Autologous Heart Valve

Tissue Engineering

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Autologous Heart Valve Tissue Engineering
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SUMMARY

Valvular heart disease is a significant cause of morbidity and mortality worldwide. Although today's valve replacement surgery is efficacious, the state-of-the-art valve prostheses used clinically have substantial limitations. These include the necessity for life-long anticoagulation therapy associated with the risk of bleeding or thromboembolism as to mechanical valve prostheses, and structural dysfunction due to progressive tissue deterioration with regard to chemically fixed biological valve prostheses. Since all currently available artificial valves cannot grow with pediatric patients, repeated replacement operations have to be performed associated with exponentially increased morbidity and mortality.

In order to overcome the limitations of contemporary heart valve prostheses, different tissue engineering concepts using various cell sources and scaffold materials have been introduced over the last 10 years. The ultimate goal of tissue engineering is to construct tissues from their cellular components which comprise the characteristics of the healthy native original. It is the perfection of architecture and function of a native, living heart valve which enables the enormous life-time performance with billions of cycles without malfunctions. For a functional engineered heart valve, besides adequate mechanical characteristics (mature extracellular matrix) and durability, the absence of immunogenic and/or inflammatory reactions is of critical importance. This goal can be achieved based on a completely autologous tissue engineering principle, also referred to as the “complete autologous heart valve tissue engineering paradigm”. The success of this paradigm is depending on three main components: (a) a biocompatible and rapidly biodegradable matrix (scaffold) which determines the three-dimensional shape and serves as an initial guiding structure for cell attachment and tissue development; (b) a cell source from which a native-analogous living tissue can be grown and which can be harvested from the future recipient without sacrificing critical anatomic structures; and (c) in vitro culture conditions which enable adequate neo-tissue formation and maturation resulting in implantable, living, autologous heart valve substitutes.

The sequence of studies comprised in this thesis addresses the principal prerequisites for the realization of the autologous heart valve tissue engineering paradigm. Chapter 2 introduces the concept of exposing tissue engineered heart valves to “biomimetic signals” and “mechanical conditioning” in vitro by using a newly developed flow bioreactor. This approach is based on the hypothesis, that in vitro exposure of the developing tissue to physical signals similar to those encountered in vivo may result in more mature engineered heart valves. In combination with a novel composite scaffold material enabling the fabrication of complex, three dimensional heart valve scaffolds, the first functional, completely autologous, tri-leaflet heart valves are demonstrated in a large animal study (chapter 3). Interestingly, the ultimate refinement and
maturation of the engineered heart valves occurred during the subsequent in vivo period of this experiment, showing an evolution of cell phenotype and extracellular matrix towards native valve tissue. This remodeling is investigated in detail in chapter 4, providing a more accurate understanding of the neo-tissue changes prior and after implantation. Based on the results of this proof of principle animal study suitable cell sources for potential human applications are investigated. Since vascular derived autologous human cells necessarily the sacrifice of intact vascular donor structures, less invasive alternative cell sources including progenitor-type cells are evaluated. For adult applications the use of human marrow stromal cells for tissue engineering of trileaflet heart valves is described in chapter 5. These cells can be routinely obtained by a simple puncture of the iliac crest representing a less invasive cell source with the potential to differentiate into various tissues. The highest medical need for a tissue engineered heart valve is in pediatric applications for the treatment of congenital cardiovascular malformations. Approximately 1% of all newborns have congenital heart defects, and many of them require open heart surgery and heart valve replacement. Since currently available artificial valve prostheses cannot grow with the young patients, repeated replacement operations have to be performed associated with exponentially increased morbidity and mortality. In chapter 6, the human umbilical cord is investigated as a readily available autologous cell source for tissue engineering, enabling the in vitro generation of living replacement materials short after birth. Ideally, a tissue engineered heart valve would be available for implantation already at the time of birth in order to prevent secondary damage to the immature ventricle. This requires cell harvest prior to birth, e.g. by ultrasound guided cordocentesis (puncture of the umbilical cord in utero). To validate this concept, umbilical cord blood derived human endothelial progenitor cells are isolated, expanded in vitro and their suitability for the in vitro engineering of living patches is demonstrated (chapter 7).

In conclusion, the results of this thesis indicate that the biomimetic approach represents a critical element for autologous heart valve tissue engineering. The cells need to be placed into the appropriate “environmental niche” to produce functional neo-tissues in vitro. It is demonstrated that this process can be initiated by bioreactors. However, so far the ultimate tissue refinement is occurring in vivo. Furthermore, various cell sources including human progenitor cells can be used for heart valve tissue engineering. Cells which can be harvested prenatally may enable the engineering of living autologous replacements for the correction of congenital heart defects ready to use at the time of birth.
waarbij het fenotype van de cellen en de compositie van de extracellulaire matrix steeds meer gaan lijken op die in de natuurlijke hartklep. Deze remodellering staat beschreven in hoofdstuk 4 en verschaf inzicht in de weefelsamenstelling van de gekweekte weefsels en de veranderingen die na implantatie optreden. Op basis van de succesvolle resultaten van de dierstudie ('proof of principle') wordt er naar geschikte celbronnen voor toekomstige toepassingen in mensen gezocht. Het verkrijgen van cellen uit de bloedvatwand vereist interventie in het intacte orgaan en er zullen dus alternatieve celbronnen gevonden moeten worden, die vaster en invasieve wijzen te verkrijgen zijn. Voor toepassing in volwassenen wordt het gebruik van beemergcellen geëvalueerd voor tissue engineering van hartkleppen, zoals beschreven in hoofdstuk 5. Deze cellen kunnen routinematig verkregen worden door middel van een punctie in het bekken en reprenteren dus een minder invasieve, alternatieve celbron met de mogelijkheid tot differentiatie in verschillende weefseltypen. De grootste behoefte aan tissue-engineered hartklepprotheses bestaat voor kinderen ter behandeling van aangeboren afwijkingen. Ongeveer 1% van alle pasgeboren hebben een hartafwijking en veel van hen hebben open-hart operaties nodig met hartklep vervanging. Omdat de huidige hartklepprotheses niet meegroeien met deze jonge patiënten, zullen zij meerdere operaties nodig die veel risico’s met zich meebrengen. In hoofdstuk 6 wordt de navelstreng geëvalueerd als mogelijke beschikbare celbron voor tissue engineering van hartkleppen, waarmee hartkleppen vervaardigd kunnen worden vlak na de geboorte. Echter, in het ideale geval zou er een autologe hartklep klaar moeten liggen bij de geboorte, zodat secundaire schade aan het ventrikel voorkomen kan worden. De cellen dienen dan voor de geboorte verkregen te worden, bijvoorbeeld door middel van echografie-gestuurde punctie van de navelstreng in de baarmoeder. Om dit concept te valideren zijn er endothel progenitor cellen geïsoleerd uit navelstreng bloed en vermenigvuldigd in vitro. Tissue engineering van patches met deze cellen is geëvalueerd, zoals beschreven in hoofdstuk 7. De resultaten beheersen dit proefschrift tonen aan dat de biomimetische aanpak een belangrijk element is voor autoloog tissue engineering van hartkleppen. De cellen moeten in de juiste omgeving geplaatst worden om functionele weefsels te kunnen vormen in vitro. Hiervoor worden bioreactoren gebruikt. De uiteindelijke verfijning van het weefsel vindt plaats na implantatie. Verder beschrijft dit proefschrift verschillende menselijke celbronnen, onder meer progenitor cellen, die gebruikt kunnen worden voor tissue engineering van hartkleppen. Cellen die verkregen kunnen worden voor de geboorte maken het mogelijk om levende autoloog weefsels te fabriceren voor de correctie van aangeboren hartafwijkingen direct na de geboorte.

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AEC</td>
<td>3-amino-9-ethyl carbazole</td>
</tr>
<tr>
<td>α-SMA</td>
<td>Alpha-smooth muscle actin</td>
</tr>
<tr>
<td>CD31/PECAM-1</td>
<td>Platelet endothelial cell adhesion molecule</td>
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<tr>
<td>EBM-2</td>
<td>Endothelial basal medium</td>
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<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
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<tr>
<td>ESEM</td>
<td>Environmental scanning electron microscopy</td>
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<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and Eosin</td>
</tr>
<tr>
<td>H&amp;S</td>
<td>Hematoxylin-Sudan</td>
</tr>
<tr>
<td>hEGF</td>
<td>Human epidermal growth factor</td>
</tr>
<tr>
<td>hFGF</td>
<td>Human fibroblast growth factor</td>
</tr>
<tr>
<td>Hum.PA</td>
<td>Human pulmonary artery</td>
</tr>
<tr>
<td>hUCC</td>
<td>Human umbilical cord cell</td>
</tr>
<tr>
<td>GAG</td>
<td>Glycosaminoglycan</td>
</tr>
<tr>
<td>MFIR</td>
<td>Mean fluorescence intensity ratio</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrixmetallo-proteinase</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>PERV</td>
<td>Porcine endogenous retrovirus</td>
</tr>
<tr>
<td>P4HB</td>
<td>Poly-4-hydroxybutyrate</td>
</tr>
<tr>
<td>PGA</td>
<td>Polylactic acid</td>
</tr>
<tr>
<td>PHA</td>
<td>Polyhydroxyalkanoates</td>
</tr>
<tr>
<td>PHO</td>
<td>Polyhydroxyoctanoate</td>
</tr>
<tr>
<td>PLA</td>
<td>Polyactic acid</td>
</tr>
<tr>
<td>PLGA</td>
<td>Copolymer of PGA and PLA</td>
</tr>
<tr>
<td>PMMA</td>
<td>Polymethylmetacrylate</td>
</tr>
<tr>
<td>R3-IGF</td>
<td>Human recombinant long-Insulin-like growth factor</td>
</tr>
<tr>
<td>SEM</td>
<td>Scanning electron microscopy</td>
</tr>
<tr>
<td>TE</td>
<td>Tissue engineering</td>
</tr>
<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
</tr>
<tr>
<td>TEHV</td>
<td>Tissue engineered heart valves</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Transforming growth factor beta</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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</table>
The contents of this chapter are partly based on


1.1 THE IDEAL HEART VALVE SUBSTITUTE - DWIGHT E. HARKENS “TEN COMMANDMENTS”

1.1.1 Valvular Heart Disease and State of the Art

Valvular heart disease is a significant cause of morbidity and mortality worldwide. In the United States, approximately 20,000 people die annually as a direct result of valvular dysfunction and 60,000 valve replacement operations are performed (Schoen 1997). Valve replacement surgery is efficacious, and the state-of-the-art valves used clinically include mechanical valves and biological valves (Fig. 1A, B).

1.1.2 Mechanical Valves

The major drawback of mechanical valves relates to the fact that these prostheses represent foreign materials, associated with the risk of infections and thromboembolic complications. To prevent thromboembolism, a life-long anticoagulation therapy is required (e.g. warfarin), showing a substantial risk of haemorrhagic or thromboembolic incidences (Hammermeister et al. 1993, Vongpatanasin et al. 1996). Apart from this, additional problems may occur, e.g. in young fertile females because of the embryo toxicity of warfarin and related substances.

1.1.3 Biological Valves

The majority of biological valve prostheses are either glutaraldehyde fixed xenografts (derived from animals) or cryopreserved homografts (derived from human donors). Glutaraldehyde-fixed or cryopreserved biological valves do not require anticoagulation medication, however, they represent non-viable prostheses suffering from structural dysfunction due to progressive tissue deterioration (Hammermeister et al. 1993, Schmidt and Baier 2000). The majority of biological valves, therefore necessitates re-replacement within 10-15 years, and because of a higher immunological competence their durability is even less in younger individuals.

1.1.4 Implications for Pediatric Cardiac Surgery

All clinically available valve prostheses basically represent non-viable structures and lack the ability to grow, to repair, or to remodel. This imposes severe problems specifically on paediatric patients. Approximately 1% of all newborns have congenital heart defects, and many of them require open heart surgery including heart valve replacement. Since currently available artificial valve prostheses cannot grow with the young patients, repeated replacement operations have to be performed associated with exponentially increased morbidity and mortality (Mayer 1995).

1.1.5 Requirements for the Ideal Heart Valve Substitute

The essential characteristics of ideal heart valve substitutes have been described already in the 1950th by Dwight E. Harken, a pioneer in heart valve surgery, and summarized as the so-called “Ten Commandments” (Harken 1989). These include durability, absence of thrombogenicity, resistance to infections, lack of antigenicity, and the potential of growth. In principle, he stated the fundamental properties of natural, living, and autologous tissues. Unfortunately, these requirements are still not met by today’s heart valve prostheses.

1.2 TISSUE ENGINEERING OF HEART VALVES

1.2.1 Overview

In order to overcome the limitations of today’s heart valve prostheses and to approximate the ideal substitute according to the commandments of Harken, researchers have developed different tissue engineering (TE) concepts using various cell sources and scaffold materials. The ultimate goal of TE is to construct tissues from their cellular components which combine most of the characteristics of the healthy native original. For a functional heart valve these
failure of the heart valves due to severe foreign body type reactions associated with a 75% mortality (Simon et al. 2003). In a further approach, specific biological matrix constituents can be used as scaffold material including collagens and fibrins (Rothenburger et al. 2002, Lee et al. 2001). These materials have the disadvantage that they are difficult to obtain from the patients in sufficient quantities. Therefore, most of these scaffolds are of animal origin. However, identification of retroviruses and prion diseases has given rise to great concern as to the risk of zoonoses. Recently, epidemiological data strongly indicates transfer of Creutzfeldt-Jakob disease from cattle onto humans via infected meat, surgical materials derived from bovine gut and drugs or vaccines prepared using fetal calf serum (Knight and Collins 2001). Porcine endogenous retroviruses (PERV) can be present in many tissues as multiple copies of PERV can be integrated into germ-line DNA. New and more infectious groups of PERV are being identified (Patience et al. 2001), as well as their capacity to infect various types of human cells in vitro (Martin et al. 1998). The use of synthetic materials as scaffolds has already been broadly demonstrated for cardiovascular tissue engineering. Initial attempts to create single heart valve leaflets were based on synthetic scaffolds, such as polylactic acid (PLA), polylactic acid (PLGA), and polyglycolic acid (PGA) (Shinoka et al. 1995, 1996). To create complete trileaflet heart valve conduits, PHA based materials (polyhydroxyalkanoates) were used (Sodian et al. 2000). These materials are thermoplastic and can therefore easily be molded into any desired three dimensional shapes. Recently, a combined polymer scaffold consisting of non-woven PGA and PHA showing promising in vivo results was introduced (Hoerstrup et al. 2000). The cells for the tissue engineering process can be isolated from donor tissues and subsequently are expanded in vitro to obtain sufficient quantities for the seeding of the heart valve scaffolds. Thereafter, the seeded constructs are grown in vitro resulting in the formation of valve-analogous neo-tissues (Zund et al. 1998). So far, most of the heart valve tissue engineering studies were based on the utilization of vascular derived cells. With regard to clinical applications, alternative human cell sources have been investigated (Schnell et al. 2001) including cells derived from bone marrow or umbilical cord (Hoerstrup et al. 2002a, Hoerstrup et al. 2002b). In contrast to vascular cells, these cells can be obtained without surgical interventions representing an easy-to-access cell source in a possible routine clinical scenario. Due to their good proliferation and progenitor qualities, these cells are expected to be an attractive alternative for cardiovascular tissue engineering applications.

1.2.2 Scaffold Materials

Synthetic polymer matrices

Synthetic biodegradable polymers such as polylactin (Shinoka et al. 1995), PGA (polylactic acid) (Shinoka et al. 1996), PLA (polylactic acid), and PLGA (copolymer of PGA and PLA) (Zund et al. 1997) have already been demonstrated to be useful in cardiovascular tissue engineering. These materials
Recently, a combined polymer scaffold consisting of non-woven PGA coated with P4HB (poly-4-hydroxybutyrate) was introduced. The combination of these polymers provides thermo-plasticity allowing moulding the scaffolds into complex three-dimensional shapes such as trileaflet heart valves (Hoerstrup et al. 2000, Hoerstrup et al. 2002). A major advantage of synthetic matrices is the fact that their biodegradation properties and biomechanics can be chemically designed according to the requirements of the particular application. Moreover, specific proteins such as growth factors may be incorporated into the scaffold structure enabling targeted promotion of specific tissue growth (Hubbell et al. 1991, Zisch et al. 2003).

**Biological matrices**

The extracellular matrix (ECM) is a complex mixture of structural and functional proteins, glycoproteins and proteoglycans arranged in a unique, tissue-specific three-dimensional ultrastructure. It serves as structural support, attachment sites for cell surface receptors, and as a reservoir for signalling factors, which modulate biological processes such as angiogenesis, vasculogenesis, cell migration and proliferation. In this manner, the ECM may serve as an appropriate scaffold for tissue repair and reconstruction (Badyylak 2002).

**Native, decellularised reseeded biological matrices**

Decellularised homo- or xenografts have successfully been reseeded with various cell types including human vascular endothelial cells and neonatal dermal fibroblasts (Bader et al. 1998, Zeltinger et al. 2001). Steinhoff and colleagues seeded autologous myofibroblast and endothelial cells on allogenic decellularised heart valve conduits and implanted them in sheep. Other studies tested decellularised porcine pulmonary valves reseeded with autologous endothelial cells also in a sheep model. This group initiated clinical studies using decellularised allografts seeded with autologous endothelial cells. The grafts were used to reconstruct the right ventricular outflow tract during aortic valve replacement according to the Ross technique (Dohmen et al. 2002).

**Native, decellularised non-seeded biological matrices**

Although not fully complying with the original concept of tissue engineering as described above, the approach to implant a scaffold into the patient relying on spontaneous endogenous cellular repopulation may represent a first step towards living replacements. Decellularised homo- or xenografts are produced by processes resulting in minimally altered valves, expressing few or non-antigenic epitopes on their surface. The cellularization of the graft takes place in vivo, most probably by circulating, marrow derived mesenchymal progenitor or stem cells (MSCs). Previous animal studies have shown the principle feasibility of this concept (O’Brien 1999, Elkins et al. 2001a, Elkins et al. 2001b) and clinical studies have also been performed lately (Elkins et al. 2001b, Elkins et al. 2003). However, no follow up results have been published so far and the first clinical applications in children resulted in dramatic structural failure of the heart valves, due to strong inflammatory responses (Simon et al. 2003). Besides the risk of immunogenic reactions the use of unfixed biological native extracellular matrices as scaffolds (in unseeded grafts, and in repopulated grafts with autologous cells) bears the risk of disease transmissions such as Creutzfeldt-Jakob disease, transmission of microorganisms or retroviruses, particularly when using xenografts. Porcine endogenous retroviruses (PERVs) have been shown to integrate into human cells (Martin et al. 1998), and Walles and co-workers were able to detect PERV-DNA by polymerase chain reaction in decellularised porcine scaffolds (Walles et al. 2003).

**Crosslinked (fixed), reseeded biological matrices**

During the process of fabrication, biological heart valve prostheses are treated, e.g. with glutaraldehyde, for chemical fixation and cross-linking of extracellular matrix proteins. This process provides sterile matrices and a reduction of their antigenic potential. From the viewpoint of bio-safety, and with regard to the above described risk of immunological and infectious complications this approach is preferable to native, decellularised matrices. The advantage of repopulation of fixed allo- and xenografts with endothelial cells of the host is a reduced risk of calcification and thromboembolism. However, fixed allo- and xenografts are incompatible with endothelialisation due to the cytotoxicity of the fixatives such as glutaraldehyde. Therefore, several detoxification procedures prior to the cell seeding were tested using glutamic acid (Lehner et al. 1997), acid-buffered urazole or citric acid (Trantina-Yates et al. 2001). Moreover, pre-seeding of the detoxified scaffolds with fibroblasts has been demonstrated to improve endothelial cell adherence (Gulbins et al. 2003).

Alternative fixation procedures, such as dyes-mediated photo-oxidation (Jansson et al. 2001, Carmagey et al. 2003), or carbodiimide-hydroxysuccinimide treatment (Wissink et al. 2000), ethanol-glycerol treatment followed by freeze-drying (Cheung et al. 2001) are currently investigated in vitro and in vivo experiments. Even though these modifications may improve the durability of the prostheses, their principle lack to grow, as a consequence of their fixed, non-living and non-degrading extracellular matrix represents a major limitation, specifically for paediatric patients.
Table 1.1: Summary of scaffold materials and their use in heart valve tissue engineering

<table>
<thead>
<tr>
<th>Matrix</th>
<th>Source</th>
<th>Pre-treatment</th>
<th>Cells</th>
<th>Conditioning</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synthetic</td>
<td>Various biocompatible, biodegradable polymers</td>
<td>Fabricated into 3D structures, sterilised by ethylenoxide</td>
<td>Seeded with autologous myofibroblasts and endothelial cells</td>
<td>Static or dynamic (bioreactor)</td>
<td>Safe, living, completely autologous, growth</td>
</tr>
<tr>
<td>Synthetic</td>
<td>Glutaraldehyde fixed</td>
<td>Acellular</td>
<td>Extensively tested, safe, non-living, calcification</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biological</td>
<td>Glutaraldehyde fixed</td>
<td>Seeded with autologous cells</td>
<td>Bioreactor or no conditioning</td>
<td>Increased cell attachment/survival</td>
<td></td>
</tr>
<tr>
<td>Biological</td>
<td>Alternative fixation procedures (e.g. photofixation, cryopreservation)</td>
<td>Acellular</td>
<td>Biosafety and immunologic situation unclear</td>
<td></td>
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<tr>
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<td>Decellularised</td>
<td>Acellular</td>
<td>Biosafety and immunologic situation unclear</td>
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1.2.3 Cells

Vascular derived cells

In most cardiovascular tissue engineering approaches cells are harvested from donor tissues, e.g. from peripheral arteries (mammary artery, radial artery) or veins (saphenous vein) which are routinely used in heart surgery. Pure endothelial and myofibroblast cell lines are obtained from mixed vascular cell populations, e.g. by labelling the cells with low-density lipoprotein (LDL) marker, and subsequent fluorescence activated cell sorting (FACS) (Shinoka et al. 1995, Hoorstrup et al. 1998). Alternatively, biopsies are digested with collagenase in order to detach endothelial cells from the surface. The rest of the tissue is cut into small pieces (1 mm) attached to culture dishes and cultivated until primary myofibroblasts grow out. It was demonstrated that cells derived from veins showed an increased proliferation in monolayer culture and an increased ECM formation when cultivated on three-dimensional structures compared to artery derived myofibroblasts (Schnell et al. 2001). Drawbacks of using vascular derived cells for TE applications are the necessity to sacrifice an intact vascular structure of the patients and their limited availability. In addition, these cells may express a considerable different phenotype than natural valvular interstitial cells (Roy et al. 2000). Moreover, vessels from patients suffering from systemic atherosclerotic vascular diseases may not be a suitable cell source at all.

Bone marrow derived cells

With regard to future clinical application of the tissue engineering concept, bone marrow cells (mesenchymal stem cells, MSCs) or progenitor cells from the peripheral blood are attractive alternative cell sources. The bone marrow is not only a source of continuous progenitors for red blood cells, platelets, monocytes, granulocytes and lymphocytes, but also of cells that meet the criteria for stem cells of non-hematopoietic tissue. These stem-like cells are currently referred to either as mesenchymal stem cells, because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as marrow stromal cells (MSCs) because they appear to arise from the complex array of supporting structures found in marrow (Prockop 1997). The use of MSCs offers several advantages: (I) they are easy to obtain; (II) they show an extensive in vitro proliferation capacity; and (III) they have a potential to differentiate into cells that can roughly be defined as mesenchymal, or marrow stromal cells (MSCs) because they appear to arise from the complex array of supporting structures found in marrow (Prockop 1997). The use of MSCs offers several advantages: (I) they are easy to obtain; (II) they show an extensive in vitro proliferation capacity; and (III) they have a potential to differentiate into various tissues. MSCs can be collected by a simple puncture of the iliac crest under local anaesthesia. Low-density fraction cells are enriched by a density gradient centrifugation using Ficoll and are further selected by their adherence to plastics by early medium change within 24 h. Conventional culture techniques are applied for cell expansion and scaffolds are seeded within 30 to 40 days after cell harvesting. MSCs including haematopoietic stem cells are capable of forming solid organ tissue cells among which functional cardiomyocytes and vascular structures have already been demonstrated (Orlic et al. 2001a, Orlic et al. 2000b, Sata et al. 2002). The TE concept suggests that the application of a favourable microenvironment will guide cellular differentiation towards phenotypes that are appropriate for autologous tissue replacement. Indeed, MSC derived human cells showed a myofibroblast-like phenotype expressing a-SMA and vimentin, while LDL receptors, desmin, CD31 and CD14 were not expressed (Hoorstrup et al. 2002, Kadner et al. 2002). These results indicate the absence of myeloid and hematopoietic cell differentiation. A similar staining pattern was reported using MSCs from sheep (Perry et al. 2003). Here, the cells stained positive also for SH2 (CD105, endoglin), a marker for mesenchymal stem cells and a subpopulation stained positive for calponin (a family of actin binding proteins). A comparable staining pattern was also reported for TEHV using valvular interstitial cells seeded onto collagen scaffolds (Taylor et al. 2002). Upon seeding of the cells on a polymeric scaffold and conditioning in a pulsatile flow environment, they produced a considerable amount of extracellular matrix proteins resulting in functional tri-leaflet heart valves (Fig. 1C). In addition,
comparable mechanical properties (uniaxial tensile strength) as found in native valve tissue or TE constructs derived from vascular cells were demonstrated (Hoerstrup et al. 2002, Kadner et al. 2002). Before implantation the tissue engineered heart valves are seeded with endothelial cells, which may be differentiated from either a subset of the aspirated MSCs (Reyes et al. 2001, Reyes et al. 2002), or from progenitor cells derived from peripheral blood (Kaushal et al. 2001, Dvorin et al. 2003, Zhao et al. 2003).

**Blood Derived Cells**

Optimally, a cell source should be easily accessible and have high growth and repair potential. A potentially promising cell source is endothelial progenitor cells (EPCs), a subpopulation of stem cells in human peripheral blood. EPCs are a unique circulating subtype of bone marrow cells differentiated from haemangioblasts, a common progenitor for both haematopoietic and endothelial cells. These cells have the potential to differentiate into mature endothelial cells. Currently, EPCs have been investigated for the repair of injured vessels, neovascularisation or regeneration of ischemic tissue (Kawamoto et al. 2002, Kocher et al. 2001, Assmus et al. 2002, Pesce et al. 2003), coating of vascular grafts (Shirota et al. 2003), endothelialization of decellularized grafts in an animal model (Kaushal et al. 2001) and seeding of hybrid grafts (Shirota et al. 2003). In a recent study it was demonstrated that differentiated human umbilical cord blood derived EPCs seeded on biodegradable scaffolds formed neo-tissue both in a biomimetic and static in vitro environment (Schmidt et al. 2004). These tissues were characterized as endothelial monolayer with related functions, e.g. the production of eNOS, indicating features of functional endothelium. Due to their stable phenotype and their growth and repair potential human umbilical cord blood derived EPCs appear to be a promising cell source for cardiovascular tissue engineering particularly for pediatric applications, where EPCs could adjust to the specific growth requirements of the cardiovascular system during childhood.

**In Vitro Culture Conditions**

Tissue formation can be enhanced by either biological and/or mechanical stimuli. Biological conditioning involves addition of protein synthesis cofactors such as e.g. ascorbic acid (Hoerstrup et al. 1999) or cytokines either directly to the growth medium or by incorporation into the scaffold material. The family of cytokines includes interleukins, hematopoietic growth factors, interferon, tumor necrosis factors and can exert multiple biological functions by interaction with specific cell surface receptors (Takehara 2000). Well-known cytokines that influence cardiovascular cells are fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β) and vascular endothelial growth factor (VEGF) (Ziegler et al. 1995; Bos et al. 1999). Besides cytokines, matrix metalloproteinases (MMPs), a family of matrix degradation enzymes, play an important role in tissue development and subsequent remodelling (Streuli 1999). Mechanical conditioning involves the application of various mechanical stimuli, such as flows, inducing shear stresses over the developing tissue and strains, being either dynamic or static in nature. Mechanical and biological stimuli do interact in a very complex way in the regulation of tissue behaviour. By mechanical stimuli, the cellular production and secretion of various cytokines is increased and -vice versa- the addition of cytokines during tissue development can enhance the effect of mechanical stimulation.

### 1.2.4 Human Native Valves - The “Golden Standard” for Tissue Engineering

The ultimate rationale of the tissue engineering concept is the creation of living neo-tissues identical or at least very close to native heart valves. So far, the majority of work has been focused on valves of the semilunar type, such as aortic or pulmonary valves; mainly because of their less complex design in comparison to the atrio-ventricular valves (i.e. absence of chordae tendineae etc.). A prerequisite to a successful development of native-analogous tissue engineered valves is an accurate understanding of the fundamentals of normal heart valves representing the “golden standard”. A description of its composition, structure and function as exemplified by the normal aortic valve is given in the following section. Interestingly, it is the tissue engineering research of recent years which has stipulated a novel interest in heart valve anatomy, biomechanics, embryology, cell biology and pathology with many important implications to the whole field of heart valve diseases.

### 1.2.5 Embryonic and Fetal Development – How does Nature do it?

When the first heart contraction takes place during embryonic development, the heart is not more than a tube consisting of a single lumen. This tube is transformed into an H-shaped outflow channel with large tissue cushions in the right and left outflow tract. These will each divide into three equal mounds of cushion material and form the origins of the aortic and pulmonary valve (Maron and Hutchins 1974). Endothelial cells lining these cushions appear to be able to differentiate into leaflet interstitial cells, regulated by local growth factors (Perry and Roth 2003). The hemodynamic environment during development of the valve cusps determines the cell shape, proliferation and fiber formation (Maron and Hutchins 1974). The cells on the ventricular side of the leaflets are flattened, due to the shearing effect of the blood flow during ventricular ejection, whereas the cells at the arterial side stay more cuboidal. The cusps grow by proliferation of cells in the downstream end, the region with low pressure and low shearing force. Cell proliferation seems to stop when the leaflets are long enough to contact the arterial wall above the sinuses during opening. Elastic fibers become prominent at the ventricular side of the leaflet, which is exposed to intermittent flexural stresses during systole. At the arterial side, exposed to the predominantly static stresses during diastole, collagenous fibers develop. At the line of closure, the leaflets consist of solely collagenous fibers, which correlate to the tensions at both sides of the leaflets. The
development of the aortic valve takes place under pressure values below 10 mm Hg, at heart rates ranging from 65-160 beats per minute and hypoxic conditions (Stock and Vacanti 2001). The acceleration of the heart rate might be a compensatory phenomenon in the absence of the Frank-Starling mechanism, as the immature fetal myocardium does not possess the ability to increase the ejection fraction in response to increasing preload. By increasing hematocrit and a shift of the hemoglobin-oxygen dissociation curve towards optimized oxygen binding characteristics the fetus compensates for the hypoxic conditions.

1.2.6 Architecture – Matrix and Cells
The load-bearing part of adult semilunar valve leaflets shows a layered architecture within the endothelial coverings, enabling the extraordinary changes in shape and dimension. The ventricularis, the layer at the inflow surface, is predominantly composed of radially aligned elastin fibers. The central layer, the spongiosa, consists of loosely arranged collagen fibers and an abundant amount of proteoglycans. The layer at the outflow surface, the fibrosa, comprises mainly circumferentially aligned collagen fibers. All collagen bundles diverge into the aortic wall, thereby transferring the gross load from the leaflet to the aortic wall. The individual layers can easily compress and shear during opening and closing of the valve. The fibrosa and ventricularis are inherently preloaded due to their attachment to each other, the fibrosa under compression and the ventricularis under tension (Vesely 1998).

Among the valvular interstitial cells three cellular phenotypes can be identified: smooth muscle cells, arranged in bundles or just as single cells (Bairati and DeBiasi 1981), and fibroblasts maintaining the extracellular matrix. Approximately 60 % of the fibroblasts represent myofibroblasts (Roy et al. 2000), cells that have phenotypic features of both fibroblasts and smooth muscle cells (Messier et al. 1994). These cellular phenotypes are situated depending on their biological and mechanical microenvironment. Myofibroblasts and fibroblasts are able to convert from one to another, triggered by either a lack of mechanical tension or the presence of continuous mechanical tension (Tomasek et al. 2002). The idea of passively functioning valve leaflets was refuted by identifying a smooth muscle cell system in the leaflets (Bairati and DeBiasi 1981), contractile properties of valvular interstitial cells (Messier et al. 1994, Taylor et al. 2000) and sensory nerve elements in the leaflets (Marron et al. 1996). Contraction within the leaflets might help to sustain the hemodynamic forces that are exerted on the leaflet during systole and diastole (Taylor et al. 2000) and represents a reactive cytoskeleton that can anchor collagen fibrils during valve closure (Messier et al. 1994).

1.2.7 Anisotropic Structure and Function
The individual layers of valve leaflets show different mechanical characteristics due to their difference in composition (Vesely and Noseworthy 1992, Scott and Vesely 1995, Scott and Vesely 1996). The fibrosa is considered to be the main load-bearing layer of the leaflet and prevents excessive stretching (Thubrikat et al. 1986). The difference in radial and circumferential extensibility, a phenomenon known as anisotropy, is not as large in this layer as it is in the ventricularis, where the radial extensibility is much larger than the circumferential extensibility (Vesely and Noseworthy 1992, Scott and Vesely 1995, Scott and Vesely 1996). The overall mechanical response of the leaflet is the summation of the mechanical properties of the individual layers. Lo and Vesely (Lo and Vesely 1995) measured a maximal extensibility of porcine aortic valve leaflets of 24 % in radial direction and 11 % in circumferential direction by whole-valve biaxial testing, a reliable way to test natural biaxial loading environment in the valve is reflected. In circumferential direction the mechanical behavior exhibits the properties of collagen bundles and in radial direction the elastin mesh is the predominant factor. The leaflet shows an extremely low compressive modulus, which is most likely influenced by the spongiosa (Vesely and Noseworthy 1992). Schoen and Levy (Schoen and Levy 1999) summarized the biomechanics of the aortic valve as follows. When the valve is nearly closed and the collagen bundles in the fibrosa are fully unfolded, collagen is the load-bearing element, enabling a stress increase while preventing a prolapse of the leaflets. The loose spongiosa layer is able to dissipate the shock of closure of the leaflets, as the hydrophilic proteoglycans in this layer readily absorb water and swell. Due to deformation of the sinus walls, resulting in an increase in volume, the pressure difference across the valve decreases. During opening of the valve, elastin extends at minimal load in the ventricularis to return the fibrosa in its original corrugated state, facilitated by the spongiosa that dissipates the arising shear stresses.

1.3 AIM OF THE THESIS
It is the fascinating perfection of architecture and function of a native, living heart valve which enables the enormous life-time performance with billions of cycles without malfunctions. The above described structural and functional complexity of normal heart valves appears to be a “conditio sine qua non” for this performance. In accordance with these observations the tissue engineering paradigm followed in this thesis is focused on maximum approximation to native heart valves as a benchmark. The aim of the thesis is (a) to achieve the generation of living, functional, autologous heart valves in vitro and to evaluate their in vivo performance; and (b) to identify and evaluate suitable human cell sources for the transfer of the heart valve tissue engineering concept to clinical applications.

1.4 OUTLINE OF THE THESIS
The sequence of studies comprised in this thesis addresses step by step principal prerequisites for the realization of the autologous heart valve tissue engineering paradigm. Chapter 2 introduces the concept of exposing tissue engineered heart valves to “biomimetic signals” and “mechanical conditioning” in vitro by using a newly developed flow bioreactor. This approach is based on the hypothesis, that in vitro exposure of the developing tissue to physical
signals similar to those encountered in vivo may result in more mature engineered heart valves. In combination with a novel composite scaffold material enabling the fabrication of complex, three dimensional heart valve scaffolds, the first functional, completely autologous, tri-leaflet heart valves are demonstrated in a large animal study (chapter 3). Interestingly, the ultimate refinement and maturation of the engineered heart valves occurred during the subsequent in vivo period of this experiment, showing an evolution of cell phenotype and extracellular matrix towards native valve tissue. This remodelling is investigated in detail in chapter 4, providing a more accurate understanding of the neo-tissue changes prior and after implantation. Based on the results of this proof of principle animal study suitable cell sources for potential human applications are investigated. Since vascular derived autologous human cells necessitate the sacrifice of intact vascular donor structures, less invasive alternative cell sources including progenitor-type cells are evaluated. For adult applications the use of human marrow stromal cells for tissue engineering of trileaflet heart valves is described in chapter 5. These cells can be routinely obtained by a simple puncture of the iliac crest representing a less invasive cell source with the potential to differentiate into various tissues. The highest medical need for a tissue engineered heart valve is in pediatric applications for the treatment of congenital cardiovascular malformations. Approximately 1% of all newborns have congenital heart defects, and many of them require open heart surgery and heart valve replacement. Since currently available artificial valve prostheses cannot grow with the young patients, repeated replacement operations have to be performed associated with exponentially increased morbidity and mortality. In chapter 6, the human umbilical cord is investigated as a readily available autologous cell source for tissue engineering, enabling the in vitro generation of living replacement materials short after birth. Ideally, a tissue engineered heart valve would be available for implantation already at the time of birth in order to prevent secondary damage to the immature ventricle. This requires cell harvest prior to birth, e.g. by ultrasound guided cordocentesis (puncture of the umbilical cord in utero). To validate this concept, umbilical cord blood derived human endothelial progenitor cells are isolated, expanded in vitro and their suitability for the in vitro engineering of living patches is demonstrated in chapter 7. Chapter 8 summarizes and discusses the results of the studies as well as future aspects of heart valve tissue engineering.
2.1 ABSTRACT

Two potential obstacles to the creation of implantable tissue engineered heart valves are inadequate mechanical properties (ability to withstand hemodynamic stresses) and adverse host-tissue reactions due to the presence of residual nondegraded polymer scaffold. In an attempt to address these problems, we developed an in vitro cell culture system that provides physiological pressure and flow of nutrient medium to the developing valve constructs. It is anticipated that in vitro physical stress will stimulate the tissue engineered heart valve construct to develop adequate strength prior to a possible implantation. Long-term in vitro development will be realized by an isolated and thereby contamination-resistant system. Longer in vitro development will potentially enable more complete biodegradation of the polymeric scaffold during in vitro cultivation. This new dynamic bioreactor allows for adjustable pulsatile flow and varying levels of pressure. The system is compact and easily fits into a standard cell incubator, representing a highly isolated dynamic cell culture setting with maximum sterility, optimal gas supply and stable temperature conditions especially suited for long-term experiments.

2.2 INTRODUCTION

The concept of tissue engineering, growing autologous cells on biodegradable polymer scaffolds, represents a promising approach to creating valve replacement tissues for cardiac surgery. Although the feasibility of in vitro formation of valve-analogous tissue and implantation in the animal model have been demonstrated in our laboratory (Shinoka et al. 1996, Zund et al. 1997) the mechanical properties of the tissue constructs and the potential adverse host-tissue reactions due to residual nondegraded polymer remain significant problems (Athanasion et al. 1996).

One approach to these problems is to create cell culture conditions for extended tissue development in vitro so that mechanically stable and reliable neo-valve-tissue would be formed with complete biodegradation of the scaffold. Such conditions would potentially include (a) physiological flow and pressure for “conditioning” the developing valve tissue construct prior to in vivo implantation and (b) strict isolation of the system for long-term maintenance of sterile culture conditions. Accordingly we developed a dynamic bioreactor capable of providing adjustable pulsatile flow and a wide range of physiological pressures. The very compact system easily fits into a standard cell incubator, is air-driven by a simple respirator, and creates a highly isolated environment which minimizes possible contact with an unsterile environment.

2.3 BIOREACTOR DESIGN AND FUNCTION

2.3.1 Pulsatile Flow Chamber ("Bioreactor")

The compact bioreactor (diameter, 15.5 cm; height, 16.8 cm), made of plexiglass (Polymethylmethacrylate, PMMA), consists of two principal chambers: the air chamber (1) is the bottom level and the cell media fluid chamber (2) is the upper level (Figs. 2.1a, b). These two chambers are separated by a silicone diaphragm (3) composed of “super stretch” silicone rubber, 0.5-mm thickness. The air chamber is connected to a respirator pump. The fluid chamber (total volume, 500 mL) is divided into two compartments. The lower compartment (2a) connects to the smaller top compartment (2b, “valve perfusion chamber”) through a tube (4). On top of this tube, a second removable silicone tube (5) is mounted and the tissue engineered valve can easily be fixed to this silicone tube by suturing. By choosing different outer diameters of the silicone tube, valve diameters ranging from 16 to 26 cm in diameter are adaptable to the system. The media flow is directed from the bottom compartment (2a) through the tube (4) to the mounted tissue engineered valve in the perfusion chamber (2b).

Pulsatility of the flow is achieved by pumping air into the lower chamber and displacing the silicone diaphragm between the liquid chamber and the bottom air chamber periodically. The media in chamber 2 is propelled up to the valve perfusion chamber. The bioreactor is connected via a valved inlet (6) and outlet (7) tubing to a separate media reservoir (total volume, 500 mL). The bioreactor as a whole consists of three plexiglass components which are fixed together by a series of stainless steel screws (8). The silicone diaphragm is positioned like a “drumskin” between the air chamber (1) and the cell-media chamber (2). The entire system can be sterilized by ethylene oxide and be reassembled with the polymer scaffold valve construct mounted within the system.

Figure 2.1: (a) Photograph of the Bioreactor, white arrow indicating position of tissue engineered heart valve. (b) Technical description.
2.3.2 Bioreactor Setting

The described system consisting of the two-chamber (air/liquid) bioreactor (1) and the connected reservoir (2) is placed into a standard humidified incubator at 37°C and 5% CO₂ (3). The air-driven system is connected through a single line to a simple respirator (4, Harvard Apparatus Inc, USA, dual phase control ventilator), which is outside of the incubator and functions as an air pump (Fig. 2.2).

![Diagram of bioreactor setting](image)

**Figure 2.2: Bioreactor setting.**

By adjusting the stroke volume and ventilation rate of the ventilator, pulsatile flows ranging from 50 to 2,000 mL/min and systemic pressures from 10 to 240 mm Hg, are established in the bioreactor at variable physiological stroke rates.

2.4 DISCUSSION

The ideal conditions for the creation of the tissue engineering of cardiovascular structures, such as heart valves, are unknown. Intuitively, we have thought that exposure of developing "tissue" to physical "signals" similar to those which the tissue will encounter *in vivo* might be beneficial. The *in vitro* formation of a mechanically (hemodynamically) competent tissue under simulated physiological conditions with complete biodegradation of the polymeric scaffold can potentially be achieved with the apparatus we have described. The use of this system may potentially enable the implantation of a hemodynamically conditioned, "pretested" and therefore reliable tissue (prior to *in vivo* implantation, the construct can be exposed to supraphysiological pressure for functional testing with regard to even extreme hemodynamic situations). Furthermore, at the time of implantation, the structure would consist only of autologous material preventing possible adverse host-tissue reactions.

A substantial precondition for the realization of the described concept is a reliable and efficient *in vitro* culture system which - being as simple as possible - allows long-term pulsatile flow conditions and prevents microbiologic contamination. Reviewing the literature, we realized that such a flow system has yet not been described. In fact, some attention has already been turned to the use of dynamic cell culture systems to study the influence of flow and cyclic stress in the field of vascular endothelial biology (Nerem and Girard 1990, Diamond et al. 1990, Mitsumata et al. 1993, Kanada and Matsuda 1993). These settings represent a situation where the response of cultured cells to a well defined mechanical environment can be evaluated, and as such they have been short term systems with very limited culture times ranging from hours to a single day; complete simulation of physiological flow is not achieved. Other systems have been used for whole vessel experiments. Benbrahim et al. (Benbrahim et al. 1994) presented a "vascular simulating device" – a compliant tubular system enabling the study of vascular cells under physiologic flow and pressure conditions.

For the purpose of tissue engineering of blood vessels, Niklason et al. established a system which creates pulsatile flow and absence of infection in long-term experiments resulting in formation of solid vascular tissue (Niklason et al. 1997). Nevertheless, an adequate setting for the developing valvular tissue has not yet been described.

We present a detailed technical description of a bioreactor we have designed for our experiments on valve tissue. The very compact and highly isolated pulsatile flow system fits in a standard incubator and is air driven by a respirator. Consequently, the bioreactor can be utilized without additional sophisticated equipment.

The "bioreactor" itself, constructed of materials such as plexiglass (chambers), silicone (membrane and tubings) and stainless steel (screws) is very robust and easy to sterilize. The transparency of plexiglass enables continuous control of the medium perfused system especially with regard to the evolving valve function of the tissue engineered heart valve and to possible microbiological contamination. Furthermore safe and easy handling of the bioreactor is made possible by its simple design. Implementation of the cell polymer construct is facilitated by the removable silicone (Fig. 2.2) on which the scaffold can be sewn before sterilization and static cell seeding. Finally the bioreactor's
capability of applying increasing flow and pressure rates from very low to high physiological values will likely allow the experimental development of cell-polymer constructs of varying degrees of "maturity."

2.5 ACKNOWLEDGEMENTS
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PROOF OF PRINCIPLE

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"Functional Living Trileaflet Heart Valves Grown In Vitro"

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3.1 ABSTRACT
Previous tissue engineering approaches to create heart valves have been limited by the structural immaturity and mechanical properties of the valve constructs. This study used an in vitro pulse duplicator system to provide a biomimetic environment during tissue formation to yield more mature implantable heart valves derived from autologous tissue.

Trileaflet heart valves were fabricated from novel bioabsorbable polymers and sequentially seeded with autologous ovine myofibroblasts and endothelial cells. The constructs were grown for 14 days in a pulse duplicator in vitro system under gradually increasing flow and pressure conditions. By use of cardiopulmonary bypass, the native pulmonary leaflets were resected, and the valve constructs were implanted into 6 lambs (weight 19 ± 2.8 kg). All animals had uneventful postoperative courses, and the valves were explanted at 1 day and at 4, 6, 8, 16, and 20 weeks. Echocardiography demonstrated mobile functioning leaflets without stenosis, thrombus, or aneurysm up to 20 weeks. Histology (16 and 20 weeks) showed uniform layered cuspal tissue with endothelium. Environmental scanning electron microscopy revealed a confluent smooth valvular surface. Mechanical properties were comparable to those of native tissue at 20 weeks. Complete degradation of the polymers occurred by 8 weeks. Extracellular matrix content (collagen, glycosaminoglycans, and elastin) and DNA content increased to levels of native tissue and higher at 20 weeks.

This study demonstrates in vitro generation of implantable complete living heart valves based on a biomimetic flow culture system. These autologous tissue-engineered valves functioned up to 5 months and resembled normal heart valves in microstructure, mechanical properties, and extracellular matrix formation.

3.2 INTRODUCTION
Valve replacement represents the most common surgical therapy for end-stage valvular heart disease, with 60,000 implantations in the United States and 170,000 worldwide (Schoen and Levy 1999). Valve replacement surgery is efficacious, and it substantially changes the natural history of valvular disease (Braunwald 1992). However, mechanical valves are associated with a substantial risk of thromboembolism, and tissue valves suffer from structural dysfunction due to progressive tissue deterioration (Schoen and Levy 1999, Vongpatanasin et al. 1996, Hammermeister et al. 1993). Because all clinically used tissue valve substitutes are nonviable, they have no potential to grow, to repair, or to remodel. Therefore, their durability is limited, especially in growing children (Mayer 1995). In an attempt to address the shortcomings of current valve options, we previously reported the feasibility of replacing a single pulmonary valve leaflet by a tissue-engineered (TE) autologous leaflet (Shinoka et al. 1996). In subsequent studies, we focused on the in vitro generation of a complete trileaflet heart valve (Stock et al. 2000). A substantial limitation was structural and mechanical "immaturity" of the constructs, which had insufficient mechanical properties and functional performance after implantation. Subsequently, more durable scaffold materials that provided better mechanical function were tested. However, because of their prolonged degradation time, they persisted in vivo and were not sufficiently replaced by autologous tissue (Sodian et al. 1999). The ideal concept of a TE heart valve includes formation of functional valve constructs on the basis of a rapidly absorbable scaffold. The scaffold provides a temporary biomechanical profile until the cells produce their own matrix proteins. The structural integrity and biomechanical profile of the TE heart valves ultimately depend on this matrix formation.

We hypothesized that in vitro exposure of the developing tissue to physical signals similar to those encountered in vivo may result in more mature TE heart valves with more favorable functional performance. Accordingly, we developed a new TE approach that made use of an in vitro pulse duplicator system and a novel rapidly bioabsorbable composite scaffold material. The present study design included 2 experimental steps: the first set of experiments was undertaken to investigate whether a biomimetic culture environment guides tissue development to more mature TE heart valves in vitro, and the in vivo study that followed was performed to assess the practical utility and performance of these valve constructs.

3.3 METHODS
3.3.1 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, molecular weight 1x10⁶, PHA 4400, Tepha Inc) 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 rapidly absorbable biopolymer that is not only strong and pliable but also thermoplastic (61°C) so that it can be molded into almost any shape. From the PGA/P4HB composite scaffold material, trileaflet valve scaffolds were fabricated by using a heat-application welding technique. The constructs were then coldgas-sterilized with ethylene oxide.
myofibroblasts, the remaining deendothelialized vessel segments were minced and cultured on P100 dishes (Corning) in DMEM (GIBCO) supplemented with 10% FBS (HyClone), penicillin, and streptomycin (GIBCO). After migration of the myofibroblasts onto the dishes (after 5 to 7 days), the cells were serially passaged and expanded in a humidified incubator at 37°C and 5% CO2. Sufficient cell numbers for cell seeding were obtained in pure culture after 21 to 28 days. The endothelial cells were characterized by the presence of CD31 (platelet endothelial cell adhesion molecule [PECAM 1]) and von Willebrand factor (vWF); the myofibroblasts, by the presence of smooth muscle actin (SMA) (Figure 3.1).

**Figure 3.1:** Immunofluorescence staining of vascular-derived cell populations used for seeding. A through C, Endothelial cells stained positively for vWF (rhodamine, red; A) and for CD31 (B) and costained positively for CD31 (rhodamine, red; C) and negatively for SMA (fluorescein, green; C), demonstrating pure endothelial cell cultures. D, In contrast, myofibroblasts stained positively for SMA (fluorescein, green) but not for CD31 or vWF.

### 3.3.3 Cell Seeding and Conditioning in an In Vitro Pulse Duplicator System

Myofibroblasts (4.5 to 5.5x10^6 per cm^2) were seeded onto the trileaflet valve scaffolds and cultured in static nutrient medium (DME, GIBCO) for 4 days in a humidified incubator (37°C, 5% CO2). Thereafter, the constructs (n=10) were seeded with endothelial cells (1.5 to 2.0x10^6 per cm^2), transferred into a pulse duplicator system (“bioreactor,” Figure 3.2), and grown under gradually increasing nutrient medium flow and pressure conditions (125 mL/min at 30 mm Hg to 750 mL/min at 55 mm Hg) for 4, 7, 14, 21, and 28 days. Controls

### 3.3.4 Animal Implants

Cells were harvested, multiplied, and seeded onto the trileaflet heart valve constructs as described above. After maturation in the bioreactor for 14 days, the TE valves were functionally tested in the system under high-pressure conditions (>150 mm Hg) for 60 minutes. Thereafter, they were implanted into the same lambs (n=6, 19±2.8 kg) from which the cells were initially harvested. Anesthesia was induced with 2 mg/kg ketamine, 0.02 mg/kg atropine, and an intravenous bolus infusion of 2 mg/kg propofol and maintained by inhalational isoflurane. The heart was exposed by a left anterolateral thoracotomy entering the chest through the third intercostal space. Systemic anticoagulation was induced with 400 IU heparin/kg. By use of femoral arterial and right atrial venous cannulation, normothermic cardiopulmonary bypass was established. With the heart beating, the main pulmonary artery was transected, and all 3 native leaflets were excised. The TE heart valve constructs were implanted by using running 5-0 monofilament sutures (Prolene, Ethicon). Heparin was reversed with 300 IU protamine/kg after weaning from bypass, and the chest was closed. No further anticoagulation was given. Echocardiography (Hewlett-Packard Sonos 1500 Cardiac Imager equipped with a 7.5-MHz phased transducer), including imaging from a long- and short-axis view, was performed after surgery and at various time intervals for up to 20 weeks. The animals were euthanized after 1 day and at 4, 6, 8, 16, and 20 weeks. Before explantation, direct pressures were measured during surgery (Digital Ultrasonic Measurement System, Sono Metrics Inc) proximal and distal to the TE construct. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85-23, revised 1985).
3.3.5 Microstructure
A representative portion of each trileaflet valve construct was examined histologically by hematoxylin and eosin stain (overall morphology) and Movat pentachrome stain (for demonstration of matrix elements, including collagen, elastin, and glycosaminoglycans (GAGs)) and by immunohistochemistry for CD31, vWF, and SMA. Additional samples were fixed in cacodylic acid (Sigma) for environmental scanning electron microscopy (ESEM).

3.3.6 Tissue Analysis
Biochemical assays were performed for analysis of cellular and extracellular components of the new tissue. Total DNA was isolated and purified by sequential organic extractions with phenol and phenol/chloroform/isoamyl alcohol and quantified by spectrophotometry (Current protocols in Molecular biology 1999). For determination of total collagen content, tissue was completely acid-digested, and total 4-hydroxyproline was measured (Bergmann and Loxley 1963). Total proteoglycan/GAG and elastin content were quantified with a BL YSCAN and FASTIN assay (Biocolor) after tissue extraction.

3.3.7 Mechanical Properties
Mechanical properties of the TE valve constructs and native valves were evaluated by use of a mechanical tester (model Mini 55, Instron Corp.). Longitudinal matrix strips were used for the test. A 75-lb/inch² maximum load cell was used, and the cross-head speed was 0.5 in/min. Young's modulus was obtained from the slope of the initial linear section of the stress-strain curve. Moreover, suture retention strength was measured.

3.3.8 Polymer Degradation Analysis
Percent residual polymer (PGA and PHA4400) in the dried tissue was determined by gas chromatography. Lyophilized tissue samples (~50 mg) were digested in a butanolysis reagent (n-butanol/concentrated HCl 9:1, containing 2 mg/mL benzoic acid as internal standard) for 2 hours at 110°C. The organic fraction of the digests was analyzed by gas chromatography (HP 5890, SPB1 column, Supelco). Standard curves were generated by using glycolide and g-butyrolactone as standards.

3.4 RESULTS
3.4.1 In Vitro
In all valves, synchronous opening and closing of the leaflets was observed in the bioreactor, under both low-pressure (35 mm Hg) and high-pressure (>150 mm Hg) conditions. Gross appearance showed the most advanced tissue formation after 14 days (Figure 3.3), without apparent differences at 21 and 28 days. All leaflets were intact, mobile, and pliable, and the valve constructs were competent during valve closure. The controls grown in static culture were fragile and began to lose structural integrity after 14 days of static culture.

Figure 3.3: TE heart valve after 14 days of conditioning in bioreactor.

Tissue Microstructure
Histology of the TE leaflets revealed cellular tissue organized in a layered fashion with a dense outer layer and lesser cellularity in the deeper portions after 14 days in the pulse duplicator (Figure 3.4). Formation of extracellular matrix was demonstrated as predominantly GAGs and some collagen. SMA-positive smooth muscle cells were detectable throughout the tissue. Tissue was maximally organized after 14 days with no further increase after longer culture duration in the pulse duplicator. The static controls showed less tissue formation and organization at all time points. ESEM demonstrated dense tissue and a confluent smooth surface with cell orientation in the direction of the flow after 7 days, whereas the controls showed a rough surface at all time points.

Tissue Analysis
Collagen content was 129% that of native valve tissue at 14 days and leveled off to 86% and 85% at 21 and 28 days, respectively. DNA content of the constructs reached 80% that of native tissue at 7 days and leveled off to 60% at 21 and 28 days. GAG content was 60% that of native valve tissue at 14 days, with no further increase at 21 and 28 days. Elastin was not detectable in any TE leaflet up to 28 days. DNA and collagen content were significantly lower in all specimens at all time points in the static controls (Figure 3.5).
Figure 3.4: A. After 14 days of pulsatile flow, leaflet portion is composed of dense fibrous tissue near surface and loose central core (hematoxylin and eosin stain, magnification x20). B. Movat stain shows GAGs (blue) and some collagen (yellow) in specimen shown in panel A. Polymer fibers are yellow-green (magnification x100). C. Static control (14 days) shows loose, poorly organized tissue (magnification x100).

Figure 3.5: Tissue components after up to 28 days of culture time in flow bioreactor. Note difference of total DNA and collagen content in TE leaflets cultured in bioreactor compared with static controls.

Mechanical Testing

Suture retention strength was >50 g after 14 days (68, 65, and 66 g at 14, 21, and 28 days, respectively) versus a maximum of 12 g at 7 days in the static controls.

3.4.2 In Vivo

All animals survived the valve replacement procedure and had uneventful postoperative courses. Echocardiography performed after surgery and at 1, 2, 4, 8, 16, and 20 weeks demonstrated mobile functioning leaflets without evidence of thrombus, stenosis, or aneurysm formation up to 20 weeks after implantation (Figure 3.6). At 16 and 20 weeks, central pulmonary regurgitation (mild to moderate) was detected. The maximum transvalvular peak-to-peak gradient was, 10 mm Hg by direct intraoperative pressure measurements in all TE valves at the time of explantation.

Figure 3.6: Echocardiography of TE valve at 8 weeks. A and B, Long-axis view of leaflets in closed (A) and opened (B) positions (* indicates TE leaflet; #, right ventricular outflow tract; and # main pulmonary artery). C and D, Short-axis view of the TE valve in closed (C) and opened (D) position

Gross appearance of all explanted trileaflet TE valves showed intact mobile leaflets with a smooth ventricular and arterial surface and no thrombus or stenosis. The TE leaflets at 4, 6, and 8 weeks appeared thicker and less pliable than the valves at 16 and 20 weeks (Figure 3.7). There was an increase of the inner diameter of the valve constructs at the level of leaflet attachments from an initial measurement of 19 to 23 mm at 20 weeks in accordance with the observed growth of the native pulmonary artery.
**Figure 3.7:** TE heart valves explanted after 6 (A) and 20 (B) weeks. Note thin and pliable leaflet at 20 weeks (C).

**Tissue Microstructure**

Histology (hematoxylin and eosin staining) showed a uniform laminated structure with progressive thinning and organization of the cuspal structure. At 16 and 20 weeks, the leaflets were layered with a loose spongy layer on the ventricular (inflow) side and fibrous layer on the arterial (outflow) side. Special stains at 20 weeks revealed collagen in the fibrous layer and GAGs in the central loose layer, whereas elastin could be detected near the inflow surface (Figure 3.8). The structure was uniform from base to edge. Coverage of the leaflet surface with CD31- and vWF-positive cells was partial, principally from the proximal attachments at 16 and 20 weeks. There was no evidence of inflammation or residual polymer at 16 to 20 weeks. ESEM demonstrated a smooth surface of the TE leaflets at both the inflow and outflow side as a smooth rounded free edge of the leaflets.

**Tissue Analysis**

Collagen content of the TE leaflets was 140% that of native tissue at 4 weeks and increased to a plateau level of ~180% after 8 weeks. DNA content of the constructs was 65% that of native tissue at 4 weeks and 6 weeks and increased to 77%, 100%, and 150% at 8, 16, and 20 weeks, respectively indicating a constant cell proliferation on the TE leaflets. GAG content increased from 90% that of native valve tissue at 4 weeks to 300% at week 16 and decreased to comparable to native values (140%) at 20 weeks. Elastin was detectable in the TE leaflets by 6 weeks.

**Mechanical Testing**

The tensile strength of all implanted TE valves leaflets was initially higher than that of native tissue and decreased over the follow-up period to be comparable to native values (130% that of native tissue at 20 weeks). In parallel, the constructs became more pliable, which was evaluated by a decrease of Young’s modulus and an increase of elongation as a percentage at maximum stress. The stress/strain curve at 20 weeks demonstrated that the mechanical properties of the new tissue strongly resembled that of native pulmonary valve tissue (Figure 3.9).

**Figure 3.8:** Histology of heart valve leaflet in vivo. A, at 6 weeks, there is early organization of tissue predominantly in outer layer (top) (magnification X50). B, cross section of leaflet at 16 weeks shows layered cellular fibrous tissue, which is more dense near outflow surface (top) (magnification X 100). C.

**Figure 3.9:** Comparison of mechanical properties of TE and native valve tissue. Stress-strain curves at 20 weeks demonstrated very similar mechanical characteristics.
Scaffold Material Bioabsorption Analysis

Scaffold material bioabsorption analysis of the valve tissue demonstrated complete biodegradation of the PGA by 4 weeks and of the P4HB by 8 weeks.

3.5 Discussion

Valve replacement surgery is efficacious and substantially changes the natural history of valvular heart disease (Braunwald 1992). However, although the overall performance of these devices is excellent, prosthesis-associated problems occur within 10 years after surgery in 30% to 35% of patients (Bloomfield et al. 1991). Mechanical valves require lifelong anticoagulation therapy (Vongpatanasin et al. 1996). Bioprosthetic valves have limited durability and may calcify prematurely, particularly in young patients (Hammermeister et al. 1993). More important, both mechanical and bioprosthetic valves are nonviable structures and do not have the ability to grow, repair, or remodel, which is a specific problem in the pediatric patient population (Mayer 1995).

TE applies the principles and methods of engineering to biological sciences in an attempt to create viable structures for replacement of deficient natural structures. (Vacanti and Langer 1999) The option of creating heart valves from autologous cells offers many potential advantages. These include elimination of unfavourable side effects of anticoagulation therapy, elimination of immune rejection, and the potential of growth, repair, and remodeling.

We previously reported the successful replacement of a single pulmonary valve leaflet by an autologous TE leaflet (Shinoka et al. 1996). These TE valve constructs were based on the rapidly bioabsorbable scaffold material PGA. A substantial limitation of the PGA-based tissue constructs is its initial stiffness and thickness, making the creation of more complex 3D TE constructs, such as a trileaflet heart valve, difficult. As an alternative scaffold material, we subsequently evaluated polyhydroxyoctanoate (PHO and PHA3836, Tepha Inc), a biocompatible, strong, and flexible polymer. Recent experiments from our laboratory with trileaflet valve constructs fabricated from porous PHO showed promising functional in vivo results. However, PHO has a prolonged bioabsorption time, which persisted in vivo, and was not sufficiently replaced by new functional tissue after 17 weeks (Sodian et al. 1999). In an attempt to create a more ideal scaffold, we developed a novel composite material consisting of a PGA mesh coated with a thin layer of P4HB. P4HB is a thermoplastic, strong, and flexible material, but it has a more rapid bioabsorption time than does PHO. This composite material combines the high porosity of PGA mesh and the added favorable mechanical properties of P4HB. Because of its thermoplasticity, it was possible to fabricate trileaflet valve scaffolds by a heat-application welding technique. In our approach to creating TE structures, the bioabsorbable materials serve as a temporary structural scaffold until the seeded cells produce their own matrix proteins. Once the scaffold is degraded, the biomechanical profile of the TE heart valves will ultimately depend on this matrix formation. In previous studies, we found that the TE constructs had either insufficient mechanical strength or functional performance. We hypothesized that growing the TE constructs in a biomimetic in vitro environment would yield more "mature" heart valve tissue with a more favourable performance in vivo. Recent studies of vascular TE demonstrated a beneficial effect of pulsatile flow with regard to TE arteries (Niklason et al. 1999). Therefore, we developed an in vitro pulse duplicator system in which the TE valves were grown under gradually increasing flow and pressure conditions, thereby providing physical signals to the developing tissues comparable to those encountered in vivo. After 14 days of in vitro culture, the valves grown in the bioreactor showed significantly higher formation of matrix proteins, a more organized histological structure, and more favorable mechanical properties than did constructs grown under static culture conditions. Six of these valve constructs were then implanted into the pulmonary position of sheep for in vivo evaluation. Echocardiography showed functioning valve constructs up to 20 weeks. However, there was mild to moderate valve regurgitation present at 16 and 20 weeks that was due to central noncoaptation. This may result from shrinkage of the cuspal tissue during the process of scaffold bioabsorption and/or the observed increase of the inner diameter of the valve constructs in accordance with the native pulmonary artery growth (4 mm over the 5-month time period). A possible solution to compensate this phenomenon may be an optimized scaffold design with an initially increased coaptive area of the polymer leaflets.

Histology showed increasing organization and layering of the leaflet structure with a fibrous layer rich in collagen and a loose layer rich in GAGs and elastin near the inflow surface as well as partial coverage with endothelium. Furthermore, the extracellular matrix analysis reflected a dynamic process of growth and remodeling, with matrix constituents comparable to native tissue at 20 weeks. Therefore, the present study suggests that remodeling of the TE heart valve occurred in vivo, yielding an organized layer and structure with many architectural features and extracellular matrix elements characteristic of the native semilunar valve (Schoen 1997). In addition to the microstructural similarities, the TE valves attained mechanical properties that at 20 weeks were almost indistinguishable from those of native valve tissue.

In summary, the present study describes a functional, living, completely autologous TE heart valve generated and conditioned in a biomimetic in vitro environment, which functioned satisfactorily in vivo up to 5 months. More important, the engineered valve leaflets gradually evolved to resemble the native pulmonary valve leaflet, as demonstrated by their histological, biomechanical, and biochemical characteristics. However, these results are very preliminary in as much as the number of implanted valves is small and the longer term fate is unknown. Our next efforts are directed at optimization of the scaffold design to incorporate sinuses of Valsalva to more closely approximate the natural shape of a semilunar heart valve and root. Moreover, the optimal cell source is still undetermined. Finally, optimization of the in vitro conditions...
with regard to growth factors, growth inhibitors, and pressure-loading conditions are areas for future studies.

3.6 ACKNOWLEDGEMENTS

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"Evolution of Cell Phenotype and Extracellular Matrix in Tissue Engineered Heart Valves during In Vitro Maturation and In Vivo Remodelling"

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4.1 ABSTRACT

Contemporary tissue valves are non-viable, and unable to grow, repair or remodel. It was postulated that tissue-engineered heart valves (TEHV) fabricated from autologous cells and a biodegradable scaffold could yield a dynamic progression of cell phenotype and extracellular matrix in vitro and in vivo, and ultimately recapitulate native valve microscopic architecture. Trileaflet valve constructs were fabricated from poly-4-hydroxybutyrate-coated polyglycolic acid, seeded with ovine endothelial and carotid artery medial cells, cultured in vitro for 4-14 days in a pulse duplicator, implanted as pulmonary valves in five lambs, and explanted at 4-20 weeks. ECM composition and collagen architecture were examined by histology (including Movat pentachrome stain and picrosirius red under polarized light), and cell phenotype by immunohistochemistry. Cells from in-vitro constructs (14 days) were activated myofibroblasts, with strong expression of α-actin (microfilaments), vimentin (intermediate filaments) and SMemb (non-muscle myosin produced by activated mesenchymal cells). Cells from in-vivo explants at 16-20 weeks were fibroblast-like, with predominant vimentin expression and undetectable levels of α-actin (similar to native valve). Collagen elaboration and cellular expression of MMP-13 (collagenase 3) were evident in vitro at 14 days. In-vivo explants had increased collagen accumulation and strong MMP-13 expression at 4-8 weeks, but less activation (decreased expression of SMemb) and patchy endothelial cells at 16-20 weeks. Moreover, the ECM architecture of 16- to 20-week explanted TEHV resembled that of native valves. Cell phenotype and ECM in TEHV prepared in vitro and implanted in vivo are dynamic, and reflect the ability of a vital tissue to remodel and, potentially, to grow.

4.2 INTRODUCTION

Each year, cardiac valve replacement using mechanical or tissue valve substitutes extends the survival and enhances the quality of life of approximately 82,000 patients with severe valvular heart disease in the United States, and 275,000 patients worldwide. Nevertheless, mechanical prosthetic valves are associated with a risk of thromboembolism, necessitating chronic anticoagulation therapy with its consequent risk of hemorrhage, whilst tissue valves suffer from dysfunction due to progressive structural tissue deterioration. A particular shortcoming of contemporary valve substitutes in the pediatric population is their failure to grow, repair and remodel. Thus, a non-obstructive, non-thrombogenic tissue valve substitute that lasts the patient’s lifetime, provides ongoing remodeling and repair of cumulative injury, and also potentially grows with the recipient is an important goal.

Tissue engineering is an approach to tissue augmentation and organ replacement in which a construct containing cells seeded onto an anatomically appropriate resorbable scaffold is cultured in vitro in a bioreactor, and then implanted (Fuchs et al. 2001, Vacanti and Langer 1999, Shinoka et al. 1996, Schoen and Levy 1999). Remodeling of the construct in vivo is intended to recapitulate normal functional architecture. Tissue-engineered heart valves (TEHV) might grow with the child, allowing valve replacement or a single stage repair of valve requiring surgery for congenital heart malformations early in childhood.

Initial attempts to fabricate TEHV prototypes have been encouraging (Shinoka et al. 1996, Schoen and Levy 1999, Sodian et al. 2000, Stock 2000, Hoerstrup et al. 2000). In one study, living heart valves prepared in vitro in a biometric flow culture system and subsequently implanted not only functioned well for up to five months, but also resembled normal heart valves histologically (Hoerstrup et al. 2000). The present study extends the analysis of the specimens generated in that previously published study by testing the hypothesis that there is a dynamic progression of cell phenotype and extracellular matrix (ECM) in vitro and in vivo, in TEHV fabricated from autologous cells and biodegradable scaffold, and that this progression ultimately yields the morphological qualities of native valves.

4.3 METHODS

4.3.1 In-vitro maturation and in-vivo remodeling of TEHV

The methods of scaffold fabrication, cell isolation, culture and seeding, and construct implantation have been described previously (Hoerstrup et al. 2000). In brief, a scaffold composed of poly-4-hydroxybutyrate-coated polyglycolic acid (a biodegradable, rapidly absorbable strong, pliable and thermoplastic polymer) was seeded with ovine carotid artery medial cells and cultured in static nutrient medium for four days. Thereafter, the evolving constructs (n = 5) were seeded with endothelial cells from the same animals, transferred into a pulse duplicator system ('bioreactor'), and matured for 14 days. The conditioned constructs were implanted into the same lambs (n = 5) from which the cells were initially harvested. TEHV were explanted at 4, 6, 8, 16 and 20 weeks. Native pulmonary valves for structural studies were obtained at surgery from healthy adult sheep (n = 3). All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 85-23,1985).

4.3.2 Morphological characterization

Cusps from native pulmonary valves and a representative cusp of each TEHV were fixed in 10% buffered formalin and embedded in paraffin. Serial sections (6 μm) were stained with hematoxylin and eosin for general morphology and special stains as discussed below.

A modified Movat pentachrome stain (Russell 1972) was carried out to demonstrate changes in composition and distribution of connective tissue elements in tissue-engineered constructs and explanted valves. Picrosirius red staining (Junqueira et al. 1979) was carried out to determine the collagen architecture and collagen types I and III. Sections were analyzed using...
polarization microscopy. Picrosirius red staining under polarized light was graded as: - = no birefringence; + = weak birefringence of few collagen fibers; and ++ = strong birefringence (Table 1).

Table 4.1: Evolution of collagen and cell phenotype in tissue-engineered heart valves during in-vitro maturation and in-vivo remodeling toward the native valve.

<table>
<thead>
<tr>
<th>Cell Marker</th>
<th>In vitro (14 days)</th>
<th>In vivo (4-8 weeks)</th>
<th>In vivo (16-20 weeks)</th>
<th>Native Valves</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>α-Actin</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Vimentin</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>+++</td>
</tr>
<tr>
<td>SMemb</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>MMP-13</td>
<td>+++</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Disoriented, primarily in fibrosa.
**Oriented, primarily in fibrosa.

4.3.3 Characterization of cell phenotype by immuno-histochemistry

Myofibroblasts were defined as cells that have two types of cytoskeletal filaments: smooth muscle α-actin (microfilaments) and vimentin (intermediate filaments) (10) (Dako Corp., Carpenteria, CA, USA). Myofibroblasts that express vimentin and α-actin are called VA-type (based on immunohistochemical staining of these filaments) (11). Cell activation was demonstrated by antibody to monoclonal SMemb (a non-muscle myosin produced by activated mesenchymal cells, also known as MHC-B) (Aikawa et al. 1993, Aikawa et al. 1998). Mouse monoclonal antibody against human MMP-13 (Oncogene Research Products, Cambridge, MA, USA) was used to determine the expression of collagenolytic enzyme collagenase 3. Endothelial cells were delineated by immunostaining with rabbit polyclonal anti-human von Willebrand factor (vWF) antibody (Dako Corp.). Immunohistochemistry was carried out using the avidin-biotin-peroxidase method after antigen retrieval. Tissue sections were treated with 0.3% hydrogen peroxide to inhibit endogenous peroxidase activity, and then incubated with primary antibodies diluted in phosphate-buffered saline supplemented with 4% of the species-appropriate normal serum. The subsequent processing was performed according to the manufacturer's recommendations (Universal Dako LSAB kit; Dako Corp.). The reaction was visualized with 3-amino-9-ethyl carbazole as substrate (AEC; Sigma Chemical Co.). Sections were counterstained with Mayer's hematoxylin solution. Adjacent sections treated with non-immune IgG served as controls for antibody specificity. Immunohistochemical staining was graded as: - = no appreciable staining; + = weak staining of <50% of cells; ++ = strong staining of 10-50% of cells; and +++ = strong in >50% of cells (Table 1).

4.4 RESULTS

The study results compared structural and morphological features of the normal ovine pulmonary valve with both constructs formed in vitro and those following in-vivo function and explantation. Figure 4.1 shows overall morphology, with connective tissue elements highlighted by Movat pentachrome stain; Figure 4.2 shows collagen fiber architecture by picrosirius red staining under polarized light; and Figure 4.3 illustrates the immunophenotype of the cells in the tissue-engineered valves.

Figure 4.1: Evolution of the extracellular matrix (ECM) towards a three-layered valve structure in TEHV in vitro and in vivo. A) Formation of ECM in vitro after 14 days in bioreactor and (B) in cusp at four-week implants as predominant proteoglycan accumulation. Elastin was not detected. C) This 20-week explant shows a three-layered structure: collagen in the fibrosa (f); proteoglycans in the spongiosa (s); and elastin (arrowheads) near the ventricular side (v), similar to the native pulmonary valve (D). The outflow
surface is indicated by the arrows. Circumferential sections; Movat pentachrome stain; original magnification x100.

Figure 4.2: Structural evolution of collagen toward functional valve architecture in TEHV in vitro and in vivo. A) In a 14-day in-vitro construct, collagen was evident as a few fibrils of weak birefringence (arrows). B) At four weeks, implants showed disorganized and disoriented accumulation of fibrillar collagen on the free edge of the leaflet (arrows); polymer residue is indicated by the arrowhead. C) At 20 weeks, explants demonstrate three-layered collagenous architecture reminiscent of the native valve (D) (f: fibrosa; s: spongiosa; v: ventricularis). Circumferential sections; picrosirius red staining under polarized light; original magnification x100.

4.4.1 Native pulmonary valves as a control

Native normal pulmonary valves from healthy adult sheep have three well-defined tissue layers, each containing cells and characteristic ECM composition and configuration, similar to that of aortic valves (Schoen 1997): (i) the fibrosa, which is composed predominantly of microscopically crimped collagen fibers arranged parallel to the free edge of the leaflet; (ii) the centrally located spongiosa, which is composed of proteoglycans and loosely arranged collagen; and (iii) the ventricularis, which is comprised of elastic fibers and loose collagen (see Fig. 4.1D). Picrosirius red staining under polarized light showed that collagen fibers were organized in a layered fashion with predominant accumulation of collagen type I in the fibrosa layer, revealed by strong red birefringence (see Fig. 4.2D). Interstitial cells of normal valves express vimentin only. Interstitial cells are not immunoreactive to α-actin, SMemb or MMP-13 antibodies, suggesting that cells in normal valves express features of quiescent fibroblast-like cells (Rabkin et al. 2001).

Figure 4.3: Dynamic progression of cell immunophenotype in TEHV toward phenotype of interstitial cells of native valves. (Upper panels) Constructs prepared in vitro (14 days) with activated myofibroblast-like cells, as determined by strong expression of α-actin and vimentin (arrows). Lower panels) Explant at 16-20 weeks, with fibroblast-like cells, showing predominant expression of vimentin (arrows) and undetectable levels of α-actin. Original Magnification x100.

4.4.2 Constructs fabricated in vitro

The formation of ECM after 14 days in the bioreactor was demonstrated by Movat staining as predominantly proteoglycan accumulation (Fig. 4.1A). This was in agreement with previously reported biochemical measurements (Hoerstrup et al. 2000). Collagen accumulation detected by picrosirius red staining was evident at 14 days as a few fibrils of weak, predominantly green, birefringence (collagen type III). The collagen fibrils were oriented along the spindle-shaped cells detected throughout the tissue (Fig. 4.2A). Immunohistochemical staining revealed that cells in the construct were activated myofibroblasts, as determined by strong expression of α-actin (microfilaments), vimentin (intermediate filaments) (Fig. 4.3, upper panels),
SMemb (produced by activated mesenchymal cells), and MMP-13 expression.

4.4.3 Explanted TEHV

Cusps at four, six and eight weeks after explantation were thick with predominant proteoglycan accumulation, especially in the central layer (Mowat). Elastin was not detected (Fig. 4.1B). Picrosirius red staining showed disorganized and disoriented accumulation of fibrillar collagen on the free edge of the leaflets (Fig. 4.2B). At 16-20 weeks, the leaflets were layered and had virtually uniform structure from base to edge. Movat staining demonstrated the presence of collagen in the fibrous layer on the pulmonary arterial (outflow) side, and proteoglycans in the central loose layer. Elastin was detected in the ventricular (inflow) side (Fig. 4.1C). Sections stained with picrosirius red and examined under polarized light further demonstrated three-layered collagenous architecture. Collagen fibers in the fibrous layer were thick, predominantly type I, densely packed, and arranged parallel to the free edge of the leaflet, reminiscent of the native valve. Collagen was almost undetectable in the central layer, and appeared loose and fragmented below the ventricular side (Fig. 4.2C and D). Some cells in explants at four to eight weeks showed weak staining for \(\alpha\)-actin and vimentin, but all cells throughout the leaflet stained strongly for SMemb and MMP-13. Explants at 16-20 weeks contained fibroblast-like cells, with predominant expression of vimentin and undetectable levels of \(\alpha\)-actin (Fig. 4.3, lower panels). Some cells were still positive for SMemb and MMP-13. Leaflets were partially covered with vWF-positive cells that were characteristic of endothelial cells (data not shown).

4.5 DISCUSSION

Recent pathological studies of contemporary tissue valve substitutes have yielded several concepts (Schoen and Levy 1999, Schoen 1999), namely that: (i) the transplanted cells are no longer viable; (ii) repopulation by recipient connective tissue or endothelial cells is minimal and ineffective; (iii) valve performance depends critically on the quality of the collagenous skeleton and its resistance to deterioration; and (iv) the non-viable donor cells (and their fragments) are the preferred loci for calcification. Tissue engineering approaches to valve substitution with living, functional cells may yield a structure that morphologically and functionally resembles a natural valve in which the cells are viable, capable of anatomically appropriate and constant renewal, remodeling and repair of the valvular ECM.

It was reported previously that the engineered valve leaflets gradually evolved to resemble the native pulmonary valve leaflet, as demonstrated by histology and biomechanical and biochemical characteristics (Hoerstrup et al. 2000). In the present study, it was further shown that the ECM and cell phenotype in TEHV prepared in vitro and implanted in vivo undergo evolution and remodeling, and eventually have cellular and matrix features of the native valve. ECM from in-vitro pre-implants and four- to eight-week explants demonstrated changes in composition, distribution and organization of connective tissue elements similar to those of valve leaflets during early development: predominant accumulation of proteoglycans; absence of elastic fibers; and weak staining for collagen (unpublished observations on sheep fetal material). Moreover, the present study results suggest that remodeling of the TEHV is evident in vivo, as shown in explants at 16-20 weeks; these specimens had a layered structure with dense fibrillar collagen characteristic of the native valve. Moreover, the immunophenotype of the cells in TEHV progressed from activated myofibroblasts in vitro to fibroblast-like cells, and ultimately to a cell phenotype of native valves in vivo. In addition, the cell phenotype changes in TEHV resembled the evolution of physiological wound healing: myofibroblasts in granulation tissue characterized by an accumulation of \(\alpha\)-actin microfilament, which progressively regress over time and are no longer present in scar fibroblasts (Darby et al. 1990). Furthermore, myofibroblasts in abnormal healing such as human hypertrophic scars (Skalli et al. 1989) contain markers of smooth muscle cell differentiation, including \(\alpha\)-actin, whereas cells in normally healing wounds do not contain this marker. Studies using and extending the paradigm described herein are prerequisite to the widespread clinical reality of tissue engineering approaches. Such techniques will provide pre-implantation 'quality control' of tissue-engineered samples grown in vitro, and morphological evaluation of implanted-explanted TEHV. It is hoped that the results of the present study and further pathological evaluation of TEHV will also shed new light on understanding the mechanisms of remodeling, growth and deterioration of TEHV, and help to identify morphological surrogates for eventual complications. Finally, such studies may identify markers of the quality and extent of tissue structural evolution that may be used for potential non-invasive assessment of maturation and healing of tissue-engineered products of diverse types.

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

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5.1 ABSTRACT

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.

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, secretationally 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.

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.

5.2 INTRODUCTION

Current options of surgical heart valve replacement comprising of mechanical or biological prostheses substantially changed the natural history of end-stage valvular heart disease (Braunwald 1992). Unfortunately, there are limitations as to the long term benefits of clinically available valve prostheses (Schoen and Levy 1999). Mechanical valves are associated with a significant risk of thromboembolism (Vongpatanasin et al. 1996), and biological valves suffer from structural dysfunction because of progressive tissue deterioration (Hammermeister et al. 1993). Contemporary clinically available valve prostheses basically represent nonviable structures and lack the potential to grow, to repair, or to remodel (Mayer 1995). Tissue engineering represents a novel scientific concept to overcome these limitations aiming at in vitro fabrication of living heart valves with a thromboreistant surface and a viable interstitium with repair and remodeling capabilities. Several groups demonstrated the feasibility of creating living cardiovascular structures by cell seeding on synthetic polymer, collagen, or xenogenic scaffold (Shinoka et al. 1996, Zund et al. 1998, Bader et al. 2000, Elkins et al. 2001). However, limitations of these tissue engineered constructs included structural immaturity and sub-optimal mechanical properties resulting in unfavorable in vivo results (Stock et al. 2000). Recently our group demonstrated the first successful tissue engineering of a completely autologous, living tissue engineered heart valve (TEHV) in a juvenile sheep model (Hoerstrup et al. 2000). These TEHV showed excellent functional performance up to 20 weeks and strongly resembled natural heart valves as to microstructure, biomechanical profile and matrix protein formation. Up to now, most approaches were based on the utilization of vascular derived cells associated with certain shortcomings. Cell harvesting before seeding necessitated the sacrifice of intact vascular structures of the donor organism. Apart from that, vascular derived cells demonstrated different characteristics compared with natural valvular interstitial cells, qualities which might be vital to the development and long term function of TEHV (Roy et al. 2000).

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 allogenic settings (Prockop 1997, Liechty et al. 2000). 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.

5.3 METHODS

5.3.1 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: 1x10⁶, 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.
5.3.2 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), penicillin (Gibco), streptomycin (Gibco), and 1000 U heparin (Roche Pharma AG, Reinach, Switzerland). Following the cell suspension was centrifuged over a Ficoll step gradient (density 1.077 g/mL) (Ficoll-Histopaque 1077, Sigma) at 1500 rpm for 10 minutes. 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 (Coming, 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.

5.3.3 Cell Seeding and In Vitro Culture

Purified human MSC (4.5 to 5.5x10^6 per cm^2) 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.

5.3.4 Analysis of MSC Cultures and Trileaflet TEHV

Flow Cytometry

A single cell suspension of 0.5 to 1x10^6 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 (Oncogene, Boston), and elastin (Sigma, St. Louis). After incubation with a secondary biotin-labeled goat-anti-mouse IgG antibody (Sigma, St. Louis) the signal was developed with the avidin-peroxidase system (ABC kit, Vector Laboratory, Burlingame CA). Before intracellular staining, permeabilization of the cells was performed by incubation with 0.1% Triton (Sigma, St. Louis) for 10 minutes. A representative portion of each valve construct was examined histologically by H&E stain (overall morphology), and Trichrome-Masson stain (for demonstration of matrix elements, including collagen, elastin, glycosaminoglycans). Immunohistochemistry was performed on frozen and paraffin embedded valve and 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 (FASTIN™ assay, Biocolor, Belfast, Ireland) were performed and compared with native human tissue (semilunar valve) (Stock et al. 2000).

Mechanical Properties

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

5.4 RESULTS

5.4.1 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 5.1a, b). Immunohistochemistry of fixed cells showed the expression of ASMA and vimentin (Figure 5.1c, d).

![Figure 5.1](image1.png)

**Figure 5.1.** Isolated MSC appeared small and rounded with a tendency to grow in clusters. Elongated cells with fibroblast-like morphology appeared after 72 hours and reached confluence after 10-14 days (a and b). Fluorescence immunohistochemistry of the cultivated MSC showed positive staining for ASMA (c) and vimentin (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 5.2).

![Figure 5.2](image2.png)

**Figure 5.2.** 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).
5.4.2 Analysis of MSC-Based TEHV

In all TEHV a synchronous opening and closing of the leaflets was observed in the bioreactor. Before explantation from 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). Gross appearance showed that all leaflets were intact, mobile, and pliable and the valve constructs were competent during valve closure (Figures 5.3 and 5.4).

Figure 5.3: Function of the TEHV in the pulse duplicator in vitro system (bioreactor; view from above on TEHV positioned in flow perfusion chamber). Note the TEHV in closed (a), intermediate (b), and fully opened position (c).

Figure 5.4: MSC based tissue engineered heart valve after 14 days in the pulse duplicator in vitro system (bioreactor). Note the thin (0.4 to 0.6 mm), intact, pliable 3 leaflets.

Histology and Immunohistochemistry of TEHV

H&E and Trichrome-masson staining of representative TEHV leaflet sections demonstrated cellular tissue organized in a layered fashion with a dense outer layer and lesser cellularity in the deeper portions after 14 days in the pulse duplicator. Immunohistochemistry showed positive staining for collagen types I, III, α-SMA, and vimentin. No positive staining was observed for desmin, collagen types II and IV. Static controls showed a loose, less organized tissue formation with irregular cellular ingrowth (Figure 5.5).

Figure 5.5: 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 (a). In contrast static controls showed less ECM deposition and less organized tissue formation (b). Immunohistochemistry showed the formation of collagen types I (c) and III (d) in the TEHV leaflet tips. Staining for ASMA (e) and vimentin (f) revealed positive signals throughout the TEHV leaflets.

Transmission and Scanning Electron Microscopy

TEM revealed cell elements typical of viable, secretionally 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 5.6).
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Figure 5.6: TEM revealed actin/myosin filaments (focal densities, see arrow, a) typical of viable, secretionally active myofibroblasts as well as collagen fibrils (♯) and elastin fiber conglomerates (*, b). SEM of the TEHV demonstrated homogenous tissue and confluent smooth surfaces with cell orientation in the direction of flow exposition (c), whereas the static controls showed a less confluent, inhomogenous surface (d).

Quantitative Biochemical Assays

The results of the quantitative biochemical assays are summarized in Figure 5.7. 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 \( >300\% \).

Figure 5.7: Quantitative biochemical assays of the extracellular matrix proteins of the TEHV showed lower values compared with human native heart valve tissue \( P<0.05 \). DNA content of the TEHV was 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 5.8). 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).

Figure 5.8: Stress-strain curves obtained by uniaxial tensile testing. In each of the investigated groups consistent mechanical behavior was observed and therefore data were graphically represented as average curves for pulsed, static, and native tissues. Tensile strength testing demonstrated comparable mechanical profiles of the MSC based TEHV to human native semilunar heart valve tissue. In contrast, static controls showed significantly weaker mechanical properties.

5.5 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 (Vongpatanasin et al. 1996, Hammermeister 1993). 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 (Mayer 1995). 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 (Shonoka et al. 1996, Zund et al. 1998, Bader et al. 2000, Elkins et al. 2001, Stock et al. 2000, Hoerstrup et al. 2000). 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 (Hoerstrup et al. 2000). 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 (Roy et al. 2000).

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 (Prockop 1997). In addition, there is evidence that these cells have unique immunological characteristics that allow persistence even in an allogenic environment (Liechty et al. 2000).

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 (Zvaifler et al. 2000, Bucala 1994). 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 (Taylor et al. 2000). 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 (Hoerstrup et al. 2000, Niklason et al. 1999, Hoerstrup et al. 2001). Pulsatile flow or fluid dynamics have a well known impact on cell morphology (Ziegler et al. 1994), proliferation, (Papadaki et al. 1996) and composition of extracellular matrix (Thoumine et al.
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 (Hoerstrup et al. 2000). 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 construct organization of native valve leaflets comprising a ventricularis, spongiosa, and fibrosa layer was not achieved (Schoen 1997). ASMA positive cells expressing vimentin were detectable throughout the constructs demonstrating myofibroblast-like cell populations as described in native semilunar valve tissue (Taylor et al. 2000, Messier et al. 1994). 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, secretationally 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 (Hoerstrup et al. 2000), 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.

5.6 ACKNOWLEDGMENTS

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Chapter 6

HUMAN UMBILICAL CORD CELLS

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“Living, Autologous Pulmonary Artery Conduits Tissue Engineered From Human Umbilical Cord Cells”

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6.1 ABSTRACT

Tissue engineering represents a promising approach to in vitro creation of living, autologous replacements with the potential to grow, repair, and remodel. Particularly in a congenital operation, there is a substantial need for such implantation materials. We previously demonstrated fabrication of completely autologous, functional heart valves on the basis of peripheral vascular cells. Presently the feasibility of creating pulmonary artery conduits from human umbilical cord cells was investigated.

Human umbilical cord cells were harvested and expanded in culture. Pulmonary conduits fabricated from rapidly bioabsorbable polymers were seeded with human umbilical cord cells and grown in vitro in a pulse duplicator bioreactor. Morphologic characterization of the generated neo-tissues included histology, transmission, and scanning electron microscopy. Characterization of extracellular matrix was comprised of immunohistochemistry. Extracellular matrix protein content and cell proliferation were quantified by biochemical assays. Biomechanical testing was performed using stress-strain and burst-stress tests. Histology of the conduits revealed viable, layered tissue and extracellular matrix formation with glycosaminoglycans and collagens I and III. Cells stained positive for vimentin and alpha-smooth muscle actin. Scanning electron microscopy showed confluent, homogenous tissue surfaces. Transmission electron microscopy demonstrated elements typical of viable proteins were significantly lower compared with native tissue; the cell content was increased. The mechanical strength of the pulsed constructs was comparable with native tissue; the static controls were significantly weaker. In vitro fabrication of tissue-engineered human pulmonary conduits was feasible utilizing human umbilical cord cells and a biomimetic culture environment. Morphologic and mechanical features approximated human pulmonary artery. Human umbilical cord cells demonstrated excellent growth properties representing a new, readily available cell source for tissue engineering without necessitating the sacrifice of intact vascular donor structures.

6.2 INTRODUCTION

Many congenital cardiac defects necessitate the use of vascular conduits (e.g., as in surgical reconstruction of the right ventricle to pulmonary artery continuity). Children who undergo these types of operations with either prosthetic or homograft materials often require multiple reinterventions related to conduit failure (Mayer 1998). All clinically available replacements basically represent nonliving, foreign materials with a limited long-term function. They lack the potential for growth and remodelling and are associated with an increased risk of thromboembolic and infectious complications. Stipulated by these shortcomings, the search for ideal replacement materials is still ongoing. The essential characteristics of such materials were described by Dwight E. Harken (Harken 1989) in the 1950s as to ideal heart valves including durability, absence of thrombogenicity, resistance to infections, lack of antigenicity, and the potential for growth (i.e., he described the fundamental characteristics of natural, autologous tissues). To meet these requirements, tissue engineering represents a new experimental approach aimed at living, autologous replacement structures. Several groups, including our own, are pursuing a tissue engineering concept in which tissue constructs are generated in vitro from autologous peripheral vascular cells by seeding onto bioabsorbable scaffolds. Some favourable results with this approach have been presented in experimental animals. Regarding large diameter vascular conduits, Shinoka and colleagues (Shinoka et al. 1998) described in vitro creation of viable pulmonary artery autografts with good functional performance in sheep. Shum-Tim and colleagues (Shum-Tim et al. 1999) demonstrated functional aortic vascular conduits on the basis of ovine carotid artery cells. Patch augmentation of pulmonary artery defects with cell-seeded bioabsorbable polymer in the sheep model was also reported (Stock et al. 2000b). Although represently milestones in this field, these studies were limited by a long persistence of the scaffold material in the circulation because of relatively slow-degrading polymer materials, and by the fact that the usage of peripheral vascular cell necessitated the sacrifice of intact vascular donor structures. In search for an alternative approach, especially with regard to a possible clinical realization, we investigated the feasibility to fabricate tissue-engineered pulmonary artery conduits on the basis of human umbilical cord cells. We used a pulse duplicator culture environment, which in previous studies has shown to accelerate tissue formation and maturation, therefore enabling the application of rapidly bioabsorbable scaffold materials (Hoerstrup et al. 2000, Hoerstrup et al. 2001).

6.3 METHODS

6.3.1 Cell Isolation and Cultivation

Human umbilical cord sections were washed with Dulbecco’s phosphate buffered saline (DPBS; Gibco, Rockville, MD), minced into 1-mm pieces and distributed in Petri dishes (Gibco BRL, Rockville, MD). Tissue sections were cultured with Dulbecco’s modified Eagle’s media (DMEM; Gibco) supplemented with 10% fetal bovine serum (HyClone, Logan, UT) and Gentamycin (Gibco). The cells were serially passaged and expanded in a humidified incubator at 37°C with 5% CO₂. Sufficient cell numbers for seeding of bioabsorbable pulmonary conduit polymer scaffolds were obtained after 3 to 4 weeks.

6.3.2 Bioabsorbable Pulmonary Conduit Scaffolds

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; molecular weight [MW], 1x10⁶; 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 that is strong, pliable, and thermoplastic (melting temperature [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 and P4HB composite scaffold material, pulmonary artery conduit scaffolds (length, 40 mm; inner diameter, 18 mm; wall thickness, 1 mm) were fabricated using a heat application welding technique. The constructs were then cold-gas sterilized with ethylene oxide.

6.3.3 Cell Seeding and In Vitro Culture

Human umbilical cord cells (4.5 to 5.5x10^6 per cm^2) were seeded onto the pulmonary conduit scaffolds and cultured in static nutrient media (DMEM, Gibco) for days in a humidified incubator (37°C; 5% CO_2). Thereafter, the constructs (n = 5) were transferred into a pulse duplicator system ("bioreactor") and grown in vitro under gradually increasing nutrient media flow and pressure conditions for an additional 14 days. Controls (n = 5) were grown in static nutrient media accordingly. The media was changed every 4 days.

6.3.4 Analysis of Human Umbilical Cord Cells

Flow Cytometry (Fluorescence Activated Cell Sorting)

Human umbilical cord cells (hUCC) (0.5x10^6; single cell suspension) in 100 µL PBS plus fetal bovine serum were incubated with saturating concentrations of monoclonal antibodies CD 31-FITC (Clone LC14 + 6 + 7; developed and provided by P. Kilshaw, University of Zurich, Zurich, Switzerland), alpha-smooth muscle actin (AMSA) (Clone 1A4, Sigma, St. Louis), desmin (Clone D33, NeoMarkers, Fremont, CA), and vimentin (Clone V9, NeoMarkers). For intracellular staining, ASMA and desmin and vimentin cells were permeabilized with ethanol and incubated with monoclonal antibodies. Then staining with a secondary FITC-conjugated IgG goat-antimouse antibody (Chemicon, Temecula, CA) was performed. 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 were 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

Human umbilical cord cells were cultivated onto glass coverslips in nutrient medium (DMEM, Gibco) and fixed in methanol. Cells were examined histologically by hematoxylin and eosin and Masson's trichrome stain.

Immunohistochemistry was performed by incubation with monoclonal mouse antibodies for ASMA (Sigma, St. Louis), vimentin (Neo-Markers, Fremont), and collagen I, III (Oncogen, Boston). Incubation with a secondary FITC-labeled goat antimouse IgG antibody (Sigma, St. Louis) to vimentin, an AlexaFluor 647-labeled goat-antimouse IgG antibody (Molecular Probes, Leiden, The Netherlands) to ASMA, and a biotin-labeled goat-antimouse IgG antibody (Sigma, St. Louis, MS) to collagen I and III was performed. The biotin-labeled antibody signal was developed with the avidin-peroxidase system (ABC kit, Vector Lab, Burlingame CA). Before intracellular staining for ASMA and vimentin, permeabilization of the cells was performed by incubation with 0.1% Triton (Sigma, St. Louis).

Analysis of the Tissue-Engineered Pulmonary Conduits Microstructure

Sections of the tissue-engineered conduits 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 Masson's trichrome staining. Immunohistochemistry was performed as described previously by incubation with monoclonal mouse antibodies for ASMA, vimentin, and collagen I and III.

Ultrastructure

Additional samples of each conduit were fixed in 2% glutaraldehyde (Sigma, St. Louis, MS) and studied by scanning electron microscopy and transmission electron microscopy.

Quantitative Tissue Analysis

As described previously, biochemical assays for total content of DNA (Current protocols in molecular biology, 1999), hydroxyproline, proteoglycan and glycosaminoglycan (BLYSCAN assay; Biocolor, Belfast, Ireland), and elastin (FASTIN assay; Biocolor) were performed and compared with native human tissue (pulmonary artery) (Hoerstrup et al. 2000).

Biomechanical Analysis

Tissue-engineered constructs and human pulmonary artery tissues were analyzed for mechanical properties using a uniaxial Instron Tensile Tester (Model 4411, equipped with a 100 N load cell and pneumatic clamps; max. pressure, 75 psig). Tests were carried out using longitudinal matrix strips of 20-mm gauge length, 5-mm width, and 1-mm thickness. The crosshead speed was 0.5 inch per minute, which corresponds to a linear strain rate of 1 minute^-1. The Young's modulus was obtained from the slope of the initial linear section of the stress-strain curve. Burst strength was measured by cannulation of the vascular constructs on a specifically designed system. They were pressurized with phosphate buffered saline (PBS, Gibco) and the hydrostatic pressure was increased by 5 mm Hg steps until vessel failure.
Statistics

Result data were expressed as mean ± standard error of the mean. We used SPSS 8.0 software for statistical analysis (SPSS Inc, Chicago, IL). An unpaired t test (Student's t test) was performed, considering a p value less than 0.05 as statistically significant.

6.4 RESULTS

After 14 days in vitro in the pulse duplicator system, gross appearance showed intact, pliable pulmonary artery conduits that were completely impermeable to fluid (Fig 6.1).

Figure 6.1: Two views (A and B) of tissue engineered pulmonary artery conduit after 14 days conditioning in the pulse duplicator bioreactor (dimensions: length, 40 mm; inner diameter, 18 mm; wall thickness, 1 mm).

6.4.1 Analysis of hUCC

Hematoxylin and eosin and Masson's trichrome staining of fixed cells showed myofibroblast-like morphology and the deposition of extracellular matrix throughout the cell cultures. Immunohistochemistry revealed intracellular expression of ASMA and vimentin (Fig 6.2). Cultured hUCC did not stain positive for the endothelial cell marker CD 31. The characterization of hUCC by flow cytometry demonstrated no significant difference in morphology compared with vascular-derived myofibroblasts from human saphenous vein as to the expression of ASMA (MFIR, 15.77), desmin (MFIR, 1.1), and vimentin (MFIR, 5.56). No positive signal was detected for CD 31 (MFIR, 1.0) among the isolated cell population.

Figure 6.2: (A) Hematoxylin-eosin and (B) Masson's trichrome staining showed cells with a fibroblast morphology and deposition of extracellular matrix proteins. Immunofluorescence staining demonstrated expression of (C) vimentin and (D) alpha-smooth muscle actin.

6.4.2 Analysis of the Tissue-Engineered Pulmonary Conduits

Tissue Microstructure

Hematoxylin and eosin and Masson's trichrome staining of representative sections of the conduit constructs demonstrated cellular tissue organized in a layered fashion and a homogenous formation of extracellular matrix predominantly comprised of glycosaminoglycans (Fig 6.3A). Immunohistochemistry showed positive staining for collagen types I (C), III (D), ASMA (E), and vimentin (F). Static controls showed a loose, less organized tissue formation with irregular cellular ingrowth (B).
Figure 6.3: (A) Hematoxylin and eosin and Masson’s trichrome staining of the conduit constructs demonstrates cellular tissue organized in a layered fashion and formation of extracellular matrix predominantly comprising glycosaminoglycans. (B) Static controls show a loose, less organized tissue formation with irregular cellular ingrowth. Immunohistochemistry shows positive staining for (C) collagen type I, (D) collagen type III, (E) alpha-smooth muscle actin, and (F) vimentin.

Ultrastructure

Scanning electron microscopy showed dense tissue formation and a confluent smooth surface (Fig 6.4A). Advanced biodegradation of the polymer scaffolds was detected by multiple hydrolytic breakage and fragmentation of the polymer fibers. In contrast, scanning electron microscopy analysis of static controls showed a nonconfluent and less homogeneous surface (B). Transmission electron microscopy revealed cell elements typical of secretionally active myofibroblasts such as collagen fibrils and elastin (C).

Quantitative Tissue Analysis

The data are summarized in Table 6.1. The cell content of the tissue-engineered constructs (DNA-assay) was almost 3 times greater compared with human pulmonary artery tissue, regardless of the in vitro conditions. In contrast, glycosaminoglycans content demonstrated values between 35% (static) and 43% (pulsed). Moreover, hydroxyproline content values were 4.8% (pulsed) and less than 1% (static) that of human tissue (hydroxyproline pulsed vs static; p < 0.05). Elastin was not biochemically detectable in any tissue-engineered construct.

<table>
<thead>
<tr>
<th></th>
<th>TE Conduit</th>
<th>Static</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>279% ± 13%</td>
<td>285% ± 17%</td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>4.8% ± 0.3%</td>
<td>0.7% ± 0.02%</td>
</tr>
<tr>
<td>Glycosaminoglycans</td>
<td>43% ± 8%</td>
<td>35% ± 1%</td>
</tr>
</tbody>
</table>

Mechanical Analysis

The mechanical properties are graphically represented in Figure 6.5, displaying a typical stress-strain curve of each of the investigated materials. The maximal tensile strength of the pulsed tissue-engineered constructs demonstrated values...
of 0.76 ± 0.15 MPa, which were comparable with human pulmonary artery tissue (0.95 ± 0.37 MPa). In contrast, the static controls showed significantly lower tensile strength (0.10 ± 0.02 MPa; p < 0.05). The evaluation of the extensibility (strain at maximal load) of the constructs revealed decreased values compared with the pulmonary artery (pulsed 0.33 ± 0.03 mm/mm; static 0.43 ± 0.09 mm/mm vs 1.61 ± 0.52 mm/mm; p < 0.05). For the Young's modulus, increased values of the pulsed tissue-engineered constructs were observed (pulsed 5.38 ± 2.6 MPa vs 1.69 ± 0.87 MPa) that corresponds to a stiffness approximately 3 times greater in comparison with human native tissue. The burst strength of the pulsed constructs was 180 mm Hg after 14 days in the bioreactor.

![Mechanical properties graphically displayed as a typical stress-strain curve of each of the investigated materials recorded at room temperature with a linear strain rate of 1 minute⁻¹ of tissue engineered (TE) pulmonary conduits (red), static controls (blue), and human pulmonary artery (Hum. PA) tissues (black).](image_url)

**Figure 6.5:** Mechanical properties graphically displayed as a typical stress-strain curve of each of the investigated materials recorded at room temperature with a linear strain rate of 1 minute⁻¹ of tissue engineered (TE) pulmonary conduits (red), static controls (blue), and human pulmonary artery (Hum. PA) tissues (black).

### 6.5 DISCUSSION

Surgical repair of various congenital cardiac defects requires the use of replacement conduits (eg, to establish right ventricle to pulmonary artery connection). Clinically available prosthetic conduits and homografts basically represent nonviable, artificial, or allogeneic materials lacking the potential for growth, repair, and remodeling (Schoen et al. 1995). This limits their long-term function, posing the substantial burden of conduit failure and related multiple reoperations on this patient population (Mayer 1995, Cleveland et al. 1992). This clinical demand has stimulated to further search for more optimal therapeutic solutions. In recent years, tremendous progress in cell biology and cell culture has led to the birth of tissue engineering (Langer and Vacanti 1993). This new experimental approach is aiming at fabrication of living replacements involving a concept in which tissue structures are generated in vitro from autologous peripheral vascular cells by seeding onto synthetic or xenogeneic bioabsorbable scaffolds (Zund et al. 1998, Bader et al. 2000, Sodian et al. 2000). In 1986, Weinberg and Bell (Weinberg et al. 1986) were the first to produce a completely biological vascular conduit from animal collagen and bovine vascular cells, unfortunately showing insufficient mechanical properties. Since then the principal feasibility of the tissue-engineering concept has been elegantly demonstrated in animal studies by successful in vivo performance of functional pulmonary and aortic autografts (Shinoka et al. 1998, Shum-Tim et al. 1999). Although representing major progress to the field, these achievements still have certain limitations regarding the ultimate goal of clinical application.

In this article we demonstrated the feasibility of tissue engineering of living pulmonary conduits on the basis of hUCC and a rapidly bioabsorbable scaffold material. In contrast to previously used materials, this scaffold offers the advantage of being thermoplastic, which allows the fabrication of threedimensional constructs using a heatwelding technique. Moreover, this scaffold has a rapid biodegradation profile adequately matching the increasing matrix production of the neo-tissues. In tissue engineering, the bioabsorbable scaffolds are designed to serve as a template to guide tissue formation and to provide initial mechanical support until the developing autologous neo-matrix replaces their function. We further used a biomimetic in vitro environment to accelerate tissue formation and maturation. The mechanical stability of tissue-engineered constructs ultimately depends on fast in vitro generation of competent, native-analogous extracellular matrix, which is crucial to in vivo function later. In previous studies, based on peripheral vascular cells, we and others have shown that pulsatile flow in vitro conditioning enhanced matrix formation, therefore allowing the use of more rapidly bioabsorbable scaffold materials (Hoerstrup et al. 2000, Niklason et al. 1999). As a consequence, at the time of implantation, mechanically more appropriate, almost completely autologous replacements were achieved minimizing the issue of adverse foreign material body reactions (Hoerstrup et al. 2000).

In the present study we selected HUCC as an alternative cell source to overcome the potential clinical limitation of using peripheral vascular cells. Human umbilical cords are readily available and easy to obtain. By means of modern cell and tissue banking technologies, human umbilical cords may be used as individual cell pools for a patient’s lifetime. The HUCC applied in the present experiments represent mixed cell populations derived from the arterial and venous components of the umbilical cords, as well as the surrounding Wharton’s jelly. Previous investigations have shown that all three cell types exhibit myofibroblast-like characteristics (Kobayashi et al. 1998), which was
confirmed in our study by flow cytometry and immunohistochemistry. In culture, the hUCC demonstrated excellent growth properties and sufficient cell numbers for seeding of the conduit scaffolds that were obtained after 3 to 4 weeks. After seeding and in vitro conditioning in the pulse duplicator bioreactor system, good tissue formation was observed after an additional 3 weeks, which resulted in intact vascular conduits with pliable, impermeable vessel walls. Microstructural and ultrastructural analysis revealed viable, layered tissues showing cell and matrix features known from the native pulmonary artery. These included expression of ASMA, vimentin, and collagen types I and III, as well as elastin. However, the quantitative matrix analysis demonstrated significantly reduced contents of typical matrix components such as collagen and glycosaminoglycans compared with native tissue values. At the same time, the tissue cellularity was significantly increased, reflecting the state of an immature, proliferative tissue with beginning matrix formation (ie, an observation we have made also in previous studies based on peripheral vascular cells [Hoerstrup et al. 2000, Hoerstrup et al. 2000]). A limitation to this study was that no endothelial cells were seeded on the developing conduit tissue. In vivo, an intact endothelium may be of crucial importance to the durability of the tissue-engineered constructs as well as to the absence of thrombus formation. However, in this first feasibility type of study, we focused on in vitro generation of mechanically competent neo-tissues and adequate extracellular matrix production. Biomechanical analysis of the tissue-engineered pulmonary conduits showed tissue strength characteristics approximating those of human pulmonary artery, making these conduits theoretically ready for implantation. However, the elastic properties were different revealing a decreased pliability of the tissue-engineered constructs, which is in accordance with the matrix-content related observation that tissue formation is still in process, specifically regarding the production of elastin. It remains to be evaluated in further in vitro and in vivo experiments whether this maturation will be sufficiently completed. Finally, this study demonstrated the favorable effect of a biomimetic in vitro environment on tissue formation and maturation. In comparison with the static controls, the “pulsed” pulmonary conduits showed significantly better mechanical profiles and tissue quality.

In conclusion, in vitro fabrication of tissue-engineered human pulmonary conduits was feasible using hUCC and a biomimetic-culture environment. The morphologic and mechanical features approximated the human pulmonary artery. The hUCC demonstrated excellent growth properties representing a new, readily available cell source for tissue engineering without necessitating the sacrifice of intact vascular donor structures. Based on these results, the next experimental efforts will be directed at in vitro generation of valved pulmonary artery conduits because of the greater clinical relevance of such replacements. Animal experiments will need to be undertaken to further evaluate this promising new concept.

6.6 ACKNOWLEDGMENTS

The authors wish to thank Manfred Welti and Jay Tracy, Laboratory for Tissue Engineering and Cell Transplantation, University Hospital Zurich, Switzerland, for their valuable work on cell culture and immunohistochemistry; and Klaus Marquard, Department of Surgical Research, University Hospital Zurich, for providing the scanning electron microscopy pictures. We further thank Dr. David P. Martin, Tepha Inc, Cambridge, MA, for his support of the scaffold materials. Finally we are grateful to Annegret Bittermann and Oliver Hoechli, Electron Microscopy Laboratory, University Zurich, Switzerland, for performing the transmission electron microscopy and for their advice and technical assistance with the fluorescence microscopy imaging.
The content of the chapter is published in

"Living patches engineered from human umbilical cord
derived fibroblasts and endothelial progenitor cells"

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A. Mol
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7.1 ABSTRACT

A major shortcoming in contemporary congenital heart surgery is the lack of viable replacement materials with the capacity of growth and regeneration. Here we focused on living autologous patches engineered from human umbilical cord derived fibroblasts and endothelial progenitor cells (EPCs) as a ready-to-use cell source for paediatric cardiovascular tissue engineering. EPCs were isolated from 20 ml fresh umbilical cord blood by density gradient centrifugation and myofibroblasts were harvested from umbilical cord tissue. Cells were differentiated and expanded in vitro using nutrient media containing growth factors. Before seeding, cell-phenotypes were assessed by immuno-histochemistry. Biodegradable patches fabricated from synthetic polymers (PGA/P4HB) were seeded with myofibroblasts followed by endothelialization with EPCs. All patches were cultured in a perfusion bioreactor. A subgroup of patches was additionally stimulated by cyclic strain. Analysis of the neo-tissues comprised histology, immuno-histochemistry, extracellular matrix (ECM) analysis and biomechanical testing. Endothelial phenotypes of EPCs before seeding were confirmed by Ac-Dil-LDL, CD 31, von-Willebrand-Factor and eNOS staining. Histology of the seeded patches demonstrated layered viable tissue formation in all samples. The cells in the newly formed tissues expressed myofibroblast markers, such as desmin and alpha-SMA. The EPCs derived neo-endothelia showed constant endothelial phenotypes (CD 31, vWF). Major constituents of ECM such as collagen and proteoglycans were biochemically detected. Stress-strain properties of the patches showed features of native-analogous tissues.

Living tissue engineered patches can be successfully generated from human umbilical cord derived myofibroblasts and EPCs. This new cell source may enable the tissue engineering of versatile, living, autologous replacement materials for congenital cardiac interventions.

7.2 INTRODUCTION

A major shortcoming in today’s surgery of congenital cardiovascular defects is the lack of living replacement materials with the capacity of growth and regeneration. Currently, pediatric surgical treatment requires the application of synthetic or non-autologous bioprosthetic materials, such as patches and vascular conduits (Mayer 1995, Schoen and Levy 1999). These materials are burdened with substantial disadvantages including obstructive tissue ingrowth and calcification of the replacement (Endo et al. 2001). These limitations and the lack of growth typically cause re-operations of paediatric patients with cardiovascular defects associated with increased morbidity and mortality. To address the above-mentioned problems cardiovascular tissue engineering is a new scientific field aiming at in vitro fabrication of living, autologous grafts with the capacity of growth, repair, and regeneration. Important functional features of ideal replacement materials for the repair of congenital defects comprise a living matrix with native-analogous biomechanical properties and a thrombo-resistant surface. In previous studies, we have demonstrated the feasibility of using human umbilical cord derived myofibroblasts as a cell source for engineering of functional paediatric tissues (Hoerstrup et al. 2002b). The umbilical cord blood is a known source for endothelial progenitor cells (EPCs) differentiated from haemangioblasts, a common progenitor for both haematopoietic and endothelial cells. These cells have the potential to differentiate into mature endothelial cells and have been successfully utilized in non-tissue engineering applications such as for the repair of injured vessels, neovascularization or regeneration of ischemic tissue (Kawamoto et al. 2001, Kocher et al. 2001, Assmus et al. 2002, Pesce et al. 2003) as well as coating of synthetic vascular grafts (Shirota et al. 2003a).

Recently, animal derived EPCs have been used for the endothelialization of decellularized grafts in animal models (Kaulash et al. 2001) and for seeding of hybrid grafts (Shirota et al. 2003b). Little is known about the feasibility and utility of umbilical cord derived EPCs for in vitro engineered endothelium in humans. Here, we investigate human umbilical cord blood derived endothelial progenitor cells (EPCs) as a source for in vitro generation of functional endothelia covering living tissue engineered patches as a simplified model for pediatric cardiovascular tissue engineering.

7.3 METHODS

7.3.1 Isolation of myofibroblasts from human umbilical cord tissue

Cells were obtained from umbilical cords of healthy individuals after informed consent was obtained from the participants. Myofibroblasts were isolated from umbilical cord tissue of newborns. Several (~8 mm³) pieces of umbilical cord tissue were washed with PBS, placed in petri-dishes and cultured in a humidified incubator (37°C, 5% CO₂) in advanced DMEM medium (Gibco, Invitrogen) supplemented with 10% fetal bovine serum (PAN Biotech, Germany), 2 mM Glutamax (Gibco) and Gentamycin (50 mg/ml, PAN). Myofibroblasts were expanded up to passage 11.

7.3.2 Isolation of EPCs from human umbilical cord blood and differentiation into endothelial phenotype

The human umbilical cord vein of newborns was punctured directly after delivery and 20 ml of fresh blood were obtained. Immediately, EPCs were isolated from mononuclear cells by density gradient centrifugation (Histopaque-1077, Sigma), followed by differential centrifugation as described by Asahara et al. (Asahara et al. 1997). Isolated cells were cultured in endothelial basal medium (EBM-2, Cambrex), containing growth factors and supplements provided by the supplier: Vascular Endothelial Growth Factor (VEGF), human Fibroblasts Growth Factor (hFGF), human recombinant long-Insulin-like Growth Factor-1 (R3-IGF), human Epidermal Growth Factor (hEGF), Gentamycin and Amphotericin (GA-1000), Hydrocortisone, Heparin,
Ascorbic Acid and 2% Fetal Bovine Serum. After 4 days, attached cells were reseeded and after 7 days the medium was changed to EBM-2 containing the same growth factors but 20% FBS. After 3 weeks of cultivating, the endothelial phenotype was confirmed by immunohistochemistry. The confluent monolayers were stained with antibodies for von Willebrand factor (vWF, phenotype was confirmed by immuno-histochemistry. The confluent CD34 (IgG 1, Clone QBEND/10, polyclonal; DAKO A/S), CD 31 (IgG1/kappa, Clone JC/70A; DAKO A/S), CD34 (IgG1, Clone QBEND/10; Serotec) and examined for uptake of Ac-LDL (Dil-Ac-LDL, Biomedical tech.) and expression of endothelial nitric oxide synthase (eNOS, polyclonal; Transduction Laboratories, Inc.).

7.3.3 Fabrication of scaffolds
Scaffolds were fabricated from a non-woven polyglycolic-acid mesh with randomized distributed fibers (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm³, porosity >95%, Albany Int.), cut into 2.3 cm² patches (Fig. 7.1) and randomized distributed fibers (PGA, thickness: 1.0 mm, specific gravity: 69 mg/cm³, porosity >95%, Albany Int.), cut into 2.3 cm² patches (Fig. 7.1) and coated with Poly-4-Hydroxybutyric acid (10 w/v P4HB, TEPHA Inc., Cambridge, MA) by dipping. Three patches each were attached to a ring-shaped support of exicator, the scaffolds were cold-gas sterilized.

7.3.4 Seeding of scaffolds
Cells were expanded as monolayers either in 175 cm² tissue culture flasks (TRP, Milian, Geneva, Switzerland) or in roller bottles (Costar, 0.5-1 cm²/bottle) using the Cellroll system (Integra biosciences) at a speed of 0.5-1 minK1. At confluency, cells were harvested by treatment with trypsin/EDTA (0.25%) and seeded onto the biodegradable PGA/P4HB patches by using fibrin as a cell carrier (Mol et al. 2004). Briefly, 3.5x10⁶ cells/100 mm² of scaffold were resuspended in a sterile thrombin solution (Sigma, 10 U/ml medium) and kept on ice in a volume that equals half of the void volume of the scaffold. The same volume of sterile, ice-cold fibrinogen solution was added and the chromophore was allowed to oxidize for 25 min. Aldehyde/perchloric acid solution (1 M) was subsequently added and the chromophore was allowed to develop at 65°C for 15 min. The absorbance of the obtained solutions was determined at 570 nm. The amount of hydroxyproline present in the hydrolysates was determined from a standard curve using known amounts of trans-4-hydroxy-L-proline (Sigma).

7.3.5 Tissue formation analysis

Histology
Samples were fixed in 4% phosphate buffered formalin (pH 7.0) and paraffin-embedded. Sections of 3–5 mm of each group were examined histologically by Eosin-Hematoxylin (H&E), Hematoxylin-Sudan (H&S) and Trichrom-Masson staining. Selected samples were characterized by immunohistochemistry. Vimentin, α-SMA, and desmin were used for analysis of the myofibroblast phenotype and vWF, CD 31 and eNOS staining to validate the phenotype of the EPC derived endothelial cells.

7.3.6 Quantification of extracellular matrix elements

Collagen
As an indicator for collagen formation, hydroxyproline content was determined from dried samples as described by Huszar et al. (Huszar et al. 1980). Briefly, tissue samples were hydrolyzed in 50–100 ml 4 M NaOH (Fluka, Switzerland) in an autoclave at a temperature of 120°C for 10 min. The solution was neutralized by adding an equal volume of 1.4 M citric acid (Fluka). ChloraminT (Riedel-de Haën (Fluka) 62 mM) was added and the samples were allowed to oxidize for 25 min. Aldehyde/perchloric acid solution (1 M) was subsequently added and the chromophore was allowed to develop at 65°C for 15 min. The absorbance of the obtained solutions was determined at 570 nm. The amount of hydroxyproline present in the hydrolysates was determined from a standard curve using known amounts of trans-4-hydroxy-L-proline (Sigma).

s-Glyco-Amino-Glycans
To further specify the extra cellular matrix, s-Glyco-Amino-Glycans were detected colorimetrically using 1,9-di-methyl-methylene blue stain (Famdale et al. 1986), following complete papain digestion of the constructs in 100 mM sodium phosphate pH 6.5, 5 mM cysteine, 5 mM EDTA and 125 mg/ml papain (Sigma).

Determination of cell number
The amount of cells growing on the constructs was indirectly determined by measuring the DNA content from the same papain digested samples using Hoechst dye (Bisbenzimide H 33258, Fluka) (Kim et al. 1988) and a fluorometer (Fluorstar, BMG, Offenburg, Germany, 355 nm excitation/460 nm emmission). Calf thymus DNA (Sigma) was used as a standard.

7.3.7 Testing of mechanical properties
Measurements of the mechanical properties were performed with freshly harvested patches (15x5x1 mm) using an uniaxial tensile tester (Instron)
equipped with a 10 N load cell. Tensile stress and strain were recorded and the Young's modulus was calculated.

7.4 RESULTS

7.4.1 Macroscopic appearance of the patches
All patches showed homogenous tissue formation and dense surfaces (Fig. 7.1).

![Image](image.png)

Figure 7.1: Tissue engineered patch based on human umbilical cord cells.

7.4.2 Phenotype of myofibroblasts
Myofibroblasts showed positive staining for vimentin and expression of α-SMA and desmin (Fig. 7.2a and b).

7.4.3 Morphology and phenotype of EPCs
EPCs could be isolated from all blood samples. After isolation, the plated cells were initially rounded. After 4 days, cells were attached and formed clusters. Two different types of EPCs were found: spindle-like shaped cells (80%) and polymorph cells (20%). Polymorph EPCs formed colonies and showed good growth under in vitro conditions. After 3 weeks, they differentiated into mature endothelial cells forming cobblestone monolayers. Before seeding onto the scaffolds, differentiated EPCs showed an endothelial phenotype. They stained positive for vWF, CD 31, CD 34, eNOS, and showed Ac-LDL-uptake. Differentiated EPCs kept this phenotype when cultivated under both cyclic strain and perfusion in the bioreactor flow system (Fig. 7.2e and f).

![Image](image.png)

Figure 7.2: Histological and immunohistological characterization. Myofibroblasts of the new tissue showed positive staining for (a) desmin and (b) α-SMA (magnification 50x). Hemalaun & Eosin staining (c) demonstrated organized tissue formation with production of extracellular matrix components; in Trichrom–Masson staining (d) collagen fibres are blue (magnifications 50x). Expression of (e) CD31 on surfaces of patches and (f) vWF, respectively confirm the endothelial phenotype of the neo-endothelium formed by EPCs (magnification 200x).

7.4.4 Histology
Histological examination of the seeded patches revealed good cell-to-polymer attachment. Myofibroblasts showed good ingrowth into the PGA/P4HB scaffolds under cyclic strain. A similar picture was observed after culturing under perfusion. EPCs demonstrated a good attachment on the neo-tissue formed by myofibroblasts in both groups of culturing conditions. H&E staining revealed organized tissue-formation with good extracellular matrix formed by myofibroblasts in the inner part of the patches (Fig. 7.2c). Trichrom–Masson-staining highlighted elements of extracellular matrix (Fig. 7.2d). An endothelial cell lining formed by differentiated EPCs was found on surfaces of all patches. Immunohistochemistry confirmed the endothelial phenotype of the neo-tissue (Fig. 7.2e and f).
7.4.5 Quantification of extracellular matrix elements

A summary of the extracellular matrix composition is given in Fig. 7.3. The collagen content of both groups were comparable (strained 4.06 mg/mg±1.92, n = 8, perfused 4.21 mg/mg±0.44, n = 8). The sulfated glycosaminoglycan content was higher in the strained than in the perfused patches (6.44 mg/mg±1.45, n = 4 versus 4.65 mg/mg±0.61, n = 5) and the cell number was significantly higher in the strained patches (3.14 mg/mg±1.02, n = 4 versus 1.24±0.35, n = 5 Student’s t-test p<0.05).

![Figure 7.3: Extracellular matrix analysis. HYP, hydroxyproline; GAG, sulphated glycosaminoglycans; DNA, deoxy nucleic acid (cell number). Averages are given in mg/mg of dry weight. The error bars represent the standard deviation.](image)

7.4.6 Tensile testing

Tissue engineered patches showed normal stress-strain profiles in both groups of culturing conditions (Table 7.1). Only strain at break showed significant differences between the groups.

7.5 DISCUSSION

For surgical treatment of congenital cardiac defects such as augmentation of the hypoplastic, stenotic right ventricular out flow tract or the pulmonary artery in tetralogy of Fallot, for the closure of a complete AVSD, enlargement of the aorta in the Norwood I procedure synthetic or bioprosthetic replacement materials are commonly used. Such non-autologous materials have been associated with substantial complications including calcifications, obstructive tissue ingrowth, infections, and thrombogenicity (Mayer 1995, Endo et al. 2001, Ben-Shachar et al. 1981). The availability of living graft materials with the potential to grow, to regenerate and to adapt to the changes in the developing cardiovascular system would have fundamental advantages over currently used replacement materials.

Table 7.1: Mechanical properties of perfused or perfused and strained tissue engineered patches

<table>
<thead>
<tr>
<th>Samples</th>
<th>Tensile strength (MPa)</th>
<th>Young’s modulus (MPa)</th>
<th>Strain at break</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strained 1</td>
<td>20</td>
<td>92</td>
<td>38</td>
</tr>
<tr>
<td>Strained 2</td>
<td>17</td>
<td>74</td>
<td>33</td>
</tr>
<tr>
<td>Strained 3</td>
<td>11</td>
<td>49</td>
<td>34</td>
</tr>
<tr>
<td>Strained 4</td>
<td>22</td>
<td>107</td>
<td>28</td>
</tr>
<tr>
<td>Strained Mean ± SD</td>
<td>18 ± .05</td>
<td>.80 ± .25</td>
<td>.33 ± .04</td>
</tr>
<tr>
<td>Perfused 1</td>
<td>30</td>
<td>.74</td>
<td>56</td>
</tr>
<tr>
<td>Perfused 2</td>
<td>17</td>
<td>.50</td>
<td>60</td>
</tr>
<tr>
<td>Perfused 3</td>
<td>17</td>
<td>.30</td>
<td>64</td>
</tr>
<tr>
<td>Perfused 4</td>
<td>88</td>
<td>.24</td>
<td>50</td>
</tr>
<tr>
<td>Perfused Mean ± SD</td>
<td>18 ± .09</td>
<td>.44 ± .23</td>
<td>.57 ± .06</td>
</tr>
</tbody>
</table>

In the present study, we investigated the feasibility to utilize human umbilical cord blood derived endothelial progenitor cells for in vitro fabrication of living autologous patches. We further investigated the influence of different in vitro culture conditions on tissue maturation and functionality of the newly formed endothelia. As demonstrated previously (Hoerstrup et al. 2002b), umbilical cord derived myofibroblasts showed excellent ingrowth into biodegradable scaffolds and organized tissue formation with the production of extracellular matrix proteins such as collagen and proteoglycans. This myofibroblasts based matrix formation represents a crucial prerequisite for the successful creation of mechanically competent, surgically implantable replacement materials.

Isolation of the endothelial progenitor cells from umbilical cord blood samples was easily performed and the cells were expandable in sufficient amounts for tissue engineering purposes. After seeding, the endothelial progenitor cells formed functional endothelial layers on the surfaces of the myofibroblast derived neo-matrices of all patches. Most importantly, the EPCs showed constant endothelial phenotype during the whole tissue engineering process and expressed functional features such as eNOS. In previous studies we and others have demonstrated that mechanical conditioning of growing tissue engineered cardiovascular structures, such as heart valves and blood vessels results in enhanced and more mature tissue formation (Niklason et al. 1999). Accordingly, the patches were cultured in a custom designed bioreactor in vitro system. We have chosen two bioreactor protocols using flow and cyclic strain as mechanical stimulation to increase the production of extracellular matrix and mechanical strength of the patches. By comparing the culture conditions, we
found higher contents of extracellular matrix proteins such as proteoglycans as well as cell number when patches were exposed to cyclic strain. However, collagen content was not significantly different. These results indicate the important influence of strain stimulation on early matrix formation as recently described by Mol et al. (Mol et al. 2003).

The mechanical profiles of all patches showed features of native cardiovascular tissues demonstrating a non-linear mechanical behaviour. In contrast, the scaffold material itself exhibits linear behaviour indicating that the measured mechanical properties are relating from the neo-tissues. However, the mechanical strength did not reach physiological values during the investigated in vitro time, restricting the current patches to low-pressure applications such as in reconstruction of the RVOT. Interestingly, we observed a higher Young's modulus representing a higher stiffness in the cyclically strained compared to perfused tissues. This difference in stiffness of the patches may result from more mature extracellular matrix formation reflected by increased proteoglycans content and a higher degree of cross-links among collagen fibres to be assessed in further experiments.

In summary, in this feasibility study we created living autologous human patches based on umbilical cord derived cells, representing a versatile replacement material for congenital cardiac surgery. The endothelial progenitor cells isolated from the umbilical cord blood showed excellent expansion capacities, constant endothelial differentiation, and formation of endothelia with functional properties. Apart from being a potent cell source for differentiated endothelial cells, human endothelial progenitor cells may enable to use tissue engineered replacement materials directly after birth, since they can be harvested prenatal, e.g. by ultrasound guided chordocentesis. This approach would provide sufficient time for the in vitro generation of autologous replacement material ready to use at or shortly after birth and is currently investigated in animal models.

7.6 ACKNOWLEDGEMENTS

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8.1 The Autologous Heart Valve Tissue Engineering Paradigm

In order to overcome the limitations of contemporary heart valve prostheses, different tissue engineering concepts using various cell sources and scaffold materials have been introduced over the last 10 years. The ultimate goal of tissue engineering is to construct tissues from their cellular components which comprise the characteristics of the healthy native original. It is the perfection of architecture and function of a native, living heart valve which enables the enormous life-time performance with billions of cycles without malfunctions. For a functional engineered heart valve, besides adequate mechanical characteristics (mature extracellular matrix) and durability, the absence of immunogenic and/or inflammatory reactions is of critical importance. This goal can be achieved based on a completely autologous tissue engineering principle, also referred to as the “complete heart valve tissue engineering paradigm” (Rabkin-Aikawa et al. 2004). The success of this paradigm is depending on three main components: (a) a biocompatible and rapidly biodegradable matrix (scaffold) which determines the three-dimensional shape and serves as an initial guiding structure for cell attachment and tissue development; (b) a cell source from which a native-analogous living tissue can be grown and which can be harvested from the future recipient without sacrificing critical anatomic structures; and (c) in vitro culture conditions which enable adequate neo-tissue formation and maturation resulting in implantable, living, autologous heart valve substitutes. The sequence of studies comprised in this thesis addresses the above mentioned principal prerequisites for the realization of the autologous heart valve tissue engineering paradigm. The “holy grail” of this approach is to achieve functional autologous tissue formation in vitro, ideally paralleled by a full disappearance of the initial biodegradable scaffold materials. Most in vivo results presented so far were based on slow degrading scaffold materials (full degradation in the order of years) of both synthetic (Sodian et al. 2000, Sutherland et al. 2005) or biological origin (Leyh et al. 2003). Although enabling implantable heart valve constructs more easily because of the mechanical properties of the scaffold material, this approach essentially creates “another type of bioprosthesis” with the potential advantage of a cell seeded surface. The essential characteristics of the ideal tissue engineered valve substitute approximating native heart valves were not realized, particularly with regard to growth and remodeling based on a fully autologous matrix. Using more rapidly degrading scaffold materials (Zund et al. 1998; Hoerstrup et al. 1998) has resulted in almost complete autologous tissue formation in vitro, hence these tissues did not reach sufficient mechanical properties to be implantable as functional heart valves withstand the hemodynamic forces of either the pulmonary or systemic circulation. As a consequence, strategies to enhance the tissue formation in vitro were investigated (Hoerstrup et al. 1999) leading to the hypothesis that “biomimetic” in vitro conditions will result in maximum tissue approximation of the engineered heart valves.

8.2 A “Biomimetic” Approach – Necessary or Nice To Have?

The concept of exposing tissue engineered heart valves to “biomimetic signals” and “mechanical conditioning” in vitro by using a flow bioreactor was introduced in this thesis. This approach was based on the hypothesis, that in vitro exposure of the developing tissue to physical signals similar to those encountered in vivo may result in more mature engineered heart valves. In combination with a rapidly biodegradable scaffold material enabling the fabrication of complex, three dimensional heart valve scaffolds, functional, autologous, tri-leaflet heart valves were demonstrated in a large animal study. Representing a proof of principle and a first step towards realization of the complete heart valve tissue engineering paradigm, several aspects need to be discussed and require further investigations:

In vitro study - Bioreactors

The results presented in this thesis clearly demonstrated that compared to conventional static culture methods, in vitro conditioning of the developing engineered heart valves in a flow bioreactor led to increased extracellular matrix production and/or mechanically more favorable neo-tissues. Furthermore, a more organized tissue formation indicated enhanced tissue maturation. However, although enabling tissues sufficiently functional for in vivo application, at the end of the in vitro process these valves did not fully approximate the matrix architecture found in their native counterparts. Critical elements such as the anisotropic arrangement of the valve leaflet layers and the production of elastin were missing. Scaffold residuals although not being mechanically relevant, were always detectable. Clearly, the bioreactor system used in this work represented a first generation setup and a true “biomimesis” was presumably not achieved both as to the mechanical conditioning itself as well as to the conditioning protocol which was based on a rather intuitive increase of mechanical loading. Whether a closer in vitro approximation to the native benchmark can be achieved by more sophisticated bioreactors and conditioning protocols is currently under investigation. Recently, new bioreactors have been developed for tissue engineering of heart valves, enabling more meticulously controlled physiological conditions (Hildebrand et al., 2004; Dumont et al., 2002). The concept of bioreactors may be further improved by using self-regulating feedback systems allowing to optimize the pressures and forces in accordance to the evolving tissue development. Moreover, monitoring of metabolic parameters (such as glucose, lactate, pH, etc.) and subsequent adjustment of the exact culture conditions may provide an optimized in vitro environment. A better understanding of the physiological hemodynamics from fetal life to adolescence will be crucial to provide flow/pressure protocols which fully mimic in vivo conditions of valvular development. A detailed understanding of the relationship between the mechanical and structural characteristics of the native valve and the stimuli (biological and mechanical) that are required to mimic these characteristics in vitro is needed. Biological stimuli, such as growth factors, either directly
supplemented to the media (Hoestrup et al., 2000c), or incorporated into the scaffolds (Rabkin and Schoen, 2002) may allow to fine-tune the production of specific matrix constituents such as the mechanically important collagen. The effect of specific mechanical regulators and their individual role in the orchestration of mechanical conditioning should also be further investigated, as e.g. laminar flow (Jockenhoevel et al., 2002) and cyclic strains (Kim et al., 1999; Mol et al., 2003) have been shown to increase particularly the production of collagen. It may be that a sequential exposure of the developing tissue to different modes of mechanical loading e.g. a modulus approach using strains initially for predominantly collagen formation followed by flows for generation of appropriate functional layering (elastin in the ventricularis and proteoglycans in the spongiosa) is necessary to achieve fully autologous heart valves, also suitable for high pressure applications. Numerical models may be valuable tools to predict tissue maturation and remodelling (Driessen et al. 2003).

In vivo study - Significance and limitation of animal model

Interestingly, the ultimate refinement and maturation of the engineered heart valves occurred during the in vivo period of the experiments, showing an evolution of cell phenotype and extracellular matrix towards native valve tissue. These findings suggest that a true native-analogous heart valve tissue may only be achieved in vivo. The question however, whether this is simply a function of a longer tissue maturation time or additional co-factors only present in vivo (e.g. erythrocytes as oxygen carriers, humoral growth factors, circadian rhythm etc.) still needs to be clarified. Furthermore, there is the question whether the appropriate native benchmark is addressed when comparing an engineered heart valve generated in vitro after relatively short tissue maturation time periods to a juvenile or adult heart valve tissue. Recent studies by Rabkin et al. have investigated the composition of heart valve tissues during embryonic and fetal development indicating similarities with in vitro engineered valve tissues (Rabkin-Aikawa et al. 2005). This leads to the conclusion that each state of tissue maturation may be best compared to its developmentally matched native benchmark.

For evaluation of cardiovascular implants such as heart valve prostheses in preclinical studies, sheep are the animal model of choice for various reasons: First, the anatomic and hemodynamic conditions are sufficiently comparable to the human situation. Second, since sheep have an increased calcium metabolism compared to humans, prosthetic implants tend to calcify rapidly. For that reason, it is widely agreed that sheep represent a "worst case model" allowing to assess degenerative processes of cardiovascular implants in a relatively short period of time (Ali et al. 1996). In addition and most importantly for tissue engineering purposes, the juvenile sheep model enables the monitoring of TEHV growth with respect to the recipient organism, since the full biological growth cycle is normally finished after 2 years. With regard to the proof of principle study included in this thesis some limitations need to be addressed: 1) Limited follow up time and number of implants: The maximum follow up time after TEHV implantation was 20 weeks, which does not cover the full animal growth as indicated above. Moreover, only 6 animals were included in the study each representing a different endpoint. Based on these numbers, statistically significant conclusions were difficult. However, there was an increase of the inner diameter of the TEHV of approximately 20% detectable over the observed period of time in accordance with the growth of the adjacent native pulmonary artery. This finding together with the absence of relevant valvular regurgitation or transvalvular pressure gradients provides some indication of functional growth. Moreover, there were no signs of TEHV calcifications in any of the implants. From studies evaluating biological heart valve prostheses it is known that first signs of degeneration are detectable few months postoperatively excluding at least early degeneration of the investigated TEHV (Walles et al. 2003; Rabkin-Aikawa et al. 2004). 2) Low pressure implantation: All TEHV investigated in the study were implanted in the pulmonary position of the heart representing the low pressure side (1/3 of systemic pressure) of the circulation. Although providing proof as to the principle feasibility of autologous heart valve tissue engineering, no conclusions can be drawn as to the functionality of the TEHV in systemic applications yet. Since in clinical reality, the majority of heart valve prostheses are implanted as aortic or mitral replacement, a substantial medical need may not be met by the tissue engineering technology.

In summary, it appears that a biomimetic approach is a "necessary" to tissue engineering of functional autologous heart valves. The cells need to be placed into the appropriate "environmental niche" to produce the "right tissue". The work included in this thesis demonstrated that this process can be initiated by bioreactors. However, so far the refinement is done by nature. Future research focused on improved bioreactor protocol may realize this refinement already in vitro. With regard to heart valve tissue engineering, in contrast to the usual saying "Do not imitate -- innovate" the motto should rather be "Imitate (nature) to innovate (valve substitutes)!!"

8.3 Alternative Human Cell Sources – Availability and Degree of Differentiation

Vascular derived autologous cells as utilized in the proof of principle sheep study (and the majority of in vivo results presented so far) necessitate the sacrifice of intact vascular donor structures and have been reported to demonstrate considerable differences compared to valvular interstitial cells (Roy et al. 2000). Therefore, less invasive alternative cell sources including progenitor-type cells were evaluated in the human cell in vitro experiments included in the second section of the thesis.

Adult Applications

For adult applications the feasibility of using human marrow stromal cells (MSC) for tissue engineering of trileaflet heart valves was investigated. These
cells were routinely obtained by a simple puncture of the iliac crest representing a less invasive cell source with the potential to differentiate into various tissues. In addition, these cells have been shown to have unique immunological characteristics, potentially allowing their applications in allogenic settings (Prokop 1997, Liechty et al. 2000). Isolation and expansion of MSC was easily performed and cell morphology and growth pattern were in accordance with previous studies focusing on mesenchymal progenitor cells (Zvaifler et al. 2000, Bucala 1994). After cellular differentiation and before seeding, the cells showed characteristics similar to human valvular interstitial cells (Taylor et al. 2000). Utilizing MSC’s in the flow bioreactor system, intact trileaflet heart valves were generated in vitro, showing functionality even under supra-physiological flow and pressure conditions. Layered tissue formation was seen and most importantly the absence of myeloid or osteoblastoid cell and tissue differentiation was confirmed. The latter being of substantial importance to exclude an osteogenic differentiation of the bone marrow-derived MSC resulting in “bone-like” tissue development detrimental for heart valve tissues. Interestingly, regarding extracellular matrix quantities, there was no significant difference between the TEHV exposed to dynamic culture conditions in the bioreactor compared to the static controls. Concomitantly, the mechanical properties were significantly higher and the morphological analysis revealed a more organized tissue formation. This indicates that tissue maturation is not a factor of matrix production alone, but also of the degree of tissue organization. The better assessment of extracellular matrix maturation needs further investigations including quantification of e.g. collagen crosslinks (Sacks and Schoen 2002; Sims et al. 2000), elastin compositions (Latif et al. 2005), and the activity of specific matrix remodeling enzymes such as metalloproteinases etc. (Rabkin-Aikawa et al. 2004). The TEHV generated in the present study, however, did not approximate the typical three layer structural composition of native valve leaflets with a ventricularis, spongiosa, and fibrosa layer, leaving room for further improvement of this approach.

**Pediatric Applications**

The highest medical need for a tissue engineered heart valve is in pediatric applications for the treatment of congenital cardiovascular malformations. Approximately 1% of all newborns have congenital heart defects, and many of them require open heart surgery and heart valve replacement (Mayer et al. 1995). Since currently available artificial valve prostheses cannot grow with the young patients, repeated replacement operations have to be performed associated with exponentially increased morbidity and mortality. In a first set of experiments addressing this issue, human umbilical cord derived cells (hUCC) were selected as an alternative cell source to overcome the potential clinical limitations of using peripheral vascular cells (even a saphenous vein is very difficult to harvest in a newborn and vein explantation may lead to extremity growth problems during later somatic development). As a readily available autologous cell source, which normally is discarded at birth, this approach may enable the in vitro generation of living replacement materials available shortly after birth. In culture, the hUCC demonstrated excellent growth properties and sufficient cell numbers for seeding of the conduit scaffolds were obtained after 3 to 4 weeks. The hUCC applied in the present experiments represented mixed cell populations derived from the arterial and venous components of the umbilical cords, as well as the surrounding Wharton’s jelly (a mucoid connective tissue). Previous investigations have shown that all three cell types exhibit myofibroblast-like characteristics (Kobayashi et al. 1998), which was confirmed in our study by flow cytometry and immunohistochemistry. In recent studies it has been further demonstrated that the Wharton’s Jelly (Wang et al. 2004) and the directly perivascular tissue contain significant amounts of mesenchymal progenitor cells (Sarugaser et al. 2005). These findings make umbilical cord tissues a promising cell source for cardiovascular tissue engineering and further applications in the field of regenerative medicine. By means of modern cell and tissue banking technologies, human umbilical cords may also be used routinely as individual cell pools for the patient’s lifetime.

Ideally, a tissue engineered heart valve would be available for implantation already at the time of birth in order to prevent secondary damage to the immature heart. This requires cell harvest prior to birth, e.g. by ultrasound guided cordocentesis (puncture of the umbilical cord in utero) providing sufficient time for the in vitro generation of autologous replacement material.

To validate the concept of prenatal tissue engineering, umbilical cord blood derived human endothelial progenitor cells (EPCs) were investigated. EPCs can be isolated from peripheral and umbilical cord blood (Hur et al. 2004, Urbich et al. 2004) representing a heterogenous population of mononuclear blood cells. The EPCs used in the current investigation were isolated and expanded from fresh human umbilical cord blood obtained at birth. Differentiation into mature endothelial cells with functional properties such as the expression of endothelial nitric oxide synthase (eNOS) was demonstrated during cell expansion. Nitric oxide is a potent agent playing a critical role in vascular physiology (vasodilatation) and prevention of atherosclerosis (Naseem 2005). In order to assess the feasibility and utility of these cells for in vitro engineered endothelia in humans, the EPCs were seeded on rectangular tissue patches as a simplified model. After seeding, the endothelial progenitor cells formed functional endothelial layers on the surface of the myofibroblast-derived neo-matrices of all patches. Most importantly, the EPCs showed constant endothelial phenotype during the tissue engineering process and continuously expressed functional features such as eNOS.

Obviously, these results were generated using a simplified model of prenatal autologous heart valve tissue engineering with certain limitations: First, for experimental simplicity and reduction of necessary cell amounts only rectangular patches were generated in vitro. Secondly, the EPCs were harvested at the time of birth during normal deliveries and not by cordocentesis during pregnancy (as in the full prenatal tissue engineering concept). This approach was chosen because of the known morbidity and mortality of cordocentesis.
(Orlandi et al. 1990) which today is only justified for genetic fetal diagnostics. Moreover, this approach allowed for sufficient amounts of umbilical cord blood for experimentally establishing the isolation and expansion protocols for EPCs. Finally, the myofibroblast-like cells (which were utilized to form a connective tissue on which the EPCs were seeded) were obtained from umbilical cord tissues at birth as described previously. Therefore, none of the cells used in this feasibility study were harvested prenatally and although having very similar if not identical properties, the realization of the complete prenatal heart valve tissue engineering approach still needs to be demonstrated. Based on the preliminary experiences with these cells it can be speculated that sufficient cell amounts rather than their progenitor potential and tissue engineering applicability will be the most difficult hurdle for realization of this concept.

Progenitor Cells

The use of not yet fully differentiated (progenitor) cells for tissue engineering as described in the studies above obviously holds a lot of promise to the field. Progenitor cells are often also referred to as “adult stem cells”, which can be misleading. In contrast to stem cells, which are derived from embryos or fetuses, progenitor cells are obtained from later stages of cellular differentiation and do not require the use of embryos. This makes them less problematic when it comes to the question whether the extraction of stem cells e.g. from embryos generated for in vitro fertilization and/or pre implantation diagnoses (normally otherwise discarded) is justified. Francois Raspail understood as early as 1825, that cells are always derived from other cells (“Omnis cellulae e cellula”), providing the basis for modern stem cell biology (Harris H, The Birth of the Cell, 1999). Working with not fully differentiated cells (and various stages of cellular differentiation) requires clear definitions to prevent misunderstandings and to enable discrimination between studies based on stem cells versus studies based on progenitors cells (“adult stem cells”). The idea of a stem cell has a natural meaning in the context of the germ line (McKay 2000): The fertilized egg (or zygote) is a “totipotent” (lat. “totus” meaning “entire”) stem cell, and generates all the cells of the organism including extra-embryonic cells such as cells of the placenta. As development proceeds, cells become channeled into particular pathways and their differentiation potential becomes more limited. At this stage, the cells are regarded as “pluripotent” (lat. “plures” meaning “several”) and can be obtained during early embryonic development. These cells contribute to all tissues that form part of the embryo and therefore often are referred to as “embryonic stem cells”. During later embryonic and fetal development, cell differentiation continues into major types of progenitor cells which persist until adult life including neuronal progenitor cells, mesenchymal progenitor cells, and hematopoietic progenitor cells (Sussman 2001). These progenitor cells are considered to be terminally committed to a specific tissue type although some studies have indicated the same developmental potential as embryonic stem cells (Eglitis and Mezey 1997). Immanent to their specific tissue system however, progenitor cells have the potential for various traits of differentiation as e.g. the hematopoietic progenitors (endothelial progenitors cells) used in the above mentioned study differentiated into mature endothelial cells.

In contrast to embryonic stem cells, progenitors can not be grown indefinitely. Moreover, due to their advanced state of maturation, progenitor cells normally need to be transplanted into an autologous situation restricting their extra-individual application. With regard to the autologous heart valve tissue engineering concept presented in this thesis, the aforementioned facts turned out not to be a limitation. Cell expansion was excellent over many cell passages with stable phenotypic qualities resulting in cell quantities sufficient for heart valve tissue engineering. Moreover, the concept of prenatal cell harvest resolves the limitations of autologous cells. To the contrary, the autologous approach increases the overall safety of the concept, since besides immunological complications, infectious risks have gained increasing importance with regard to allogenic or xenogenic applications in the times of prion or retroviral diseases (Martin et al. 1998; Walles et al. 2003).

8.4 Towards Clinical Use – Outlook

Only few clinical applications of “tissue engineered heart valves” have been reported so far. None of these applications followed the complete autologous heart valve tissue engineering paradigm; that is non-autologous replacements based on non degradable scaffold materials were implanted (Elkins et al. 2001b, Elkins et al. 2003). In one study, first results with decellularized heart valves of either xenogenic or allogenic scaffolds seeded with endothelial cells from saphenous veins have been presented (Dohmen et al. 2002). However, during clinical follow up neither the initial seeding efficiency nor tissue remodeling or growth has been demonstrated so far. Only longer term observations and more meticulous tissue analysis of these implants will prove a potential advantage compared to contemporary heart valve prostheses. First clinical applications of xenogenic decellularized heart valves in children resulted in dramatic structural failure due to strong inflammatory responses and a 75% mortality (Simon et al. 2003).

In the light of these results the risks and pitfalls in transferring a new technology to clinical applications are emphasized. To prevent catastrophic failure it is important to precisely define the clinical indications addressed by a new methodology such as heart valve tissue engineering. In general, heart valve replacement with today’s mechanical and biological valve prostheses is efficacious and safe. It therefore will be hard to establish a competing technology, before many years of safety studies have been performed. In pediatric cardiac surgery, however, the situation is profoundly different. As of today, there is no clinically available implantation material with the potential of growth. A growing tissue engineered autologous heart valve would therefore address an unmet medical need. Since contemporary artificial heart valve prostheses have to be replaced several times in accordance with the children’s body growth, substantial and increasing morbidity and mortality has to be faced with each reoperation (Mayer et al. 1995). Provided that a tissue engineering
solution can prevent only a single reoperation (normally after 4-5 years) a substantial advantage compared to state of the art would have been achieved. This is why a tissue engineered heart valve for pediatric applications may enter the clinic much easier than a valve for adult applications. In fact, ethical committees and authorities will most probably permit such a new approach under the aspect of a “life-saving” technology (www.FDA.gov/orphan). A strict precondition however, is the establishment of valid animal model data with sufficient animal numbers and follow up periods. Furthermore, the study protocols need to be in accordance with the guidelines required by the authorities in order to qualify for a “safety study”. In preparation of first clinical applications (pilot study), laboratory processes and logistics according to good manufacturing practice (GMP) need to be established to provide maximum “in process control and safety”. The following shortly summarizes such scenario in the light of pediatric heart valve tissue engineering: After diagnosis of the heart valve malformation (e.g. through prenatal echocardiography), cells need to be harvested (either prenatally or at birth) by a medical team. Thereafter, the cells have to be transferred quickly and safely to an appropriate laboratory for screening (exclusion of infections etc.) and subsequent cell expansion. Biodegradable heart valve scaffolds need then to be fabricated according to the patients individual specifications (valve diameter measured e.g. by ultrasound). Thereafter, the custom-made scaffolds are seeded with the patient’s autologous cells and grown in vitro (bioreactors). In parallel, the medical team will schedule the operation to the time point when the tissue engineered heart valve is expected to be ready for implantation. With regard to clinical reality (scheduled operations often are postponed due to emergencies, patients are not ready for operation due to an otherwise harmless infection etc.) the tissue engineered heart valves need to come with a certain storage life time (at least 48h) to enable flexibility regarding the clinical requirements. Postoperative follow up comprises intensive care with meticulous diagnostics (invasive pressure monitoring, postoperative echocardiography, etc.) followed by regular pediatric cardiology assessment after hospital discharge. All the components of this sequence of actions have to comply with previously defined standardized protocols. After successful initiation of such a pilot study, multicenter studies in various selected qualified medical centers (pivotal studies) have to be started according to the same principles potentially resulting in a stepwise introduction and establishment of the new heart valve tissue engineering technology into routine clinical practice.

Based on the thereby generated clinical data, broader indications can be taken into account. The first clinical applications however, should be focused on the low pressure system such as in reconstruction of the right ventricular outflow tract (e.g. replacement of the pulmonary valve). In this situation, the TEHV are exposed only to 1/3 of the systemic pressure conditions providing a hemodynamically less challenging environment. In a next step, high pressure solutions will have to be established in children (e.g. replacement of the aortic valve). Improved bioreactor systems and conditioning protocols may be necessary (as discussed previously) to enable autologous heart valves sufficiently safe and functional e.g. for aortic valve replacements. Further increasing clinical experiences with pediatric applications may finally provide sufficient clinical data (and from a regulatory standpoint may be the only way) to justify entering into adult applications.
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CURRICULUM VITAE

Simon P. Hoerstrup was born on October 20, 1966 in Cologne, Germany. From 1988 to 1995 he studied medicine at the University of Cologne with electives in Switzerland (University of Berne) and the USA (Baylor College of Medicine and Texas Heart Institute, Houston; Harvard Medical School, Boston). After graduation, he received his Medical Doctor (M.D.) degree (magna cum laude) from the University of Cologne and started his clinical education in Cardiovascular Surgery at the University Hospital Zürich, Switzerland. From 1998-2000, he received a research grant by the German Research Foundation and Harvard Medical School and was research fellow at Children’s Hospital Boston, Harvard Medical School. In 2001, he received his “Habilitation” (Privatdozent Dr. med.) and “Venia Legendi” from the Medical Faculty of the University of Zürich, Switzerland. In 2003, he was appointed part-time professor at the department of Biomedical Engineering of the Eindhoven University of Technology. Currently, he is director, Cardiovascular Research, Clinic for Cardiovascular Surgery and director, Division of Regenerative Medicine, Department of Surgical Research, University Hospital and University of Zürich, Switzerland.