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

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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;108:1729-1734.)

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.¹ 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.¹ 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,² lamb,³–⁶ and human clinical applications⁷ using biodegradable scaffolds. The key benefit from using such scaffolds is that they degrade in vivo, thereby avoiding the long-term presence of foreign materials⁸ 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 angiogenesis⁹–¹¹ and endothelialization of artificial grafts using bone marrow cells (BMCs).¹² 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-L-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; Instron) 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 Medical University 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 of 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 thicknesses 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 detected 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-Flk-1 [1:200], anti–human-Tie-2 [1:1000], anti–human-VEGF [1:200], anti–human-angiopoietin-1/4 [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 guanidine 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’-TTCTGATACAGTTCTTCCTGAGG-3’; antisense, 5’-CGAAGTGGAAGTCTAGCTGATG-3’) and the dog S17 ribosomal subunit (sense, 5’-GAAGCCGCGCCGAGCTATACA-3’; antisense, 5’-GAGGCTGAGTAGCCTTGCTG-3’) as previously described.13,14 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.9,11,15–18 We examined them for expression of Flk-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).

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**Figure 2.** Seeded BMCs and circulating bone marrow–derived cells. To detect vascular endothelial cells, sections were stained with anti–Flk-1 antibody (red). A, CFDA- and Flk-1–positive cells adhering onto poly-L-lactic acid fiber that comprises the scaffold. Yellow cells in merged image indicate seeded bone marrow–derived cells that express endothelial cell lineage markers. B, CD31-negative, Flk-1–positive cells (red) in a TEVA. Red cell in the merged image is an endothelial cell that migrated from the bloodstream into a TEVA as it does not express CFDA. Scale bar=20 μm.

**Figure 3.** Immunofluorescence labeling with endothelial cell (EC) lineage cell markers. Immunostaining of the TEVAs with Flk-1, Tie-2, and CD31 at 4 weeks after implantation. CFDA-positive cells (green) lodged in a TEVA also expressed Flk-1–, Tie-2–, and CD31-antigens (red). Original magnification: Flk-1, ×200; Tie-2 and CD31, ×100.

**Figure 4.** Expression of VEGF and angiopoietin-1 (Ang-1) in TEVAs. A and B, Expression of VEGF (red) and Ang-1 (red) in 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 VEGF_{121}, VEGF_{164}, and VEGF_{188}, respectively.

**Figure 5.** Endothelialization of the TEVAs and seeded BMCs contribution.
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 produc-
tion of SMCs (red) and collagen fibers (blue), and Victoria
blue–van Gieson revealed proliferation of elastic fibers (Fig-
ures 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,
adhore to, proliferate in, and differentiate in the scaffold to
dcstruct components of a new vessel wall. Furthermore, we
observed the presence of endothelial cells, presumably der-
ded from the bone marrow, circulating in the bloodstream to
the scaffold.9,10 These cells have the potential to differen-
tiate 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
infections. Compared with the technique that was
reported in which cell culture was required,7 this technique
should be extremely useful. As large numbers of cells can be
obtained from BMCs without culture, fewer steps in the
preparation of TEVAs would mean a lower risk of contami-
nation and less work and time. Fewer materials for culture
would mean cost benefits, and the technique can be applied
even in emergency cases in a clinical setting, as TEVAs can
be prepared in a few hours.

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 differ-
etiate 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 differen-
tiated 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 cover-
age 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 histo-
genesis 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
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 fash-
ion 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 well-
being, inasmuch as TEVAs might reduce the instance of
complications caused by incompatible materials and lead to
a reduced likelihood of further surgery.

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