Tissue Engineering of Functional Trileaflet Heart Valves From Human Marrow Stromal Cells

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Background—We previously demonstrated the successful tissue engineering and implantation of functioning autologous heart valves based on vascular-derived cells. Human marrow stromal cells (MSC) exhibit the potential to differentiate into multiple cell-lineages and can be easily obtained clinically. The feasibility of creating tissue engineered heart valves (TEHV) from MSC as an alternative cell source, and the impact of a biomimetic in vitro environment on tissue differentiation was investigated.

Methods and Results—Human MSC were isolated, expanded in culture, and characterized by flow-cytometry and immunohistochemistry. Trileaflet heart valves fabricated from rapidly bioabsorbable polymers were seeded with MSC and grown in vitro in a pulsatile-flow-bioreactor. Morphological characterization included histology and electron microscopy (EM). Extracellular matrix (ECM)-formation was analyzed by immunohistochemistry, ECM protein content (collagen, glycosaminoglycan) and cell proliferation (DNA) were biochemically quantified. Biomechanical evaluation was performed using Instron™. In all valves synchronous opening and closing was observed in the bioreactor. Flow-cytometry of MSC pre-seeding was positive for ASMA, vimentin, negative for CD 31, LDL, CD 14. Histology of the TEHV-leaflets demonstrated viable tissue and ECM formation. EM demonstrated cell elements typical of viable, secretionally active myofibroblasts (actin/myosin filaments, collagen fibrils, elastin) and confluent, homogenous tissue surfaces. Collagen types I, III, ASMA, and vimentin were detected in the TEHV-leaflets. Mechanical properties of the TEHV-leaflets were comparable to native tissue.

Conclusion—Generation of functional TEHV from human MSC was feasible utilizing a biomimetic in vitro environment. The neo-tissue showed morphological features and mechanical properties of human native-heart-valve tissue. The human MSC demonstrated characteristics of myofibroblast differentiation.

Key Words: tissue engineering ■ valves ■ cells ■ prosthesis
with natural valvular interstitial cells, qualities which might be vital to the development and long term function of TEHV. In search for alternative cell sources, especially with regard to future routine clinical realization of the tissue engineering concept, we identified human marrow stromal cells (MSC) as a promising candidate. The usage of MSC may offer several advantages in 1) easy collection by a simple bone marrow puncture avoiding the sacrifice of intact vascular structures, 2) showing the potential to differentiate into multiple cell lineages, and 3) demonstrating unique immunological characteristics allowing persistence in allogeneic settings. In the present study we investigated the feasibility of creating functional tissue engineered heart valves on the basis of human MSC and the influence of a biomimetic in vitro environment on tissue formation and cell differentiation.

Methods

Bioabsorbable Trileaflet Valve Scaffold

Nonwoven polyglycolic-acid mesh (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm³, Albany Int.) was coated with a thin layer of poly 4-hydroxybutyrate (P4HB, MW: 1·10⁶, Tepha Inc., Cambridge, MA) by dipping into a tetrahydrofuran solution (1% wt/vol P4HB). Following solvent evaporation, a continuous coating and physical bonding of adjacent fibers was achieved. P4HB is a biologically derived rapidly absorbable biopolymer which is strong, pliable, and thermoplastic (Tm 61 °C) so it can be molded into almost any shape. Complete biodegradation of the combined material occurs after 4 to 6 weeks. From the PGA/P4HB composite scaffold material trileaflet heart valve scaffolds were fabricated using a heat application welding technique. The constructs were then cold gas sterilized with ethylene oxide.

Cell Isolation and Cultivation

Human MSC were obtained by bone marrow punctures (iliac crest) from healthy individuals (n=5, mean age 35±5 years) after informed consent was obtained from each participant. Human MSC were isolated from 10–15 mL of bone marrow aspirate and resuspended in 20 mL Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone, Gibco), penicillin (Gibco), streptomycin (Gibco), and 1000 U heparin (Roche Pharma AG, Reinach, Switzerland). Following the cell suspension centrifugation over a Ficoll step gradient (density 1.077 g/mL) (Ficoll-Histopaque 1077, Sigma) at 1500 rpm for 10 minute. The interface fraction was collected and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone), penicillin (Gibco), streptomycin (Gibco), in tissue flasks (Corning, Inc). The nonadherent cells floated off while MSC adhered, spread, and grew. Medium was replaced at 24 and 72 hours and every 6 days following. Cells were serially passaged and expanded in a humidified incubator at 37 °C with 5% CO₂. Sufficient cell numbers for cell seeding on bioabsorbable polymer scaffolds were obtained after 21–28 days.

Cell Seeding and In Vitro Culture

Purified human MSC (4.5 to 5.5·10⁶ per cm³) were seeded onto the trileaflet valve scaffolds and cultured in static nutrient medium (DMEM, Gibco) for 7 days in a humidified incubator (37 °C, 5% CO₂). Two groups of seeded heart valve constructs were investigated. In group A, the constructs (n=5) were transferred into a pulse duplicator system (“bioreactor”) and grown under gradually increasing nutrient media flow and pressure conditions for additional 14 days (125 mL/min at 30 mm Hg (days 1 to 4); 250 mL/min at 40 mm Hg (days 5 to 7); 500 mL/min at 50 mm Hg (days 8 to 10); 750 mL/min at 75 mm Hg (days 11 to 14)). In group B (controls, n=5) the seeded heart valve constructs were grown in static nutrient media (absence of pulsed nutrient media flow) accordingly. The media was changed every 4 days.

Analysis of MSC Cultures and Trileaflet TEHV

Flow Cytometry

A single cell suspension of 0.5 to 1·10⁶ MSC in 100 μL PBS was incubated with saturating concentrations of monoclonal antibodies CD 31-FITC (Sigma, St. Louis), LDL-Dil (Biomedical Technologies Inc, Stoughton, MA), CD 14-FITC (Beckon Dickinson, San Jose, CA). For intracellular staining, cells were permeabilized with ethanol for 30 minute and incubated with monoclonal antibodies against ASMA (Sigma, St. Louis) and vimentin (NeoMarkers, Fremont). After washing, staining with a secondary FITC-conjugated IgG goat-anti-mouse antibody (Chemicon, Temecula, CA) was performed for 30 minute. Forward and side scatter gates were set to exclude debris and 10 000 gated events were counted per sample. Corresponding isotype and positive controls were performed for each antibody. Cells were analyzed with the flow cytometer FACS-Calibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) and data were analyzed 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

MSC cultivated onto glass coverslips were examined histologically by hematoxylin and eosin (H&E) and Trichrome-masson staining. In addition, immunohistochemistry was performed by incubation with monoclonal mouse antibodies for ASMA (Sigma, St. Louis), vimentin (NeoMarkers, Fremont), desmin (NeoMarkers, Fremont), collagen I, II, III, IV (Oncogen, Boston), and elastin (Sigma, St. Louis). After incubation with a secondary biotin-labeled goat-anti-mouse IgG antibody (Chemicon, Temecula, CA) was performed for 30 minute and incubated with saturating concentrations of monoclonal antibodies against ASMA (Sigma, St. Louis) and vimentin (NeoMarkers, Fremont). After washing, staining with a secondary FITC-conjugated goat-anti-mouse antibody (Chemicon, Temecula, CA) was performed for 30 minute. For intracellular staining, cells were permeabilized with ethanol for 30 minute and incubated with monoclonal antibodies against ASMA (Sigma, St. Louis) and vimentin (NeoMarkers, Fremont). Following the cell suspension centrifugation over a Ficoll step gradient (density 1.077 g/mL) (Ficoll-Histopaque 1077, Sigma) at 1500 rpm for 10 minute. The interface fraction was collected and cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (HyClone), penicillin (Gibco), streptomycin (Gibco), in tissue flasks (Corning, Inc). The nonadherent cells floated off while MSC adhered, spread, and grew. Medium was replaced at 24 and 72 hours and every 6 days following. Cells were serially passaged and expanded in a humidified incubator at 37 °C with 5% CO₂. Sufficient cell numbers for cell seeding on bioabsorbable polymer scaffolds were obtained after 21–28 days.

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static control sections. Tissue sections were stained using standard indirect immunoperoxidase avidin-biotin or immunofluorescence (FITC) techniques with monoclonal antibodies for α-smooth muscle actin (ASMA, Sigma, St. Louis), vimentin (NeoMarkers, Fremont), desmin (NeoMarkers, Fremont) as well as collagen I, II, III, IV (Oncogene, Boston) and elastin (Sigma, St. Louis).

**Scanning and Transmission Electron Microscopy**

Samples of each trileaflet valve construct were fixed in 2% glutaraldehyde (Sigma, St. Louis) for scanning electron microscopy (SEM) and transmission electron microscopy (TEM).

**Quantitative Biochemical Matrix Analysis**

As described previously, biochemical assays for total content of DNA, hydroxyprolin, proteoglycan/glycosaminoglycan (GAG) (BLYSCAN™ assay, Biocolor, Belfast, Ireland), and elastin (FAS-TIN™ assay, Biocolor, Belfast, Ireland) were performed and compared with native human tissue (semilunar valve).11

**Mechanical Properties**

Tissue engineered valve leaflet constructs and human native tissue valves were analyzed for mechanical properties by using a mechan-

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**Figure 2.** Flow cytometry analysis of MSC demonstrated no significant difference in the expression of ASMA (black) and vimentin (black) compared with vascular myofibroblasts (gray). No positive signals were detected for LDL (black) and CD31 (black) compared with endothelial cells (gray), and CD14 (black) compared with peripheral blood mononuclear cells (gray).
ical tester (Instron, Instron Corp., Canton, MA). Longitudinal matrix strips were used for the test. A 75 psig maximum load cell was used and the cross head speed was 0.5 inch per minute. The Young’s modulus was obtained from the slope of the initial linear section of the stress-strain curve.

Statistics

Result data were expressed as mean ± standard error of the mean. We used SPSS 8.0 software for statistical analysis. An unpaired t test (Student’s t test) was performed, considering a P<0.05 as statistically significant.

Results

Analysis of Isolated MSC

Morphology of the isolated cells initially appeared small and rounded with a tendency to grow in clusters. Nonadherent cells were removed by medium change at 24 hours and every 4 days thereafter. Elongated cells with fibroblast-like morphology appeared after 72 hours and reached confluence after 10–14 days (Figure 1a, b).

Immunohistochemistry of fixed cells showed the expression of ASMA and vimentin (Figure 1c, d). Staining for the deposition of collagen I and III showed positive signals. In contrast, no signal was observed following antibody staining for desmin, collagen II, IV, and elastin.

Flow Cytometry analysis of MSC demonstrates no significant difference in the expression of ASMA (MFIR 3.66) and vimentin (MFIR 12.59) compared with vascular-derived myofibroblasts. No positive signal was detected for CD 14 (MFIR 1.13), CD 31 (MFIR 1.1), and LDL (MFIR 1.94) among the isolated cell population (Figure 2).

Analysis of MSC-Based TEHV

In all TEHV a synchronous opening and closing of the leaflets was observed in the bioreactor. Before explantation form the pulse duplicator at day 14, the TEHV was tested in the transparent system under both low (0.125 L/min at 30 mm Hg for 60 minute) and high flow and pressure conditions (4.5 L/min at 200 mm Hg for 60 minute).
Histology and Immunohistochemistry of TEHV

Figure 5. Trichrome-masson staining of TEHV leaflets demonstrated ECM and organized layered tissue formation with a dense outer layer and less cellularity in the deeper portions (Fig. 5a). In contrast static controls showed less ECM deposition and less organized tissue formation (Fig. 5b). Immunohistochemistry showed the formation of collagen types I (Fig. 5c) and III (Fig. 5d) in the TEHV leaflet tips. Staining for ASMA (Fig. 5e) and vimentin (Fig. 5f) revealed positive signals throughout the TEHV leaflets.

appearance showed that all leaflets were intact, mobile, and pliable and the valve constructs were competent during valve closure (Figures 3 and 4).

Transmission and Scanning Microscopy

TEM revealed cell elements typical of viable, secretationally active myofibroblasts such as actin/myosin filaments as well as collagen fibrils and elastin fiber networks. SEM showed dense tissue formation and a confluent smooth surface with cell orientation in flow direction. Advanced biodegradation of the polymer scaffold was detected by multiple hydrolytic breakage and fragmentation of the polymer fibers. In contrast, SEM analysis of static controls showed a nonconfluent and less homogeneous surface (Figure 6).

Quantitative Biochemical Assays

The results of the quantitative biochemical assays are summarized in Figure 7. The extracellular matrix proteins of the TEHV showed significantly lower values compared with human native heart valve tissue (P<0.05). Collagen (hydroxyproline) content reached 25% and glycosaminoglycan content 37% that of the native human controls. There was no elastin detectable in the TEHV constructs. DNA content of the TEHV was significantly higher (P<0.01) compared with human native heart valve tissue reaching value of >300%.

Mechanical Properties

The biomechanical profile of the MSC-TEHV leaflets was comparable to those of native human semilunar valve tissue as to specific tensile strength (max. stress) and strain at maximum load (max. strain) (stress-strain curves, see Figure 8). Compared with the static controls, the MSC-TEHV leaflets were significantly stronger (max stress: 92±12% versus 15±2% of native human valve tissue, P<0.05) and less pliable (Young’s modulus: 139±14% versus 12±4% of native human valve tissue, P<0.05).

Discussion

As applied to the development of heart valve replacements, tissue engineering merges aspects of cell biology and engineering in an attempt to overcome the limitations of currently available valve options, such as thromboembolism in mechanical prostheses and structural dysfunction in biological heart valves.3–4 All clinically available valve substitutes basically represent nonviable structures and lack the potential to repair, to remodel, and to grow; the later imposing substantial problems specifically to the pediatric cardiac surgery patient population.5 The principal aim of heart valve tissue engineering—that is in vitro fabrication of living heart valves with a thromboresistant surface and a viable interstitium—has been demonstrated by considerable experimental work.6–11 Recently, our group reported the first successful tissue engineering of a completely autologous and living TEHV in a juvenile sheep model showing excellent functional performance and strong resemblance to natural heart valves as to morphological and biomechanical features.11 However, this and previous studies were based on the utilization of vascular derived cells (VC) having the disadvantage to necessitate the sacrifice of intact vascular structures of the donor organism. Moreover, VC demonstrated considerable differences compared with native valvular interstitial cells, qualities which might be vital to the development and long term function of TEHV.12

Given these problems it appeared that the ideal cell source for TEHV is still undetermined. Therefore, we investigated the feasibility to create functional TEHV on the basis of MSC, which are known to exhibit traits of multipotent cells. MSC can be routinely collected from patients, easily isolated from human bone marrow, and can be induced in vitro to differentiate into different mesenchymal cell types.13 In addition, there is evidence that these cells have unique immunological characteristics that allow persistence even in an allogenic environment.14
In the present study, isolation of the MSC was easy to perform. Initial cell morphology appeared small and rounded and cells started to grow in a colony-forming pattern followed by myofibroblast-like cell development. Identical morphological characteristics and growth pattern were described for mesenchymal precursor cells by other studies using a comparable isolation procedure. Characterization of the MSC populations before TEHV scaffold seeding revealed features of myofibroblast-like differentiation such as expression of ASMA, vimentin and the deposition of collagen types I and III. A similar staining pattern was reported for human valvular interstitial cells. Furthermore, desmin, CD 14, CD 31, and LDL were not detected indicating the absence of myeloid and endothelial cell differentiation. Collagen type II was also not observed, implying the absence of osteoblastoid differentiation of the MSC.

In vitro exposure of tissue engineered cardiovascular cell seeded constructs to biomimetic flow conditions has been demonstrated to significantly enhance tissue maturation and mechanical properties. Pulsatile flow or fluid dynamics have a well known impact on cell morphology, proliferation, and composition of extracellular matrix. We previously reported application of an in vitro pulse duplicator bioreactor system providing a gradually increasing flow and pressure environment to grow completely autologous implantable heart valves revealing the important impact of biomimetic in vitro conditioning on tissue maturation. Based on this experience with vascular-derived cells, we hypothesized that utilizing the pulse duplicator bioreactor to grow TEHV constructs based on human MSC might guide cell differentiation and tissue formation into the direction of native heart valve tissue.

In fact, after tissue culture in the bioreactor for 14 days, the MSC based valve constructs showed intact, mobile and pliable leaflets and functional competence during valve closure even under supra-physiological flow and pressure conditions. Histology of the TEHV leaflets revealed advanced biodegradation of the scaffold replaced by a viable tissue organized in a layered fashion with extracellular matrix proteins characteristic of heart valve tissue such as collagen I and III, and glycosaminoglycans. However, the typical three-layered structural composition of native valve leaflets comprising a ventricularis, spongiosa, and fibrosa layer was not achieved. ASMA positive cells expressing vimentin were detectable throughout the constructs demonstrating myofibroblast-like cell populations as described in native semilunar valve tissue. Myeloid, osteoblastoid, and endothelial cell differentiation of the isolated MSC was not observed in the neo-tissue. There was no positive staining for CD 14, collagen type II, CD 31, LDL, and desmin. The
ultra-structural analysis of the TEHV supported this observation demonstrating cell elements typical of viable, secretionally active myofibroblasts such as actin/myosin filaments as well as collagen fibrils and elastin.

The quantitative ECM protein analysis revealed values significantly lower compared with human native valve tissue. Concomitantly, the cell content was significantly increased, possibly reflecting the high cellular turn-over of growing tissue still in process to complete tissue development. Whether this process of tissue remodeling and maturation will be continuing under physiological conditions, as seen in previous animal studies, needs to be determined in future longer-term experiments. The histology of the valve constructs not exposed to pulsatile flow (static controls) showed a loose, less organized tissue formation and SEM demonstrated a less confluent and less homogeneous surface structure. Interestingly, there was no significant difference between the pulsed TEHV and the static controls as to the quantitative ECM analysis. This may emphasize the fact, that the biomimetic in vitro environment did not increase the absolute amount of matrix protein formation but rather influenced the degree of tissue organization and maturation. This would be in accordance with the observation that the mechanical properties of the controls showed significantly weaker tissue properties as to the pulsed valve constructs.

Compared with native human semilunar heart valves the mechanical evaluation of the pulsed MSC based TEHV demonstrated a strong resemblance in the biomechanical profiles, theoretically making these TEHV suitable for in vivo implantation.

In summary, the present study demonstrated the feasibility of creating functional TEHV on the basis of human MSC utilizing a biomimetic in vitro culture environment. The trileaflet TEHV showed mechanical properties and morphological features resembling native heart valves. MSC isolation was easily performed without necessitating the sacrifice of intact vascular structures. The MSC demonstrated characteristics of myofibroblast differentiation showing their possible progenitor potential. It appears that human MSC represent a promising cell source for cardiovascular tissue engineering purposes, especially in regard to future routine clinical application. However, these results are preliminary inasmuch as this study was limited to in vitro experiments aiming primarily at a proof of principle of this concept. Our next efforts are directed at optimization of the MSC isolation techniques as well as the in vitro culture conditions. Application of growth factors, growth inhibitors and optimized flow and pressure loading conditions will be areas of future studies.

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