Engineered Living Blood Vessels: Functional Endothelia Generated From Human Umbilical Cord-Derived Progenitors

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Background. **Tissue-engineered living blood vessels (TEBV) with growth capacity represent a promising new option for the repair of congenital malformations. We investigate the functionality of TEBV with endothelia generated from human umbilical cord blood–derived endothelial progenitor cells.**

Methods. **Tissue-engineered living blood vessels were generated from human umbilical cord–derived myofibroblasts seeded on biodegradable vascular scaffolds, followed by endothelialization with differentiated cord blood–derived endothelial progenitor cells. During in vitro maturation the TEBV were exposed to physiologic conditioning in a flow bioreactor. For functional assessment, a subgroup of TEBV was stimulated with tumor necrosis factor-α. Control vessels endothelialized with standard vascular endothelial cells were treated in parallel. Analysis of the TEBV included histology, immunohistochemistry, biochemistry (extracellular matrix analysis, DNA), and biomechanical testing. Endothelia were analyzed by flow cytometry and immunohistochemistry (CD31, von Willebrand factor, thrombomodulin, tissue factor, endothelial nitric oxide synthase).**

Results. **Histologically, a three-layered tissue organization of the TEBV analogous to native vessels was observed, and biochemistry revealed the major matrix constituents (collagen, proteoglycans) of blood vessels. Biomechanical properties (Young's modulus, 2.03 ± 0.65 MPa) showed profiles resembling those of native tissue. Endothelial progenitor cells expressed typical endothelial cell markers CD31, von Willebrand factor, and endothelial nitric oxide synthase comparable to standard vascular endothelial cells. Stimulation with tumor necrosis factor-α resulted in physiologic upregulation of tissue factor and downregulation of thrombomodulin expression.**

Conclusions. **These results indicate that TEBV with tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord progenitor cells. Thus, blood-derived progenitor cells obtained before or at birth may enable the clinical realization of tissue engineering constructs for pediatric applications.**

(Ann Thorac Surg 2006;82:1465–71)

Currently available replacement materials for the repair of congenital malformations lack the potential of growth and remodeling and consequently require reoperations with increased morbidity and mortality during the pediatric patients' lifetime. Furthermore, thrombogenicity and graft occlusion still represent a major limitation of currently used cardiovascular grafts [1]. As these are either synthetic or bioprosthetic [2], patients have to deal with severe side effects, such as lifelong anticoagulation therapy and increased risks for infections and thromboembolism [3]. Autologous living replacement materials with functional bioactive endothelium closely mimicking its native counterpart could avoid these severe complications. A promising strategy to generate such viable autologous cardiovascular replacements with growth and regeneration potential in vitro is by tissue engineering. In vitro as well as in vivo studies on tissue-engineered cardiovascular constructs have shown promising results [4–6]. Recently, bone marrow–derived progenitor cells have been successfully applied in cardiovascular tissue engineering [7, 8], and the first clinical experiences have been reported [9]. Furthermore, peripheral blood–derived ovine endothelial progenitor cells have been successfully used for the coating of vascular expanded polytetrafluoroethylene grafts (ePTFE) [10], and the first clinical trials have been carried out using antibody-coated coronary stents for endothelial progenitor cell capture in vivo [11].

For congenital applications, the autologous tissue replacement should ideally be available at or shortly after

Accepted for publication May 11, 2006.

Presented at the Forty-second Annual Meeting of The Society of Thoracic Surgeons, Chicago, IL, Jan 30–Feb 1, 2006.

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Published by Elsevier Inc

0003-4975/06/$32.00
doi:10.1016/j.athoracsur.2006.05.066

birth to prevent secondary damage to the immature heart. Thus, cells would have to be harvested during pregnancy. Here, bone marrow–derived cells may not be the optimal choice as prenatal harvest is difficult and bears high risks. The umbilical cord appears to be a more promising cell source as prenatal cell harvesting could be realized by cordocentesis without harming intact fetal structures. In previous studies, we have demonstrated the feasibility of using human umbilical cords as a cell source for the engineering of pediatric tissues [12, 13]. Furthermore, we reported that human umbilical cord blood–derived endothelial progenitor cells formed endothelia-like structures on the surfaces of biodegradable vascular grafts [14].

Here, we investigate the structural and functional characteristics of complete living tissue–engineered blood vessels (TEBV) fabricated from Wharton's jelly with endothelia generated from human umbilical cord blood–derived endothelial progenitor cells (EPC).

Material and Methods

Cell Harvest and Isolation

Cells were harvested after delivery from fresh human umbilical cord tissue and blood of healthy individuals after informed consent was obtained from the participants (approved by the Ethics Committee STV1–2005).

HUMAN UMBILICAL CORD–DERIVED MYOFIBROBLASTS. Pieces (approximately 8 mm^3) of umbilical cord tissue were obtained from Wharton's jelly by excision biopsy and placed in culture dishes as described before [12]. Outgrowing cells were expanded up to the fifth passage.

HUMAN UMBILICAL CORD BLOOD–DERIVED ENDOTHELIAL PROGENITOR CELLS. Endothelial progenitor cells were isolated using density gradient centrifugation (Histopaque-1077, Sigma Chemical Company, St. Louis, MO) from the mononuclear fraction of 20 mL of fresh blood that was obtained by puncturing the human umbilical cord vein directly after delivery. Endothelial progenitor cells were cultured and differentiated in endothelial basal medium (EBM-2, Cambrex, Walkersville, MD), containing growth factors and supplements (vascular endothelial growth factor, human fibroblasts growth factor, human recombinant long-insulin-like growth factor-1, human epidermal growth factor, gentamicin and amphotericin, hydrocortisone, heparin, ascorbic acid, and 2% fetal bovine serum) [14].

STANDARD VASCULAR ENDOTHELIAL CELLS. Human umbilical cord vein–derived endothelial cells (HUVEC) were isolated using a collagenase installation technique. The umbilical cord veins were incubated in 0.2% collagenase (Collagenase A, Roche Diagnostics GmbH, Mannheim, Germany) dissolved in serum-free medium. After 20 minutes, the cell suspension was centrifuged. Cells were cultured and expanded in EBM-2 medium containing the above mentioned growth factors and supplements.

Fabrication of Tissue-Engineered Blood Vessels

Biodegradable tubular scaffolds (n = 8; inner diameter 0.5 and length 3 cm) were produced by heat application welding technique from a nonwoven polyglycolic-acid mesh (thickness, 1.0 mm; specific gravity, 69 mg/cm^3; Albany Int, Albany, NY) and dip-coated with poly-4-hydroxybutyric acid (1% wt/vol P4HB, TEPHA Inc, Cambridge, MA). After sterilization with ethanol, human umbilical cord-derived myofibroblasts (3.5×10^6 cells/cm^2) were seeded on the inner surfaces of the vascular tubes using fibrin as a cell carrier [15]. After 14 days of culturing under static conditions in EBM-2 medium containing the above mentioned growth factors, vessels (n = 4) were endothelialized with differentiated cord blood–derived EPC (1.5×10^6 cells/cm^2). Control vessels (n = 4) were endothelialized with HUVEC. After endothelialization, all TEBV were kept at humidified incubator conditions (37°C, 5% CO_2) for an additional 24 hours and implanted into a pulse duplicator system [16]. Briefly, the pulse duplicator system consisted of a bioreactor connected by means of a silicon tubing with a medium reservoir. The pulsatile flow of the nutrient medium (EBM-2 medium containing the above mentioned growth factors) was generated by periodic expansion of a highly elastic membrane, deflated and inflated using an air pump. Tissue-engineered vessels were connected in parallel to the medium reservoir–bioreactor circulation. The pulsed flow of nutrient medium was directed through their inner lumen, exposing them to flow and shear stress (125 mL/min at 30 mm Hg) for 7 subsequent days.

Exposure to Inflammatory Stimuli

For functional plasticity assessment of endothelial cells in response to inflammatory stimuli, representative samples of TEBV were exposed to tumor necrosis factor-α (Sigma). Endothelial progenitor cell–derived endothelia were exposed to tumor necrosis factor-α (10 ng/mL medium) for 20 hours. Controls endothelialized with standard vascular endothelial cells as well as monolayers of both cell types seeded into chamber slides (Falcon) were treated in parallel.

Analysis of Tissue-Engineered Blood Vessels

HISTOLOGY AND IMMUNOHISTOCHEMISTRY. After fixation in 4% phosphate-buffered formalin (pH 7.0) and paraffin-embedding, 5- to 7-μm sections of TEBV were examined histologically by hematoxylin and eosin and Masson's trichrome staining. Cell phenotypes of the neotissues were validated by immunohistochemistry using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, AZ) with Ventana reagents for the entire procedure. Primary antibodies against the following antigens were applied: vimentin (clone 3B4), desmin (clone D33), CD31 (clone JC/70A), von Willebrand factor (vWF; affinity-purified rabbit antibodies; all from DakoCytomation, Glostrup, Denmark), α-smooth muscle actin (clone 1A4; Sigma), and endothelial nitric

oxide synthase type III (affinity-purified rabbit antibodies; BD Transduction Laboratories, San Diego, CA). Primary antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. For endothelial nitric oxide synthase, the signal was enhanced with the amplification kit.

QUANTITATIVE EVALUATION OF EXTRACELLULAR MATRIX ELEMENTS. As an indicator for collagen, the content of hydroxyproline was determined from dried samples. [17]. Sulfated glycosaminoglycans were detected colorimetrically using papain-digested samples and 1,9-dimethyl-methylene blue [18]. Human saphena vein tissue served as native vascular control.

DETERMINATION OF CELL NUMBER. Cell numbers were determined from the papain digests after 50× dilution in a Tris buffer (10 mmol/L Tris, 100 mmol/L NaCl, 1 mmol/L EDTA, pH 7.4) and labeling of the DNA using Hoechst dye (Bisbenzimide H33258, Fluka, Buchs, Switzerland) [19]. After 10 minutes of incubation in a dark environment, the fluorescence was measured by fluorometry (Fluostar, BMG, Offenburg, Germany, 355 nm excitation/460 nm emission). The amount of DNA was calculated from a standard curve using calf thymus DNA (Sigma) and compared with the amount of DNA in native vascular tissue.

SCANNING ELECTRON MICROSCOPY. Representative samples of TEBV of all groups were fixed in 2% glutaralaldehyde for 24 hours. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP microscope (Zeiss, Jena, Germany).

ASSESSMENT OF PHYSIOLOGIC PROPERTIES OF ENDOTHELIA. Monolayers of stimulated and unstimulated differentiated EPC were trypsinized and analyzed for the expression of tissue factor (TF) and thrombomodulin (TM) by flow cytometry (FACS). All samples were stored at 4°C following trypsinisation. Flow cytometry analysis was performed using antibodies against TF (Clone1, Acris, Hiddenhausen, Germany), TM (Clone 1009, DakoCytomation, Glostrup, Denmark), CD31, and vWF. Primary antibodies were detected with fluorescein isothiocyanate–conjugated goat anti-mouse antibodies (Boehringer Mannheim, Indianapolis, IN). Analysis was performed on a Becton Dickinson FACScan (Sunnyvale, CA). Irrelevant isotype-matched antibodies (IgG1MOPC-21, Sigma Chemical) served as negative controls, and stimulated as well as unstimulated HUVEC served as positive controls. Fixation and permeabilization for detection of vWF was performed using 70% ethanol. Geometric mean fluorescence intensity ratios (MFIR) were calculated as $MFI_{\text{antibody of interest}}/MFI_{\text{corresponding control antibody}}$.

EVALUATION OF MECHANICAL PROPERTIES. Mechanical properties of TEBV tissues were analyzed using a uniaxial tensile tester (Instron 4411, Boston, MA) equipped with a 10-N load cell. The crosshead speed was set to correspond to an initial strain rate of 1/min. The recorded tensile force and displacement were transformed into stress–strain curves.

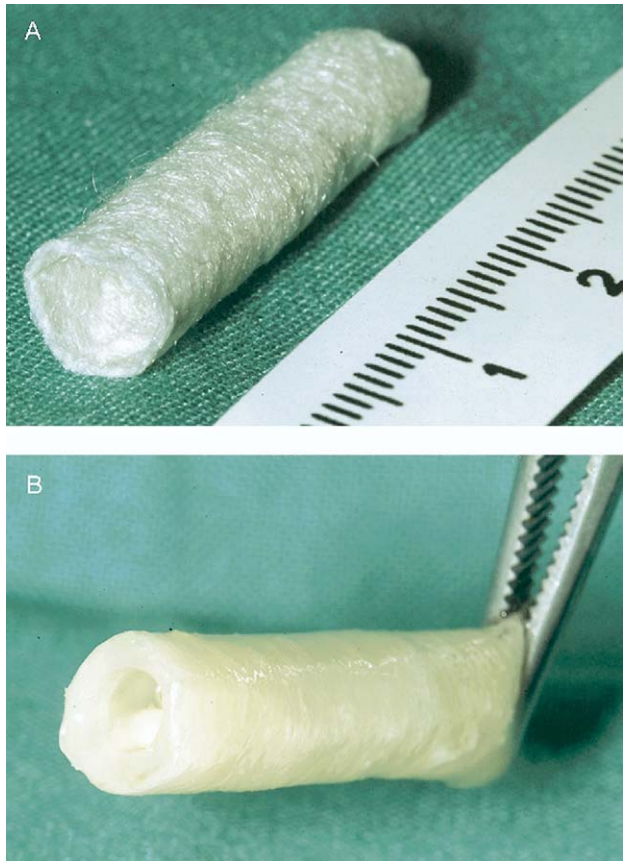

Fig 1. Macroscopic appearance of tissue-engineered blood vessels. Vascular scaffolds of 0.5 cm diameter and 3 cm length fabricated from nonwoven polyglycolic acid mesh and poly-4-hydroxybutyric acid before seeding (A) and after in vitro culturing (B). Tissue-engineered blood vessels were intact, pliable, and densely covered with cells (B). The lumen was open, and the wall thickness was homogeneous at about 0.3 cm.

Results

Analysis of Tissue-Engineered Blood Vessels

MACROSTRUCTURE. Figure 1A shows the vascular scaffold of about 3 cm length and 0.5 cm diameter before seeding. After in vitro culturing, the surfaces were covered densely with cells, and the lumen was open (Fig 1B). A homogeneous wall thickness of 0.3 cm was observed. Tissue-engineered blood vessels were macroscopically intact and pliable. Furthermore, they were amenable for suturing manipulation.

TISSUE MICROSTRUCTURE. Histology revealed a cellular tissue in the hematoxylin and eosin staining (Fig 2A) with the beginnings of collagen synthesis as demonstrated by Masson's trichrome staining (data not shown). Morphologically, a three-layered tissue architecture with an outer layer consisting of loosely arranged cells was observed. Cells in the middle layer were organized more compactly, and those in the inner monolayer were longitudinally oriented (Fig 2A). Immunohistochemistry revealed expression of α-smooth muscle actin in the middle layer (Fig 2B) and of CD31 in the inner layer (Fig 2C).

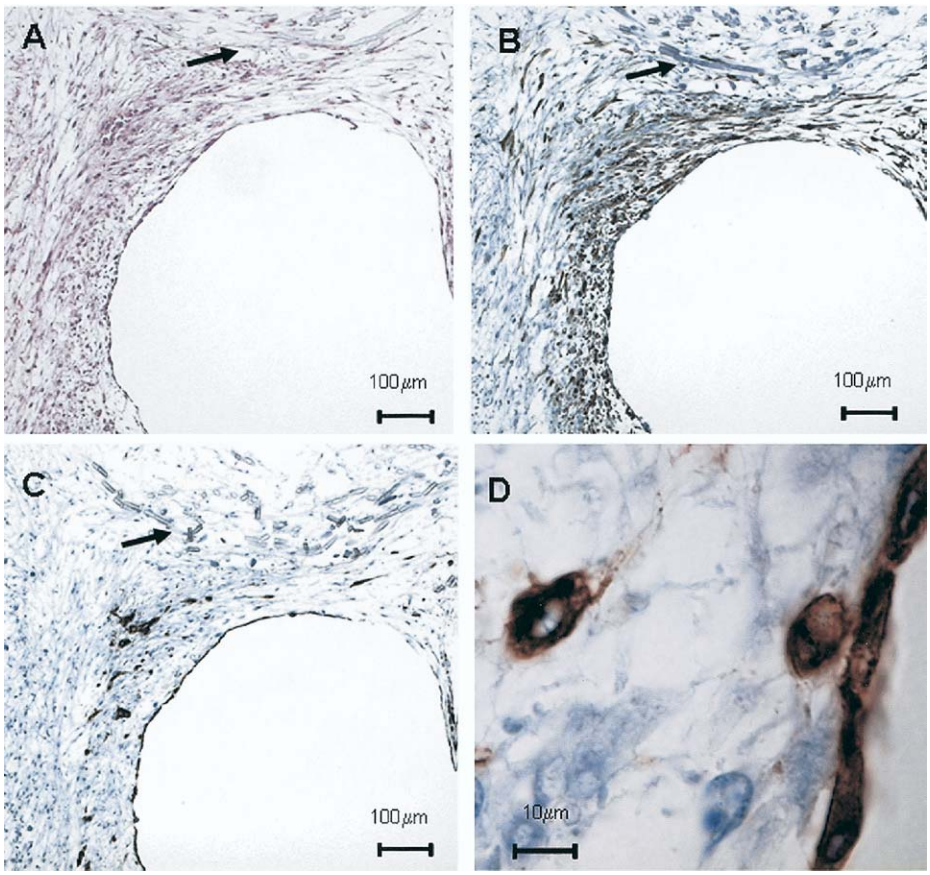

Fig 2. Histology and immunohistochemistry of tissue-engineered blood vessels. (A) Hematoxylin and eosin staining revealed a cellular tissue with an outer layer consisting of loosely arranged cells and a middle layer organized more compactly. The innermost layer consisted of endothelial cells. (B) Cells in the middle layer expressed α-smooth muscle actin. (C) A CD31-positive cell layer lined the lumen of the tissue-engineered blood vessels. Additionally, some cells expressing CD31 could be found in the middle layer. (D) Higher magnification of CD31-positive cells distant from the luminal cell lining. Arrows point at remnants of scaffold.

Additionally, in the middle layer some cells expressing CD31 were found (Fig 2C). The distance of these cells from the inner endothelial layer is visualized by higher magnification (Fig 2D). Vimentin could be seen throughout the whole tissue, whereas desmin could not be detected (data not shown). No difference in tissue formation or phenotype was found between TEBV endothelialized with differentiated EPC and HUVEC (data not shown).

SURFACE MORPHOLOGY. Scanning electron microscopy demonstrated smooth endothelialized inner surfaces for all TEBV. Both EPC-derived (Fig 3A) and HUVEC-derived neoendothelia (Fig 3B) were intact.

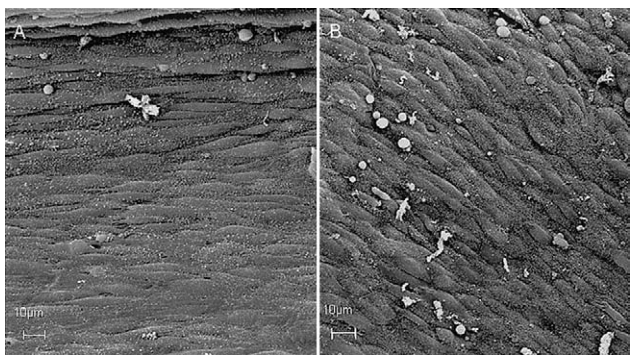

Fig 3. Morphology of the inner surfaces. Scanning electron micrographs show smooth inner surfaces of the tissue-engineered blood vessels densely covered with endothelial cells derived from endothelial progenitor cells (A) and human umbilical vein endothelial cells (B).

EXTRACELLULAR MATRIX ANALYSIS. Biochemistry revealed the major extracellular matrix constituents (Fig 4). Up to 62% of native tissue glycoaminoglycans (7.4 μg/mg) was present in the TEBV (4.55 ± 1.67 μg/mg). The cell number expressed as total DNA amount in the TEBV (3.87 ± 2.30 μg/mg) was 90% that of native tissue (4.30 μg/mg). However, the average amount of hydroxyproline was only 0.5 ± 0.32 μg/mg, corresponding to 1% of the amount found in native tissue (50 μg/mg).

ASSESSMENT OF PHYSIOLOGIC PROPERTIES OF ENDOTHELIA. Figure 5 shows the antigene expression pattern of differentiated EPC compared with HUVEC determined by flow cytometry. Differentiated EPC (grey curves) expressed the typical endothelial cell markers CD31, TM, and vWF (data not shown for the latter). Furthermore, they lacked the expression of TF. Human umbilical vein endothelial cells demonstrated a similar expression pattern. Stimulation of differentiated EPC with tumor necrosis factor-α (black curves) resulted in a tendency of upregulation of TF (MFIR-TF unstimulated, 0.50 ± 0.36 versus MFIR-TF stimulated, 1.56 ± 0.32) and downregulation of TM (MFIR-TM unstimulated, 1.57 ± 1.10 versus MFIR-TM stimulated, 0.96 ± 0.09). The same tendency could be observed with HUVEC (MFIR-TF unstimulated, 0.73 ± 0.20 versus MFIR-TF stimulated, 1.39 ± 0.77) and down-

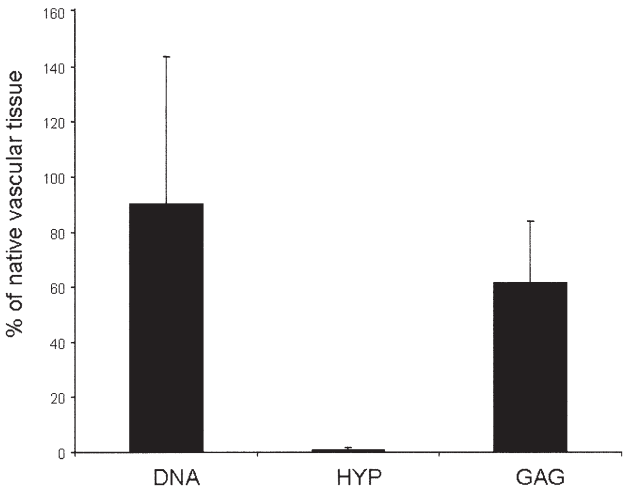

Fig 4. Quantification of extracellular matrix elements and cell number. Total cell number (represented as DNA) in tissue-engineered blood vessels was 90% of native tissue values; glycosaminoglycans (GAG) and collagen (hydroxyproline, HYP) were 62% and 1%, respectively.

regulation of TM (MFIR-TM unstimulated, 1.20 ± 0.56 versus MFIR-TM stimulated, 1.02 ± 0.75). These findings were consistent in three independent experiments. Furthermore, immunohistochemistry demonstrated constant expression of endothelial nitric oxide synthase and vWF in the unstimulated and stimulated TEBV (data not shown).

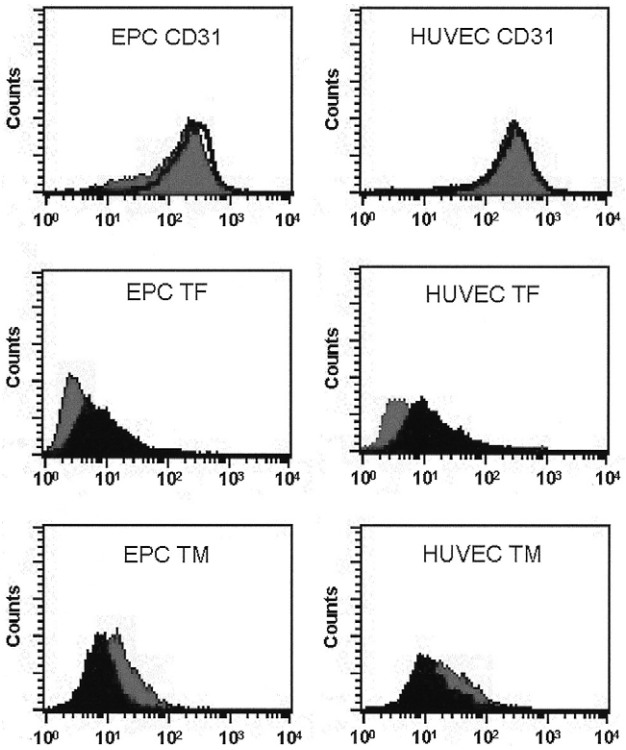

Fig 5. Functional plasticity and bioactivity of endothelial cells. Both endothelial progenitor cell–derived endothelial cells (EPC) and human umbilical vein endothelial cells (HUVEC) expressed CD31 and thrombomodulin (TM) but lacked the expression of tissue factor (TF) (grey). After stimulation with tumor necrosis factor-α (black) the expression of thrombomodulin was decreased whereas the expression of tissue factor was upregulated. CD31 expression was not affected.

Table 1. Mechanical Properties of Tissue-Engineered Blood Vessels

Cell Type	Young's Modulus (MPa)	Tensile Strength (MPa)	Strain at Maximal Stress (%)
TEBV endothelialized with EPC	1.99 ± 0.43	0.22 ± 0.06	0.18 ± 0.03
TEBV endothelialized with HUVEC	2.08 ± 0.93	0.16 ± 0.06	0.17 ± 0.10

EPC = endothelial progenitor cells; HUVEC = human umbilical vein endothelial cells; TEBV = tissue-engineered blood vessels.

BIOMECHANICAL PROPERTIES. Stress–strain curves of TEBV showed profiles that were comparable to those of native tissues. Table 1 summarizes the mechanical properties, which were similar for TEBV endothelialized with EPC-derived endothelial cells and HUVEC. For both groups of TEBV, the Young's modulus was about 2.0 MPa, the tensile strength was 0.19 MPa, and the strain at maximal stress was 0.18%.

Comment

Successful tissue engineering of entire blood vessels from umbilical cord–derived cells could be demonstrated. The generated vessels showed a three-layered tissue architecture similar to the structure of native blood vessels, with an endothelium-lined intima, a media containing smooth muscle cells, and an adventitia with connective tissue. The outer layer of the TEBV showed characteristics of connective tissue with the beginning stages of collagen and glycosaminoglycan production. Cells in the middle layer expressed α-smooth muscle actin, a marker of smooth muscle cells, indicating the development of a media. This stratified architecture with specific cell phenotypes may be related to the transdifferentiation of Wharton's jelly–derived cells as the presence of mesenchymal progenitor cells with multilineage potential in the Wharton's jelly of human umbilical cords is reported [20, 21]. The applied flow and shear stress or growth factors in the medium may enhance this effect. The lack of desmin indicates that the transdifferentiation into smooth muscle cells and the tissue maturation has not been completed yet.

The inner layer was formed by functional EPC-derived endothelial cells, which expressed typical markers of endothelial cells such as vWF, CD31, endothelial nitric oxide synthase, and TM. Furthermore, the absence of TF and the presence of TM indicate that the neoendothelia may have nonthrombogenic characteristics. Biologically active TF plays an important role in the pathogenesis of thrombosis [22]. Thrombomodulin has been demonstrated to be an important modulator of endothelial thromboresistance [23]. The physiologic response to tu-

mor necrosis factor-α [23] demonstrates the functional plasticity of the endothelia. This is analogous to native endothelia, whose phenotypic characteristics "vary in space and in time" as well as in response to inflammatory stimuli [24].

The presence of endothelial cells in the middle layer (Fig 2C) may be explained by the beginning of vascular endothelial growth factor–guided migration [25, 26] of the endothelial cells from the inner lumen surface to the outer layers to form a network providing a basis for the vasa vasorum.

Beginning collagen synthesis was detected in TEBV, even though it was still low at 1% of the native tissue value. This may indicate beginning tissue maturation, which will continue in vivo [5], but might restrict the TEBV to low-pressure applications. Alternatively, collagen content could be increased before implantation by exposing the neotissues to higher mechanical stimulation in a biomimetic system [27] or by diminishing the static culture time for the benefit of the bioreactor phase as it has been reported that the collagen content increases with the time of applied pulsatile stress [5, 6, 16]. However, the mechanical characteristics of the TEBV suggest that the neotissues would function also in vivo. Corresponding in vivo tests in an animal model will be performed.

In summary, these results indicate that TEBV with a three-layered tissue architecture and functional endothelia similar to native blood vessels can be successfully generated from human umbilical cord progenitor cells. Thus, umbilical cord–derived progenitor cells obtainable before or at birth combined with the possibility to manufacture scaffolds of any diameter will allow the fabrication of living autologous TEBV tailored to the anatomy of the patient. This approach may enable the realization of the tissue engineering concept for pediatric applications and will be investigated in future in vivo studies.

This work was financially supported partly by the Swiss National Research Foundation. The authors thank Olivier Gilléron (Symetis AG, Zurich, Switzerland) for supplying the scaffold material, Astrid Morger (Department of Surgical Research, USZ) for her support in the histologic examination, the Laboratory for Special Techniques (Institute for Clinical Pathology, USZ) for the immunohistochemical examination, and Klaus Marquardt (EMZ, University of Zurich) for to the scanning electron microscope investigations.

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DISCUSSION

DR CHRISTOPHER A. CALDARONE (Toronto, Ontario, Canada): This is a very, very interesting topic, and I just had some questions that may seem very basic.

In terms of the harvest of the umbilical cord cells, is tissue required or is it just a blood draw? In other words, would this be compatible with a prenatal patient where you obviously can't injure the umbilical cord?

DR SCHMIDT: Currently, both blood and tissue is required. Umbilical cord blood is already used in a standard procedure for genetic analysis. In addition to the blood, we think it would be possible to obtain small pieces of the umbilical cord tissue by biopsy from the Wharton's jelly using advanced fetoscopic technology.

DR CALDARONE: And this is a routine procedure?

DR SCHMIDT: A routine procedure is only the harvest of the blood. But there are promosing animal studies, papers that show you can obtain fetal tissue without high risk for the patient.

DR CALDARONE: It's fascinating.

The other thing I wanted to ask is, was there any contractile function to this engineered blood vessel?

DR SCHMIDT: We haven't tested that. We only investigated the expression of SMA (smooth muscle actin) indicating contractile elements of the cells.

DR SUNJAY KAUSHAL (Baltimore, MD): You showed very elegantly the histology, showing that they're smooth muscle cell–positive as well as the endothelial markers. Did they have, the bioengineered vessels, do they have any extracellular matrix? In particular, did they have elastin formation in those blood vessels that you engineered?

DR SCHMIDT: There are several papers demonstrating that elastin is not produced under in vitro culture conditions only. But when tissue-engineered constructs were implanted in vivo, elastin was produced. So we haven't investigated the amount of elastin because of our experience in previous studies.

DR KAUSHAL: My second question relates to how you optimized the in vitro culture system. I would presume that you would have a lot of variability depending on the time of incubation.

DR SCHMIDT: We have several experiences from previous and recent studies. There we observed that after 21 days tissue formation in vitro is sufficient. For further tissue maturation the construct can be implanted in vivo, where the tissue maturation will continue.

DR FRANK A. PIGULA (Boston, MA): So with this construct, you have not examined its fate after implantation?

DR SCHMIDT: No, we haven't yet.