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In Vitro Heart Valve Tissue Engineering

Dörthe Schmidt, Anita Mol, Jens M. Kelm, and Simon P. Hoerstrup

Summary

Heart valve replacement represents the most common surgical therapy for end-stage valvular heart diseases. A major drawback all contemporary heart valve replacements have in common is the lack of growth, repair, and remodeling capabilities. To overcome these limitations, the emerging field of tissue engineering is focusing on the in vitro generation of functional, living heart valve replacements. The basic approach uses starter matrices of either decellularized xenogeneic or biopolymeric materials configured in the shape of the heart valve and subsequent cell seeding. Moreover, in vitro strategies using mechanical loading in bioreactor systems have been developed to improve tissue maturation. This chapter gives a short overview of the current concepts and provides detailed methods for in vitro heart valve tissue engineering.

Key Words: Heart valves; Bioreactor; In vitro tissue maturation; Endothelial cells; Myofibroblasts; Stromal marrow cells; Endothelial progenitor cells; Scaffolds.

1. Introduction

Currently available heart valve prostheses for the treatment of advanced heart valve diseases represent non-living foreign material and therefore are inherently different from the tissue they replace. Thus, they are associated with substantial morbidity and mortality with regard to increased risks of thromboembolism, increased rates of infections, immunological reactions, and related prosthesis malfunctions. Heart valve tissue engineering represents a promising strategy to overcome today's lack of living autologous replacements with the capacity of growth, regeneration, and self-repair.

A series of studies have been undertaken to determine whether tissue engineering principles could be used to develop living valve substitutes with a thromboresistant surface and a viable interstitium with repair, remodeling,

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and growth capabilities. Several groups demonstrated the feasibility of creating living cardiovascular structures by cell seeding on synthetic polymers, collagen, or xenogeneic scaffolds *(1–4)*. As to heart valve tissue engineering, significant milestones comprise the successful replacement of a single pulmonary valve leaflet by a tissue-engineered autologous leaflet *(5)*, followed by in vitro-generated complete autologous trileaflet heart valves based on ovine *(3,6)* and human stromal marrow cells *(7, see* **Fig. 1***)*.

Two principle strategies have been developed to fabricate living autologous heart valve replacements. The first strategy requires an ex vivo phase generating the tissue-engineered heart valve by in vitro cell technologies *(8)*. The second strategy circumvents the in vitro tissue-culture phase by direct implantation of natural tissue-derived heart valve matrices for potential cell ingrowth and remodeling in vivo *(9)*. Matrices used for the latter approach include decellularized tissues derived from pericardium or valves, cell-free porcine small intestine submucosa *(9)*, or synthetic biodegradable polymeric scaffolds, such as collagen or fibrin gels. Although first clinical trials have been initiated *(10,11)* and decellularized scaffolds implanted in humans have demonstrated ingrowth of host cells, a strong inflammatory response and fatal valve failures were reported *(12)*.

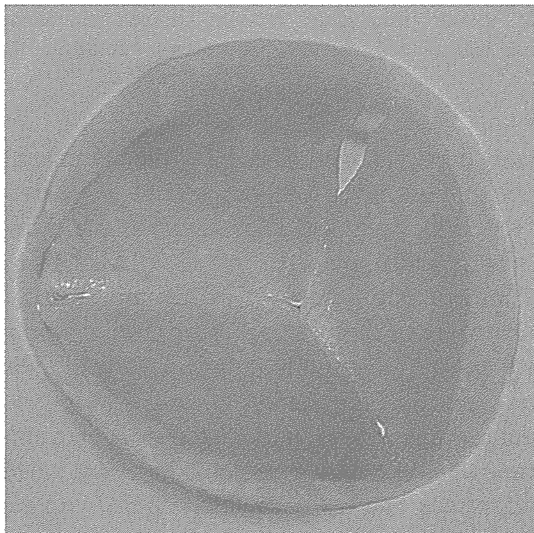

Fig. 1. Heart valve tissue engineered from human marrow stromal cells *(7)*.

The process of in vitro engineering of heart valves (*see* **Fig. 2**) requires the harvest of autologous cells. After isolation, cells are expanded in vitro. After a sufficient number of cells for the tissue engineering approach are obtained, cells are seeded onto biodegradable heart valve scaffolds. To guide tissue maturation and formation, the seeded constructs are implanted into a bioreactor system, applying mechanical stimulation to the growing tissues. Mimicking a physiological environment with regard to flow and pressure in vitro has been shown to result in improved tissue maturation and implantable autologous heart valve *(3,13,14)*.

2. Materials

2.1. Cell Culture

1. Medium for myofibroblasts: Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Invitrogen) and gentamycin (50 μg/ml; Pan Biotech GmbH, Aidenbach, Germany).
2. Medium for endothelial cells: Endothelial basal medium (EBM™-2; Cambrex, Walkersville, MD) containing vascular endothelial growth factor (VEGF), human fibroblast growth factor (hFGF), human recombinant long-insulin-like growth factor-1 (R-3-IGF-1), human epidermal growth factor (hEGF), gentamycin and amphotericin (GA-1000), hydrocortisone, heparin, ascorbic acid (all form Cambrex), and 20% FBS (Invitrogen).

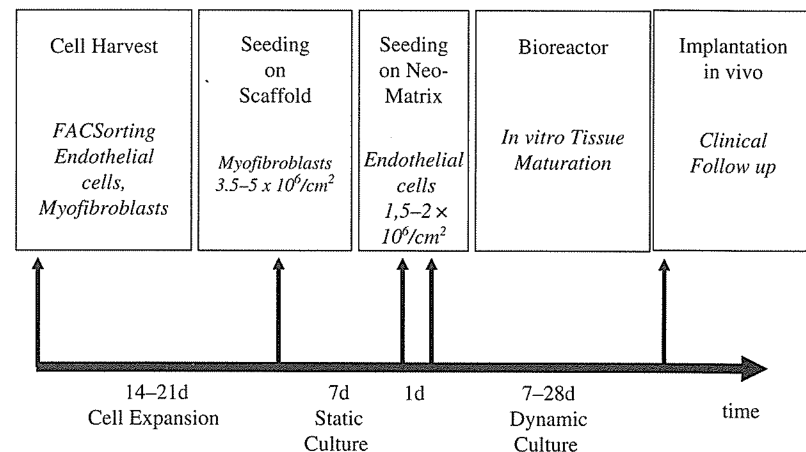

Fig. 2. Schematic of the in vitro protocol for generating heart valves.

3. Medium for bone marrow cell isolation: DMEM (Invitrogen) supplemented with 10% FBS, streptomycin (Invitrogen) and 1000 U heparin (Roche Pharma AG, Reinach, Switzerland).
4. Density gradient for isolation of umbilical cord blood-derived cells or bone marrow-derived cells: Ficoll–Histopaque-1077 (Sigma Chemical Company, St. Louis, MO).
5. Washing solution for tissue biopsies: Washing solution based on phosphate buffer solution (PBS; Kantonsapotheke, Zurich, Switzerland) containing Genatmycin 200 μg/ml (Pan Biotech GmbH) and Amphotericin B 2.5 μg/ml (Pan Biotech GmbH).
6. Collagenase solution for isolation of endothelial cells from vessels: Endothelial basal medium (EBM™-2; Cambrex) containing collagenase (final concentration 2 mg/ml; Sigma Chemical Company).

2.2. Characterization of Cells by Flow Cytometry

1. Fluorescence Activated Cell Sorting (FACS) buffer: PBS (Kantonsapotheke) containing 2% bovine serum albumin.
2. Primary antibodies against CD31 (clone JC/70A), von Willebrand factor (vWF; affinity-purified rabbit antibodies), vimentin (clone 3B4), desmin (clone D33; all from DakoCytomation, Glostrup, Denmark), α-smooth muscle actin (α-SMA; clone Sigma Chemical Company), CD34 (AC136; Miltenyi, Bergisch Gladbach, Germany), and secondary antibody (Fluorescein isothio-cyanate-conjugated (FITC); BD Bioscience, San Jose, CA).
3. 4% formalin (Kantonsapotheke).
4. 0.1% Triton-X 100 solution (Sigma Chemical Company).
5. 5 ml polystyrene round-bottom tubes (Becton Dickinson Labware, Franklin Lakes, NJ).
6. FacsScan (Becton Dickinson, Sunnyvale, CA).

2.3. Fabrication of Heart Valves

1. Heart valve scaffolds (*see* **note 1**).
2. Single-cell solution of myofibroblasts containing $3.5–5 \times 10^6$ cells/cm^2 scaffold.
3. Endothelial cell solution containing $1.5–2 \times 10^6$ cells/cm^2 scaffold.
4. Neubauer counting chamber (Brand, Germany).
5. DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen) and gentamycin (50 μg/ml; Pan Biotech GmbH).
6. Polystyrene 6-well flat-bottom culture plates (Costar 3516, Cambridge, MA).

2.4. In Vitro Maturation of Heart Valve Tissue

1. Biomimetic system (*see* **note 2**).
2. Culture medium for the tissue maturation with special regard to the development of extracellular matrix is DMEM (Invitrogen) supplemented with 10% FBS (Invitrogen), gentamycin (50 μg/ml; Pan Biotech GmbH) and ascorbic acid (0.25 mg/ml medium; Sigma Chemical Company).

3. Methods

3.1. Cell Culture

Several alternative human cell sources have been investigated for their use in heart valve tissue engineering. Among the most promising are vascular-derived cells, bone marrow-derived cells, blood-derived cells, and umbilical cord-derived cells (*see* **Fig. 3**), particularly for pediatric applications. In most cardiovascular tissue engineering approaches, cells are harvested from vascular structures, such as mammary, radial artery, and saphenous vein ***(15)***.

The obtained mixed-cell population consisting of myofibroblasts and endothelial cells is sorted by fluorescence-activated cell sorting, and the pure-cell populations are cultivated and used for the fabrication of the replacements ***(16)***. Alternatively, to detach the endothelium from the luminal layer, the vessel biopsies are digested with collagenase, and the endothelial cells are harvested. Afterward, the tissue is minced into small pieces, and outgrowing myofibroblasts are cultured.

3.1.1. Isolation of Human Myofibroblasts

1. The vessel tissue is minced into several pieces (~3 mm × 3 mm).
2. Five to seven tissue pieces are placed into a cell-culture dish (3.5 cm in diameter) and cultured in DMEM medium containing 10% FBS and gentamycin.
3. Outgrowing cells are expanded using standard cell-culture protocols.

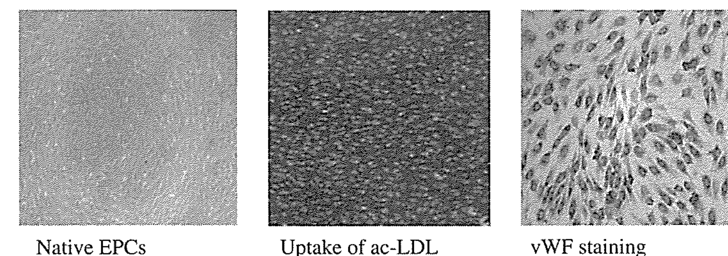

Fig. 3. Human endothelial progenitor cells isolated from human umbilical cord blood form cobblestone monolayers after 21 days demonstrate typical characteristics of endothelial cells ***(24)***.

3.1.2. Human Endothelial Cell Isolation

3.1.2.1. Isolation of Human Endothelial Cells from Human Vessels

1. Directly after vessel harvest, the biopsies are rinsed with the washing solution.
2. Afterward, one end of the vessel is closed using clamps, and the collagenase solution is filled into the lumen of the vessel. Afterward, the other opening is closed as well.
3. After 20 min of incubation at 37°C in a humidified incubator (5% CO_2), clamps are removed for liberating the cell suspension containing the endothelial cells.
4. The cell suspension is collected and transferred into a centrifugation tube for centrifugation at $30 \times g$ for 8 min at room temperature.
5. After the supernatant is removed carefully, the cell pellet is resuspended, and the isolated cells are expanded in EBM-2 medium containing the supplements, growth factors, and 20% FBS.

3.1.2.2. Isolation of Human Umbilical Cord Blood-Derived Endothelial Progenitor Cells

1. Obtained fresh human umbilical cord blood (~20 ml) has to be layered onto 15 ml density gradient that is placed in a 50-ml centrifugation tube.
2. After centrifugation for 30 min at room temperature, endothelial progenitor cells (EPCs) are isolated from mononuclear cells using antibodies against CD34 and FACS sorting.
3. Isolated cells are cultured in endothelial medium containing the above-mentioned growth factors and supplements but 2% FBS using 6-well plates.
4. After 4 days, attached cells are trypsinized and reseeded, and after 7 days, the medium is changed to endothelial medium containing the same growth factors but 20% FBS (*see* **note 3**).

3.1.3. Isolation of Human Bone Marrow-Derived Cells

1. Human marrow stromal cells are isolated from 10–15 ml bone marrow aspirate (e.g., from iliaca crest) and resuspended in 20 ml bone marrow cell isolation medium.
2. Following the cell suspension is centrifuged over a density gradient at 1500 rpm (100g) for 10 min.
3. The interface fraction is collected and cultured into medium for myofibroblasts (*see* **note 4**).
4. Medium has to be replaced after 24 and 72 h and every further 6 days.

3.2. Characterization of Cells by Flow Cytometry

Isolated cells are characterized using flow cytometry. Therefore, cells are detached from the culture flasks using trypsin.

1. About 500,000 detached cells are needed per sample. All samples are stored at 4°C following trypsinization and placed in a 5 ml polystyrene round-bottom tube.

2. For detection of intracellular antigens, cells are fixed and permeabilized. Therefore, 2 ml 4% formalin is slowly added and cells are left at room temperature for 30 min (*see* **note 5**).
3. After fixation, cells are centrifuged and resuspended in 100 µl 0.1% Triton-X 100.
4. After incubation for 3–5 min at room temperature, cells are washed with 1 ml ddH_2O and centrifuged at $250 \times g$ for 6 min. Afterward, the supernatant is removed.
5. Cells are resuspended in the primary antibody solution (primary antibody is diluted 1:100 in 100 µl FACS buffer).
6. After incubating for 30 min, 1 ml FACS buffer is added, and the sample is centrifuged at $250 \times g$ for 6 min.
7. As the primary antibody is not directly labeled, steps 6 and 7 have to be repeated using the FITC-labeled secondary antibody (dilution, 1:100) before FACS analysis can be performed on the FacsScan.

3.3. Fabrication of Heart Valves

For the fabrication of heart valves, sterilized scaffolds (*see* **Fig. 4**) are seeded with cells. The seeding procedure is performed sequentially.

1. Human myofibroblasts are trypsinized from the culture flasks, wherein they have been expanded and suspended to create a single-cell solution.
2. The cell number of the solution is determined by direct counting using the Neubauer counting chamber.
3. The cell suspension is centrifuged at 1000 rpm (100g) at 20°C for 5 min, the supernatant is removed, and fresh culture medium is added. The cell concentration has to

Fig. 4. Stented heart valve based on a non-woven PGA coated with 1% poly-4-hydroxybutyrate (P4HB) *(17)*.

be adjusted to the surface area ($3.5–5 \times 10^6$ cells resuspended in $50\,\mu l$ medium/cm^2 scaffold).

4. Seeding is performed in polystyrene 6-well flat-bottom culture plates (Costar 3516).
5. Therefore, scaffolds are placed in the center of the wells, and the cells are evenly distributed into the scaffolds.
6. Myofibroblasts are seeded into the constructs that are statically cultured followed by endothelial cell seeding after 4 days.
7. After 2 h culturing in a humidified incubator (37°C, 5% CO_2) that allows attachment of the cells to the scaffolds, 5 ml medium/well is added for further culture.
8. After 4 days, endothelial cells are trypsinized and seeded into the constructs using the same seeding protocol, but $1.5–2 \times 10^6$ cells are resuspended in $50\,\mu l$ medium/cm^2 scaffold.

3.4. Maturation of Heart Valve Tissue

Tissue maturation of the heart valves can be performed in vitro using biomimetic systems (*see* **Fig. 5**). After a static culture phase, seeded constructs are implanted into the bioreactor and conditioned for 14 days. The bioreactor protocol depends on the requirements that the generated tissue has to match in vivo. For instance, maturation of heart valves for application in a high-pressure system requires other components of the extracellular matrix compared with those for low-pressure systems. Thus, the conditioning protocol has to be adapted to the needs of tissue components. The diastolic pulse duplicator *(17)*, for example, was developed to apply increasing strain onto the growing tissue during culture time (*see* **Fig. 5**), aiming at valve substitutes for systemic pressure application.

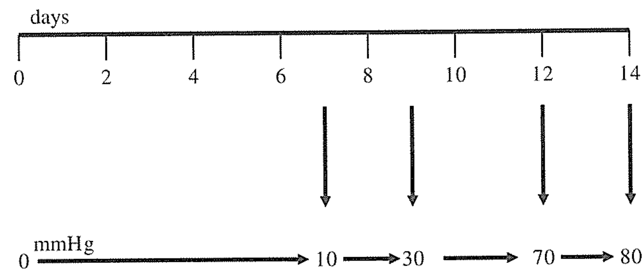

Fig. 5. Conditioning protocol for tissue maturation in the diastolic pulse duplicator based on Mol et al. *(17)*. Dynamic transvalvular pressure increases gradually from 0 to 80 mmHg within 14 days of culturing.

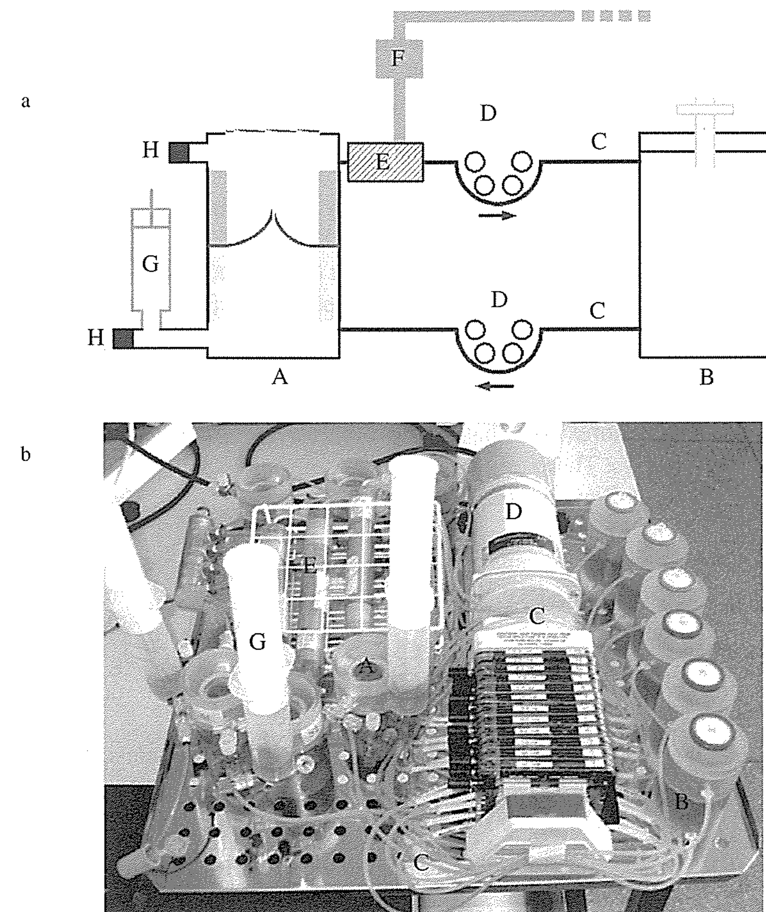

Fig. 6. Example of a bioreactor *(17)*. (a) The schematic drawing and its function of one diastolic pulse duplicator and (b) a photograph of six systems used simultaneously. The system consists of a bioreactor (A), in which the valve is implanted, and a medium container (B). These are connected through two parallel tubing series (C) running through a roller pump (D). For mechanical stimulation, a tube is placed in a polycarbonate cylinder (E) in which compressed air is released through a magnetic valve (F) resulting in compressing and decompressing the tubing and therewith in a pressure difference over the heart valve leaflets. A syringe (G) serves as a compliance chamber. The pressure difference across the leaflets is monitored using pressure sensors (H).

Notes

1. Heart valve scaffolds can be produced based on several matrices including synthetic and biological. Several synthetic biodegradable polymers, such as polyglactin *(1)*, polyglycolic acid (PGA) *(5)*, polylactid acid (PLA) *(18)*, polyhydroxyalkanoates (PHA) *(19)*, and a copolymer of PGA and PLA (PGLA) *(20)*, have been investigated. Another approach for fabricating complete, trileaflet heart valves is the use of PGA coated with poly-4-hydroxybutyrate (P4HB) *(3,7)*. This biologically derived and rapidly absorbable copolymer is strong and pliable. Owing to its thermoplasticity, it can be molded into almost any shape, and complete degradation has been observed within 8 weeks after implantation *(3)*. Other studies in heart valve tissue engineering were based on biological scaffolds such as xenogenic or allogeneic decellularized fixed heart valves *(21)*.
2. Tissue formation of the seeded heart valves takes place in a biomimetic in vitro culture system (bioreactor) that mimics the physiological environment of their native counterparts. Therefore, different types of bioreactors have been developed, such as pulsatile flow reactors (*13,14,22*) or diastolic pulse duplicators *(17)* as shown in Fig. 6.
3. After isolation, the plated cells are initially rounded. After 4 days, cells are attached and they form clusters. Two different types of EPC can be found: spindle like-shaped cells (80%) and polymorph cells (20%). Spindle-like cells die out during the first days, whereas polymorph EPCs form colonies and show good growth under in vitro conditions. After 3 weeks, they differentiate into mature endothelial cells forming cobblestone monolayers (*23,24*).
4. The non-adherent cells float off, whereas marrow stromal cells adhere, spread, and grow.
5. Antibodies against CD31 or vWF are used for endothelial cell detection and CD34 for progenitor cells. Myofibroblasts are characterized by the intracellular antigens vimentin, α-SMA, and desmin.

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