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Prenatally Fabricated Autologous Human Living Heart Valves Based on Amniotic Fluid–Derived Progenitor Cells as Single Cell Source

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Background—A novel concept providing prenatally tissue engineered human autologous heart valves based on routinely obtained fetal amniotic fluid progenitors as single cell source is introduced.

Methods and Results—Fetal human amniotic progenitors were isolated from routinely sampled amniotic fluid and sorted using CD133 magnetic beads. After expansion and differentiation, cell phenotypes of CD133– and CD133+ cells were analyzed by immunohistochemistry and flowcytometry. After characterization, CD133– derived cells were seeded onto heart valve leaflet scaffolds (n=18) fabricated from rapidly biodegradable polymers, conditioned in a pulse duplicator system, and subsequently coated with CD133+ derived cells. After in vitro maturation, opening and closing behavior of leaflets was investigated. Neo-tissues were analyzed by histology, immunohistochemistry, and scanning electron microscopy (SEM). Extracellular matrix (ECM) elements and cell numbers were quantified biochemically. Mechanical properties were assessed by tensile testing. CD133– derived cells demonstrated characteristics of mesenchymal progenitors expressing CD44 and CD105. Differentiated CD133+ cells showed features of functional endothelial cells by eNOS and CD141 expression. Engineered heart valve leaflets demonstrated endothelialized tissue formation with production of ECM elements (GAG 80%, HYP 5%, cell number 100% of native values). SEM showed intact endothelial surfaces. Opening and closing behavior was sufficient under half of systemic conditions.

Conclusions—The use of amniotic fluid as single cell source is a promising low-risk approach enabling the prenatal fabrication of heart valves ready to use at birth. These living replacements with the potential of growth, remodeling, and regeneration may realize the early repair of congenital malformations. (*Circulation. 2007;116[suppl I]:I-64–I-70. *)

Key Words: prenatal fetal progenitor cells  ■  tissue engineering  ■  heart valves  ■  amniotic fluid
autologous heart valves before birth is the amniotic fluid as it contains fetal progenitor cells including hematopoietic progenitor cells\(^9\) and mesenchymal progenitor cells.\(^10\) Moreover, it is routinely obtained in low-risk procedure for genetic diagnostics. Here, a novel concept is introduced using prenatally obtained amniotic fluid as a single fetal cell source for the fabrication of living autologous heart valves prior to birth.

### Methods

#### Amniotic Fluid Sampling

Amniotic fluid sampling was performed for diagnostic purposes (chromosomal abnormalities) between 16 to 18 weeks of gestation, gestational age being determined according to first trimester crown rump length. Patients were informed (written and orally) that amniotic cells would be used for research purposes as described in this article (approved by the Ethics Committee STV20–2006). After ultrasound assessment of fetal biometry and amniotic fluid index, ultrasound guided abdominal amniocentesis was performed under sterile conditions using a 22 G needle.

#### Cell Isolation and Differentiation

Cells were isolated from 4.5 mL of amniotic fluid (n = 9, mean gestation age 16 weeks, mean maternal age 37 years) that was not used for the diagnostics described above. Thereafter, amniotic fluid was centrifuged at 350 g at room temperature for 10 minutes. After removing the supernatant, cells were sorted using a CD133 cell isolation kit (Miltenyi Biotech) according to the manufacturer’s instructions. Briefly, cells were resuspended in phosphate buffered saline supplemented with 0.5% bovine serum (PAN Biotech). Blockering reagent (100 \(\mu\)L/10\(^6\) cells) was added and cells were labeled with CD133 MicroBeads (100 \(\mu\)L MicroBead dispersion/10\(^6\) cells). After incubation at 4°C for 30 minutes, cells were washed by adding 10 to 20\(\times\) the labeling volume of buffer and centrifugation at 350g for 10 minutes. The resuspended cell solution was injected into magnetic columns for cell separation. After CD133– cells were collected, the columns were washed with buffer and removed from the magnetic field. Subsequently, CD133+ cells were flushed out using a plunger supplied with the columns.

For proliferation and differentiation CD133– and CD133+ cells were cultured, and nonattached cells were removed after 2 days. To initiate endothelial differentiation, CD133+ cells were exposed to basalmatrix (EBM™2, Cambrex), containing growth factors and supplements, namely vascular endothelial growth factor (VEGF), human fibroblasts growth factor (hFGF), human recombinant long-insulin-like growth factor-1 (R-3-IGF-1), human epidermal growth factor (hEGF), gentamycin and amphoteracin (GA-1000), hydrocortisone, heparin, ascorbic acid, and 20% fetal bovine serum. In contrast, the CD133– cell fraction was cultured in basalmedium supplemented with hFGF, R-3-IGF-1, hEGF, GA-1000, ascorbic acid, and 20% fetal bovine serum.

#### Immunochemistry

CD133– and CD133+ amniotic fluid-derived cells were cultured in chamber slides, and immunohistochemistry was performed as described before\(^9\) using the following primary antibodies: transcription factor Oct-3/4 (affinity purified goat antibodies; Santa Cruz Biotechnology), CD44 (clone G44–26), endothelial nitric oxide synthase type III (eNOS) (affinity purified rabbit antibodies; both from BD Biosciences), CD34 (clone QBEND 10; Serotec), vimentin (clone 3B4), desmin (clone D33), CD31 (clone JC/70A), von Willebrand factor (affinity purified rabbit antibodies; all from DakoCytomation), and \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA, clone 1A4; Sigma).

#### Flowcytometry (FACS)

For quantification of antigen expression, FACS-analysis was performed using antibodies against CD44 (fluorescein isothiocyanate [FITC]-conjugated, Clone MEM-85), CD105 (FITC-conjugated, Clone MEM-226; both from Immunotech), CD34 (FITC-conjugated, Clone Ac136; Miltenyi), CD31 (Clone 158 to 2B3; NeoMarkers) CD141 (thrombomodulin; Clone 1009; DakoCytomation) and von Willebrand factor (vWF;Clone 3F2-A9; BD Biosciences). Irrelevant isotype-matched antibodies (FITC-conjugated IgG2a, clone 713; FITC-conjugated mouse IgG2b, clone PFR-02; both from Immunotech; IgGlMOPC-21, Sigma Chemical Company) served as controls. Unconjugated primary antibodies were detected with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibodies (Boehringer Mannheim). For detection of vWF, cells were fixed and permeabilized using an inside stain kit (Miltenyi, Biotech) following the manufacturer’s instructions. Analysis was performed on a Becton Dickinson FACScan.

#### Genotypy of Cells

DNA was extracted from amniotic fluid–derived cells directly after cell harvest as well as from cell cultures of CD133– and CD133+ cells using the InstaGene Matrix (BioRad) according to the manufacturer’s instructions. In parallel, maternal DNA was extracted from peripheral blood samples using the EZI Blood Kit (Qiagen). Fetal origin of the cells and absence of maternal contamination was determined by comparing fetal and maternal microsatellite marker profiles obtained by quantitative fluorescent PCR.\(^12,13\) 13 autosomal and 1 sex chromosome–specific STR markers as well as the deletion polymorphism at the Amelogenin locus were amplified by multiplex PCR. The PCR products were separated by capillary electrophoresis (ABI 310) and analyzed using the GeneScan and Genotyper software packages (Applied Biosystems).

#### Engineering of Heart Valve Leaflet Tissues

Heart valve leaflets (2.3 cm\(^2\), n = 18) were cut from rapidly biodegradable nonwoven polyglycolic-acid meshes (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm\(^3\), Albany Int) and dip-coated with Poly-4-Hydroxybutyric acid (1% wt/vol P4HB, TEPHA Inc.). Before evaporation of the solvent, 3 leaflet scaffolds each were attached to a ring-shaped device (20 mm diameter) fabricated from nonbiodegradable Fastacryl (Vertex-dental) using a trileaflet-shaped heart valve mold. Leaflet scaffolds were positioned on the mold, and the ring-shaped device was placed on top. By dissolving the surface layer of the device the leaflet scaffolds were fixed to the edges of the rings. After evaporation of the solvent, the mold was removed and resulted valves were sterilized in 70% ethanol. Sterile scaffolds were seeded with CD133+ derived cells (3.5×10\(^6\) cells/cm\(^2\)) using fibrin as a cell carrier and positioned in a Diastolic Pulse Duplicator, described in detail before.\(^15\) Continuous perfusion was applied (4 mL/min) using the basal medium containing the above mentioned growth factors and supplements for CD133– and CD133+ cell culture. After 7 days, 2 groups were formed with n = 9 leaflets each: to 1 group cyclic straining (15 mm Hg) was applied in addition to perfusion whereas the other was exposed to perfusion only. CD133+ derived cells were kept under humidified incubator conditions (37°C, 5% CO\(_2\) and expanded up to passage 7.

After an additional 14 days, the leaflets were coated with CD133+ derived cells (1.5×10\(^6\) cells/cm\(^2\)) on both sides. They were kept under static conditions for 24 hours and subsequently cultivated for an additional 7 days exposed to the same mechanical conditions but in medium containing the above mentioned growth factors and supplements for CD133+ cell culture. Thereafter, heart valves were explanted from the bioreactor and neotissues were analyzed.

#### Analysis of Generated Heart Valve Leaflets

**Assessment of Functionality**

The opening and closing behavior of heart valve leaflets was visualized in a custom-built in vitro set-up consisting of a computer-controlled pump, representing the ventricle, and a systemic circulation.\(^16\) Physiological systemic flow profiles were generated and images of the opening and closure behavior were obtained using a...
high-speed video camera (Phantom v9.0; Vision Research Inc) up to half of systemic conditions.

**Evaluation of Tissue Organization and Phenotypes**

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 histology using Hematoxylin-Eosin (H&E), Trichrom-Masson, and Movat pentachrome staining as to morphology and tissue organization. Cell phenotypes were displayed by immunohistochemistry using the primary antibodies and detection kits described above. Deparaffinization and antigen retrieval (CD31: predigestion with protease 1, all other antibodies: heating with cell conditioner 1) was performed on the Ventana Benchmark staining system (Ventana Medical Systems).

**Quantitative Assessment of Extracellular Matrix Elements and Cell Number**

Hydroxyproline, an indicator for collagen 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 TNE-buffer (10 mmol/L Tris, 100 mmol/L NaCl, 1 mmol/L EDTA, pH7.4) and labeling of the DNA using Hoechst dye (Bisbenzimide H33258, Fluka). Native human heart valve tissues served as controls.

**Evaluation of Mechanical Properties**

Mechanical properties of leaflet tissues (15×5×1 mm) were analyzed using an uniaxial tensile tester (Instron 4411), equipped with a 10N load cell, at strain rate of 1 minute . The recorded tensile force and displacement were transformed into stress-strain curves.

**Scanning Electron Microscopy**

Representative samples of tissue engineered leaflets were fixed using 2% glutaraldehyde. After preparation, samples were sputtered with gold and investigated with a Zeiss Supra 50 VP Microscope (Zeiss). Native heart valve leaflets served as controls.

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

**Results**

**Morphology and Phenotype of Cells**

In total 2.6×10⁶ ± 1.2×10⁵ cells were isolated from 4.5 mL amniotic fluid whereas 1% represented CD133 + cells. After 2 days, about half of the amniotic fluid–derived CD133− amniotic fluid–derived cells were attached to the culture dishes (Figure 1A). After an initial phase of slow growth, cells started...
to proliferate and formed a confluent layer after 10 days (Figure 1B). Immunohistochemistry revealed expression of CD44 (Figure 1C) and vimentin (Figure 1D) and a lack of Oct-3/4, CD34, vH9251-SMA, and desmin (data not shown). Furthermore, neither expression of eNOS (Figure 1E) nor CD31 or vWF (data not shown) could be detected after 28 days. In contrast, CD133 vH11001 cells were initially round and demonstrated cluster-like formation after 2 days (Figure 1F). After 10 days, cells formed a cobblestone-like morphology (Figure 1G) and expressed CD44 (Figure 1H) and vimentin (Figure 1I). Oct-3/4 and CD34 were not detected (data not shown). After 28 days, cells started to express eNOS (Figure 1J) but lacked the expression of CD31 and vWF (data not shown).

FACS analysis confirmed these staining patterns by displaying the expression of CD44 for most of the CD133 vH11002 cells (Figure 2A) and vH11001 partly for CD133 vH11001 cells (Figure 2B). Additionally, a fraction of CD133 vH11002 cells demonstrated expression of CD105 (Figure 2C), whereas only a small amount of CD133 vH11001 cells was positive for CD105 (Figure 2D). CD34 was neither expressed by CD133 vH11002 cells (Figure 2E) nor by CD133 vH11001 cells (Figure 2F). Furthermore, no CD141 expression could be observed on CD133 vH11002 cells (Figure 2G) in contrast to the CD133 vH11001 cell fraction (Figure 2H). CD31 and vWF were not detectable in CD133 vH11002 cells and CD133 vH11001 cells, respectively (Figure 2I and 2L).

**Figure 3.** Confirmation of fetal genotype and exclusion of maternal contamination by quantitative fluorescent PCR. The autosomal STR marker profiles (only D13S258, D21S143, D18S499, and D13S305 shown in Figure) obtained from the starting material (amniotic fluid cells after harvest) were identical to those obtained from CD133 vH11002 and CD133 vH11001 cells, but were clearly different from the maternal blood profiles. Furthermore, no contamination (indicated by additional maternal peaks) was detected A. In male fetal DNA the polymorphism at the Amelogenin locus (AMXY) on both sex chromosomes exhibited 2 alleles (AMXY [Y] and AMXY [X]) with equal dosage (similar peak height/area). In addition, the presence of only 1 allele for all X-chromosome specific STR markers (only P39 and DXS981 shown in Figure) as well as 1 allele of the Y-chromosome specific STR marker DYS448 is in agreement with a male profile without maternal contamination (B).

**Genotype of Cells**
Quantitative fluorescent PCR revealed different autosomal STR profiles in all amniotic fluid cell-derived DNAs when compared with the corresponding profiles of maternal blood-derived DNAs (Figure 3A). Furthermore, in male fetal DNA only 1 allele for the X-chromosome specific STR-markers could be detected. Additionally, the Y-chromosome specific markers were positive (Figure 3B). No maternal admixture as evidence of contamination was found in the subsequent cell cultures thus confirming the pure fetal origin of CD133 vH11002 and CD133 vH11001 cells.

**Analysis of Tissue Engineered Heart Valve Leaflets**

**Macroscopic Appearance**
Figure 4 demonstrates a photograph of a tissue engineered heart valve after 28 days of culturing in the Diastolic Pulse Duplicator. The neotissue appeared to have homogeneous thickness with smooth surfaces. The leaflets were intact and pliable demonstrating a sufficient opening and closing behavior (Figure 5) under low-pressure conditions (systolic pressure of ventricle = 25 mm Hg; enddiastolic pressure of ventricle = 2 mm Hg; systolic pressure of artery = 25 mm Hg; end diastolic pressure artery = 4 mm Hg).

**Histology and Immunohistochemistry**
Leaflets of all groups showed cellular tissue with production of ECM, independent of mechanical stimulation. H&E stain-
ing revealed tissue formation organized in a layered manner with dense outer layers and lesser cellularity in the inner part (Figure 6A). Expression of eNOS demonstrated an endothelial cell lining on the surfaces of the tissue engineered leaflets (Figure 6B). Trichrom-Masson staining highlighted collagen predominantly at the outer part (Figure 6C), whereas in the center loosely arranged ECM substance was detected, characterized as predominantly GAG in the Movat staining (Figure 6D). Additionally, vimentin was detected throughout the tissue, whereas α-SMA was not expressed (data not shown).

**ECM Elements and Mechanical Properties**

Mechanical conditioning showed no significant quantitative and qualitative impact on the production of ECM elements or mechanical properties. The Table summarizes the content of ECM elements in the tissue engineered heart valve leaflets. The amount of GAG in tissue engineered heart valve leaflets reached values up to 80% of native values (strained leaflets 12.5±0.81 μg/mg; perfused 10.8±1.31 μg/mg; native 16.18 μg/mg). The amount of hydroxyproline was up to 5% of native values (strained leaflets 2.51±0.79 μg/mg; perfused 1.27±0.36 μg/mg; native 47.21 μg/mg). The cell number in all tissue engineered tissues, detected as DNA content, was comparable to that of native tissue (strained leaflets 4.12±0.77 μg/mg; perfused 3.47±1.70 μg/mg; native 4.11 μg/mg). The production of ECM elements was reflected in the mechanical properties, in specific, tensile strength: strained leaflets 0.05±0.02 MPa; perfused leaflets 0.07±0.03 MPa, strain at maximal stress: strained leaflets 0.61±0.11; perfused leaflets 0.65±0.20, Young modulus strained leaflets 0.16±0.07 MPa; perfused leaflets 0.19±0.09 MPa.

**Scanning Electron Microscopy**

Tissue engineered heart valve leaflets demonstrated surfaces densely covered with extracellular matrix elements producing cells when seeded with CD133+ only (Figure 7A). When coated with CD133+ cells an endothelium-like formation was observed on the leaflet surfaces (Figure 7B). Magnification of a native heart valve leaflet surface is presented as Figure 7C.

**Discussion**

In many congenital heart malformations corrective intervention is mandatory directly or shortly after birth to prevent secondary damage to the immature heart. Since such heart malformations are often detectable already during pregnancy (eg, by routine ultrasound examination), the ideal pediatric tissue engineering paradigm would comprise prenatal cell harvest to provide time for the in vitro fabrication of an autologous living implant that is ready to use at birth. Amniotic fluid represents an attractive fetal cell source for pediatric cardiovascular tissue engineering as it enables easy prenatal access to fetal progenitor cells from all 3 germ layers.
in a low-risk procedure. Furthermore, previous studies have shown promising results based on these cells with regard to noncardiovascular tissue engineering.20

Here, we demonstrate that living autologous heart valve leaflets were successfully engineered using amniotic fluid–derived cells, namely CD133+/H11001, a noncommitted progenitor cell population with the capacity to differentiate into endothelial cell lines, and CD133−.

Both CD133+/H11001 and CD133− cells demonstrated excellent growth capacity. When analyzed, they showed characteristics of fetal multipotential but not pluripotent progenitor cells as indicated by the expression of CD44 and the lack of Oct-3/4, respectively. Furthermore, their fetal origin was confirmed by gene analysis. The staining pattern of CD133− cells, namely the expression of CD44 and CD105 and a lack of CD34, reflects a mesenchymal progenitor cell type. When used for the fabrication of autologous heart valve leaflets, CD133− exhibited characteristics of fibroblast-like cells by producing extracellular matrix elements.

Most of the isolated CD133+ cells expressed CD44 but lacked the expression of CD34. During cell expansion and differentiation using vascular endothelial growth factors, CD133+ cells showed characteristics of functional endothelial cells as indicated by the expression of CD141 and eNOS. This staining pattern suggests that CD133+ cells might be multipotential adult progenitor cells with the capacity to differentiate into endothelial cells under well-defined conditions.21,22 Furthermore, the expression profiles may indicate that the CD133+ population is not homogenous. When seeded onto the surfaces of engineered heart valve leaflets, CD133+ cells formed functional endothelial layers. CD31 and vWF were not expressed during the culture time period indicating that the differentiation process might not have been completed, suggesting modification of the culture conditions such as growth factor concentrations. Moreover, the functionality of the endothelial-like layers has to been proven in vivo.

The stress-strain profiles of tissue engineered heart valve leaflets showed nonlinear mechanical behavior indicating that the measured mechanical properties were mainly attributed to the newly formed tissues as the initial scaffold material itself exhibited linear behavior. The mechanical properties did not reach physiological values1 during the investigated in vitro cultivation time period. This might be explained by the uncompleted collagen production suggesting further improvement of the mechanical loading protocol in vitro.23,24 However, under conditions simulating the right ventricular outflow tract tissue engineered heart valve leaflets demonstrated sufficient opening and closing behavior indicating that the engineered constructs might function in a low-pressure environment as pulmonary valve replacements.

In summary, here we demonstrated the feasibility to generate living autologous heart valve leaflets in vitro using amniotic fluid as a single cell source. The cell populations required for heart valve tissue, that is myofibroblasts-fibroblast-like cells and endothelial cells could be successfully differentiated and expanded. The premature state of amniotic fluid-derived fetal multipotential progenitor cells may in addition represent an advantage regarding their regenerative capacity. This might be
associated with an increased potential to form heart valve tissues that approximate their native counterparts in architecture and cell phenotypes under well defined conditions. However, the not fully differentiated stage might be a potential risk for tumor development by uncontrolled cell growth or differentiation. In this feasibility study focusing on the evaluation of a new fetal cell source for the prenatal fabrication of heart valve tissues, a nonbiodegradable material was used for fixation of heart valve leaflets for technical simplicity. However, for in vivo studies the ring structure can be easily changed to biodegradable polymer materials. Furthermore, the applicability of the engineered heart valve tissues as to high pressure application has to be investigated using, e.g., numeric flow-structure simulation. These important aspects have to be considered in future in vivo studies as well as their functionality with respect to long-term durability and adequate growth behavior of this new class of replacements.

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Disclosures

None.

References


Figure 7. Surface morphology. In SEM, tissue engineered heart valve leaflets based only on amniotic fluid–derived CD133+ cells demonstrated surfaces covered with extracellular matrix elements producing cells (A) but an endothelium-like formation appeared after coating with CD133+ cells (B). C presents a magnification of a native heart valve leaflet surface.