Conclusions

Patients' lifetime. Furthermore, today's heart valve prostheses frequently necessitating several re-operations over the patient's lifetime.1 Furthermore, today's heart valve prostheses and lack the capacity of growth. Replacements with growth potential ready for use at birth. Combined with the use of cell banking technology, this approach may be applied also for postnatal applications. (Circulation. 2006;114[suppl I]:I-125–I-131.)

Key Words: chorionic villi endothelial progenitor cells heart defects (congenital) heart valves prenatal progenitor cells tissue engineering

Many congenital cardiac heart valve defects require surgical repair. Although today’s surgical strategies have substantially improved morbidity and mortality of children with cardiovascular malformations, there is a principle lack of autologous living replacement materials. Currently used heart valve replacements comprise both synthetic or bioprosthetic prostheses and lack the capacity of growth, frequently necessitating several re-operations over the patients' lifetime.1 Furthermore, today’s heart valve prostheses are associated with adverse side effects such as life-long anticoagulation therapy and increased risks for infections and thromboembolism.1,2 These disadvantages may be potentially avoided by valve substitutes that more closely mimic their native counterparts as to adequate mechanical function, durability, growth potential, as well as the absence of immunogenic, thrombogenic, and/or inflammatory reactions.3

Tissue engineering is a promising strategy to meet these requirements by in vitro fabrication of autologous living heart valve replacements, as has been demonstrated in previous animal studies.4–6 Transfer of the methodology from animal to human systems revealed promising results using human cell sources such as bone marrow or umbilical cord tissues.7–9 However, an ideal cell source enabling the fabrication of heart valve replacements ready for use in the early neonatal period has not been identified yet. Early surgical corrections of congenital heart malformations are critically important to prevent secondary damage to the infant’s immature heart. Therefore, such a cell source would allow cell harvest in the

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pre-natal period after the cardiac defect is detected (eg, by ultrasound) to have the implant ready for use at or soon after birth. Furthermore, the ideal cell source should be accessible easily and allow cell harvest without substantial risks for both the mother and the child and without sacrifice of intact infantile donor tissue.

A promising cell source is the human placenta enabling prenatal access to infant tissue. Particularly, its chorionic villi provide extra-embryonically situated fetal mesenchymal cells, including progenitor cells, that are routinely obtained for prenatal genetic diagnostics by biopsy. The obtained tissue samples could also serve as a cell source for tissue engineering. Theoretically, one specimen could then be used for both diagnostics and the tissue engineering application.

In preliminary studies, we have demonstrated the feasibility of using umbilical cord blood-derived endothelial progenitor cells as a prenatally accessible cell source for the endothelialization of tissue engineered patches because prenatal ultrasound-guided percutaneous umbilical cord blood sampling is already a routine and well-established procedure. Here, we present a new concept using human prenatal progenitor cells derived from chorionic villi and umbilical cord blood as exclusive cell source for the fabrication of living autologous heart valve leaflets as a further step toward the clinical realization of the pediatric tissue engineering approach.

**Methods**

**Cell Harvest and Isolation**

After informed consent was obtained from the participants, ~5 mg of tissue were obtained from accessory chorionic villus sampling of male infants that was harvested routinely for diagnostic purposes. Chorionic tissues were sampled using a transabdominal procedure between 11 and 13 weeks of gestation. Gestational age was confirmed according to the embryonic length assessment. After cleaning the skin with standard surgical preparation solution, an 18-gauge needle, 6 inches long, was inserted carefully into the mid chorionic bed under continuous sonographic guidance. Through the 18-gauge needle placed in the chorionic bed, a second (20-gauge) needle was carefully inserted for aspiration of chorionic villi; 10 to 30 mg of chorionic tissue material were then aspirated and put into medium for further processing.

After routine genetic diagnostic assessment was performed, cells were isolated from the remaining chorionic villi of male infants by digestion. Briefly, chorionic villi were washed with serum-free medium and transferred to a centrifugation tube. Tissues were completely covered with collagenase (Collagenase A; Roche Diagnostics GmbH, Mannheim, Germany) solved in serum-free medium and transferred to a centrifugation tube. Tissues were incubated at 37°C. After 60 minutes, cells were centrifuged and the supernatant discarded. Cells were suspended in trypsin/EDTA (PAN Biotech GmbH, Aidenbach, Germany) and incubated for 10 minutes at 37°C. Afterward, cells were centrifuged again. After discarding the supernatant, cells were resuspended, cultured in chamber slides. After confluence, cells were washed with nuclease-free medium and transferred to a centrifugation tube. Tissues were cultured in chamber slides. After confluence, cells were washed with phosphate-buffered saline and fixed with 70% ethanol. Immunohistochemistry was performed using the Ventana Benchmark automated staining system (Ventana Medical Systems, Tucson, Ariz). Primary antibodies against the following antigens were applied: vimentin (clone 3B4), desmin (clone D33), CD31 (clone IC/70A), Ki-67 antigen (clone MB-1), von Willebrand factor (affinity purified rabbit antibodies; all from DakoCytonation, Glostrup, Denmark); α-smooth muscle actin (α-SMA, clone 1A4; Sigma Chemical Company, St. Louis, Mo) and endothelial nitric oxide synthase type III (eNOS) (affinity purified rabbit antibodies; BD Transduction Laboratories, San Diego, Calif). Primary antibodies were detected with the Ventana iVIEW DAB detection kit, yielding a brown reaction product. For eNOS, the signal was enhanced with the amplification kit. Slides were counterstained with hematoxylin and glass-coverslipped.

**Flowcytometry (FACS)**

For quantification of antigen expression FACS analysis was performed using antibodies against vimentin (clone 3B4), desmin (clone D33), α-smooth muscle actin (α-SMA, clone 1A4; all from DakoCytonation). Cells were fixed and permeabilized using an inside staining kit (Miltenyi, Biotech, Bergisch Gladbach, Germany) following the manufacturer’s instructions. Primary antibodies were detected with fluorescein isothiocyanate-conjugated goat anti-mouse antibodies (Boehringer Mannheim, Indianapolis, Ind). Analysis was performed on a Becton Dickinson FACScan (Sunnyvale, Calif). Irrelevant isotype-matched antibodies (IgG1MOPC-21; IgG2a, cloneUPC-10; both Sigma Chemical Company) were used as negative controls. Human vascular-derived fibroblasts served as positive controls.

**Genotyping of Cells**

DNA was extracted from trypsinized cell cultures using the In StaGene Matrix (BioRad, Hercules, Calif) according to the manufacturer’s instructions. Fetal origin of the cells was determined by demonstration of male sex by quantitative fluorescent polymerase chain reaction and using the following microsatellite markers (STR markers): AMXY, P39, DXS981, DYS448, DXS6854, X22, XH-PRT, DXS996, and DXS1238E.

**Processing of Tissue Engineered Heart Valve Leaflets**

Scaffolds for heart valve leaflets (n=12) were produced from a rapidly biodegradable non-woven polyglycolic-acid mesh (thickness: 1.0 mm, specific gravity: 69 mg/cm3; Albany Int, Albany, NY) dip-coated with poly-4-hydroxybutyric acid (1% wt/vol P4HB; TEPHA Inc, Cambridge, Mass).

Three scaffold leaflets each were assembled to heart valves using a ring-shaped device (diameter 20 mm). After sterilization in 70% ethanol, chorionic villi-derived cells were seeded onto the scaffolds (3.5×106 cells/cm2) using fibrin as a cell carrier. Constructs were positioned in a strain-perfusion bioreactor and perfused (4 mL/min) with endothelial basal medium containing the above mentioned growth factors and supplements. After 7 days, 2 groups were formed with n=6 leaflets: one group was exposed to mechanical stimulation by cyclic straining (15 mm Hg) in addition to perfusion and the other to perfusion only. After an additional 14 days, leaflets were endothelialized with differentiated EPCs (1.5×106 cells/cm2) on both leaflet sides and cultivated for an additional 7 days under exposure of the same mechanical conditions. Thereafter, heart valves were explanted from the bioreactor and neo-tissues were analyzed.

**Analysis of Tissue Engineered Heart Valve Leaflets**

**Histology and Immunohistochemistry**

Representative samples of all leaflets were fixed in 4% phosphate-buffered formalin (pH 7.0) and paraffin-embedded. Sections of 5 to...
7 μm were examined by hematoxylin and eosin, Trichrom-Masson, and Movat pentachrome staining. Morphology and tissue organization of engineered heart valve leaflets were compared with native neonatal pulmonary heart valve leaflets. Cell phenotypes were validated by immunohistochemistry performed as described. For paraffin sections, antigen retrieval was performed for all antibodies by heating with cell conditioner 1 (Ventana), except for CD31, in which predigestion with protease 1 (Ventana) for 4 minutes was required.

Quantitative Evaluation of Extracellular Matrix Elements and Cell Number
As an indicator for collagen, hydroxyproline content was determined of dried tissue samples. Sulfated glycosaminoglycans (GAG) were detected colorimetrically using papain-digested samples and 1,9-dimethyl-methylene blue. Cell numbers were determined from the same papain digests after 50× dilution in Tris [hydroxymethyl]aminomethane-sodium chloride-ethylene diamine tetracetic acid (TNE): 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). Native heart valve tissues served as controls.

Evaluation of Mechanical Properties
Mechanical properties of leaflet tissues (15×5×1 mm) were analyzed by using an uniaxial tensile tester (Instron 4411), equipped with a 10-N load cell. The crosshead speed was set to correspond to an initial strain rate of 1 minute⁻¹. The recorded tensile force and displacement were transformed into stress-strain curves.

Scanning Electron Microscopy
Representative samples of both tissue-engineered and native leaflets were fixed in 2% glutaraldehyde for 24 hours. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP Microscope.

Statistical Analysis
The data were analyzed using SYSTAT version 10 (SPSS, Chicago, Ill). Analysis of variance was performed to estimate the means and the significance of the categorical factors (straining and perfusion only).

The authors had full access to the data and take full responsibility for their integrity. All authors have read and agree to the manuscript as written.

Results
Morphology and Phenotype of Cells
Chorionic villi-derived cells were attached to the culture dishes 24 hours after isolation (Figure 1A) and started to proliferate (Figure 1B). Cells showed fibroblast–myofibroblast-like morphology. Immunohistochemistry (Figure 2) and FACS analysis (data not shown) revealed expression of vimentin (Figure 2A) and in subpopulations α-SMA (Figure 2B) but absence of desmin (Figure 2C). This staining pattern reflects a fibroblast–myofibroblast-like phenotype (VA type) comparable to interstitial cells detected in native
heart valve leaflets. No changes in phenotype were detected after cryopreservation (Figure 2D-F).

After differentiation, EPC showed typical cobblestone morphology and expressed CD31, von Willebrand factor, and eNOS (data not shown).

**Genotype of Cells**

Sex chromosome specific quantitative fluorescent polymerase chain reaction revealed only one allele for the x-chromosome specific STR markers. Additionally, the y-chromosome–specific markers were positive. This male genotyping result confirmed the fetal origin of the cells (Figure 3).

**Analysis of Tissue-Engineered Heart Valve Leaflets**

**Macroscopic Appearance**

A macroscopic picture of the leaflets assembled as a trileaflet heart valve after 28 days in culture is presented in Figure 4. The leaflets were intact and pliable. Cells densely covered all leaflets.

**Histology and Immunohistochemistry**

Leaflets of all groups showed cellular tissue with production of extracellular matrix (ECM), independent of mechanical stimulation. Hematoxylin and eosin staining revealed tissue formation organized in a layered manner with dense outer layers and lesser cellularity in the inner part (Figure 5A) compared with native neonatal leaflets (Figure 5B). Trichrom-Masson staining highlighted collagen predominantly at the outer layers (Figure 5C), whereas in the center loosely arranged ECM substance was detected, characterized as predominantly GAG in the Movat staining (Figure 5E). In contrast, native leaflets demonstrated a more homogenous contribution of collagen fibers (Figure 5D) and GAG (Figure 5F). Cells expressed vimentin throughout all tissue engineered leaflets (Figure 5G) and native neonatal leaflets (Figure 5H) but lacked α-SMA expression in both tissue-engineered leaflets (Figure 5I) and in native neonatal leaflets (Figure 5J). Also, desmin was not detected (data not shown).

Expression of CD31 demonstrated an endothelial cell lining, highlighted brown on the surfaces of both tissue-engineered (Figure 5K) and native neonatal leaflets (Figure 5L). Expression of von Willebrand factor and eNOS confirmed the presence of functional endothelial cells (data not shown).

**Quantification of ECM Elements**

Figure 6 summarizes the content of extracellular matrix of leaflets relative to those of native heart valve tissues. The amount of GAG was comparable to that of native tissue or, in some samples, even higher (strained leaflets 5.5 ± 0.73 μm/mg; perfused 5.13 ± 0.74 μm/mg; native 3.98 ± 1.07 μm/mg). The amount of hydroxyproline reached up to 14% of native values (strained leaflets 4.62 ± 2.11 μm/mg; perfused 3.10 ± 1.54 μm/mg; native 54.04 ± 6.25 μm/mg). The cell number, detected as DNA content, was up to 68% of native tissue values (strained leaflets 2.78 ± 0.72 μm/mg; perfused 2.62 ± 1.10 μm/mg; native 4.11 ± 1.46 μm/mg). Mechanical conditioning showed no significant quantitative impact on the production of ECM elements.

**Mechanical Properties**

Two representative stress–strain curves of strained (solid line) and perfused (dashed line) tissue engineered leaflets are presented in Figure 7. Mechanical stimulation resulted in stronger tissues as indicated by tensile strength (0.24 ± 0.04 MPa versus 0.10 ± 0.05 MPa; P < 0.001), as well as strain at maximal stress (0.68 ± 0.18 versus 0.46 ± 0.14; P < 0.052). Furthermore, mechanically stimulated leaflets were less pliable than those exposed to perfusion only as indicated by the higher E-modulus (0.59 ± 0.17 MPa versus 0.33 ± 0.17 MPa; P < 0.03).

**Scanning Electron Microscopy**

Figure 8A shows a micrograph of the unseeded biodegradable leaflet scaffold. After 21 days of culturing in the bioreactor, seeded leaflets were completely covered with chorionic villi-derived prenatal cells (Figure 8B). Cells demonstrated complete ingrowth into the biodegradable scaffold. After endothelialization, tissue engineered heart valve leaflets demonstrated smooth surfaces (Figure 8C) comparable to native heart valve surfaces (Figure 8D). Magnification of the neo-endothelia and those of neonatal pulmonary heart valve leaflets are presented in Figure 8E and 8F, respectively.

**Discussion**

Pediatric cardiovascular tissue engineering represents a promising scientific concept to provide living heart valve
replacements with the capacity of growth for the repair of congenital malformations. For this concept, cells are ideally harvested prenatally to enable the use of the engineered substitutes at or soon after birth, to prevent secondary damage to the newborn’s immature heart. So far, a major obstacle for the clinical realization of this concept is the lack of prenatal cell sources enabling cell harvest without substantial risks for the unborn child or mother and sacrifice of intact donor tissue.

Human chorionic villi represent a unique prenatal cell source providing minimally invasive access to fetal extra-embryonic mesenchyme sprouted out from the multi-lineage trophoblast. Villous development starts between days 12 and 18 after conception by cell sprouting and continues with local vasculogenesis out of the mesenchymal precursor cells. In the present study, cells were prenatally harvested from mesenchymal villi obtained for routinely diagnostic assessment. Today, this procedure is well-established in the clinical routine with acceptable risks for the mother and the unborn child. Besides excellent expansion capacity, isolated cells exhibited phenotypic profiles of a fibroblast–myofibroblast lineage, indicating similarity to human adult valvular interstitial cells. After cryopreservation, cells showed stable phenotypes demonstrating the potential of cell banking for subsequent postnatal application.

When using chorionic villi-derived cells for the fabrication of heart valve leaflets, resulting tissues showed features of proliferating tissues with beginning collagen production and content of GAG approximating native values. Furthermore, in response to biomimetic conditioning cells expressed predominantly vimentin in the tissue-engineered heart valve leaflets.
Thus, cells exhibited phenotype profiles similar to those of native neonatal pulmonary heart valve leaflets. The observed change in α-SMA expression with regard to pre-seeding cells may be related to and enhanced by the progenitor potential of the chorionic villi-derived cells.

Isolated umbilical cord blood-derived EPC differentiated into mature functional endothelial cells during cell expansion as described before and formed functional endothelial layers on the surfaces of tissue engineered leaflets also under mechanical stress. This observation indicates that the human umbilical cord blood-derived differentiated EPC would also function as a non-thrombogenic cell layer in an in vivo environment within a range of mechanical stresses.

All stress–strain profiles showed non-linear mechanical behavior similar to native leaflet tissues. In contrast, the initial scaffold material itself exhibited linear behavior demonstrating that the measured mechanical properties were mainly attributed to the newly formed tissues. Exposure of

Figure 7. Comparison of mechanical properties of strained and perfused leaflets: Mechanical properties of two representative samples of strained (solid line) and perfused (dashed line) tissue engineered leaflets are displayed as stress–strain curves. Strained leaflets were stronger but less pliable than perfused leaflets. The dotted lines represent the E-modulus.

Figure 8. Surface morphology: Scanning electron micrographs show the surfaces of unseeded scaffolds (A) and cellular distribution throughout the polymer 21 days after seeding (B). After endothelialization tissue-engineered leaflets demonstrated smooth surfaces (C) comparable to those of native heart valve leaflets (D). Magnifications revealed parallel cell alignment of neo-endothelial (E) and of native heart valve leaflet endothelia (F).
the tissue engineered leaflets to mechanical stimulation (cyclic strain) resulted in stronger but less pliable tissues compared with only perfused tissues, in agreement with other studies. This observation indicates the positive effect of mechanical stimulation on the mechanical strength and may be related to a higher degree of cross-links among the collagen fibers. The mechanical strength of tissue engineered leaflets did not reach physiological values during the investigated in vitro cultivation time period. This may be explained by the uncompleted collagen production suggesting further improvement of the mechanical loading protocol in vitro. This will be investigated in future studies and the functionality of the engineered tissues in vivo will have to be demonstrated.

In summary, this study demonstrates the feasibility to use prenatally available human progenitors as an exclusive cell source for the fabrication of heart valve leaflets. The successful use of chorionic villi-derived mesenchymal progenitors and umbilical cord blood-derived endothelial progenitor cells may enable the clinical realization of autologous cardiovascular replacements with the capacity of growth ready for use at birth, as both can be accessed prenatally. Chorionic villi-derived mesenchymal progenitors were obtained from viable fetal specimens that are routinely sampled for genetic diagnostics, whereas umbilical cord blood-derived endothelial progenitor cells can be harvested by prenatal percutaneous ultrasound guided cord blood sampling.

Furthermore, prenatal progenitor cells that are not fully differentiated might have a higher potential to form heart valve tissues that are similar to the native counterparts in architecture and cell phenotypes. However, there is a potential risk that their immature stage might lead to tumor development by uncontrolled cell growth or differentiation via genetic alterations. This important aspect has to be considered and investigated in future in vivo studies. Using cell banking technology, the presented approach may be applied for postnatal applications as well.

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Disclosures

None.

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Living Autologous Heart Valves Engineered From Human Prenatally Harvested Progenitors

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