Human Umbilical Cord Cells: A New Cell Source for Cardiovascular Tissue Engineering

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Background. **Tissue engineering of viable, autologous cardiovascular constructs with the potential to grow, repair, and remodel represents a promising new concept for cardiac surgery, especially for pediatric patients. Currently, vascular myofibroblast cells (VC) represent an established cell source for cardiovascular tissue engineering. Cell isolation requires the invasive harvesting of venous or arterial vessel segments before scaffold seeding, a technique that may not be preferable, particularly in pediatric patients. In this study, we investigated the feasibility of using umbilical cord cells (UCC) as an alternative autologous cell source for cardiovascular tissue engineering.**

Methods. **Human UCC were isolated from umbilical cord segments and expanded in culture. The cells were sequentially seeded on bioabsorbable copolymer patches ($n = 5$) and grown in vitro in laminar flow for 14 days. The UCC were characterized by flow cytometry (FACS), histology, immunohistochemistry, and proliferation assays and were compared to saphenous vein–derived VC. Morphologic analysis of the UCC-seeded copolymer patches included histology and both transmission and scanning electron microscopy. Characterization of the extracellular matrix was performed by immunohistochemistry and quantitative extracellular matrix protein assays. The tissue-engineered UCC patches were biomechanically evaluated using uniaxial stress testing and were compared to native tissue.**

Results. **We found that isolated UCC show a fibroblast-like morphology and superior cell growth compared to VC. Phenotype analysis revealed positive signals for α-smooth muscle actin (ASMA), desmin, and vimentin. Histology and immunohistochemistry of seeded polymers showed layered tissue formation containing collagen I, III, and glycoaminoglycans. Transmission electron microscopy showed viable myofibroblasts and the deposition of collagen fibrils. A confluent tissue surface was observed during scanning electron microscopy. Glycoaminoglycan content did not reach values of native tissue, whereas cell content was increased. The biomechanical properties of the tissue-engineered constructs approached native tissue values.**

Conclusions. **Tissue engineering of cardiovascular constructs using UCC is feasible in an in vitro environment. The UCC demonstrated excellent growth properties and tissue formation with mechanical properties approaching native tissue. It appears that UCC represent a promising alternative autologous cell source for cardiovascular tissue engineering, offering the additional benefits of using juvenile cells and avoiding the invasive harvesting of intact vascular structures.**

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Currently available synthetic or bioprosthetic replacements for repairing congenital cardiac defects show certain disadvantages that limit long-term benefits. Prosthetic replacements lack growth potential and can become obstructed by tissue ingrowth or calcification, leading to the need for multiple replacements [1, 2]. All synthetic material is thrombogenic and, after implantation, the risk of thrombembolic and infectious complications potentially increases [3]. Cryopreserved homograft tissue appears to offer advantages over prosthetic replacements by theoretically providing some viability with a potential ability for growth [4–6]. Nevertheless, these tissues are subject to enhanced calcification after implantation in children, and donor scarcity remains a significant problem for pediatric patients [7–9].

Tissue engineering attempts to create viable replacement structures with the potential to grow, repair, and remodel, which would be of great benefit, especially for pediatric patients. The basic principle of tissue engineering involves the isolation and seeding of cells on biodegradable three-dimensional scaffolds and subsequent cultivation under dynamic or static conditions.

By using scaffolds of synthetic polymer, collagen, or xenogenic pulmonary artery conduits, patch material, trileaflet heart valves, and vessel grafts were already generated and successfully tested under in vitro and in

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vivo conditions [10–14]. Recently, the first clinical application of a tissue-engineered pulmonary artery graft was reported with encouraging results after implantation in a pediatric patient [15].

Presently, autologous vascular-derived myofibroblasts represent an established cell source for cardiovascular tissue engineering. These cells demonstrate good cell polymer attachment and tissue formation, resulting in functioning cardiovascular constructs. Before scaffold seeding, vascularly derived myofibroblasts are isolated from segments of aorta, carotis, or saphenous vein segments. This requires invasive harvesting of intact vascular structures, which may not be preferable, especially in pediatric patients. In search of an alternative approach, we investigated the feasibility of using autologous umbilical cord vascular cells for tissue engineering of cardiovascular constructs.

Material and Methods

Cell Isolation and Cultivation

Human umbilical cord and saphenous vein sections (2 to 3 cm in length) were washed with Dulbecco's phosphate buffered saline (Gibco, Rockville, MD) and minced into 1-mm pieces, which 100 × 15-mm Petri dishes (Gibco). Tissue sections were cultured with Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone) and Gentamycin (Gibco). Medium was replaced at 24 and 72 hours and every 6 days thereafter. Daily progress was monitored by phase-contrast microscopy. The cells were serially passaged and expanded in a humidified incubator at 37°C with 5% CO_2. Cell numbers sufficient for seeding of polymer scaffolds ($n = 5$) were obtained after 21 to 28 days.

Polymer Scaffolds

Nonwoven polyglycolic-acid mesh (PGA) (Albany Int) was coated with a thin layer of poly-4-hydroxybutyrate (P4HB, MW: 1×106, PHA 4400, Tepha Inc, Cambridge, MA) by dipping into a tetrahydrofuran solution (1% wt/vol P4HB). After solvent evaporation, a continuous coating and physical bonding of adjacent fibers was achieved. P4HB is a biologically derived biopolymer that shows complete biodegradation after 4 to 6 weeks. Polymer scaffold patches (5 × 2 cm; $n = 5$) were cut from the PGA/P4HB composite material and were cold gas sterilized with ethylene oxide.

Cell Seeding and In Vitro Culture of Polymer Constructs

Bioabsorbable polymer scaffolds were seeded with an approximate density of 4.5 to 5.5 × 10^6 UCC per square centimeter and cultured for 7 days in a humidified incubator (37°C, 5% CO_2). Afterward the constructs were exposed for 14 days to a continuous laminar flow of 250 to 300 mL/min DMEM supplemented with 10% fetal bovine serum (HyClone), Gentamycin (Gibco) in a humidified incubator (37°C, 5% CO_2).

Analysis of UCC Cultures

FLOW CYTOMETRY. Single cell suspension of UCC and VC were prepared for FACS. We incubated 0.5 × 10^6 cells in 100 μL of PBS plus BSA with saturating concentrations of monoclonal antibodies CD 31-FITC (Clone LCI4 + 6+7, kindly provided by P. Kilshaw), ASMA (Clone 1A4, Sigma, St. Louis), desmin (Clone D33, NeoMarkers, Fremont) and vimentin (Clone V9, NeoMarkers, Fremont). For intracellular staining (ASMA, desmin, vimentin) cells were permeabilized with ethanol for 60 minutes at room temperature and incubated with monoclonal antibodies for 30 minutes. Following washing, staining with a secondary FITC-conjugated IgG goat-antimouse antibody (Chemicon, Temecula, CA) was performed for 30 minutes. Forward and side scatters were set to exclude debris and 10,000 gated events were counted per sample. Corresponding irrelevant isotype-matched and positive controls were performed for each antibody. Cells were analyzed with the flow cytometer FACS-Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA). Data analysis was performed with the CELL QUEST software program (Becton Dickinson Immunocytometry Systems, San Jose, CA). Expression levels were calculated as mean fluorescence intensity ratio (MFIR) defined as mean fluorescence intensity of the studied antibodies divided by mean fluorescence intensity of corresponding isotype controls.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY. The UCC were cultivated onto glass coverslips in nutrient medium (DMEM). After 2 to 3 days, cells were washed with PBS and fixed in methanol for 10 minutes. Cells were examined histologically by hematoxylin and eosin and Trichrome-masson stain. Immunohistochemistry was performed by incubation with monoclonal mouse antibodies for ASMA (Sigma, St. Louis), vimentin (NeoMarkers, Fremont), and collagen I, III (Oncogen, Boston). Incubation with a secondary FITC-labeled goat-antimouse IgG antibody (Sima, St. Louis) to vimentin, a AlexaFluor 647–labeled goat-antimouse IgG antibody (Molecular Probes, Leiden, NL) to ASMA, and a biotin-labeled goat-antimouse IgG antibody (Sima, St. Louis) to collagen I and III was performed. The biotin-labeled antibody signal was developed with the avidin-peroxidase system (ABC kit, Vector Laboratories, Burlingame, CA). Before intracellular staining for ASMA and vimentin permeabilization of the cells was performed by incubation with 0.1% Triton (Sigma, St. Louis) for 10 minutes.

MTT ASSAY. The UCC ($n = 4$) and VC ($n = 4$) were seeded in 24-well plates with a density of 5 × 10^4 cells/ml per well and cultured with DMEM. MTT assays were performed on days 1, 2, 4, 6 and 7. Quantities of 250 μL of medium and 20 μL of MTT solution (Sigma) were added to each sample and incubated for 1 hour, followed by isopropanol for 5 minutes. MTT absorbency was measured at 570 nm and growth curves developed.

Analysis of UCC-Seeded Polymer Constructs

HISTOLOGY AND IMMUNOHISTOCHEMISTRY. Sections of UCC-seeded polymer scaffolds were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Paraffin sections were cut at 5-μm thickness and studied by hematoxylin and eosin and Trichrome-masson stain. Immunohistochemistry was performed as described above by incubation with monoclonal mouse antibodies for ASMA, vimentin, and collagen I and III.

SCANNING AND TRANSMISSION ELECTRON MICROSCOPY. Additional samples of MSC-seeded polymer strips were fixed in 2% glutaraldehyde (Sigma, St. Louis) and studied by scanning electron and transmission electron microscopy.

BIOCHEMICAL ASSAYS. Samples of UCC constructs ($n = 10$) were analyzed by biochemical assays for cellular and extracellular components and compared to human pulmonary artery. Total DNA was isolated and purified by sequential organic extractions with phenol and phenol/chloroform/isoamyl alcohol and quantitated by spectrophotometry [16]. Total proteoglycan/glycosaminoglycan content were quantitated with a BLYSCAN assay (Biocolor, Belfast, Ireland) after tissue extraction.

BIOMECHANICAL TESTING. Longitudinal strips (20 mm $\times$ 5 mm $\times$ 1 mm) of tissue-engineered constructs and human pulmonary artery were uniaxial stress tested with an Instron tensile analyzer (model 4411) equipped with a 100-N load cell and pneumatic clamps (max. pressure 75 psig) (Instron Corporation, Canton, MA). The cross head speed was 0.5 inch/min (corresponding to a linear strain rate of 1 min^{-1}). The mechanical properties were analyzed for maximal stress and strain at maximal load. Modulus AutYoung was calculated from the slope of the linear section of the stress–strain curve.

Results

Histology and Immunophenotyping of UCC

Hematoxylin and eosin and Trichrome-masson staining of fixed cells showed elongated cells with fibroblast-like morphology as well as deposition of extracellular matrix throughout the cell culture. Immunohistochemistry revealed intracellular expression of ASMA and vimentin, along with formation of collagen I and III (Fig 1). Cultured UCC did not stain positive for the endothelial cell marker CD 31.

Flow Cytometry

Results of the FACS analysis of UCC are shown in Figure 2. The UCC characterization by flow cytometry demonstrates no significant difference in morphology for UCC (Fig 2A) compared to VC (Fig 2B). There was no difference detected for the expression of ASMA (MFIR 15.77) (Fig 2C), vimentin (MFIR 5.56) (Fig 2D) or desmin (MFIR 1.1) (Fig 2E), compared to VC. No positive signal was detected for CD 31 (MFIR 1.0) among the isolated cell population (Fig 2F).

MTT Assay

The cell growth potential of UCC is shown in Figure 3. Compared to VC, UCC demonstrated excellent cell growth.

Histology and Immunohistochemistry of UCC-Seeded Polymer Constructs

Hematoxylin and eosin and Trichrome-masson staining of the UCC-seeded polymer patches showed a layered tissue formation and a dense upper layer with deposition of extracellular matrix proteins. Irregular cellular ingrowth was observed in the less cellularized, deeper parts of the polymer patches. Immunohistochemistry of UCC-seeded constructs showed positive staining for ASMA and vimentin. Extracellular matrix analysis demonstrated deposition of collagen I and III (Fig 4C and 4D).

Transmission and Scanning Electron Microscopy

Transmission electron microcopy of sections of UCC-seeded patches showed secretionally active myofibroblasts with deposition of collagen fibrils. Scanning electron microscopy demonstrated good cell–polymer attachments. Cells formed a confluent surface and grow well into deeper parts of the patch scaffolds. Degradation of the scaffold was studied by multiple breakages and fragmentation of the polymer fibers (Fig 4A and 4B).

Biochemical Assays

The DNA assay demonstrated an increased number of cells in the UCC constructs compared to those in the human pulmonary artery. After 3 weeks in culture, samples contained 361% more DNA than did pulmonary artery (1.77 $\pm$ 0.32 μg/mg vs 0.49 $\pm$ 0.03 μg/mg). In contrast, the tissue-engineered patches reached only 34% of the amount of glycoaminoglycans measured in native pulmonary artery (3.16 $\pm$ 0.91 μg/mg vs 9.16 $\pm$ 0.36 μg/mg).

Biomechanical Testing

The maximal uniaxial tensile stress testing of the tissue-engineered constructs showed values of 1.25 $\pm$ 0.45 MPa, which are comparable to those of native pulmonary artery (0.95 $\pm$ 0.37 MPa). Evaluation of the distensibility of UCC patches reached reduced values for the strain at maximal load of 25% compared to pulmonary artery (0.401 $\pm$ 0.14 mm/mm vs 1.611 $\pm$ 0.52 mm/mm). The tissue-engineered samples were also 317% less pliable than pulmonary artery (5.375 $\pm$ 0.348 MPa vs 1.694 $\pm$ 0,52 MPa), a tissue that contains high amounts of elastin.

Comment

Living tissue-engineered constructs appear to be an attractive new concept for cardiovascular surgery, especially for the repair of congenital cardiac defects. The creation of viable substitutes with the ability to regenerate, remodel, and grow would have substantial advantages over currently available materials and prostheses.

In the present study, we evaluated the feasibility of

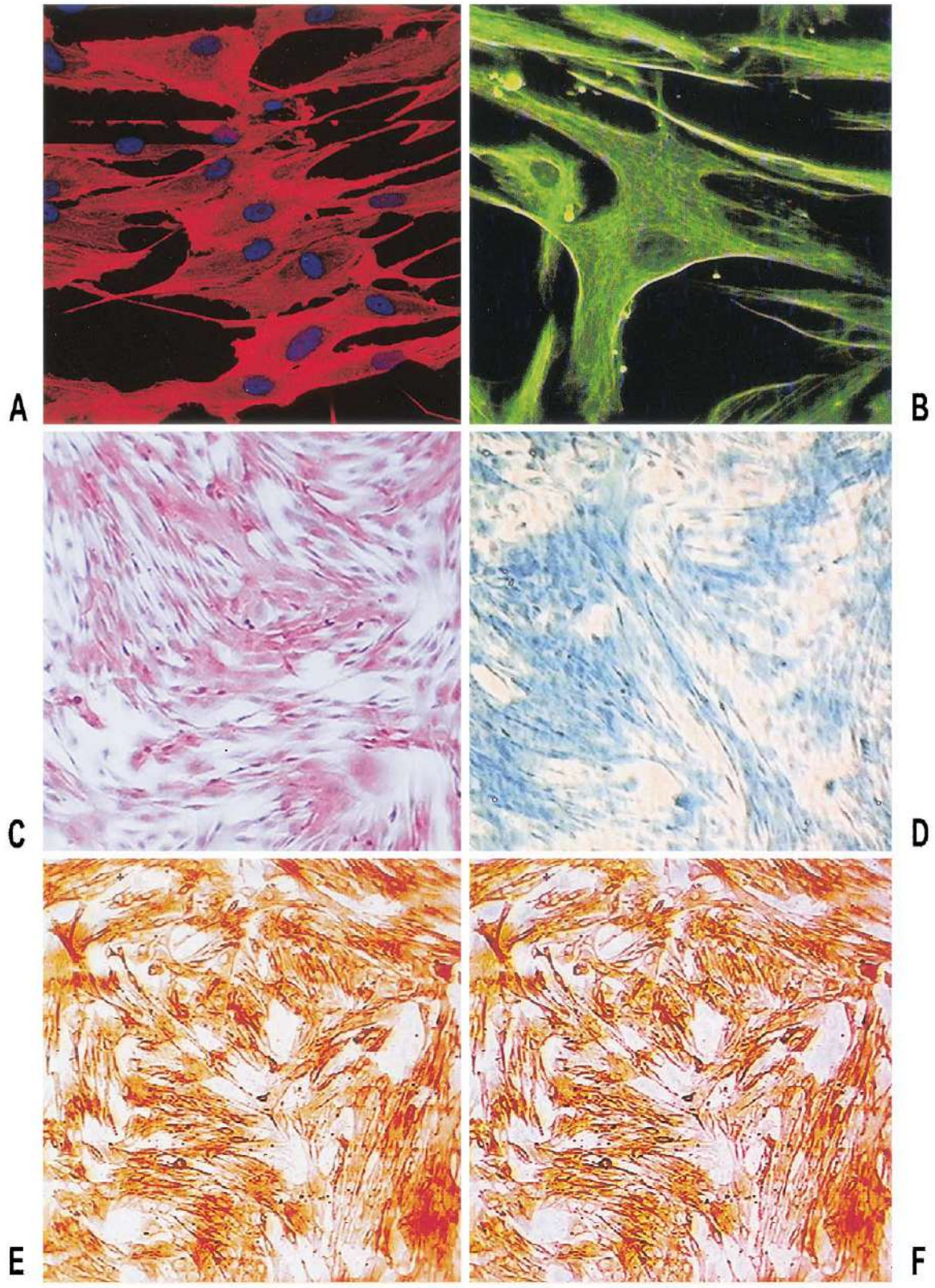

Fig 1. Histology and immunohistochemistry of umbilical cord cells. Immunofluorescence staining demonstrated expression of ASMA (A) and vimentin (B). Hematoxylin and eosin (C) and Trichrome-masson (D) staining show cells with fibroblast morphology and deposition of extracellular matrix proteins. Staining with monoclonal antibodies against collagen I (E) and collagen III (F) revealed positive signals of cultured umbilical cord cell cultures.

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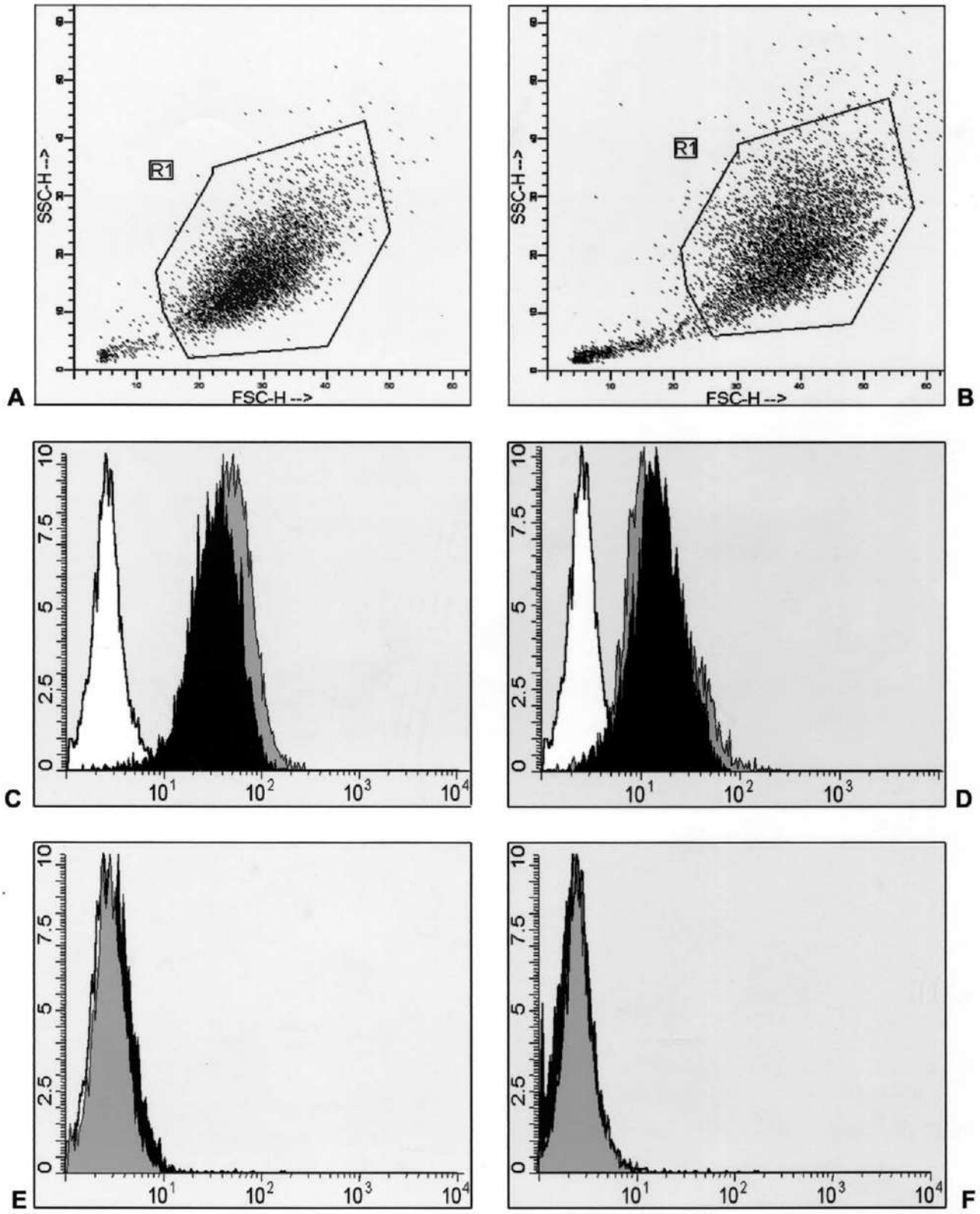

Fig 2. Flow cytometry of umbilical cord cells. Flow cytometry demonstrated no significant difference in the cell morphology for umbilical cord cell (A) and for vascular myofibroblast cell (B) populations. No difference was detected in expression level of ASMA (C), vimentin (D), desmin (E), and CD 31 (F) between umbilical cord cells (black peak) *and vascular myofibroblast cells* (gray peak). *Corresponding isotype controls are shown as white peaks.* FSC = *forward scatter count;* SSC = *side scatter count.*

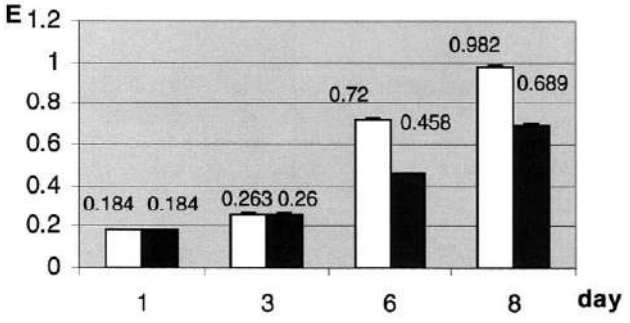

Fig 3. MTT assay of umbilical cord cell. Open squares: UCC = *umbilical cord cell (n = 4); Filled squares:* VC = *vascular myofibroblast cells (n = 4).*

using an alternative autologous cell source—namely, human UCC—for tissue engineering of cardiovascular constructs. Umbilical cord was chosen as a cell source because it is easy to obtain and avoids the invasive harvesting of intact vascular structures, which might be a particular advantage in pediatric patients.

The isolated UCC represent a mixed cell population deriving from umbilical cord artery, vein, and surrounding Wharton's jelly. A study by Kobayashi and colleagues [17] demonstrated that all three cell types exhibit myofibroblast-like characteristics. These findings were confirmed by this study; flow cytometry and immunohistochemistry analysis of the isolated UCC showed positive signals for ASMA, desmin, and vimentin that were comparable to those of vascular cells derived from saphenous vein. The absence of endothelial cells in the outgrowth cell population was confirmed by negative staining for CD 31. The importance of using arterial versus venous myofibroblast for tissue engineering is still unclear. By using both cell lines, several groups report the successful generation of heart valves, pulmonary conduits, and patch material [15, 18–22]. There was no difference in function and tissue formation between the constructs tested in vivo.

Compared to vascular-derived cells, UCC demonstrated an excellent cell growth, making these cells an attractive candidate for tissue engineering. After seeding, UCC attached well to the bioabsorbable polymer scaffolds and formed a confluent homogenous surface. Histology revealed a layered tissue formation and growth pattern comparable to that previously described for tissue-engineered constructs seeded with vascularly derived cells REF. After 3 weeks of cultivation, UCC continued to express myofibroblast characteristics and stained strongly positive for ASMA and vimentin. Transmission electron microscopy revealed viable myofibroblast and collagen fibrils. Further analysis demonstrated deposition of collagen I and III, which are important extracellular matrix components of cardiovascular structures. Compared to native cardiovascular tissue samples, a lower amount of glycoaminoglycan content was shown in the extracellular matrix protein analysis of the UCC-seeded scaffolds. In contrast, we studied an increased amount of cells in the UCC constructs compared to native tissue. Similar results were previously obtained for vascular cell–derived constructs and were reported for tissue-engineered heart valves [18]. It appears that the tissue-engineered constructs represent immature growing tissue with a high cell turnover and reduced amounts of extracellular matrix proteins.

We believe that in vitro tissue engineering of cardiovascular structures requires exposure of the cell-seeded constructs to a biomimetic environment. Generation of tissue- engineered heart valves and vascular conduits from saphenous vein–derived myofibroblasts under a continuous pulsatile flow showed improved tissue orga-

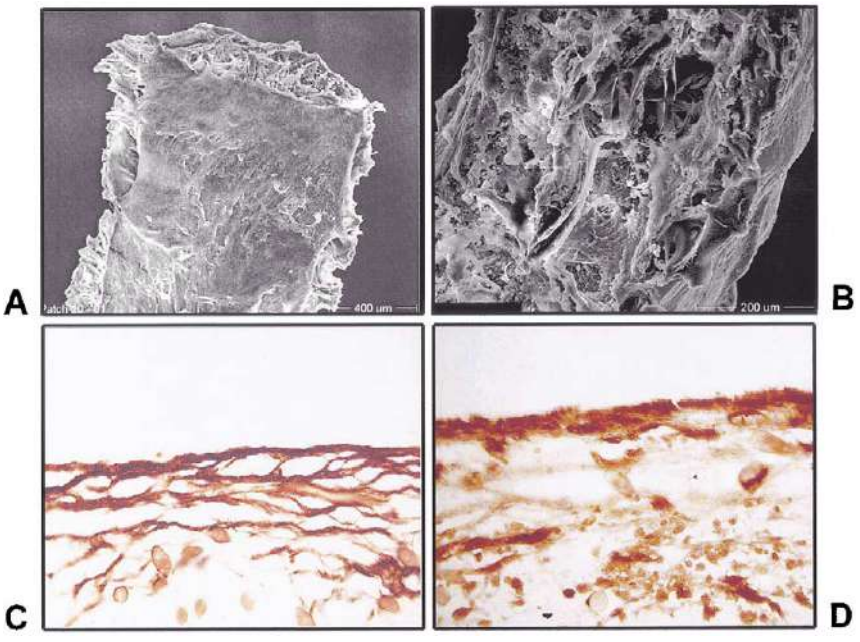

Fig 4. Scanning electron microscopy and immunohistochemistry of umbilical cord cell constructs. Scanning electron microscopy of sections of umbilical cord cell polymer constructs shows tissue formation with confluent surface (A). Cell attached well to the polymer and growth into deeper sections of patches. Degradation of scaffold was studied by multiple breakages and fragmentation of polymer fibers (B). Immunohistochemistry demonstrated deposition of collagen I (C) and III (D) in constructs.

nization and maturation compared to those in static controls [14, 18, 21]. After implantation of tissue-engineered heart valves in the pulmonary position in sheep for up to 20 weeks, Hoerstrup and colleagues [18] report development of a tissue structure strongly resembling that of native semilunar valve leaflets. Given these results, improved tissue formation and maturation of UCC-seeded constructs after exposure to an in vivo environment can be anticipated. Biomechanical stress testing revealed values of the tissue-engineered patches comparable to those of native tissue, which may allow future in vivo testing of UCC constructs in an animal model.

In the present study, the feasibility of using UCC as an alternative autologous cell source for the tissue engineering of cardiovascular constructs was demonstrated. Human UCC show typical myofibroblast characteristics with excellent cell growth. After seeding, cells attached well to the polymer scaffold and formed a layered, confluent tissue with a extracellular matrix staining positive for collagen I and III. Mechanical testing showed stress properties of the tissue-engineered constructs that were comparable to those of native cardiovascular tissue. The immaturity of the newly formed, growing tissue was reflected by an increased cell content in the presence of reduced amounts of extracellular matrix proteins as well as by increased stiffness of the tissue.

In conclusion, it appears that UCC represent an attractive cell source for use in cardiovascular tissue engineering by offering the possibility of working with juvenile cells and avoiding invasive harvesting of intact vascular structures.

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