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Functional tissue engineering of human heart valve leaflets

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Contents

Summary

The aortic heart valve, situated between the aorta and the left ventricle, is one of the four valves in the heart controlling the blood flow (chapter 1). It consists of three leaflets, with sufficient coaptation areas to ensure closure of the valve. The valve opens and closes about 3.7 billion times in a lifetime, requiring strength, flexibility, and durability of the tissue. For this imposing function, the leaflets have a highly organized and specific tissue structure. When valve function is impaired, for example by calcification of the leaflets or inherited diseases, a live-threatening situation occurs as insufficient oxygen is being supplied to the body. A common treatment is the replacement of the aortic valve with either a mechanical valve or a bioprosthetic valve. The durability of a mechanical valve is excellent. However, patients having such a valve implanted, require lifelong anticoagulation therapy to prevent thrombo-embolism, the formation of blood clots on the surface of the valve leaflets. This treatment itself results in increased risks of internal bleedings. Patients with a bioprosthetic valve, for example a glutaraldehyde-treated porcine valve or a cryopreserved human donor valve, do not face anticoagulation therapy. The drawback of this type of heart valve replacement is that they are less durable caused by enhanced calcification and immune reactions of the body. All heart valve replacements share the major disadvantage that they are non-living structures and, therefore, not able to grow, repair and remodel in response to a changing environment, features which are of particular importance in children.

The ideal heart valve replacement is autologous, engineered from material of the patient itself to prevent immune reactions, and living to be able to grow, repair and remodel. The emerging field of tissue engineering focuses on the engineering of such organs. The principle of tissue engineering is to seed and culture cells from the recipient onto a carrier material, the scaffold, which degrades while the tissue is developing outside the body (in-vitro). As soon as the tissue properties are sufficient to withstand in-vivo conditions, the tissue can be implanted into the recipient. Heart valve replacements can be engineered according to this principle (chapter 2). Ten years ago, the first successful replacement of one of the pulmonary valve leaflets by a tissue engineered leaflet was reported in lambs. Nowadays, complete tissue engineered valves are shown to be fully functional in the pulmonary position in animal studies. The pulmonary valve is, however, exposed to lower pressures and flows than the aortic valve. A valve, able to resist the pressures and flows in the aortic position has not yet been created, using the principle of tissue engineering and a relatively fast degrading

scaffold material. The ultimate goal of the studies described in this thesis is to engineer such valve leaflet tissues, based on human saphenous vein cells and a relatively fast degrading scaffold material (PGA/P4HB).

To stimulate tissue formation outside the body, engineered heart valves are generally cultured in bioreactors, exposing the developing tissue to mechanical conditioning by application of e.g. flows and pressures that mimic the conditions in the body. Optimizing the conditioning protocol represents a useful tool towards the development of an aortic heart valve replacement. The bioreactors described so far by other research groups are all flow-driven and aim to mimic the opening and closing behavior of the native heart valve. The hypothesis underlying the present thesis is that not the flow over the leaflets, but the strains inside the leaflets have a dominant effect on tissue formation and development. These strains are highest during diastole, when the valve is closed, induced by the pressure of blood resting onto the closed leaflets.

The hypothesis was tested by engineering simple rectangular tissues, which were dynamically strained during culturing. Tissue formation and mechanical properties improved when larger strains were used (chapter 3). Subsequently, the hypothesis was extended to the more complex geometry of the aortic valve, with the three leaflets fixed to a stent, representing the aortic wall. To dynamically strain the leaflets, a dynamic pressure difference was applied over the leaflets. To ensure a compact and non-porous structure for efficient conditioning, the cells were embedded in fibrin gel and subsequently transferred to the scaffold, in which the gel polymerized (chapter 4). Next, a novel bioreactor system was developed (chapter 5) to apply a dynamic pressure difference over the leaflets and to provide the tissue with fresh nutrients and oxygen via a circulation system (perfusion). By fixation of the leaflets to the stent and the potential of the tissue to contract, a constant prestrain developed within the leaflets. This prestrain, combined with continuous perfusion, resulted in tissue engineered leaflets with excellent tissue formation, exceeding the values mentioned by others in similar studies and with mechanical properties more or less similar to native values. When exposing them to physiological aortic valve flow conditions, the leaflets were shown to stay intact (chapter 6). An important difference is, however, that native aortic valve leaflets display highly anisotropic behavior, indicating that the mechanical properties differ in different directions, related to their organized tissue structure. When tissue-engineered leaflets are dynamically strained during culturing, tissue formation itself is not further enhanced, but some tissue anisotropy developed, as well as improved tissue organization.

In conclusion, the strain-based approach to tissue engineer heart valve leaflets is very promising. Prestrain, combined with continuous perfusion, results in excellent tissue properties, whereas additional dynamic straining represents a valuable method to induce anisotropy in the tissue. This is the first study to describe tissue-engineered leaflets, based on a relatively fast degrading scaffold material, to possess sufficient tissue and mechanical properties to be able to function in the aortic valve position. The obtained results are very promising and efforts to test the functionality of the engineered leaflets in animal studies, towards clinical implementation, should be certainly made (chapter 7).

Chapter 1

Introduction

The contents of this chapter are partly based on A. Mol, C.V.C. Bouten, F.P.T. Baaijens, G. Zund, M.I. Turina and S.P. Hoerstrup (2004), *Tissue engineering of semilunar heart valves: current status and future developments*, Journal of heart valve disease; **13**: pp 272-280 and A. Mol and S.P. Hoerstrup (2004), *Heart valve tissue engineering - where do we stand?*, International journal of cardiology; **95 suppl 1**: pp S57-S58.

1.1 The human aortic valve

The aortic heart valve, situated at the outlet of the left ventricle, is one of the four valves in the heart controlling the blood flow in the body. This particular valve guides the blood flow into one direction, the aorta, and prevents back flow of blood into the heart. The aortic valve opens and closes approximately 103.000 times each day and about 3.7 billion times in a lifetime (Thubrikar, 1990). In order to create an aortic valve replacement it is essential to understand its function, anatomy and morphology. Furthermore, knowledge of the way the heart valve develops and its composition will result in better guidelines to the concept of functional tissue engineering of human aortic heart valves. This first chapter reviews the anatomy and function, as well as the development of the aortic valve (section 1.1) and into more detail the development, composition and properties of the individual leaflets of the valve (section 1.2) as this thesis has its main focus on the leaflets. The currently used heart valve replacements are summarized (section 1.3) and the promising concept of tissue engineering to create a living heart valve replacement is discussed (section 1.4). The chapter ends with a short description of the aim of this thesis (section 1.5) and its outline (section 1.6).

1.1.1 Anatomy and function

Fig. 1.1 shows the aortic valve and its components, when the aorta is cut open at the site of the valve.

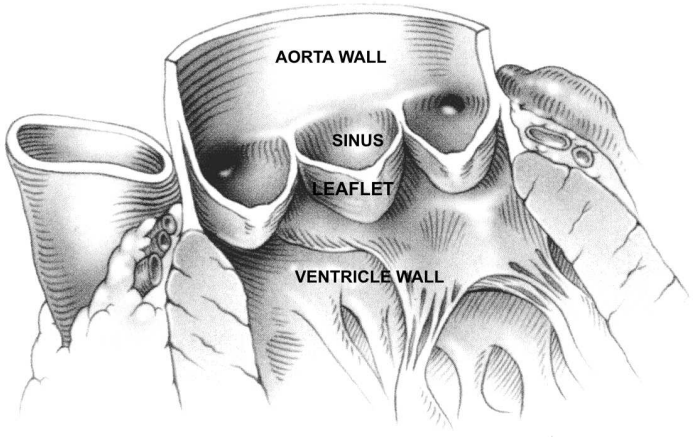


Figure 1.1: The anatomy of the aortic valve, situated at the outlet of the left ventricle, showing the three leaflets and the sinuses. Two of the three sinuses contain the orifices of the coronary arteries.

The aortic valve consists of three leaflets, also called cusps due to their bulgy shape. Behind each leaflet, the aortic wall expands to form dilated pouches, the sinuses. In two of the three sinuses, the orifices of the coronary arteries are located, supplying the heart with fresh oxygenated blood. The line of attachment of the leaflets to the aortic wall is the aortic valve ring.

A drawing of the typical pressure curves during one cardiac cycle is shown in Fig. 1.2. During diastole, the ventricle is filling with blood, resulting in an increasing ventricular pressure. As soon as the ventricular pressure equals the pressure in the aorta, the aortic valve will open to release the blood into the aorta. The blood flow through the valve during systole, gives rise to large shear stresses at the ventricular surfaces of the leaflets. When the ventricle has emptied its blood into the aorta, the pressure drops and the valve closes.

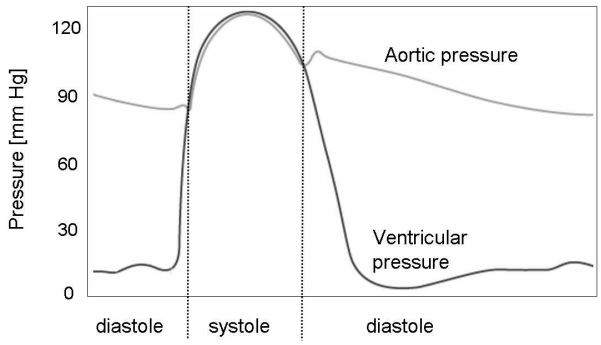


Figure 1.2: Typical aortic and ventricular pressures during one cardiac cycle. The ventricle fills with blood during diastole, resulting in an increasing ventricular pressure. The valve is closed during this filling phase. When the ventricular pressure exceeds the aortic pressure, the valve opens and blood can flow freely into the aorta (systole). When the ventricle has emptied, the valve closes and the pressure in the ventricle drops. The ventricle starts to fill with blood again, initiating a new cardiac cycle.

The pressure difference over the valve leaflets during diastole induces large deformations in the leaflets. A prolapse of the leaflets is prevented by substantial coaptation of the valve, indicating contact of adjacent leaflets up to 40% of their surfaces (Schoen and Levy, 1999). The part of the leaflet that is visible from ventricular perspective is the part that bears the load of the aortic pressure and is referred to as the load-bearing part of the leaflet. The part that coapts against the other two leaflets is called the coaptation surface. The free boundary of the leaflet is called the free edge with a thickening in the center of each leaflet, the nodulus of Arantius. The radius of the aortic valve in adults is about 12 mm (Swanson and Clark, 1974).

1.1.2 Development

The heart is the first organ to form in the embryo. When the first heart contraction takes place, the heart is no more than a muscular tube with a single endothelial lumen (Maron and Hutchins, 1974; Schroeder *et al.*, 2003). This tube is transformed into an H-shaped outflow channel with large tissue cushions in the right and left outflow tracts (Maron and Hutchins, 1974; Stock and Vacanti, 2001a). These cushions form by localized expansion of the extracellular matrix. After five weeks, the chambers of the heart are in the correct position and unilateral blood flow is provided in the heart (Schroeder *et al.*, 2003). The central luminal part of the cushions will form the valve leaflets (described in section 1.2.1), while the peripheral part forms the wall of the sinuses (Anderson *et al.*, 2003).

The complete development of the aortic valve takes place under pressure values below 10 mm Hg, at heart rates ranging from 65 to 160 beats per minute, and hypoxic conditions (Stock and Vacanti, 2001a). 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 The aortic valve leaflets

Every biological tissue is composed of cells, embedded in a protein matrix (extracellular matrix), consisting of mainly collagen, elastin and proteoglycans. The ratio of those components, produced by the cells, is different for each tissue type, depending on its function. Collagen is the main load-bearing protein and the formed fibers give mechanical strength to the tissue. Elastin serves to provide elasticity to the tissue and is important for proper functioning of the collagen fibers. The largest part of the extracellular matrix is composed of proteoglycans. Proteoglycans can form gels of varying pore sizes by binding water and form the ground substance of a tissue.

1.2.1 Development of the leaflets

The valve leaflets are formed in the sixth week of embryonic development (Teal *et al.*, 1986). The contractions and unilateral blood flow cause localized expansions in the cushions, resulting in the transformation of a subpopulation of endocardial cells. These endocardial cells transform into mesenchymal cells in response to soluble protein signals (Nolan, 1998). Furthermore, mesenchymal cells from the myocardium migrate into the cushions to initiate reshaping into valve leaflets. A major constituent of the cushion matrix is the proteoglycan hyaluronan, which is crucial for the swelling of the cushions and appears to play a significant role in valve morphogenesis by interaction with other matrix molecules and is referred to as 'the biological glue bringing it all together'. Type VI collagen binds to hyaluronan and weaves the extracellular

matrix of the cushions, facilitating cell migration and invasion. Hyaluronan, in association with other proteoglycans, affects growth factor signaling to a large extent and also affects the specific transformation of cells to facilitate maturation of the valve leaflets (Schroeder *et al.*, 2003).

The hemodynamic environment during the development of the valve leaflets has shown to largely influence the formation of the specific structure of the leaflets. 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 remain more cuboidal. The leaflets grow by proliferation of cells in the downstream end, the region exposed to 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, which is 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 equal tensions at both sides of the leaflets (Maron and Hutchins, 1974).

1.2.2 Composition and structure-function properties

The load-bearing part of adult aortic valve leaflets shows a layered architecture within the endothelial coverings (Fig. 1.3), enabling the extraordinary changes in shape and dimension to take place. 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 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 on 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).

The individual layers of valve leaflets show different mechanical characteristics due to their differences in composition (Vesely and Noseworthy, 1992; Scott and Vesely, 1995, 1996). The fibrosa is considered to be the main load-bearing layer of the leaflet, and prevents excessive stretching (Thubrikar *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 greater than the circumferential extensibility (Vesely and Noseworthy, 1992; Scott and Vesely, 1995, 1996). The overall mechanical response of the leaflet is a summation of the mechanics of the individual layers. Lo and Vesely (1995) measured a maximal extensibility of porcine aortic valve leaflets of 24% in the radial direction and 11% in the circumferential direction by whole-valve biaxial testing - this is a reliable method of testing in which the natural biaxial loading environment in the valve is reflected. In the circumferential direction, the mechanical behavior exhibits the properties of collagen bundles, whilst in the radial direction the elastin mesh is the

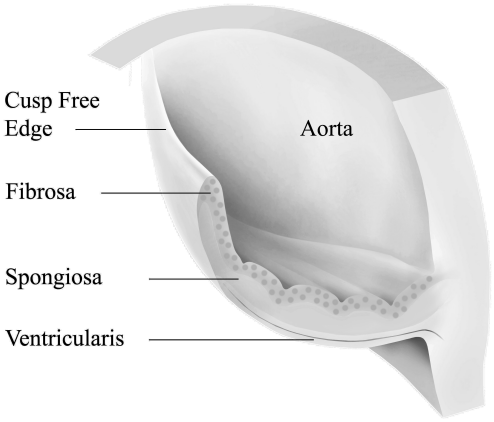


Figure 1.3: Configuration of the fibrosa, spongiosa, and ventricularis within the aortic valve leaflet.

predominant factor. The leaflet shows an extremely low compressive modulus, which is most likely facilitated by the spongiosa (Vesely and Noseworthy, 1992).

Simply examining the function and structure of collagen does not explain the highly non-linear, viscoelastic stress-strain characteristics and anisotropic behavior of the valve leaflets. Uniaxial tensile tests of circumferential and radial leaflet strips with and without digestion of elastin display a great impact of elastin on the stress-strain curves (Vesely, 1998). Without elastin, the overall stiffness of the leaflets increased (Lee *et al.*, 2001). This indicates that elastin is indeed a functional element in the aortic valve and its role should not be underestimated due to its low content compared to collagen, being 13% versus 50% by dry weight respectively (Vesely, 1998; Lee *et al.*, 2001).

Elastin is not only limited to the ventricularis, but is present throughout the leaflets as a complex network of sheets, tubes, and fibers. It functions as a 'housekeeper', restoring the collagen fiber geometry to its original configuration between loading cycles (Vesely, 1998). In the fibrosa, a matrix of elastin surrounds the collagen bundles, storing energy as it becomes stretched during diastole. This energy is used to return the collagen back to its original structure at systole. The elastin in the ventricularis consists of a large, very dense sheet. The size of the elastin layer is representative for the forces needed to hold the leaflet in its resting structure and to allow the valve closure to progress smoothly to a point at which the collagen takes over the load (Scott and Vesely, 1996). The mechanisms through which forces are transferred between collagen and elastin are not yet properly understood (Scott and Vesely, 1995, 1996). Either elastin fibers connected between different collagen fibers help to return the collagen bundles to their crimped state, or elastin fibers attached to different parts of the same collagen fiber (Scott and Vesely, 1995).

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, which results 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.

1.2.3 Cells in the aortic valve leaflets

The cells present in the aortic valve leaflets between the endothelial linings are referred to as valvular interstitial cells. Among the valvular interstitial cells, three cellular phenotypes can be identified: (i) smooth muscle cells, arranged in bundles or just as single cells (Bairati and DeBiasi, 1981; Cimini *et al.*, 2003), (ii) fibroblasts maintaining the extracellular matrix, and (iii) cells that have phenotypic features of both fibroblasts and smooth muscle cells, referred to as myofibroblasts (Messier *et al.*, 1994; Tomasek *et al.*, 2002; Taylor *et al.*, 2004). The unique characteristics of the myofibroblasts, expressing both skeletal and cardiac contractile proteins, may be central to the lifelong durability of the valve (Taylor *et al.*, 2004). In adult valve leaflets, the more quiescent fibroblast phenotype is predominating among the valvular interstitial cells, characterized by the expression of vimentin and low expression of α-SMA. During development and adaptation due to diseases, the phenotype of the cells is shifted towards a more activated myofibroblast phenotype, characterized by vimentin and α-SMA coexpression and secretion of proteolytic enzymes that are capable of degrading the matrix, mediating matrix remodeling (Rabkin-Aikawa *et al.*, 2004). Various types of proteolytic enzymes (MMPs) and their tissue inhibitors (TIMPs) are present in normal valve leaflets in a specific profile and on specific locations (Dreger *et al.*, 2002). When the cells change their phenotype (f.e. during development and adaptation to higher loads), this balance will be shifted towards one enabling matrix remodeling.

The distribution of the various phenotypes among the valvular interstitial cells in the aortic valve leaflet is depending on the biological and mechanical microenvironment. In the normal adult aortic valve leaflet, fibroblasts are mainly found in the ventricularis, while myofibroblasts and smooth muscle cells are segregated in the fibrosa (DellaRocca *et al.*, 2000). A comparable distribution pattern can be found in the vascular wall with myofibroblasts and smooth muscle cells in the medial layer and fibroblasts in the adventitial layer. In the case of hypertension, fibroblasts of the adventitia convert into myofibroblasts to adapt to the higher mechanical loads (Sartore *et al.*, 1999). This same adaptation process might explain the distribution pattern of cellular phenotypes in the aortic valve leaflets. Due to the pressure difference over the leaflets, more smooth muscle-like cells are expected in the fibrosa compared to the ventricularis. Pressure levels may influence differences between pulmonary valve leaflets and aortic valve leaflets as well (DellaRocca *et al.*, 2000). In disagreement

with the phenotype distribution in the human aortic valve as described above (DellaRocca *et al.*, 2000), the phenotype distribution in porcine aortic valve leaflets was reported to be different, with smooth muscle cells and myofibroblasts more prevalent in the ventricularis than in the fibrosa by Bertiplaglia *et al.* (2003). The mechanism behind this difference has not yet been elucidated.

The idea of passively functioning aortic valve leaflets was refuted by identifying a smooth muscle cell system in the leaflets (Bairati and DeBiasi, 1981). The smooth muscle cells are terminally differentiated and arranged in small bundels in the ventricularis, running circumferentially, or as individual cells (Cimini *et al.*, 2003). Furthermore, contractile properties of valvular interstitial cells were observed (Filip *et al.*, 1986; Messier *et al.*, 1994; Taylor *et al.*, 2000), as well as 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 leaflets during systole and diastole, either by assisting the valve in opening or to provide support during diastole (Taylor *et al.*, 2000; Cimini *et al.*, 2003), and represents a reactive cytoskeleton that can anchor collagen fibrils during valve closure (Messier *et al.*, 1994).

The endothelial cells covering the surfaces of the leaflets provide a protective, non-thrombogenic layer. It is remarkable that the endothelial cells on the leaflets are aligned perpendicular to the direction of the flow, in contrast to the alignment of endothelial cells in the direction of the flow in blood vessels. This indicates that a mechanical force, other than flow, induces this endothelial cell alignment on the leaflets. As the alignment is similar to the alignment of the collagen fibers, it has been hypothesized that the pressure stresses during diastole, responsible for the alignment of the collagen fibers, are responsible for the alignment of the leaflet endothelial cells as well. The only spot where the endothelial cells align in the direction of the flow is at the nodulus (Deck, 1986).

1.3 Heart valve replacements

Diseases can affect any of the four heart valves, although diseases of the aortic valve occur most frequently, associated with high mortality rates (Schoen and Levy, 1999). The most common treatment for end-stage valvular diseases is surgical replacement of the valve with either a mechanical or a bioprosthetic valve, each having its own advantages and disadvantages.

1.3.1 Mechanical and bioprosthetic valves

Mechanical heart valve replacements display good structural durability, but are associated with a risk of prosthetic valve endocarditis and high rates of thromboembolic complications caused by their non-physiological surface and flow abnormalities. Lifelong anticoagulation therapy is necessary in these patients, which is associated with a substantial risk of spontaneous bleeding and embolism, particularly in patients aged over 70 years (Senthilnathan *et al.*, 1999).

Bioprosthetic heart valve replacements are either of animal origin (xenografts), such as porcine aortic valves and bovine pericardial valves, or they may be taken from human donors (homografts). Xenografts are chemically crosslinked - this inhibits autolysis, enhances the mechanical stability, and creates the possibility of having valves of different size directly available. However, these valves differ in many respects from native valves, for example in their opening and closing behavior due to the chemical pre-treatment (Schoen and Levy, 1999). Explanted xenografts were shown to be stiffer in the radial direction and less stiff in the circumferential direction compared to native porcine valves (Adamczyk and Vesely, 2002). The risk of thromboembolic complications is much lower, but the valve's durability is limited. Structural failure is strongly age-dependent, making xenografts attractive for the elderly, but less suitable for children and young adults (Schoen and Levy, 1999).

1.3.2 Risks in xenotransplantation

One important aspect in xenotransplantation is the risk of zoonoses - human diseases caused by infectious agents from animals (Takeuchi, 2000) - which might even be facilitated by the mandatory immunosupression (Weiss *et al.*, 2000; Moza *et al.*, 2001). The identification of porcine endogenous retroviruses and prionic diseases has given rise to great concern. Recently, epidemiological data have strongly indicated transfer of Creutzfeldt-Jakob disease from cattle into humans either via infected meat, via surgical materials derived from bovine gut, or via drugs or vaccines prepared using fetal calf serum (Knight and Collins, 2001). Porcine endogenous retroviruses (PERV) can be present in all organs, 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; Specke *et al.*, 2001). There may be other infectious agents from animals that can be transferred into humans by xenotransplantation, though as yet their existence has not been proven.

1.3.3 Homografts

Cryopreserved aortic donor valves are the heart valve replacements closest to the natural valve, not being thrombogenic and with a low risk of infection. They are not chemically crosslinked and exhibit good mechanical properties, which prolongs their lifetime (Lee *et al.*, 2001). However, their disadvantages are their limited availability, a more difficult implantation technique (Senthilnathan *et al.*, 1999), and failure associated with a specific immune response, especially in young children (Rajani *et al.*, 1998).

The use of cryopreserved pulmonary homografts as aortic heart valve replacements has been shown to result in early failure as the fiber structure of the pulmonary valve is less resistant to the hemodynamic environment in the aortic position compared to cryopreserved aortic valves (Koolbergen *et al.*, 2002). Furthermore, these valves were shown to suffer from gross regurgitation in vitro, highlighting their unsuitability as aortic valve replacements (Jennings *et al.*, 2002). A controversial issue

regarding cryopreserved homografts concerns the viability of the inherited endothelial and interstitial cells. A lack of viable cells after implantation was stated (Schoen and Levy, 1999), as well as the long-term survival of cellular elements (Angell *et al.*, 1998). In general, although cryopreservation reduces cellularity, the expression of strong allogeneic antigens could still be demonstrated, and this might trigger the immune system of the host, resulting in graft rejection (Oei *et al.*, 2002).

1.4 Tissue engineering - a new discipline

Several attempts have been made to create functional heart valve replacements with the ability to grow, repair, and remodel using the concept of tissue engineering (Fig. 1.4). In tissue engineering, the patient's own cells, isolated, for example, from a blood vessel and expanded using standard cell culture techniques, are seeded onto an appropriate carrier, termed the scaffold, in the shape of a heart valve. Subsequent stimulation, transmitted via the culture medium (biological stimuli) or via 'conditioning' of the tissue in a bioreactor (mechanical stimuli), promotes tissue development, resulting in a completely autologous, functional, and living heart valve implant.

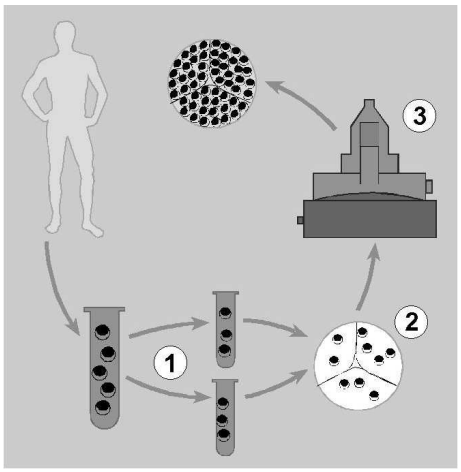


Figure 1.4: Tissue engineering of heart valves. 1) Isolation of cells from a blood vessel of the patient and separation of myofibroblasts and endothelial cells. 2) Seeding of myofibroblasts onto a scaffold material in the shape of a trileaflet heart valve and subsequent seeding of endothelial cells onto the surfaces. 3) The cell/scaffold construct is placed into a bioreactor to stimulate tissue development.

Heart valve tissue engineering represents a promising approach towards autologous, living, and functional heart valve replacements with the ability to grow, repair, and remodel. Mechanical conditioning of the developing tissue in a bioreactor

has rendered heart valve replacements, which have shown excellent functionality in sheep when implanted in the pulmonary position (Hoerstrup *et al.*, 2000a). The pulmonary position is, however, a low-pressure environment and as most heart valve replacements concern the aortic valve, a high-pressure environment, the tissue properties of the neo-tissue have to be optimized further to serve as aortic heart valve replacements.

1.4.1 The scaffold

The heart valve scaffold may be either based on biological or synthetic materials. Donor heart valves or animal derived valves depleted of cellular antigens can be used as a scaffold material. Removing the cellular components results in a material composed of essentially extracellular matrix proteins that can serve as an intrinsic template for cell attachment (Samouillan *et al.*, 1999). In general, non-fixed acellularized valve leaflets have shown recellularization by the host, as demonstrated in dogs (Wilson *et al.*, 1995) and sheep (Elkins *et al.*, 2001). However, first clinical applications of this concept in children resulted in rapid 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 (Lee and Mooney, 2001; Rothenburger *et al.*, 2002). These materials have the disadvantage that they are difficult to obtain from the patient in sufficient quantities. Therefore, most of these scaffolds are of animal origin. In this context, identification of retroviruses and prionic diseases has given rise to great concern as to the risk of zoonoses (see section 1.3.2).

An ideal synthetic scaffold for tissue engineering applications must be at least 90% porous (Agrawal and Ray, 2001), and must possess an interconnected pore network, as this is essential for cell growth, nutrient supply, and removal of metabolic waste products. Besides being biocompatible, biodegradable, and reproducible, the scaffold should also display a cell-favorable surface chemistry and match the mechanical properties of the native tissue. The rate of degradation should be proportional to the rate of tissue formation and controllable in order to ensure mechanical stability over time (Hutmacher, 2001; Hutmacher *et al.*, 2001).

The use of synthetic materials as scaffolds has already been broadly demonstrated for heart valve tissue engineering. Initial attempts to create single heart valve leaflets were based on synthetic scaffolds, such as polyglactin, PGA (polyglycolic acid), PLA (polylactic acid) and PLGA (copolymer of PGA and PLA). To create complete trileaflet heart valve conduits, PHA based materials (polyhydroxyalkanoates) were used (Sodian *et al.*, 2000a). These materials are thermoplastic and can therefore be easily molded into any desired three dimensional shape. A combined polymer scaffold consisting of non-woven PGA and P4HB (poly-4-hydroxybutyrate) has shown promising results in sheep, when placed in the pulmonary position (Hoerstrup *et al.*, 2000a).

1.4.2 The cells

In most cardiovascular tissue engineering approaches cells are harvested from donor tissues. From peripheral arteries, for example, mixed vascular cell populations consisting of myofibroblasts and endothelial cells can be obtained. Out of these, pure viable cell lines can be easily isolated by cell sorters (Hoerstrup *et al.*, 1998) and the subsequent seeding onto the biodegradable scaffold is undertaken in two steps. First the myofibroblasts are seeded and grown in-vitro and second, the endothelial cells are seeded on top of the generated neo-tissue leading to the formation of a native leaflet-analogous histological structure (Zund *et al.*, 1998).

With regard to clinical applications, several vascular human cell sources have been investigated (Schnell *et al.*, 2001). Recently, cells derived from bone marrow or umbilical cord have been successfully utilized to generate heart valves and conduits invitro as well (Hoerstrup *et al.*, 2002a,c), reviewed by Rezai *et al.* (2004). 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 potential, these cells are expected to be an attractive alternative for cardiovascular tissue engineering applications. Apart from the use of so called progenitor cells, the research on stem cells and their differentiation pathways is still in its infancy and a drawback is the possible immunogenicity of these cells. This may be solved by genetic engineering (Odorico *et al.*, 2001), although by itself still being in an experimental phase.

1.4.3 Conditioning strategies

Tissue formation can be stimulated by either biological or mechanical conditioning. Biological conditioning involves addition of cytokines either directly to the growth medium or by incorporation into the scaffold material. Cytokines are a group of regulatory molecules that function as mediators of cell communication and can exert multiple biological functions by interaction with specific cell surface receptors. The family of cytokines includes interleukins, hematopoietic growth factors, interferons, tumor necrosis factors, and growth factors (Takehara, 2000). Well-known cytokines that influence vascular cell behavior 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, regulating cell behavior, matrix metalloproteinases (MMPs) play an important role in tissue development and subsequent remodeling (Streuli, 1999). When cytokines are directly applied to the growth medium, the effect is short term and should be repeated several times. When incorporated into a biodegradable scaffold material, slow release can be obtained by coupling of the cytokine release to the degradation rate.

Mechanical conditioning involves the application of various mechanical stimuli in a bioreactor, such as flows, inducing shear stresses over the developing tissue, and strains, being either dynamic or static in nature. For engineering of heart valves, the most commonly used bioreactor is a pulse duplicator system, in which the normal

opening and closing behavior of the valve is mimicked (Hoerstrup *et al.*, 2000b,c). In this type of bioreactor, the tissue is exposed to increasing flow rates and pressures. Recently, new bioreactors have been developed for tissue engineering of heart valves, in which the exact physiological conditions of a heart valve in vivo can be applied (Dumont *et al.*, 2002; Hildebrand *et al.*, 2004; Rutten *et al.*, 2005).

Mechanical and biological stimuli do interact in a very complex way in the regulation of tissue behavior. By mechanical stimuli, the production and secretion of various cytokines by the cells are increased and the other way around, the addition of cytokines during tissue development can increase the effect of mechanical conditioning. TGF-β is, for example, one of the cytokines that plays a key role in this process (O'Callaghan and Williams, 2000). The complex interaction of biological and mechanical stimuli on vascular cells and the surrounding extracellular matrix is shown in Fig. 1.5.

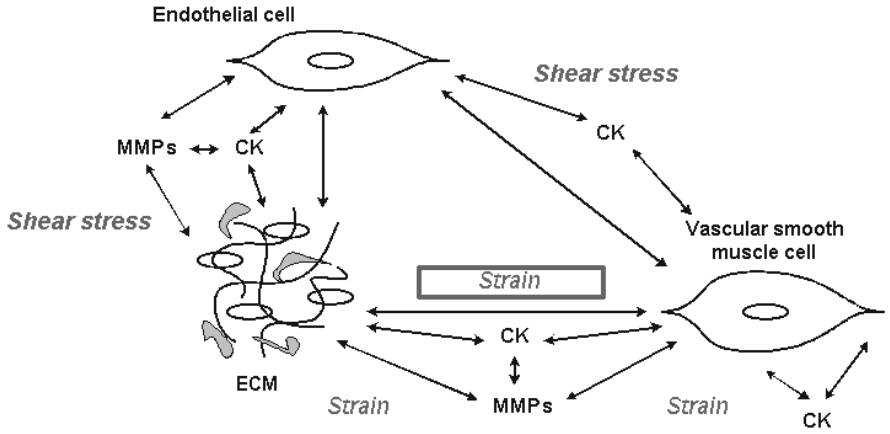


Figure 1.5: The complexity between biological and mechanical stimuli on cells and their surrounding extracellular matrix. The cells can influence each other directly via cell-cell contact or via secretion of cytokines (CK). The extracellular matrix (ECM) is influenced by the cells via secretion of matrix metalloproteinases (MMPs) and does on its turn influence the cells via receptors on the cells. The main focus of this thesis is on the direct influence of strain on the vascular smooth muscle cells and their matrix, represented in the block.

1.5 Aim of the thesis

The aim of the studies described in this thesis is to improve the properties of the developing tissue via mechanical conditioning to ultimately obtain heart valve leaflet

replacements capable of withstanding the large stresses and strains at the aortic position. This work focuses on the use of a strain-based approach, in combination with minimized flows, instead of the more commonly known flow-based approach to tissue engineer heart valves. This thesis deals with the initial phase of tissue engineering, the stimulation of tissue development by the seeded vascular cells. Seeding with endothelial cells, stabilizing the tissue for implantation, will have to be performed in future studies.

1.6 Outline

This chapter has reviewed the basic understanding, essential for functional tissue engineering of human heart valves. Chapter 2 gives an overview of what has been done so far in the emerging field of tissue engineering of heart valves. To optimize tissue formation and subsequent mechanical properties of tissue engineered heart valve leaflets, the hypothesis of using strains during culturing was first tested on engineered leaflet tissue-equivalents with a simple rectangular geometry as described in chapter 3. This approach was shown successful and was extended to the more complex geometry of a trileaflet heart valve. In order to mechanically load the neo-tissue efficiently, a compact structure is necessitated. With the use of fibrin as a cell carrier, as described in chapter 4, this goal was achieved. Chapter 5 describes the concept of functional tissue engineering of heart valve leaflets using strains, describing the development of a novel bioreactor system and methods to apply and calculate the strains in the engineered valve leaflets. The feasibility of this approach is demonstrated by some examples of tissue-engineered leaflets, cultured using this strain-based approach. The effects of the separate types of strain, e.g. compaction-induced prestrain, either or not in combination with continuous medium circulation, and dynamic strains, is elucidated in chapter 6. Preliminary results of the functionality of the engineered valve leaflets when exposed to physiological flow conditions are described in chapter 6 as well. The final chapter 7 summarizes the obtained results and discusses further aspects for future studies towards tissue engineering of clinically applicable aortic heart valve replacements.

Chapter 2

Ten years of heart valve tissue engineering

The contents of this chapter are partly based on A. Mol, C.V.C. Bouten, F.P.T. Baaijens, G. Zund, M.I. Turina and S.P. Hoerstrup (2004), *Tissue engineering of semilunar heart valves: current status and future developments*, Journal of heart valve disease; **13**: pp 272-280 and A. Mol and S.P. Hoerstrup (2004), *Heart valve tissue engineering - where do we stand?*, International journal of cardiology; **95 suppl 1**: pp S57-S58.

2.1 Tissue engineered heart valves: an overview

Heart valve replacement represents the most common surgical therapy for end-stage valvular heart diseases. A major drawback of all currently available heart valve replacements is the lack of growth, repair, and remodeling capacities. Living cardiovascular structures, such as heart valves, can be engineered in-vitro using the principle of tissue engineering. Autologous cells, expanded and isolated in the laboratory, are seeded onto an appropriate carrier structure, the scaffold. The cells start to develop a neo-matrix, while the scaffold material is degrading, ultimately rendering a completely autologous, functional, and living implant. The first tissue engineered heart valve leaflets were implanted successfully in pulmonary position in sheep in 1995 (Shinoka *et al.*, 1995, 1996). This chapter gives an overview of the achievements within this very fast growing field. Where do we stand now, ten years later, and what are the future challenges?

2.1.1 Tissue engineered valves using biological scaffolds

Donor heart valves or animal-derived valves depleted of cellular antigens, which make them less immunogenic, can be used as a scaffold material. Removing the cellular components results in a material composed of essentially extracellular matrix proteins that can serve as an intrinsic template for cell attachment. Examples of acellularization techniques are freeze-drying (Curtil *et al.*, 1997), treatment with trypsin/EDTA (Steinhoff *et al.*, 2000; Leyh *et al.*, 2003; Kasimir *et al.*, 2003), detergent treatment (Bader *et al.*, 1998; Booth *et al.*, 2002; Kim *et al.*, 2002; Bertiplaglia *et al.*, 2003; Kasimir *et al.*, 2003), and multi-step enzymatic procedures (Zeltinger *et al.*, 2001). The use of trypsin has shown to render incomplete acellularization and structural alterations of the matrix (Kasimir *et al.*, 2003), although others have reported complete absence of cellular components and maintenance of matrix integrity (Leyh *et al.*, 2003). Detergent treatment resulted generally in complete acellularization and preservation of the matrix (Kim *et al.*, 2002; Kasimir *et al.*, 2003). Discrepancies in the outcome using various acellularization techniques are most probably due to proteases present in the native tissue, as their activation leads to autolysis of extracellular matrix proteins, resulting in damage to the structure and function of the matrix scaffold. Therefore, suitable protease inhibitors should be used (Booth *et al.*, 2002). To remove any residual DNA and RNA from the matrix, nuclease digestion steps are desirable (Bader *et al.*, 1998; Booth *et al.*, 2002; Bertiplaglia *et al.*, 2003).

The maintenance of mechanical properties depends on the acellularization method used (Samouillan *et al.*, 1999) and on the degree of crosslinking, which stabilizes the collagen structure but decreases the ability of tissue ingrowth. In general, non-fixed acellularized valve leaflets have been shown to promote remodeling of the prosthesis by neovascularization and recellularization by the host, as demonstrated in dogs (Wilson *et al.*, 1995) and sheep (Goldstein *et al.*, 2000; Elkins *et al.*, 2001), and to possess sufficient mechanical integrity to withstand physiologic conditions after implantation, even in aortic position. The amount of cellular repopulation has been shown to differ between studies, and is thought to depend on the source of the matrix as well as

the acellularization technique used (Leyh *et al.*, 2003). However, colonization of the matrices with valvular interstitial-like cells have been reported in all of these studies. Endothelialization - preservation of the subendothelial cellular and matrix components of the implanted valves - was described in both dogs (Wilson *et al.*, 1995) and sheep (Leyh *et al.*, 2003). This endothelialization, as well as the growth, repair, and remodeling capabilities, might be further optimized by seeding cells onto the matrices beforehand, according to the concept of tissue engineering.

All studies using acellular matrices seeded with endothelial cells, showed a confluent layer covering the leaflets when cultured in-vitro (Curtil *et al.*, 1997; Bader *et al.*, 1998; Kim *et al.*, 2002) and after implantation (Steinhoff *et al.*, 2000). Acellularized aortic valve leaflets, when seeded with their original valvular interstitial cells, demonstrated all phenotypes present in the native valve leaflet after in-vitro culturing, indicating the potential of cultured valvular interstitial cells to differentiate into various phenotypes (Bertiplaglia *et al.*, 2003). The cellularity of the leaflets did not reach physiological levels after in-vitro culturing, either statically (Curtil *et al.*, 1997; Steinhoff *et al.*, 2000) or dynamically in a bioreactor, in which the opening and closing of the valve was simulated (Zeltinger *et al.*, 2001). Three months after implantation the leaflets were thickened, which might represent excessive extracellular matrix formation and cellular proliferation (Steinhoff *et al.*, 2000). Further studies are required to examine the cause of this thickening and to exclude the risk of valve failure after implantation. Although the use of acellularized matrices as a scaffold material might be promising for future clinical use, important disadvantages include the infectious risk when using animal-derived materials (as described in section 1.3.2), as well as immunological complications. In fact, recent first clinical applications of this concept in children resulted in rapid failure of heart valves due to severe foreign body-type reactions associated with a 75% mortality (Simon *et al.*, 2003).

To create a heart valve replacement consisting solely of autologous tissue, the scaffold material should degrade while the valve tissue is developing. Collagen is one of the biological materials that show biodegradable properties and is used as a foam (Rothenburger *et al.*, 2002), gel or sheet (Hutmacher *et al.*, 2001), sponge (Taylor *et al.*, 2002), and even as a fiber-based scaffold (Rothenburger *et al.*, 2001). However, collagen has the disadvantage that it is difficult to obtain from the patient, and nowadays most collagen scaffolds are of animal origin. Due to the slow degradation of collagen, scaffold material will still be present at the moment of implantation. This, on the one hand could lead to the risk of zoonoses and on the other hand to immunological reactions and inflammation.

Fibrin is another biological material, which displays good controllable biodegradable properties. As fibrin gels can be produced from the patient's blood to serve as autologous scaffold, no toxic degradation or inflammatory reactions are expected (Lee and Mooney, 2001). Three-dimensional structures can be produced by injection molding of the cell-gel mixture, followed by enzymatic polymerization of fibrinogen. Degradation is controlled by adding aprotonin, a proteinase inhibitor that slows down or can even stop fibrinolysis (Ye *et al.*, 2000; Jockenhoevel *et al.*, 2001b,a). Immobilization of growth factors in specific areas has also shown to be feasible (Schense and Hubbell, 1999). As a scaffold material, fibrin also has its disadvantages. The

diffusion and washout of substances into the surrounding medium seems to be reduced compared to other porous matrices (Jockenhoevel *et al.*, 2001a). Fibrin tends to shrink and has poor overall mechanical properties (Ye *et al.*, 2000; Jockenhoevel *et al.*, 2001b). Fixation of the gel using poly-L-lysine has shown to prevent shrinkage and the inner tension that subsequently arises resulted in more pronounced collagen formation and improved mechanical properties (Jockenhoevel *et al.*, 2001a). Besides its potential as a biological scaffold material, fibrin gel can also be used as a cell carrier in porous synthetic scaffolds (Ameer *et al.*, 2002).

2.1.2 Tissue engineered valves using synthetic scaffolds

Synthetic materials have been used as scaffold materials for engineering various types of tissue (Hutmacher *et al.*, 2001; Lee and Mooney, 2001; Stock and Vacanti, 2001b). Several attempts to create tissue-engineered heart valve leaflets were also based on synthetic scaffolds, such as polyglactin, polyglycolic acid (PGA), polylactic acid (PLA), PLGA (a co-polymer of PGA and PLA) and polyhydroxyalkanoates (PHAs).

A highly porous synthetic material can be obtained by either fabricating a woven or non-woven fiber mesh or using a salt-leaching technique (Agrawal and Ray, 2001; Hutmacher, 2001). Polyglactin, PGA and PLA are members of the aliphatic polyester family, which degrade by cleavage of the polymer chains following hydrolysis of their ester bonds. The resultant monomer is either secreted in the urine or enters the tricarboxylic acid cycle (Agrawal and Ray, 2001). To fabricate scaffolds for heart valve leaflets, combinations of aliphatic polyesters have been used as well, including woven polyglactin and non-woven PGA meshes (Shinoka *et al.*, 1995, 1996; Shinoka, 2002) and layers of PLGA and non-woven PGA meshes (Breuer *et al.*, 1996; Zund *et al.*, 1997; Kim *et al.*, 2001). The major limitations of an aliphatic polyester are its thickness, initial stiffness, and non-pliability, all of which make the fabrication of a trileaflet heart valve a difficult process.

The PHA family consists of polyesters built up from hydroxyacids, which are produced as intracellular granules by various bacteria (Kessler and Witholt, 2001). To create trileaflet heart valve conduits, polyhydroxyoctanoate (PHO) (Sodian *et al.*, 2000a,b,c,d) and poly-4-hydroxybutyrate (P4HB) are used (Sodian *et al.*, 2000b). These materials possess thermoplastic properties and can, therefore, be easily molded into any desired shape (Sodian *et al.*, 2002a). The general drawback of PHAs is their slow degradation. Combinations of aliphatic polyesters and PHAs have shown promising results (Hoerstrup *et al.*, 2000a; Stock *et al.*, 2000; Rabkin *et al.*, 2002), in particular the use of PGA coated with P4HB, which combines the high porosity of PGA with the thermoplastic properties of P4HB (Hoerstrup *et al.*, 2000a; Rabkin *et al.*, 2002).

In order to obtain pure cell populations for seeding fluorescence-activated cell sorting (FACS) can be used (Shinoka *et al.*, 1995; Hoerstrup *et al.*, 1998). A mixed cell population, isolated from a blood vessel, is labeled with an acetylated low-density lipoprotein marker, which attaches solely to endothelial cells. The endothelial cells can then be easily separated from the other vascular-derived cells, which comprise a mixture of smooth muscle cells, myofibroblasts and fibroblasts, by using FACS. Using

a mixed cell population does not result spontaneously in an endothelial lining covering the leaflets and, therefore, the endothelial cells must be seeded sequentially after initial neo-matrix formation has occurred, only at the surface of the cell-polymer construct (Sodian *et al.*, 2000a). The source of the vascular-derived cells, whether from an artery or a vein, has been shown to influence the eventual tissue-engineered construct. Constructs seeded with venous cells were superior to those seeded with arterial cells with respect to collagen formation and mechanical stability (Schnell *et al.*, 2001). Considering the source of endothelial cells, no endothelial lining of the construct was observed when cells were obtained from a vein (Sodian *et al.*, 2000a). As the vein is the most promising, most easily accessed cell source for clinical use in the near future, further studies should be conducted to identify differences between endothelial cells from arteries and veins. Culturing the constructs in a dynamic environment, for example generated by a bioreactor, resulted in more pronounced and organized tissue formation when compared to static culture (Hoerstrup *et al.*, 2000a; Rabkin *et al.*, 2002).

2.2 Future research focuses

The search for the optimal scaffold material will continue, as even the most promising currently available material - a non-woven PGA mesh coated with P4HB - is still not ideal. This material degrades quickly, but in an uncontrollable manner, whereas degradation of the ideal scaffold should occur on demand in order to ensure mechanical stability over time. Acidic byproducts arise during the degradation process, which might be cytotoxic. Furthermore, the mechanical properties of this scaffold do not meet the load-bearing properties of the native aortic valve. A promising concept might be the use of a basic structure, prepared from a slowly degrading material and preferably matching the collagen fibers in the leaflets, combined with a faster-degrading material providing the cells with a large attachment area.

A completely different approach is engineering tissues without the use of a scaffold material. Flexible and elastic tissue layers were reported by folding cultured cell layers and subsequent framing to provide inner tension (Ye *et al.*, 2000; Hoerstrup *et al.*, 2002b). However, the mechanical properties of these tissues are not yet sufficient to allow implantation.

Although veins represent the most promising cell source for clinical use in the near future, one should focus on the senescence of the cells and their ability to divide, as the number of divisions must be sufficiently large to bulk the tissue into its original size and to allow remodeling once implanted in the body. A future approach might be the use of vascular cells from young donors, as these can be genetically engineered to remove unwanted gene expression patterns, thereby preventing an immune response of the host (Curtis and Riehle, 2001). Another approach might be the use of stem cells, obtained from fetal or adult tissues (Perry and Roth, 2003). Recently, the use of, for example, animal (Perry *et al.*, 2003) and human bone marrow and umbilical cord cells (Hoerstrup *et al.*, 2002a,c; Kadner *et al.*, 2004) was shown to be applicable in cardiovascular tissue engineering (extensively reviewed by Rezai *et al.* (2004)).

The field of research with regard to stem cells and their differentiation pathways is still in its infancy, the major drawback being the immunogenicity of these cells. In theory, this problem could be solved by the use of genetic engineering (Odorico *et al.*, 2001), although this approach by itself is currently in an experimental phase. Hence, for accelerated clinical application, the use of autologous cells appears to be most appropriate.

The tissue-engineered heart valves implanted into animals were all implanted in the pulmonary position, as their mechanical properties did not allow placement in the aortic position. A more thorough understanding is needed 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. Tissue-engineered valves in animals ultimately displayed a native-analogous structure, caused by remodeling of the valve in-vivo. It would be preferable to achieve such native-analogous features prior to implantation. Biological stimuli, such as the addition of growth factors to the medium, for example, basic fibroblast growth factor and ascorbate (Hoerstrup *et al.*, 2000c), or the incorporation of growth factors into the scaffold - the so-called 'third generation' biomaterials (Rabkin and Schoen, 2002) - help to stimulate collagen production. The effect of mechanical regulators on collagen production should also be further investigated, as laminar flow (Jockenhoevel *et al.*, 2002) and cyclic strains (Kim *et al.*, 1999) - and in particular large cyclic strains (Mol *et al.*, 2003) - have been shown to increase the amount of collagen, resulting in improved mechanical properties of the engineered tissues. Numerical models, which can be used to predict the remodeling of the collagen architecture in tissue engineering applications, might also be valuable in the generation of mechanically improved tissue-equivalents (Driessen *et al.*, 2003).

The concept of bioreactors can be improved by using a controllable, self-regulating loop to measure the pressures and forces that the tissue withstands during its development and to subsequently increase the load on the valve to further improve its mechanical properties. Using a bioreactor, it should be possible to monitor the exact culture conditions (such as pH, pO_2, and pCO_2) and directly adjust these parameters to provide an optimal environment. Another approach might be to apply fetal conditions within the bioreactor instead of mimicking the physiological environment in the adult human body.

Clearly, many disciplines are involved within the field of tissue engineering and only a synergetic scientific effort will lead to optimal progress. Likewise, it is clear that for every facet in this process, further research is still needed. However, in conclusion it can be stated that much progress has been already made in the past ten years, and that the results obtained thus far show great promise with regard to the future clinical application of heart valve tissue engineering.

Chapter 3

The relevance of large strains in engineered valve leaflet tissue-equivalents

The contents of this chapter are based on A. Mol, C.V.C. Bouten, G. Zund, C.I. Guenter, J.F. Visjager, M.I. Turina, F.P.T. Baaijens and S.P. Hoerstrup (2003), *The relevance of large strains in functional tissue engineering of heart valves*, Thoracic and cardiovascular surgeon; **51**: pp 78-83.

3.1 Introduction

In the emerging field of tissue engineering, organ replacements are fabricated on the basis of a biodegradable carrier - the scaffold - seeded with the patient's own cells. The carrier, which can either consist of a biologically derived material or a synthetic polymer, provides the developing tissue with sufficient mechanical properties during the in-vitro culturing period. Despite the success of this approach, problems arose concerning the mechanical properties of engineered tissues that serve a predominantly biomechanical function, such as heart valves. Increasing evidence suggests mechanical conditioning as a valuable method to improve the mechanical properties of engineered tissues prior to implantation (Butler *et al.*, 2000). Tissue-engineered heart valves cultured in a bioreactor with mechanical stimulation have shown functionality for prolonged periods of time when placed in the pulmonary position in animal models (Hoerstrup *et al.*, 2000a). Still, these heart valve replacements appear to lack mechanical strength to withstand stresses arising at the aortic position. Optimizing the in-vitro mechanical conditioning protocol might help solving this problem.

In current bioreactors, two separate mechanical stimuli are applied simultaneously to the tissue to mimic opening and closing behavior of the native heart valve. Increasing levels of pulsatile flow (125 to 750 ml/min) and pressure (30 to 55 mm Hg) were applied (Hoerstrup *et al.*, 2000a), resulting in dynamic (shear) stresses and strains. Studying the effect of each separate stimulus on tissue development will provide new data and insights into the optimal in-vitro conditioning protocol. The present study addresses the effect of one of these stimuli, cyclic strain, on tissue development and mechanical properties in engineered heart valve leaflet tissue-equivalents.

3.2 Materials and methods

3.2.1 Preparation of valve leaflet tissue-equivalents

Human venous myofibroblasts were harvested from the saphenous vein and expanded using regular cell culture methods (Schnell *et al.*, 2001). Rectangular shaped scaffolds (1.0 x 4.0 cm) composed of non-woven polyglycolic-acid meshes (PGA; thickness 1.0 mm; specific gravity 69 mg/cm^3; Cellon S.A.) were coated with a thin layer of poly-4-hydroxybutyrate (P4HB; MW: 1 X 10^6; TEPHA Inc., Cambridge, MA) and cold-gas sterilized (Hoerstrup *et al.*, 2002a). To facilitate cell attachment, the scaffolds were incubated in culture medium composed of DMEM (Gibco BRL), supplemented with 10% FBS (HyClone) and 0.1% gentamycin (PAN) for 24 hours. Venous myofibroblasts (passage 5-6) were seeded onto the scaffolds at a density of 5 X 10^6 cells per square centimeter in three steps with intervals of two hours.

3.2.2 Tissue culture and mechanical conditioning

After seeding, the constructs were statically cultured in a humidified incubator (37°C, 5% CO_2) for 7 days. To promote matrix production, L-ascorbic acid 2-phosphate (Sigma) was added to the culture medium (Hoerstrup *et al.*, 1999, 2000c). After that, the tissue constructs (n=3) were placed in a custom-built straining device (Fig. 3.1) for exposure to increasing cyclic strains, starting at 2% up to a maximum of 7, 9, and 10% for an additional 14 days. Corrections were performed for the permanent deformation of the scaffold material during straining (see section 3.2.3). About three-quarters of the medium (DMEM, supplemented with 10% FBS, 0.3% gentamycin, 1% amphotericin-B (PAN) and L-ascorbic acid 2-phosphate) was changed every 4 days. Controls (n=3) were grown under static conditions accordingly.

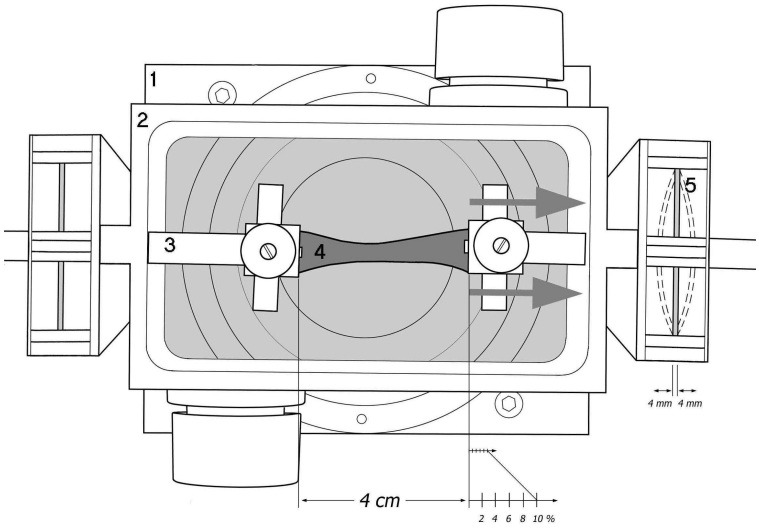


Figure 3.1: The straining device, consisting of a frame (1) that holds a ventilated culture chamber (2) with stainless steel clamps (3) for the tissue (4) and silicone membranes (5) to maintain sterility. One of the clamps is connected to a computer-driven linear actuator to apply cyclic straining to the tissue (represented in the figure as percentages of the original construct length). The complete set-up is placed in a humidified incubator during culturing.

3.2.3 Scaffold deformation under mechanical conditioning

To determine the permanent deformation of the scaffold, rectangular unseeded scaffolds (3.5 X 0.35 X 0.1 cm, n=3 per tested group) were pre-wetted for 24 hours in

culture medium and subsequently strained with 5, 10, or 15% cyclic strain for 24 hours in the straining device in culture medium. The difference in length of the scaffold before and after straining was determined, resulting in an equation describing the deformation behavior at various strains, which was used to adjust the straining protocol every 2-4 days.

3.2.4 Qualitative evaluation of tissue formation

After 21 days of culturing, representative pieces of the constructs were fixed in 4% phosphate-buffered formalin and embedded in paraffin. Sections were cut at 5 μm thickness and studied by Hematoxylin and Eosin (H&E) and Trichrome Masson stains. Additional samples were prepared for scanning electron microscopy (SEM) and transmission electron microscopy (TEM). For SEM, samples were fixed in 2% glutaraldehyde (Fluka), gradient-dehydrated at critical point and sputtered in AuPd. For TEM, samples were fixed for 2-3 days in 2.5% glutaraldehyde in phosphate buffer (0.1M, pH 7.4) and further fixed in osmium tetroxide, dehydrated in graded alcohol and embedded in epon. Ultrathin sections were cut (60-80 nm) and contrasted with acetate and lead citrate. To visualize viability and cell orientation, a representative portion of the constructs was stained with the viable CellTracker Green (CTG) / Propidium Iodide (PI) staining (Molecular Probes) and visualized using confocal laser scanning microscopy (CLSM; Leica TCS-SP; Heidelberg, Germany). This staining can be applied several times in one construct to monitor viability and orientation of the cells during culturing (Breuls *et al.*, 2003).

3.2.5 Quantitative evaluation of tissue formation

Biochemical assays for total DNA content, hydroxyproline, and glycosaminoglycans were performed on lyophilized samples of each construct (minimum of n=3 per assay per construct) and the values of the strained constructs were compared to those of statically cultured constructs. Total DNA content was determined according to the QIAmp DNA Mini Kit (Qiagen; Basel, Switzerland). Hydroxyproline was determined as described by Hoerstrup *et al.* (1999) with some minor modifications. In short, lyophilized samples were hydrolyzed under pressure using 2M NaOH and subsequently oxidized using a chloramin-T solution. Ehrlich's reagent was added and after development of the chromophore for 2 hours at 37°C, the absorbence was read at 570 nm. Absorbency readings were plotted against a standard curve. The glyosaminoglycans content was determined using a modification of the assay described by Farndale *et al.* (1986). In short, lyophilized samples were digested in papain solution overnight at 60°C. The digestion was aborted by heating the samples to a temperature of 95°C for one hour. Dimethylmethylene blue color reagent was added and the absorbence was read at 540 and 595 nm and the value at 595 nm was extracted from the value at 540 nm. The absorbency readings were plotted against a standard curve to determine the amount of glycosaminoglycans in the constructs.

3.2.6 Evaluation of mechanical properties

The mechanical properties of the tissue constructs (minimum of n=3 per construct) were analyzed using an uniaxial tensile tester (Instron, model 4411, equipped with a load cell of 10 N). A constant strain rate of the original sample length per minute was used for all samples. Engineering stress-strain curves up to breakage were obtained by normalizing the load-displacement data for the initial sample surface. The ultimate tensile strength for each sample was assessed from the stress-strain curves to obtain mean values for the constructs. The stiffness of the tissue was measured by calculating the Young's modulus (the slope of the curve at the interval of 15-20% strain). Sample data were averaged to obtain construct values.

3.2.7 Statistics

Results are expressed as the mean percentage values of the strained constructs compared to the unstrained constructs, which were set at 100%, $\pm$ the standard error of the mean. Unpaired t-tests were used to elucidate differences between the strained and unstrained constructs, considering a p-value less than 0.05 as statistically significant.

3.3 Results

3.3.1 Scaffold deformation under mechanical conditioning

Deformation of the PGA/P4HB coated non-woven meshes is described by the equation: $y = 0.25x^2 - 0.14x - 0.02$, where y represents the percentage deformation of the applied strain and x represents the percentage applied strain with a R^2 of 1. For mechanical conditioning of the tissue constructs, each strain increase was corrected for this deformation, resulting in straining protocols varying from 2-7%, 2-9%, and 2-10%.

3.3.2 Qualitative evaluation of tissue formation

The H&E staining showed more tissue formation and organization in strained constructs compared to statically cultured constructs (Fig. 3.2(a), 3.2(b)). The statically cultured constructs showed tissue formation mainly at the surface, which was less dense compared to the strained constructs, while the strained constructs showed more ingrowth into the inner parts of the scaffold. Trichrome Masson stainings showed extracellular matrix formation, mainly consisting of glycosaminoglycans and collagen in all tissue constructs. The amount of matrix formation was larger in the strained constructs (Fig. 3.2(c), 3.2(d)). SEM images of the surfaces of the constructs show a smoother and denser surface layer for the strained constructs compared to the statically cultured construct (Fig. 3.3(a), 3.3(b)). TEM images show viable cells and the presence of extracellular matrix, more pronounced and organized in the strained constructs (Fig. 3.4(a), 3.4(b)). Viability was high as shown by the CLSM images. The

cells were oriented randomly in the statically cultured constructs versus orientation mainly in the direction of strain in the strained constructs (Fig. 3.5(a), 3.5(b)).

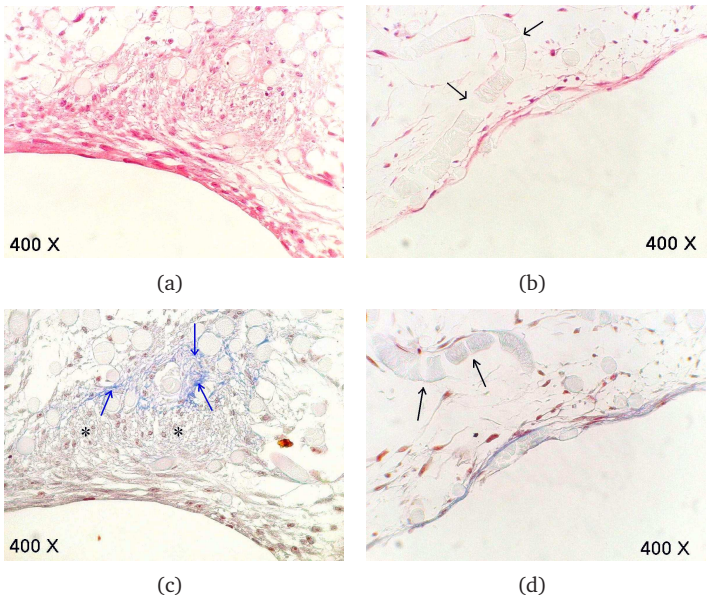
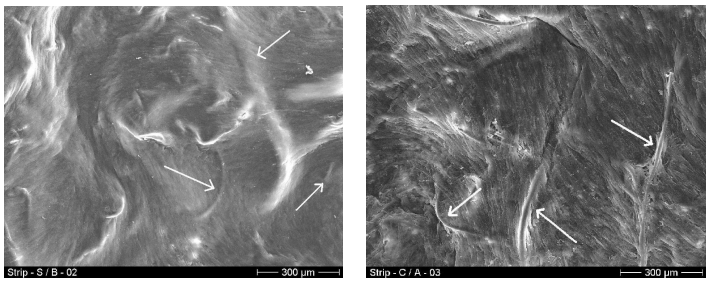


Figure 3.2: Histology of the tissue engineered constructs. The H&E staining shows more ingrowth and a denser surface layer for the construct exposed to a maximum of 10% strain (a) compared to the statically cultured construct (b). Trichrome Masson staining shows more extracellular matrix formation for the construct cultured with a maximum of 10% strain (c) compared to the statically cultured construct (d). The matrix consists mainly of glycosaminoglycans (*) and collagen (blue arrows). Degradation of the PGA fibers was visible by defragmentation of the fibers (black arrows).

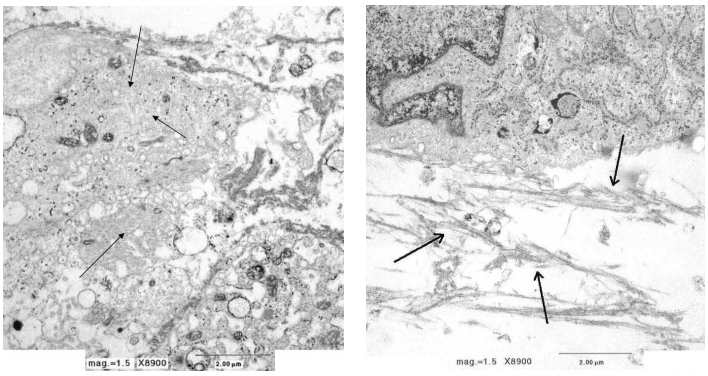
3.3.3 Quantitative evaluation of tissue formation

The quantitative tissue properties are shown in Fig. 3.6. Compared to statically cultured constructs, the total DNA content was 168±67% in the constructs cultured with maximum strains of 7%, 212±47% in the constructs cultured with maximum strains of 9%, and 227±6% in the constructs cultured with maximum strains of 10%. The differences were statically significant for strains of 9% and larger ($p<0.005$). Hydroxyproline content increased from 116±54% to 171±23% in the constructs strained with respectively a maximum of 7 and 9% versus statically cultured constructs. The difference with statically cultured constructs was statistically significant by a maximum strain of 9% ($p<0.01$). The amount of glycosaminoglycans increased from



(a) (b)

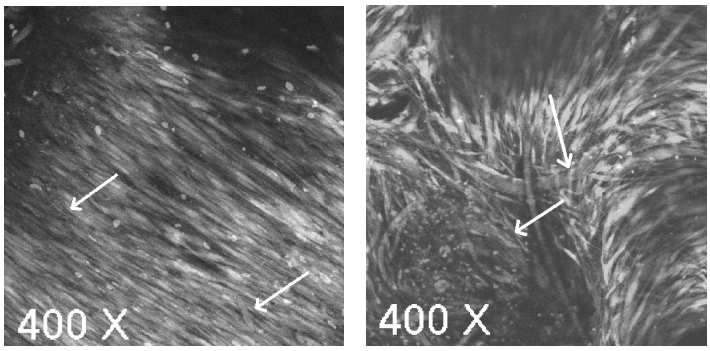
Figure 3.3: SEM images showing a smoother and denser surface layer for the construct cultured with maximally 10% strain (a) compared to the statically cultured construct (b). Rests of PGA fibers underlying the newly formed tissue are visible (white arrows).



(a) (b)

Figure 3.4: TEM images showing viable cells and more pronounced matrix formation in the construct cultured with a maximum of 10% strain (a) compared to the statically cultured construct (b). Immature collagen fibers could be identified (arrows).

109±15% at a maximum of 9% strain to 185±37% at a maximum strain of 10% compared to statically cultured constructs. The difference in glycosaminoglycans content of strained constructs with statically cultured constructs was significant at a maximum of 10% strain ($p<0.01$).



(a) (b)

Figure 3.5: CLSM images showing high viability and oriented cells in the construct strained with a maximum of 10% (a) and randomly organized cells in the statically cultured construct (b). Rests of the fibers were shown with this method as well (arrows).

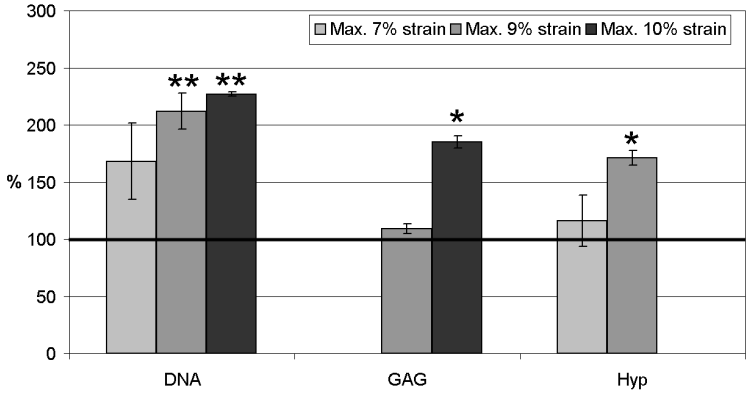


Figure 3.6: Quantitative tissue properties as determined by biochemical assays for the amount of DNA, glycosaminoglycans (GAG) and hydroxyproline (Hyp), expressed as percentage to the unstrained constructs ($\star\star$ represents $p<0.005$ and $\star$ $p<0.01$).

3.3.4 Evaluation of mechanical properties

The mechanical properties of the constructs with respect to unstrained constructs are shown in Fig. 3.7. The ultimate tensile strength of the strained constructs increased from 120$\pm$42% in the constructs cultured under a maximum strain of 7% to 141$\pm$39% for maximum strains of 9%, and 255$\pm$55% for maximum strains of 10% when compared to statically cultured constructs. The ultimate tensile strength was

significantly larger compared to statically cultured constructs for strains larger than 9% ($p<0.05$ for 9% strain and $p<0.005$ for 10% strain). The Young's modulus increased from $93\pm45\%$ to $200\pm75\%$ and $308\pm151\%$ for constructs cultured under maximum strains of 7, 9, and 10% respectively, compared to statically cultured constructs. The difference with statically cultured constructs was significant at strains larger than 9% ($p<0.05$ for 9% strain and $p<0.005$ for 10% strain).

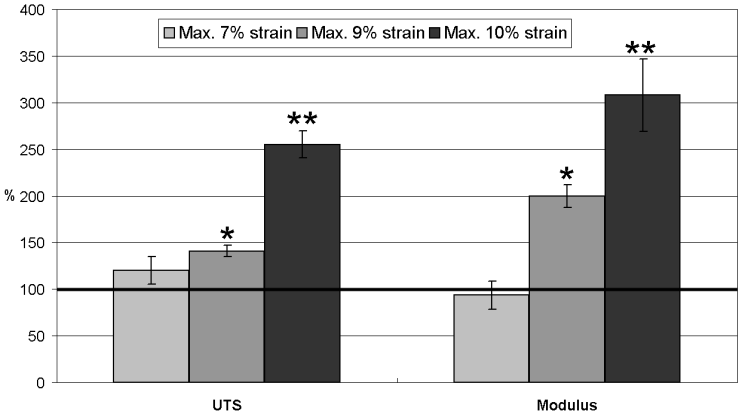


Figure 3.7: The ultimate tensile strength (UTS) and modulus of the constructs, expressed as percentage to the unstrained constructs ($\star\star$ represents $p<0.005$ and $\star$ $p<0.05$).

3.4 Discussion

Exposure of engineered smooth muscle tissue to cyclic strains of 7% for prolonged periods of time has already shown its benefits compared to static culture (Kim *et al.*, 1999; Kim and Mooney, 2000). In this study, engineered valve leaflet tissue-equivalents cultured under increasing cyclic strains up to 10% were superior over statically cultured tissues with respect to the amount and organization of newly developed tissue, mechanical properties, smoothness and density of the surface layer, and cell orientation. The effect was more pronounced and statistically significant when larger cyclic strains (9-10%) were used. Thus, we conclude that large strains may play an essential role in early tissue development - in addition to what has been hypothesized so far and to what is currently done by applying flows and pressures that result in minimal tissue strains (Hoerstrup *et al.*, 2000a; Stock and Vacanti, 2001a).

The effect of flow on tissue development by myofibroblasts is not completely clear, as some state improved tissue development (Jockenhoevel *et al.*, 2002), while others state an inverse effect on cell proliferation (Ueba *et al.*, 1997; Karlon *et al.*, 2002). From a co-culture study of endothelial cells and myofibroblasts, it was shown that

exposure of endothelial cells to flow stabilized the underlying myofibroblasts by reducing cell proliferation (Nackman *et al.*, 1998). By measuring the biosynthetic activity in porcine heart valve leaflets with and without exposure to flow, it has been demonstrated that flow over the leaflets resulted in biosynthetic levels closer to native values. The biosynthetic activity in heart valve leaflets that were not exposed to flow was increased (Weston and Yoganathan, 2001). This further indicates a stabilizing effect of flow on tissue development. We hypothesize that exposure to flow is desired when seeding the engineered tissue with endothelial cells in a later phase of the tissue engineering process to provide a protective and non-trombogenic layer over the newly developed tissue. At this stage, the endothelial cells may stabilize the tissue under the exposure of flow to prepare the tissue for implantation.

A new bioreactor is being developed in which the hypothesis of using large strains in early tissue development will be tested on a trileaflet heart valve. For this purpose, the elastic properties should mimic the deformation in the leaflets by applying pressures existing in the native valve at diastole. Visualization of the orientation, density, and remodeling of the formed collagen network in the newly developed tissue is another issue that is being investigated at the moment, as this network will provide the mechanical strength to the tissue. Numerical models are being used to predict the formation and remodeling of this network under different mechanical conditions for tissue engineering applications (Driessen *et al.*, 2003).

The undesired permanent deformation of the used scaffold has implications for the interpretations of the results in this study. The exact time lapse of this deformation, as well as the effect of the newly developed tissue in combination with the degradation of the scaffold, on this deformation is not known. Despite the corrections performed for the deformation in the calculated strain protocols, it is possible that the cells actually experienced less or more strains than mentioned over the culturing period. Furthermore, straining might accelerate the degradation process of the scaffold as more scaffold surface is exposed to the culture medium when strained, although this was qualitatively not observed. Studies are being addressed to improve the used scaffold to obtain elastic properties comparable to the native valve tissue, thereby, preventing large permanent deformations.

In the present study, we applied increasing strains, starting at relatively low levels of 2%. Considering the beneficial effect on tissue formation, we speculate that conditioning protocols that initiate at even higher levels of straining could be more effective. Further investigations have to elucidate the optimal straining regime, i.e. in terms of strain magnitudes, time of straining, and possible strain-relieve periods.

3.5 Acknowledgements

This work was supported in part by a grant (A. Mol) from the Netherlands Organization for Scientific Research (NWO, grant number R 90-179).

Chapter 4

Fibrin as a cell carrier in cardiovascular tissue engineering applications

The contents of this chapter are based on A. Mol, M.I. van Lieshout, C.G. Dam - de Veen, S. Neuenschwander, S.P. Hoerstrup, F.P.T. Baaijens and C.V.C. Bouten (2005), *Fibrin as a cell carrier in cardiovascular tissue engineering applications*, Biomaterials; **26**: pp 3113-3121

4.1 Introduction

In tissue engineering, the seeding method of the scaffold influences the final outcome of engineered tissues to a large extent. The initial distribution of cells throughout the scaffold and the amount of cells lost during seeding influences ultimate tissue development and organization. Currently, several seeding methods are used, being either static or dynamic in nature. Static seeding is performed by injecting a concentrated cell suspension into the scaffold. Dynamic seeding procedures involve stirring or agitation of cells in suspension together with the scaffold. The dynamic seeding methods render a more uniform cell distribution throughout the scaffold with larger cell yields when compared to static seeding (Kim *et al.*, 1998; Burg *et al.*, 2000). In these comparison studies, however, the static seeding method involved only a single seeding step, while static seeding using multiple seeding steps has shown to render large cell yields and acceptable cell distribution as well (Zund *et al.*, 1999). This multiple-step static seeding procedure lowers the risk of destroying nascent cell-polymer interactions by physical forces created by dynamic seeding methods (Sutherland *et al.*, 2002). In cardiovascular tissue engineering, static seeding methods are most commonly used.

Seeding of a scaffold is a complex and time-consuming process as several seeding steps are necessary for proper static seeding. For more efficient and less time-consuming seeding, involving only one single seeding step, cells can be encapsulated in gels, for example collagen and fibrin gels. The gel can subsequently be molded into the desired structure. In this way, blood vessels (Stegemann and Nerem, 2003) and heart valves (Jockenhoevel *et al.*, 2001a) are being engineered. By encapsulating cells into a gel, newly synthesized extracellular matrix components can accumulate in the extracellular space, rather than diffusing into the surrounding medium (Ye *et al.*, 2000). Reported limitations of this tissue engineering approach are the shrinkage of the gel/cell construct and the weakness of the tissue (Jockenhoevel *et al.*, 2001a). However, as soon as gel contraction is impaired by fixation of the developing tissue, improved tissue properties were obtained due to internal strains (Hoerstrup *et al.*, 2002b). Fixation of the tissue during engineering of cardiovascular structures having a complex geometry, such as heart valves, is rather difficult. Therefore, combining the advantages of cells encapsulated in a gel with the structural properties of a fiber-based scaffold or sponge may lead to optimized tissue formation and organization and a tissue remaining its shape. This approach was shown to be successful in tissue engineering of cartilage, where the cells were encapsulated in either fibrin gel (Ameer *et al.*, 2002) or alginate gel (Marijnissen *et al.*, 2002) and injected into a fiber mesh, cardiac muscle, where the cells were encapsulated in matrigel and injected into collagen sponges (Radisic *et al.*, 2003) and urinary bladder wall by encapsulation of cells into a collagen gel and subsequent pouring of the gel onto a fiber-based scaffold (Nakanishi *et al.*, 2003).

In this study, the feasibility of fibrin gel as a cell carrier combined with a biodegradable polymer fiber mesh is tested for engineering cardiovascular tissue and compared to the conventional static seeding procedure. The advantage of fibrin gels over the other described gels is that it can be obtained autologous. Furthermore, cells en-

trapped in fibrin gels were reported to produce more collagen (Grassl *et al.*, 2002; Neidert *et al.*, 2002) and elastin (Long and Tranquillo, 2003) compared to cells entrapped in collagen gel. The tendency of shrinkage of the gel might in this case be a desired property as it leads to inner tension within the developing tissue, resulting in a larger amount of collagen when the gel is fixed and, therefore, prevented from shrinkage (Jockenhoevel *et al.*, 2001a). Another advantage of this approach is the compact structure that arises directly after seeding, providing a sufficient basis for mechanical loading, without the risk of losing cells and freshly formed extracellular matrix. Mechanical conditioning of the neo-tissue was shown to be relevant in cardiovascular tissue engineering to enhance tissue formation (Kim *et al.*, 1999; Niklason *et al.*, 1999; Hoerstrup *et al.*, 2000a; Jockenhoevel *et al.*, 2002; Mol *et al.*, 2003; Stegemann and Nerem, 2003).

Fibrin is known to degrade within several days by cell-associated enzymatic activities when no degradation inhibitors are used (Ye *et al.*, 2000). Therefore, in tissue engineering applications using cells encapsulated in a fibrin gel, degradation inhibitors are often used to preserve the scaffold function of the fibrin (Ye *et al.*, 2000; Jockenhoevel *et al.*, 2001a; Grassl *et al.*, 2002, 2003). The effect of the concentration of the inhibitors on collagen formation in the gels differed among studies. Beneficial results were reported (Ye *et al.*, 2000) as well as adverse effects (Grassl *et al.*, 2002) and no effects at all (Grassl *et al.*, 2003). When the cells encapsulated in fibrin gel are injected into a fiber-based scaffold, the relevance of degradation inhibitors is questioned. As the fibrin gel only serves to obtain initial uniform cell distribution and to improve the seeding efficiency, the fibrin is allowed to degrade while the fiber-based scaffold provides structural integrity to the developing tissue. Furthermore, the advantage of fibrin to render a compact tissue structure, enabling accumulation of extracellular matrix components, is mainly important in the initial phase of culturing. The newly formed extracellular matrix can replace the fibrin over time when degradation is not inhibited. Culturing with and without fibrin degradation inhibitors is compared in this study, to determine its influence on final tissue outcome.

Human saphenous vein cells are used in this study as they have shown to be favorable in tissue engineering of cardiovascular structures (Schnell *et al.*, 2001) and as they represent an easy-accessible cell source for future clinical use. A non-woven PGA fiber mesh coated with polycaprolactone is used as a scaffold material. Qualitative tissue analyses are performed for all tested methods up to a culture period of 6 weeks and quantitative analyses are performed after 4 weeks of culturing. Furthermore, the medium used for culturing is analyzed for soluble matrix components.

4.2 Materials and methods

4.2.1 Scaffold preparation and sterilization

Rectangular shaped non-woven polyglycolic acid (PGA) meshes (1-1.5 cm^2, density 72.76 mg/cm^3, thickness 1.2 mm, Cellon, Luxembourg) were coated with polycaprolactone (PCL, MW=80000, Sigma, USA) to provide structural integrity to the mesh.

A 2% w/v PCL in chloroform solution was used and the scaffold was dipped in the coating solution and subsequently in distilled water to quench the coating around the fibers. This process was repeated three times. A scanning electron microscopy image (SEM, Philips XL30 Esem-FEG, the Netherlands) of the coated fiber mesh is shown in Fig. 4.1.

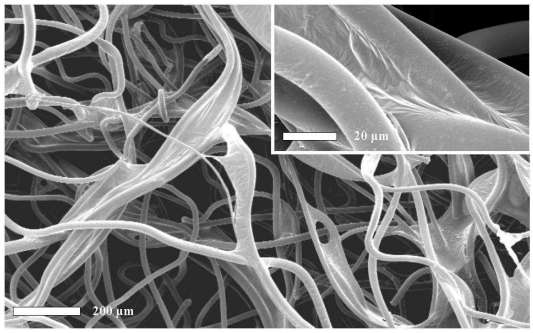


Figure 4.1: SEM images of the PGA mesh coated with PCL at low (large image) and high (small image) magnification. The PCL coating provides structural integrity to the mesh by fusion of the randomly oriented PGA fibers at crossing points.

After vacuum drying overnight the scaffolds were placed in 70% ethanol for 3-4 hours to obtain sterility. The ethanol was allowed to evaporate overnight and the scaffolds were washed three times in phosphate-buffered saline (PBS, Sigma, USA). The scaffolds were placed in culture medium overnight, consisting of DMEM Low Glucose (Cambrex, Belgium) supplemented with 10% fetal bovine serum (FBS, Biochrom, Germany), 1% L-glutamine (Biochrom, Germany), 0.3% gentamycin (Biochrom, Germany), and L-ascorbic acid 2-phosphate (Sigma, USA), further referred to as standard culture medium, to facilitate cell attachment.

4.2.2 Cell harvesting and culturing

Human venous myofibroblasts were harvested from the vena saphena magna and expanded using regular cell culture methods (Schnell *et al.*, 2001). The medium used to culture those cells consisted of DMEM Low Glucose supplemented with 10% FBS, 1% L-glutamine and 0.1% gentamycin.

4.2.3 Cell seeding

Two methods were used to seed the cells onto the scaffolds: (A) dripping of a concentrated cell suspension onto both sides of the scaffolds, referred to as the conventional seeding method and (B) seeding the cells by using fibrin as a cell carrier. For both seeding methods, a density of 2.5 X 10^6 cells per 100 mm^3 of scaffold was used.

Conventional seeding procedure

For method A (n=6), cells were resuspended in standard culture medium in an amount that equals half the void volume of the scaffold (0.5 X height X width X thickness). This concentrated cell suspension was dripped onto one side of the scaffold and the cells were allowed to attach to the scaffold for 45 minutes. The scaffold was turned over and the same procedure was followed for the other side. After another 45 minutes medium was added.

Seeding procedure using fibrin

For method B (n=12), cells were resuspended in a sterile bovine thrombin solution (10 IU thrombin/ml medium; Sigma, USA) in an amount that equals half the void volume of the scaffold. Subsequently, the cells in the thrombin solution were added to an equal amount of sterile bovine fibrinogen solution (10 mg actual protein/ml medium; Sigma, USA). These concentrations were shown to be optimal in terms of mechanical stability of the fibrin gel. The cells were resuspended in thrombin as cell clumping was noticed when using fibrinogen to suspend the cells, indicating interaction of the cells with fibrinogen. This phenomenon has been described by others via, for example, the $\alpha\nu\beta3$ integrin, a known fibrinogen receptor on various cell types, such as fibroblasts, endothelial cells, and smooth muscle cells (Hsueh *et al.*, 1998; Yee *et al.*, 1998; Herrick *et al.*, 1999). After carefully mixing, the fibrin solution containing the cells was dripped onto the scaffold. The coagulation time of the fibrin gel was determined using a coagulometer (Amelung, Germany) without cells and was shown to vary from 20 to 40 seconds. The construct was allowed to polymerize for 75 minutes before medium was added. Within this method, two types of media were used to prepare the thrombin and fibrinogen solutions, either the standard culture medium (method B1, n=6) or the standard culture medium supplemented with the fibrin degradation inhibitor ε-amino-n-caproic acid (ACA; 2 mg/ml medium; Sigma, USA) (method B2, n=6).

4.2.4 Tissue culture

All tissue constructs were cultured statically up to 6 weeks. The constructs seeded by method A and B1 were cultured in standard culture medium. The constructs seeded by method B2 were cultured in standard culture medium supplemented with the fibrin degradation inhibitor ACA. About 2/3 of the medium was changed every 3 days, in order to not remove all of the endogenous produced biochemical substances, such as growth factors. The removed medium was stored at $-20^{\circ}\mathrm{C}$ for further analysis as described in section 4.2.7.

4.2.5 Qualitative evaluation of tissue formation

Tissue formation was analyzed qualitatively over time by histology. Small pieces of the constructs were taken after 2, 4, and 6 weeks of culturing and fixed in phosphate-buffered formalin (Fluka, USA). Subsequently, the pieces were embedded in paraffin.

Sections were cut at 5 μm thickness and studied by Haematoxylin and Eosin (H&E) staining for general tissue morphology and Trichrome Masson staining for extracellular matrix formation. For the Trichrome Masson staining a commercially available kit was used (Sigma, USA). The sections were analyzed by light microscopy. Sections of the constructs from week 4 and 6 were additionally stained by a picrosirius red staining according to Junqueira *et al.* (1979) and analyzed by polarization microscopy. Sirius Red binds to collagen in a parallel fashion and enhances the normal birefringence of collagen fibers (the degree to which they retard polarized light), which can be detected using polarization microscopy (Junqueira *et al.*, 1979; Pickering and Boughner, 1991). In this way also very thin fibrils, undetectable in normal light microscopy, become visible.

4.2.6 Quantitative evaluation of tissue formation

Tissue development was analyzed quantitatively after 4 weeks by biochemical assays. The constructs were cut into tiny pieces, mixed, and lyophilized. The amount of DNA - as an indicator for cell number - was determined using a commercially available kit (Sigma, USA). DNA was isolated from the samples by treatment with digestion buffer containing protease K. After several centrifuge steps using DNA-binding columns, the trapped DNA was eluted in buffer. The amount of DNA was subsequently determined by measuring the absorbance of the DNA in solution at 260 nm. The ratio of absorbance at 260 and 280 nm was within the range of 1.6-1.9 for all samples. The amount of DNA was divided by the dry weight of the tissue samples to obtain the amount of DNA per mg dry tissue.

The hydroxyproline assay - as an indicator for collagen - followed the protocol provided by Huszar *et al.* (1980). In short, the tissue samples were hydrolyzed in 4 M NaOH (Fluka, USA) in an autoclave at a temperature of $120°\mathrm{C}$ for 10 minutes. The solution was neutralized by adding 1.4 M citric acid (Fluka, USA). Chloramin-T (62 mM) was added and the samples were allowed to oxidize for 25 minutes. Aldehyde/perchloric acid solution (1 M) was subsequently added and the chromophore was allowed to develop at $65°\mathrm{C}$ for 15 minutes. The absorbance of the obtained solutions was determined at 570 nm. The amount of hydroxyproline present in the solutions was determined from a standard curve, prepared in addition to the samples. The amount of hydroxyproline was divided by the dry weight of the tissue to obtain the amount of hydroxyproline per mg dry tissue.

4.2.7 Analysis of culture medium

The frozen medium was analyzed to determine the amount of soluble collagen that leaked out of the constructs. For this analysis a commercially available kit was used, the SirCol^{TM} soluble collagen assay (Biocolor, Northern Ireland). In short, 1 ml of SirCol dye reagent was added to 100 μl of the medium samples, which binds soluble collagen during a 30 minutes period of shaking and forms a precipitate. The precipitate was subsequently pelleted by centrifugation, after which the unbound dye solution was removed. Alkali reagent was added to dissolve the bound dye and the

absorbance was measured at 540 nm. The assay is performed for the medium samples of the constructs engineered by method A and method B1 over a time period up to four weeks of culturing as well as for control medium, where no constructs were cultured in, but further treated similar. The amount of soluble collagen was determined from a standard curve prepared in addition to the samples. The amount of soluble collagen in the control medium was subtracted from the amount in the medium the constructs were cultured in, in order to correct for variations in the amount of soluble collagen initially present in the culture medium, and expressed in μg/ml medium. Furthermore, the reduced sample size due to the removal of pieces for histology was taken into consideration and corrected for.

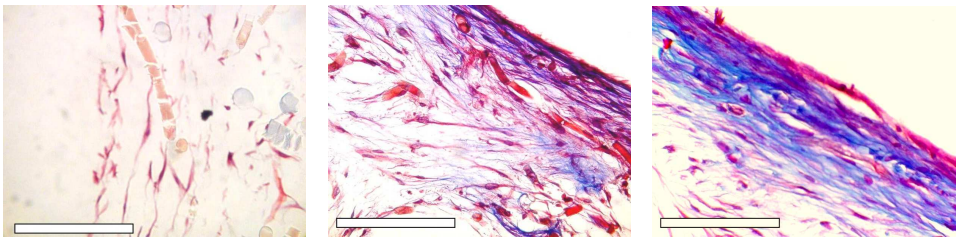
4.2.8 Statistics

Results are expressed as mean values $\pm$ standard error of the mean. One-way ANOVA analysis was used to determine significant differences between the three tested groups for hydroxyproline and DNA content, followed by Bonferroni post-hoc tests between groups in case of a significant result. Student t-tests were used to elucidate differences in the accumulative amount of soluble collagen in the medium between groups A and B1 at each time point. Differences were considered significant at a p-value < 0.05.

4.3 Results

4.3.1 Qualitative evaluation of tissue formation

Tissue formation, as analyzed by histology over time for group B1, is shown in Fig. 4.2. Fig. 4.3 shows tissue formation after four weeks of culturing for all groups.



(a) (b) (c)

Figure 4.2: Trichrome Masson stained sections of constructs from group B1 after two (a), four (b), and six (c) weeks of culturing. The bars in the images represent scales of 175 μm. After two weeks, predominantly cells (purple) were present. Collagen deposition (blue) was observed after four weeks and increased further after six weeks of culturing. Fragmented scaffold remnants could be identified (orange-red).

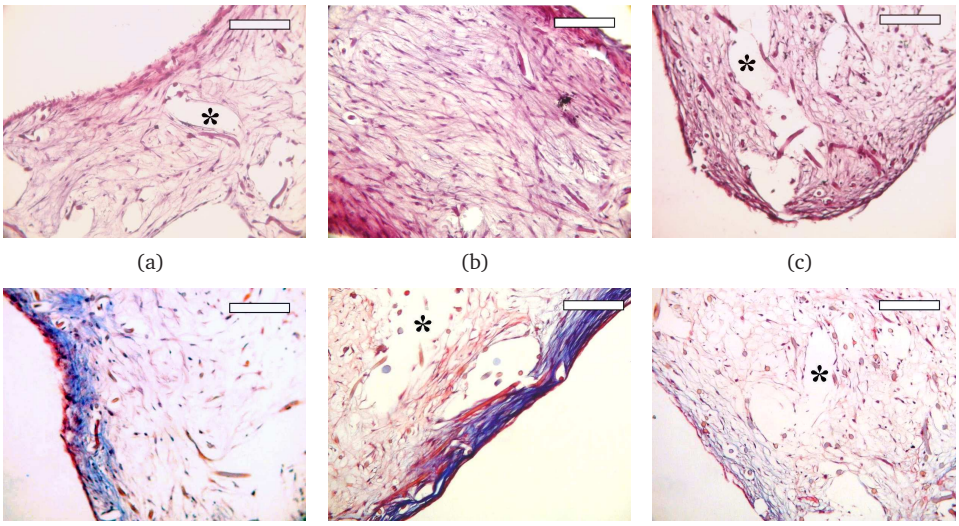


Figure 4.3: Heamatoxylin and Eosin (H&E) stained sections for general tissue morphology (a, b, c) and Masson Trichrome (MT) stained sections for extracellular matrix formation (d, e, f) of constructs from group A (a, d), group B1 (b, e), and group B2 (c, f) after four weeks of culturing. The bars in the images represent scales of 175 μm. Cells were present throughout the whole construct (dark purple in H&E, purple in MT). Tissue formation was observed mainly at the surface of the constructs (purple in H&E), consisting mainly of collagen (blue in MT). Best tissue formation was observed for group B1, followed by group A with abundant amounts of collagen. In constructs from group B2, many cells could be identified, however, hardly any collagen deposition could be observed. The vacuolar regions (asterisk) are cutting artifacts due to polymer remnant.

After two weeks of culturing, all constructs showed a homogeneous distribution of cells throughout the scaffold. However, there was not much extracellular matrix at this time point (Fig. 4.2(a)). After four weeks of culturing, the amount of extracellular matrix increased drastically, in particular for experimental group B1 (Fig. 4.2(b), 4.3(b), 4.3(e)), followed by experimental group A (Fig. 4.3(a), 4.3(d)). The amount of extracellular matrix formation increased even further after six weeks of culturing (Fig. 4.2(c)), although the difference was not as large as the increase between week two and four. It was obvious that although cells were present throughout the entire constructs, extracellular matrix formation was limited to the surfaces. The tissue development in experimental group B1 (Fig. 4.2(c)) and experimental group A did not differ after six weeks of culturing. In samples of experimental group B2 hardly any extracellular matrix formation could be identified after four (Fig. 4.3(c), 4.3(f))

and six weeks of culturing, although many cells could be identified.

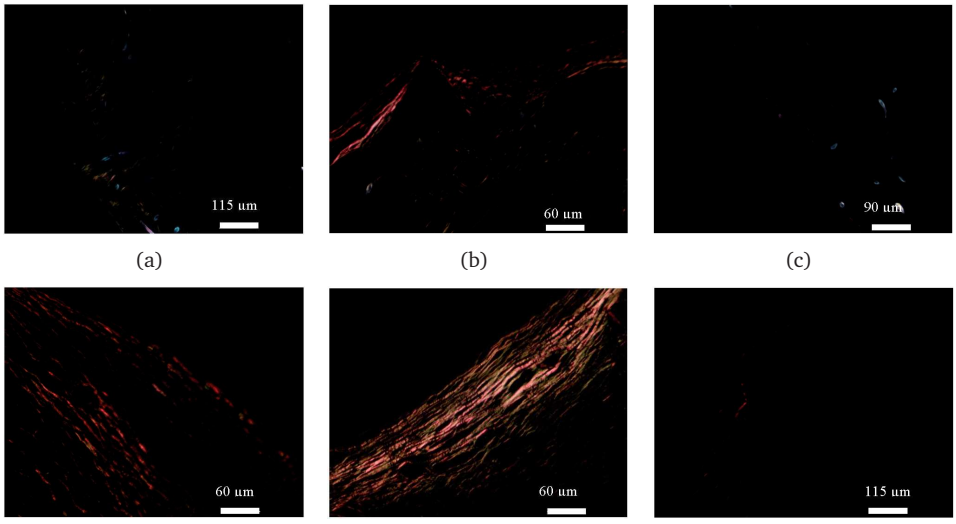


Figure 4.4: Picrosirius red stained sections of constructs from group A (a, d), B1 (b, e), and B2 (c, f) after four (a, b, c) and six (d, e, f) weeks of culturing analyzed by polarized light microscopy. Scale bars in the images represent the magnification. Some birefringent fibers could be identified in constructs of group B1 after four weeks, while in constructs of group A and B2 hardly any fibers could be identified. After six weeks, in both groups A and B1 birefringent fibers were present, although the density and total amount was larger for group B1 compared to group A. No birefringent fibers could be observed after six weeks of culturing in constructs from group B2.

The Picrosirius Red stainings are shown in Fig. 4.4. Some birefringent fibers are present after four weeks of culturing in experimental group B1 (Fig. 4.4(b)). At this time point, hardly any birefringent fibers could be identified in the constructs from group A (Fig. 4.4(a)). After six weeks, birefringent fibers were identified in both groups A (Fig. 4.4(d)) and B1 (Fig. 4.4(e)), although the amount and density of birefringent fibers was larger in group B1 compared to group A. In constructs from experimental group B2 (Fig. 4.4(c), 4.4(f)), hardly any birefringent fibers could be identified after four and six weeks of culturing. The polarization colors of the collagen fibers are depending on several factors. Larger collagen fiber diameter, better alignment of the fibers, and more tightly packed collagen molecules show longer wavelengths (more towards orange-red) (Dayan *et al.*, 1989; Rabau and Dayan, 1994), as well as fibers of increasing age (Pickering and Boughner, 1991). The birefringence color of the fibers is also reported to be dependent on the type of collagen present

(Junqueira *et al.*, 1979; Dayan *et al.*, 1989). In this study, only the amount and density of the birefringent fibers was addressed and referred to as a measure of maturity.

4.3.2 Quantitative evaluation of tissue formation

The results of the biochemical assays after four weeks of culturing are represented in Fig. 4.5. ANOVA analysis revealed no significant differences in cell number (DNA, p=0.15) between the groups. For the amount of collagen (hydroxyproline), one of the three groups seemed to be different according to the ANOVA analysis (p=0.042). However, pairwise comparison probabilities from subsequent Bonferroni post-hoc tests did not reveal significant differences (p=0.112 for group A versus B1, p=1.000 for group A versus B2, and p=0.065 for group B1 versus B2).

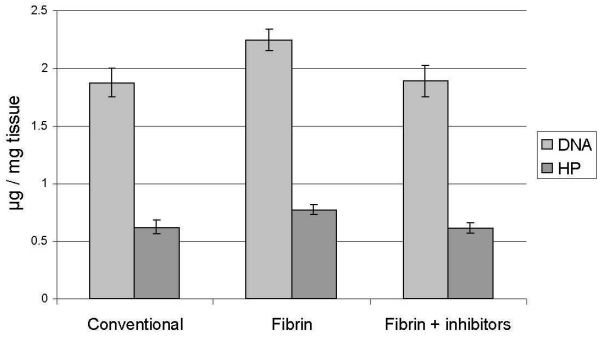


Figure 4.5: Amount of DNA and hydroxyproline in constructs from all groups (conventional=A, fibrin=B1 and fibrin + inhibitors=B2) after four weeks of culturing. The error bars indicate standard error of the mean. No significant differences could be identified between the groups for the amount of DNA using one-way ANOVA (p=0.15). For the amount of hydroxyproline one-way ANOVA revealed a small significant difference between the three groups (p=0.042). However, subsequent Bonferroni post-hoc tests showed no significant differences between the three groups (p=0.112 for group A versus B1, p=1.000 for group A versus B2, and p=0.065 for group B1 versus B2).

4.3.3 Medium analysis

The medium analyses for soluble collagen leaching out of the constructs are shown in Fig. 4.6. The accumulated amount of soluble collagen present in the medium was significantly larger at all time points in experimental group A as compared to group B1, indicating a larger loss of soluble collagen into the surrounding medium in group A.

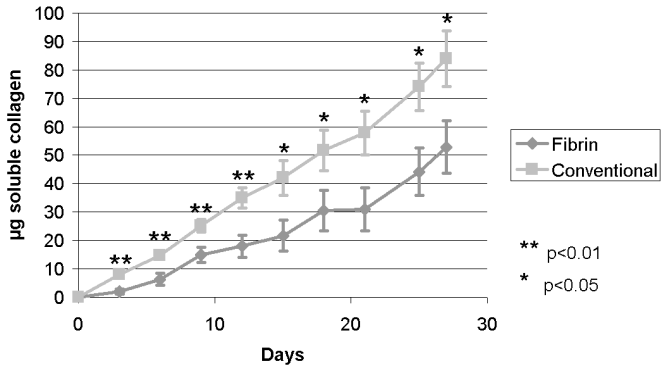


Figure 4.6: Accumulated amount of soluble collagen in the medium over four weeks of culturing for constructs of group A (conventional) and B1 (fibrin). The error bars indicate standard error of the mean. Student t-tests revealed significantly larger amounts of soluble collagen diffusing out of the constructs into the medium at all time points when using the conventional seeding method.

4.4 Discussion

Static seeding methods have shown to be suitable for cardiovascular tissue engineering (Zund *et al.*, 1999). Due to multiple seeding steps in this procedure, it is a time consuming and complex seeding method. Gels have been demonstrated to be excellent cell carriers, making the seeding procedure less time-consuming, as only one seeding step is necessary, and more efficient. Combining the advantages of a gel as a cell carrier, providing a homogeneous cell distribution and less loss of freshly formed extracellular matrix into the surrounding medium, with the advantages of a fiber-based scaffold, providing structural integrity to the developing tissue, offers a promising approach within tissue engineering applications. The use of fibrin as a cell carrier combined with a fiber-based scaffold for cardiovascular tissue engineering applications is tested in this study and compared to conventional static seeding. Tissue development is analyzed qualitatively after two, four, and six weeks and quantitatively after four weeks of culturing.

For both fibrin and conventionally seeded constructs tissue development increased over the culture period. The extracellular matrix deposition occurred rather late, between week two and four (Fig.4.2). The overall tissue formation after six weeks did not seem to be different among the two groups. After four weeks, however, the amount of extracellular matrix appeared to be larger in the fibrin seeded constructs compared to the constructs seeded by dripping (Fig. 4.3), although this difference was not significant (Fig. 4.5). It is obvious that the extracellular matrix was formed mainly at the surfaces of the constructs, while cells were distributed throughout the whole

construct. This might be due to a lack of diffusion of nutrients into the inner parts of the constructs and can be prevented by perfusion of the constructs immediately after seeding (Radisic *et al.*, 2003). Although the absolute amount of hydroxyproline was not larger, the collagen fibers in the constructs seeded with fibrin seemed more mature, as more and denser packed birefringent fibers could be observed, compared to the collagen fibers in the constructs seeded by the conventional seeding method after four weeks as shown by the picrosirius red stainings (Fig. 4.4). From these results, it can be stated that the use of fibrin does not increase tissue formation compared to seeding by dripping. However, it does seem to render more mature collagen fibers in a shorter period of time. This quicker development of extracellular matrix might be due to the contractile property of the fibrin over time, creating inner tension in the scaffold. Furthermore, the faster extracellular matrix formation might be due to decreased loss of soluble matrix components into the surrounding medium when fibrin was used, as is the case for soluble collagen (Fig. 4.6). An issue for further studies is the possible alteration in types of extracellular matrix proteins produced and in expression of cell surface proteins by the use of fibrin. For human dermal fibroblasts, no fibrin concentration dependent change in cell surface protein expression was noted (Cox *et al.*, 2004), but a comparison to cells not in fibrin gel was not made.

When fibrin is used as a scaffold material, fibrin degradation inhibitors are often used to prevent rapid degradation of the fibrin. Whether this is also beneficial in case of using fibrin as a cell carrier was determined by comparing tissue development in fibrin seeded constructs cultured with the fibrin degradation inhibitor ACA and without this fibrin degradation inhibitor. The number of cells in the newly formed tissues was not significantly different between both groups as shown by the DNA assay (Fig. 4.5). Although the amount of hydroxyproline tended to be lower when the fibrin degradation inhibitor was used, this was not significant (Fig. 4.5). This is in contradiction to the histology results (Fig. 4.3, 4.4), in which hardly any collagen fibers could be identified. As the fibrin degradation inhibitor ACA is a lysine-analogue (Rath and Pauling, 1995), it might act as a competitive residue preventing cross-linking of collagen molecules, explaining the absence of collagen fibers. The formation of hydroxyproline is obviously not hampered. Therefore, when using fibrin as a cell carrier, this particular fibrin degradation inhibitor should be avoided. Future studies are needed to elucidate the exact relation between ACA and the maturity of collagen and the use of other fibrin degradation inhibitors.

4.5 Conclusions

Seeding scaffolds using fibrin as a cell carrier offers a lot of advantages over the conventional static seeding method in tissue engineering of cardiovascular structures. It provides a fast and efficient seeding method, involving only one seeding step, rendering a compact structure. It is anticipated that the obtained structure can be efficiently mechanically loaded, which has shown to be a prerequisite in tissue engineering of cardiovascular structures. Furthermore, the compact structure prevents the loss of soluble matrix components into the surrounding medium, resulting in a more mature

extracellular matrix. Fibrin can be obtained completely autologous from the patient and degrades fast over time, serving solely as an initial entrapment system for the cells. Other promising features are loading the fibrin gel with growth factors, for example TGF-β1 (Huang *et al.*, 2002) to obtain controlled release of this growth factor, or cross-linking bifunctional peptides into the fibrin gel to obtain a bioactive gel (Schense and Hubbell, 1999).

Chapter 5

Tissue engineering of human heart valve leaflets using a strain-based conditioning approach

The contents of this chapter are based on A. Mol, N.J.B. Driessen, M.C.M. Rutten, S.P. Hoerstrup, C.V.C. Bouten and F.P.T. Baaijens, *Tissue engineering of human heart valves using a strain-based conditioning approach*, Annals of biomedical engineering; -submitted -.

5.1 Introduction

In cardiovascular tissue engineering, stimulation of tissue formation by mechanical conditioning has proven to be a useful tool for development of functional cardiovascular structures enabling growth, repair, and remodeling. The main ways of conditioning a developing tissue are by applying either flow or strain, or a combination of both. Various bioreactors have been developed in the past years to apply specific conditioning protocols to growing cardiovascular structures.

Bioreactors that use flow as the main mechanical stimulus are, for example, the bioreactors developed by Williams and Wick (2004) and Narita *et al.* (2004) to engineer blood vessels. Furthermore, a pulse duplicator system has been developed by Hoerstrup *et al.* (2000a,b) to grow heart valves as well as blood vessels, modified by Sodian *et al.* (2001, 2002b) to include the seeding procedure. Bioreactors that use strains as the main mechanical cue are e.g. the bioreactors developed by Niklason *et al.* (1999, 2001) and Seliktar *et al.* (2000) for tissue engineering of blood vessels. In these bioreactors, the tissue is exposed to dynamic strains by applying intraluminal pulsatile pressures via an inflatable silicone tube. Niklason *et al.* (1999, 2001) apply dynamic strains of about 5%, while Seliktar *et al.* (2000) use larger strains of about 10%. For myocardial tissue, a comparable bioreactor is developed by Gonen-Wadmany *et al.* (2004) applying dynamic strains (0-12%) to the developing tissue by pulsatile inflation of a silicone bulb, to which the tissue is attached. The flow applied to the tissues cultured in these strain-based bioreactors can be kept to a minimum and are mainly induced by medium circulation or the movement of the tissue itself. In another type of bioreactors, the physiological environment of the cardiovascular structure is mimicked, including both flow and strain. These bioreactors can be used for testing native tissues, as well as for tissue engineering and subsequent functionality testing. A bioreactor in which the physiological pressure waveforms, present in a blood vessel, can be applied is the system developed by McCulloch *et al.* (2004). A bioreactor in which physiological pressure waveforms can be applied in combination with physiological flows is developed by Thompson *et al.* (2002). As blood vessels are exposed to longitudinal strains in-vivo as well, Mironov *et al.* (2003) developed a bioreactor that exposes developing blood vessels to a physiological environment including dynamic longitudinal strains. Hildebrand *et al.* (2004), Dumont *et al.* (2002), and Rutten *et al.* (2005) have developed bioreactors, in which the exact physiological conditions of a heart valve in-vivo can be applied, with the latter providing the possibility to visualize valve function using MRI. Despite these efforts, the question still remains to what extent the developing tissue should be exposed to mechanical cues and which mechanical cues are optimal for tissue development.

The cells in the tissue engineered structures are responsible for the formation of the extracellular matrix and via mechanical conditioning they can be stimulated to produce larger amounts of extracellular matrix. Apart from the nature and magnitude of the mechanical cues, the cellular (pheno)type as well as the culture conditions (either 2D or in a 3D environment) affect the cellular responses to mechanical loading (Dethlefsen *et al.*, 1996; Ueba *et al.*, 1997; Watase *et al.*, 1997; Kim *et al.*, 1999; Chapman *et al.*, 2000; O'Callaghan and Williams, 2000; Kim and Mooney, 2000; Jocken-

hoevel *et al.*, 2002; Lee *et al.*, 2002; Engelmayr *et al.*, 2005). Human saphenous vein cells were chosen as a cell source for tissue engineering of human heart valve leaflets. Not only do they represent an easily accessible cell source, they were also shown to be more sensitive to mechanical cues compared to human arterial derived cells (Dethlefsen *et al.*, 1996; Schnell *et al.*, 2001). Although the optimal conditioning procedure for this specific cell type still has to be determined, increased extracellular matrix formation has been demonstrated in engineered valve leaflet tissue-equivalents cultured with these cells using dynamic strains (Mol *et al.*, 2003). It is, therefore, hypothesized that the optimal conditioning protocol involves dynamic strains to stimulate matrix production by the seeded vascular cells. An initial low shear stress environment combined with exposure of the developing tissue to physical stimuli has been suggested by Barron *et al.* (2003) to be advantageous for initial tissue development. Shear stresses, by application of flows, will most likely start to play a significant role at a later stage when the leaflets are seeded with endothelial cells to provide a non-thrombogenic surface layer, stabilizing the underlying tissue prior to implantation (Nackman *et al.*, 1998; Weston and Yoganathan, 2001).

The goal of this study is to set up a basic concept to engineer human heart valve leaflets using dynamic strains in combination with minimized flows to stimulate extracellular matrix formation. This is a novel approach as all currently used conditioning approaches for tissue engineering of heart valves concentrate on the application of pulsatile flows, with none or limited tissue straining. For this purpose, a new bioreactor system is developed, the Diastolic Pulse Duplicator (DPD), to deliver dynamic strains to the heart valve leaflets by applying a dynamic pressure difference over the closed leaflets. Requirements for such a bioreactor system are: 1) simplicity in its use, 2) sized small to save on culture medium and incubator space, 3) usage of biocompatible materials, 4) maintenance of sterility over prolonged periods of time, and 5) the ability to monitor and control the applied transvalvular pressure. As a stented valve geometry is used in this study, compaction-induced prestrain develops in the leaflets as tissue compaction, common in growing and healing tissues, is constrained by the rigid stent. Medium is circulating in the DPD at low speed (4 ml/min) to provide oxygen and fresh nutrients to the developing tissue and to remove waste products.

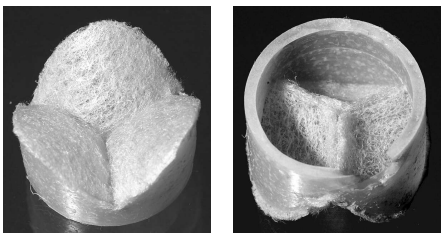
The feasibility of the strain-based approach for human heart valve tissue engineering is demonstrated. Tissue formation and mechanical properties of leaflets exposed to dynamic strains in the DPD and compaction-induced prestrain by the stent were compared with leaflets exposed to compaction-induced prestrain only. The latter leaflets were cultured in the DPD with low-speed medium circulation without application of a dynamic pressure difference. As controls, unloaded rectangular-shaped valve leaflet tissue-equivalents, in which compaction was not constrained, were used. The valve scaffolds were prepared from a non-woven PGA fiber mesh, coated with P4HB. Fibrin was used as a carrier for the human saphenous vein cells to ensure homogeneous cell distribution throughout the scaffold and to render a compact structure suitable for mechanical conditioning (Mol *et al.*, 2005). The levels of compaction-induced prestrain were estimated, as well as the strain distribution in the leaflets resulting from the dynamic transvalvular pressure applied in the DPD, the latter using numerical analyses.

5.2 Materials and methods

5.2.1 Preparation of the leaflet tissues

The heart valve scaffold

Trileaflet heart valve scaffolds were fabricated on a Fastacryl® stent. The two components, Fastacryl powder and fluid (PMMA and MMMA, Vertex-dental, the Netherlands) were mixed, poured into a mold, and allowed to polymerize for 30 minutes. After complete polymerization, the stent was released from the mold. Anatomically shaped leaflets, including coaptation areas, were cut out of non-woven polyglycolic acid meshes (PGA; thickness 1.0 mm; specific gravity 69 mg/cm^3; Cellon, Luxembourg). The leaflets were coated with a thin layer of poly-4-hydroxybutyrate (P4HB; MW 1x10^6; TEPHA Inc., Cambridge, USA) as described before (Hoerstrup *et al.*, 2000a). Before evaporation of the solvent, the leaflets were positioned onto a teflon mold in the shape of a trileaflet heart valve. The fastacryl stent was placed on top. By action of the solvent, dissolving the surface layer of the stent, the leaflets were fixed to the stent. After evaporation of the solvent, the valve scaffold including the stent was removed from the mold (Fig. 5.1). The valve scaffolds were further dried under vacuum overnight to remove solvent remnants, after which they were sterilized using ethylene oxide.



(a) (b)

Figure 5.1: Photographs of the valve scaffold, bottomview (a) and topview (b). The scaffold is fabricated from a non-woven PGA mesh, coated with P4HB and includes a fastacryl stent fixed to the leaflets. The leaflets contain coaptation areas to ensure closure of the valve.

Seeding procedure

Cells harvested from the human vena saphena magna and expanded using regular cell culture methods were used (Schnell *et al.*, 2001). The medium to culture these cells consisted of DMEM Advanced (Gibco, USA), supplemented with 10% Fetal Bovine Serum (FBS; PAN Biotech, Germany), 1% GlutaMax (Gibco, USA), and 0.1% gentamycin (PAN Biotech, Germany). The medium used for seeding and subsequent tissue culture contained 0.3% gentamycin and additional L-ascorbic acid 2-phosphate

(0.25 mg/ml; Sigma, USA) to promote extracellular matrix production. The scaffolds were placed in medium overnight before seeding to facilitate cell attachment by deposition of proteins. The seeding was performed per leaflet using fibrin as a cell carrier (Mol *et al.*, 2005). Briefly, the cells were suspended in a sterile thrombin solution (10 IU/ml medium; Sigma, USA) in a volume that equals half the void volume of the scaffold (0.5 X length X width X thickness). The cells in thrombin were mixed with an equal amount of sterile fibrinogen solution (10 mg actual protein/ml medium; Sigma, USA) and dripped onto the scaffold. The fibrin solution was taken up by the scaffold and remained inside due to polymerization of the fibrin gel. The leaflets were seeded with a density of 4-5 million cells (passage 6-7) per cm^2 of scaffold. The seeded valve scaffolds were allowed to polymerize for 20 minutes in an incubator ($37^{\circ}\mathrm{C}$ and 5% CO_2) before placement into the DPD.

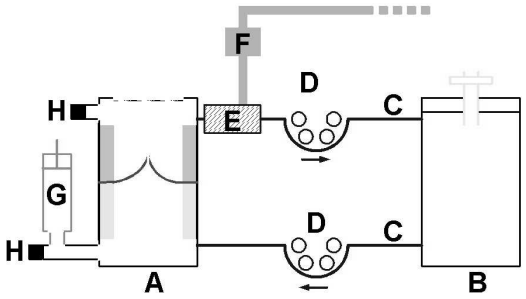
5.2.2 The Diastolic Pulse Duplicator (DPD)

Description of the DPD

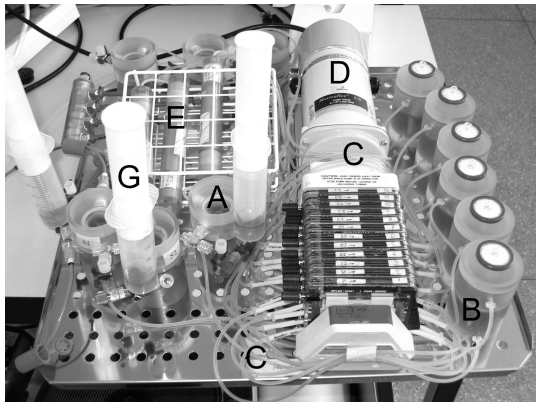
Each DPD consists of two components as shown in Fig. 5.2(a): a bioreactor (height=9 cm, diameter=6 cm), in which the valve is cultured, and a medium container of similar dimensions, both fabricated from polycarbonate (KUBRA Kunststoffen, The Netherlands). The bioreactor itself consists of two parts, the upper part containing a glass window (Melles Griot BV, The Netherlands) for visualization of the valve, and a lower part, which can be screwed together. Silicone rings (van der Heijden, The Netherlands) were used to seal all components. The bioreactor and the medium container are connected via two parallel silicone tubing series (Rubber, the Netherlands). Polypropylene connector parts (Neolab, Milispec Int., The Netherlands) were used to secure the tubing. Both tubing series run through a roller pump (Masterflex®, Cole-Parmer, USA). Part of the upper tubing serie consists of a thicker silicone tube placed in a polycarbonate cylinder, with a connection to tubing suitable to withstand air under pressure (Festo, The Netherlands). This compressed-air tubing is connected to a compressed-air tap (7 bar). The air pressure is reduced to 2 bar and runs through a proportional magnet valve (Festo, The Netherlands) into the polycarbonate cylinder. The complete DPD is sterilized by ethylene oxide and is placed inside an incubator, together with the roller pump. Up to six DPDs can be placed onto one shelf of a normal sized incubator as shown in Fig. 5.2(b).

Functioning of the DPD

About 75 ml of medium is circulating from the medium container, through the bioreactor, and back into the medium container, via the roller pump at very low speed (4 ml/min) to supply fresh nutrients to and to remove waste products from the developing valve tissue. A sterile filter (0.2 μm, Schleicher & Schuell Bioscience, Germany) is placed in the lid of the medium container to oxygenate the circulating medium. About 25 ml of medium is present in the bioreactor and the tubing series, and the remaining 50 ml is present in the medium container and can be changed easily. To



(a)



(b)

Figure 5.2: Schematic drawing of one DPD and its function (a) and a photograph of six DPD systems in use simultaneously (b). One DPD consists of a bioreactor [A], in which the valve is cultured, and a medium container [B]. They are connected to each other via two parallel tubing series [C], which run through a roller pump [D]. Part of the upper tubing consists of a thicker tube placed in a polycarbonate cylinder [E]. Compressed air can be released into this cylinder, via a magnet valve [F], to compress and decompress the tube, resulting in a pressure difference over the leaflets. A syringe [G], placed on the bioreactor, serves as a compliance chamber. The pressures upstream and downstream of the leaflets is monitored using pressure sensors [H].

apply a dynamic pressure difference over the leaflets, the silicone tube in the polycarbonate cylinder is compressed and decompressed by the air coming from the proportional magnet valve. The transvalvular pressure generated over the valve leaflets is controlled via a programmable multi-IO-card using LabView software (National Instruments, USA). The shape and frequency of the pressure wave is programmed for an optimal tranvalvular pressure. A syringe, connected to the lower part of the biore-

actor, filled with 10 ml of medium and 40 ml of air, serves as compliance chamber. Pressure sensors (BD, Belgium), connected to both the lower and upper part of the bioreactor, are used to measure the pressure upstream (at the ventricular side) and downstream (at the arterial side) of the leaflets. Using the same multi-IO-card and software, the dynamic pressure difference could be monitored and logged.

Sterility and biocompatibility

To prove maintained sterility over several weeks, the DPD was sterilized by ethylene oxide and filled with 75 ml of culture medium without the addition of gentamycin. The system was fully functional for a period of three weeks. During the testing period, a culture flask filled with culture medium, without gentamycin, served as control. The medium in the medium container was changed every 3-4 days as well as two thirds of the medium in the culture flask. The medium was checked microscopically for contamination and was stored at $-20^{\circ}\mathrm{C}$ until further use.

In order to ensure that the materials used in the DPD are suitable for cultivation of heart valves and that no toxic components are being released, a biocompatibility test was performed using the medium that was stored during the sterility test. Human saphenous vein cells were seeded into 24 wells plates (30.000 cells/well) and left overnight to attach and spread in an incubator in normal cell culture medium. The next day, the normal cell culture medium was replaced by the medium of the sterility test that had circulated through the DPD and the control medium (five wells per test group). The medium stored after the first four medium changes, as well as the control medium from the culture flask, was used. The cells were allowed to grow for three subsequent days, after which the metabolic activity of the cells, as a measure for viability, was determined using an MTT test (Mosmann, 1983). Briefly, MTT (Sigma, USA) in solution (5 mg/ml in PBS) was diluted in medium and added to the cells. After one hour of incubation at $37^{\circ}\mathrm{C}$ and 5% CO_2, metabolic active cells had converted the MTT salt into purple crystals located inside the cells. Isopropanol (VWR International, USA), containing 10% formic acid (Sigma, USA) was added to the cells to release and dissolve the purple crystals and the optical density of the solution was determined. The results are expressed as percentages with respect to the control, which was set at 100%.

5.2.3 Tissue culture and mechanical conditioning

Three experimental groups of valve leaflet tissues were engineered to compare tissue formation and to show the feasibility of the strain-based approach. The first group comprises rectangular shaped valve leaflet tissue-equivalents. They were cultured statically in a cell culture flask for up to four weeks and could compact freely, serving as non-loaded controls. The second and third group consisted of valve leaflets, all cultured in the DPD. Directly after placement in the DPD, all leaflets were exposed to medium circulation at low speed (4 ml/min) to supply fresh nutrients to the developing tissue. Valve leaflets were cultured up to four weeks, exposed to continuous medium circulation and prestrain due to compaction constrained by the stent (group

2). Additional valve leaflets were exposed to dynamic strains at 1 Hz in addition to the prestrain and continuous medium circulation (group 3). The pressures upstream and downstream of the valves, as well as the dynamic transvalvular pressure, were recorded every three hours. The valve leaflets of group 3 were cultured up to two, three and four weeks. The medium in the medium container, as well as two thirds of the medium in the culture flask, for group 1, was replaced every three to four days.

5.2.4 Evaluation of tissue formation

Qualitative tissue analysis

Tissue formation in all groups was analyzed by histology after four weeks. Representative pieces were fixed in phosphate-buffered formalin (Fluka, USA) and embedded in paraffin. Sections were cut at 5 μm thickness and studied by Haematoxylin and Eosin (H&E) staining for general tissue morphology and Trichrome Masson staining for collagen formation, as collagen is the main load-bearing component of the extracellular matrix.

Mechanical testing

The mechanical properties of circumferential strips of the engineered leaflet tissues of the three groups after four weeks of culturing were determined by uniaxial tensile tests. As thickness measurements of the fresh strips are practically difficult, the thickness was determined from representative histology sections. Stress-strain curves were obtained using an uniaxial tensile tester (Instron, Belgium, model 4411, equipped with a load cell of 10 N) with a constant strain rate of 1.7% per second. To get insight into the evolution of mechanical properties in time, the mechanical properties of circumferential strips of dynamically strained leaflets after two and three weeks of culturing were additionally determined.

5.2.5 Estimation of strains in the leaflets

To estimate the amount of prestrain in the leaflets, due to compaction constrained by the stent, the difference in size of the leaflets in the stent after culturing and after release from the stent was determined. The leaflets, having an initially bulged shape in the stent, did straighten during culturing due to free compaction of the neo-tissue. The initial circumferential size of the leaflets was 23 mm, whereas in fully straightened leaflets this size was reduced to 20 mm, indicating that the leaflets could compact freely up to 13% during culturing. The circumferential size of the leaflets was measured after release of the leaflets from the stent of the valves exposed to prestrain and medium circulation after four weeks. This measured size was divided by the maximally straightened size of 20 mm to calculate the amount of prestrain in the leaflets. This value was subsequently extracted from 1 and multiplied by 100% to obtain the amount expressed in a percentage of prestrain.

Finite element analyses were used to estimate the amount and distribution of the dynamic strains in the leaflets resulting from the applied dynamic transvalvular pressure. The finite element mesh of the stented valve geometry is shown in Fig. 5.3. The configuration shown in this figure was assumed to be stress-free and because of symmetry only 1/2 of a valve leaflet was used in the finite element analyses. At the symmetry edge, nodal displacements in the normal direction were suppressed, whereas at the fixed edge all nodal displacements were set to zero. At the free edge, a contact surface was defined to model coaptation of adjacent leaflets. The transvalvular pressure p_{tv} was subsequently applied to the downstream surface of the leaflets. In this study, the finite element package SEPRAN was used (Segal, 1984).

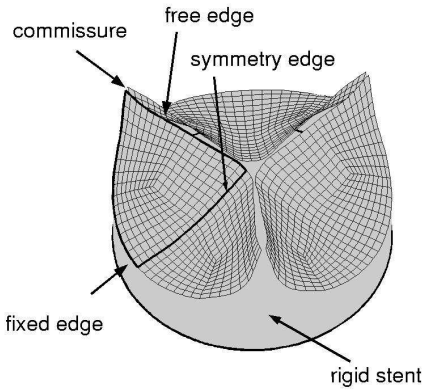


Figure 5.3: Finite element mesh of the stented valve geometry. Because of symmetry only 1/2 of a leaflet is used in the finite element analyses. This part of the geometry is discretized using 224 hexahedral elements.

The valve tissues were modeled as an incompressible generalized Neo-Hookean material:

$$\boldsymbol{\sigma} = -p\boldsymbol{I} + G(\boldsymbol{B} - \boldsymbol{I}) \tag{5.1}$$

with $\boldsymbol{\sigma}$ the Cauchy stress, p the hydrostatic pressure, $\boldsymbol{I}$ the unity tensor and G the shear modulus of the material. The left Cauchy-Green deformation tensor was defined as $\boldsymbol{B} = \boldsymbol{F} \cdot \boldsymbol{F}^T$ with $\boldsymbol{F}$ the deformation gradient tensor. To describe the (potential) non-linear behavior of the leaflet tissues, the following expression for G was used:

$$G = G_0 \left(\frac{\mathrm{I}_1(\boldsymbol{B})}{3} \right)^n \tag{5.2}$$

with G_0 and n material parameters and $\mathrm{I}_1(\boldsymbol{B}) = \mathrm{trace}(\boldsymbol{B})$ the first invariant of $\boldsymbol{B}$. The parameter of n is used to control the degree of non-linearity: n>0 results in strain

hardening, $n<0$ in strain stiffening and for $n=0$ the classical Neo-Hookean model is obtained. The material parameters were obtained by fitting the constitutive law (Eq. 5.1) to the mean results before failure of the uniaxial tensile tests of the leaflet tissues exposed to dynamic strains after two, three, and four weeks of culturing.

5.3 Results

5.3.1 The DPD

The medium, without addition of antibiotics, that had circulated in the DPD did not show any macroscopic or microscopic signs of contamination for the complete test period. The system was easy to handle and the risk of contamination during medium replacement was minimal. The results of the biocompatibility test, performed for the first four medium changes during the sterility test, are shown in Fig. 5.4. The metabolic activity of the cells cultured with the four tested media were all within 80 to 100% when compared to the controls, indicating the DPD to be biocompatible and suitable for cultivation of human heart valves.

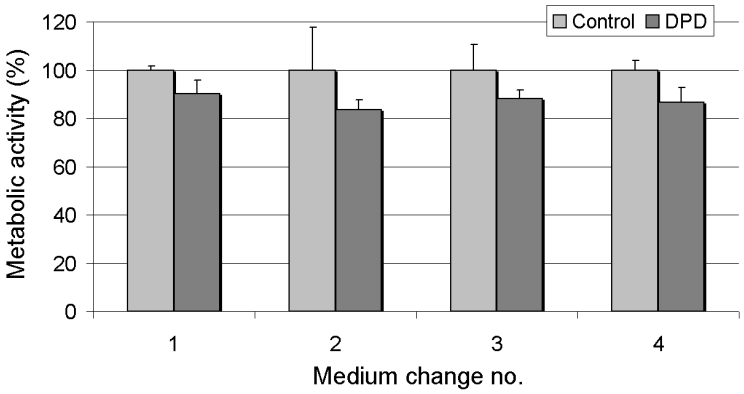


Figure 5.4: Metabolic activity of cells cultured with the first four changes of the medium that has circulated in the DPD during the sterility test. The tested medium (dark gray bars) was compared to control medium (light gray bars), which were set at 100%. The metabolic activity was in all cases 80-100% compared to the controls and, therefore, the DPD can be considered as being biocompatible. The error bars represent the standard error of the mean.

5.3.2 Dynamic straining protocol

A representative transvalvular pressure curve,measured during culturing is shown in Fig. 5.5. The permanent transvalvular pressure, present over the valve leaflets, is

referred to as the DC offset, which is the average value of the difference in pressure upstream and downstream of the valve. The cyclic transvalvular pressure is defined as the peak-to-peak transvalvular pressure value. The maximum transvalvular pressure value, including the DC offset and the cyclic transvalvular pressure, is referred to as the applied dynamic transvalvular pressure.

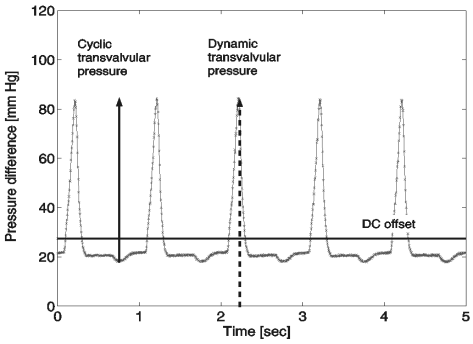


Figure 5.5: A representative transvalvular pressure curve measured during culturing of the valves. The definitions of the DC offset, the cyclic transvalvular pressure difference, and the dynamic transvalvular pressure difference are represented.

The dynamic transvalvular pressure was averaged for each day, resulting in dynamic transvalvular pressures increasing gradually from 0 to about 80 mm Hg within the first two weeks of culturing. The last two weeks of culturing, the pressures were lowered, due to the expected loss of the support function of the scaffold, and kept constant at about 37 mm Hg.

5.3.3 Evaluation of tissue formation

Histology

Sections stained with H&E and Trichrome Masson (Fig. 5.6) showed superior tissue formation in the leaflets (Fig. 5.6(a), 5.6(b), 5.6(d), 5.6(e)) when compared to the non-loaded rectangular leaflet tissue-equivalents (Fig. 5.6(c), 5.6(f)). The tissue of the leaflets cultured with additional dynamic strains (Fig. 5.6(a), 5.6(d)) appeared to be more homogeneous and denser when compared to the leaflets exposed to prestrain only (Fig. 5.6(b), 5.6(e)). Collagen could be identified in the leaflets, either cultured using prestrain or additional dynamic strains, after four weeks of culturing (Fig. 5.6(e), 5.6(d)).

Mechanical tests

Representative stress-strain curves for all groups after four weeks of culturing are shown in Fig. 5.7(a). The non-loaded leaflet equivalents showed linear behavior,

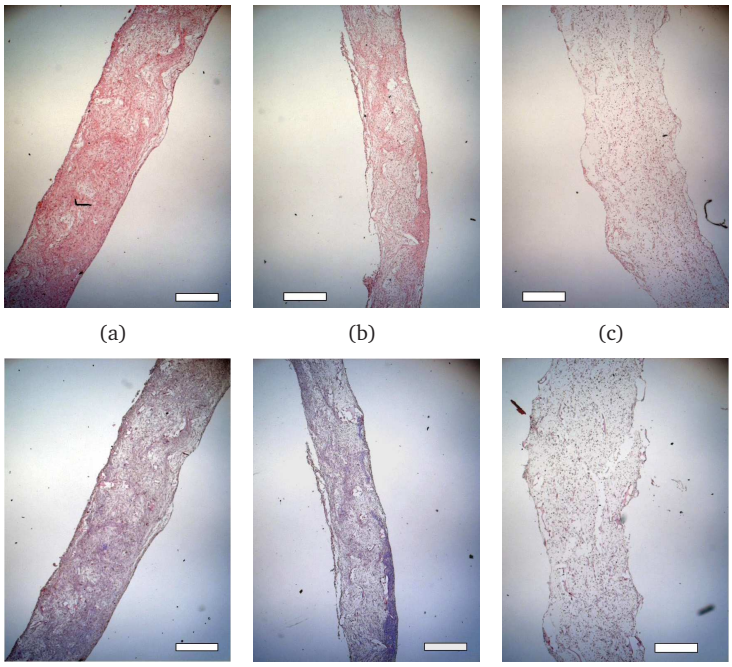
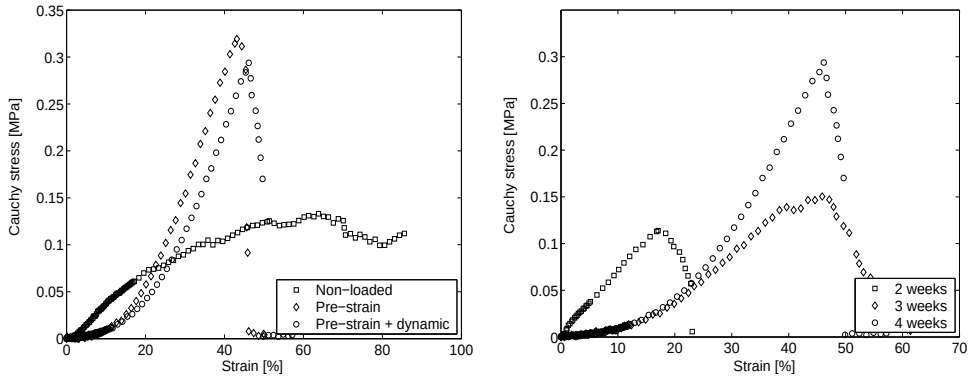


Figure 5.6: Representative sections of the valve leaflet tissues after four weeks of culturing stained with H&E (a,b,c) and Trichrome Masson (d,e,f). The bars in the images represent scales of 350 μm. The dynamically strained leaflets (a,d) and the leaflets exposed to prestrain only (b,e) showed superior tissue development when compared to the non-loaded valve leaflet tissue-equivalents (c,f). The dynamically strained leaflet tissue seemed more homogeneous and denser as compared to the leaflets exposed to prestrain only. Collagen, stained blue, could be identified in the leaflets (d,e).

while the leaflets, exposed to compaction-induced prestrain and either with or without additional dynamic strains, showed more tissue-like non-linear behavior. The evolution of mechanical properties with increasing culture time is shown in Fig. 5.7(b) for the leaflets exposed to dynamic strains. After two weeks of culturing, the mechanical behavior was linear, representing scaffold-like behavior. After three and four weeks, the tissue showed more non-linear mechanical behavior, representative for tissue contribution.



(a) (b)

Figure 5.7: Stress-strain curves after four weeks of culturing of non-loaded leaflet tissue-equivalents, leaflets exposed to prestrain, and leaflets exposed to additional dynamic strains (a) and the evolution of mechanical properties over time for dynamically strained leaflets (b). The non-loaded leaflet tissue-equivalents showed linear mechanical behavior, while the leaflet tissues showed tissue-like non-linear behavior after four weeks of culturing (a). Dynamic straining of the leaflet tissues during culturing resulted in increased non-linear tissue-like mechanical behavior with time (b).

5.3.4 Estimation of strains in the leaflets

Prestrain

The amount of prestrain in the leaflets of the valves exposed to prestrain only after four weeks of culturing, assuming maximal straightening of the leaflets, was shown to vary between 3 and 5%.

Dynamic strains

The constitutive law (Eq. 5.1) fitted the results of the uniaxial tensile tests quite reasonable. Table 5.1 summarizes the input parameters for the finite element model, the mean absolute error of the fits, and the estimated mean dynamic strains after two, three and four weeks of culturing, based on a transvalvular pressure difference of 37 mm Hg. The mean dynamic strains in the leaflets increased from 8% after two weeks to about 20% after four weeks of culturing. The dynamic strain distribution in the leaflets after four weeks of culturing is shown in Fig. 5.8, with in Fig. 5.8(a) and 5.8(b) the strain distribution within one leaflet at respectively the upstream and downstream surface and in Fig. 5.8(c) the estimated range of overall dynamic strains within a leaflet.

Culture time [weeks]	**Thickness [mm]**	G_0 **[kPa]**	n **[-]**	**Mean absolute error of fit [kPa]**	**Mean dynamic strain [%]**
2	0.80	188	0.0	0.95	8
3	0.59	51	8.6	3.93	24
4	0.63	59	9.2	4.61	20

Table 5.1: Summary of the input parameters for the finite element analyses of the dynamically strained leaflets after two, three, and four weeks of culturing. The parameter n has been set to zero for the leaflets after two weeks of culturing, due to the observed linear behavior. Furthermore, the mean absolute errors of the fits of the constitutive law (Eq. 5.1) to the results of the uniaxial tensile tests and the resulting estimated mean dynamic strains are represented.

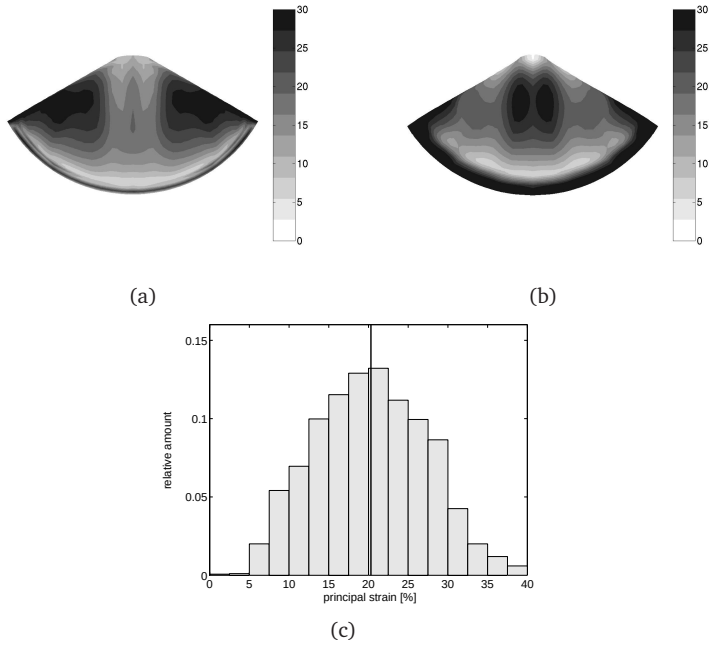


Figure 5.8: The dynamic strain distribution after four weeks of culturing at the upstream (a) and downstream (b) surfaces of the leaflets at an applied dynamic transvalvular pressure difference of 37 mm Hg. The grayscale represents the amount of dynamic strains in %. The relative amount of the overall dynamic strains found in the leaflet and the mean value are shown in the histogram (c).

5.4 Discussion

Mechanical stimulation of tissue formation is a well-known technique in tissue engineering of cardiovascular structures to improve tissue formation and organization. Various conditioning approaches are being employed in bioreactor systems, from flow-based to strain-based to even mimicking the exact physiological environment in the body. For tissue engineering of blood vessels, flow-based as well as strain-based approaches are used, however, for tissue engineering of heart valves, only flow-based approaches have been described. The optimal conditioning protocol depends on several factors, such as the sensitivity of the cell phenotype and source (i.e. animal or human) to mechanical cues, the scaffold used, the transfer of the mechanical cues from the scaffold to the cells, and the magnitude and type of mechanical cue.

In this study, we focused on tissue engineering of human heart valve leaflets engineered from human saphenous vein cells seeded using fibrin as a cell carrier onto PGA/P4HB trileaflet heart valve scaffolds (Fig. 5.1). For this particular cell type, being more sensitive to mechanical stimulation as compared to human arterial derived cells, we previously have shown a large impact of cyclic straining during culturing on tissue formation (Mol *et al.*, 2003). In this former study, the amount of extracellular matrix formation increased when larger strains were used. Flow is most likely not necessitated in the early phase of tissue development, but might start to play a significant role as soon as the leaflets are to be seeded with endothelial cells in a later phase. Flow might then stabilize the tissue via signaling by the endothelial cells and prepare the tissue for subsequent implantation. This study describes a strain-based approach to tissue engineer human heart valve leaflets.

In order to expose the developing heart valve leaflets to dynamic strains, a pressure difference had to be applied over the leaflets, mimicking the diastolic phase in the heart. A novel bioreactor, the Diastolic Pulse Duplicator (Fig. 5.2), is developed for this purpose, which can expose the developing tissue to increasing amounts of dynamic strains. Besides application of dynamic strains, the leaflets cultured in this study were exposed to prestrain, due to tissue compaction constrained by the stented geometry. The flow was kept low (4 ml/min) and served solely to provide the tissue with sufficient fresh nutrients and to remove waste products. The DPD was very easy to handle, sized small with a total medium volume of only 75 ml per valve, proven to be biocompatible (Fig. 5.4), and sterility could be maintained over prolonged periods of time.

Rectangular non-loaded valve leaflet tissue-equivalents were shown to render much less tissue formation after four weeks of culturing when compared to the superior tissue formation in the leaflets, exposed to either prestrain only as well as to additional dynamic strains (Fig. 5.6). The tissue of the leaflets exposed to dynamic strains appeared to be more homogeneous and denser packed as compared to leaflets exposed to prestrain only, however, this concerned only qualitative observations by histology. The mechanical properties (Fig. 5.7) of the leaflets exposed to dynamic strains showed increased non-linear tissue-like behavior over time, indicating increasing amounts of tissue and collagen. After four weeks of culturing, the non-loaded rectangular shaped leaflet tissue-equivalents showed linear behavior, representative

for scaffold behavior. The leaflets, exposed to prestrain alone as well as to additional dynamic strains showed non-linear behavior after four weeks of culturing, correlating with the larger amounts of tissue found in the leaflets as compared to the non-loaded leaflet tissue-equivalents. The continuous medium circulation in the DPD will most likely contribute to the improved tissue formation in the leaflets as well. This contribution will be elucidated in future studies as well as possibilities to monitor tissue development during culturing by the identification of biochemical markers in the circulating medium. Furthermore, the application of larger flows in the DPD, necessitated later in the process of tissue engineering of heart valves after endothelial cell seeding, has to be studied.

Prestrain alone obviously resulted in abundant amounts of tissue. The prestrain is estimated to be in the range of 3 to 5% and might already be sufficient for optimized tissue formation. However, dynamic straining might further enhance tissue organization and, furthermore, might represent a valuable tool to maintain the bulged shape of the leaflets by some deformation of the scaffold and the tissue. Furthermore, for future applications towards tissue engineering of stentless human heart valves, dynamic strains will be of larger importance as the prestrains in stentless valves will be much less. It is, therefore, of importance to correlate the amount and distribution of the induced strains to the tissue properties in future studies, which should be determined not only qualitative as in this study, but quantitative as well.

To get insight into the amount of dynamic strains applied to the leaflets, the dynamic strain distribution was estimated. The material properties of the leaflets determine the amount of dynamic strains at a given dynamic transvalvular pressure. The transvalvular pressure was monitored in the DPD during culturing (Fig. 5.5). Theoretically, the amount of dynamic strains can be monitored using markers on the leaflets and subsequent imaging of the leaflets being strained, followed by image analyses. As this is a time-consuming and in practice rather difficult method, we have chosen to estimate the dynamic strains in the leaflets by finite element analyses. The material parameters of the developing leaflets, determined by fitting the constitutive law (Eq. 5.1) to the mean results of the uniaxial tensile tests before failure, were used to serve as input for the finite element analyses. The mean dynamic strain in the valve leaflets cultured in this study, as an example for future use, were estimated to vary from 8% at two weeks to 20% at four weeks of culturing (Fig. 5.8 and Table 5.1). The estimated dynamic strains increased between week two and three, while the applied dynamic transvalvular pressures were similar, indicating loss of scaffold support, resulting in a decreased stiffness of the tissues, as shown by a lower shear modulus after three weeks of culturing. For the stented valve geometry, the amount of applied dynamic strains in this study might be too large for optimized tissue formation, when taking the prestrain into account, but application of large dynamic strains in the DPD was shown feasible. As currently the strains in the leaflets are determined afterwards, a future research focus for optimization of the Diastolic Pulse Duplicator is the development of a non-invasive method to determine tissue strains directly during culturing and to integrate this feature in a feedback loop to control the magnitude of the strains.

In the finite element analysis, an initial stress-free configuration of the leaflets is

assumed, which is not completely true as the valve leaflets show compaction during culturing. In future studies, this point will be addressed and the compaction behavior will be incorporated into the model. The material properties of the leaflets are assumed to be homogeneous and isotropic, which might not be the case after a certain culture period due to the influence of prestrain in mainly the circumferential direction and the local dynamic strain distribution in the leaflets. Therefore, leaflets to be cultured in the DPD in further studies will be mechanically tested in both circumferential and radial direction to identify possible anisotropic properties. In case of anisotropic properties, an extended model should be used for the finite element analyses as described by Driessen *et al.* (2004).

In conclusion, the strain-based approach to tissue engineer human heart valve leaflets has proven its feasibility using the Diastolic Pulse Duplicator, offering new possibilities towards tissue engineering of functional human aortic heart valve replacements.

5.5 Acknowledgements

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Chapter 6

Tissue-engineered human aortic heart valve leaflets

6.1 Introduction

Ten years ago, the first successful replacement of one of the pulmonary valve leaflets by a tissue engineered leaflet in a sheep model was reported (Shinoka *et al.*, 1995, 1996), opening an emerging field of research for heart surgeons, biologists, chemists, physicists, and biomedical engineers. From then on, rapid progress was made in the development of new scaffold materials, the search for cell sources, and methods of conditioning of the developing tissue, all with the common goal to improve tissue maturation and mechanical properties to engineer a complete functional, living heart valve replacement.

Mechanical conditioning has demonstrated to be a valuable tool to improve tissue properties when compared to static culture (Kim and Mooney, 2000; Seliktar *et al.*, 2001; Hoerstrup *et al.*, 2002a; Isenberg and Tranquillo, 2003; Mol *et al.*, 2003; Engelmayr *et al.*, 2005). Using an in-vitro pulsatile flow environment for culturing, trileaflet autologous heart valves were shown to function properly in sheep when implanted into the pulmonary position (Hoerstrup *et al.*, 2000a). However, up to now there are no reports of engineered heart valve leaflet tissues, based on fast degrading polymer scaffolds, capable of sustaining the large flows and pressures at the aortic site of the heart. The focus of this study is to increase the mechanical properties of engineered human heart valve leaflets via optimized mechanical conditioning in an attempt to fulfil this demanding challenge.

Up to now, tissue engineered heart valves were mainly cultured in flow-driven pulse duplicator bioreactors with limited tissue straining (Hoerstrup *et al.*, 2000a,b). Based on this principle, various bioreactors have been further developed, in which the physiological environment of a heart valve can be mimicked, including both flows and pressures (Dumont *et al.*, 2002; Hildebrand *et al.*, 2004; Rutten *et al.*, 2005). However, no functional heart valves were reported to be tissue engineered in those bioreactors yet.

It is questionable whether a physiological stimulus is the right conditioning strategy for engineering heart valves. The overall question, to what extent the tissue should be conditioned during culturing and what the optimal type, magnitude, and timing of the stimulus should be, has still not been answered. We have recently developed a novel approach to mechanically condition tissue engineered heart valve leaflets, focusing on the induction of strains within the tissues. This is based on the hypothesis that tissue strains mediate collagen formation and reorganization, thereby improving tissue strength (Mol *et al.*, 2003). To culture heart valve leaflets using this strain-based approach, the 'Diastolic Pulse Duplicator' (DPD) has been developed, a bioreactor, in which tissue strains are induced by applying a dynamic pressure difference over the valve leaflets. Flow is in this approach of less importance and serves solely to provide fresh nutrients to the developing tissue (perfusion). Culturing of engineered leaflets in the DPD with exposure to a dynamic pressure difference has resulted in improved and tissue-like mechanical properties. Interestingly, also perfusion in combination with prestrain induced by the stented geometry, has shown reasonable mechanical properties (chapter 5).

This study will elucidate in detail the individual influences of the culture condi-

tion, either static or perfused in the DPD, as well as the influence of prestrain, and additional dynamic straining on tissue development and mechanical properties. Tissue development was studied qualitatively as well as quantitatively and the mechanical properties were evaluated. The evolution of the leaflet properties over time (up to four weeks) was investigated and comparisons were made between the different groups of engineered leaflets after four weeks of culturing.

A non-woven PGA mesh coated with P4HB was used as scaffold, as previously established for the successful implantation of a tissue engineered valve in pulmonary position in sheep. Since an initial compact and non-porous structure is a prerequisite for direct loading without the risk of washing out cells and freshly formed tissue, fibrin was used as a cell carrier (Mol *et al.*, 2005). Stented human valve leaflets, cultured statically in a culture flask and in the DPD, exposed to solely perfusion or additional dynamic strains, were engineered, as well as strips, which could compact freely during culturing. The tissue properties of all leaflets obtained in the DPD showed a good quality and exceeded values reported in literature based on this type of scaffold. Therefore, some of the valve leaflets were tested for functionality under exposure to flow conditions mimicking the in-vivo aortic valve environment up to 24 hours. This study is, to our knowledge, the first to describe human tissue engineered valve leaflets, based on a fast degrading synthetic polymer, with sufficient tissue and mechanical properties to sustain the conditions at the aortic site in the body.

6.2 Materials and methods

6.2.1 Scaffold

Trileaflet stented heart valve scaffolds were prepared as described in section 5.2.1. In short, the stents were prepared from Fastacryl® (Vertex-dental, the Netherlands). Anatomically shaped leaflets were cut out of PGA sheets (thickness 1.0 mm; specific gravity 69 mg/cm^3; Cellon, Luxembourg) and coated with a thin layer of P4HB (provided by Symetis Inc., Switzerland) as described before (Hoerstrup *et al.*, 2000a). The leaflets were directly positioned onto a teflon mold to render the specific bulged valve leaflet shape. The stent was placed on top of the leaflets and by subsequent solvent evaporation, the leaflets were fixed to the stent. The scaffolds were dried under vacuum overnight and sterilized by soaking in 70% ethanol for 3-4 hours. The ethanol was allowed to evaporate overnight, after which the scaffolds were washed in phosphate-buffered saline (PBS). Besides valve-shaped scaffolds, rectangular strips (2 X 1 cm) were prepared in the same manner without a stent.

6.2.2 Seeding procedure

Cells, harvested from the human vena saphena magna, were used and expanded using regular cell culture methods (Schnell *et al.*, 2001). The medium to culture these cells consisted of DMEM Advanced (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS; Biochrom, Germany), 1% GlutaMax (Gibco, USA) and 0.1% gentamycin

(Biochrom, Germany). The medium used for seeding and subsequent tissue culture contained 0.3% gentamycin and additional L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma, USA) to promote extracellular matrix production. The scaffolds were placed in medium overnight before seeding to facilitate cell attachment by deposition of proteins. The seeding was performed per leaflet using fibrin as a cell carrier (Mol *et al.*, 2005). Briefly, the cells were suspended in a sterile thrombin solution (10 IU/ml medium; Sigma, USA) in a volume that equals half the void volume of the scaffold (0.5 X length X width X thickness). The cells in thrombin were mixed with an equal amount of sterile fibrinogen solution (10 mg actual protein/ml medium; Sigma, USA) and dripped onto one side of the scaffold. The fibrin solution was taken up by the scaffold and remained inside due to polymerization of the fibrin gel. The leaflets were seeded with a density of 4-5 million cells (passage 7) per cm^2 of scaffold. The seeded valve scaffolds were allowed to polymerize for 20 minutes in an incubator ($37^{\circ}C$ and 5% CO_2) before placement into either the Diastolic Pulse Duplicator or culture flasks. Rectangular strips were seeded accordingly and were placed in culture flasks.

6.2.3 Tissue culture and mechanical conditioning

The engineered tissues were divided into four groups (Table 6.1): 1) dynamically strained leaflets, 2) perfused leaflets, 3) static valve leaflets, and 4) strips. In the stented leaflet tissues (group 1, 2, and 3), prestrain developed as a result of compaction constrained by the stent. The leaflet tissues were cultured under continuous perfusion in the Diastolic Pulse Duplicator (DPD) (group 1 and 2) or statically in culture flasks (group 3 and 4). Furthermore, some of the valve leaflets were exposed to additional dynamic strains at a frequency of 1 Hz in the DPD (group 1). The strips (group 4) could freely compact during culturing, serving as unloaded controls.

Group no	Description	Culture conditions	Mechanical conditioning	Culture time	Number of leaflets (n)
1	dynamically strained leaflets	perfused in DPD	prestrain and dynamic strains	2, 3 and 4 wks	3 (2 + 3 wks) 6 (4 wks)
2	perfused leaflets	perfused in DPD	prestrain	4 wks	12
3	static valve leaflets	static in flask	prestrain	4 wks	6
4	strips	static in flask	none	4 wks	6

Table 6.1: Overview of the experimental groups in this study. The influence of prestrain, induced by compaction constrained by the stent, as well as continuous perfusion and additional dynamic strains could be elucidated using this experimental setup. An n of 1 comprises a single engineered leaflet tissue.

The DPD has been extensively described in chapter 5 and was sterilized by autoclaving. In short, the DPD consists of a bioreactor, in which the valve leaflets are placed after seeding, and a medium container. The leaflets are continuously perfused via a roller pump circuit at 4 ml/min. Additionally, dynamic strains can be applied to the leaflets by applying a pressure difference over the leaflets, controlled via a compressed-air system, compressing and decompressing a cylinder placed in the perfusion circuit, at a frequency of 1 Hz. The medium in the medium container (about two thirds of the total amount of medium) was replaced every 3-4 days (group 1 and 2). Two thirds of the medium of the statically cultured tissues (group 3 and 4) in the culture flasks was also replaced every 3-4 days. For the strained leaflets (group 1), the pressure difference over the leaflets was increased to 15 mm Hg after one week of culturing and further increased to 30 mm Hg after two weeks of culturing. In the last two weeks of culturing the pressure difference was 25 mm Hg.

6.2.4 Tissue compaction, prestrain, and dynamic strains

Shrinkage of the tissue due to contractile forces developing within the tissue, referred to as tissue compaction, influences the ultimate geometry of the leaflet tissues. Total tissue compaction after release from the stent was calculated and expressed as percentage of initial volume for the leaflets of group 1 after two, three, and four weeks of culturing to get insight into changes in compaction over time and of all groups after four weeks of culturing to elucidate differences between the groups. The leaflets in the stent, having an initially bulged shape, could compact freely until the leaflets were completely flattened. The circumferential size of the leaflet after release from the stent was extracted from the maximally flattened size in the stent. This value was subsequently extracted from 1 and multiplied by 100 to obtain the amount of prestrain expressed as a percentage. The range of dynamic strains in the leaflets, induced by the dynamic pressure difference, was estimated by computational analyses, as described in chapter 5, modeling the valve leaflet tissue as an incompressible generalized Neo-Hookean material. As input for this model, the mechanical properties of the valve leaflet tissues were necessitated as described in section 6.2.7. The range of dynamic strains in the engineered leaflets was estimated after two, three, and four weeks of culturing and expressed in percentages.

6.2.5 Qualitative evaluation of tissue formation

Before analysis, the engineered tissues were photographed for macroscopic appearance. Tissue formation and organization was studied by histology after two, three, and four weeks for leaflets of group 1 to gain insight into tissue formation over time and after four weeks for comparisons. Furthermore, cell orientation and viability were studied for leaflets of group 1 and 2 after four weeks of culturing.

Histology

Representative samples of the tissues were fixed in phosphate-buffered formalin (Fluka, USA) and embedded in paraffin. Sections were cut at 5 μm thickness and studied by Hematoxylin and Eosin staining (H&E) for general tissue morphology, Trichrome Masson for the appearance of collagen, and Verhoeff - van Gieson for the appearance of elastin. The stained sections were analyzed by regular light microscopy.

Cell orientation and viability

Leaflets of group 1 and 2 after four weeks of culturing were stained with the viable CellTracker Green (CTG) and Propidium Iodide (PI) (Molecular Probes), directly after withdrawal from the DPD, and visualized using confocal laser scanning microscopy (CLSM, Zeiss 510, Germany) as described by Breuls *et al.* (2003). CTG stains living cells green and the nuclei of dead cells red. Cell orientation could be visualized throughout the leaflet. The ratio of living cells to dead cells in a representative tissue volume was used as a measure for viability.

6.2.6 Quantitative evaluation of tissue formation

Tissue formation was analyzed quantitatively by biochemical assays for DNA - as an indicator for cell number -, glycosaminoglycans (GAGs), and hydroxyproline - as an indicator for collagen content. Similar to the qualitative tissue analyses, tissue formation in the leaflets of group 1 was quantified after two, three, and four weeks. The leaflets from the other groups were evaluated after four weeks of culturing. The amount of DNA, GAGs, and hydroxyproline was expressed as μg per mg dry tissue. To gain insight into the amount of GAGs and hydroxyproline produced per μg DNA - as an indicator for extracellular matrix synthesis activity by the cells - the GAG and hydroxyproline content per mg were divided by the amount of DNA per mg. Besides the biochemical assays, the amount of collagen cross-links in leaflets from group 2 and 4 after four weeks of culturing were analyzed and expressed in pmol per mg dry tissue. The amount of collagen cross-links correlated to the amount of hydroxyproline represents a quantitative measure of maturity of the collagen network.

DNA/GAG assay

To determine the amount of DNA and GAGs in the tissue samples (about 2 mg dry weight), the samples were digested in papain solution (100 mM phosphate buffer, 5 mM L-cystein, 5 mM EDTA and 125-140 μg papain per ml) overnight at 60°C. The samples were centrifuged after digestion and part of the supernatant was used for the DNA assay and part for the GAG assay. The amount of DNA in the engineered leaflets was determined using the Hoechst dye method (Cesarone *et al.*, 1979). In short, the samples were diluted in TE buffer (10 mM Tris, 1 mM EDTA, pH 7.4) and 100 μl was pipetted into a black 96-wells plate (Corning, USA) in duplo. A similar amount of working solution containing the Hoechst dye (10 mM Tris, 1 mM EDTA, 2 M NaCl and 2.5 μg Hoechst dye per ml) was added to each well. The plate was incubated

at room temperature for 10 minutes, protected from light, to allow binding of the Hoechst dye to the DNA. Subsequently, the fluorescence was measured (excitation: 355 nm, emission 460 nm). The amount of DNA in the samples was determined from a standard curve prepared from calf thymus DNA (Sigma, USA) in addition to the samples. The GAG content was determined using a modification of the assay described by Farndale *et al.* (1986). Briefly, 40 μl of each sample was pipetted in duplo into a flat bottom 96 wells plate. 150 μl of DMMB color reagens (46 μM dimethylmethylene blue, 40.5 mM glycin, 40.5 mM NaCl, pH 3.0) was added to each well and the plate was gently shaken. The absorbencies at 540 and 595 nm were read within 5 to 10 minutes before precipitation and extracted from one another. The amount of GAG in the samples was determined from a standard curve prepared from chrondroitin sulfate from shark cartilage (Sigma, USA) in addition to the samples.

Hydroxyproline assay

For the hydroxyproline assay, a modified version of the protocol provided by Huszar *et al.* (1980) was followed. In short, the tissue samples (about 2 mg dry weight) were hydrolyzed in 4 M NaOH (Fluka, USA) for 10 minutes at $120^{\circ}\mathrm{C}$. The hydrolyzed samples were subsequently neutralized by addition of a similar amount of 1.4 M citric acid (Fluka, USA) and centrifuged. Chloramin-T (62 mM) was added to the supernatant and the samples were allowed to oxidize for 25 minutes at room temperature. For color development, aldehyde/perchloric acid solution (1 M) was added and the samples were incubated for 15 minutes at $65^{\circ}\mathrm{C}$. The absorbency was read at 550 nm. The amount of hydroxyproline in the samples was determined from a standard curve prepared from trans-4-hydroxyproline (Sigma, USA) in addition to the samples.

Cross-link analysis

The quantification of collagen cross-links was used as a measure for tissue maturation. The amount of hydroxylysylpyridinoline (HP) and lysylpridinoline (LP) collagen cross-links were quantified as described by Bank *et al.* (1997), using high-performance liquid chromatography (HPLC). Tissue samples (about 3 mg dry weight) of group 2 (four randomly taken samples) and 4 (three randomly taken samples) were hydrolyzed in 6 M HCl at $110^{\circ}\mathrm{C}$ for 20 hours. After drying, the samples were subsequently dissolved in water containing pyridoxine, serving as internal standard, and further diluted in HFBA in acetonitile. The samples were injected into the HPLC system and the cross-links as well as the internal standard were eluted at room temperature at a flow rate of 1 ml/min. The fluorescence was subsequently measured.

6.2.7 Evaluation of mechanical properties

The mechanical properties of the engineered tissues were determined by uniaxial tensile tests in both circumferential and radial direction to elucidate anisotropic properties. The thickness of the leaflets was determined from representative histology

sections. Stress-strain curves were obtained using an uniaxial tensile tester (custom-built, equipped with a load cell of 20 N) and a constant strain rate of 1.7% per second. From the stress-strain curves, the ultimate tensile strength (UTS) was determined, as well as the elongation at break as a percentage of the initial length. The modulus was calculated, defined as the slope of the linear portion of the curve (in most cases above 25% strain). After studying anisotropic behavior, the mechanical test data was averaged per leaflet and subsequently averaged per group. To get insight into the evolution of mechanical properties with time, the mechanical properties of the leaflets of group 1 were determined after two, three, and four weeks of culture. Furthermore, the mechanical properties of the leaflets of all groups were determined after four weeks of culturing in order to elucidate differences between the tested groups. Correlations between the mechanical properties and the amount of DNA, GAGs, and hydroxyproline in the leaflet tissues were elucidated.

6.2.8 Functionality test

The opening and closing behavior of leaflets of group 2 was visualized in a custom designed 'valve exerciser' (Rutten *et al.*, 2005) in PBS at flow values mimicking the physiological aortic valve environment for 24 hours. The leaflets from this group were chosen for their optimal macroscopic appearance, as described in section 6.3.1. Images were obtained with a high-speed video camera (Kodak HS4540, USA). The flow, aortic pressure, left ventricular pressure, and transvalvular pressure were monitored using flow (Transonic, USA) and pressure sensors (Radi Medical Systems, Sweden). Data acquisition was performed using LabView software (National Instruments, USA).

6.2.9 Statistics

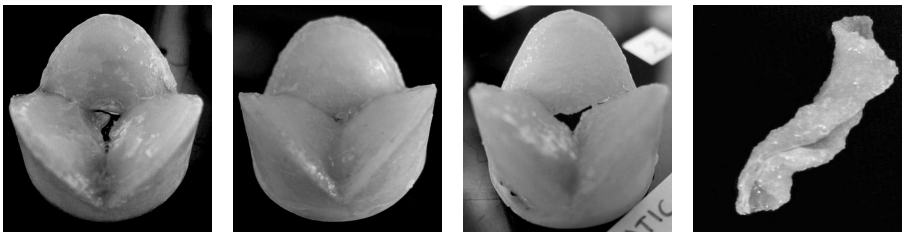
All quantitative data was averaged per leaflet (2-3 samples per leaflet) and subsequently averaged per group and represented as the average value of each group $\pm$ the standard error of the mean, unless otherwise stated. Comparisons between groups were performed by one-way ANOVA using Bonferroni post-hoc tests (STATGRAPHICS Plus 5.1, Statistical Graphics Corp., USA) to determine significant differences ($p<0.05$). For the uniaxial tensile tests, student t-tests were used to elucidate differences between the properties in radial and circumferential direction within each group. The correlation between the mechanical properties and the amount of hydroxyproline, GAG, and DNA was studied by linear regression analysis.

6.3 Results

6.3.1 Macroscopic appearance

All leaflet tissues were intact after four weeks of culturing and showed dense tissue formation, depicted in Fig. 6.1. In case of the stented leaflets, the individual leaflets were (partly) grown together from the commissures towards the center of the valve.

The leaflets of group 2 grew together over their full length (Fig. 6.1(b)), while in the leaflets of group 1 (Fig. 6.1(a)) and 3 (Fig. 6.1(c)) the tips were free for compaction, whereby they retracted some. All leaflets kept their shapes, although they lost their initially bulged shape and became flattened in the stent. The tissues of group 4, which could compact freely during culturing lost their shape completely (Fig. 6.1(d)).



(a) (b) (c) (d)

Figure 6.1: Appearance of the engineered leaflet tissues after four weeks of culturing: the strained leaflets (a), the perfused leaflets (b), the static valve leaflets (c) and one of the strips (d). The leaflets were intact and kept their shape, while the strips deformed completely. Dense tissue formation was observed.

6.3.2 Tissue compaction, prestrain, and dynamic strains

The leaflets of group 1 showed a significant decrease in volume after three (24±3% of its initial volume) and four weeks (26±1% of its initial volume), compared to two weeks of culturing (47±2% of its initial volume, $p<0.001$) as shown in Fig. 6.2. When tissue compaction and the resulting tissue volume for all groups was compared after four weeks of culturing (not shown), it was evident that the decrease in volume of the leaflets of group 1 was most pronounced (26±2% of its initial volume) when compared to leaflets of group 2 (37±1% of its initial volume, $p<0.001$), group 3 (33±0.4 of its initial volume, $p<0.05$), and group 4 (35±2% of its initial volume, $p<0.01$). The amount of prestrain in the leaflets did not change over time and was not different between the tested groups (not shown), being 4±1%. The dynamic strains (average ± standard deviation) applied in the DPD, based on the mechanical properties of the leaflets of group 1 after two, three, and four weeks of culturing (see section 6.3.5) and the applied pressure difference over the leaflets, was 2±1% after two weeks, 6±3% after three weeks and 4±2% after four weeks of culturing.

6.3.3 Qualitative evaluation of tissue formation

The CTG/PI stainings and subsequent visualization by CLSM showed a high viability for both leaflets from group 1 and 2 after four weeks of culturing (not shown). In the leaflet from group 2, the cells were oriented in several directions at various locations,

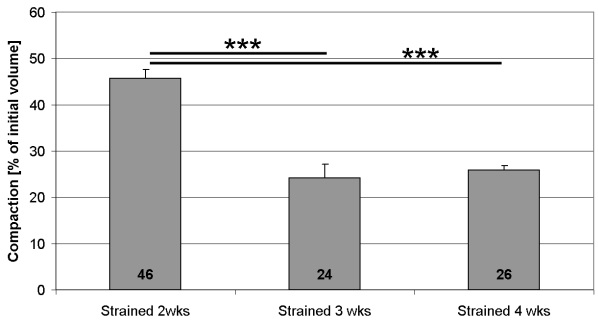


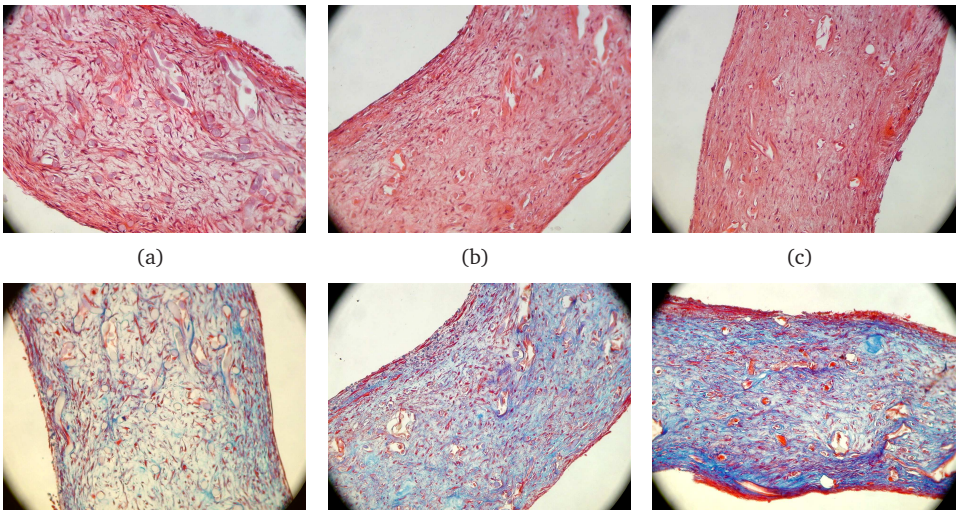
Figure 6.2: Tissue compaction, expressed as percentage of initial volume, resulted in a significant decrease in volume of the dynamically strained leaflets between week two and three. After three weeks there was no additional decrease in volume ($\star\star\star$ represents $p<0.001$).

with no overall orientation identifiable. In the leaflet from group 1, the orientation also differed at various locations, however, in general the cells along the midline of the leaflets, from the fixed edge towards the nodulus, were oriented radially and further inwards the leaflet they oriented towards the commissure points.

Histology sections show the morphology of the leaflets of group 1 after two (Fig. 6.3(a), 6.3(d)), three (Fig. 6.3(b), 6.3(e)), and four (Fig. 6.3(c), 6.3(f)) weeks of culturing. H&E stainings (Fig. 6.3(a), 6.3(b), 6.3(c)) show homogeneous tissue throughout the full thickness of the leaflets. The amount of tissue seemed to increase mainly between week two and three. Trichrome Masson stainings (Fig. 6.3(d), 6.3(e), 6.3(f)) show that a large amount of the formed tissue consisted of collagen (stained blue). Fig. 6.4 shows H&E stainings of leaflets from group 2 (Fig. 6.4(a)), group 3 (Fig. 6.4(b)), and group 4 (Fig. 6.4(c)) after four weeks of culturing. The tissue in those three groups, in particular in group 3 and 4, seemed less homogeneously distributed throughout the leaflet. No elastin was detected in the Verhoeff - van Gieson stainings in any of the groups (not shown).

6.3.4 Quantitative evaluation of tissue formation

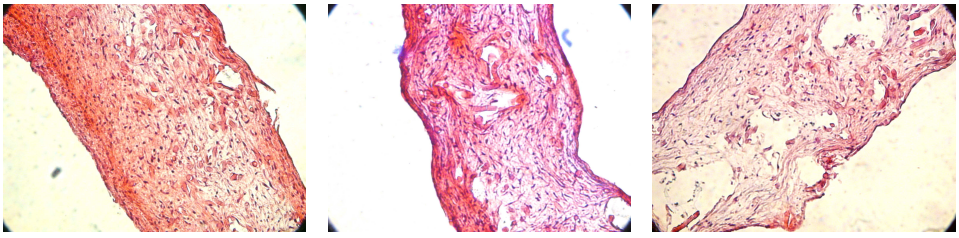
An overview of the amount of DNA, GAG, and hydroxyproline in the four groups after four weeks of culturing is shown in Fig. 6.5. The amount of DNA in the leaflets of group 1 did not change with increasing culture time (not shown). After four weeks of culturing, group 3 and 4 contained similar amounts of DNA (2.28 ± 0.13 μg/mg and 2.59 ± 0.09, respectively), more when compared to group 1 (1.63 ± 0.16 μg/mg, $p<0.001$ and $p<0.05$ respectively) and group 2 (1.77 ± 0.11 μg/mg, $p<0.001$ and $p<0.05$ respectively). The amount of GAGs in the leaflets of group 1 increased between two and three weeks (from 23.7 ± 0.8 to 32.1 ± 1.0 μg/mg, $p<0.01$) and was stable thereafter (not shown). Group 1 obviously contained the largest amount of



(a) (b) (c)

(d) (e) (f)

Figure 6.3: Tissue morphology of the strained leaflets after two (a,d), three (b,e), and four (c,f) weeks of culturing. H&E staining (a,b,c) shows general tissue morphology, which was homogeneous throughout the full thickness of the leaflets. Trichrome Masson (c,d,e) stained collagen blue and demonstrated a homogeneous distribution of collagen. Tissue formation seemed to increase between week two and three of culturing. All images were taken at a 200-fold magnification.



(a) (b) (c)

Figure 6.4: Tissue morphology after four weeks, stained by H&E, for the perfused leaflets (a), the static valve leaflets (b), and the strips (c). The tissue development in the latter two groups seemed less homogeneous when compared to the strained leaflets (Fig. 6.3(c)). All images were taken at a 200-fold magnification. The vacuoles represent cutting artifacts due to polymer remnants.

GAGs (33.1±1.1 μg/mg, $p<0.001$ for all groups) after four weeks of culturing, followed by group 2 (23.0±1.0 μg/mg, $p<0.001$ compared to group 3 and $p<0.01$ compared to group 4). Group 3 and 4 contained similar amounts of GAGs (16.3±1.0 μg/mg and 17.4±0.5 μg/mg respectively). The amounts of hydroxyproline in the leaflets of group 1 increased between two and three weeks (from 10.7±0.5 to 16.7±0.6 μg/mg, $p<0.05$) and was stable thereafter (not shown). The amount of hydroxyproline in group 1 (14.5±1.1 μg/mg), group 3 (12.1±0.7 μg/mg), and group 4 (13.8±0.6 μg/mg) were similar. The hydroxyproline content was largest in group 2 (18.8±0.8 μg/mg, $p<0.01$ versus group 1 and $p<0.001$ versus group 3 and 4).

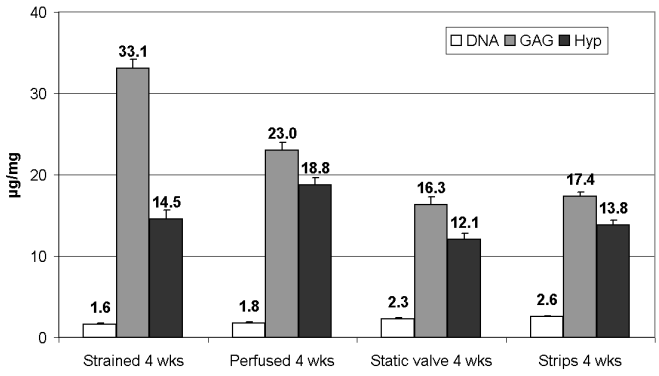


Figure 6.5: The amount of DNA, GAGs, and hydroxyproline (Hyp) in the leaflets after four weeks of culturing. The amount of DNA in the strained and perfused leaflets was similar and less when compared to both the static valve leaflets and the strips. The amount of GAGs was largest in the strained leaflets, followed by the perfused leaflets. The static valve leaflets and the strips contained the least amounts of GAGs. Hydroxyproline content was largest in the perfused leaflets. The strained leaflets, the static valve leaflets, and the strips all contained similar amounts of hydroxyproline. The significance levels are not shown, see text for details.

To detect any changes in extracellular matrix synthesis activity of the cells, the amount of GAGs and hydroxyproline produced per μg DNA were determined for all groups after four weeks of culturing, shown in Fig. 6.6. With increasing culture time, the amount of GAG and hydroxyproline produced per μg DNA was similar (not shown). The synthesis activity for the production of hydroxyproline was similar for leaflets of group 1 (9.7±1.0 μg/μg) and group 2 (11.2±0.9 μg/μg), both larger when compared to group 3 (5.4±0.4 μg/μg, $p<0.05$ and $p<0.001$ respectively) and group 4 (5.4±0.3 μg/μg, $p<0.05$ and $p<0.001$ respectively). The synthesis activity for the production of GAGs was largest for leaflets of group 1 (21.2±2.3 μg/μg), followed by group 2 (13.7±1.24 μg/μg, $p<0.01$). The amount of GAGs produced per μg DNA in group 3 (7.2±0.4 μg/μg) and group 4 (6.7±0.2 μg/μg) was similar, but significantly

lower when compared to group 1 ($p<0.001$) and 2 ($p<0.01$).

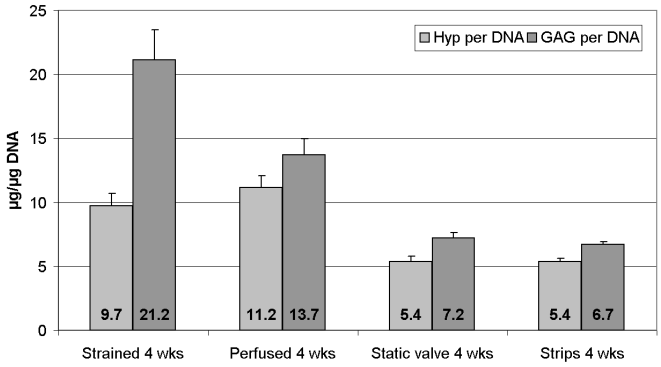


Figure 6.6: The amount of hydroxyproline (Hyp) and GAGs produced per μg DNA in the four groups after four weeks of culturing. The synthesis acitivity for the production of hydroxyproline was similar in the strained and perfused leaflets, but larger when compared to both the static valve leaflets and the strips. The amount of GAGs produced per μg DNA was largest in the strained leaflets, followed by the perfused leaflets. The static valve leaflets and the strips had the lowest synthesis acitivity for the production of GAGs. The significance levels are not shown, see text for details.

The collagen cross-link analysis (not shown) revealed more HP (hydroxylysylpyridinoline) cross-links in leaflets from group 2 (51.6$\pm$1.6 pmol/mg) when compared to group 4 (36.9$\pm$2.7 pmol/mg, $p<0.05$). The amount of LP (lysylpridinoline) cross-links was not significantly different between both groups (1.70$\pm$0.15 pmol/mg for group 2 and 1.58$\pm$0.06 for group 4). The ratio of HP versus LP cross-links was also not different (33$\pm$5 and 25$\pm$1 respectively). The amount of cross-links per μg hydroxyproline was determined as the ratio of the mean hydroxyproline content and the mean amount of HP cross-links in both groups and was 2.75 and 2.89 pmol HP/μg hydroxyproline respectively.

6.3.5 Evaluation of mechanical properties

To detect anisotropy in the leaflets, tensile tests were performed in both circumferential and radial direction and the properties in both directions were compared for each group. In group 2, 3, and 4 after four weeks of culturing, no anisotropy could be identified. In group 1 some anisotropy was observed at various time points as shown in Fig. 6.7. The UTS in circumferential direction was significantly larger when compared to the radial direction after two weeks of culturing (1.26$\pm$0.15 MPa versus 0.77$\pm$0.08 MPa respectively, $p<0.05$). After three weeks of culturing, the properties in both directions were similar and after four weeks, the modulus in circumferential

direction was significantly larger when compared to the radial direction (7.38±0.37 MPa versus 4.80±0.47 MPa respectively, $p<0.01$).

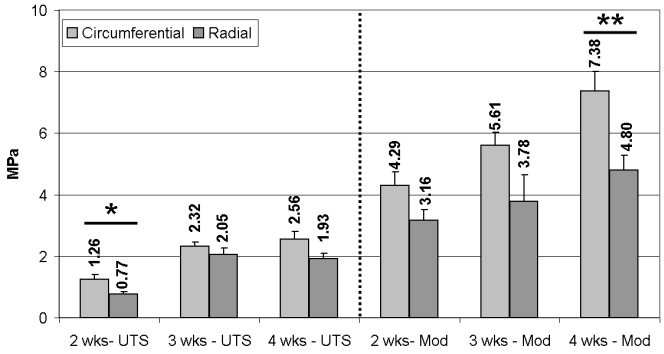


Figure 6.7: The UTS and modulus for both circumferential and radial direction for the strained leaflets after two, three, and four weeks of culturing. The UTS was significantly larger in circumferential direction after two weeks of culturing and the modulus was significantly larger in circumferential direction after four weeks of culturing ($\star$ represents $p<0.05$, $\star\star$ represents $p<0.01$).

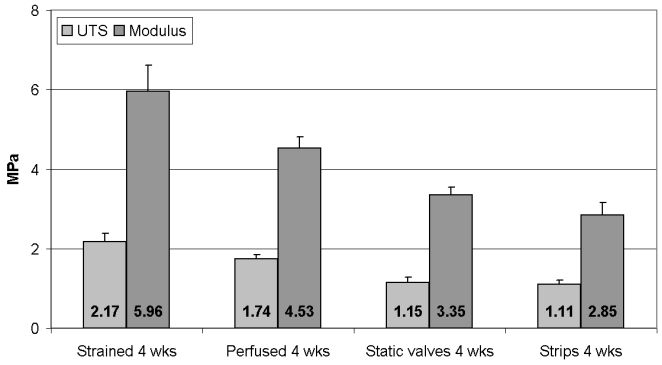


Figure 6.8: The UTS and modulus for all groups after four weeks of culturing. The strained and the perfused leaflets were stronger when compared to the static valve leaflets and the strips. The strained leaflets were stiffer when compared to the static valve leaflets and the strips and the perfused leaflets were stiffer compared to the strips. The significance levels are not shown, see text for details.

The averaged mechanical properties of the tissues of all groups (averaged per leaflet and subsequently averaged per group) after four weeks of culturing are shown in Fig. 6.8. The UTS in group 1 increased between week two and three (from 1.26±0.15 to 2.32±0.14 MPa, $p<0.05$) and was stable thereafter, while the modulus did not change significantly over time (not shown). After four weeks of culturing, no significant differences were found between group 1 and 2, as well as between group 3 and 4. The UTS in group 1 (2.17±0.22 MPa) as well as group 2 (1.74±0.11 MPa) was larger when compared to group 3 (1.15±0.13 MPa, $p<0.001$ and $p<0.05$ respectively) and 4 (1.11±0.10 MPa, $p<0.001$ and $p<0.05$ respectively). The leaflets of group 1 showed an increased modulus (5.96±0.64 MPa) when compared to group 3 (3.35±0.20 MPa, $p<0.001$). Both group 1 and 2 (4.53±0.19 MPa) showed an increased modulus when compared to group 4 (2.85±0.30 MPa, $p<0.001$ and $p<0.01$ respectively). For the elongation at break, no differences could be elucidated between the groups (53±3% in group 1, 54±4% in group 2, 45±6% in group 3, and 57±4% in group 4). Fig. 6.9 shows representative stress-strain curves for all groups after four weeks of culturing, demonstrating typical non-linear tissue-like behavior in all groups.

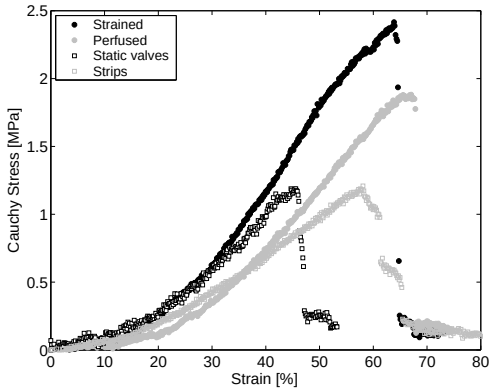


Figure 6.9: Representative stress-strain curves for all four groups after four weeks of culturing. The leaflet tissues of all groups show typical non-linear mechanical behavior, representative for tissue-like behavior.

The correlation between the UTS or modulus and the amounts of hydroxyproline, GAGs, and DNA in the tissues was studied (not shown). Positive correlations were found for the amount of hydroxyproline and the modulus ($R^2=0.11$, $p<0.05$) and the UTS ($R^2=0.28$, $p<0.01$), as well as for the amount of GAGs and the modulus ($R^2=0.57$, $p<0.001$) and the UTS ($R^2=0.54$, $p<0.001$). The amount of DNA was negatively correlated with both the modulus ($R^2=0.23$, $p<0.01$) and the UTS ($R^2=0.14$, $p<0.05$).

6.3.6 Functionality test

For the functionality test of the leaflets of group 2, the free edges of the leaflets had to be cut from each other as they were grown together. The leaflets tended to retract some after cutting before placement into the 'valve exerciser'. Images taken during one opening and closing cycle are shown in Fig. 6.10 and the applied flow and resulting pressure curve in Fig. 6.11.

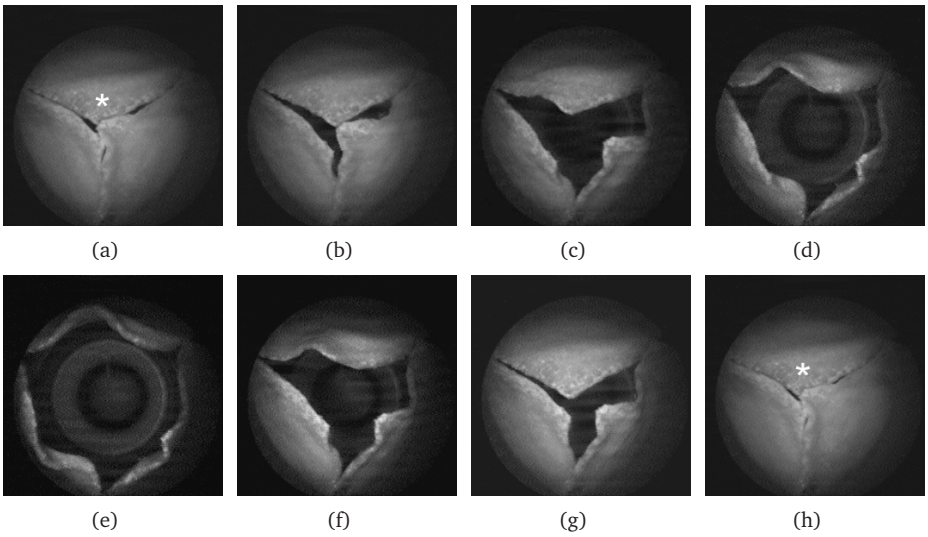
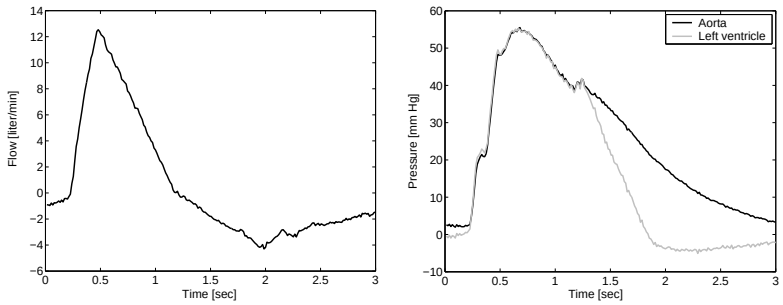


Figure 6.10: Images obtained during testing of the perfused leaflets at physiological adult flow conditions during one opening and closing cycle. One of the tips of the leaflets showed some prolapse when the valve was closed ($\star$).

The coaptation of the leaflets was minimal and after some time in the 'valve exerciser' at physiological adult aortic valve flow values, the tips of the leaflets tempted to prolapse. The leaflets were, however, able to sustain the aortic valve in-vivo flow environment for the 24 hours tested. Leakage was observed in the flow and pressure curve due to minimal coaptation and the subsequent prolapse of one of the tips of the leaflets. After 24 hours of exposure to physiological conditions mimicking the in-vivo aortic valve environment, the leaflets were all intact and no large deformations were seen. Subsequent tissue analysis showed no clear differences with non-tested leaflets from the same group.

6.4 Discussion

Ten years of intensive research in tissue engineering of heart valves has resulted in many improvements in tissue development and mechanical properties, mainly by me-



(a) (b)

Figure 6.11: Applied flow (a) and resulting pressure curves (b) during testing of the perfused leaflets at physiological flow conditions. The represented flow is scaled to mimic physiological flows. Backflow could be observed by the drop of the flow below zero. The pressure curve shows the pressures measured at the aortic and the left ventricular site of the valve. Leakage was observed as the aortic valve pressure drops when the valve is closed.

chanical conditioning methods. However, leaflets withstanding the large strains and stresses in the aortic valve position, based on a fast degrading polymer scaffold, have not been reported yet. In this study, the influences of various mechanical stimuli have been studied in order to engineer leaflets that do fulfill this goal. To elucidate the influence of prestrain, perfusion, and additional dynamic straining and the evolution of the tissue over time, tissue development as well as mechanical properties were studied for the experimental groups described in Table 6.1, cultured up to four weeks.

Evolution of tissue properties over time

Tissue compaction after release of the leaflets from their stents increased 1.9-fold between two and three weeks of culturing, resulting in a decrease of tissue volume, and was stable thereafter (Fig. 6.2). Cells seeded in gels (either fibrin or collagen) and non-woven PGA meshes, are known to compact very rapidly (Kim and Mooney, 1998; Girton *et al.*, 1999; Neidert *et al.*, 2002; Hiraoka *et al.*, 2003; Berglund *et al.*, 2003; Grassl *et al.*, 2003; Cummings *et al.*, 2004). Reinforcement of the gels, as well as coating of the PGA meshes, has shown to prevent tissue compaction to a large extent (Kim and Mooney, 1998; Hiraoka *et al.*, 2003), indicating that a proper scaffold with sufficient mechanical properties is able to at least partly resist the contractile forces in the developing tissue. In this study, the increase in compaction between two and three weeks of culturing might have been caused by the loss of mechanical integrity of the scaffold material, which might be further enhanced by the straining (Engelmayr *et al.*, 2003). After three weeks compaction was stable, most probably due to the large amount of tissue formed to prevent further compaction.

Similar to tissue compaction, the overall amount of tissue increased between two and three weeks of culturing (Fig. 6.3). Hydroxyproline and GAG contents significantly increased (Fig. 6.5), as well as the UTS (Fig. 6.8), indicating a stronger tissue with increased amounts of tissue. Increases in collagen content with culture time were reported in other studies as well (Hoerstrup *et al.*, 2000c; Grassl *et al.*, 2003; Ross and Tranquillo, 2003). The increase in collagen was not reflected in the modulus. The tissue development and mechanical properties did not increase further after three weeks of culturing. The amount of DNA did not increase over time, in contrast to many other studies (Hoerstrup *et al.*, 2000c; Ozawa *et al.*, 2000; Ross and Tranquillo, 2003; Shi and Vesely, 2004). This might be influenced by the straining as discussed in the next subsection. These results indicate that a culture time of three weeks might already be appropriate for optimal tissue development. Although, the tissue might still improve in its organization and maturation with increasing culture time, not necessarily representing an increase in the absolute amount of tissue.

Effect of mode of conditioning

The stented leaflets all kept their shape, although they flattened in the stent, while the strips completely deformed (Fig. 6.1). Tissue compaction resulted in a 1.3-fold decrease in volume in the strained leaflets when compared to the other groups. It has been reported that increasing numbers of cells enhance tissue compaction (Baguneid *et al.*, 2004), although that was not the case in this study (Fig. 6.5). Cyclic straining is described to enhance tissue compaction (Seliktar *et al.*, 2001; Stegemann and Nerem, 2003), which might explain the increased compaction in the strained leaflets. However, opposite results have been reported as well (Isenberg and Tranquillo, 2003; Cummings *et al.*, 2004).

The tissue in the strained leaflets was very dense and homogeneously distributed throughout its full thickness (Fig. 6.3), as well as in the perfused leaflets, although to some lesser extent (Fig. 6.4). Tissue formation in the static valve leaflets and the strips was more pronounced at the surfaces, although ingrowth could also be demonstrated in those tissues. Improved tissue distribution by perfusion is most likely caused by enhanced nutrient supply and removal of metabolic waste products (Carrier *et al.*, 2002; Radisic *et al.*, 2003; Kofidis *et al.*, 2003). Additional straining might have further increased this beneficial effect (Engelmayr *et al.*, 2005).

Collagen and GAGs were abundantly present in the tissues, however, no elastin was detected. Elastin has been reported in many engineered tissues, although most of these were based on rat cells (Kim *et al.*, 1998; Kim and Mooney, 1998, 2000; Isenberg and Tranquillo, 2003; Ross and Tranquillo, 2003; Shi and Vesely, 2004). Just sparsely was elastin reported in engineered tissues from human cells (Long and Tranquillo, 2003). GAG formation (Fig. 6.5) was largest for the strained leaflets (2-fold compared to the static valve leaflets and the strips) and was obviously upregulated by the additional dynamic strains as observed by an increased amount of GAGs produced per μg DNA (3-fold when compared to the static valve leaflets and the strips, Fig. 6.6). Such a large upregulation of GAG production has not been stated in many studies (Narita *et al.*, 2004; Xing *et al.*, 2004) and most probably the dynamic compo-

nent plays a large role in this process. GAG formation might be initiated to protect and embed the matrix components. Prestrain only resulted in increased GAG formation when it was accompanied by perfusion (1.3-fold), indicating a larger effect of perfusion than prestrain on the GAG production. Obviously, collagen formation (Fig. 6.5) was only enhanced by prestrain in combination with perfusion (1.4-fold). However, the synthesis activity of the cells to produce collagen was increased in the strained leaflets as well (about 2-fold, Fig. 6.6). Many studies have reported increased collagen formation as a response to dynamic conditioning, some of them with increased collagen synthesis by the cells (Hoerstrup *et al.*, 2002a,b; Engelmayr *et al.*, 2005; Jeong *et al.*, 2005) and others just because of more cells (Sodian *et al.*, 1999, 2000d; Mol *et al.*, 2003). The reason that total collagen formation was not larger in the strained leaflets in this study might be due to the lack of rest for the cells to produce collagen as by the straining they are already stimulated to produce more GAGs. It may be speculated that by straining at a lower frequency or by incorporating rest periods, collagen formation would be enhanced. The amount of collagen cross-links was larger in the perfused leaflets when compared to the strips. This was not due to more mature tissue, but to more collagen in general as the amount of cross-links per collagen molecule was in the same range for both groups.

Obviously, less collagen formation does not necessarily result in less optimal mechanical properties as the UTS and the modulus were similar for both the strained and perfused leaflets (Fig. 6.8). Similar stiffness with less collagen is most probably caused by improved tissue organization. The cells in the strained leaflets were oriented more into a specific direction. This might be related to the dynamic strains, studied extensively by others (Sodian *et al.*, 1999, 2000d; Kim and Mooney, 2000; Neidert *et al.*, 2002; Gonen-Wadmany *et al.*, 2004; Wagenseil *et al.*, 2004). As collagen alignment is reported to be correlated to cell orientation (Wang *et al.*, 2003), this would indicate orientation of the collagen in the strained leaflets as well. Furthermore, the mechanical behavior in the strained leaflets tended to exhibit anisotropic characteristics (Fig. 6.7). Prestrain only resulted in increased mechanical properties when accompanied by perfusion (1.6-fold). The overall increase in mechanical properties in the perfused and strained leaflets showed a moderately strong correlation with GAG fomation and less with collagen formation, in contrast to other studies that report very strong linear relationships for collagen (Neidert *et al.*, 2002; Engelmayr *et al.*, 2005) and none for GAGs (Engelmayr *et al.*, 2005).

The leaflets with increased mechanical properties were shown to contain less DNA (about 1.4-fold) when compared to the leaflets that were mechanically weaker (Fig. 6.5). The effects of mechanical conditioning on the amount of DNA are divers among studies. Increased amounts were reported (Sodian *et al.*, 1999, 2000d; Stegemann and Nerem, 2003; Mol *et al.*, 2003; Jeong *et al.*, 2005), as well as no differences (Hoerstrup *et al.*, 2002a; Isenberg and Tranquillo, 2003; Engelmayr *et al.*, 2005), and a decreased amount (Hoerstrup *et al.*, 2002b). The phenotype of the cells might have a large influence on the reported results. Vascular cells are known to be able to differentiate from a quiescent fibroblast-like phenotype into a more active myofibroblast phenotype, with increased proliferative and matrix synthesis activity, in response to mechanical conditioning (Stegemann and Nerem, 2003). Obviously, the balance

between proliferation and extracellular matrix synthesis is influenced by mechanical conditioning as well. In this study, the cells in the static valve leaflets and the strips do mainly proliferate and are less stimulated to produce matrix, while in the strained and perfused leaflets, the cells are mainly producing matrix and are much less proliferative. The changes in cell phenotype should be a focus for future studies as it can affect tissue development to a large extent.

The tremendous increase in tissue properties and mechanical behavior of the engineered leaflets, when compared to studies using the similar scaffold material (Hoerstrup *et al.*, 2000c, 2002a; Kadner *et al.*, 2002), might be related to the sterilization techniques used, although this issue has to be elucidated in further studies. The contribution of the scaffold remnants to the mechanical properties of the tissues is not known. Scaffold remnants were still present after four weeks of culturing, however, their contribution to the mechanical properties is expected to be minimal as stiffness values as low as 0.15 MPa for PGA/P4HB scaffolds after three weeks of incubation have been reported (Engelmayr *et al.*, 2003).

Functionality

Absolute values for the mechanical properties of native human aortic valve leaflets differ between studies (Clark, 1973; Stradins *et al.*, 2004), although, it is known that they exhibit highly anisotropic and non-linear mechanical behavior, being stiffer and stronger in the circumferential direction. The strained and perfused leaflets in this study showed much less anisotropy, none for the perfused and a tendency towards stiffer and stronger properties in circumferential direction for the strained leaflets. The average UTS of the strained leaflets was 2.17 MPa. This is within the range for native human aortic valve leaflets, stated by Stradins *et al.* (2004), but below the values reported by Clark (1973). The average modulus of the strained leaflets was 5.96 MPa, correlating with the circumferential modulus for native human aortic valve leaflets, stated by Clark (1973), but far below the circumferential modulus reported by Stradins *et al.* (2004). The modulus did, however, exceed the modulus in radial direction described by both (Clark, 1973; Stradins *et al.*, 2004).

The hydroxyproline content of native human aortic valve leaflets is described to vary with age, with values of 77 μg/mg for persons under 20 years and 37 μg/mg for persons over 60 years (Bashey *et al.*, 1967). In the engineered leaflets values up to 19 μg/mg were measured, between 25 and 50% of the reported native values. The amount of collagen cross-links in the perfused leaflets was about 32% of native human aortic valve leaflets and the ratio of cross-links per collagen molecule was in the same range (not published; Balguid, 2005). The amount of GAGs and DNA in the native human aortic valve is not stated in literature. Measurements in our laboratory have elucidated the values for porcine aortic valve leaflets to be 7.7 and 0.7 μg/mg, respectively. The amount of GAGs (33 μg/mg) and DNA (1.6 μg/mg) in the strained leaflets exceeded those amounts.

The mechanical properties and the amounts of the various tissue components in the engineered leaflets do still not completely mimic the properties of adult native human aortic valve leaflets. The tissue engineered leaflets showed more similarities

with neonatal human aortic leaflet tissue with indistinct layers, predominant accumulation of GAGs, some collagen, and almost undetectable elastin (Rabkin-Aikawa *et al.*, 2004). With regard to their mechanical properties, they should be able to resist the flows present in the adult aortic valve environment. Therefore, the perfused leaflets were tested for functionality in the 'valve exerciser' under physiological flow conditions, mimicking the in-vivo environment of the adult aortic valve. The leaflets showed to sustain the large flows for the 24 hours tested, showing some prolapse due to insufficient coaptation. Due to this prolapse and the subsequent leakage, the aortic pressure gradually decreased to zero, instead of maintaining a stable level around 80 mm Hg. Hence, before these leaflets can be tested for functionality in animal studies, a larger coaptation area should be incorporated to ensure proper closure. Via computational modeling, an optimum between tissue compaction and coaptation of the leaflets might be determined, resulting in an optimized geometry.

6.5 Conclusions

The influence of the mode of conditioning on tissue formation and mechanical properties are summarized in Table 6.2.

Group	**Conditioning mode**	**DNA**	**GAG**	**Hyp**	**UTS**	**Mod**	**Anisotropy**
dynamically strained leaflets	PS, PERF, and DYN	+	+++	+	++	++	+/-
perfused leaflets	PS and PERF	+	++	++	++	++	-
static valve leaflets	PS	++	+	+	+	+	-
strips	none	++	+	+	+	+	-

Table 6.2: Influence of the mode of conditioning on tissue and mechanical properties in the different groups of engineered leaflet tissues. Used abbreviations: PS=prestrain, PERF=perfusion, DYN=dynamic strains, Hyp=hydroxyproline, UTS=ultimate tensile strength, and Mod=modulus.

Mechanical conditioning has shown to represent a valuable tool to guide tissue development in tissue engineered heart valve leaflets towards functional and living heart valve leaflets appropriate for systemic pressure application, such as in aortic valve replacement. Preventing free compaction, resulting in prestrain, has shown to render tissues that remain their shape and, in combination with continuous perfusion, excellent tissue formation was demonstrated with mechanical properties close to those of native human aortic heart valve leaflets. Additional dynamic straining represents a useful tool to induce tissue orientation in the leaflets towards anisotropic mechanical

behavior, characteristic for aortic valve leaflets. By optimizing the dynamic straining protocol, the tissue development in the strained leaflets might be even further enhanced. To our knowledge, this is the first study to describe tissue engineered human heart valve leaflets, based on a fast degrading polymer, suitable to withstand the large stresses and strains arising at the aortic valve position.

Chapter 7

General discussion

7.1 Strain-based tissue engineering of heart valves

This section summarizes, discusses and compares the obtained results in the various chapters of this thesis. Furthermore, discrepancies between studies are discussed. The section ends with the general conclusions that can be drawn from this thesis.

7.1.1 The hypothesis

Although tissue engineered trileaflet heart valves, based on fast degrading PGA/P4HB scaffolds, have shown excellent functionality in sheep in pulmonary position (Hoerstrup *et al.*, 2000a), the challenge still remains to engineer heart valve tissues that are capable to function in the high-pressure aortic valve environment. The bioreactors developed over the years for conditioning the engineered heart valves are all flow-driven, either or not in combination with some tissue straining (Hoerstrup *et al.*, 2000a,b; Dumont *et al.*, 2002; Hildebrand *et al.*, 2004; Rutten *et al.*, 2005).

In contrast, this thesis concentrates on tissue straining with minimized flows as it is hypothesized that large tissue strains are beneficial for tissue development and evolution through strain-based mediation of the load-bearing tissue components. Minimal flows and large strains were chosen, since it is believed that strains induced by the large transvalvular pressure during diastole, mainly in circumferential direction, influence ultimate heart valve structure and composition to a larger extent when compared to the stresses induced by the blood flow during systole, exerted mainly in radial direction (Deck, 1986). Also, predictions of the collagen fiber architecture matched the architecture in native valve leaflets when diastole, rather than systole, was simulated (Boerboom *et al.*, 2003). In the human embryo, collagen formation is hypothesized to be induced by the strains arising during diastole as well (section 1.2.1). Furthermore, the vascular cells used for tissue engineering of the valve leaflets are also in-vivo not directly exposed to the shear stresses induced by the blood flow due to the protective layer of endothelial cells. The dominant mechanical stimulus that these cells receive is dynamic straining. Another example of a possible advantage of using low or minimized flows in tissue engineering is that low flow rates, in contrast to large flow rates, were shown to induce matrix remodeling in veins (Fernandez *et al.*, 2004), a feature desirable for initial tissue development. However, the veins used in the latter study did contain an intact endothelial cell layer. The benefits of mechanical strains on the proliferative capacity of cells and the production of extracellular matrix have been extensively described (Williams, 1998) and did also apply to engineered smooth muscle tissues (Kim *et al.*, 1999; Kim and Mooney, 2000). In the latter studies tissue strains of 7% were applied, while in this thesis it was hypothesized that larger strains may further increase this beneficial effect.

Larger flows might start to play an essential role in a later phase of the tissue engineering process, following seeding of endothelial cells onto the leaflets to provide a protective non-thrombogenic layer as flow over endothelial cells has reported to stabilize the underlying cells (Nackman *et al.*, 1998; Weston and Yoganathan, 2001). For initial tissue development flows might very well be not necessitated (Barron *et al.*, 2003) or even be contra-productive. This thesis work focuses only on the initial tissue

development phase, the tissue stabilization phase after seeding of endothelial cells has to be addressed in future work.

The hypothesis of using large strains to improve tissue properties was tested on rectangular shaped valve leaflet tissue-equivalents as described in chapter 3. Exposing the developing tissues to increasing cyclic strains up to 10% at a frequency of 1 Hz resulted in improved tissue formation, organization, and mechanical properties after three weeks of culturing. This improvement was more pronounced with increasing strains. The hypothesis to use tissue strains to improve initial tissue formation was hereby proven to be useful and the step towards a more complex geometry could be made.

7.1.2 Prerequisites

To extent the hypothesis (large tissue strains in combination with minimized flows being beneficial for tissue development) to 3D engineered human heart valve leaflets, several prerequisites had to be fulfilled. The most easy and efficient way to apply strains to a trileaflet heart valve geometry is by applying a transvalvular pressure difference over the valve leaflets, thereby mimicking the diastolic phase in the heart.

In order to apply a pressure difference over tissue engineered valve leaflets, the leaflets should have an initial compact and non-porous structure, because otherwise pressure differences can hardly be applied and the cells and freshly formed extracellular matrix might be washed out of the scaffold. Embedding the cells in a fibrin gel and subsequent transfer into the fiber mesh scaffold, before polymerization of the gel, resulted in a compact structure suitable for mechanical conditioning as hypothesized in chapter 4 and demonstrated in chapter 5 and 6. Fibrin gel was chosen as a cell carrier instead of collagen because fibrin can be obtained autologous from the blood of the patient, while autologous collagen is hard to derive. Furthermore, cells in fibrin gel were shown to render more collagen when compared to cells in a collagen gel (Grassl *et al.*, 2002; Neidert *et al.*, 2002). The use of fibrin as a cell carrier had several advantages besides the generation of a compact structure suitable for mechanical loading as described in chapter 4. The seeding procedure was fast and efficient, involving only a single seeding step, while multistep procedures are necessitated for efficient seeding when no cell carrier is used (Zund *et al.*, 1999). The compact structure prevented the loss of freshly formed soluble matrix components into the surrounding medium, resulting in a faster development of the extracellular matrix, being more mature in nature when compared to tissues seeded by the conventional method. Other groups using fibrin as a scaffold material report the use of fibrin degradation inhibitors to prevent the loss of fibrin (Jockenhoevel *et al.*, 2001a; Ye *et al.*, 2000; Grassl *et al.*, 2002, 2003). This was shown to have adverse effects in the study described in chapter 4 of this thesis, it even resulted in an inhibitory effect on tissue formation, speculated by inhibition of collagen cross-linking and subsequent tissue maturation.

Another prerequisite to strain engineered valve leaflets in a trileaflet heart valve geometry is the development of a bioreactor system to apply a transvalvular pressure difference over the leaflets, inducing the desired tissue strains. A novel bioreactor was developed to fulfill this goal, referred to as the 'Diastolic Pulse Duplicator' (DPD) as

described in chapter 5. The DPD was demonstrated to be easy in its use with just 75 ml of medium required for one system, sized small to save on incubator space, sterile for several weeks, and to consist of biocompatible materials. As the part where the valve is located in is a closed system with no possibility for gas exchange, a perfusion circuit was included to supply fresh oxygenated medium to the tissue and to remove metabolic waste products (perfusion). A pressure difference could be applied using a compressed-air system compressing and decompressing an elastic tube in the perfusion circuit, which could be controlled using LabView software. The pressures above and below the valve could be monitored during culturing. The amount of strain, induced by the applied transvalvular pressure, was estimated by numerical analyses, whereby the tissues were modeled as incompressible generalized Neo-Hookean materials. As the amount of strains induced by the pressure difference depends on the composition of the developing tissues, the mechanical properties as well as the thickness of the tissues served as input for the analyses. The feasibility of the use of the DPD to engineer human heart valve leaflets using the strain-based conditioning approach is demonstrated in chapter 5.

7.1.3 Mode of conditioning

Once the prerequisites for strain-based tissue engineering of human heart valve leaflets were fulfilled, the search towards optimal mechanical conditioning protocols was continued. Various conditioning cues could be applied in the DPD and to optimize the protocol further, it was of importance to elucidate the relative and combined effects of each of them. First of all, as a stented heart valve geometry was used, prestrains were induced in the developing tissues as compaction was constrained by the stent. Second, the tissues cultured in the DPD were exposed to continuous perfusion of medium at low speed (4 ml/min), and third, the developing leaflet tissues could additionally be exposed to dynamic strains by a dynamic transvalvular pressure difference. Chapter 6 describes the effects of these different conditioning cues.

Prestrain resulted in improved tissue properties and mechanical behavior, only when accompanied by continuous perfusion, indicating the importance of continuous supply of fresh nutrients and the removal of metabolic waste products. Additional dynamic straining resulted in increased GAG formation, most probably to protect and embed the formed matrix components, while collagen formation was less when compared to leaflets that were perfused only. The mechanical properties, however, were not influenced by the lower collagen content, the UTS and modulus were similar to the perfused leaflets. This might point to improved tissue organization in the strained leaflets, demonstrated by a more particular cell orientation (also described in chapter 3) and the tendency towards anisotropic mechanical behavior when additional dynamic straining was used. From this study, it was concluded that prestrain in combination with perfusion improved tissue development, while additional dynamic strains might be useful to further optimize tissue organization and maturation.

7.1.4 Discrepancies between studies

As described in chapter 5 and 6, prestrain of about 5% already resulted in sufficient tissue development. Additional dynamic straining of about 5% over the applied 5% prestrain has shown no additional increase in collagen, while in chapter 3 it is described that increasing strains, at least larger than 7%, resulted in increased collagen formation. An important issue in the interpretation of the results described in chapter 3 is, however, the large permanent deformation of the tissue during culturing, also reported by Kim and Mooney (2000), which might have resulted in strains applied of a lesser amplitude as stated. Furthermore, due to these large deformations, the period within one straining cycle that the tissue is actually exposed to straining might also be less. This large deformation, induced by straining the developing tissues, was not noticed in the engineered tissues in chapter 5 and 6. An explanation for this might be, on the one hand, the use of fibrin as a cell carrier (chapter 4) and subsequent contraction of the cells in the gel and, on the other hand, the use of a different geometry for the engineered leaflet tissues (chapter 5 and 6). The cells in the leaflets in chapter 5 and 6 are, first of all, continuously exposed to prestrain. Second, during dynamic conditioning, the cells in the leaflets were most probably exposed to the dynamic strains for a larger part of the straining cycle when compared to the largely deformed tissues described in chapter 3 and the distribution of the applied strains in the leaflet differed locally as was not the case in the tissues described in chapter 3. These differences might explain the discrepancy in collagen formation by dynamic straining between the studies described in chapter 3 and 6.

Increased tissue formation induced by dynamic straining in the study described in chapter 3 is clearly caused by an increase of cell number. The synthesis activity of the cells to produce extracellular matrix was not increased, it even tended to decrease. This is in contrast to the study described in chapter 6, in which the synthesis activity of the cells to produce both GAGs and collagen was largely upregulated due to straining, either prestrain only or in combination with additional dynamic strains. This might result from a difference in phenotype of the cells. Dynamic mechanical conditioning is known to induce phenotype shifts (Stegemann and Nerem, 2003), but then the shifts found and their effects should be similar in both studies. Not much is known regarding the influence of the use of fibrin as a cell carrier and the presence of prestrain in the developing tissue on the phenotype and the subsequent proliferative and matrix synthesis activity of the cells. Also the influence of continuous perfusion of the tissue on the phenotype of the cells is not known, although no large effects are expected as the strained tissues from chapter 3 were also perfused by inducing movement of the surrounding medium by straining. From the discrepancy in extracellular matrix synthesis activity of the cells between the two studies, the hypothesis arose that the balance between proliferation and matrix synthesis is largely influenced by the mode of conditioning and possibly by the use of fibrin as a cell carrier as well. The cells in the study described in chapter 3 show mainly increased proliferative activity when exposed to dynamic straining, while the cells in the study described in chapter 6 showed increased matrix synthesis activity and much less proliferation when exposed to prestrain in combination with perfusion and additional dynamic strains.

There is a tremendous increase in mechanical properties, and most probably also in tissue properties, between the studies described in chapter 5 and 6, although the conditioning approach was similar. Similar cells and scaffolds were used, as well as the same conditioning system and the studies were performed by the same person. The one large difference between those two studies is the sterilization method used to sterilize both the scaffolds and the bioreactor systems. In the study described in chapter 5, the scaffolds as well as the bioreactor systems were sterilized by ethylene oxide, while in the study described in chapter 6, the scaffolds were sterilized using 70% ethanol and the bioreactor systems by autoclaving. The mechanical properties were much less for the tissues described in chapter 5, when compared to chapter 6. This might indicate possible inhibitory effects of ethylene oxide on tissue development. It is known that ethylene oxide is a very toxic component and that the sterilized goods should be degassed for a sufficient amount of time. Periods up to three days have been stated (Prabhakar *et al.*, 2003). Ethylene oxide have been reported to induce poor tissue development when not degassed properly (Gulbins *et al.*, 2004). The lack of an appropriate degassing period might be the reason for the inferior mechanical properties in chapter 5 as compared to chapter 6.

7.1.5 General conclusions

The strain-based approach to tissue engineer heart valve leaflets in combination with the use of fibrin as a cell carrier has shown to be a promising approach in the field of tissue engineering of heart valves. The tissue properties and mechanical behavior of the valve leaflets obtained using this approach were approaching those of native human aortic valve leaflets, except they did not display the highly anisotropic properties found in the native leaflet and were less flexible. The ultimate tensile strength and modulus were within the range reported for native human aortic valve leaflets, although different values of those were mentioned in literature (Clark, 1973; Stradins *et al.*, 2004). The collagen content was 25-50% and the amount of collagen cross-links about 32% compared to native human aortic valve leaflets. The amount of DNA and GAG in the engineered leaflets were exceeding those found in native porcine aortic valve leaflets (no data was available for human aortic valve leaflets).

The amount of the various tissue components in the perfused leaflets was compared to the amounts reported by others in similar studies of engineered tissues based on either collagen and fibrin gels as a scaffold or synthetic biodegradable polymers. Regarding DNA content, studies performed with human cells have reported values varying from 0.65 μg/mg after six weeks of culturing with venous derived cells (Schnell *et al.*, 2001) to 2.7 μg/mg after two weeks with bone marrow derived cells (Kadner *et al.*, 2002). The DNA content of the perfused leaflets in this thesis was 1.8 μg/mg, correlating well with the content found by others. The GAG content was not stated in many studies, but was reported to be 1.4 μg/mg after two weeks of culturing using canine aortic smooth muscle cells (Narita *et al.*, 2004) up to 5.8 μg/mg after two weeks of culturing using human venous derived cells (Kadner *et al.*, 2002). The amount in the perfused leaflets in this thesis was 23 μg/mg, clearly exceeding the values reported in literature. The amount of hydroxyproline in studies performed

with human cells varied from 0.43 μg/mg after four weeks of culturing (Jockenhoevel *et al.*, 2002) up to 6.1 μg/mg after eight weeks of culturing (Hoerstrup *et al.*, 2002b). The perfused leaflets in this thesis contained 18.8 μg/mg hydroxyproline. From these comparisons, it is demonstrated that the large amount of tissue obtained using the strain-based approach in combination with the use of fibrin as a cell carrier and the avoidance of ethylene oxide sterilization exceeds all reported values provided by others for the amount of collagen and GAGs in engineered tissues.

When the mechanical properties are compared with values stated in literature based on the same type of scaffold (PGA/P4HB) it was clear that the perfused leaflets in this thesis contained a larger ultimate tensile strength, being 2.17 MPa, versus 0.76 MPa stated by others after three weeks of culturing (Hoerstrup *et al.*, 2002a). The largest value for the ultimate tensile strength in engineered tissues was reported to be 3.6 MPa after two weeks of culturing of lamb arterial smooth muscle cells on a sandwiched PGA/polyglactin scaffold (Shinoka *et al.*, 1995), although this scaffold degrades much slower when compared to the PGA/P4HB scaffolds. A large part of the resulting mechanical properties might, therefore, still be attributed by the scaffold, which was not the case in the studies described in this thesis. The modulus of engineered tissues from human umbilical cord cells on PGA/P4HB, cultured for three weeks, was reported to be 5.38 MPa (Hoerstrup *et al.*, 2002a), very well correlating with the modulus found in the strained leaflets in this thesis, being 5.96 MPa. The variance in the modulus of engineered tissues reported in literature was huge, with a lowest value of 0.06 MPa in collagen based tissue-equivalents (Seliktar *et al.*, 2001) and a largest value of 5.38 MPa (Hoerstrup *et al.*, 2002a). It has to be noted that comparisons of the modulus between studies is fairly difficult as the method to determine the modulus affects the calculated value to a large extent.

Preliminary functionality tests of the engineered leaflets showed promising results for further studies. Exposure to physiological flows, mimicking the in-vivo environment of the adult native aortic valve leaflets, showed to induce no rupture or mass deformations. The coaptation of the leaflets was, however, minimal, resulting in a prolapse of the tips of the leaflets during diastole and subsequent leakage. Despite various issues still to be solved, this is to our knowledge the first report of tissue engineered human leaflets, based on a fast degrading polymer scaffold, that contains sufficient tissue and displays sufficient mechanical behavior to be used as aortic heart valve leaflets. Future animal studies will elucidate possible steps towards clinical implementation of heart valves engineered using the described strain-based approach.

7.2 Towards clinically implementable tissue engineered heart valves

This section describes the focuses for future research regarding the engineered leaflets in this thesis, which still are many. Furthermore, future animal studies are discussed, as well as remarks towards clinical implementation.

7.2.1 Focuses for future research

Alternative cell sources

An important issue in tissue engineering in general is whether the cells that are used develop a similar phenotype as found in the native tissue. This is hypothesized to have large impact on the long-term functioning of the implanted tissue (Butcher and Nerem, 2004). Safest would be to use cells originating from the tissue for which a replacement is being engineered. In case of heart valves, valve biopsies to obtain valvular interstitial cells have been shown feasible (Maish *et al.*, 2003). For clinical implementation, however, the risk of post-operative problems is unacceptable. Both arterial and venous cells, derived from blood vessels, have been widely used in tissue engineering of heart valves as they are closely related to the cells in a heart valve. Venous cells are easier to obtain from a patient when compared to arterial cells (Butcher and Nerem, 2004), but an intact vascular structure always has to be sacrificed.

Lately, a lot of progress has been made in the elucidation of differentiation pathways of cells from other sources with a possible promising use in cardiovascular tissue engineering applications. Bone marrow cells, for example, can be very easily obtained by a biopsy, avoiding the sacrifice of an intact structure (Kadner *et al.*, 2002), and were shown to be able to behave similar to vascular cells. The extracellular matrix formation by endothelial progenitor cells (EPCs), obtained from peripheral blood, was demonstrated to not be sufficient to be used as a single cell source yet, however, they were shown to transdifferentiate into endothelial cells on PGA/P4HB meshes (Dvorin *et al.*, 2003). Microvessel formation was observed when seeded together with vascular cells on PGA/PLLA matrices (Wu *et al.*, 2004). As EPCs do represent a very valuable cell source as no biopsy or operation is required, future studies should elucidate whether those cells can be induced to produce more extracellular matrix. Their capacity to induce microvessel formation is of particular importance for tissue engineering of the valve conduit wall as this concerns a rather thick tissue.

For use in neonatal applications, cells from human amniotic fluid represent a novel cell source with a lot of potential and products ready for implantation as soon as the child is born. The cells, obtained via routine ultrasonography-guided amniocentensis, were demonstrated to expand very easily. They were shown to express markers specific for fibroblasts and myofibroblasts and to adhere and attach to PGA/P4HB (Kaviani *et al.*, 2001, 2003). Also, cells from the human placenta represent a source of mesenchymal cells that expand easily in culture and attach to PGA/P4HB scaffolds (Kaviani *et al.*, 2002).

There are obviously several promising alternative cell sources, avoiding the sacrifice of an intact vascular structure, as is the case for the engineered leaflets in this thesis, however, many issues are still to be resolved regarding their differentiation pathways, their ability to produce extracellular matrix, and the stability of their induced phenotype.

Scaffold improvements

The scaffold used in this thesis, PGA/P4HB, has been shown very well suitable, FDA-approval has been initiated and, therefore, may be appropriate for future clinical studies, although there are still some issues to be resolved. As demonstrated, the scaffold is just partly resistant to the forces of compaction, resulting in valve leaflets that lose their bulged shape and contain none to minimal coaptation. As the initial geometry determines the ultimate shape, the geometry should be enlarged to include the effect of compaction in order to end up with sufficient coaptation surfaces in the leaflets. Furthermore, the valve leaflets to be implanted should contain elastic properties to prevent deformations when exposed to physiological conditions in the body. Although no massive deformations were observed in the leaflets, engineered in this thesis, after exposure to physiological flow conditions, these were just preliminary short-term results. As no elastin could be detected in the leaflets, it might be wise to incorporate elastic properties into the scaffold. The incorporation of, for example, an elektrospun P4HB sheet in between two thin PGA/P4HB meshes might be a promising approach to induce elastic properties into the scaffold and is worthwhile studying.

Incorporation of biochemical factors into the scaffold (bioactive scaffolds) represents another focus for future research, guiding tissue properties locally, which might be valuable in obtaining anisotropic tissue properties and the formation of the specific three-layered structure, found in native valve leaflets. Biochemical factors to be incorporated are, for example, TGF-β, insulin and plasmin. A combination of these three biochemical factors have shown to largely upregulate collagen production (Neidert *et al.*, 2002).

Bioreactor and conditioning improvements

The bioreactor used in this thesis to engineer heart valve leaflets, the 'Diastolic Pulse Duplicator' (DPD), can still be further optimized, for example by incorporation of a feed-back loop to control the transvalvular pressure applied over the leaflets, as well as the ability to monitor tissue strains in the leaflets during culturing and subsequent automatized adjustment of the transvalvular pressure to induce the desired strains. Furthermore, the costs for production of this bioreactor system should be decreased to make the system available for use by other research groups and for future clinical studies.

With regard to the dynamic conditioning protocol, there are several issues worthwhile studying. In this thesis the nature of conditioning was mainly studied, aspects such as magnitude, frequency, and duration should still be studied into more detail to render 'laws for tissue formation'. The frequency used in this thesis to apply the transvalular pressure difference was 1 Hz, mimicking more or less the in vivo frequency of heart valve loading. It has been demonstrated that the frequency plays an important role in influencing the balance between proliferation and extracellular matrix synthesis of cells. A higher loading rate (2 Hz) has been shown to be favorable for cell proliferation, while a lower loading rate (0.5 Hz) is preferred for matrix synthesis (Xing *et al.*, 2004). The period of one cycle wherein the tissue is actually strained has also shown to influence tissue formation. Short stretch times, exposing the tissue for

a short time (6-12%) of one straining cycle to strains, have been demonstrated to be beneficial for improvement of mechanical properties of engineered tissues (Isenberg and Tranquillo, 2003). Furthermore, the shape of the transvalvular pressure curve applied over the leaflets is reported to influence tissue formation. When the waveforms are more physiological-like with smooth transitions, proliferation and protein synthesis of vascular cells was enhanced when compared to peaky-waveforms with fast transitions (Narita *et al.*, 2004).

Endothelialization of the tissue

Seeding of endothelial cells onto the surfaces of the leaflets to provide a protective non-thrombogenic layer over the leaflet surfaces has not been performed in this study, but should be studied for future clinical implementation. The hypothesis that endothelial cells stabilize the underlying tissue by application of flow in order to prepare the tissue for subsequent implantation has to be tested. It has been reported that the flow should not be too large after seeding to enhance endothelial cell retention and to maintain a confluent lining (Baguneid *et al.*, 2004).

The functionality of the endothelial cell layer should be studied as endothelial cells exert a very specific function in regulating the behavior of the underlying cells. An interesting issue is, furthermore, the orientation of endothelial cells seeded on the surfaces of the leaflets as a response to physiological flows. The endothelial cells on the native aortic valve were shown to be oriented circumferentially, perpendicular to the blood flow over the leaflets (Deck, 1986), in contrast to the endothelial cells lining blood vessels.

Extended tissue analysis techniques

There is a continuous balance between synthesis and degradation of extracellular matrix proteins in tissues. Shifts in this balance can not be noted when only total content of proteins is measured, as is the case in this thesis. For example, when hydroxyproline contents are comparable between experimental groups, it might be that the synthesis is upregulated, but that simultaneously degradation is also upregulated. Mechanical straining is known to increase the activity of metalloproteinases (MMPs) (Seliktar *et al.*, 2001), enzymes that are capable of degrading extracellular matrix components. Furthermore, MMPs were shown to be upregulated in pathological conditions in valve leaflets (Rabkin *et al.*, 2001), as well as in tissue engineered constructs after implantation (Stock *et al.*, 2001). Therefore, elucidating this balance is worthwhile to further improve tissue formation. New techniques to follow collagen fiber formation and orientation in-vitro will be very helpful as well to study the influences of mechanical conditioning.

The use of proteomic tools, such as two-dimensional gel electrophoresis and the ProteinChip technology, enables us to create protein profiles (Bruneel *et al.*, 2003; Opitz *et al.*, 2004; Nair *et al.*, 2004), while the microarray technology is useful to study gene expression profiles (Feng *et al.*, 1999; Klapperich and Bertozzi, 2004). These novel technologies may enable comparisons between engineered tissues and

their native counterparts, to study into more detail the pathway through which mechanical conditioning stimulates tissue formation. Furthermore, these are helpful techniques to gain more insight in how to optimize this process and to study the development of the tissues after implantation.

Sterilization methods

As already indicated in section 7.1.4, the sterilization method might have a large impact on tissue formation. Due to the possible toxic effects of ethylene oxide, alternative sterilization methods should be studied. The bioreactor system used in this thesis was sterilized by autoclaving, which is a generally accepted sterilization method. The scaffolds, however, were sterilized using 70% ethanol, which is not a widely accepted sterilization method. For future clinical implementation, an alternative sterilization method should be used. Alternative methods comprise, for example, gamma irradiation and plasma sterilization.

Gamma irradiation has shown to alter the properties of PGA, mainly by increasing its cristallinity (Prabhakar *et al.*, 2003), making it more brittle, as well as by quicker weight loss (Chu *et al.*, 1995). However, cell growth and matrix production by cells seeded onto gamma-irradiated meshes have shown to be not influenced. It even seemed that the cells were less dedifferentiated (Prabhakar *et al.*, 2003). Gamma irradiation of P4HB was also shown to be feasible, although some molecular weight loss was observed at increasing irradiation doses (Martin and Williams, 2003). Gamma irradiation is, however, a costly process and requires an isolated site for safe operation. Plasma sterilization is an effective surface treatment as it uses UV photons, among others, to destroy micro-organisms (Moisan *et al.*, 2001). However, it still has to be seen whether this is a suitable method for sterilizing scaffolds and whether the UV photons do not disrupt the surface of the polymer.

7.2.2 Future animal studies

As the properties of the engineered leaflets in this thesis were of very good quality, the next step would be to test them for functionality in the aortic valve position in an animal study. The commonly used animal model for testing of heart valves is the sheep, due to the resemblance of its heart valves in terms of mechanical properties and haemodynamic flow parameters to humans (Rashid *et al.*, 2004). Furthermore, the risk of calcification is large in sheep as well as in humans. Using lambs instead of adult sheep, incorporates the possibility to study growth of the valves.

Before the step to animal studies can be made with the leaflets engineered in this thesis, several issues have to be solved. First of all, the leaflets have to be engineered completely autologous. Cells from the lamb, in which the valve will be implanted, should be used. It has to be kept in mind that cellular responses of cells from different species on mechanical conditioning might vary (Seliktar *et al.*, 2001). Also the age of the cells has shown to influence tissue formation (Grassl *et al.*, 2003). Fibrin, which is used as a cell carrier, should also be obtained from the same lamb. Furthermore, it has to be demonstrated that there are no fibrin leftovers in the leaflets before im-

plantation, as those might be pro-thrombic (Neidert *et al.*, 2002; Grassl *et al.*, 2003). The leaflets, as they have been engineered in this thesis, can not be implanted containing the bone cement stent. Therefore, for near future animal studies, the stent of bone cement will be replaced by a rigid stent of a PGA non-woven mesh coated with P4HB. When using a larger concentration of coating, the stent becomes rigid, while still allowing cell ingrowth. When this approach is successful, future studies should address the fabrication of tissue engineered stentless heart valves incorporating the sinuses for even better performance.

After implantation, it is of importance that remodeling of the heart valve leaflets is followed. It is known that the remodeling process takes up to months after implantation (Stock *et al.*, 2001). The tissue should stabilize itself without signs of overgrowth and increased matrix production. Increased matrix production might be noted in the first few months after implantation (Stock *et al.*, 2000, 2001), however, it should return to normal stable levels thereafter. The phenotype of the cells should be studied as the phenotype should shift to a more quiescent phenotype (Rabkin-Aikawa *et al.*, 2004), indicative for a stabilized quiescent tissue. This has been demonstrated to appear after 20 weeks of implantation (Rabkin *et al.*, 2002). Of interest is also the origin of the cells in the tissues after months of implantation, whether they are still persisting from the seeding or whether they have been attracted in vivo. There are indications that the cells do persist from the seeding (Shinoka, 2002). Growth of the valve with the animal is of importance for future use in children. Growth, however, should be distinguished from dilatation (Shinoka *et al.*, 1998; Sodian *et al.*, 2000a), which in practice is very difficult.

The use of animals in research should be very well thought through and approval has to be obtained from ethical committees. Furthermore, the animals should be treated according to specific guidelines, for example the ones provided by the National Institutes of Health (publication nr. 85-23, revised 1985).

7.2.3 Clinical implementation remarks

Despite promising results in animal studies, extrapolation of results obtained in animal studies to human applications has been shown full of risks. A striking example is the use of the SYNERGRAFT^{TM} decellularized porcine valve replacement. While they were shown to function very well in animal studies, they have led to early failure when implanted in pulmonary position in children (Simon *et al.*, 2003). In contrast to the animal studies, no spontaneous endothelialization or repopulation of the matrix with the patient cells was observed and a severe inflammatory response was induced, resulting in encapsulation of the valve matrix. In this case, the commonly used sheep model has failed to predict the failure in humans. Endothelial cells from humans were shown to need a living underlying compound for normal growth and survival in the blood stream, while endothelial cells from animals do not (Zund *et al.*, 1998).

Having valves available off-the-shelf will always remain a disadvantage for tissue engineered heart valves as it takes time to expand the cells and to culture the heart valve in-vitro. For neonatal applications, it might, however, be possible to have engineered heart valves available as soon as the child is born.

The ideal properties of tissue engineered heart valve replacements are listed below, based on the 'Ten Commandments of Satisfactory Prosthetic Aortic Valves' provided by Harken *et al.* (1962) and the list provided by Schoen and Levy (1999):

- It must not propagate emboli (endothelialized surface and resistant to calcification)
- It must be chemically inert and not damage blood elements (absence of deleterious immunological and inflammatory processes)
- It must offer no resistance to physiological flows
- It must close promptly
- It must remain closed during the appropriate phase of the cardiac cycle (large effective coaptation area)
- It must have lasting physical and geometrical features (appropriate heterogeneity, anisotropy, and amount of extracellular matrix as well as cellular potential for extracellular matrix synthesis, remodeling, and repair)
- It must be inserted in a physiologic site, generally the normal anatomic site
- It must be capable of permanent fixation (stable geometry and mechanical properties but potential growth with patient, resistant to tissue overgrowth)
- It must not annoy the patient
- It must be technically practical to insert

The success of each product and of tissue engineering in general is depending on the ability to prove that the products are both safe and effective. No clear safety and efficiency standards are available yet, but efforts to do so are ongoing. Basically, standards should be considered for the following seven areas: 1) cell and tissue sourcing, 2) cell and tissue characterization, 3) biomaterials testing, 4) quality assurance, 5) quality control, 6) nonclinical assessment, and 7) clinical evaluation (Omstead *et al.*, 1998).

In conclusion, clinical implementation of the leaflets engineered in this thesis is still far away, however, the obtained results in-vitro are very promising and efforts should be certainly made towards clinical implementation.

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Samenvatting

De aortaklep, gesitueerd tussen de aorta en het linker ventrikel, is een van de vier kleppen in het hart welke de bloedstroom in het lichaam in de juiste richting leiden (hoofdstuk 1). De klep bestaat uit drie vliezen met ruime coaptatie om volledige sluiting van de klep te garanderen. De aortaklep opent en sluit zich ongeveer 3.7 biljoen maal in een mensenleven en dit vereist sterke, flexibele en duurzame vliezen. Wanneer de klep niet goed functioneert, bijvoorbeeld door ziekten, verkalking van de vliezen of een aangeboren afwijking, ontstaat een levensbedreigende situatie omdat er niet voldoende zuurstofrijk bloed het lichaam in gepompt kan worden. Binnen de cardiochirurgie is het vervangen van de aortaklep dan ook een veel voorkomende behandeling.

Tot op heden wordt voornamelijk gewerkt met twee typen hartklep prothesen: de mechanische hartklep en de biologische hartklep prothese, beiden met hun eigen voor- en nadelen. De levensduur van een mechanische klep is zeer goed, echter patiënten die dit type prothese geïmplanteerd hebben gekregen moeten hun leven lang antistollingsmedicijnen innemen teneinde de kans op trombo-embolisme, de vorming van bloedstolsels op het oppervlak van deze kleppen, te verkleinen. Deze behandeling op zijn beurt levert gezondheidsrisico's op, zoals een verhoogde kans op interne bloedingen. De biologische hartklep prothese, bijvoorbeeld de varkensklep of de humane donorklep, kent dit probleem niet, echter deze kleppen zijn minder duurzaam door versnelde calcificatie en afstotingsmechanismen van het lichaam. Een groot nadeel van alle hartklepprothesen in het algemeen is dat het om niet levend materiaal gaat. Deze kleppen kunnen niet groeien, zichzelf niet herstellen bij schade en zich niet aanpassen aan de behoeften van het lichaam wat vooral van belang is voor de groep jonge patiënten.

De ideale hartklep prothese is autoloog, vervaardigd van lichaamseigen materiaal om afstotingsreacties te vermijden, en levend, zodat de klep kan meegroeien en zich kan aanpassen gedurende het leven van de patiënt. Binnen het onderzoeksveld van tissue engineering houdt men zich bezig met het vervaardigen van lichaamseigen organen. Het principe van tissue engineering is het kweken van lichaamseigen cellen op een dragermateriaal, de zogenaamde scaffold, welke afbreekt terwijl het weefsel zich ontwikkelt buiten het lichaam (in-vitro). Zodra het weefsel toereikende eigenschappen bevat om in het lichaam (in-vivo) te functioneren kan de prothese in de patiënt worden geïmplanteerd. Ook hartklep prothesen kunnen volgens dit principe vervaardigd worden (hoofdstuk 2). Tien jaar geleden zijn de eerste succesvolle im-

plantaties verricht van enkele autologe tissue-engineerde hartklepvliezen in lammetjes. Inmiddels worden complete tissue-engineerde hartkleppen vervaardigd, welke functioneel blijken te zijn als vervanging van de pulmonaalklep in dierstudies. De pulmonaalklep staat echter aan minder hoge drukken en stroming bloot vergeleken met de aortaklep. Een klep die de hoge drukken en stromingen in de aortaklep positie kan weerstaan, gebruik makend van een snel afbrekend scaffold materiaal, is tot op heden nog niet middels tissue engineering vervaardigd. Het doel van de studies in dit proefschrift is het vervaardigen van tissue-engineered weefsel dat wel aan deze eisen voldoet. Dit proefschrift richt zich op tissue engineering van de vliezen van de hartklep, vervaardigd van cellen geïsoleerd uit een stukje bloedvat uit het been en een snel afbrekend scaffold materiaal.

Om weefselvorming buiten het lichaam te stimuleren worden hartkleppen gekweekt in zogenaamde bioreactoren, waarin het weefsel wordt blootgesteld aan mechanische factoren, zoals stroming en druk, zoals dat in het lichaam ook gebeurt. Optimalisatie van het conditioneringsprotocol speelt een grote rol in het streven naar een tissue-engineerde aortaklep. De bioreactoren die beschreven zijn door diverse onderzoeksgroepen zijn allemaal stromingsgestuurd om het openen en sluiten van de klep na te bootsen. De hypothese die ten grondslag ligt aan dit proefschrift is dat niet zozeer de stroming, maar juist de rekken in de vliezen die ontstaan wanneer de klep gesloten is door de druk van het bloed dat op de vliezen rust, de belangrijkste factor is die weefselvorming stimuleert.

Deze hypothese is getoetst middels het vervaardigen van simpele rechthoekige weefsels en deze cyclisch te rekken tijdens het kweken. Weefselvorming en mechanische eigenschappen bleken te verbeteren wanneer hogere rekken werden gebruikt (hoofdstuk 3). De hypothese is vervolgens uitgebreid naar de complexe geometrie van een aortaklep, met de drie vliezen gefixeerd aan een stent welke de aortawand representeert. Om verzekerd te zijn van een compacte en niet-poreuze structuur voor efficiënte conditionering werden de cellen eerst in een fibrine gel gemengd en vervolgens in de scaffold gebracht (hoofdstuk 4). Tevens is er een nieuw bioreactor systeem ontwikkeld (hoofdstuk 5) om een drukverschil over de vliezen aan te brengen en het weefsel voortdurend van medium met verse voedingsstoffen en zuurstof te voorzien via een circulatie systeem (perfusie). Doordat de vliezen gefixeerd waren aan de stent en weefsel van nature contraheert, ontstond er een constant aanwezige voorrek in de weefsels. Deze voorrek in combinatie met perfusie resulteerde in hartklepvliezen met uitstekende weefselvorming, de waarden overtreffend die worden genoemd in vergelijkbare studies. Tevens kwamen de mechanische eigenschappen in de buurt van wat de aortaklep vliezen van nature hebben en bleven ze intact wanneer blootgesteld aan de fysiologische stromingen die in de aortaklep positie voorkomen (hoofdstuk 6). Een duidelijk verschil is echter dat de natuurlijke aortaklep vliezen anisotroop gedrag vertonen, dat wil zeggen dat de mechanische eigenschappen verschillen met de richting waarin je ze meet, gerelateerd aan de zeer specifieke en georganiseerde weefselstructuur. Wanneer de tissue-engineerde vliezen dynamisch gerekt werden tijdens het kweken is aangetoond dat de weefselvorming niet zozeer verder werd gestimuleerd, maar dat er meer organisatie en oriëntatie in het weefsel ontstond, wat resulteerde in meer anisotrope eigenschappen.

Uit dit proefschrift kan geconcludeerd worden dat de rek-gerichte benadering zeer veelbelovend is voor tissue engineering van hartklep vliezen. Constant aanwezige voorrek in combinatie met perfusie leidt tot uitstekende weefseleigenschappen, terwijl dynamisch rekken een waardevolle methode representeert om anisotropie in het weefsel aan te brengen. Dit is de eerste studie die tissue-engineerde hartklep vliezen beschrijft, gebaseerd op een snel afbrekend scaffold materiaal, met voldoende weefsel eigenschappen om te kunnen functioneren in de aortaklep positie. Deze resultaten zijn veelbelovend en de volgende stap naar het testen van de functionaliteit van de vliezen in diermodellen, alvorens klinische implementatie, moet zeker gemaakt gaan worden (hoofdstuk 7).

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Anita Mol,
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Curriculum Vitae

Anita Mol was born on December 9, 1975 in Nijmegen, the Netherlands. In 1994, she finished her secondary education (VWO) at the Liemers College in Zevenaar. After four years of study at the Fontys Hogeschool Eindhoven, she obtained her bachelor degree in Medical Imaging (MBRT) in 1998. Internships for this study were performed at the department of radiology of the Hofpoort Hospital in Woerden, the department of radiotherapy of the Catharina Hospital in Eindhoven, the department of nuclear medicine of the Laurentius Hospital in Roermond, and an Erasmus internship at the University of Ulster in Belfast, Northern-Ireland. Her graduation project concerned a research project to decrease the amount of scatter radiation in the contralateral breast during breast radiotherapy and was performed at the department of radiotherapy of the Catharina Hospital in Eindhoven in collaboration with the department of radiotherapy of the VU Hospital in Amsterdam. Due to her broad interest in research, she started a two year follow-up study in Biological Health Sciences at the University of Maastricht. She obtained her master degree in 2000. Her graduation project involved a mutation analysis of the ATRX gene in various patient groups suffering from mental retardation and functional analyses of found mutations. In 2001, she started her PhD at the department of Biomedical Engineering at the Eindhoven University of Technology in close collaboration with the Clinic for Cardiovascular Surgery of the University Hospital Zurich in Switzerland, which resulted in this thesis.