced migration of vascular smooth muscle cells in vitro.
factor regulates endothelial cell survival through the phosphatidylinositol
3′-kinase/Akt signal transduction pathway; requirement for Flk-1/KDR
21. Davis S, Aldrich TH, Jones PF, et al. Isolation of angiopoietin-1, a ligand
for the TIE2 receptor, by secretion-trap expression cloning. Cell. 1996;
87:1161–1169.
23. Frangogiannis NG, Michael LH, Entman ML. Myofibroblasts in
reperfused myocardial infarcts express the embryonic form of smooth
expression of vascular smooth muscle myosin heavy chain isoforms.
chain isoform in embryonic smooth muscle and its expression during
vascular development and in arteriosclerosis. J Biol Chem. 1991;266:
3768–3773.
28. Suri C, Jones PF, Patan S, et al. Requisite role of angiopoietin-1, a ligand
for the TIE2 receptor, during embryonic angiogenesis. Cell. 1996;87:
1171–1180.
formation and vasculogenesis in Flk-1–deficient mice. Nature. 1995;376:
62–66.
31. Asahara T, Takahashi T, Masuda H, et al. VEGF contributes to postnatal
neovascularization by mobilizing bone marrow-derived endothelial pro-
First Evidence That Bone Marrow Cells Contribute to the Construction of Tissue-Engineered Vascular Autografts In Vivo
Goki Matsumura, Sachiko Miyagawa-Tomita, Toshiharu Shin’oka, Yoshito Ikada and Hiromi Kurosawa

Circulation. published online September 8, 2003;
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2003 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/early/2003/09/08/01.CIR.0000092165.32213.61

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/