Functional endothelium on tissue engineered small diameter vascular grafts

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Functional endothelium on tissue engineered small diameter vascular grafts

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en
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Summary

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In coronary and peripheral artery disease the native arteries are occluded or damaged, and thus arterial revascularization needs to be performed. Worldwide the major treatment is performing bypass graft procedures. Another medical need for vascular grafts is in patients with renal failure who depend on dialysis. Autologous arteries and veins, as well as synthetic grafts, are currently used as grafts for replacing small diameter blood vessels. However, some of these grafts have a limited life time. For example, saphenous veins, used as coronary bypass grafts, demonstrated a patency of only 57% after 10 years. In addition, vascular substitutes are increasingly in demand as the number of patients who need follow-up surgery and have run out of native graft material is increasing. Tissue engineered vascular grafts (TEVGs) could offer a good alternative to overcome the limitations in small vessel grafting by creating viable constructs with repair and remodeling capabilities.

Recently, large improvements have been made in the development of mechanically strong human TEVGs. Less research focused on the development of a functional endothelial cell (EC) layer on human TEVGs. The endothelium is a highly active layer involved in tissue homeostasis, regulation of vascular tone and growth regulation of other cell types. In addition, thrombosis, which is one of the causes of graft failure, is proactively inhibited by an intact and quiescent endothelium. Therefore, the main focus of the present thesis was the development of a functional EC layer on human TEVGs. These TEVGs were based on a PGA/P4HB scaffold seeded with human saphenous vein myofibroblasts (MF).

For the development of the endothelialized TEVGs, ECs and MFs have to be co-cultured. However, this co-culture is not trivial as these cells require different stimuli. In this thesis, first a 3D co-culture model was developed, which was used to optimize the co-culture conditions for human saphenous vein ECs and MFs. It was demonstrated that ECs did not survive in DMEM culture medium, but that ECs need a specific EC medium. Using this medium, it was demonstrated that a confluent EC layer could be cultured on strong cardiovascular constructs, when the ECs were seeded after 3 or 4 weeks of tissue development.

When in co-culture, ECs influence the phenotype of the cells in their environment. In the present thesis, this was demonstrated by a change in growth and αSMA expression of MFs due to co-culture with ECs. In addition, it was shown that the extracellular matrix composition of 3D cardiovascular construct was influenced by a layer of ECs. Functional ECs also need to be non-thrombogenic. Using the 3D co-culture model, in combination with a blood perfusion model system, it was demonstrated that the ECs indeed reduced the thrombogenicity of TE cardiovascular constructs.

Finally, a bioreactor system was developed in which small diameter TEVGs could be cultured and endothelialized. One day after EC seeding, the cell layer was nearly

confluent and the ECs had a cobblestone morphology. The seeded ECs were shear stress conditioned using a culture medium supplemented with xanthan gum to achieve a blood-analog viscosity. Xanthan gum is a stable thickener and low concentrations already result in high viscosities and shear-thinning behavior. The use of xanthan gum ensured that a physiological shear stress could be induced in the grafts using a physiological flow rate. It was shown that xanthan gum did not affect the growth of ECs, their alignment due to shear stress and their vasodilating properties. When shear stress was applied to the seeded ECs of the TEVGs, the ECs proliferated into a confluent layer. In addition, the ECs elongated and aligned in the direction of flow. In contrast, the cells did not form a confluent layer when no flow was applied.

In conclusion, the current thesis evaluates several EC functions using different model systems. In addition, a bioreactor system was developed and used to culture small diameter human TEVGs. After optimization of the culture conditions, a functional EC layer was created on these grafts, which was able to withstand a physiological shear stress. This functional EC layer is an important step towards the clinical use of these TEVGs.

Chapter 1

Introduction

1.1 Blood vessels

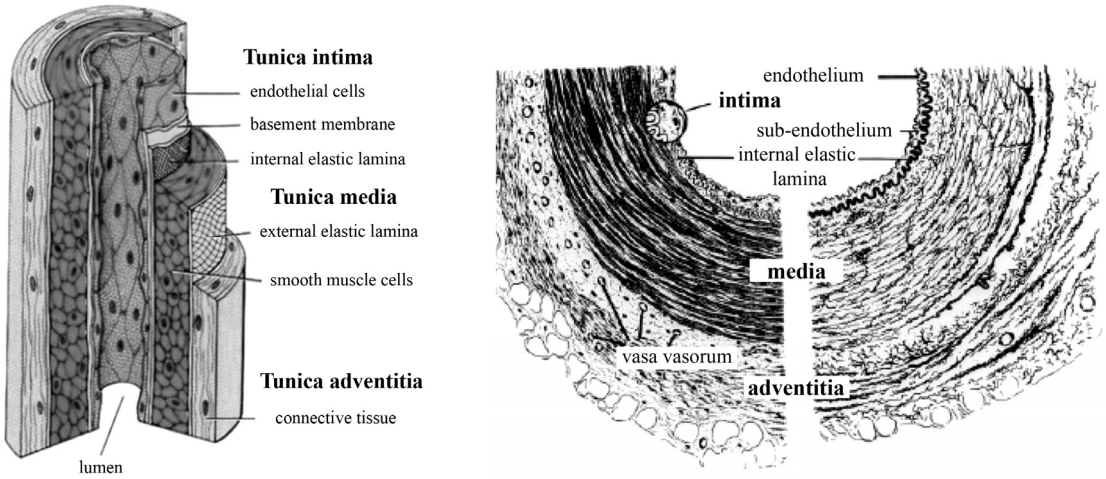
1.1.1 Anatomy of a blood vessel

Blood vessels are tubular structures consisting of three concentric layers (Figure 1.1A-B). From the outside to the inside of the vessel, these tubes are the tunica adventitia, tunica media, and tunica intima.

The tunica adventitia is a fibrous connective tissue, mainly containing adventitial fibroblasts in a matrix of collagen I and III, elastin fibers and glycoproteins. Due to the high collagen content, the tunica adventitia supplies most of the mechanical strength of the vessel. A network of small blood vessels that supply nutrients to the vessel wall, the vasa vasorum (Figure 1.1B), and a network of nerves that create neural control, the nervi vasorum, are also present in this layer. The external elastic membrane serves as a boundary layer between the fibroblasts in the adventitia and the smooth muscle cells (SMC) in the tunica media.

The tunica media contains mainly SMCs and elastin fibers. The SMCs are orientated circumferentially and are the responsive elements that set the vascular tone and regulate blood flow. The elastin fibers in this layer play an important role in the visco-elastic behavior of the vessel.

The tunica intima, positioned at the lumen, consists of a confluent monolayer of endothelial cells (ECs), residing on a basement membrane. The ECs are elongated cells, which are aligned in the direction of the main flow. Underneath this layer, the basement membrane is present, which is a sub-endothelial fibro-elastic connective tissue layer. Furthermore, an organized layer of internal elastic membrane provides stability to the ECs.



A **B**

Figure 1.1: Schematic drawing of a blood vessel (A). Schematic overview of histological cross-section (B) of a muscular artery (left) and elastic artery (right). Adapted from Patel et al. (Patel et al., 2006).

1.1.2 Endothelial cells

ECs, as the inner lining of blood vessels, are strategically located between blood and the vascular smooth muscle. In adults, the endothelium consists of approximately ten trillion (10^{13}) cells, covers approximately 700 m^2 and weighs about 1 kg (Galley and Webster, 2004). ECs are highly metabolically active, since they release many humoral factors (Figure 1.2). The endothelium plays an important role in many physiological functions, including the control of vasomotor tone, blood cell trafficking, hemostatic balance, permeability, and innate and adaptive immunity. ECs are heterogeneous and vary in phenotype depending upon the size, function, and location of the vessel (Aird, 2007a; Thorin and Shreeve, 1998).

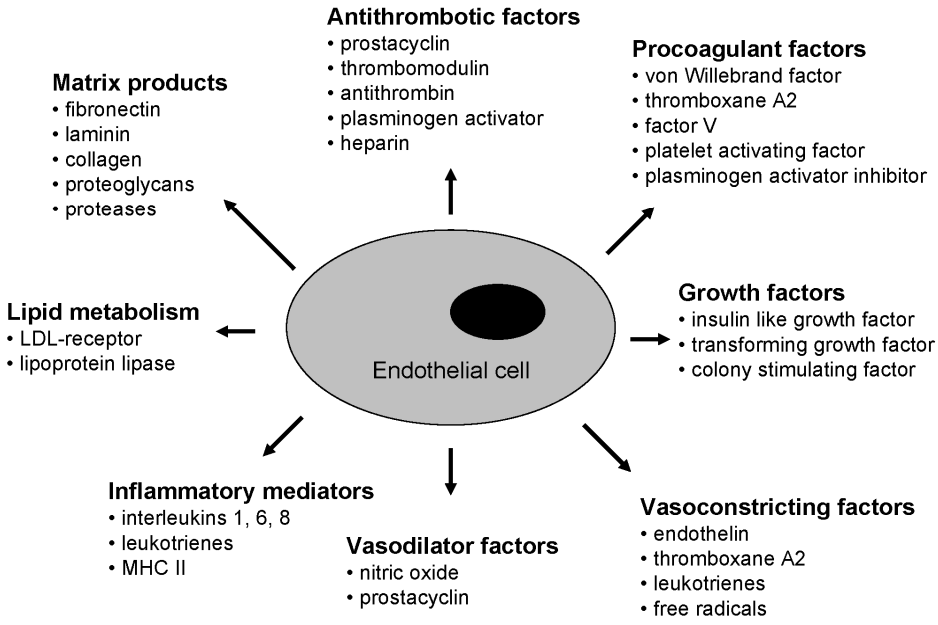


Figure 1.2: Endothelial cells release humoral factors that control vascular relaxation and contraction, thrombogenesis and fibrinolysis, and platelet activation as well as inhibition. Adapted from Galley and Webster (Galley and Webster, 2004).

ECs are involved in the coagulation cascade by expressing molecules such as thrombomodulin (TM) and tissue factor (TF). ECs also synthesize heparan sulfate proteoglycans that bind antithrombin III, which neutralizes and inhibits thrombin. Furthermore, ECs secrete nitric oxide (NO) and prostacyclin (PGI_2), which suppress platelet adhesion (Mitchell and Niklason, 2003). When coagulation does occur, ECs secrete tissue-type plasminogen activator (t-PA) and plasminogen activator inhibitor (PAI-1) which control fibrinolysis.

The endothelium is also involved in the modulation of vasoactivity. ECs release mediators which act as vasodilators, such as NO and PGI_2. The cells can also release vasoconstrictive agents, like endothelin and thromboxane A2 (Luscher and Barton, 1997). The ECs furthermore regulate vascular structure and protect the vessel wall from activation of vascular SMCs (Luscher and Barton, 1997).

1.1.3 Hemodynamic forces

Blood vessels are constantly subjected to and influenced by biomechanical forces inherently present due to the pulsatile nature of blood flow and pressure. This blood flow induces a hydrostatic pressure, a tensile strain in radial direction of the blood vessel, and a shear stress at the endothelial surface of the blood vessel. Although both hydrostatic pressure and stretch forces influence blood vessel physiology, shear stress is the main regulator of EC function and phenotype.

The shear stress ranges from approximately 0.1 to 0.6 Pa in the venous system and from approximately 1 to 7 Pa in the arterial vascular network and is dependent on the shape and diameter of the vessels (Malek *et al.*, 1999). In small diameter blood vessels, such as the coronary artery and radial artery, average shear stresses of 0.7 Pa (Doriot *et al.*, 2000) and 0.82 Pa (Girerd *et al.*, 1996), respectively were reported. Shear stress has been shown to influence vessel wall remodeling (Girerd *et al.*, 1996; Tronc *et al.*, 1996). Specifically, chronic increases in blood flow, and consequently shear stress, such as seen in the radial artery of dialysis patients (Girerd *et al.*, 1996), lead to expansion of the vessel radius returning the mean shear stress to its baseline level. The opposite effect occurs when the shear stress is decreased.

Under physiological arterial hemodynamic shear stress, the ECs are aligned in the direction of flow, in contrast with a low shear stress where the ECs have a cobblestone-shaped morphology. Many of the humoral factors released by the ECs, such as vasoconstrictors, vasodilators, growth regulators, and antithrombotic factors, are influenced by shear stress (Barakat and Lieu, 2003). In general, ECs can switch their phenotype from a quiescent atheroprotective phenotype under physiological and elevated levels of shear stress to an atherogenic phenotype at low shear stress (Malek *et al.*, 1999).

1.2 Arterial revascularization

In coronary artery disease (CAD) and peripheral artery disease (PAD) the native arteries are occluded or damaged, and thus arterial revascularization needs to be performed.

In 2005, 16 million people were suffering from CAD in the USA (Rosamond *et al.*, 2008). In CAD, the coronary arteries, which supply blood to the heart, are occluded due to a build up of atherosclerotic plaque. Occlusion of these arteries leads to oxygen deprivation of the heart muscle, which can lead to chest pain or a myocardial infarction. Besides the large number of CAD patients, there are also 8 million people suffering from PAD (Rosamond *et al.*, 2008). Narrowing of arteries in the leg leads to a cramping pain caused by an inadequate supply of blood to the affected muscle. Worldwide the major treatment for revascularization in CAD and PAD is bypass grafting. In 2005, approximately 470,000 coronary artery bypass graft (CABG) procedures were performed in the USA on patients suffering from cardiovascular disease (Rosamond *et al.*, 2008).

Another medical need for arterial grafts is in patients with renal failure who depend on dialysis. This procedure requires frequent access to the peripheral circulation, which is usually facilitated by the creation of an arteriovenous shunt in the arm (Ethier *et al.*, 2008). In 2006, approximately 350,000 patients with advanced and permanent kidney failure were on hemodialysis (United States Renal Data System, 2008). The shunt however is subject to repeated cannulation and has a limited lifetime. In approximately 40% of the patients, an arteriovenous graft (AVG) was inserted to allow continued hemodialysis (United States Renal Data System, 2008).

Autologous arteries, such as the internal mammary artery and radial artery, are used for treatment of CAD (Campbell and Campbell, 2007; Motwani and Topol, 1998; Raja *et al*., 2004). In addition, autologous veins, such as the saphenous vein, are used for the treatment of CAD and PAD. These veins have also been used for the creation of AVG in hemodialyis patients (Haimov *et al.*, 1980). Although saphenous vein grafts are widely used, thrombosis, occlusion and aneurysm formation (Berardinelli, 2006; Motwani and Topol, 1998; Raja *et al.*, 2004; Verma *et al.*, 2004) occur frequently. Saphenous veins, used for CABG, demonstrated a patency of 57% after 10 years (Sabik III *et al.*, 2005). The 5-year saphenous vein graft failure rate of lower extremity peripheral grafts is reported to be 30-50% (Owens *et al.*, 2008). Unfortunately, due to age, vascular disease, or previous harvest, many patients have no suitable autologous arteries or veins (Amiel *et al.*, 2006; Jankowski and Wagner, 1999; McKee *et al.*, 2003). Due to increased operating time for saphenous vein harvesting, and the need to preserve the vein for peripheral vascular or coronary arterial revascularization the focus for hemodialysis vascular access has shifted from autologous vessels to other vascular substitutes (Berardinelli, 2006).

Synthetic grafts, such as expanded polytetrafluoroethylene (ePTFE) or Dacron (polyethylene terephthalate fiber) have been used successfully to bypass large-diameter arteries, with a high flow and a low resistance. However, the patency rates have been disappointing when they were used to replace small diameter (< 5 mm) arteries, such as the coronary and the infragenicular vessels (Sapsford *et al.*, 1981; Steinthorsson and Sumpio, 1999). This is mainly caused by increased thrombogenicity and accelerated intimal thickening, which lead to early graft stenosis and occlusion (Ao *et al.*, 2000; Sayers *et al.*, 1998). In CAD, synthetic grafts are therefore rarely used. This is in strong contrast with hemodialysis vascular access, where PTFE grafts are most common. Although relatively easy to place and ready to use, these grafts still have high rates of stenosis, thrombosis, and infection (Roy-Chaudhury *et al.*, 2006). Tissue engineered blood vessels could offer a good alternative to overcome the limitations in small vessel grafting by creating viable constructs with repair and remodeling capabilities.

1.3 Tissue engineering

1.3.1 Tissue engineering of vascular grafts

Tissue engineering (TE) has the potential to overcome some of the previously mentioned shortcomings, as these TE constructs consist of autologous living cells which are able to adapt to their environment (Vacanti and Langer, 1999). A common paradigm for TE uses cells which are isolated from a patient (Figure 1.3). These cells are seeded onto a scaffold, which provides a temporary skeleton to support the growing tissue and provides the desired shape until the cells produce their own extracellular matrix (ECM). Ideally, such a scaffold is biodegraded while the new tissue is forming around it. These seeded constructs are placed in a bioreactor system, where they are mechanically and/or biochemically stimulated. These stimuli enhance tissue formation and development. In cardiovascular TE, ECs are seeded after a certain culture period, and subsequently conditioned. This results in an autologous tissue which can be, ideally, implanted into the patient.

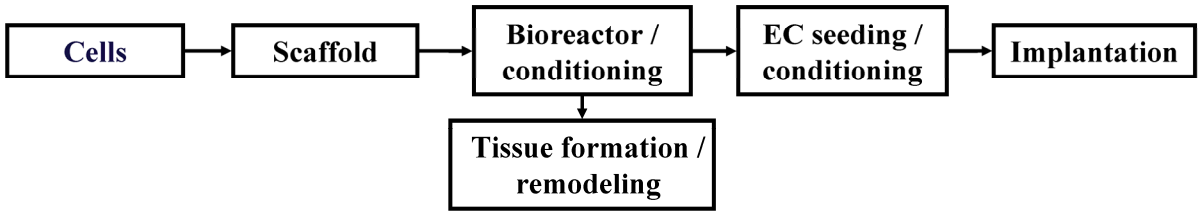


Figure 1.3: Tissue engineering paradigm including cells, scaffold, bioreactor conditioning, EC seeding, and implantation.

Small diameter blood vessels and heart valves have been the focus of tissue engineering research in the cardiovascular field. The ideal tissue engineered vascular graft (TEVG) has many desirable characteristics (Table 1.1). At a minimum, it must contain appropriate mechanical properties, like burst pressure and compliance. Furthermore, it should have the correct elasticity to prevent aneurysm formation. In addition, a TEVG should allow complete healing without any immunological reaction, should remodel according to cues from the environment, and even have the ability to grow when placed in children. Finally, a TEVG should contain a stable functional endothelium. This endothelium should be able to withstand the shear forces of blood, should have non-thrombogenic and mechanotransducing properties. Hemodialysis grafts have some additional challenges, as the grafts have to be punctured several times a week and have to withstand higher hemodynamic loads, because the flow in these grafts is very high.

Table 1.1: Characteristics of an ideal artificial arterial substitute (Campbell and Campbell, 2007).

- Appropriate mechanical properties
  - Burst pressure
  - Elasticity
  - Compliance
  - Suturability
- Biocompatible and biostable
  - Non-inflammatory
  - Non-toxic
  - Non-carcinogenic
  - Non-immunogenic
- Capable of remodeling
- Thromboresistant
- Infection resistant
- Vasoactive
- Easy to handle
- Easily available in different specifications
- Ease of manufacturing
  - Cheap
  - Short time
  - "Off the shelf" availability

In the last 2 decades, there has been extensive research on the development of small diameter TEVGs. Several approaches have been investigated, such as using collagen (Berglund *et al.*, 2003) or fibrin scaffolds (Isenberg *et al.*, 2006), using a cell sheet-based technique (L'Heureux *et al.*, 2006), and using biodegradable scaffolds (Niklason *et al.*, 2001; Poh *et al.*, 2005; Stekelenburg *et al.*, 2008). Berglund *et al*. developed collagen-based blood vessels seeded with human SMCs and ECs. However, without a cross-linked support sleeve the vessels had poor mechanical properties, i.e. burst pressures of 100 mmHg (Berglund *et al.*, 2003). Isenberg *et al*. created fibrin based media-equivalents from rat aorta cells, which resulted in burst pressures of approximately 241 mmHg (Isenberg *et al.*, 2006).

L'Heureux *et al*. developed a completely autologous approach called sheet-based TE (L'Heureux *et al.*, 2006). Dermal fibroblasts were obtained from a small skin biopsy and grown into sheets, which were rolled around a support mandrel to form a tubular structure. After a culture period of 24 weeks, the resulting TEVGs had burst pressures in excess of 3000 mmHg. In a first clinical safety study, these TEVGs were implanted as arteriovenous shunts in hemodialysis patients. The results are encouraging as the TEVGs were functioning well for hemodialysis access with follow-ups of 12 months (L'Heureux *et al.*, 2007b).

Several biocompatible and biodegradable polymers have been used as scaffolds for the construction of TEVGs. Polyglycolic acid (PGA), which is bioabsorbed within 6-8 weeks, is most commonly used. Niklason *et al*. seeded PGA scaffolds with bovine arterial smooth muscle and endothelial cells and created TEVGs which had burst pressures of 2150 mmHg (Niklason *et al.*, 1999). The grafts were implanted as autologous saphenous artery interposition grafts which remained patent for one month. Despite these encouraging results, when human cells were used in the same model system, the TEVGs lacked appropriate mechanical properties (Poh *et al.*, 2005).

To increase the mechanical properties of the scaffold, copolymers have been produced by combining PGA with other polymers, such as polylactic acid (Shin'oka *et al.*, 2005) and poly-4-hydroxybutyrate (P4HB)(Hoerstrup *et al.*, 2001). Shin'oka *et al*.

constructed biodegradable TEVGs made of a polycaprolactone-polylactic acid copolymer reinforced with woven PGA with 12-24 mm diameters. These TEVGs were used to reconstruct the low-pressure pulmonary outflow tract in pediatric patients (Shin'oka *et al.*, 2005). This is one of the first studies to demonstrate the feasibility of the successful usage of TEVGs in the clinic. The application of this method for the replacement of small diameter blood vessels still has to be investigated.

A co-polymer of PGA-P4HB, seeded with ovine myofibroblasts (MF) and ECs, was used by Hoerstrup *et al*. for the creation of TEVGs. A bioreactor system, which applied a direct flow through the vascular lumen, was used for 4 weeks and resulted in grafts with sufficient suture strength and burst pressures of 336 mmHg (Hoerstrup *et al.*, 2001). Human saphenous vein-derived MFs have been used by Mol *et al.* for tissue engineering of human heart valves (Mol *et al.*, 2006). These cells were characterized as a mixture of vimentin and vimentin/α-smooth muscle actin positive cells. It has been demonstrated that vein-derived MFs are superior to aortic derived cells with respect to collagen formation and mechanical stability of tissue engineered constructs (Schnell *et al.*, 2001). Stekelenburg *et al*. seeded PGA-P4HB scaffolds with these vein-derived MFs using a fibrin gel as a cell carrier. The scaffolds were cultured around a silicone tube and dynamically conditioned for 4 weeks. The combination of fibrin and dynamic conditioning resulted in TEVGs with burst pressures of 900 mmHg (Stekelenburg *et al.*, 2008).

In general, these studies indicate that the creation of mechanically strong TEVGs is feasible and that more research is necessary to create a functional endothelial layer.

1.3.2 Endothelial cells on vascular grafts

In some of the aforementioned studies, the TEVGs were not seeded with ECs, in others ECs were seeded, but the resistance to physiological shear stress was not demonstrated. Niklason *et al*. showed that the application of only a perfusion flow revealed a rounded EC morphology and less than complete EC coverage on bovine TEVGs (Niklason *et al.*, 2001). The clinically used grafts presented by Shin'oka *et al*. did not contain an EC layer prior to implantation. Although the 2 months old explant showed some endothelium-like cells, it is questionable whether a similar small diameter graft would stay patent (Shin'oka *et al.*, 2005).

Many studies have shown that the patency of synthetic grafts is improved by EC seeding (Hoenig *et al.*, 2006; Laube *et al.*, 2000; Meinhart *et al.*, 2001; Seifalian *et al.*, 2002). Endothelialized small diameter (6-7 mm) ePTFE grafts showed a 7 year patency rate of 62.8% when implanted in infrainguinal positions (Meinhart *et al.*, 2001). A similar approach was used for CABG and showed a patency rate of 90.5% after a mean postoperative follow-up of 27.7 months (Laube *et al.*, 2000). Despite the relatively short follow-up period of the latter study, endothelialized ePTFE grafts seem a promising alternative for saphenous vein grafts. However, ePTFE grafts still lack the capacity to remodel and grow, so the use of endothelialized TEVGs is worthy of investigation.

In animal models, high patency rates were observed after implantation of EC seeded TE grafts with a follow up of several weeks (Borschel *et al.*, 2005; Dardik *et al.*, 1999; Niklason *et al.*, 1999; Swartz *et al.*, 2005). However, it is suggested that host derived re-endothelialization is occurring and that the seeded endothelium is no longer present at the time of explantation (Borschel *et al.*, 2005; Swartz *et al.*, 2005). It is commonly assumed that re-endothelialization of vascular grafts is slow and almost never complete in humans (Berger *et al.*, 1972; Rahlf *et al.*, 1986). Therefore, the favorable outcome in animal studies may not be indicative of clinical success.

In vitro conditioning of EC covered constructs with shear stress appears to prevent *in vivo* loss of endothelium. The application of shear stress for EC retention has been studied in several systems. Applying a shear stress too early in the culture process results in an incomplete coverage (Hoerstrup *et al.*, 2001). *In vitro* shear stress conditioning, by slowly increasing the shear stress over the course of several days, increases EC retention (Kaushal *et al.*, 2001) and reduces neointima formation after implantation (Dardik *et al.*, 1999).

1.4 Rationale and outline

Recently, strong human TEVGs have been developed in our group, with burst pressures of 900 mmHg (Stekelenburg *et al.*, 2008). The grafts were based on a PGA scaffold coated with P4HB and seeded with human saphenous vein myofibroblasts (MF) in a fibrin gel. Although these grafts had sufficient mechanical properties, they did not have an EC layer and were therefore not suitable for *in vivo* application.

The present thesis focuses on the development of a functional EC layer on human TEVGs. As a first step, the growth of saphenous vein ECs on saphenous vein MF seeded rectangular cardiovascular constructs was investigated to create a 3D co-culture model system (Chapter 2). This model system was used to investigate the influence of ECs on the ECM production of MFs (Chapter 3). One of the functions of ECs is their capacity to influence the phenotype of smooth muscle cells (SMC) in the vessel wall. To investigate whether human saphenous vein ECs were capable of influencing the SMC characteristics of human saphenous vein MFs, layers of ECs and MFs were co-cultured. Afterwards, the expression of several SMC markers was analyzed using RT-PCR and immunohistochemical assays (Chapter 4). Functional ECs need to be non-thrombogenic. Therefore, a method was developed to investigate the thrombogenicity of endothelialized cardiovascular constructs (Chapter 5). Shear stress conditioning is a method to improve orientation and retention of ECs in TEVGs. However, often a high non-physiological flow rate is necessary to create a physiological shear stress. Increasing the viscosity of the culture medium can overcome this. Therefore, the use of xanthan gum as a viscosity increasing additive was investigated in detail (Chapter 6). A bioreactor system was developed for the creation of TEVGs. With this system, ECs can be seeded in TEVGs and they can be conditioned using a physiological shear stress (Chapter 7). In the last chapter, a general discussion about the results is presented and conclusions are drawn (Chapter 8).

Chapter 2

Development of a 3D co-culture model system for the investigation of vascular cell interactions

2.1 Introduction

Tissue engineering (TE) of small diameter (<5 mm) blood vessels is a promising approach to develop viable alternatives for autologous vascular grafts (L'Heureux *et al.*, 2006; Niklason *et al.*, 2001; Stekelenburg *et al.*, 2008). Several different approaches have resulted in tissue engineered vessels with sufficient mechanical properties for implantation. The development of a functional, confluent endothelial layer, resistant to shear forces is necessary for tissue engineered vascular grafts, but has proven a challenge (Niklason *et al.*, 2001).

The successful creation of a confluent EC layer on vascular grafts depends on several factors, such as cell seeding density (Salacinski *et al.*, 2001), seeding method (Pawlowski *et al.*, 2004) and the recipient surface, i.e. coating. The type of material and coating affect attachment of ECs, but also their ability to stretch and proliferate (Foxall *et al.*, 1986; Kaehler *et al.*, 1989; Zhang *et al.*, 1995). Although seeding of ECs is feasible on several prostheses, attachment and shear-stress resistance remains a challenge.

Many research groups have examined whether modifications of prosthetic graft surfaces can either stimulate self-endothelialization or allow preseeded cells to remain attached better (Gulbins *et al.*, 2004; Salacinski *et al.*, 2001). Coatings of prosthesis materials with natural occurring extracellular matrix (ECM) proteins such as fibronectin, collagen or fibrin appreciably improve adhesion and subsequent proliferation of endothelial cells (Consigny and Vitali, 1998; Foxall *et al.*, 1986; Kaehler *et al.*, 1989; Zhang *et al.*, 1995). It is expected that, in contrast to prosthetic grafts, the attachment and proliferation of ECs on TE grafts is easier, as they already consist of ECM proteins.

Strong small diameter TE vascular grafts are being cultured by us with burst pressures of 900 mmHg (Stekelenburg *et al.*, 2008). The constructs are based on a polyglycolic acid (PGA) scaffold coated with poly-4-hydroxybutyrate (P4HB) and seeded with human myofibroblasts (MF) in a fibrin gel. These grafts mainly consist of collagen and glycosaminoglycans. It is expected that these proteins provide sufficient cell recognition sites for ECs to attach and proliferate into a confluent layer. On the other hand, degradation products of PGA are known to influence cell behavior (Higgins *et al.*, 2003) and hamper EC growth (Dvorin *et al.*, 2003; Musey *et al.*, 2002). Furthermore, ECs seeded on fibrin matrices can grow in capillary-like tubular structures when stimulated by angiogenic growth factors (van Hinsbergh *et al.*, 2001).

The goal of the present study was to seed human saphenous vein ECs on human saphenous vein MF seeded PGA/P4HB strips and investigate the capacity of the ECs to form a confluent monolayer on such 3D constructs. In this 3D cardiovascular co-culture model system the interaction between ECs and MFs could be studied. As growth medium differs for the two cell types growth capacity of ECs in tissue engineering medium was studied first.

2.2 Materials and Methods

2.2.1 Cell Culture

Human endothelial cells (ECs) and myofibroblasts (MF) were harvested from the same fresh discarded vein segment of the human saphenous vein (Chapter 3) and expanded using standard culture methods up to passage 7 (Mol *et al.*, 2006; Schnell *et al.*, 2001). The MFs were characterized as a mixture of vimentin and vimentin/actin type MFs. The culture medium for MFs consisted of DMEM Advanced (Invitrogen, Netherlands), supplemented with 10% FBS, 1% GlutaMax (Gibco) and 0.1% gentamycin (Biochrom, Germany). The EC culture medium consisted of EGM-2 EC medium supplemented with growth additives (Cambrex, Belgium), containing hydrocortisone (0.04%), human fibroblast growth factor B (0.4%), vascular endothelial growth factor (0.1%), R^3-insulin-like growth factor 1 (0.1%), ascorbic acid (0.1%), human epidermal growth factor (0.1%), gentamicin sulfate amphotericin-B (0.1%) and heparin (0.1%) and 20% Fetal Bovine Serum (FBS; Greiner, Austria), further referred to as EC medium. The medium used for EC-MF tissue constructs, referred to as TE medium, was the same as for MFs, with 0.3% instead of 0.1% gentamicin, and supplemented with L-ascorbic acid 2-phosphate (0.25 mg ml^{-1}; Sigma).

2.2.2 Endothelial cell proliferation experiment

Ideally, one type of culture medium has to be used for the co-culture model, as TE medium appears optimal for TE of cardiovascular tissues (Mol *et al.*, 2006; Stekelenburg *et al.*, 2008), but may not sustain EC growth. Therefore, growth of vein-derived ECs was studied in this medium. ECs (passage 6) were seeded in 25 cm^2 flasks (n=4, 5000 cells cm^{-2}) in EC medium. After 1 day, the medium of 2 flasks was changed to TE medium whereas the others were kept on EC medium. To analyze the growth of the cells in both culture media, photographs were taken daily using a digital camera mounted on a microscope. The number of cells was determined by manually counting the cells in the images.

2.2.3 Construct fabrication and tissue culture

For the creation of the co-culture model, rectangular shaped scaffolds (n=12, 20 x 7 mm) composed of fast degrading nonwoven polyglycolic acid (PGA) (thickness 1.0 mm, specific gravity 70 mg cm^{-3}; Cellon, Bereldange, Luxembourg) were coated with poly-4-hydroxybutyrate (P4HB) (Symetis Inc, Zürich, Switzerland). The ends of the constructs were glued in a 6 well plate using a 20% solution of polyurethane (PU) (DSM, Netherlands) in tetrahydrofuran, leaving a 15 x 7 mm remaining surface for cell seeding (Figure 2.1). The solvent was allowed to evaporate overnight. The constructs were sterilized by placing them in 70% ethanol for 4 hours. Afterwards, the constructs were washed with PBS and placed in EC medium overnight. MFs of passage 7 were

seeded at a density of $3.3 \cdot 10^4$ cells mm^{-3} scaffold using bovine fibrin as cell carrier (Mol *et al.*, 2005). Three days later, ECs (passage 7) were added (Figure 2.2) by dripping the cell solution on the top of the constructs, resulting in a density of $5 \cdot 10^4$ cells cm^{-2}. Non EC-seeded samples served as control.

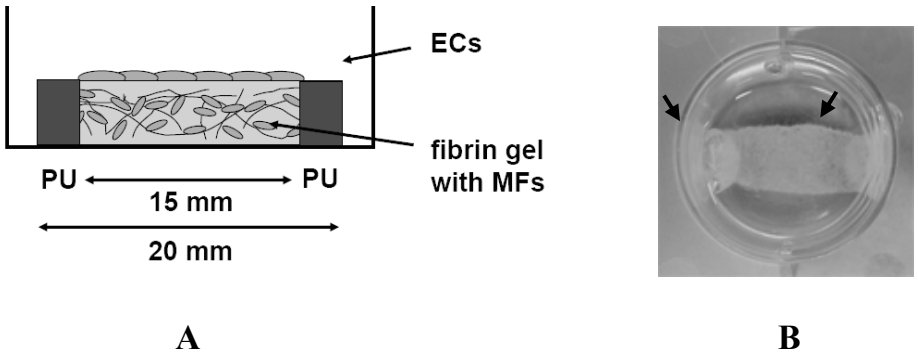


Figure 2.1: Schematic drawing (A) and top view photograph (B) of construct with polyurethane glue (PU, arrows).

2.2.4 Qualitative tissue analyses

At several time points (day 4, 7, 10, 14, 21), samples (n=2) were removed from the wells and examined for the presence of ECs (Figure 2.2). The samples were stained for 45 minutes with FITC UEA-1 lectin (Sigma), which is specific for ECs (Hormia *et al.*, 1983), and simultaneously with Cell Tracker Orange (CTO, Invitrogen, USA), which stains viable ECs and MFs. The constructs were analyzed using a confocal laser scanning microscope (CLSM; Axiovert 100M, Zeiss, Göttingen, Germany). The FITC UEA-1 lectin and CTO were excited at 488 and 543 nm, respectively and its emission was recorded between 505 and 530 and above 570, respectively. To visualize the EC layer, Z-projections and Y-projections of the z-stacks were produced.

Tissue morphology was further studied by histology. Samples were fixed in phosphate-buffered formaldehyde (3.7%) and embedded in paraffin. Cross sections (10μm) of the short axis were stained with hematoxylin and eosin (H&E).

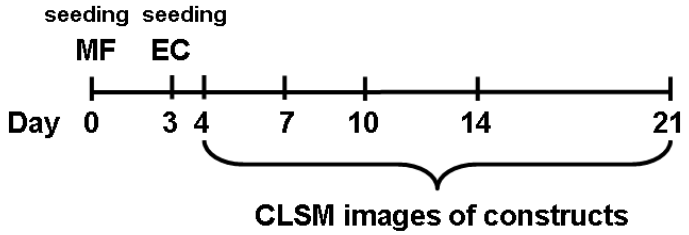


Figure 2.2: Experimental protocol for qualitative tissue analysis.

2.2.5 Quantitative tissue formation analysis

To quantify tissue formation after three weeks (n=2), the amount of DNA, sulfated glycosaminoglycans (GAGs), and hydroxyproline, was measured and expressed in mg per dry weight of tissue. For the analyses, lyophilized tissue samples were digested using a papain solution (100 mM phosphate buffer, 5 mM L-cysteine, 5 mM ethylenediaminetetraacetic acid (EDTA), and 125 to 140 µg papain ml^{-1}) at 60°C for 16 hours. The Hoechst dye method (Cesarone *et al.*, 1979) with a reference curve of calf thymus DNA (Sigma) was used to determine the DNA content. Using a modification of the assay described by Farndale *et al*. (Farndale *et al.*, 1986) and a shark cartilage chondroitin sulfate reference (Sigma), the sulfated GAG content was determined. The hydroxyproline content was determined with an assay modified from Huszar *et al*. (Huszar *et al.*, 1980) and a reference of trans-4-hydroxyproline (Sigma). The ratio of hydroxyproline to collagen was assumed to be 0.13. To obtain a measure for the amount of matrix components produced per cell, the collagen and GAG content were normalized for the amount of DNA.

2.3 Results

2.3.1 Influence of culture medium on EC proliferation

The growth of ECs cultured in EC medium and TE medium was investigated. The cells in TE medium ceased to proliferate after 1 day (Figure 2.3) and almost all cells had died after 5 days (Figure 2.4A), whereas the cells in EC medium proliferated and formed a confluent layer (Figure 2.4B). Thus, TE medium was not conducive to EC proliferation and survival.

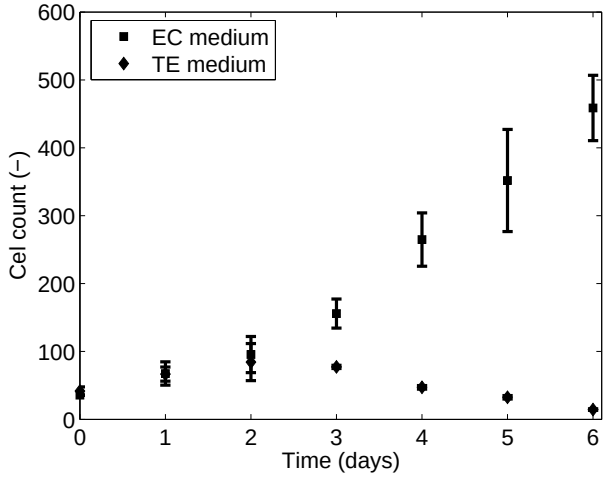


Figure 2.3: Growth curves of ECs cultured in EC medium and TE medium (mean ± s.d.). At day 1 the EC medium was changed to TE medium. The declining curve of TE medium shows that the ECs did not survive.

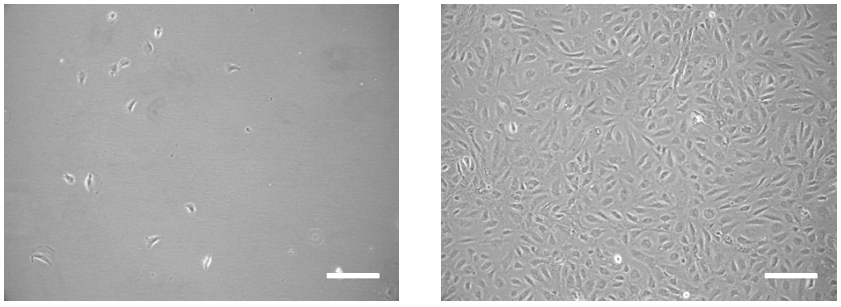


Figure 2.4: Representative phase contrast images of ECs (day 6) in TE medium (A) and EC medium (B). The ECs have grown confluent in the EC medium, but did not survive in TE medium. Scale bars represent 50 µm.

2.3.2 Endothelial cells on TE constructs

For visualization of the developing EC layer, projections of z-stacks were made with the CLSM. Due to the flat morphology of the ECs, the CTO staining of the ECs

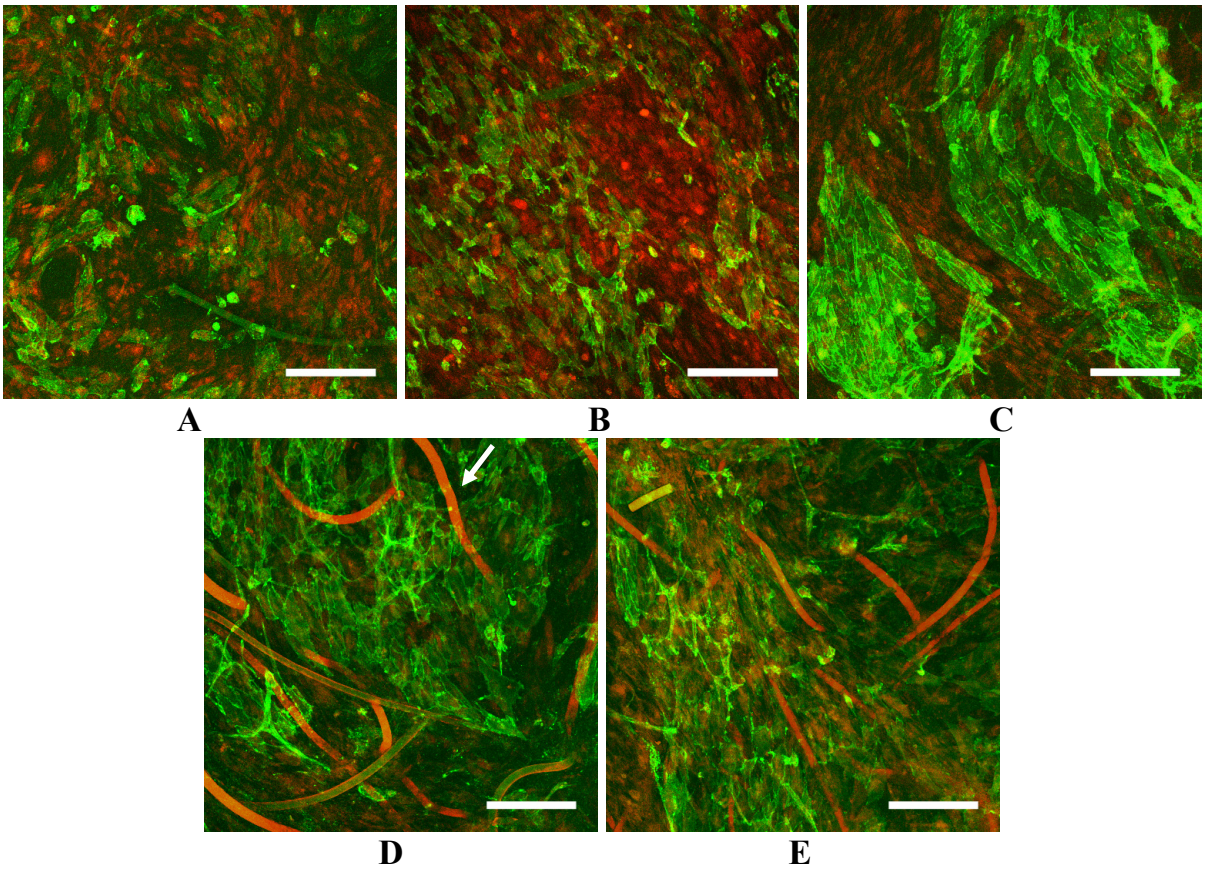


Figure 2.5: Z projections of EC layer at day 4, 7, 10, 14, and 21 (A, B, C, D, E), showing FITC UEA-1 stained ECs (green) and CTO stained MFs (red) and PGA fibers (autofluorescence: red). More confluent patches of ECs were found on day 14 and 21. Scale bars represent 200 µm, arrow indicates PGA fiber.

was limited. One day after EC seeding (day 4), 40% of the surface was covered with single ECs (Figure 2.5A). At day 7, more groups of ECs were found, suggesting proliferation of the cells (Figure 2.5B). Large parts of the constructs were covered with ECs at day 10 (Figure 2.5C). At days 14 and 21 EC coverage ranged from 60-100% (Figure 2.5D-E).

In addition to patches of confluent ECs on the surface, FITC-UEA-1 positive EC tube-like-structures (TLS) were also found below the surface. TLS were first seen at day 10, but they were more pronounced at day 14 and 21. They were up to 500 µm long and were found up to 80 µm below the surface of the construct (Figure 2.6A-C). The TLS were detected at different positions. TLS were found directly situated under an EC monolayer or in areas without EC coverage. Furthermore, the ECs sometimes formed TLS which were situated on top of the constructs. Some TLS seem to be connected to the overlying EC monolayer (Figure 2.6D), suggesting that they originated from that layer. Figure 2.7 shows several examples of TLS found in the constructs. In most cases, the TLS had side branches and were organized in small networks. Using higher magnifications, it was not possible to detect a clear lumen in the TLS (Figure 2.8).

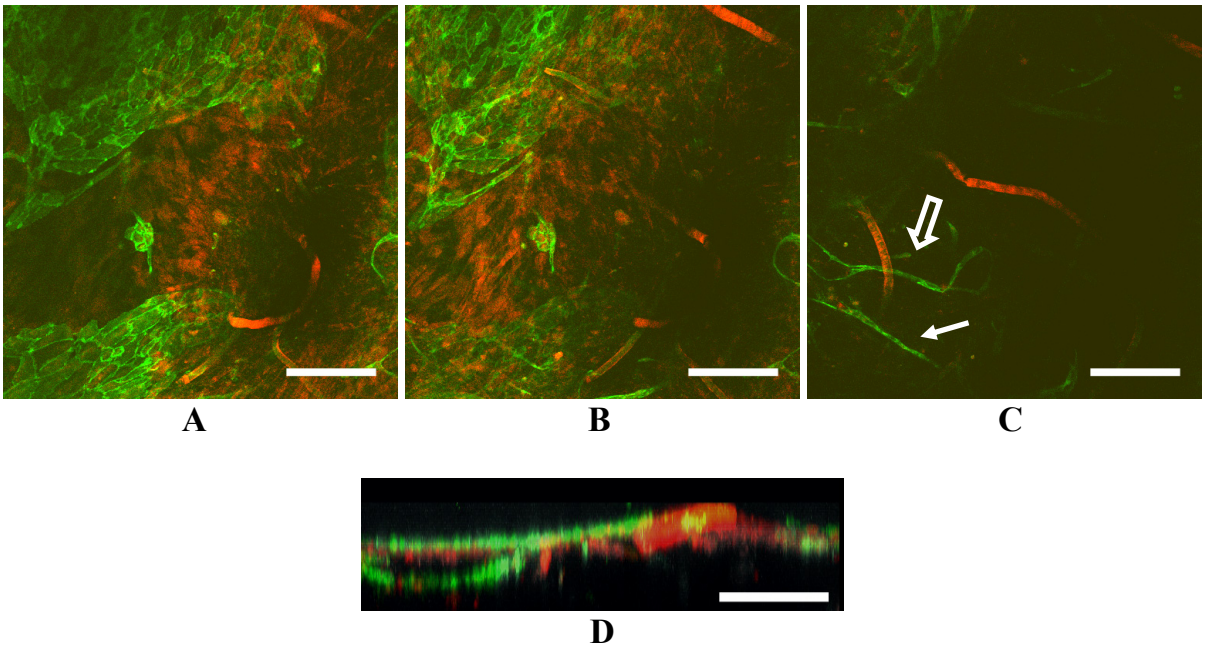


Figure 2.6: CLSM images of construct at the surface (A) and 20 and 80 µm (B, C) deeper into the tissue, showing typical examples of TLS formed by the ECs (green) in a MF (red) layer. At the surface (A) patches of confluent ECs can be seen. The TLS is situated 80 µm below the surface of the construct (C), but is not seen at a depth of 20 µm (B). One TLS was situated below a patch of ECs (closed arrow) and the other in the MF layer without an EC layer present (open arrow). A sideway projection of the TLS (closed arrow) shows the connection to the EC monolayer (D). Scale bars represent 200 µm.

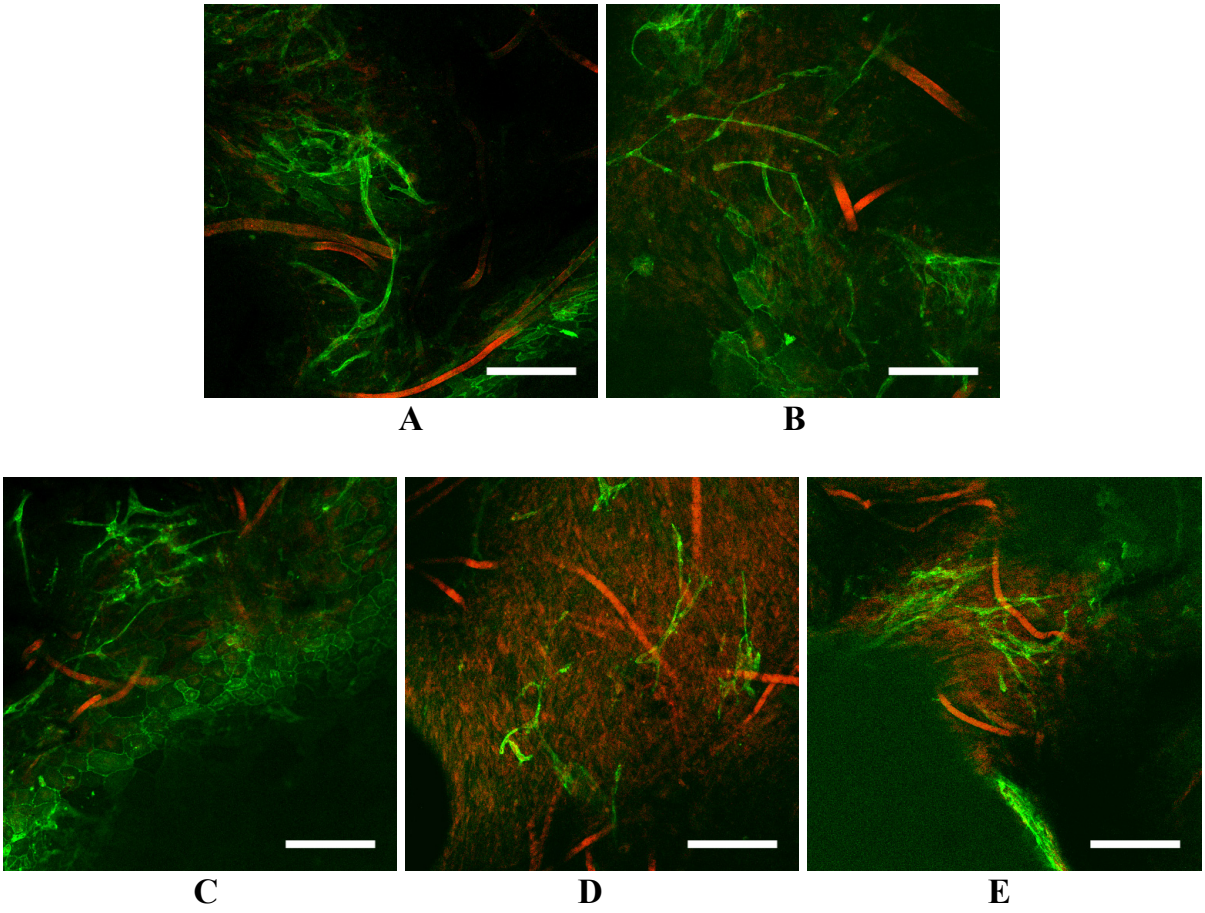


Figure 2.7: Several TLS (green) found at day 15 (A, B) and day 22 (C, D, E) in the MF (red) layer. PGA fibers (autofluorescence: red) were also present. Scale bars represent 200 µm.

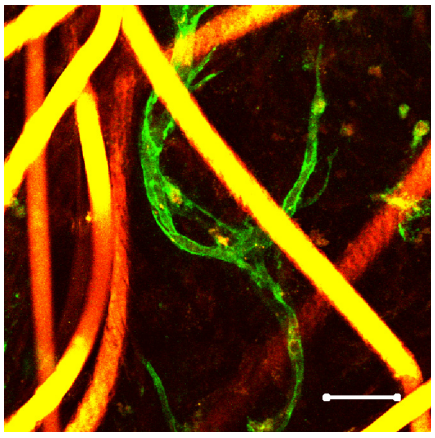


Figure 2.8: Higher magnification of TLS (green) in between autofluorescent PGA fibers (yellow/red). Scale bar represents 50 µm.

2.3.3 Extracellular matrix composition

When the constructs were taken out of the 6 well plates after 3 weeks, they had a very loose structure. They were almost transparent with some of the scaffold still visible. Hardly any compaction had occurred, indicating that the cells had not produced much ECM. This was also reflected by the quantitative analyses of the ECM components, where hardly any GAGs and collagen were found in the constructs (Figure 2.9A-B). The average collagen content was 13 µg/mg, equaling approximately 4% of the amount in human coronary arteries (Ozolanta *et al.*, 1998).

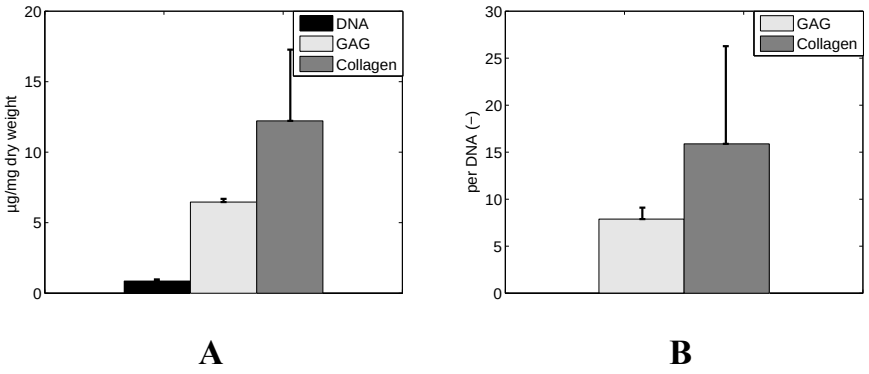


Figure 2.9: ECM composition of tissue strips (day 21) per dry weight (A) and per DNA (B) (mean ± s.d.). The amounts of GAGs and collagen were very low, indicating little tissue formation.

2.3.4 Histological analysis

Due to the low tissue density and the stiff scaffold remnants, it was not possible to obtain histological paraffin sections from the 4 to 15 days old constructs. Because of degraded scaffold material, it was only possible to obtain sections of the last group (day 21). H&E staining revealed that only at the top of the constructs some tissue was present (Figure 2.10). The rest of the construct contained sparse cells and a lot of scaffold remnants.

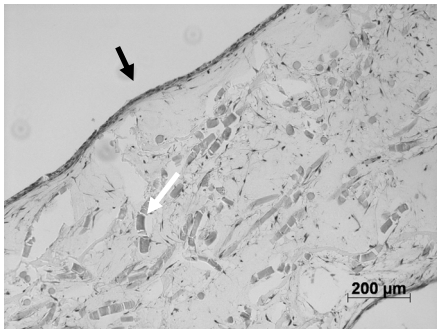


Figure 2.10: H&E stained section showing only tissue formation at the top of the construct (black arrow) and a lot of scaffold remnants (white arrow).

2.4 Discussion

In the current study, human saphenous vein ECs were seeded on MF seeded PGA/P4HB strips in order to investigate the capacity of these cells to form a confluent monolayer on such constructs. A proliferation experiment showed that the ECs did not grow in TE medium, therefore it was necessary to culture the constructs in EC medium for the whole experiment. However, the developed tissue did not resemble that of previous studies (Boerboom *et al.*, 2008; Stekelenburg *et al.*, 2008). ECM formation was sparse and consequently the tissue had a very loose structure. Poor tissue formation was probably caused by changing the culture medium. However, the ECs did seem to proliferate on the surface of the construct, but did not always form a confluent monolayer. Remarkably, the ECs started to invade the constructs and formed small networks of tube like structures (TLS) below the surface. The TLS were up to 500 μm long and found up to 80 μm deep.

Advanced DMEM supplemented with ascorbic acid, in our case referred to as TE medium, is often used for cardiovascular TE experiments (Mol *et al.*, 2006; Stekelenburg *et al.*, 2008) as it is optimized for collagen production (Hoerstrup *et al.*, 2000). Several culture media, including regular DMEM, have been used to successfully culture human saphenous vein ECs (Karim *et al.*, 2006; Terramani *et al.*, 2000), however it was shown in the current study that the ECs were unable to survive in TE medium. Compared to EC medium, TE medium lacks specific growth factors such as VEGF and hEGF, which are known to stimulate EC growth. Supplementing these growth factors to TE medium did not improve the EC viability (data not shown). In addition, the approximately 5-fold higher glucose concentration of the TE medium might negatively influence EC survival as a high glucose concentration is known to inhibit EC proliferation (Stout, 1982) and stimulate EC apoptosis (Risso *et al.*, 2001). In order to get EC survival, it was therefore necessary to culture the constructs in EC medium for the entire duration of the experiment.

After 3 weeks, hardly any tissue had developed in the constructs. The growth factors added to the EC medium, which on the one hand stimulated EC growth, might on the other hand have inhibited collagen formation by the MFs. It is for instance known that a combination of FGF and heparin, which were both supplemented to the EC medium, inhibited collagen production in human keloid fibroblasts (Tan *et al.*, 1993). The low glucose concentration of the EC medium might have two effects on the TE constructs. First, fibroblasts are known to produce less collagen in a low glucose concentration compared to a high glucose concentration (Tang *et al.*, 2007). Second, although monolayers of MFs could survive in EC medium (data not shown), it might be possible that the glucose level in the center of the constructs is not enough for proper cell function. In order to get enough tissue formation in the co-culture model, it is suggested to culture the constructs for a longer period in TE medium and seed ECs after a longer culture period.

The ECs seeded on the surface of the constructs attached properly and started proliferating. Several patches of confluent ECs could be seen after 7 days, however the ECs did not always reach a 100% confluency in the following days. Also after 7 days, tube-like-structures (TLS) started to form and were clearly visible 3 days later. Fibrin and collagen gels seeded with ECs of different origin are often used to investigate the formation of TLS *in vitro* (Koolwijk *et al.*, 1996; Sieminski *et al.*, 2005). In these experiments, ECs would not form TLS spontaneously, but a combination of several growth factors had to be added to get TLS formation. Tumor necrosis factor-α (Koolwijk *et al.*, 1996) or a phorbol esther (Sieminski *et al.*, 2005) in combination with bFGF and VEGF had to be supplemented to the culture medium to induce TLS formation. As in the current experiment EC medium was also supplemented with several growth factors, it might be that this culture medium itself induced TLS formation. However, when ECs were seeded on PGA/P4HB strips which were filled with fibrin gel only, i.e. without MFs, they proliferated and formed a confluent EC layer, without forming TLS (data not shown). It is therefore suggested that the TLS formation is caused by signals from the MFs. Due to the initial construct thickness of 1 mm and the low glucose medium, it is likely that the MFs in the center have a limited availability of oxygen and nutrients and were therefore producing growth factors which signal the ECs to form TLS and hamper the formation of a confluent monolayer.

Although the formation of TLS was not the objective of this study, it is interesting that the ECs form these structures in the presence of MFs and their appearance might be useful for future vascularization of TE constructs. Tissue engineering of thick constructs is currently limited by the diffusion of nutrients to the cells in the center. Metabolically active cells must be situated within 150–200 μm of a blood supply in order to function properly (Colton, 1995). Therefore small-diameter blood vessels, with wall thicknesses ranging from 300 to 1000 μm, as well as their TE replacements, require some degree of microvasculature for proper oxygenation and nutrient exchange (Colton, 1995; Folkman and Hochberg, 1973). Recently, researchers have shown that the *in vitro* prevascularization of engineered muscle tissue constructs improved the vascularization, blood perfusion and survival of these constructs after transplantation in mice (Levenberg *et al.*, 2005). Tremblay *et al*. showed that the presence of capillary-like structures in an endothelialized reconstructed human skin prior to grafting markedly increased the speed of vascularization by inosculation of its capillary network with the host's vasculature (Tremblay *et al.*, 2005).

In conclusion, the seeded ECs did not always form a confluent monolayer on the TE constructs, but formed TLS and invaded the constructs, which was probably caused by the MFs. The MFs did not create enough ECM, resulting in weak tissue constructs. The low ECM production of the MFs was caused by the EC medium, which was necessary for EC survival. It is suggested that the tissue development has to be improved in order to create an endothelialized 3D model system, with which EC and MF interactions can be studied. Therefore, it is suggested to culture the TE constructs in TE medium for several weeks and then seed the ECs using EC medium.

Chapter 3

The influence of endothelial cells on the ECM-composition of 3D engineered cardiovascular constructs

The contents of this chapter are based on R.A.A. Pullens, M. Stekelenburg, F.P.T. Baaijens and M.J. Post (2008), *The influence of endothelial cells on the ECM-composition of 3D engineered cardiovascular constructs,* Tissue engineering and regenerative medicine, in press

3.1 Introduction

In 2004, approximately 425,000 coronary bypass graft procedures were performed in the USA on patients suffering from cardiovascular disease (Rosamond *et al.*, 2007). Internal mammary arteries and saphenous veins are the current graft material of choice. However, the saphenous vein grafts have a limited life time (Raja *et al.*, 2004) as is shown by a patency of 57% after 10 years (Sabik III *et al.*, 2005). Several studies indicate that a disrupted endothelial cell (EC) layer is one of the reasons for this low patency rate (Manchio *et al.*, 2005; Sellke *et al.*, 1996). Vascular substitutes are increasingly in demand as the number of patients who need follow-up surgery and have run out of native graft material is increasing. The same is true for arteriovenous shunt material for vascular access in dialysis patients (Berardinelli, 2006). Tissue engineering (TE) of small diameter (<5 mm) blood vessels is a promising approach to develop viable alternatives for autologous vascular grafts (L'Heureux *et al.*, 2006; Niklason *et al.*, 2001). Such TE grafts should provide sufficient mechanical support and should also contain a functional EC layer (Mitchell and Niklason, 2003). The endothelium is a highly active layer involved in tissue homeostasis, regulation of vascular tone and growth regulation of other cell types. In addition, thrombosis, which is one of the causes of graft failure, is proactively inhibited by an intact and quiescent endothelium (Mitchell and Niklason, 2003).

Many studies have shown that seeding of ECs on synthetic grafts improves the patency of these grafts (Hoenig *et al.*, 2006; Seifalian *et al.*, 2002). In several animal models, high patency rates could be observed after implantation of EC seeded TE grafts with follow ups of several weeks (Borschel *et al.*, 2005; Dardik *et al.*, 1999; Niklason *et al.*, 1999; Swartz *et al.*, 2005). However, it is unknown whether this was the result of seeded endothelium or host derived re-endothelialization (Swartz *et al.*, 2005). Because in humans re-endothelialization of vascular grafts is slow and almost never complete (Berger *et al.*, 1972; Rahlf *et al.*, 1986), the favorable outcome in animal studies may not be indicative of clinical success. To overcome this problem, it is suggested that the EC layer of a TE human graft should be confluent prior to implantation. This layer should also be able to withstand the shear forces of blood. Because ECs will upregulate junction and adhesion molecules after several days of confluency (Lampugnani *et al.*, 1997), it is hypothesized that prior to implantation of the TE vascular grafts, ECs need to be cultured longer than the commonly used 1-3 days (Borschel *et al.*, 2005; Niklason *et al.*, 1999).

However, in co-culture with ECs, smooth muscle cells (SMC) may appreciably reduce their synthetic activity (Powell *et al.*, 1996), possibly leading to poor tissue composition and reduced mechanical strength. The *in vitro* results that support this relationship seem to depend on the cell source. For instance, in a 2D co-culture system, SMC proliferation was stimulated by bovine ECs, but was down regulated by human ECs (Imegwu *et al.*, 2001). In addition, the collagen production of bovine SMCs was reduced in the presence of endothelium (Powell *et al.*, 1997). Similar results for bovine cells were found in a 3D culture system (Williams and Wick, 2005).

Recently, strong small diameter TE vascular grafts were cultured in our group with burst pressures up to 900 mmHg (Stekelenburg *et al.*, 2008). The constructs were based on a polyglycolic acid (PGA) scaffold coated with poly-4-hydroxybutyrate (P4HB) and seeded with human myofibroblasts (MF) in a fibrin gel. The goal of the present study was to create a confluent EC layer on 3D rectangular tissue strips, with a similar tissue composition, and determine whether the ECs have an influence on the proliferation and the production of extracellular matrix (ECM) of human saphenous vein MFs. To achieve this goal, the influence on the tissue development of the EC culture medium and the additional co-culturing of ECs was investigated. For this purpose, rectangular PGA/P4HB scaffolds were seeded with human saphenous vein MFs and cultured for 5 weeks, while being longitudinally constrained. After a culture period of 3 or 4 weeks, human saphenous vein ECs were seeded on top of the constructs and co-cultured for 2 or 1 weeks, respectively. Afterwards, the confluency of the endothelial layer was visualized and the mechanical properties and tissue composition of the constructs were analyzed.

3.2 Materials and methods

3.2.1 Cell isolation and culture

Endothelial cells (ECs) and myofibroblasts (MF), were harvested from the same fresh discarded vein segment of the human saphenous vein, obtained from a patient undergoing coronary bypass surgery, according to the Dutch guidelines of secondary use material. ECs were isolated using an adapted enzymatic digestion method (Terramani *et al.*, 2000). In brief, the vein segment was rinsed in phosphate buffered saline (PBS) and incubated for 10 minutes in an antibiotics solution containing PBS supplemented with 2.5 μg ml^{-1} amphotericin B and 200 μg ml^{-1} gentamycin (Biochrom, Germany). After infusion of a 0.2% type I collagenase solution (Sigma, USA), the vein segment was clamped at both ends and incubated at room temperature for 20 minutes. After incubation, the cell suspension was collected and pelleted by centrifugation at 250 rcf for 5 min. The cell pellet was resuspended in EC medium. EC medium consisted of EGM-2 endothelial cell medium (Cambrex, Belgium) supplemented with 20% Fetal Bovine Serum (FBS; Greiner, Austria) and the EGM-2 kit containing hydrocortisone (0.04%), human fibroblast growth factor B (0.4%), vascular endothelial growth factor (0.1%), R^3-insulin-like growth factor 1 (0.1%), ascorbic acid (0.1%), human epidermal growth factor (0.1%), gentamicin sulfate amphotericin-B (0.1%) and heparin (0.1%).

Cells from the vessel wall were isolated using an explant technique. Vein pieces were placed in 6 well plates and the outgrowth cells were collected. These cells were expanded using regular cell culture methods (Schnell *et al.*, 2001), and characterized as a mixture of V (vimentin) and VA (vimentin/actin) type MFs (Mol *et al.*, 2006).

The culture medium of MFs consisted of DMEM Advanced (Invitrogen, Netherlands), supplemented with 10% FBS, 1% GlutaMax (Gibco) and 0.1% gentamycin. The medium used for MF seeding and subsequent tissue culture, referred

to as TE medium, was the same as for MFs, with 0.3% in stead of 0.1% gentamicin, and supplemented with L-ascorbic acid 2-phosphate (0.25 mg ml^{-1}; Sigma).

3.2.2 Scaffold preparation and seeding

Rectangular shaped scaffolds (n=30, 30 x 9 mm) composed of rapid degrading nonwoven polyglycolic acid (PGA) (thickness 1.0 mm, specific gravity 70 mg cm^{-3}; Cellon, Bereldange, Luxembourg) were coated with poly-4-hydroxybutyrate (P4HB) (Symetis Inc, Zürich, Switzerland). The scaffolds were placed in 6 wells plates and the outer 5 mm of the long axis of the strips were glued to the well using a 20% solution of polyurethane (PU) (DSM, Netherlands) in tetrahydrofuran, leaving a 20 x 9 mm area for cell seeding (Figure 3.1A). The cell-seeded rectangular scaffold strips will be referred to as TE constructs. The solvent was allowed to evaporate overnight. The constructs were sterilized by placing them in 70% ethanol for 4 hours. Afterwards, the constructs were washed with PBS and placed in TE medium overnight. Seeding of the MFs was performed at a density of $2.5 \cdot 10^6$ cells (passage 7) per 100 mm^3 scaffold using bovine fibrin as cell carrier (Mol *et al.*, 2005). Seeding of ECs (passage 7) was performed after 3 or 4 weeks (see next section) by dripping a cell solution on the constructs, resulting in a density of $1 \cdot 10^4$ cells cm^{-2}.

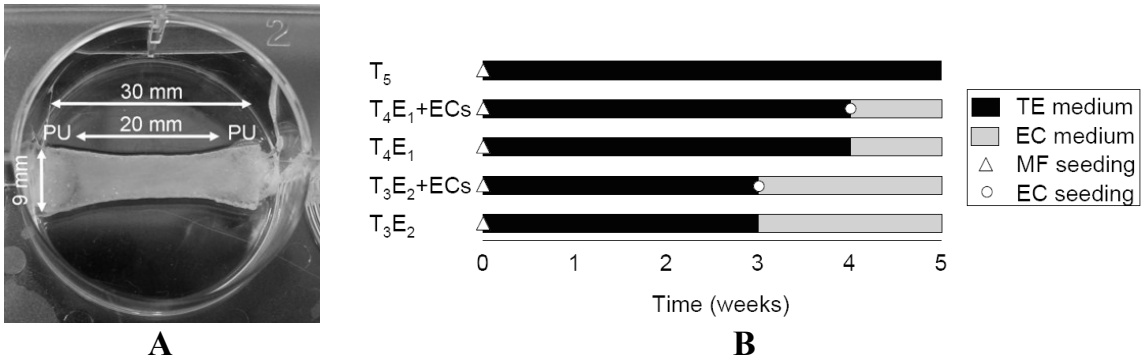


Figure 3.1: TE construct showing the glued (PU) outer edges. The area left for cell seeding is 20 x 9 mm (A). Definition of groups showing the control group cultured in TE medium for 5 weeks (T_5), the groups seeded and cultured with ECs for 1 and 2 weeks (T_4E_1+ECs and T_3E_2+ECs) and the control groups in which only the medium was changed without seeding ECs (T_4E_1 and T_3E_2) (B).

3.2.3 Tissue culture

After seeding of the MFs, the 6 wells plates were placed on a shaker (50 rpm) in an incubator to allow mixing of the TE medium (T). The constructs were divided into 5 groups (n=6) (Figure 3.1B), and cultured for 5 weeks. The culture medium was changed to EC medium (E) at the moment of EC seeding, as studies showed that ECs did not survive in TE medium (Chapter 2). To investigate whether this medium influences the tissue development, three control groups were defined. In one group the

TE medium was not changed to EC medium (T_5) and in the other two groups the medium was changed to EC medium after 3 or 4 weeks without seeding of the ECs, further referred to as T_3E_2 and T_4E_1. To test the influence of the ECs, constructs were cultured for 3 or 4 weeks in TE medium after which ECs were seeded and cultured on the constructs using EC medium, further referred to as T_3E_2+ECs and T_4E_1+ECs.

3.2.4 Qualitative tissue analyses

After the five week culture period, the EC seeded constructs were stained for 45 minutes with FITC UEA-1 lectin (5 μM, Sigma), for visualization of the ECs (Hormia *et al.*, 1983), and Cell Tracker Orange (10 μM, CTO, Invitrogen, USA), for visualization of MFs and ECs. Due to the flat morphology of the ECs, the CTO concentration in these cells was limited. Afterwards, the constructs were detached from the wells and analyzed using a confocal laser scanning microscope (CLSM; Axiovert 100M, Zeiss, Göttingen, Germany). The FITC UEA-1 lectin and CTO were excited at 488 and 543 nm, respectively and the emissions were recorded between 505 and 530 and above 570, respectively. To visualize the EC layer, Z-projections of z-stacks were produced.

Tissue morphology in all groups was further studied by histology. Samples were fixed in phosphate-buffered formaldehyde (3.7%) and embedded in paraffin; 10 μm sections were stained with hematoxylin and eosin (H&E) for general tissue morphology and Masson Trichrome (MTC) for collagen visualization. To analyze the EC layer of the constructs, immunohistochemistry was performed. The sections were stained with the EC specific markers monoclonal mouse anti-human CD31 and polyclonal rabbit anti-human vWF (Dako, Denmark). Afterwards the sections were stained with goat anti-mouse Alexa-488 and goat anti-rabbit Alexa-555 secondary antibodies. DAPI staining was used to stain cell nuclei. Control sections incubated with only the secondary antibodies were completely negative. Images were taken using a fluorescent microscope (Axiavert 200, Zeiss) mounted with a monochrome Axiocam, using appropriate filters and post-hoc color definition.

3.2.5 Quantitative tissue formation analyses

To quantify tissue formation, the amount of DNA, sulfated glycosaminoglycans (GAGs), and hydroxyproline, was measured and expressed in mg per dry weight of tissue. For the analyses, lyophilized tissue samples were digested using a papain solution (100 mM phosphate buffer, 5 mM L-cysteine, 5 mM ethylenediaminetetraacetic acid (EDTA), and 125 to 140 μg papain ml^{-1}) at 60°C for 16 hours. The Hoechst dye method (Cesarone *et al.*, 1979) with a reference curve of calf thymus DNA (Sigma) was used to determine the DNA content. Using a modification of the assay described by Farndale *et al*. (Farndale *et al.*, 1986) and a shark cartilage chondroitin sulfate reference (Sigma), the sulfated GAG content was determined. The hydroxyproline content was determined with an assay modified from Huszar *et al*. (Huszar *et al.*, 1980) and a reference of trans-4-hydroxyproline (Sigma). The ratio of

hydroxyproline to collagen was assumed to be 0.13. To obtain a measure for the amount of matrix components produced per cell, the collagen and GAG content were normalized for the amount of DNA.

It was recently demonstrated that collagen cross-links are important for the biomechanical tissue properties of heart valves and TE constructs (Balguid *et al.*, 2007), therefore the constructs cross-link content was also determined. For the analysis the digested samples were hydrolysed in a 6M HCl (Merck, Germany) solution. The acid hydrolysates were used to measure the number of the mature collagen cross-links hydroxylysyl pyridinoline (HP), which is the main type of collagen cross-links present in cardiovascular tissue, by HPLC as described previously (Bank *et al.*, 1997). The number of HP cross-links was expressed per collagen triple helix.

3.2.6 Evaluation of mechanical properties

To determine the mechanical properties of the constructs, the strips were subjected to uniaxial tensile tests. The thickness of the constructs was measured with a Digimatic Micrometer (Mitutoyo America Corporation, Aurora, USA). Tensile tests were performed with a tensile tester equipped with a 20N load cell (Kammrath-Weiss, Dortmund, Germany). Stress-strain curves were obtained at a strain rate equal to the initial sample length per minute. The stress was defined as the force divided by the deformed cross-sectional area. The ultimate tensile strength (UTS) was determined from the curves. The slope of the linear part of the curve represented the modulus of elasticity of the tissue.

3.2.7 Statistics

Quantitative data were averaged per group, and represented as average ± standard error of the mean. Using a two-way ANOVA analyses, the influence of the culture medium and the presence of ECs were determined. Post-hoc comparisons using contrast analysis were used to determine significant differences ($p<0.05$) between groups. Group differences were determined when either the culture medium or the presence of ECs was different. All statistical analyses were performed using SPSS v.14.0 software (SPSS Inc., Chicago, IL, USA).

3.3 Results

3.3.1 Qualitative tissue analyses

During the first 3 weeks of the tissue engineering experiment, the shape of the constructs did not change. In the last 2 weeks the width of constructs decreased, due to tissue compaction. In the EC seeded group T_4E_1+ECs, a continuous, connecting monolayer of UEA-1 (Figure 3.2A), CD31 (Figure 3.2B-C) and vWF (Figure 3.2D-E) positive ECs was found. Similar results were found in the T_3E_2+ECs group, indicating a stable EC layer for 1 to 2 weeks.

All constructs consisted of dense tissue with abundant amounts of collagen. However, the groups cultured for 2 weeks on EC medium had a less dense ECM structure, (T_3E_2+ECs (Figure 3.3) and T_3E_2), compared to the ones cultured in TE medium, suggesting a negative influence of the EC medium on tissue development. A cell layer of MFs was present on top of the tissue constructs. No differences in tissue structure were observed between the EC seeded groups and their control groups (data not shown). Similar H&E and MTC stainings were found in the T_5 control group and the T_4E_1+ECs group (Figure 3.3).

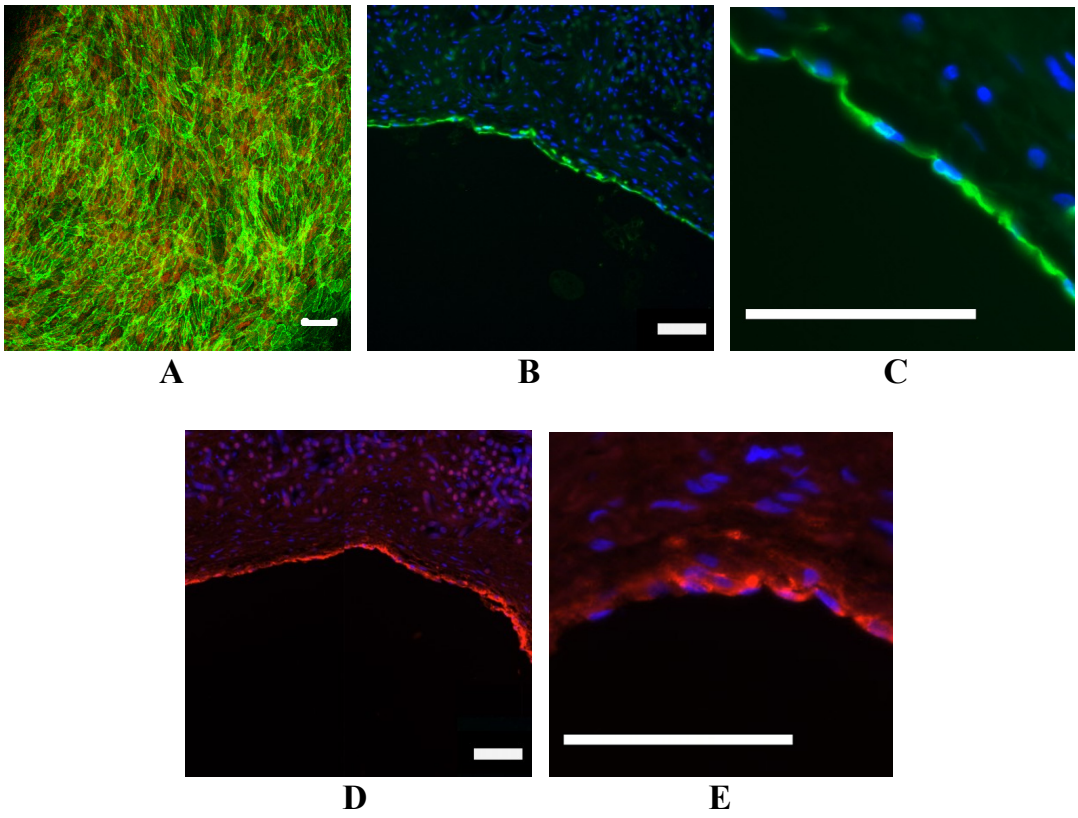


Figure 3.2: Characterization of the EC monolayer after 1 week of culture (T_4E_1+ECs). Projection of CLSM images of UEA-1 lectin stained ECs (green) and general cell staining CTO (red) (A). Immunohistochemistry staining of CD31 (B, C, green) and vWF (D, E, red), counterstained with DAPI (blue), showing the continuous endothelial lining. Scale bars represent 100 µm.

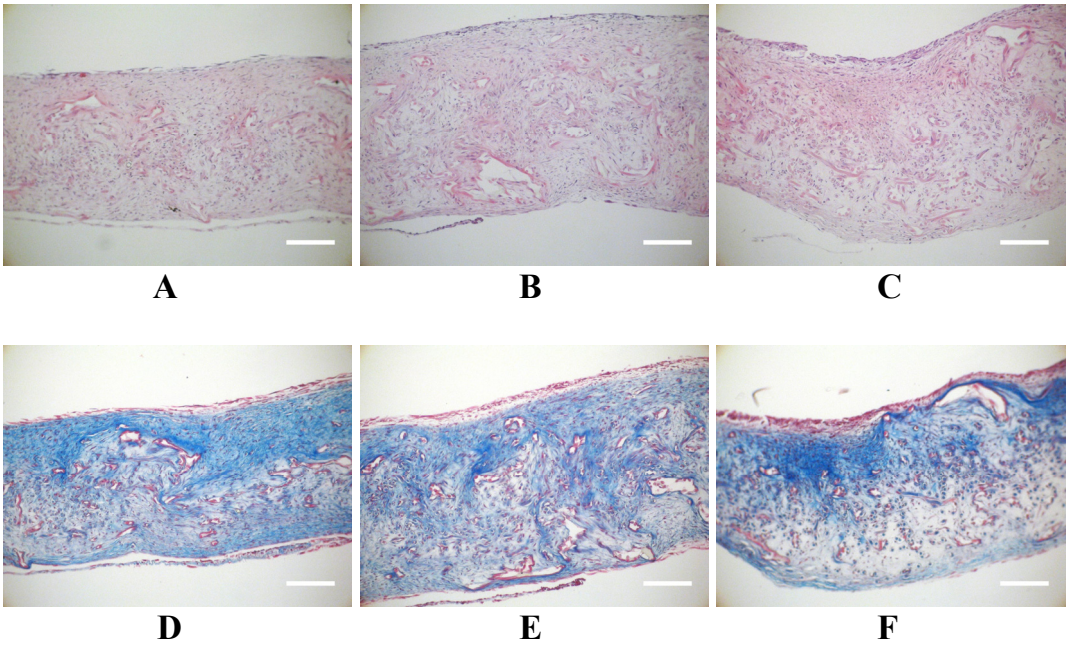


Figure 3.3: Hematoxylin and eosin (H&E) staining (A, B, C) and masson trichrome staining (MTC) staining (D, E, F) of control group T_5 (A, D), and EC seeded groups T_4E_1+ECs (B, E) and T_3E_2+ECs (C, F). H&E staining shows homogenous tissue formation. A cell layer containing less collagen was present on top of the constructs. Collagen, stained blue in MTC staining, was abundantly present in the tissues. The T_3E_2+ECs group had a less homogeneous ECM structure. Scale bars represent 200 µm.

3.3.2 Quantitative tissue formation analyses

The cells produced large amounts of collagen. The average collagen content was 190 µg/mg, equaling approximately 60% of the amount in human coronary arteries (Ozolanta *et al.*, 1998). The amount of DNA and GAGs was significantly affected by the EC medium (Table 3.1, $p<0.05$ and $p<0.001$). The DNA content of the T_4E_1 group and the GAG content of the T_3E_2 and T_4E_1 groups were higher than the control group T_5 (Figure 3.4A-B, $p<0.05$ and $p<0.005$), suggesting that EC medium supports cell proliferation and GAG production after a certain period of exposure to TE medium. After 2 weeks culture in EC medium however, the amount of collagen was lower ($p<0.05$) than after 1 week (T_3E_2 and T_4E_1, Figure 3.4C). Normalization of GAG and collagen amounts to DNA content provided matrix production per cell. Although differences were found in the GAG content, no differences were found in GAG production per cell between the different groups (Figure 3.4D). In contrast, the collagen production per cell was decreased in the T_3E_2 group (Figure 3.4D, $p<0.005$).

Culturing the constructs in EC medium has a stimulating effect on the number of HP cross-links per triple helix in the TE constructs (Table 3.1, $p<0.005$). The highest amount was found in the T_3E_2 group (Figure 3.5). This result suggests that EC medium alters the balance between collagen production and collagen maturation.

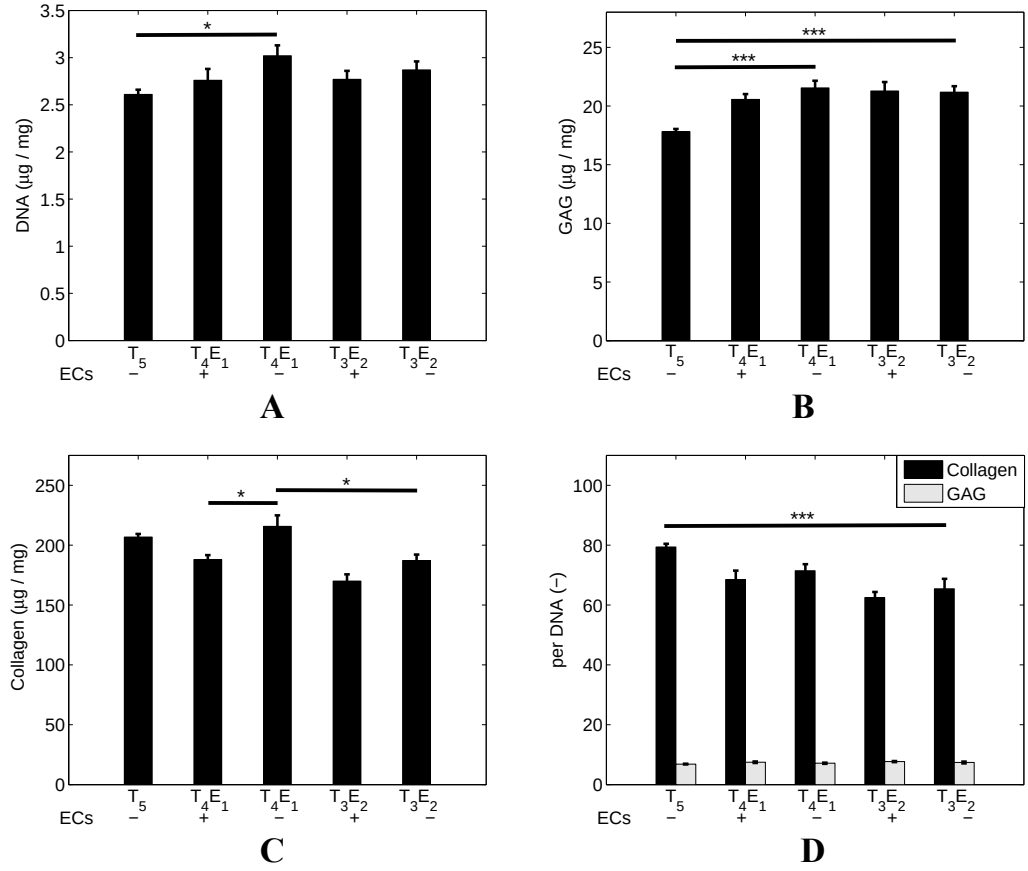


Figure 3.4: The amounts of DNA, GAG and collagen per dry weight (A, B, C) and the amount of collagen and GAG per DNA (D) of all groups (mean ± s.e.m.). Culturing in EC medium for 1 week increased the DNA amount in the constructs (A). EC medium also increased the GAG amounts in the T_4E_1 and T_3E_2 groups (B). When EC medium was used for 2 weeks, the collagen amount decreased (C). Co-culturing with ECs did not influence the DNA and GAG amounts (A, B), but decreased the collagen amount. Culturing for 2 weeks in EC medium lowered the collagen production per cell (D). Group differences were determined when either the culture medium or the presence of ECs was different. ($p<0.05$; *** $p<0.005$)*

Co-culture of ECs reduced the collagen content, compared to respective controls (Table 3.1, $p<0.001$). The collagen amount was significantly lower ($p<0.05$) in the T_4E_1+ECs group, compared to T_4E_1-ECs. However, when normalized for the amount of DNA, this difference was not present (Figure 3.4D), suggesting that the production of collagen per cell was not affected by the presence of endothelium. The presence of ECs had no influence on the DNA, GAG content and the HP cross-links per triple helix (Figure 3.4A-B, Figure 3.5, Table 3.1).

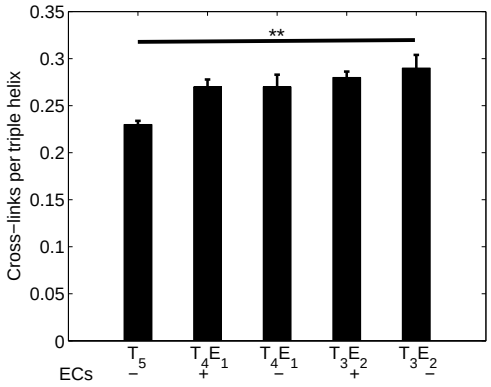


*Figure 3.5: The amounts of HP cross-links per triple helix collagen of all groups (mean ± s.e.m.). EC medium increased the amount of HP cross-links per triple helix. (** p<0.01)*

Table 3.1: F-values and p values of two-way ANOVA analysis. The presence of EC medium had a significant influence on the ECM composition (except for the GAG / DNA) and the mechanical properties. The presence of ECs had an influence on the collagen amount per dry weight only. (NS: not significant)

	EC medium		**Endothelial cells**	
	F	**Sig.**	**F**	**Sig.**
DNA (μg /mg)	4.44	p<0.05	3.54	NS
GAG (μg /mg)	14.23	p<0.001	0.63	NS
Collagen (μg /mg)	10.54	p<0.005	19.05	p<0.001
GAG / DNA (-)	1.77	NS	1.92	NS
Collagen / DNA (-)	11.45	p<0.001	3.17	NS
Cross-links per triple helix	4.34	p<0.005	0.01	NS
Young's Modulus	12.97	p<0.001	0.41	NS
UTS	7.49	p<0.005	1.63	NS

3.3.3 Mechanical properties

The constructs were relatively strong and stiff, indicated by an average UTS and Young's modulus of 2 MPa and 11 MPa, respectively (Figure 3.6). The constructs cultured in EC medium for 2 weeks (T_3E_2) had lower Young's moduli than the control constructs T_5 ($p<0.005$), suggesting that a shorter culture period in EC medium is advised. Seeding and co-culturing of ECs (T_3E_2+ECs and T_4E_1+ECs) did not have an additional effect on the mechanical properties (Table 3.1).

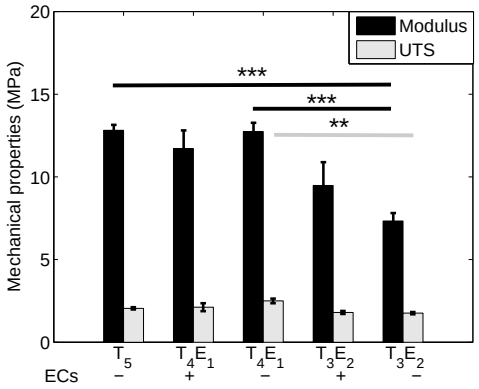


*Figure 3.6: Mechanical properties of the constructs of all groups (mean ± s.e.m.). Culturing for 2 weeks in EC medium lowered the Young's modulus and UTS compared to 1 week EC medium (T_3E_2-ECs vs T_4E_1-ECs). (** $p<0.01$; *** $p<0.005$)*

3.4 Discussion

The development of a functional, shear resisting endothelial layer is one of the major issues limiting the successful application of tissue engineered vascular grafts (Mitchell and Niklason, 2003). To investigate the influence of human ECs on MF in a 3D environment, MF seeded constructs were cultured, using static conditioning, for 3 or 4 weeks. ECs were then seeded and co-cultured for 2 or 1 week, respectively. After 1 week of co-culture, a confluent monolayer of ECs was found, which was maintained for another week. Compared to the often applied 1-3 days EC co-culture period (Borschel *et al.*, 2005; Niklason *et al.*, 1999), this longer co-culture period is likely to have a positive outcome on cell retention when shear stress is applied, because ECs will upregulate junction and adhesion molecules after several days of confluency (Lampugnani *et al.*, 1997). Co-culturing an EC layer longer than 1-3 days results in a lower thrombogenicity, which was shown in TE vessels implanted in sheep (Swartz *et al.*, 2005), and HUVECs cultured on a layer of ECM (Remy-Zolghadri *et al.*, 2004).

Pilot studies have shown that TE constructs develop hardly any tissue when cultured exclusively in EC medium (Chapter 2). Therefore, it was decided to culture the constructs for 3 or 4 weeks in TE medium before seeding the ECs and changing to EC medium. Control groups were defined to investigate whether the EC medium alone would still influence the tissue formation. In the present study, changing the culture medium to EC medium increased the proliferation of the MFs. In addition, it reduced their collagen production which resulted in weaker constructs, despite an increase in cross-links per collagen triple helix. The growth factors and the low glucose concentration of the EC medium are likely responsible for these changes.

Co-culturing ECs on the TE constructs caused only small differences in ECM composition. No significant differences were found in DNA and GAG content between the EC seeded groups and their controls. However, two way ANOVA analysis revealed a significant influence of EC seeding on collagen content. This could indicate that ECs lower the synthetic activity of the MFs, creating a more quiescent tissue. Despite this

lower collagen amount, the mechanical properties of the constructs were not altered by the EC co-culture period. In addition to the collagen amount, the cross-links are also important for the mechanical properties, but they were not influenced by the ECs. It is expected that the small decrease in collagen amount was not enough to reduce the mechanical properties.

To our knowledge the present study is the first to investigate the influence of human saphenous vein ECs on MFs in a 3D co-culture system. The influence of ECs on SMCs, however, has been studied more often. Several studies with 2D systems, in which cell types were separated by a microporous membrane have found a stimulating effect on SMC proliferation (Powell *et al.*, 1996), however, an inhibiting effect has also been reported (Campbell and Campbell, 1986; Imegwu *et al.*, 2001). The decreased collagen content found in the present study is consistent with other studies (Fillinger *et al.*, 1997; Powell *et al.*, 1997). Obviously, these 2D co-culture systems do not represent the *in vivo* situation. Recently, several 3D co-culture systems have been developed where animal ECs were directly cultured on the SMCs (Lavender *et al.*, 2005; Niwa *et al.*, 2007) or on SMCs seeded in a tubular PGA construct (Williams and Wick, 2005). In these PGA constructs, which were subjected to shear stress, similar results for the collagen content were found, however, they found a significant increase in SMC proliferation (Williams and Wick, 2005). This difference can be explained by the fact that in the present study no shear was applied to the ECs. It is likely that mechanical conditioning such as shear stress further influences the tissue development in these constructs in an endothelium dependent manner (Nackman *et al.*, 1998). Secondly, although a similar co-culture period was used, in the present study the moment of EC seeding was later, which might have reduced the stimulatory effect of the ECs on MF proliferation and the inhibitory effect on collagen production. In addition, a higher amount of MFs was seeded and a fibrin gel was used, which might have reduced the initial proliferation rate.

In conclusion, strong rectangular cardiovascular constructs were created by seeding human saphenous vein ECs and MFs on a PGA/P4HB scaffold. A confluent endothelial layer could be maintained for up to 2 weeks. The use of specific EC medium was crucial for the development of this layer, as ECs did not survive in TE medium, normally used for tissue culture. However, the EC medium altered the collagen production of the cells, resulting in weaker constructs, especially when EC medium was used for 2 weeks. Co-culturing ECs for 1 or 2 weeks had an influence on the collagen content, but there was no additional affect on the mechanical properties. The 1 week co-cultured constructs had a confluent EC layer and good mechanical properties. This setup can be used for the development of a shear resistant EC layer on human TE vascular grafts.

Acknowledgements

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Chapter 4

Influence of endothelial cells on smooth muscle cell characteristics of myofibroblasts

4.1 Introduction

Tissue engineering of small diameter (<5 mm) blood vessels is a promising approach to develop viable alternatives for autologous vascular grafts (L'Heureux *et al.*, 2006; Niklason *et al.*, 2001; Stekelenburg *et al.*, 2008). For a good performance, such tissue engineered vascular grafts (TEVG) should have sufficient mechanical strength and contain a functional endothelial cell (EC) layer (Mitchell and Niklason, 2003). One of the functions of the EC layer is communication with smooth muscle cells (SMC) in the underlying vessel wall (Campbell and Campbell, 1986; Luscher and Barton, 1997). For example, ECs keep the SMCs in a quiescent non-proliferative contractile state under physiological levels of shear stress, but when shear stress is decreased ECs stimulate SMC proliferation (Malek *et al.*, 1999). Modulation from a contractile to a synthetic SMC phenotype is associated with changed expressions of proteins, including α-smooth muscle actin (αSMA), smooth muscle myosin heavy chain (MHC), calponin and vimentin (Owens *et al.*, 2004; Worth *et al.*, 2001).

In vitro 2D co-culture experiments have been used to study interactions between ECs and SMCs. A stimulating effect of ECs on SMC proliferation was demonstrated (Waybill and Hopkins, 1999). In addition, co-culturing ECs on SMCs changed the phenotypic expression of SMCs. In the co-cultures, an increased gene expression of platelet-derived growth factor and transforming growth factor (TGF) was found, compared to pure SMC cultures (Heydarkhan-Hagvall *et al.*, 2003). In addition, the collagen production of co-cultured SMCs is down-regulated compared to a monoculture of SMCs (Powell *et al.*, 1997). In those studies, SMCs and ECs are typically plated on opposite sides of a microporous membrane. Although these studies have provided valuable information about EC-SMC interactions such a system is not ideal. In TE, these cells are not separated by a synthetic biomaterial, but the ECs are often seeded directly onto the vessel wall, containing SMCs. Powell *et al*. compared the bilayer membrane system to ECs directly cultured on top of SMCs and showed that SMCs had typical hill-and-valley growth in direct co-culture, but this was absent in the bilayer culture (Powell *et al.*, 1998).

Recently, several research groups have used such a direct cell-cell contact co-culture system (Helenius *et al.*, 2004; Lavender *et al.*, 2005; Niwa *et al.*, 2007; Wallace *et al.*, 2007). Those studies demonstrated that when co-cultured, both ECs and SMCs alter the expression of fibrinolytic and coagulation factors, compared to EC and SMC monocultures (Helenius *et al.*, 2004). Lavender *et al*. have examined conditions necessary to maintain a confluent layer of porcine ECs on a layer of porcine SMCs (Lavender *et al.*, 2005). Although the ECs stayed confluent for 10 days, the SMC layers were unstable and sometimes contracted during the experiments. Wallace *et al*. have used this system to co-culture human aortic SMCs and human umbilical vein ECs (Wallace *et al.*, 2007). They demonstrated that the SMCs, when co-cultured with ECs, increased the expression of calponin, suggesting SMC differentiation towards a contractile phenotype. It was suggested that more differentiated contractile SMCs are desired for TEVGs, to allow the vessel to fully respond to vasoactive mediators.

In our group, human saphenous vein myofibroblasts (MF) were used for the creation of small diameter TEVGs (Stekelenburg *et al.*, 2008). These cells have also been used to create cardiovascular TE strips, on which a confluent EC layer was cultured (Chapter 3). In view of the interaction between ECs and SMCs in co-culture the question was asked whether these human saphenous vein ECs influence the SMC characteristics of human saphenous vein MFs. Therefore, the goal of the present study was to compare the growth pattern and expression of αSMA, MHC and vimentin of MFs cultured in a monolayer to MFs co-cultured with ECs.

4.2 Materials and methods

4.2.1 Culturing human vena saphena cells

Human saphenous vein ECs and MFs were expanded using standard culture methods up to passage 7 (Chapter 3). The culture medium for MFs consisted of DMEM Advanced (Invitrogen, Netherlands), supplemented with 10% Fetal Bovine Serum (FBS; Greiner), 1% GlutaMax (Gibco) and 1% penicillin/streptomycin (Lonza, Belgium), further referred to as 2D medium. The EC culture medium consisted of EGM-2 medium supplemented with growth additives (Lonza), and 20% FBS.

4.2.2 Experimental conditions

To determine the influence of ECs on the growth pattern and expression of αSMA, MHC, and vimentin of MFs, MFs (7000 cells cm^{-2}) were seeded in 6 well plates for RT-PCR analyses. For immunofluorescent staining, MFs were also seeded on gelatin (0.1%) coated glass slides, which had been placed in 24 well plates. The cells were cultured in 2D medium for 5 days and then divided in different groups (Table 4.1). In the first (MF_{2D}, n=2) and second group (MF_{EC}, n=2), MFs were cultured for a subsequent 5 days in 2D or EC medium, respectively. Additionally, a co-culture group (MF-EC, n=2) was created by seeding ECs (6000 cells cm^{-2}) onto the MFs. This group was cultured in EC medium. Culture medium was changed every 2 days.

Table 4.1: Experimental groups

Groups	EC seeding	Culture medium	6 wells (N)	24 wells (N)
MF_{2D}	-	2D medium	4	4
MF_{EC}	-	EC medium	4	4
MF_{COND}	-	Conditioned EC medium	2	2
MF-EC	+	EC medium	4	4

To investigate whether the ECs affected the MFs due to cell-cell contact or due to a secreted soluble factor, the experiment was repeated using a different batch of MFs and ECs and extended with a fourth group (Table 4.1). In this group (MF_{COND}, n=2), the MFs were cultured in 2D medium for 5 days, after which the culture medium was

changed to conditioned EC medium. EC medium was conditioned by a confluent EC layer for 2 days prior to use.

4.2.3 RNA isolation and RT-PCR

To measure the expression of SMC markers using RT-PCR of the initial experiment, the MFs of the 6 wells were trypsinized and cell pellets were created by centrifugation (5 min, 1500 rpm). RNA extraction was performed with an RNeasy Mini Kit (Qiagen, Netherlands), according to the protocol given by the manufacturer. RNA concentration and purity was measured using the NanoDrop spectrophotometer. RNA with an A260/280 ratio of 1.9-2.1 was considered pure and accepted for further analysis. The integrity of the total RNA was checked by gel electrophoresis with ethidium bromide staining. Human aorta RNA (Westburg, Netherlands) was used as positive control for the SMC markers.

For the reverse transcription (RT) step total RNA was incubated with a mixture of random primers (20 ng μl^{-1}, Promega), dNTPs (5 mM, Invitrogen) in First-Strand Buffer (Invitrogen) at 72° C for 6 minutes and then cooled down to 37° C to allow primer annealing. An enzyme mix of M-MLV enzyme (Moloney Murine Leukemia Virus Reverse Transcriptase, 200 U ml^{-1}, Invitrogen), dithiothreitol (0.1 M, Invitrogen) and RNase-free water was added, and incubated at 37° C for 1 hour. Finally, the enzyme was inactivated by heating the reaction to 95° C for 5 minutes. RT-PCR was performed using primers for the contractile proteins αSMA and MHC and cytoskeletal protein vimentin (Table 4.2). GAPDH was used as a reference gene.

Table 4.2: Primers used for RT-PCR

Primers	Genbank	Sequence (5' → 3')		Product
αSMA	X13839	Forward:	CTG GAC TCT GGA GAT GGT G	155 bp
		Reverse:	GCA GTA GTA ACG AAG GAA TAG C	
MHC	NM002474	Forward:	ACG ACA ACT CCT CAC GAT TC	187 bp
		Reverse:	TCA CTT CTC ATC TTC TCC TTG G	
Vimentin	NM003380	Forward:	AGG ACC AGC ATG TCC AAA TC	95 bp
		Reverse:	GGC AGC CAC ACT TTC ATA TT	
GAPDH	NM002046	Forward:	TGT TGC CAT CAA TGA CCC CTT	201 bp
		Reverse:	CTC CAC GAC GTA CTC AGC G	

Taq Polymerase (Invitrogen), PCR Buffer (Invitrogen) and $MgCl_2$ (Sigma) were added to cDNA samples, together with the appropriate primer pair, and dNTPs (5 mM) and incubated in a PCR Thermocycler (Bio-Rad). The thermal profile was 95° C for 1 minute, followed by 35 cycles of 30 seconds at 95° C, with 1 minute annealing (T = 60° C) intervals followed by 30 seconds elongation at 72° C. An additional 10 minute elongation period at 72° C was included after completion of the last cycle. The PCR products were analyzed by gel electrophoresis with ethidium bromide staining.

4.2.4 Immunofluorescent stainings

For immunohistochemical analysis, the cells on the glass slides were fixed using formaldehyde (3.7%, 10 min), washed and permeabilized by 0.5% Triton X-100 (Merck, Germany) in PBS. Two different staining combinations were performed. First, the cells were stained with a combination of monoclonal IgG2 mouse anti-human antibody against αSMA (Dako, Denmark) and monoclonal IgM mouse anti-human antibody against vimentin (Abcam, UK). Second, the antibody against αSMA was combined with an EC specific monoclonal IgG1 mouse anti-human antibody against CD31 (Dako), to visualize the interaction of the MFs and ECs. Afterwards, the specific stainings were visualized with a goat anti-mouse IgG2 Alexa 488 secondary antibody (Invitrogen) for αSMA and a goat anti-mouse IgM and IgG1 Alexa 555 (Invitrogen) for vimentin and CD31, respectively. In addition, DAPI (Invitrogen) was added to stain cell nuclei. Images were taken using a fluorescent microscope (Axiovert 200, Zeiss).

4.3 Results

4.3.1 Morphological appearance

Although the MFs in the MF2D group (Figure 4.1A) and in the MF-EC group (Figure 4.1B) had a similar confluency, morphologically the cells in the MF-EC group

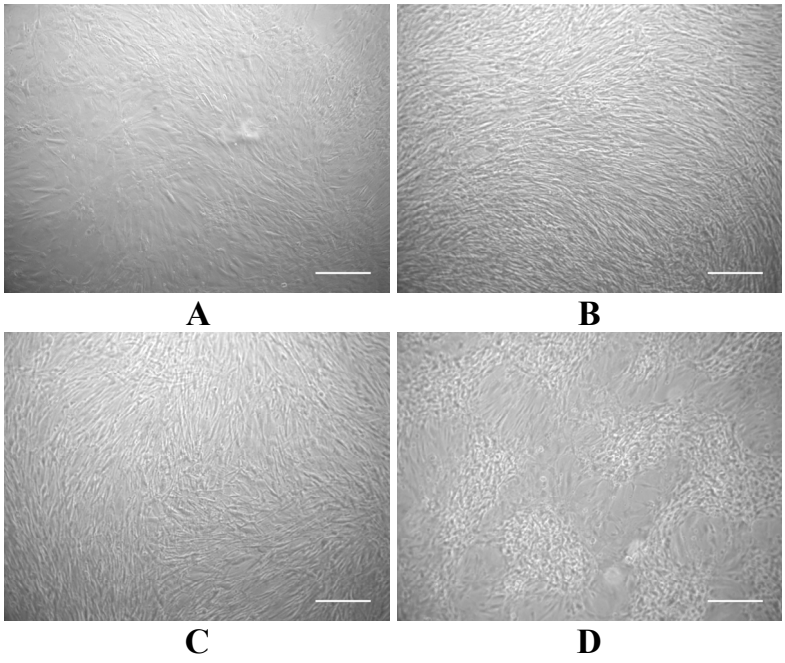


Figure 4.1: Brightfield images displaying the cell morphology in the MF_{2D} (A), MF_{EC} (B), MF_{COND} (C), and MF-EC (D) groups. The MFs in the MF_{EC} and MF_{COND} groups had an elongated and sprout-like appearance compared to the MF_{2D} group. In contrast to the MF_{2D}, MF_{EC}, and MF_{COND} groups, the cells in the MF-EC group formed clusters. Scale bars represent 300 μm.

were more elongated and had a more sprout-like appearance, compared to the cells in MF_{2D}.

The MFs in the MF_{COND} group displayed a similar appearance as in the MF-EC group (Figure 4.1C). As a result of the changed morphology, the cell density appeared higher in the MF-EC and MF_{COND} groups, suggesting that the EC medium stimulated proliferation. In contrast, the MFs in the co-culture MF-EC group (Figure 4.1D) were not confluent, but appeared to form clusters. The ECs also formed patches on top of the MF clusters.

4.3.2 Gene expression

The cells in the 6 wells plates of the initial experiment were analyzed for the gene expression of αSMA, MHC and vimentin. Commercially available human aorta RNA was used as positive control. The gene expressions of αSMA and vimentin were positive in the MF_{2D}, MF_{EC} and MF-EC groups (Figure 4.2). In the MF_{2D} and MF_{EC} groups no MHC expression was found, however in the co-culture group a positive expression of MHC was found.

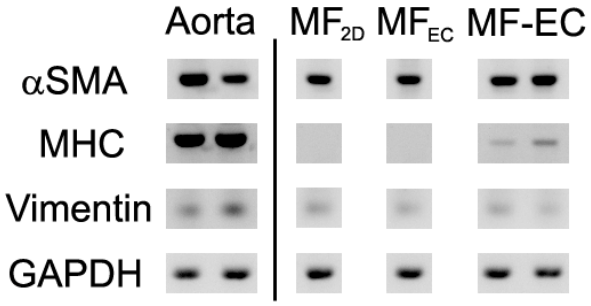


Figure 4.2: Representative results of RT-PCR analysis of the initial experiment. Positive αSMA and vimentin expressions were found in the MF_{2D}, MF_{EC} and MF-EC groups. In contrast to the MF_{2D} and MF_{EC} groups, the cells in the MF-EC group had a positive MHC expression.

4.3.3 Immunofluorescent staining

In the MF_{2D} group, approximately 65% of the cells were positive for αSMA (Figure 4.3A). All the MFs were vimentin positive. Comparison of the αSMA staining in the MF_{EC} and MF_{COND} groups (Figure 4.3B-C) to the MF_{2D} group showed a comparable amount of αSMA positive cells. However, the MFs in the MF_{EC} and MF_{COND} had a different shape, which was also seen in the brightfield images (Figure 4.1). The MFs were more elongated and had a more sprout-like appearance. In the MF-EC group, clear clusters of αSMA positive cells could be seen (Figure 4.3D and 4.4). In these clusters, the cell nuclei density was larger compared to other parts of the culture.

A double stain of CD31 and αSMA clearly demonstrated that the ECs had not formed a confluent layer, but had formed patches (Figure 4.4). In addition, the clusters of αSMA positive MFs co-localized with the patches of CD31 positive ECs.

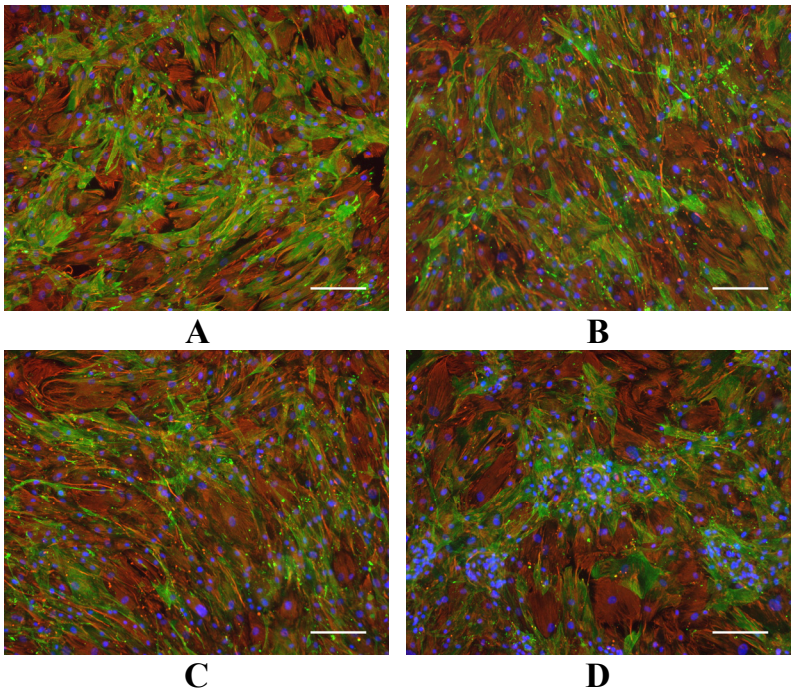


Figure 4.3: MFs stained with αSMA (green), vimentin (red) and DAPI (blue) in the MF_{2D} (A), MF_{EC} (B), MF_{COND} (C), and MF-EC (D) groups. Approximately 65% of the MFs were αSMA positive in the MF_{2D}, MF_{EC}, and MF_{COND} groups. In the MF-EC group clusters of αSMA positive MFs were seen. Scale bars represent 200 µm.

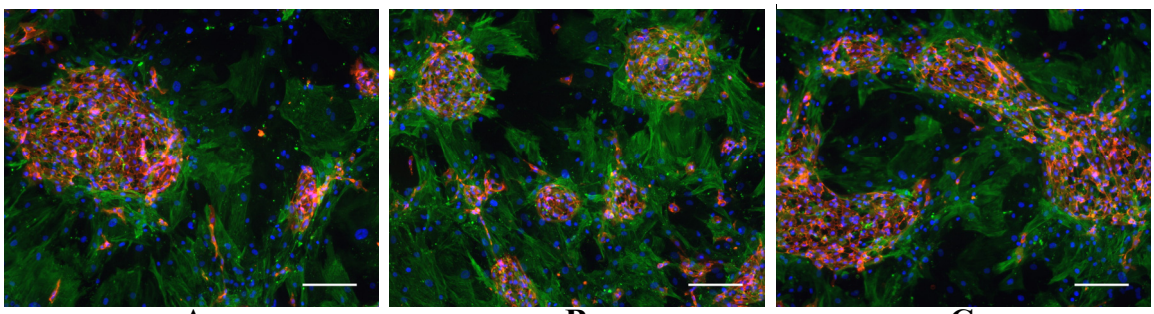


Figure 4.4: Typical examples of MF and EC interaction in the MF-EC group (A-C) stained with αSMA (green), CD31 (red) and DAPI (blue). The αSMA positive MFs have formed clusters, which were co-localized with patches of CD31 positive ECs. Scale bars represent 200 µm.

4.4 Discussion

In the present study, human saphenous vein ECs were co-cultured on top of human saphenous vein MFs, to investigate the influence of ECs on the SMC characteristics of the MFs. In the presence of ECs, the MFs formed clusters. In these clusters the majority of MFs were αSMA positive. On top of these clusters patches of ECs were present. This clustering of MFs was triggered by direct cell-cell contact with the ECs, as the MFs in the conditioned EC medium group stayed a homogenous monolayer. In addition, RT-PCR experiments of the initial experiment demonstrated an increased gene expression of MHC in the co-culture MF-EC group.

The co-culture system used in this study allowed direct contact between the different cell types as opposed to other, indirect co-culture systems in which the cell populations interact through factors secreted into the cell culture medium. Microporous membrane systems have been widely used for studying direct cell-cell contact interactions, and have demonstrated amplified effects compared with conditioned media experiments (Fillinger *et al.*, 1997). However, when such a bi-layer culture separated by a microporous membrane was compared to a co-culture model where cells were directly grown on top of each other, differences were found (Powell *et al.*, 1998). The increased cell-cell contact lowered the amount of TGF-β1 protein and stimulated hill-and-valley growth.

In the current study, due to EC co-culturing, clusters were formed by the MFs. These clusters might be an early form of hill-and-valley growth. Hill-and-valley formation is normally used as a phenotypic marker for SMCs and can occur when SMCs are cultured in a monolayer. In contrast, the cells used in the current study were defined as MFs and did not show hill-and-valley formation when cultured in 2D medium. The cells were derived from the human saphenous vein by an outgrowth procedure. In this isolation technique, the whole saphenous vein vessel wall was used without separating the media and adventitia. So although the total cell population was defined as MFs (Mol *et al.*, 2006), of which approximately 65% was αSMA positive, this might have been a mixed population of perivascular fibroblasts and SMCs, which are both known to grow out of these explants (Grenier *et al.*, 2003). There is no consensus definition of an MF and immunohistochemical distinction from a SMC is common but questionable. In fact, it has been argued that these cells can only be distinguished at ultrastructural level by transmission electron microscopy (Eyden, 2008). In a mixed population of MFs and SMCs, these cells could have reacted differently to the EC co-culture conditions. In accordance, it is has been shown that HUVECs upregulate adhesion molecules when ECs are co-cultured with SMCs but not with fibroblasts (Chiu *et al.*, 2003).

In the current study, the ECs did not proliferate into a confluent layer on the MFs, but formed patches. Lavender *et al*. have shown that ECs seeded on proliferating SMCs formed patches, compared to ECs seeded on quiescent SMCs, which grew confluent (Lavender *et al.*, 2005). Hence, the level of differentiation of MFs presumably affects EC growth and migration. It might well be that the MFs were still

too active for the ECs to form a confluent layer, although the MFs were grown confluent. Activity of the MFs can also influence the expression of SMC markers. For example, quiescent SMCs did have a positive protein expression of smooth muscle myosin, compared to a negative expression in proliferating SMCs (Lavender *et al.*, 2005). Despite these effects of differentiation status of the MFs, the patches of ECs did alter the growth of the MFs. The ECs and MFs formed coinciding clusters. This was likely caused by either cell-cell contact with ECs or the paracrine effect of short lived substances released by the ECs as MFs cultured in conditioned EC medium did not form these clusters. Another effect of the EC-MF co-culture was that the gene expression screening of the initial experiment showed an increase in MHC expression in the co-culture group. Although this could indicate a more contractile phenotype of the MFs due to co-culturing with ECs, this observation should be considered preliminary as the increased expression was not found in the repeated experiment.

In the present study, no further attempts were made to change the differentiation status of ECs or MFs by for instance shear stress or cyclic stress. Several studies have indicated the importance of shear stress for EC function (Chiu *et al.*, 2003; Nackman *et al.*, 1998) in co-culture with SMCs. In an EC-SMC co-culture, proliferation of the SMCs was stimulated but this effect disappeared in the presence of shear stress. (Nackman *et al.*, 1998). Likewise, ECs without shear stress show higher expression of adhesion molecules and chemokines during co-culture with SMCs (Chiu *et al.*, 2003; Chiu *et al.*, 2004). When placed under shear stress however no differences were observed between ECs with and without SMCs. For future co-culture studies, the model should therefore be extended to include shear stress to the ECs and perhaps cyclic stretch to MFs.

In conclusion, human saphenous vein ECs influence the growth pattern of MFs, which can be seen by co-clustering of αSMA positive cells and CD31 positive cells. This influence was triggered by direct cell-cell contact or local paracrine interaction as these growth pattern changes were not occurring when the MFs were cultured in conditioned EC medium. Additional studies will have to be performed to investigate whether the ECs are indeed increasing the contractile behavior of the MFs.

Acknowledgements

We thank Kang Yuen Rosaria-Chak, Emmy Manders, Anne Vrancken and Elise Huisman from the Eindhoven University of Technology for performing the experiments. This research was performed in the Dutch Program for Tissue Engineering from the Dutch Technology Foundation (STW) and applied science division of NWO

Chapter 5

Thrombogenicity measurements on endothelialized cardiovascular constructs

The contents of this chapter are based on R.A.A. Pullens, M.M.E. Lamers, M. Stekelenburg, J.W.M. Heemskerk, F.P.T. Baaijens and M.J. Post (2008), *Method for measuring platelet adhesion on tissue engineered cardiovascular constructs,* manuscript in preparation

5.1 Introduction

Tissue engineered vascular grafts (TEVG) should contain a confluent, adherent, and non-thrombogenic endothelial cell (EC) layer. This is important as a mechanically strong, but thrombogenic TEVG is likely to fail as a result of thrombosis. Thrombosis occurs when blood comes in contact with surfaces other than the endothelium, in particular collagen (Sarkar *et al.*, 2007). ECs not only act as a physical barrier between blood and these subendothelial tissues, but also proactively inhibit thrombosis (Mitchell and Niklason, 2003). ECs are involved in the coagulation cascade by expressing molecules, such as thrombomodulin (TM) and tissue factor (TF), and release substances which neutralize thrombin. Furthermore, ECs secrete nitric oxide (NO) and prostacyclin (PGI_2), which suppress platelet adhesion (Mitchell and Niklason, 2003) and tissue plasminogen activator (tPA) and plasminogen activator inhibitor (PAI-1) to control fibrinolysis.

There is indirect evidence that the provision of an endothelial layer confers non-thrombogenicity on the TEGVs. Human umbilical vein ECs (HUVECs) and endothelial progenitor cells (EPCs) on TEVGs expressed the anti-coagulant anchor TM and endothelial nitric oxide synthase (NOS-3) which produces the platelet inhibitor nitric oxide (Schmidt *et al.*, 2006). EC-seeded collagen modules for tissue engineering (TE) expressed high levels of TM and low levels of the pro-coagulant TF (She *et al.*, 2007).

Thrombogenicity of TE surfaces can also be studied by exposure to blood components, most commonly platelet or leukocyte concentrates (Curwen *et al.*, 1982; Furukawa *et al.*, 1999; Grunkemeier *et al.*, 1998; McGuigan and Sefton, 2007). Bos *et al*. compared platelet adhesion on surfaces with immobilized albumin-heparin conjugates and surfaces seeded with ECs and showed a reduction in platelet adhesion on EC seeded surfaces under static conditions (Bos *et al.*, 1999).

The use of whole blood, however, offers conditions that mostly reflect the *in vivo* situation as all platelet, cell and major parts of the coagulation and plasmin cascades are represented. End point measurements include fibrin formation and deposition, thrombin formation and platelet adhesion (Cauwenberghs *et al.*, 2007; Diquelou *et al.*, 1994; Hedeman Joosten *et al.*, 1998; Kirchhofer *et al.*, 1993; Lecut *et al.*, 2004). Endothelialized fibrin based TEVGs for instance showed low platelet adhesion and no fibrin deposition when exposed to whole blood (Isenberg *et al.*, 2006). Likewise, EC seeding of sheet based TEVGs drastically inhibited platelet adhesion and activation upon exposure to heparinized whole blood (L'Heureux *et al.*, 1998).

Developing TEVGs, based on a PGA/P4HB scaffold seeded with human saphenous vein myofibroblasts (MF) using a fibrin gel, has resulted in mechanically strong vascular grafts (Hoerstrup *et al.*, 2001; Stekelenburg *et al.*, 2008). Endothelialization of such cultured cardiovascular rectangular constructs was feasible and resulted in a confluent monolayer (Chapter 3). However, the non-thrombogenic behavior of the resulting EC layer was not studied.

Therefore, the goal of the current study was to investigate whether human saphenous vein ECs grown on MF seeded PGA/P4HB strips reduce platelet adhesion.

Whole blood perfusion experiments were performed, using a custom made flow chamber. To study the effect of fibrin as a cell carrier, platelets suspended in PBS were used to examine the platelet adhesion on MF monolayers and MF-EC co-cultures with and without fibrin.

5.2 Materials and methods

5.2.1 Culturing human vena saphena cells

Human saphenous vein ECs and myofibroblasts (MFs) were expanded using standard culture methods up to passage 7 (Mol *et al.*, 2006; Schnell *et al.*, 2001). The MFs were characterized as a mixture of vimentin and vimentin/actin type MFs (Chapter 4). The culture medium for MFs consisted of DMEM Advanced (Invitrogen, Netherlands), supplemented with 10% Fetal Bovine Serum (FBS; Greiner, Austria), 1% GlutaMax (Gibco) and 1% penicillin/streptomycin (P/S; Lonza, Belgium), further referred to as 2D medium. The EC culture medium consisted of EGM-2 medium supplemented with growth additives (Lonza), 1% P/S, and 20% FBS, further referred to as EC medium. The medium used for culturing EC-MF tissue constructs, referred to as TE medium, was the same as for MFs, but supplemented with L-ascorbic acid 2-phosphate (0.25 mg ml^{-1}; Sigma).

5.2.2 Static platelet adhesion

Platelet adhesion on MF monolayers and MF-EC co-cultures with and without fibrin was investigated under static conditions. MFs ($5 \cdot 10^3$ cells cm^{-2}, passage 7) were seeded in 6 well plates using either 2D medium (n=14) or by using fibrin as a cell carrier (n=14). The fibrin provided a 3D environment for the MFs. The cells were cultured for 7 days in 2D culture medium, which was changed every 2-3 days. After 7 days, half of the wells (n=7) of the 2D medium and fibrin seeded wells were seeded with ECs ($7 \cdot 10^3$ cells cm^{-2}, passage 7). All groups were subsequently cultured in EC medium for 7 days and medium was changed every 2-3 days. Table 5.1 shows the experimental groups (n=7 per group).

Table 5.1: Test groups in static and dynamic adhesion experiment. MFs seeded with or without fibrin, with or without ECs seeded on top. 3D constructs seeded with or without ECs.

Group	Type	Condition	EC	N
MF	Mono	Static	-	7
MF + EC	Mono	Static	+	7
MF_{fibrin}	Fibrin	Static	-	7
MF_{fibrin} + EC	Fibrin	Static	+	7
MF_{const}	3D construct	Dynamic	-	5
MF_{const} + EC	3D construct	Dynamic	+	5

Platelets were isolated from buffy coats from human blood (Sanquin, Netherlands). The buffy coat was diluted ten fold with PBS and then centrifuged for 10 minutes at 4500 rpm. The supernatant containing platelets (30 ml) was taken and incubated with 90 µM carboxyfluorescein succinimidyl ester (CFSE, Sigma) at room temperature for 10 minutes to label the platelets. After staining, the solution was centrifuged (1000 rpm) for 10 minutes and the resulting platelet pellet was suspended in PBS.

Medium was removed from each well and after rinsing with PBS, 2 ml of the platelet suspension was added to each well and incubated at 37° C, 5% CO_2 for 1 hour under static conditions. After incubation, the platelet suspension was removed and the wells were rinsed twice with PBS to remove unbound platelets. Images were taken using a fluorescent microscope and the adherent platelets were counted manually.

5.2.3 Tissue engineered constructs

Rectangular shaped scaffolds (n=10, 30 x 8 mm) composed of nonwoven polyglycolic acid (PGA; thickness 1.0 mm, specific gravity 70 mg cm^{-3}; Cellon, Bereldange, Luxembourg) were coated with poly-4-hydroxybutyrate (P4HB; Symetis Inc, Zürich, Switzerland) in tetrahydrofuran (THF). The THF was allowed to evaporate overnight. The two outer edges of the long axis of the scaffolds were glued on stainless steel rings using a 20% solution of polyurethane (PU; DSM, Netherlands) in THF, leaving a 20 x 8 mm remaining surface for cell seeding (Figure 5.1). The rings were placed in wells of 6 well plates. The constructs were sterilized by UV radiation for 45 minutes and subsequent soaking in 70% alcohol for 3 hours. Afterwards, the constructs were washed with PBS and placed in TE medium overnight. The rectangular tissue engineered constructs will further be referred to as TE strips.

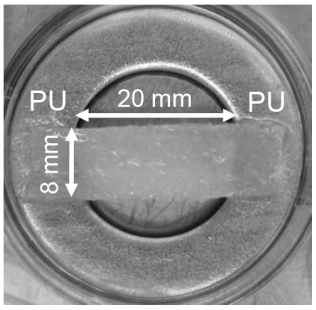


Figure 5.1: TE strip shortly after seeding. The scaffold is glued (PU) to a stainless steel ring.

MFs were seeded in the TE strips using fibrin as a cell carrier (Mol *et al.*, 2005). Briefly, MFs were suspended in TE medium containing thrombin (10 IU ml^{-1}, Sigma). This cell suspension is mixed with an equal volume of TE medium containing fibrinogen (10 mg ml^{-1}) and this fibrin/cell suspension ($20 \cdot 10^6$ cells ml^{-1}) was mixed until onset of the polymerization process of the gel and subsequently dripped onto the TE strips (150 µl $strip^{-1}$). The 6 well plates were placed in an incubator at 37° C and 5% CO_2 to allow the fibrin gel to further polymerize for 30 minutes. Afterwards the

wells were filled with 6 ml TE medium, and placed on a shaking table to allow gentle mixing of the TE medium. During the 4 week culture period, the TE medium was changed every 2-3 days. After 4 weeks of culturing, ECs (passage 7) were seeded onto half of the TE strips (n=5) with a cell density of 10^6 cells cm^{-2} using EC medium. The EC seeded constructs were cultured for 7 days in EC medium, and the non EC seeded TE strips were cultured in TE medium.

5.2.4 Composition of TE strips

The EC layer of the TE strips was studied using an EC specific FITC UEA-1 lectin (Sigma) staining and confocal microscopy (Zeiss, CLSM 510) as described previously (Chapter 2). The amount of DNA, sulfated glycosaminoglycans, and collagen of the TE strips was determined by previously described biochemical assays (Chapter 2). Furthermore, the tissue morphology was studied by staining paraffin sections (10 μm) with Hematoxylin and Eosin (H&E) and Masson trichrome (MTC; Sigma, USA)

5.2.5 Dynamic platelet adhesion

Whole blood perfusion experiments, for the determination of dynamic platelet adhesion, were performed as described previously (Cauwenberghs *et al.*, 2007; Siljander *et al.*, 2004) using custom made flow chambers (Figure 5.2). Blood (10 ml) from healthy volunteers was collected and 1 ml saline containing 20 μl D-Phe-Pro-Arg chloromethyl ketone (40 μM PPack, Calbiochem, San Diego, CA, USA) was added. Coagulation was prevented by hourly addition of 10 μl ml^{-1} PPack (20μM). CFSE (90 μM) was used to label the platelets.

The EC seeded (n=5) and non EC seeded TE strips (n=5) were removed from the stainless steel rings and pieces (10 x 5 mm) of the strips, were placed in a custom made flowchamber (Figure 5.2). Depending on the thickness of the TE strips, a modified flowchamber was used with either a 700 or 800 μm deep cavity. The chamber was covered with a coverglass, placed in a holder and attached to a pump. Shear stress was calculated using the equation for parallel plate flow: $\tau = 6\ \mu Q / wh^2$ with τ = shear stress, μ = fluid viscosity ($4 \cdot 10^3$ Pa/s), Q = blood flow rate, w = channel width, and h = channel height. The blood was placed in a syringe and perfused through the flow chamber at a continuous flow rate of 10 ml h^{-1} for 6 minutes, resulting in a shear stress of 1.5 Pa. After perfusion, the flow chamber was rinsed at the same flow rate for 6 minutes with PBS, to remove all non-adhered platelets. The TE strips were removed from the flow chamber and placed on a clean coverglass for analysis with the fluorescence microscope. Photos were taken of the surface (> 9 per strip) and adhered platelets were counted manually.

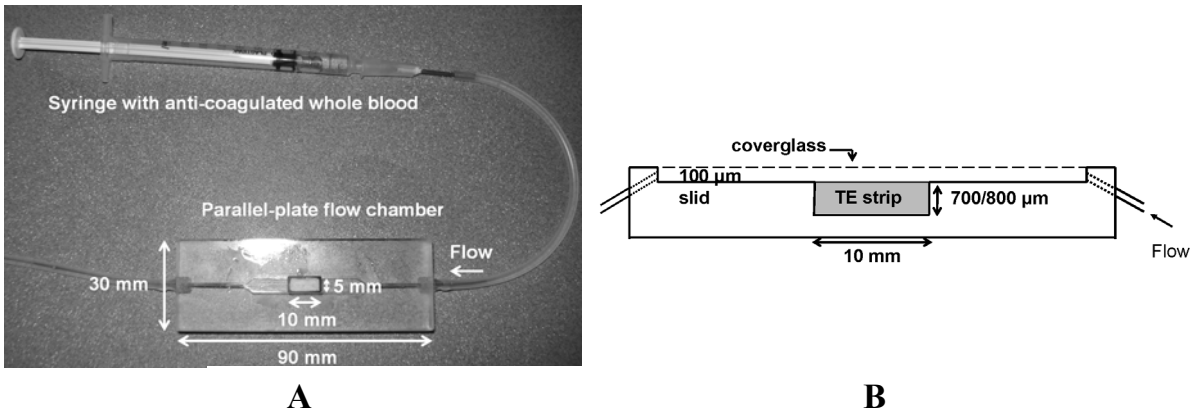


Figure 5.2: Parallel-plate flow chamber connected to syringe with anti-coagulated blood (A) and schematic overview of flow chamber (B). The TE strips had a length and width of 10 mm and 5 mm, respectively.

5.2.6 Statistical analyses

Differences between groups were determined by one way ANOVA analysis with a Bonferroni post hoc test. P-values of $p \leq 0.05$ were considered significant.

5.3 Results

5.3.1 Static platelet adhesion

Static platelet adhesion was examined in several MF cultures and MF-EC cultures (Figure 5.3A-D). The MFs without fibrin (Figure 5.3A) had formed a homogenous layer of elongated cells. The cell density of the MFs seeded in fibrin seemed lower (Figure 5.3C), compared to the fibrin free culture. When ECs were seeded onto the MF cultures (Figure 5.3B & D), they formed clusters of cells and reached an estimated confluency of 80%.

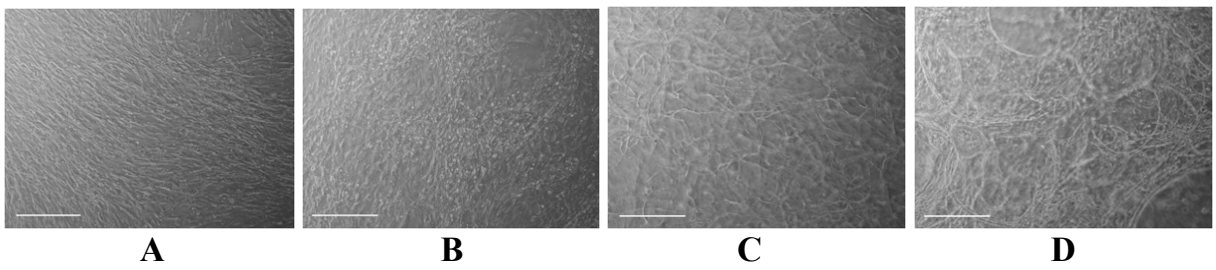


Figure 5.3: Images of cell cultures. The MFs had formed an almost confluent layer (A). ECs co-cultured on the MFs (B). MFs seeded in fibrin without (C) or with (D) ECs co-cultured on top. In co-cultures, ECs did not form a confluent layer. Scale bars indicate 300 µm.

The adherent fluorescent platelets were visualized (Figure 5.4A-D) and counted (Figure 5.4E). Co-cultures with ECs had lower numbers of adherent platelets, both in the fibrin ($p<0.001$) or fibrin-free conditions ($p<0.01$). Platelet adhesion on MFs in fibrin was higher than on MFs in a monolayer ($p<0.05$). The platelets were distributed equally over the surface (Figure 5.4A-D).

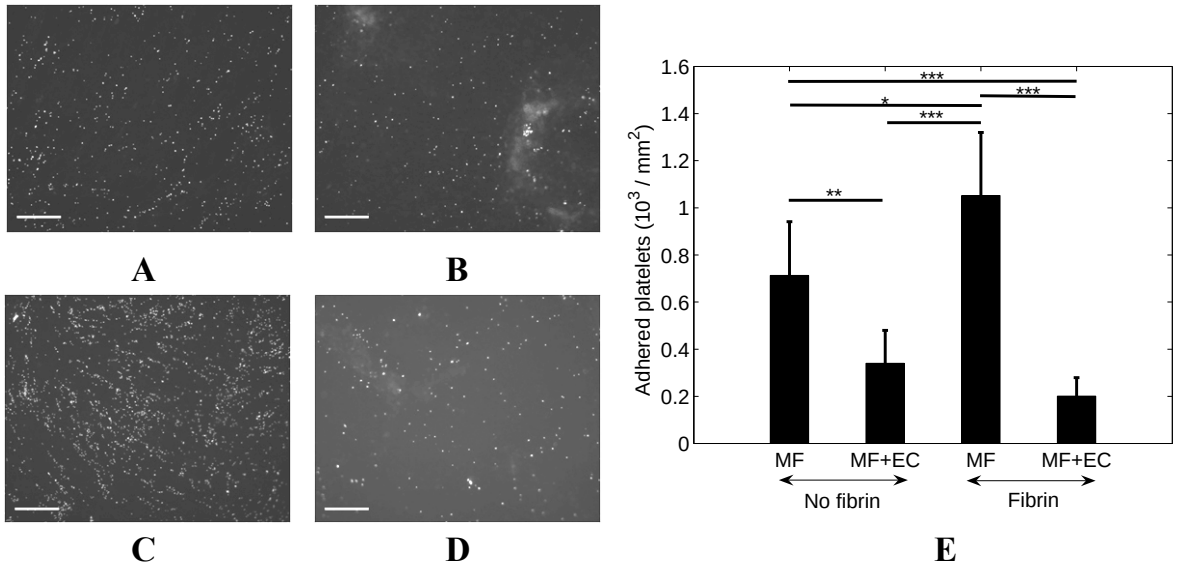
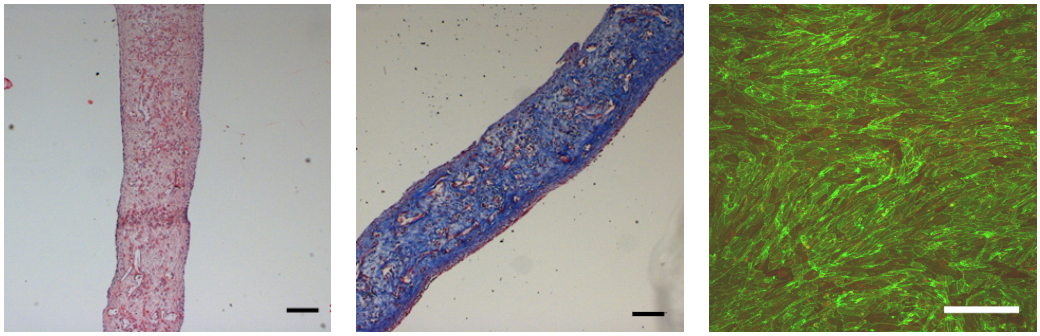


Figure 5.4: Fluorescent microscope images of adherent platelets on the MFs without (A, B) and with fibrin (C, D) with co-cultured ECs (B, D). The number of adherent platelets was quantified per mm^2 (E) (mean ± s.d.). When ECs were co-cultured on MFs, both with and without fibrin, the number of adherent platelets decreased $p<0.001$ and $p<0.01$, respectively. Platelet adhesion on MFs in fibrin was higher than on MFs in a monolayer ($p<0.05$). Scale bars indicate 200 μm. ($p<0.05$, ** $p<0.01$, *** $p<0.01$)*

5.3.2 Composition of TE strips

During culture, the surface of the TE strips, with or without ECs, became smoother and the strips became less transparent. Compaction occurred, resulting in a decreased width of the TE strips. The average DNA, glycosaminoglycan and collagen content (mean ± s.d.) of the TE strips were 2.7 ± 0.5 μg mg^{-1}, 25.7 ± 2.8 μg mg^{-1} and 129.8 ± 10.9 μg mg^{-1}, respectively. H&E and MTC staining (Figure 5.5A-B) indicate a solid tissue, with collagen abundantly present. This ECM composition was similar to previously cultured tissues (Chapter 3). A confluent monolayer of ECs was present on the constructs (Figure 5.5C). Half of the cells had a cobblestone morphology, the other half was slightly elongated.



A B C

Figure 5.5: H&E (A) and MTC (B) staining of TE strip showing good tissue formation. Collagen (B, blue) is present throughout the total construct. A comparable staining was found in the EC seeded strips. The EC seeded TE strips had a confluent layer of UEA-1 positive ECs (C, green). Scale bars indicate 300 µm (A, B) and 200 µm (C).

5.3.3 Dynamic platelet adhesion

TE strips with or without ECs were exposed to blood flow with fluorescently labeled platelets, to determine platelet adhesion under dynamic flow circumstances (Figure 5.6A-B). The amount of adherent platelets on the TE strips was higher ($p=0.05$) than on the EC seeded TE strips (Figure 5.6C). Platelet aggregates were also more abundant in the absence of ECs (data not shown).

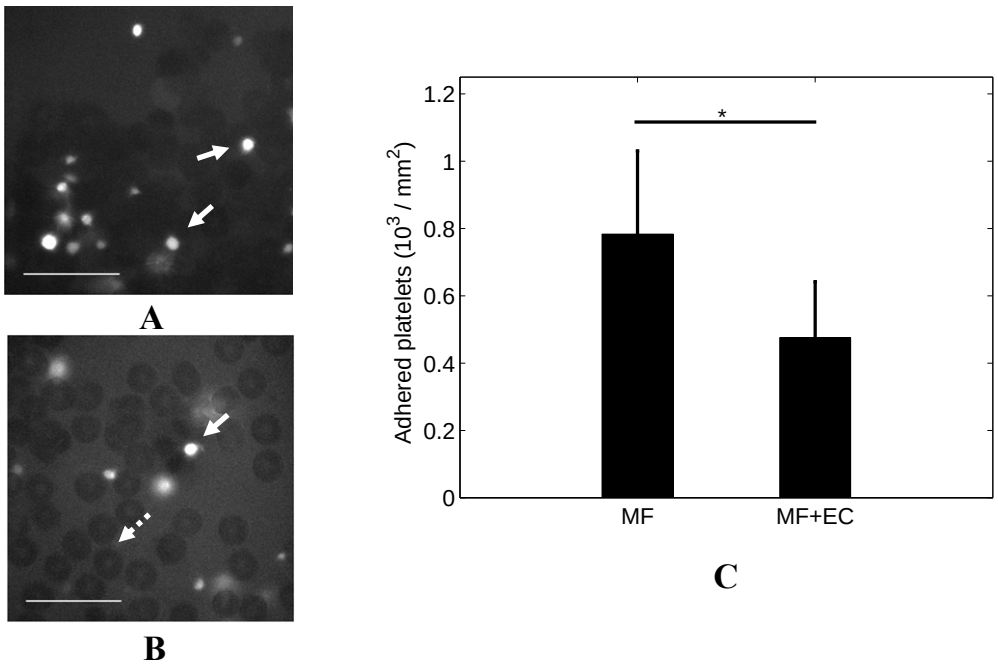


Figure 5.6: Adherent platelets after whole blood flow on TE strips without (A) and with ECs (B). The amount of platelets per mm^2 is determined (C) (mean ± s.d.). A lower number of platelets adhered to the EC seeded TE strips ($p \leq 0.05$). Solid and dashed arrows indicate platelets and red blood cell, respectively. Scale bars indicate 50 µm.

Despite rinsing with PBS, some red blood cells were still visible. Approximately, 10% of the adherent platelets on the non EC seeded TE strips had pseudopods (Figure 5.7), suggesting an activated state, compared to less than 1% on the EC seeded TE strips.

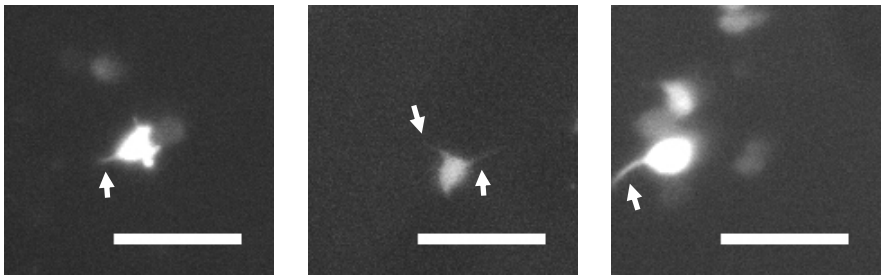


Figure 5.7: On the non EC seeded TE strips, approximately 10% of the platelets had pseudopods (arrows), suggesting an activated state. Scale bars indicate 20 μm.

5.4 Discussion

A non-thrombogenic, shear resistant, confluent EC layer is necessary for a good performance of TEVGs. Cells from the human saphenous vein can be used for the development of TEVGs (Poh *et al.*, 2005; Stekelenburg *et al.*, 2008). The present study focused on measuring platelet adhesion, as a measure for thrombogenicity, to ECs seeded on a MF monolayer, on MFs seeded in fibrin, and on 3D tissue constructs. Co-cultures of MFs with ECs had lower numbers of adherent platelets, both in the fibrin ($p<0.001$) or fibrin-free conditions ($p<0.01$). To investigate whether the ECs could also reduce the thrombogenicity of TE 3D constructs, whole blood perfusion experiments were performed. Less platelets adhered to the endothelialized TE strips, compared to the TE strips. Furthermore, the platelets which did adhere to the endothelialized constructs had a round non activated state, whereas 10% of the platelets on the non-endothelialized constructs appeared activated.

In the static monolayer experiments, the maximum number of platelets that adhered to the MFs seeded in a fibrin gel was approximately $1.4 \cdot 10^3$ platelets mm^{-2}. Compared to static platelet adherence on several biomaterials (Motlagh *et al.*, 2007; Rodrigues *et al.*, 2006; Vanickova *et al.*, 2006), this number is relatively low. Approximately $1.5 \cdot 10^5$ platelets mm^{-2} adhered to ePTFE (Motlagh *et al.*, 2007), about $1.5 \cdot 10^4$ platelets mm^{-2} (Vanickova *et al.*, 2006) to fibrinogen, and approximately $1.5 \cdot 10^4$ platelets mm^{-2} (Rodrigues *et al.*, 2006) to monolayers of alkanethiolates on gold. In these studies, often platelet rich plasma (PRP) or a buffer supplemented with $CaCl_2$ and/or $MgCl_2$ was used, to activate the platelets. Because in the current study, the platelets were suspended in PBS without additional additives was used, this has probably resulted in a lower amount of attached platelets.

A 2-fold reduction in platelet adhesion was found when ECs were seeded on a monolayer of MFs. ECs seeded on a fibrin gel containing MFs, resulted in a 5-fold

reduction, suggesting a non-thrombogenic phenotype. The difference between the fibrin and the non-fibrin groups, might be explained by the incomplete coverage of the ECs seeded on the monolayer of MFs. The ECs formed patches, leaving some MFs and fibrin exposed to the platelets. Although no difference was found in EC coverage of the fibrin and the MF monolayer, the ECs on the fibrin could have been more quiescent. Lavender *et al*. have shown that ECs seeded on proliferating SMCs formed patches, compared to ECs seeded on quiescent SMCs, which grew confluent (Lavender *et al.*, 2005). Hashi *et al*. (Hashi *et al.*, 2007) incubated monolayers of human aorta SMCs and ECs with PRP and found a 4-fold reduction in adherent platelets in the presence of ECs. ECs seeded on albumin conjugates reduced the number of adherent platelets 2-4 fold after static incubation (Bos *et al.*, 1999). Our results were therefore in line with these observations.

In the current study, the tissue composition of cardiovascular TE strips, which were cultured for 5 weeks, was comparable to previously cultured TEVGs (Stekelenburg *et al.*, 2008) and heart valves (Mol *et al.*, 2006). These TE strips contained a confluent EC layer and, as such, could be used as a model system for performing thrombogenicity measurements. For these experiments, platelet adhesion was determined under dynamic conditions. Anti-coagulated blood was perfused over the TE strips, using a pump and a custom made parallel plate flow chamber. This flow chamber was designed with a 100 μm slid, however, because the TE strips had a variable thickness, the size of the slid might have varied. This might have resulted in differences in applied shear stress, which can influence platelet adhesion (Diquelou *et al.*, 1994). However, assuming similar thickness variability in both groups, the platelet adhesion results have been affected in the same way.

Platelet adhesion on endothelialized TE strips was 2-fold lower compared to TE strips without ECs. A similar decrease was found when ECs were seeded on several types of Dacron surfaces (Prasad and Krishnan, 2005). Another study showed that ECs furthermore lowered thrombin and fibrin formation when seeded on ePTFE grafts (Hedeman Joosten *et al.*, 1998).

In the present study, the EC layer of the TE strips was not conditioned with shear stress. It is well known, that shear stress conditioning of ECs seeded in TEVGs is necessary to create a shear resistant EC layer. Shear stress is also important in the non-thrombogenic behavior of ECs. ECs upregulate several molecules such as NO and PGI_2, which inhibit platelet aggregation (Barakat and Lieu, 2003; Traub and Berk, 1998). It is predicted that the platelet adhesion on shear conditioned cardiovascular TE strips will decrease even more, enhancing the non-thrombogenic behavior.

The current study focused on platelet adhesion and activation. However, besides activation of cellular components of the blood, thrombosis also involves the activation of the coagulation cascade and the complement cascade. Although, the ECs indeed lowered the amount of adherent platelets to monolayers of MFs and cardiovascular TE strips, this data is not sufficient to declare the EC layer non-thrombogenic. For future studies, some elements of the coagulation and complement system should be measured. For example, the expression of TM and TF of the EC layer could be studied (Schmidt

et al., 2006), which should be high and low, respectively, when the layer is un-activated and confluent. Besides extending the present work with molecular expression studies, different end point measurements could be performed using the blood perfusion system. These end point measurements, like thrombin activation and fibrin generation and deposition, can give additional information on the non-thrombogenic behavior of the EC seeded surface.

In conclusion, a model has been developed in which platelet adhesion, as a measure for thrombogenicity on 3D vascular constructs can be measured. Using this model, it was shown that the presence of a confluent EC layer lowered the thrombogenicity of human cardiovascular TE constructs.

Acknowledgements

We thank Moniek Lamers from the Eindhoven University of Technology for performing the experiments. This research was performed in the Dutch Program for Tissue Engineering from the Dutch Technology Foundation (STW) and applied science division of NWO.

Chapter 6

Medium with blood-analog mechanical properties for cardiovascular tissue culturing

The contents of this chapter are based on C.N. van den Broek, R.A.A. Pullens, O. Frøbert, M.C.M. Rutten, W.F. den Hartog and F.N. van de Vosse (2008), *Medium with blood-analog mechanical properties for cardiovascular tissue culturing,* Biorheology, in press

6.1 Introduction

Tissue engineering (TE) of small diameter (<5 mm) blood vessels is a promising approach to develop viable alternatives for autologous vascular grafts (L'Heureux *et al.*, 2006; L'Heureux *et al.*, 2007a; Niklason *et al.*, 2001; Stekelenburg *et al.*, 2008). Such TE grafts should provide sufficient mechanical support and should also contain a functional endothelial cell (EC) layer (Mitchell and Niklason, 2003). *In vitro* conditioning of EC covered constructs with cyclic shear appears to prevent *in vivo* loss of endothelium. Several bioreactor systems have been developed in which shear stresses can be applied to vascular grafts (Buttemeyer *et al.*, 2003; Hoerstrup *et al.*, 2001; Isenberg *et al.*, 2006; L'Heureux *et al.*, 2006). The TE grafts in these bioreactors however, are typically perfused with culture medium having a viscosity lower than blood, thus inducing wall shear stresses below physiological levels when physiological flow rates are applied (Isenberg *et al.*, 2006; Narita *et al.*, 2004).

Human blood is a non-Newtonian fluid, which shows shear-thinning behavior under high flow velocities. Several supplements have been investigated to create a fluid with blood-like mechanical properties. Examples of such substances are polyacrylamide (Mann and Tarbell, 1990; Moravec and Liepsch, 1983), glycerol (Brookshier and Tarbell, 1993; Moravec and Liepsch, 1983), and xanthan gum (XG) (Brookshier and Tarbell, 1993; Gijsen *et al.*, 1999; Mann and Tarbell, 1990). For use in arterial culture, however, the supplement should not affect the cell and tissue biology.

Previously, among others, Gijsen *et al*. (Gijsen *et al.*, 1999) used XG to create a non-Newtonian solution with blood-analog shear-thinning behavior for use in a large artery model. XG is a polysaccharide produced by the bacterium *Xanthomonas campestris* found on cabbage. It is a very stable thickener and low concentrations already result in high viscosities and shear-thinning behavior (Rocks J.K., 1971; Sanderson G.R., 1981). It is expected that the addition of XG to a culture medium will not lead to large changes in osmolality, because of its high molar mass (approximately 2×10^6 g mol^{-1}, (Katzbauer B., 1998)) and the low concentration that is needed to increase fluid viscosity to blood viscosity levels. In human studies, XG has been documented as a safe additive to food without adverse dietary or physiological effects (Eastwood *et al.*, 1987). Babbar and Jain proved the biocompatibility of XG for cultures of bacteria and fungi (Babbar and Jain, 2006).

The aim of this study was to develop a culture medium with blood-analog mechanical properties, based on the addition of XG, which does not significantly influence cell and tissue biology. Such a culture medium could be used to apply more physiological boundary conditions to a cultured vessel than is possible with current culture media.

Viscosity measurements were performed to determine the XG concentration needed to create a culture medium with non-Newtonian shear-thinning behavior comparable to that of blood. To examine the effect of XG on cell biology both the osmolality of the XG supplemented culture medium and the effect of the culture medium with XG on cell growth in EC monolayers were investigated. It has been well

established that, after a certain time, EC align in the direction of flow when exposed to laminar shear stress (Dewey, Jr. *et al.*, 1981). Therefore, the shear-sensing property of ECs was studied by examining EC alignment after 24 hours of physiological shear stress. It was also investigated whether XG possesses any intrinsic vasoactive properties or affects arterial smooth muscle and other EC functions.

6.2 Materials and methods

6.2.1 Preparation of an XG solution

XG (Fluka Biochemika, 95465) was sterilized by exposure to UV light for 90 minutes. The XG was added to culture media or a physiological salt solution at different concentrations (Table 6.1) and dissolved at 37° C at constant agitation for a period of approximately 24 hours until clear solutions were obtained.

Table 6.1: Xanthan gum concentration and solutions used for the different experiments

Experiment	Solution	XG concentration (g l^{-1})
Viscosity measurements	- Culture medium (DMEM)	0.0, 0.1, 0.2, …, 1.0
	- EC medium	0.0, 0.1, 0.2, …, 1.0
Osmolality	- Culture medium (DMEM)	0.0 and 0.7
Culture of EC monolayer	- EC medium	0.0 and 0.66
EC alignment	- EC medium	0.66
Intrinsic vasoactive properties and effect on cell function	- Physiological salt solution	0.0 and 0.69

6.2.2 Viscosity measurements

The culture medium should have shear-thinning behavior comparable to that of blood for physiological shear rates of 100-500 s^{-1} in the larger arteries (> 1 mm diameter). A porcine epicardial coronary artery, such as the proximal part of the left anterior descending coronary artery (LAD), has an inner diameter of approximately 3 mm, and a flow rate (at rest) of 60 ml min^{-1}. The required fluid viscosity to induce a mean physiological wall shear stress (1.5 Pa) is estimated considering the culture medium as a Newtonian solution. Consequently, the fluid viscosity should be about 4×10^{-3} Pa s for a wall shear rate of approximately 200 s^{-1}. Around this mean shear rate the medium should show non-Newtonian, blood-analog shear-thinning behavior.

Viscosity measurements were performed with a regular culture medium, as used in organ culture and an EC medium for use in the culture of ECs. The regular culture medium (Dulbecco's Modified Eagle's Medium (DMEM), BioWhittaker) was supplemented with 2% fetal bovine serum (FBS), 1% penicillin/streptomycin, 1% L-glutamine, 0.2% amphotericin, 5 mg l^{-1} vancomycin as used in arterial culture. The EC medium consisted of EGM-2 medium supplemented with growth supplements (Lonza,

Belgium) and 20% FBS (Greiner). We examined both media with stepwise increasing XG concentrations from 0.1, 0.2,…, 1.0 g l^{-1}. A cone-plate test (Macosko C.W., 1994) was performed at 37° C to determine the viscous behavior of the DMEM and EC medium solutions with different concentrations of XG (ARES, Rheometric Scientific). The shear rate varied from 1000 s^{-1} to 10 s^{-1} decreasing by 6 steps per decade. Each measurement took 4 s and was repeated twice. The viscosities measured at a shear rate of 200 s^{-1} for the different XG concentrations were fitted with a quadratic function. From this fit, the XG concentration needed to increase medium viscosity to 4×10^{-3} Pa s was determined.

6.2.3 Osmolality

The osmolality of DMEM alone and DMEM supplemented with XG [0.7 gL^{-1}] was measured at 37°C using a vapor-pressure osmometer (Knauer). A calibration curve was determined using different concentrations of NaCl. The osmolality of both solutions was determined from 3 samples and averaged.

6.2.4 Effect of XG on cell morphology and growth

ECs were harvested from fresh discarded segments of the human saphenous vein, following a previously described method (Chapter 3). The cells were expanded *in vitro* with standard methods using EC medium. At passage 5, the EC were seeded in 25 cm^2 flasks (n = 48) at a concentration of 5000 cells cm^{-2}. After 1 day, the flasks were divided into 2 groups, one where the cells were kept on EC medium, and one where the EC medium was supplemented with XG [0.66 g l^{-1}] (EC-XG medium), at a concentration at which the viscosity was 4×10^{-3} Pa s at a shear rate of 200 s^{-1}. At days 2, 4, 6, and 8, phase contrast images were made of flasks from each group (n = 6) and the cells were trypsinized. The number of cells was determined using a nucleocounter (Chemometec).

Quantitative data were averaged per group per time point, and represented as mean ± standard deviation. Using a variance-weighted two-way ANOVA analysis, the influence of time and XG medium were determined. Post-hoc comparisons using the Bonferroni correction were used to determine significant differences ($p < 0.05$) between groups per time point. All statistical analyses were performed using SPSS v.15.0 software (SPSS Inc., Chicago, IL, USA).

6.2.5 Effect of XG on endothelial cell alignment

ECs were seeded (1.6×10^4 cells cm^{-2}) on 0.1% gelatin coated glass slides (n = 8). The ECs were cultured overnight in EC medium, after which the medium was changed to EC-XG medium. The slides were divided into a static and a shear stress group. ECs in the shear stress group (n = 4) were subjected to a steady laminar shear stress with a physiological value of 1.5 Pa induced by the EC-XG medium using a parallel-plate flow chamber. Flow was generated by a roller pump, which was configured such that

pulsations were minimized. After 24h of culture at zero-flow (n = 4) and physiological shear stress (n = 4) EC monolayers were fixed in formaldehyde (3.7%) for 10 minutes. To test for changes in shear thinning behavior of the XG medium due to the roller pump, the viscosity of the used culture media were analyzed. The EC monolayers were stained with rhodamin-conjugated Phalloidin (Sigma) and DAPI (Sigma), labeling the EC actin filaments and cell nuclei, respectively. Images were made with a 40x objective using a fluorescence microscope (Zeiss).

6.2.6 XG and endothelium dependent relaxation

To test whether XG possesses any intrinsic vasoactive properties or affects vasomotion by smooth muscle and ECs, rings of a porcine coronary artery were stimulated by inducing either vasoconstriction by direct smooth muscle stimulation or vasodilation by indirect relaxation of the smooth muscle cells through an endothelium-dependent pathway. Ideally, vasoconstrictor and vasodilator properties remain unchanged upon addition of XG to the surrounding saline.

Hearts from Landrace-Yorkshire hogs were obtained at a local slaughterhouse. Immediately after sacrifice the aorta was cannulated and the coronary circulation perfused with a physiological salt solution (PSS, containing (mM) 119 NaCl, 25 $NaHCO_3$, 4.7 KCl, 1.2 $MgSO_4$, and 1.5 $CaCl_2$) with 5.5 mM glucose, gassed with 5% CO_2 in O_2 and buffered with HEPES (20 mM). The LAD was carefully dissected and the proximal 3 - 4 cm of the artery was left intact. Arterial rings were mounted on two steel wires for isometric force measurement (Multi Myograph System 610M, Danish Myo Technology, Denmark). One wire was attached to an isometric force transducer and the other to a displacement unit, permitting control of the internal circumference of the preparation. The organ bath contained PSS, gassed with 5% CO_2 in O_2. The coronary arteries were allowed to equilibrate in PSS to 37° C, and stepwise stretched to 3.9 kPa of passive tension, which we found, in preliminary experiments, was optimal for maximal force development. After a stabilizing period in the organ bath, artery tone was induced via receptors by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$, 10^{-5} M). In some experiments the EC layer was removed with a small cotton stick. Successful removal was evaluated by adding the endothelium-dependent vasodilator bradykinin (Bk, 10^{-7} M). Total inhibition of relaxation to Bk was indicative of successful mechanical removal of ECs.

Potential vasoactive properties of XG (0.69 g l^{-1}, the concentration at which medium viscosity is 4×10^{-3} Pa s at a shear rate of 200 s^{-1}) were tested by: 1) the effect of adding XG on preconstriction with 10^{-5} M $PGF_{2\alpha}$ in arterial segments with and without ECs; 2) the effect of adding XG on endothelial function with Bk concentration response curves. Bk concentration varied from 10^{-10} to 10^{-7} M increasing in two steps per decade. Results are presented as mean ± s.e.m. Because coronary artery tension varied between segments the steady state tension induced by $PGF_{2\alpha}$ was used as an internal standard (100%). To test for differences between preconstriction with $PGF_{2\alpha}$ a Student's T-test was used. Differences in Bk concentration response curves were evaluated using repeated measures two-way analysis of variance. Differences were considered statistically significant when $p < 0.05$.

6.3 Results

6.3.1 Viscosity measurements

Viscosity increased with increasing XG concentration (Figure 6.1). Moreover, the solution demonstrated shear-thinning behavior (Figure 6.2). From the fit in Figure 6.1 it can be shown that the concentration of XG in DMEM and EC medium should be 0.69 g l^{-1} and 0.66 g l^{-1} respectively to create a medium viscosity of 4×10^{-3} Pa s at a shear rate of 200 s^{-1}. This would result in an average wall shear stress of 1.5 Pa for 3 mm diameter grafts. After subjecting XG medium to 48h of flow generated with a roller pump, no differences in shear thinning behavior were found (data not shown).

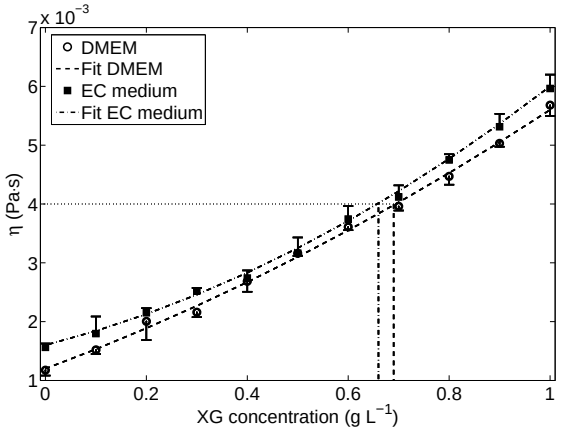


Figure 6.1: XG concentration in DMEM and EC medium versus the viscosity at a shear rate of 200 s^{-1} (mean ± s.d.). The data are fitted with a quadratic function $R^2 = 0.993$ and 0.999 for the fit of the XG concentration vs. DMEM and EC medium, respectively.

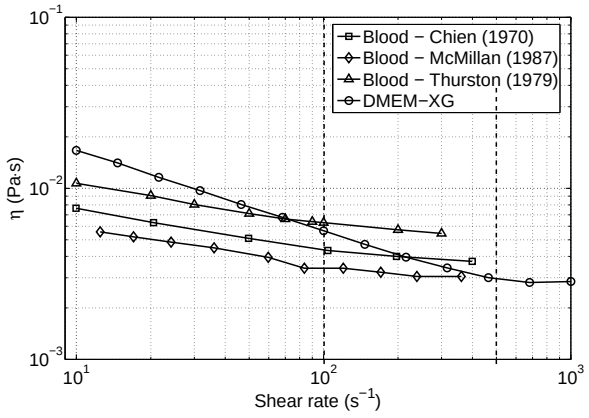


Figure 6.2: Shear rate versus the viscosity of DMEM supplemented with XG [0.7 g l^{-1}], compared to literature derived curves for blood (Chien et al., 1970; McMillan et al., 1987; Thurston, 1979). The two vertical dashed lines indicate the range of physiological shear rates for larger arteries (> 1 mm diameter).

6.3.2 Osmolality

The osmolality of DMEM was 317 ± 10 mOsm kg^{-1}, compared to an osmolality of 285 ± 2 mOsm kg^{-1} for DMEM supplemented with XG.

6.3.3 Effect of XG on cell morphology and growth

To examine the effect of XG on cell growth, XG was added to the culture medium and EC numbers were investigated. No differences were found in the EC shape and size, between the normal EC medium and EC-XG medium (Figure 6.3), suggesting that the EC are not influenced by the presence of XG. In both groups, the EC growth was rapid in the first days and leveled off towards confluence, suggesting contact inhibition of growth (Figure 6.4). The two-way ANOVA analysis of the growth curves (Figure 6.4) revealed a significant effect of time on cell density ($p < 0.001$), but the presence of XG did not have that significant effect.

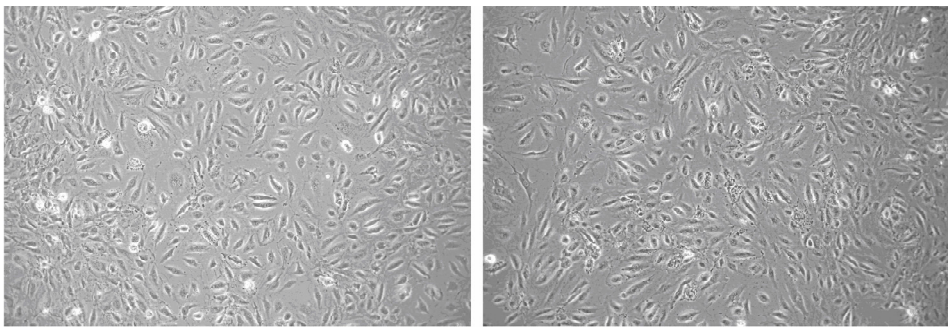


Figure 6.3: Typical images of EC monolayers after 8 days of culture in EC medium (A) and EC-XG medium (B). No difference in EC shape and size could be observed.

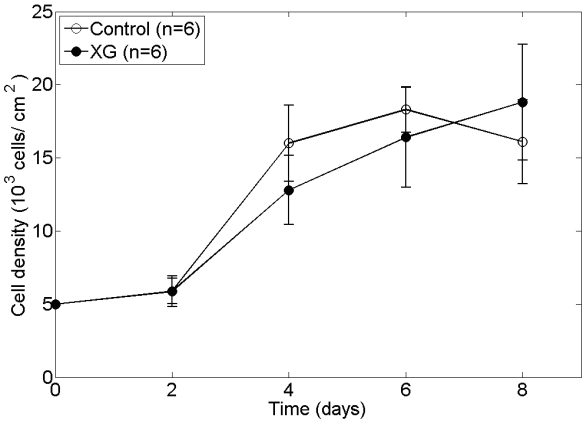
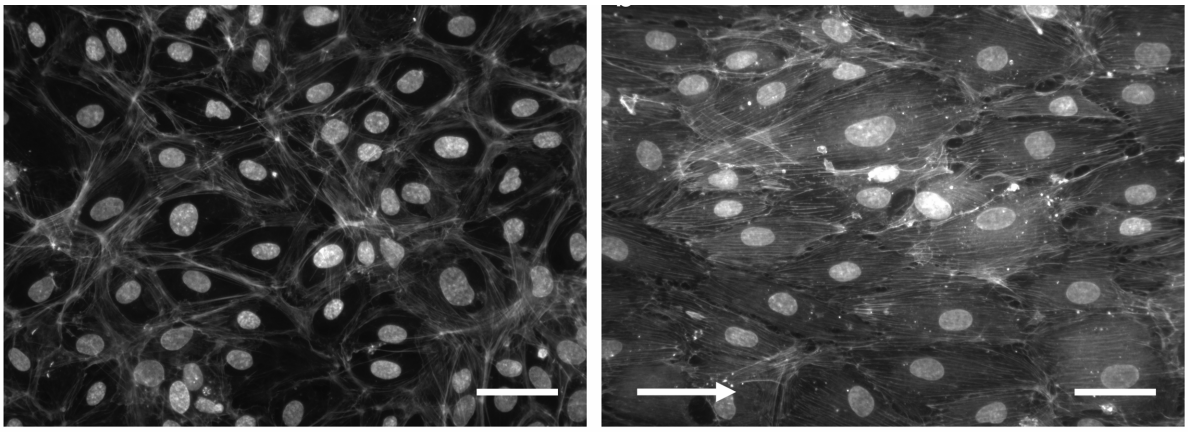


Figure 6.4: Time versus the cell density in a human EC culture in EC and EC-XG media (mean ± s.d.). No significant difference in cell density was found.

6.3.4 Effect of XG on endothelial cell alignment

When ECs were cultured with EC-XG medium under zero-flow conditions, ECs were oriented randomly (Figure 6.5A). After 24h at a shear stress of 1.5 Pa with EC-XG medium, the ECs and the associated actin filaments elongated and aligned in the direction of the flow (Figure 6.5B). Moreover, the actin cytoskeleton organization changed; more actin filaments can be observed in the central part of the EC, in contrast to the ECs in the control group (zero flow) where actin filaments were concentrated at the outer edges of the cells.



A **B**

Figure 6.5: F-actin filaments and cell nuclei in ECs cultured under static conditions (A); after 24h under a physiological laminar shear stress of 1.5 Pa (B). ECs are oriented randomly (A) and in the direction of the flow (B). The scale bars represent 50 μm, the arrow indicates the direction of flow.

6.3.5 Intrinsic and extrinsic vasoactive properties of XG

To investigate whether XG possesses any intrinsic vasoactive properties or affects smooth muscle and EC function, rings of a porcine coronary artery were stimulated by addition of $PGF_{2\alpha}$ (vasoconstriction) and Bk (endothelium-dependent vasodilation). There was no effect of XG on the coronary artery tension of arterial rings in PSS preconstricted with 10^{-5} M $PGF_{2\alpha}$ in arterial segments with or without EC (Figure 6.6). The tension is presented relative to the steady state tension induced by 10^{-5} M $PGF_{2\alpha}$. Figure 6.7 represents the effect of adding XG on the Bk-induced vasodilation of preconstricted arterial rings in PSS, as a measure of EC function. As in Figure 6.6, arterial tension is presented relative to the steady state tension induced by 10^{-5} M $PGF_{2\alpha}$. XG did not alter Bk-induced vasodilation significantly.

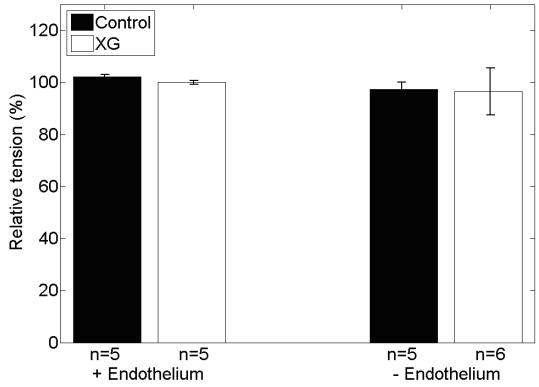


Figure 6.6: The effect of XG compared to normal medium on preconstriction with $PGF_{2\alpha}$ in coronary artery segments with endothelium and without endothelium (mean ± s.d.). No significant differences in arterial tension were found.

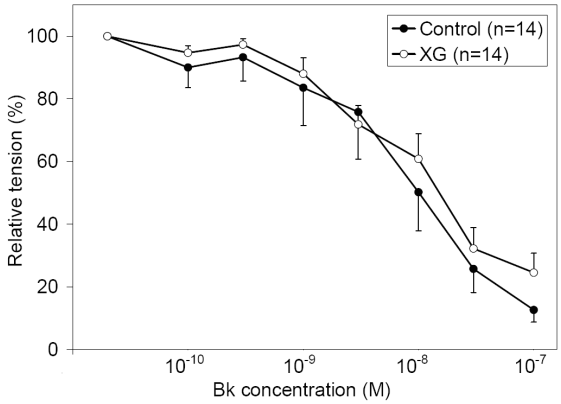


Figure 6.7: Addition of XG compared to normal medium on Bk concentration response curves in coronary artery segments after preconstriction with 10^{-5}M $PGF_{2\alpha}$ (mean ± s.d.). No significant difference in arterial tension was found.

6.4 Discussion

We investigated XG for its suitability as a viscosity enhancing agent for use in cardiovascular tissue culture. The main findings of this study were that: 1) XG-medium has blood-analog mechanical properties; 2) the osmolality of XG-medium is within a physiologically acceptable range; 3) addition of XG to EC medium does not influence EC growth; 4) ECs align in the direction of flow after 24h of shear stress induced by XG-medium, and 5) XG does not change vasomotion.

Viscosity measurements were performed to study the effect of XG on medium viscosity. Medium viscosity indeed increased with increasing XG concentration. Also, the solution shows shear-thinning behavior. The viscosity measurements performed on blood as found in the literature exhibit a large spread. Still, the DMEM-XG solution, though showing more pronounced shear-thinning behavior than blood, has viscosities

within the same range as the different blood measurements at physiological shear rates. For DMEM with supplements, the XG concentration should be 0.69 g l^{-1}. With this XG concentration, a medium viscosity of 4×10^{-3} Pa s for an average shear rate of 200 s^{-1} is created and the required mean physiological wall shear stress, estimated to be approximately 1.5 Pa (Lehoux and Tedgui, 2003; Malek *et al.*, 1999), is induced at physiological flow rates. For the EC-XG medium the required XG-concentration to create this increased medium viscosity is 0.66 g l^{-1}, so less XG is needed, probably due to the higher concentration of FBS in the EC medium. Because of this, viscosity measurements should be performed to determine the optimal concentration for each specific medium. A culture medium with blood-like shear-thinning behavior is of specific importance for physiologically pulsatile flow in arteries, as the oscillatory shear stress amplitude is attenuated by the shear-thinning behavior. Also, in situations of low shear rates as in veins and venules, the shear-thinning behavior is more pronounced. Therefore, the use of XG-medium is of particular interest in culturing arteries and veins at physiological pulsatile flows rates.

In TE and organ culture, for which the XG supplemented culture medium may be used, it is of importance that the medium viscosity is constant over time. After subjecting XG medium to 48h of flow (1.5 Pa shear stress) generated with a roller pump, no differences in shear thinning behavior were found (data not shown). As culture medium is usually replaced every 2-3 days, these results indicate that physiological shear stresses are maintained during culturing. It should be noted, however, that if degradation of XG occurs this will probably not be caused by the shear stress, but by the pump in which higher shear stresses may occur. Consequently, degradation of the XG polymer chains leading to a change in viscosity may happen when a different pump is used.

To date, dextran, exhibiting Newtonian behavior in solution, is the most widely used viscosity enhancing additive in artery culture (Chesler *et al.*, 1999). However, Sen *et al*. (Sen *et al.*, 2002) reported that medium osmolality is increased to 385 mOsm kg^{-1} for a dextran concentration of 150 g l^{-1}, which, in that study, was needed to increase medium viscosity to blood viscosity levels, whereas osmolalities between 260 mOsm kg^{-1} and 320 mOsm kg^{-1} are acceptable for most cells (Freshney R.I., 1994). This was corroborated by the finding that dextran concentrations above 50 g l^{-1} were detrimental to cell proliferation and affected cell metabolism (Sen *et al.*, 2002). Other examples of supplements that have been reported to increase medium viscosity for use in cultures of fungi, bovine embryos, and neural stem cells, respectively, are, alginate (Gromada and Fiedurek, 1997), hyaluronic acid potassium salt (Stojkovic *et al.*, 2002), and (carboxy)methylcellulose (Sen *et al.*, 2002). However, a study at our laboratory showed that, when added to DMEM, the osmolalities of the solutions were outside the physiologically acceptable range (data not shown). Therefore, they have not been investigated further. The osmolality of DMEM-XG [0.7 g l^{-1}] is 285 ± 1.9 mOsm kg^{-1}, which is within the physiologically acceptable range and comparable to blood plasma osmolality (about 290 mOsm kg^{-1}, (Boron W.F. and Boulpaep E.L., 2005)). DMEM alone has an osmolality of 317 mOsm kg^{-1}. Therefore, all DMEM-XG solutions up to a concentration of at least 0.7 g l^{-1} will be physiologically compatible. Accordingly, there

was no significant difference between the growth of human saphenous vein ECs in normal EC medium or EC medium supplemented with XG. Moreover, the shape and size of the ECs did not change. From those results it can be concluded that the proliferation rates are not compromised due to the added XG.

The response of ECs to fluid shear stress was studied by subjecting ECs to physiological shear stress in a parallel-plate flow chamber. After 24h of shear, EC alignment in the direction of flow was prominent, whereas statically cultured ECs which were in the presence of EC-XG medium did not align, which is in agreement with previous investigations (Dewey, Jr. *et al.*, 1981; Remuzzi *et al.*, 1984). Also, the change in arrangement of the actin filaments; i.e. the increase of aligned actin filaments in the center of the ECs, was similar to a study in which ECs were subjected to 1.5 Pa shear stress induced with medium which had not been supplemented with a viscosity-enhancing substance (Galbraith *et al.*, 1998). Therefore, it can be concluded that XG does not influence the response of ECs to shear stress. In addition, as it has been suggested that the EC glycocalyx surface layer is necessary for ECs in mechanosensing (Florian *et al.*, 2003; Yao *et al.*, 2007), it can be suggested that, the functioning of the glycocalyx layer is not affected by the XG.

To investigate whether XG possesses any intrinsic pharmacological properties, porcine coronary rings were tested for their constrictive and dilative capacity in presence of XG in PSS. XG had no significant influence on $PGF_{2\alpha}$-induced preconstriction in arterial segments with and without endothelium. Moreover, it did not affect Bk-induced vasodilation significantly. From those tests it can be concluded that XG does not influence endothelial and smooth muscle cell activity.

In conclusion, this study shows that, unlike standard culture media, XG as a blood-analog culture medium has rheological properties suitable for use in vessel culture and tissue engineering to induce physiological wall shear stresses under physiological flow conditions.

Acknowledgements

We thank Margit Nielsen (University of Aarhus, Denmark) and Kang Yuen Rosaria-Chak (Eindhoven University of Technology, The Netherlands) for technical help.

Chapter 7

Endothelialization of human tissue engineered vascular grafts

The contents of this chapter are based on R.A.A. Pullens, M. Stekelenburg, F.P.T. Baaijens and M.J. Post (2008), *Flow-aligned Human Endothelial Cells on Tissue Engineered Blood Vessels*, submitted

7.1 Introduction

In 2005, approximately 470,000 coronary bypass graft procedures were performed in the USA on patients suffering from cardiovascular disease (Rosamond *et al.*, 2008). Saphenous vein grafts are commonly used for these procedures, although they have a limited life time (Raja *et al.*, 2004), demonstrated by a patency of 57% after 10 years (Sabik III *et al.*, 2005). Vascular grafts are increasingly in demand as the number of patients who need follow-up surgery and have run out of native graft material is increasing. The same holds for arteriovenous shunt material for vascular access in dialysis patients (Berardinelli, 2006). Tissue engineering (TE) of small diameter (<5 mm) blood vessels is a promising approach to develop viable alternatives for autologous vascular grafts (L'Heureux *et al.*, 2006; L'Heureux *et al.*, 2007a; Niklason *et al.*, 2001; Stekelenburg *et al.*, 2008). Such TE grafts should provide sufficient mechanical support and contain a functional endothelial cell (EC) layer (Mitchell and Niklason, 2003). Several causes for autologous graft failure have been proposed including injuries related to graft harvesting, adaptation to the arterial circulation, and activation of the inflammatory and coagulation pathways (Akowuah *et al.*, 2003). Some of these failure modes are caused by a disrupted EC layer (Manchio *et al.*, 2005; Sellke *et al.*, 1996).

Many studies have shown that the patency of synthetic grafts is improved by EC seeding (Hoenig *et al.*, 2006; Seifalian *et al.*, 2002). In animal models, high patency rates were observed after implantation of EC seeded TE grafts with a follow up of several weeks (Borschel *et al.*, 2005; Dardik *et al.*, 1999; Niklason *et al.*, 1999; Swartz *et al.*, 2005). However, it is suggested that host derived re-endothelialization is occurring and that the seeded endothelium is no longer present at the time of explantation (Borschel *et al.*, 2005; Swartz *et al.*, 2005). It is commonly assumed that re-endothelialization of vascular grafts is slow and almost never complete in humans (Berger *et al.*, 1972; Rahlf *et al.*, 1986). Therefore, the favorable outcome in animal studies may not be indicative of clinical success. *In vitro* conditioning of EC covered constructs with shear appears to prevent *in vivo* loss of endothelium. The application of shear stress for EC retention has been studied in several systems. *In vitro* shear stress conditioning, by slowly increasing the shear stress over the course of several days, increases EC retention (Kaushal *et al.*, 2001) and reduces neointimal thickness after implantation *in vivo* (Dardik *et al.*, 1999). Several bioreactor systems have been developed in which shear stresses can be applied to vascular grafts (Buttemeyer *et al.*, 2003; Hoerstrup *et al.*, 2001; Isenberg *et al.*, 2006; L'Heureux *et al.*, 2006). As regular culture medium was used in these systems, the physiological shear stresses had to be induced by high non-physiological flows (>200 ml/min). Depending on the diameter of the vessels such high flows can become turbulent, which will inhibit EC alignment (Isenberg *et al.*, 2006). The average flow in the left anterior descending coronary artery is approximately 55 ml min^{-1} (Berne and Levy, 2001; Marcus *et al.*, 1999). Physiological shear stresses can be applied to TEVGs using these physiological flows by increasing the viscosity of the culture medium. Adding xanthan gum to the medium will increase its viscosity. We have shown that xanthan gum supplemented medium is a

blood-analog culture medium, which has rheological properties suitable for use in TE, without altering EC proliferation or EC flow induced alignment (Chapter 6).

Recently, strong vascular grafts have been developed in our group, with burst pressures of 900 mmHg (Stekelenburg *et al.*, 2008). The grafts were based on a polyglycolic acid (PGA) scaffold coated with poly-4-hydroxybutyrate (P4HB) and seeded with human myofibroblasts (MF) in a fibrin gel. The grafts were cultured for 4 weeks around a silicon tube. Although these grafts had sufficient mechanical properties, they did not have an EC layer and were therefore not suitable for *in vivo* application. The goal of the present study was to develop endothelialized PGA/P4HB based TEVGs, and to test the retention, survival and phenotypical changes of ECs under shear stress in a newly custom-built bioreactor system with physiological flow.

7.2 Materials and methods

7.2.1 Cell Culture

Human endothelial cells (ECs) and myofibroblasts (MF) were harvested from the same fresh discarded vein segment of the human saphenous vein, following a previously described method (Chapter 3). Two cell batches were mixed and expanded using regular culture methods (Schnell *et al.*, 2001) up to passage 7. The MFs were previously characterized as a mixture of vimentin and vimentin/actin type MFs (Mol *et al.*, 2006). The medium for culturing ECs, further referred to as EC medium, consisted of EGM-2 medium supplemented with growth additives (Lonza, Belgium), 20% Fetal Bovine Serum (FBS; Greiner, Austria) and 1% penicillin/streptomycin (P/S; Lonza, Belgium). The culture medium of MFs consisted of Advanced DMEM (Invitrogen, Netherlands), supplemented with 10% FBS, 1% GlutaMax (Gibco), and 1% P/S. The medium used for MF seeding and subsequent tissue culture, referred to as TE medium, was the same as for MFs, but supplemented with L-ascorbic acid 2-phosphate (0.25 mg ml^{-1}; Sigma).

7.2.2 Construction of rectangular tissue constructs

To account for experimental variability, in each experiment tissue strips were cultured to serve as internal controls. Rectangular shaped scaffolds (n=12, 30 x 6 mm) composed of nonwoven polyglycolic acid (PGA; thickness 1.0 mm, specific gravity 70 mg cm^{-3}; Cellon, Bereldange, Luxembourg) were coated with poly-4-hydroxybutyrate (P4HB; Symetis Inc, Zürich, Switzerland) in tetrahydrofuran (THF). The P4HB coating provided structural stability to the PGA mesh by fusion of the crossing fibers. The THF was allowed to evaporate overnight. The two outer edges of the long axis of the scaffolds were glued on stainless steel rings using a 20% solution of polyurethane (PU; DSM, Netherlands) in THF, leaving a 20 x 6 mm remaining surface for cell seeding (Figure 7.1). The rings were placed in 6 well plates. The constructs were sterilized by UV radiation for 45 minutes and subsequent soaking in 70% alcohol for 3 hours.

Afterwards, the constructs were washed with PBS and placed in TE medium overnight. The rectangular tissue engineered constructs will further be referred to as TE strips.

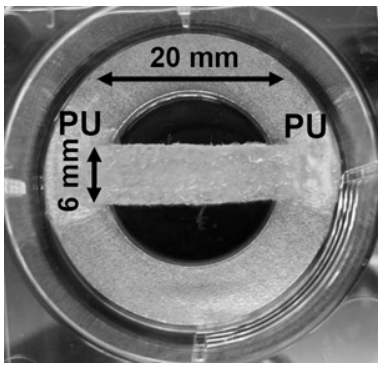
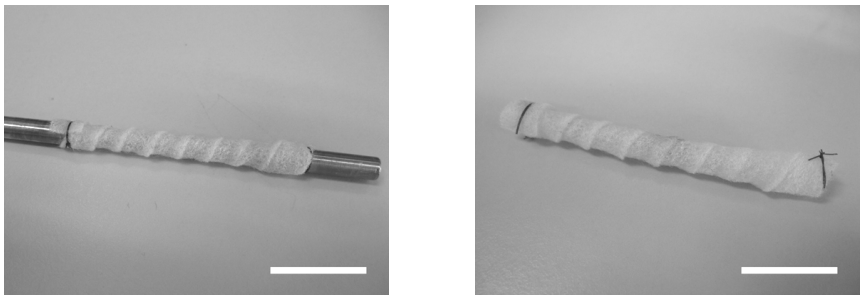


Figure 7.1: Rectangular TE strips on stainless steel ring in 6 well plate.

7.2.3 Construction of vascular grafts

Vascular grafts were made according to a previously described method (Stekelenburg *et al.*, 2008). Briefly, a sheet of non-woven PGA fiber mesh of 120 x 8 x 1 mm was wrapped around a rod with a diameter of 3 mm (Figure 7.2A). The constructs were then dip-coated in 1 w/v% P4HB in THF and the solvent was allowed to evaporate overnight. The inner diameter of the resulting grafts was 3 mm, the length approximately 3.5 cm and the wall thickness 1 mm (Figure 7.2B).



A **B**

Figure 7.2: A sheet of PGA fiber mesh wrapped around an iron rod after coating with P4HB (A) and after removal from the rod (B). Scale bars indicate 2 cm.

7.2.4 Bioreactor system

The tubular constructs were slid over a silicone tube and connected to the two stainless steel rods of the sterile bioreactor using sutures (Figure 7.3A-B). The silicone tube could be removed without detaching the vascular graft (Figure 7.3C-D). The tubular constructs were sterilized by UV radiation for 45 minutes and subsequent soaking in 70% alcohol for 3 hours. The constructs were then rinsed with sterile PBS and the bioreactors were filled with TE medium overnight.

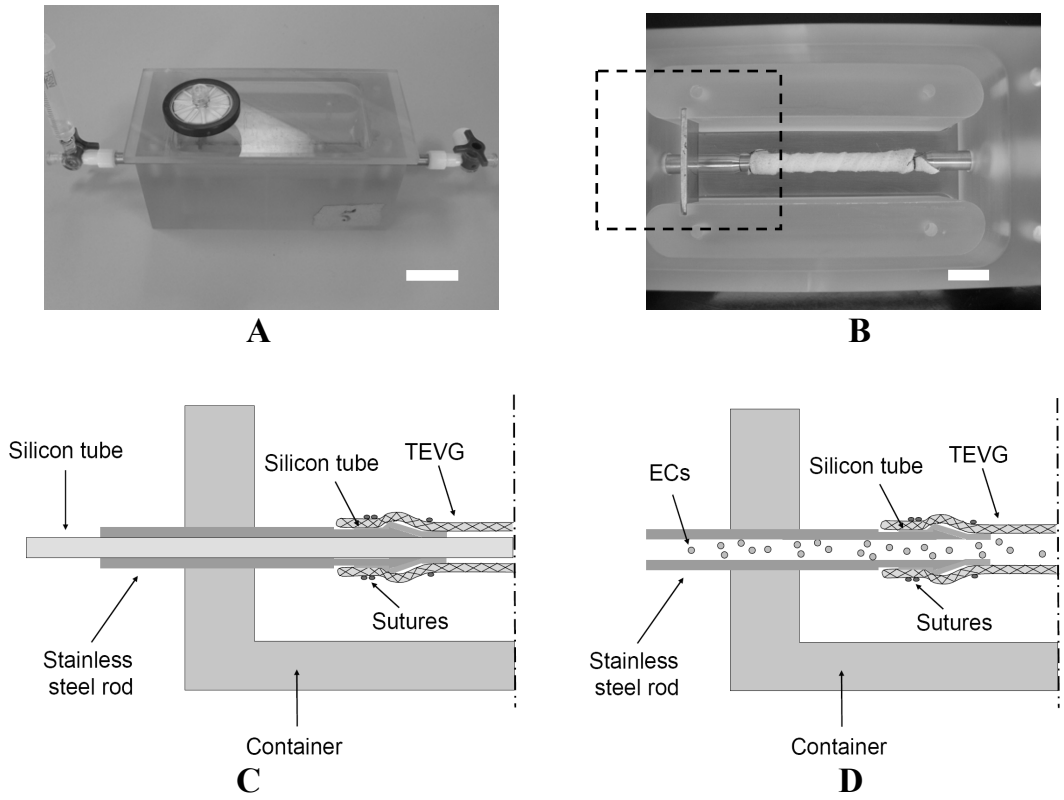


Figure 7.3: Bioreactor system (A). PGA/P4HB scaffold in bioreactor system prior to MF seeding (B). Dashed rectangle indicates area of schematic overviews (C, D). Schematic overview of TEVG connection during the first 4 weeks of culturing (C) and during EC seeding (D). Scale bars indicate 2 cm (A) and 1 cm (B).

7.2.5 Myofibroblast seeding

MFs were seeded in the TE strips and the tissue engineered vascular grafts (TEVG) using fibrin as a cell carrier (Mol *et al.*, 2005). The cell containing fibrin gel ($15\text{-}20 \cdot 10^6$ cells ml^{-1}) was mixed until onset of the polymerization process of the gel and subsequently dripped onto the TE strips and tubular constructs (150 µl/strip and 1000 µl/tube). Using this method, no cell-solution is lost as the scaffold absorbs the solution while the fibrin rapidly polymerizes. In this way, a homogeneous cell distribution throughout the scaffold is ensured (Mol *et al.*, 2005). The well plates and bioreactors were placed in an incubator at 37° C and 5% CO_2 to allow the fibrin gel to further polymerize for 30 minutes. Afterwards the wells and bioreactors were filled with 6 and 35 ml TE medium, respectively, and placed on a shaking table to allow mixing of the TE medium. During the 4 week culture period, TE medium was changed twice a week.

7.2.6 Endothelial cell seeding and conditioning

After 4 weeks of culture, ECs were seeded on the TE strips and in the TEVGs. The ECs on the TE strips were seeded by dripping an EC suspension containing $1.4 \cdot 10^5$ ECs ml^{-1}. The EC medium was changed every 2 days.

For seeding ECs in the TEVGs, first, the silicone tubes were gently pulled out of the bioreactors, leaving the TEVGs attached to the stainless steel rods (Figure 7.3D). After removal of the silicone tubes, the TEVGs were flushed with EC medium. Subsequently, the TEVGs were filled with an EC suspension containing approximately $5 \cdot 10^5$ cells ml^{-1}. The bioreactors were closed and placed in a rotation device and rotated for 3 hours at 0.5 rpm (Figure 7.4A). After this period, the TEVGs were gently flushed with fresh EC medium to remove non-attached ECs. The bioreactors were placed back in the incubator for further culture.

The TEVGs were divided in 4 groups (Figure 7.5). The first group (N1, n=4) was cultured for 1 day after EC seeding to test the seeding efficiency. The second group (N4, n=3) was cultured for 4 days after EC seeding, without the application of flow. In the third (L4, n=6) and fourth (H4, n=4) group, the bioreactors were attached to a medium reservoir and a rollerpump (Figure 7.4B) to apply a flow, inducing shear stress to the ECs. The Hagen-Poiseuille equation: $\tau = 4\mu Q / 4\pi r^3$ with τ = shear stress, μ = fluid viscosity, Q = medium fluid flow rate, and r = radius of the TEVG was used to calculate the shear stresses. The applied shear stress was gradually increased up to 0.5 Pa and 1.25 Pa, for the low (L4) and high (H4) flow groups, respectively. A syringe was attached to the bioreactor as a compliance vessel. The medium used for the application of flow was EC medium supplemented with xanthan gum to increase the viscosity to $4 \cdot 10^3$ Pa s^{-1} (Chapter 6). This culture medium had bloodlike shear thinning behavior and ensured that the applied shear stresses occur at physiological flows.

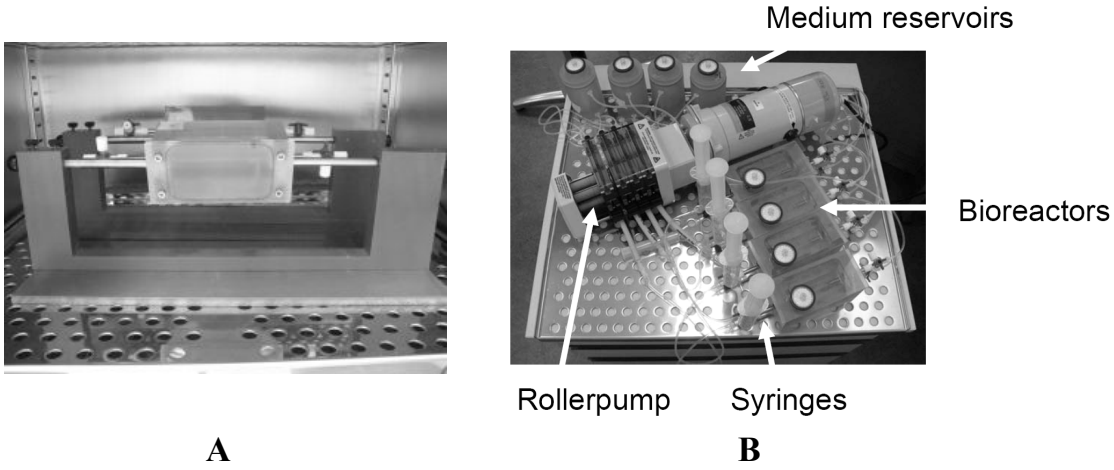


Figure 7.4: Bioreactors were placed in rotation devices for EC seeding (A) and were attached to a roller pump and medium reservoirs (B) for the application of shear stress.

Regular EC medium was used on the outside of the vessels for the EC culture period. TEVGs were only analyzed when no leakage occurred during the application of flow. Due to practical limitations, the vessels had to be cultured in three consecutive experiments. To account for experimental variability, TE strips which were cultured for a similar period were used as internal controls.

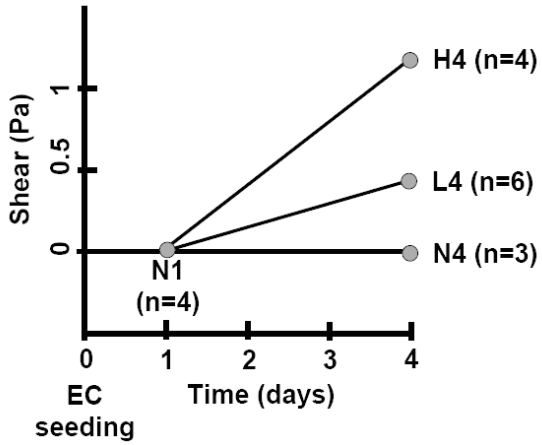


Figure 7.5: Definition of experimental groups. To test the EC seeding efficiency, TEVGs were sacrificed 1 day after EC seeding (N1, n=4). The remaining TEVGs were cultured 3 days longer, without flow (N4, n=3) or with increasing flows creating a final shear stress of 0.5 Pa (L4, n=6) or 1.25 Pa (H4, n=4).

7.2.7 Ultrasound measurements

After the EC culture period, the lumens of the TEVGs were visualized in the bioreactors using an ultrasound scanner with a linear probe (Esaote Europe, Netherlands) combined with an arterial analyzer (ART.LAB, Esaote Europe). The TEVGs were then removed from the bioreactor system and several rings were cut for further analysis (Figure 7.6).

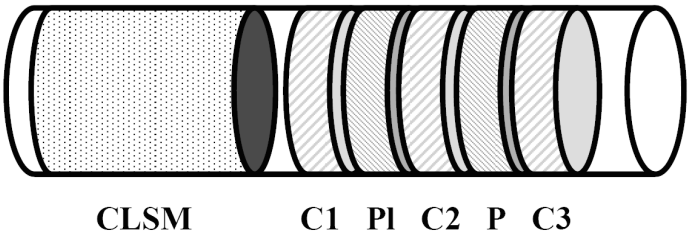


Figure 7.6: Schematic drawing of TEVG analyses. One part is used for CLSM measurements of the EC layer. Several rings were cut for mechanical testing (C1-3), other rings were fixed in formaldehyde (3.7%) for imbedding in technovit plastic (Pl) and paraffin (P).

7.2.8 Endothelial cell visualization

Approximately 1 cm of each vessel (Figure 7.6, CLSM) was stained for 45 minutes with FITC UEA-1 lectin (Sigma), which is specific for ECs (Hormia *et al.*, 1983), and Cell Tracker Orange (CTO, Invitrogen, USA), which stains viable cells. These dyes were diluted in EC medium to a final concentration of 5 μM and 10 μM, respectively. After staining, the ring segment was opened using a scalpel and placed between two cover glasses to get an en face view of the inside of the vessel. The constructs were visualized using a confocal laser scanning microscope (CLSM; Axiovert 100M, Zeiss, Göttingen, Germany). As the surface of the constructs was not flat, z-projections of z-stacks were produced, to visualize the EC layer.

7.2.9 Histology

Tissue morphology in all groups was studied by histology. Rings of the TEVGs were fixated in phosphate-buffered formaldehyde (3.7%) and embedded in technovit plastic (Pl) and paraffin (P) (Figure 7.6). Technovit sections (10 μm) were stained with toluidine blue for general tissue staining. On these sections, the inner and outer perimeters of the TEVGs were measured. The inner and outer diameters of the TEVGs were calculated from these perimeters assuming circular TEVGs. By subtracting the inner diameters from the outer diameters, the average wall thicknesses of the TEVGs were determined. The paraffin sections (10 μm) were stained with Masson Trichrome (MTC) for collagen visualization.

To analyze the EC layer of the TEVGs, immunohistochemistry staining, using the EC specific marker monoclonal mouse anti-human CD31 (Dako, Denmark), was performed. To determine the phenotype of the MFs in the TEVGs, sections were stained with monoclonal IgG2 mouse anti-human antibody against smooth muscle cell marker αSMA (Dako) and monoclonal IgM mouse anti-human antibody against the cytoskeletal protein vimentin (Abcam, UK). Afterwards, the specific stainings were visualized with a goat anti-mouse IgG2 Alexa 488 secondary antibody (Invitrogen) for αSMA and a goat anti-mouse IgM Alexa 555 (Invitrogen) for vimentin. Control sections incubated with only the secondary antibodies were completely negative (data not shown).

7.2.10 Evaluation of mechanical properties

To determine the mechanical properties of the TE strips and TEVGs, the TE strips and tissue rings (Figure 7.6, C1-3) were subjected to uniaxial tensile tests. Tensile tests were performed with a tensile tester equipped with a 20N load cell (Kammrath-Weiss, Germany). Stress-strain curves were obtained at a strain rate equal to the initial sample length per minute. The stress was defined as the force divided by the deformed cross-sectional area. The ultimate tensile strength (UTS) was determined from the curves. The slope of the linear part of the curve represented the Young's modulus. From the tensile tests pressure-strain curves were calculated (Stekelenburg, 2006).

7.2.11 ECM composition

The mechanically tested strips and rings were used for the determination of the ECM composition of the TE strips and TEVGs, respectively. To quantify tissue formation, the amount of DNA, sulfated glycosaminoglycans (GAGs), and hydroxyproline, was measured and expressed in mg per dry weight of tissue. For the analyses, lyophilized tissue samples were digested using a papain solution (100 mM phosphate buffer, 5 mM L-cysteine, 5 mM ethylenediaminetetraacetic acid (EDTA), and 125 to 140 μg papain ml^{-1}) at 60°C for 16 hours. The Hoechst dye method (Cesarone *et al.*, 1979) with a reference curve of calf thymus DNA (Sigma) was used to determine the DNA content. Using a modification of the assay described by Farndale *et al*. (Farndale *et al.*, 1986) and a shark cartilage chondroitin sulfate reference (Sigma), the sulfated GAG content was determined. The hydroxyproline content was determined with an assay modified from Huszar *et al*. (Huszar *et al.*, 1980) and a reference of trans-4-hydroxyproline (Sigma). The ratio of hydroxyproline to collagen was assumed to be 0.13. To obtain a measure for the amount of matrix components produced per cell, the collagen and GAG content were normalized for the amount of DNA. Because of variability between the experiments, the absolute values of the TEVGs could not be combined. Therefore, the ECM components of the TEVG were normalized against the ECM components of the corresponding TE strips.

7.2.12 Statistical analysis

Quantitative data were averaged per group, and represented as average ± standard deviation. ANOVA analysis with Bonferroni post-hoc comparisons were used to determine group differences. All statistical analyses were performed using SPSS v.15.0 software (SPSS Inc., Chicago, IL, USA).

7.3 Results

7.3.1 Morphological appearance

During the culture of TEVGs, the apparent scaffold wrappings disappeared (Figure 7.7), resulting in a smooth outer surface of the TEVGs. After removal of the silicone tube and EC seeding, most TEVGs compacted in circumferential direction. As these TEVGs were still attached to the stainless steel rods and thus constrained in longitudinal direction, small tears at the attachment points were observed in 30% of the TEVGs, resulting in leakage during the application of flow. The application of flow resulted in open TEVGs, in contrast to the N4 group where some TEVGs partially collapsed.

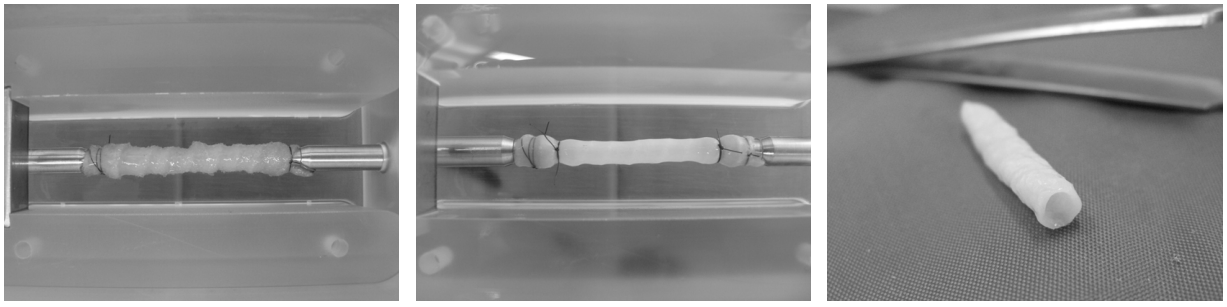


Figure 7.7: Example of TEVG after MF seeding (A) and after 5 weeks of culture, before (B) and after removal from the bioreactor (C). With time the scaffold wrappings disappeared, resulting in a smooth TEVG surface.

7.3.2 Influence shear on lumen dimension

Ultrasound measurements were performed to visualize the vessels in the bioreactors. The collapsed TEVGs of the N1 and N4 groups (Figure 7.8A-B) had a reduced lumen. The application of flow in the L4 and H4 groups, resulted in average pressures of 5 and 22 mmHg. Due to flow application, the lumens of TEVGs from the L4 and H4 groups (Figure 7.8C-D) had constant lumens, which were estimated at 2.5 to 3 mm. There was hardly any wall movement observed when flow was applied.

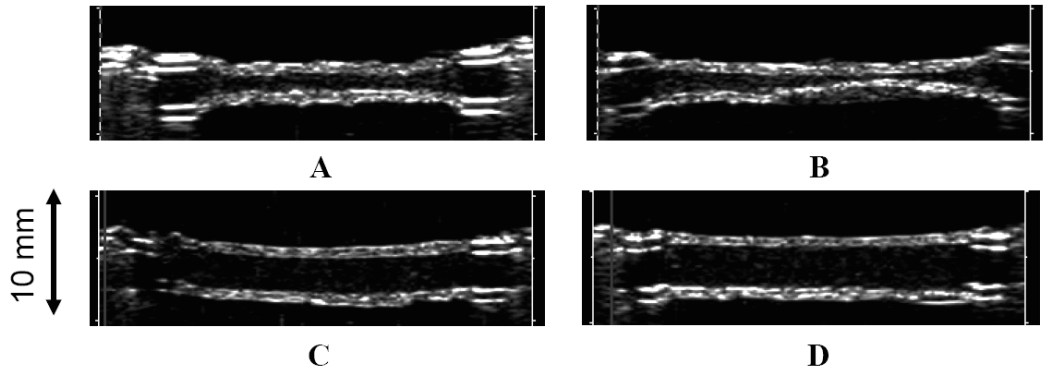


Figure 7.8: Ultrasound measurements of the TEVGs of the N1 and N4 groups (A, B) showed flattened lumens. The application of flow resulted in open TEVGs in the L4 and H4 groups (C, D).

Technovit sections, stained with toluidine blue showed a comparable dense tissue structure in all groups (Figure 7.9). The wall thicknesses of the TEVGs were inhomogeneous, due to the scaffold wrapping process. The technovit sections were also used to determine the inner and outer diameters of the TEVGs. The inner diameters of the flow groups were larger ($p<0.05$) than the no flow groups (Table 7.1). Likewise, the outer diameters of the L4 and H4 groups were larger ($p<0.05$ and $p<0.01$, respectively) than the N1 group. Consequently, there were no differences between the average wall thicknesses of the different TEVGs.

*Table 7.1: Dimensions of the different TEVG groups (mean ± s.d.). The inner diameters of L4 and H4 groups were increased compared to the N1 and N4 groups. The outer diameters were increased compared to the N1 group. The average wall thickness was similar in all groups. Differences with N1 and N4 groups are indicated with * and #, respectively. Single or double symbols indicate p<0.05 and p<0.01.*

	Groups			
	N1	**N4**	**L4**	**H4**
Inner diameter (mm)	2.47±0.12	2.39±0.14	2.82±0.16 * ##	2.94±0.13 ** ##
Outer diameter (mm)	3.73±0.09	3.86±0.18	4.18±0.16 **	4.11±0.18 *
Average wall thickness (mm)	0.63±0.06	0.66±0.10	0.68±0.11	0.66±0.11

7.3.3 Influence shear on EC alignment

A CLSM was used to visualize the EC layers of the TEVGs. Due to the flat morphology of the ECs, the CTO staining of the ECs was limited. One day after EC seeding, 70-90% of the surface was covered with ECs (Figure 7.9, N1). The ECs had typical cobblestone morphology. The ECs did not form a confluent layer in the N4 group (Figure 7.9). Some cells looked damaged and were rounded up. In the L4 and H4 groups (Figure 7.9), the ECs had an elongated shape and were mainly aligned in the direction of flow. However, in both groups patches were found where the ECs did have an elongated shape, but were lying oblique to the direction of flow (data not shown). The EC layer of the L4 and H4 groups was similar.

7.3.4 Histological analysis

An immunohistochemical CD31 staining confirmed the partial confluency in the N1 group, the damaged EC layer in the N4 group and the full confluency of the L4 and H4 groups (Figure 7.9). When an EC layer was present, it always was a layer of single cells (Figure 7.10A). MTC staining showed a dense tissue in all groups, however collagen was not abundantly present (Figure 7.10B). Vimentin was present throughout all constructs, however the amount of αSMA was limited (Figure 7.10C). No apparent differences were found between the different groups.

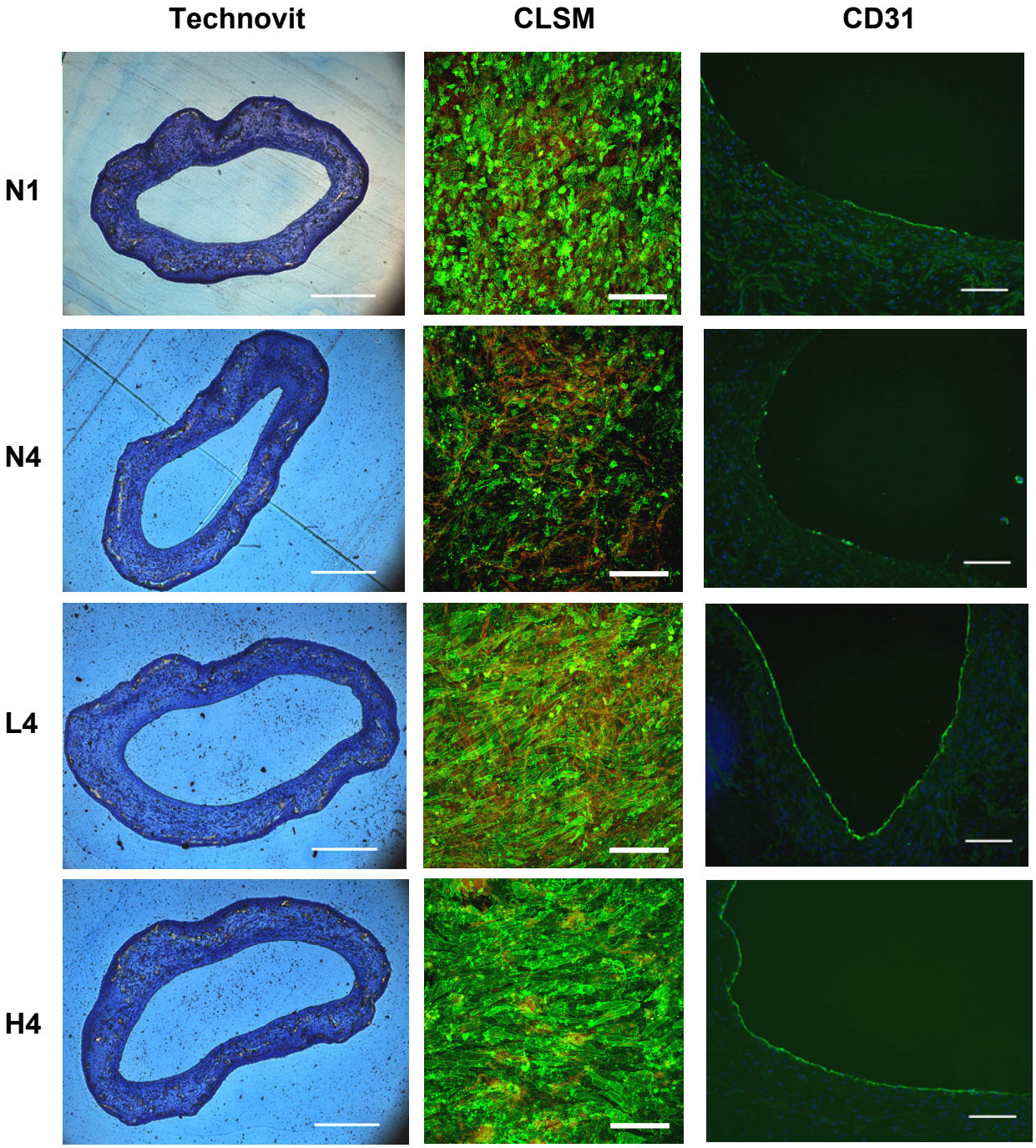
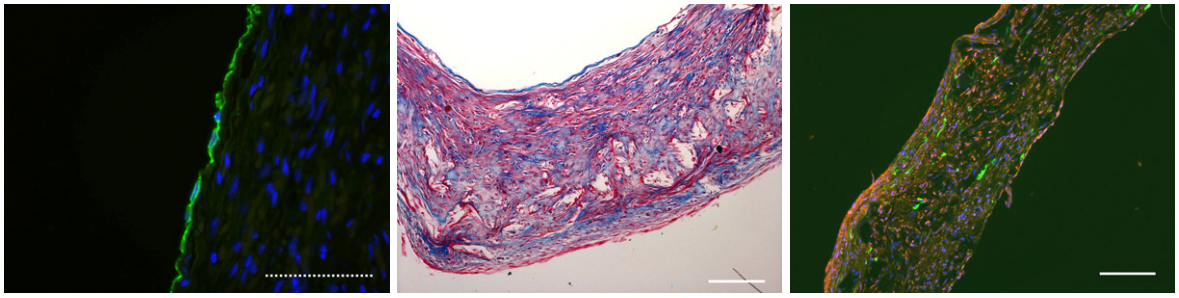


Figure 7.9: Qualitative analysis of TEVGs of all groups (N1, N4, L4, H4). Technovit sections stained with toluidine blue showed dense tissue structure and an inhomogeneous wall thickness. The non-circular shape is caused by cutting of the rings. The L4 and H4 groups had larger inner diameters. Typical en face CLSM images of ECs (green) on TEVGs. Autofluorescent PGA and CTO-stained MFs were seen in red. Round ECs were present on 70% of the surface after seeding (N1). In the N4 group, the ECs looked damaged. In the L4 and H4 groups, the ECs were aligned in the direction of flow. Flow direction was from left to right. CD31 staining further confirmed the EC surface coverage. Scale bars represent 1000 µm for the technovit sections and 200 µm for the CLSM and CD31 images.



A **B** **C**

Figure 7.10: Tissue sections of group H4 with different stainings. CD31 (green) staining (A). MTC staining, indicating collagen in blue (B). Immunofluorescent staining of αSMA (green), vimentin (red) and DAPI (blue) staining (C). The EC seeded side is left (A), top (B), and right (C). Dashed scale bar represents 100 µm and solid scale bars represent 200 µm.

7.3.5 No influence of shear on mechanical properties of TEVGs

The Young's moduli and UTS of the TEVGs of all groups were around 1 MPa and 0.35 MPa, respectively (Table 7.2). Estimated burst pressures were calculated from the tensile tests of the rings. The average burst pressure of the groups was approximately 600 mmHg. No significant differences in estimated burst pressures were found between the groups.

Table 7.2: Mechanical properties of the different TEVG groups (mean ± s.d.). The mechanical properties of all groups were similar.

	Groups			
	N1	**N4**	**L4**	**H4**
Young's Modulus (MPa)	0.91±0.05	0.72±0.24	0.91±0.26	1.00±0.27
UTS (MPa)	0.41±0.08	0.37±0.03	0.32±0.05	0.33±0.02
Estimated burst pressures (mmHg)	663±158	600±91	598±114	530±55

7.3.6 No influence of flow on ECM composition

In each experiment TE strips were cultured which served as controls for corresponding TEVGs that were cultured simultaneously. This was necessary as collagen production varies between experiments (cell batch), but is fairly constant within one experiment. The average DNA, GAG and collagen amounts (mean ± s.d.) of all TE strips were 2.92 ± 0.38 µg mg^{-1}, 14.19 ± 0.51 µg mg^{-1}, and 123.47 ± 34.80 µg mg^{-1}, respectively. When normalized to the TE strips, all TEVGs had similar GAG and collagen amounts. The DNA contents of L4 and H4 groups were higher ($p<0.05$) compared to the N1 group (Figure 7.11A). The collagen content per DNA was lower in

all groups, which were cultured for 4 days after EC seeding (Figure 7.11B), but no differences were found between the flow and no flow groups.

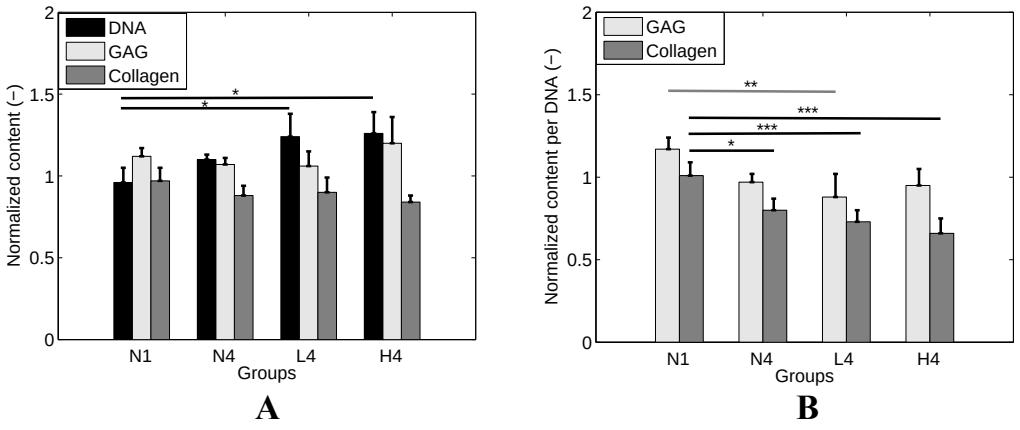


Figure 7.11: ECM composition of the TEVGs, normalized to the corresponding TE strips per dry weight (A) and per DNA (B) (mean ± s.d.). The DNA content of the L4 and H4 groups were higher than the N1 group. The collagen per DNA of the L4 and H4 groups was lower than the N1 group. (p<0.05, ** p<0.01, *** p<0.005)*

7.4 Discussion

Tissue engineered vascular grafts (TEVGs) require a functional EC layer, which is able to resist the shear forces of blood (L'Heureux *et al.*, 2007a; Mitchell and Niklason, 2003). The goal of the present study was to investigate whether ECs could be seeded in PGA/P4HB based human TEVGs and whether they would align in the direction of flow and withstand physiological shear stresses. TEVGs were created based on a PGA/P4HB scaffold seeded with human saphenous vein MFs using fibrin as a cell carrier (Stekelenburg *et al.*, 2008). After 4 weeks of culture, human saphenous vein ECs were rotationally seeded in the TEVGs. These ECs were flow conditioned using xanthan gum supplemented culture medium for 3 days, inducing shear stresses up to 1.25 Pa at physiological flows.

Several studies have indicated the importance of conditioning ECs with shear stress to create an EC layer which can withstand physiological shear stress (Buttemeyer *et al.*, 2003; Dardik *et al.*, 1999). Preconditioning an EC layer with a shear stress of 2.5 Pa for 3 days resulted in retention of a confluent EC monolayer on grafts 24 hours after implantation in rats (Dardik *et al.*, 1999). EC seeding on expanded polytetrafluoroethylene grafts combined with application of a 1.5 Pa shear stress for 14 days, resulted in open grafts 6 weeks after implantation in pigs (Buttemeyer *et al.*, 2003). Isenberg *et al*. showed that fibrin-based media-equivalents, cultured from rat SMCs, could be endothelialized and that the ECs could be aligned using a shear stress of 1.3 Pa (Isenberg *et al.*, 2006). In contrast, ECs seeded on a biological graft which were not conditioned were unable to withstand a shear stress of 0.93 Pa (Baguneid *et al.*, 2004). Niklason *et al*. showed that the application of only a perfusion flow revealed

a rounded EC morphology and less than complete EC coverage on bovine TEVGs (Niklason *et al.*, 2001), which is similar to the N1 and N4 groups of the current study. In the present study, we gradually increased the shear stress to a maximum of 1.25 Pa. The ECs formed a confluent monolayer and were able to withstand this physiological shear stress. In most cases, the ECs were aligned in the direction of flow, however, some patches of elongated ECs were found lying oblique to the direction of flow. As the walls of the TEVGs were sometimes inhomogeneous, due to the initial wrappings of the scaffold, this might have caused disturbances in the laminar flow profile, which in turn could have caused the oblique alignment.

In bioreactors used for the development of vascular grafts regular culture medium with low viscosity is being used. Under these conditions, it is often impossible to create physiological shear stresses at physiological flows (Isenberg *et al.*, 2006; Narita *et al.*, 2004). For example, a bioreactor system was developed by Narita *et al*. which could reproduce a wide range of pulsatile flows with a completely physiological pressure profile (Narita *et al.*, 2004). However, the shear stresses ranged from 0.001 - 0.035 Pa which is unlikely to be beneficial for EC retention *in vivo* (Dardik *et al.*, 1999). On the other hand, to apply physiological shear stresses super-physiological flows are needed (Buttemeyer *et al.*, 2003; Isenberg *et al.*, 2006; L'Heureux *et al.*, 2006). The application of high flows can result in turbulent flow profiles, which negatively affects elongation and alignment of ECs (Isenberg *et al.*, 2006). In the present study, xanthan gum was supplemented to the culture medium to increase its viscosity (Chapter 6). With xanthan gum it was possible to create physiological shear stresses at physiological flows.

The TEVGs developed in the present study were statically cultured for 5 weeks and had estimated burst pressures up to 600 mmHg. These values were lower than TEVGs cultured in a similar way by Stekelenburg *et al*. (Stekelenburg *et al.*, 2008), which had burst pressures of 900 mmHg. These TEVGs were dynamically strained, in contrast to the current TEVGs. However, this has not caused the differences in burst pressure, as in pilot studies using the current bioreactor system, TEVGs were created which reached burst pressures of 1100 mmHg (data not shown). Differences in cell source might explain the observed differences in burst pressure. It is well known that cells from different donors behave differently in TE experiments (Klinger *et al.*, 2006). Furthermore, the applied cell isolation technique can also induce cell batch differences, as the cells originated from different outgrowths. In this isolation technique, the whole saphenous vein vessel wall was used and it is known that perivascular fibroblasts and SMCs grow out at different time points (Grenier *et al.*, 2003). This may have resulted in a mixed population of fibroblasts and SMCs, of which the ratio can differ per batch. These different cell ratios will likely cause differences in the collagen productive capacity of the total cell population and might also explain the burst pressure differences. Compared to human left internal mammary arteries (LIMA), the TEVGs had lower mechanical properties. For example, the Young's modulus and ultimate tensile stress of LIMAs have been reported to be 8.0 ± 3.0 MPa and 4.1 ± 0.9 MPa, respectively (Stekelenburg *et al.*, 2008).

The estimated burst pressures of the human TEVGs were several times higher then the average blood pressure in humans. Although L'Heureux *et al*. (L'Heureux *et al.*,

2007a) have recently proposed that burst pressures similar to that of saphenous veins (i.e. >1700 mmHg) are a minimal criteria for TEVGs, it is unknown whether the burst pressures presented in the current study would also be sufficient.

The ECM composition of the TEVGs of the 4 groups was similar. Only the DNA contents of the L4 and H4 groups was increased compared to the N1 group. There was, however, no significant difference found between groups L4 and H4 and the N4 group. The application of shear stress is known to increase the proliferation of SMCs (Haga *et al.*, 2003). Furthermore, the EC medium contains mitogenic growth factors, like bFGF, and is known to cause a DNA increase in similar cardiovascular constructs (Chapter 3). The TEVGs had a similar DNA and GAG content as human LIMAs, which have been reported to be 3.5 ± 0.9 μg mg^{-1} and 12.3 ± 2.8 μg mg^{-1}, respectively (Stekelenburg *et al.*, 2008). In contrast, the amount of collagen of the TEVGs was only 47% of the human LIMAs (Stekelenburg *et al.*, 2008). This low collagen content is probably the main cause of the relatively low burst pressures.

The TEVGs were cultured around silicone tubes for 4 weeks, resulting in inner diameters of 3 mm. After removal of this silicone tube, the TEVGs further compacted and the diameter decreased to about 2.5 mm. Due to flow conditioning, the diameter of the TEVGs returned to the original 3 mm. Although a syringe was used as compliance to lower the pressures generated by the rollerpump, the L4 and H4 TEVGs were still loaded with average pressures of 5 and 22 mmHg, respectively. The application of flow at these pressures probably caused the enlarged diameter compared to N1 and N4 groups. At these relatively low pressures, it was not expected that the diameter of the TEVGs would increase. This might be caused by a lack of elastin in the ECM composition. In native arteries, elastin fibers dictate mechanics at low strains before collagen fibers are loaded and also provide the elastic components that prevent dynamic tissue creep (Long and Tranquillo, 2003). However, although the importance of elastin is recognized *in vivo*, its role in TEVGs prior to implantation is not resolved. The tissue remodeling process will continue after implantation and might be sufficient for proper graft functioning (Hoerstrup *et al.*, 2006). It is also possible that the dilation of the TEVGs is caused by shear stress mediated vasodilation. ECs stimulated with shear stress, produce several vasoregulators, like NO and prostacylcin (Barakat and Lieu, 2003; Davies, 1995), which can cause vasodilation (Paniagua *et al.*, 2001; Tronc *et al.*, 1996). However, due to the low α-SMA expression, it seems unlikely that the TEVGs have a large relaxation capacity. Additional studies including longer flow conditioning periods and *in vivo* implantations will provide more insight into the significance of these diameter changes. In addition, our methodology can also be used to long term fatigue testing under physiological pulsatile flow.

Although a shear resistant EC layer is one of the prerequisites for a TEVG, several other functions of ECs should be evaluated. For example, the thrombogenicity of the endothelialized TEVGs should be tested, as thrombogenic TEVGs are likely to fail by thrombosis before failure by dilation or rupture. Furthermore, the interaction of the ECs with the MFs in the TEVG should be investigated, for example by testing vasodilation through an endothelium-dependent mechanism.

In conclusion, human TEVGs were created from PGA/P4HB seeded with human saphenous vein MFs and ECs. After 5 weeks of culture, including 3 days of shear stress conditioning, the burst pressure of these TEVGs was 600 mmHg, and the TEVGs contained a confluent aligned EC layer that could withstand physiological shear stresses. Applying physiological flows at physiological pressures to these TEVGs for a longer period of time will elucidate the fatigue resistance and dilation of the TEVGs.

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Chapter 8

General discussion

8.1 Summarizing remarks

Autologous arteries and veins, as well as synthetic grafts, are currently used as bypass grafts for replacing small diameter blood vessels. Although the arteries perform well, lower patency rates have been reported for the veins and synthetic grafts. Tissue engineered vascular grafts (TEVGs) could offer a good alternative to overcome the limitations in small vessel grafting by creating viable constructs with repair and remodeling capabilities. Design criteria for TEVGs have been formulated for benchmarking and regulatory purposes. In general, these criteria are based on the properties of native vessels, although it is difficult to create a TEVG which is equal to a native vessel. It is even questionable whether this is necessary, as in animal models the growth and remodeling capacity of TEVGs has been demonstrated (Hoerstrup *et al.*, 2006; L'Heureux *et al.*, 2006; Niklason *et al.*, 2001). However, it is agreed upon that a set of minimal criteria has to be achieved before TE vessels can be used in a clinical setting.

At a minimum, such TEVGs must contain appropriate mechanical properties, like burst pressure, compliance, and elasticity. In addition, TEVGs should allow complete healing, and should remodel according to cues from the environment. Finally, a TEVG should contain a stable functional endothelium. This endothelium should be able to withstand the shear forces of blood, and should have both non-thrombogenic and mechanotransducing properties.

Recently, large improvements have been made in the development of mechanically strong human TEVGs (L'Heureux *et al.*, 2006; Stekelenburg *et al.*, 2008). Less research focused on the development of a functional endothelial cell (EC) layer on human TEVGs. Therefore, the main focus of the present thesis was the development of a functional EC layer on human TEVGs. The TEVGs were based on a PGA/P4HB scaffold seeded with human saphenous vein myofibroblasts (MF) (Stekelenburg *et al.*, 2008).

For the development of endothelialized TEVGs, ECs and MFs have to be co-cultured. However, this co-culture is not trivial as these cells require different stimuli. In this thesis, first a 3D co-culture model was developed, which was used to optimize the co-culture conditions for human saphenous vein ECs and MFs (Chapter 2 and 3). It was demonstrated that ECs did not survive in DMEM culture medium. In addition, it was demonstrated that a confluent EC layer could be cultured on strong cardiovascular constructs, when the ECs were seeded after 3 or 4 weeks of tissue development (Chapter 3).

When in co-culture, ECs influence the phenotype of the cells in their environment. In the present thesis, this was demonstrated by a change in growth and αSMA expression of MFs due to co-culture with ECs (Chapter 4). In addition, it was shown that the extracellular matrix (ECM) composition of 3D cardiovascular construct was influenced by a layer of ECs (Chapter 3). However, the EC medium also had a large influence (Chapter 3). Functional ECs also need to be non-thrombogenic. Using the 3D co-culture model, it was demonstrated that the ECs indeed reduced the thrombogenicity of TE cardiovascular constructs (Chapter 5).

Finally, a bioreactor system was developed in which small diameter TEVGs could be cultured and endothelialized. The seeded ECs were shear stress conditioned using a culture medium supplemented with xanthan gum to achieve a blood-analog viscosity. It was shown that xanthan gum did not affect the growth of ECs, their alignment due to shear stress and their vasodilating properties (Chapter 6). This bioreactor system was used to create human TEVGs and it was shown that the seeded ECs elongated and could resist a physiological shear stress, which is also necessary for a functional EC layer (Chapter 7).

The significance and limitations of our findings will be discussed in relation to current state of the art. In addition, the implications for future research and applicability to clinical practice will be addressed.

8.2 Model system characteristics

In this thesis, several model systems were developed to study the functionality of ECs. The characteristics of these model systems, including the used culture medium and the advantages and disadvantages of the systems will be discussed. In addition, the bioreactor system and the cultured TEVGs will be discussed.

8.2.1 Culture medium

In tissue engineering (TE) experiments, the type of culture medium has a large influence on cell growth and tissue development. DMEM supplemented with ascorbic acid, which was used in our studies, is commonly used for TE experiments, as this culture medium stimulates collagen production by smooth muscle cells (SMC) and MFs (Hoerstrup *et al.*, 2006; Mol *et al.*, 2006; Niklason *et al.*, 2001; Stekelenburg *et al.*, 2008). For animal-derived ECs, DMEM can also be used (Hoerstrup *et al.*, 2006; Niklason *et al.*, 2001). However, for human ECs often other culture media are used, such as M199 medium (L'Heureux *et al.*, 1998) and EGM-2 medium (Karim *et al.*, 2006; Schmidt *et al.*, 2006). It was shown in the present thesis that human saphenous vein ECs did not grow in DMEM, and that EGM-2 medium had to be used instead (Chapter 2). This was comparable to a study by Karim *et al*. who compared different culture media, such as EGM-2, DMEM, and M199 medium, for co-culturing human saphenous vein ECs and MFs and also chose EGM-2 medium as this showed the best results for the ECs (Karim *et al.*, 2006).

Although the MFs did survive in EGM-2 medium, these cells were stimulated by the growth factors in this medium. This resulted in an increased amount of DNA and in decreased amounts of collagen and GAGs in the TE constructs compared to DMEM cultured constructs (Chapters 2 and 3). When measuring EC-MF interactions, the influence of the EGM-2 medium on the MFs always had to be taken into account. Despite the stimulating effect of the culture medium, ECs were able to influence the phenotype of the MFs. However, the culture medium itself might have influenced the EC-MF interactions.

8.2.2 Different model systems

In this thesis, different model systems have been used to investigate the EC functions. Cells have been cultured in monolayers or co-cultured in 2D (Chapter 2, 4, 5). In addition, cells were co-cultured in a 3D configuration as rectangular TE constructs (Chapter 2, 3, 5) or as vascular grafts (Chapter 7). These systems all have their own advantages and disadvantages.

In general, the advantages of monolayer cultures and 2D co-cultures are that they are relatively simple to perform, require a short culture time, are easily controllable and enable a quick screening of cell reactions. The important drawback is that 2D cultures are less realistic, as most cells reside in a 3D environment *in vivo*, which is particular true for the MFs. In this thesis, cell monolayers were used to investigate the EC growth under different medium conditions and to investigate EC alignment under to flow. The alignment was representative of what was observed in 3D culture and we therefore suggest that 2D studies are applicable to study this EC function. The 2D co-culture model was used to investigate the influence of ECs on the phenotype of the MFs (Chapter 4). Although results were in accordance with other studies, the MF phenotype could also be influenced by a 3D environment. Therefore future studies should investigate the interaction of ECs on MFs in a 3D environment.

The advantage of a 3D co-culture is the more native-like environment, which ensures a more realistic response of the cells. The drawbacks are that, due to their complexity, the cultures are more prone to experimental variability and that their culture times are often increased. The 3D co-culture model, consisting of rectangular TE constructs, was developed (Chapter 3) to study several conditions for culturing TEVGs, such as culture medium and culture period. Although this 3D co-culture system gave valuable information about the EC growth and the interaction of ECs with MFs, the application of shear stress was not possible. Shear stress could only be applied when the TE constructs were removed from the 6 well plates and placed in a flowchamber as was used in the thrombogenicity study (Chapter 5). The TEVGs were developed to resemble blood vessels and as such they can be used as a model system, which will be discussed in the next section.

8.2.3 Development of tissue engineered vascular grafts

Endothelialized TEVGs were successfully created, however it was not always possible to apply shear stress due to leakiness of some TEVGs (30%). After removal of the silicone tube and seeding of the ECs, the grafts compacted in circumferential direction, which was seen as a decreased diameter. However, this also caused small tears in the tissue at the attachment points, as that tissue was not able to compact. This resulted in leakage in approximately 30% of the TEVGs. Some grafts started to leak immediately, while others failed after 1 or 2 days of flow application. It is evident that these attachment points are a drawback of the presented bioreactor system. The profile of the attachment points could be improved to decrease the step up in diameter from silicone tube to steel fitting, which might enhance tissue strength. The manufacturing processes of the tubular scaffold could also be improved. The scaffold is made by

wrapping the PGA sheet around an iron rod and then coating in P4HB, resulting in inhomogeneous wall thicknesses. If the scaffold could be manufactured in a tubular structure in stead of a sheet, this might improve the homogeneity of the TEVGs.

This thesis focused on the development of a functional EC layer on human TEVGs. Although this is an important criterion, the success of a TEVG is also determined by its mechanical properties and fatigue resistance. In the present thesis, the mechanical properties of the TEVGs were assessed by circumferential tensile tests, which were used to calculate burst pressures. These calculated burst pressures were demonstrated to be a good comparison to regular burst pressure measurements (Stekelenburg, 2006). The current endothelialized TEVGs had calculated burst pressures in the order of 600 mmHg after a culture period of 4 weeks (Chapter 7). The collagen content of the TEVGs was approximately 44% of native coronary arteries (Ozolanta *et al.*, 1998).

Using a similar approach, Stekelenburg *et al*. demonstrated TEVGs with burst pressures of 900 mmHg (Stekelenburg *et al.*, 2008). However, they used a dynamic conditioning protocol, which is known to increase the mechanical properties of TEVGs (Seliktar *et al.*, 2000). Klinger *et al*. reported burst pressures of 500 mmHg in TEVGs after a 8 week culture period (Klinger *et al.*, 2006). Comparable to the TEVGs in this thesis, Klinger *et al*. used a PGA scaffold, but they did not coat it with P4HB and did not seed the cells using a fibrin gel. In addition, they used genetically modified cells to reach burst pressures of 500 mmHg. The proliferative arrest of these cells was overruled by ectopic expression of telomerase via human telomerase reverse transcriptase (hTERT) gene transfection. In comparison, TEVGs seeded with non-modified cells reached only burst pressures of 100 mmHg (Poh *et al.*, 2005). Using a different approach, namely sheet based tissue engineering, L'Heureux *et al*. recently reported human TEVGs with burst pressures exceeding 3500 mmHg (L'Heureux *et al.*, 2006). However, the culture period of these grafts was approximately 24 weeks.

8.3 Cell sources

The cell type and its source are important parameters for the development of TEVGs. In this thesis, human saphenous vein derived myofibroblasts and endothelial cells were used. These cell types as well as alternative cell sources will be discussed in this section.

8.3.1 Myofibroblasts

For the experiments described in this thesis all human cells were isolated from pieces of discarded saphenous vein. The collagen formation and mechanical properties of TE constructs derived from venous cells were demonstrated to be superior to those of aortic tissue derived constructs (Schnell *et al.*, 2001). After the isolation of the ECs using a collagenase treatment (Chapter 3), the vessels were cut in small pieces and MFs grew out (Schnell *et al.*, 2001). These cells were collected in several batches, cultured up to passage 6 and then used for the experiments. These cells were all vimentin positive and a subpopulation was αSMA positive (Chapter 5). Based on these markers

the cells were defined as MFs (Mol *et al.*, 2006), and have been used to successfully culture heart valves (Mol *et al.*, 2006) and small diameter vascular grafts (Stekelenburg *et al.*, 2008). However, defining those cells as MFs, based on those markers, might have been incorrect as there are many other cell types which are αSMA and vimentin positive and look like fibroblastic cells (Eyden, 2008). In fact, it has been argued that these cells can only be distinguished at ultrastructural level by transmission electron microscopy (Eyden, 2008). In addition, the saphenous vein wall normally does not contain MFs, but fibroblasts and SMCs, which will both grow out of the those vessel wall pieces (Grenier *et al.*, 2003). It is therefore more likely that the experiments were performed with a mixed population of fibroblasts and SMCs.

Initially, it was assumed that the cell population was equal between different cell batches of the same donor. However, there were some differences between the cell batches. The proliferation rate of the cells differed from vial to vial even when they were of the same passage and derived from the same donor. In addition, when cardiovascular constructs were cultured, the resulting tissue properties, such as mechanical properties and ECM composition, were different. It is therefore advised to investigate whether there are phenotypic differences between the different batches of isolated cells. In addition, it should be studied whether there is a correlation with the ECM composition of the resulting tissue.

The developed TEVGs are intended for use in the clinical setting, resulting in the use of autologous cells. Therefore, a study relating cell phenotype and ECM composition should also be performed to get a better understanding of patient specific differences. Not surprisingly, it seems that the ECM composition of TE constructs is depending on the cell donor. However, it is unknown what causes these differences. In this thesis, the same passage number and seeding density of the MFs were used for the experiments. Both of these parameters can influence the resulting tissue composition and should be optimized for every patient.

8.3.2 Endothelial cells

For endothelialization of the TEVGs, ECs isolated from the saphenous vein were used. It could be argued that, for implantation in the arterial circulation, the use of arterial ECs is preferred, as these ECs are already more accustomed to the arterial hemodynamic conditions. It is indeed known that ECs in arteries and veins express unique molecular markers (Aird, 2007b). For example, ephrinB2 is a marker, which is expressed in arteries only, and as such identifies a difference between arterial and venous ECs (Shin *et al.*, 2001). Despite these phenotypic differences, studies in avian models suggest that the arterial and venous phenotypes maintain a certain degree of plasticity (Moyon *et al.*, 2001; Othman-Hassan *et al.*, 2001). Cell culture studies in which flow induces an arterial like phenotype in venous ECs suggest that the hemodynamic environment can influence venous and arterial identity (Dai *et al.*, 2004; Tsukurov *et al.*, 2000). In a related model, transplantation of the pulmonary valve into the aortic position in humans resulted in new EC expression of the arterial marker ephrinB2 (Rabkin-Aikawa *et al.*, 2004). These results suggest that venous ECs can be

used for arterial implantation especially when the cells are properly conditioned in vitro prior to implantation.

Similar to the MFs, similar EC seeding densities and passages were used on the TE constructs and TEVGs. The cells were seeded at approximately 70-80% confluency, such that the ECs could proliferate into a confluent layer. It is possible that lower seeding concentrations might have given similar results, however this was not studied in this thesis.

8.3.3 Alternative cell sources

In most cardiovascular TE approaches cells are harvested from vascular structures. Besides the use of artery-derived cells (Hoerstrup *et al.*, 2001; Niklason *et al.*, 2001), cells from saphenous veins, which were used in the present thesis, have been used regularly (Mol *et al.*, 2006; Poh *et al.*, 2005; Stekelenburg *et al.*, 2008). However, harvesting cells from vascular structures requires the sacrifice of an intact native tissue. The dermal fibroblasts which are used by L'Heureux *et al*. for the generation of sheet based grafts require only a small skin biopsy, however the ECs were isolated from a peripheral vein (L'Heureux *et al.*, 2007a). To address the requirement of the use of a native vessel, research has focused on alternative cell sources such as bone marrow-derived cells, umbilical cord derived cells, and blood-derived cells. The umbilical cord cells are of particular of interest for pediatric purposes.

The bone marrow is an attractive cell source for a variety of cell types. Shin'oka *et al*. constructed biodegradable TEVGs made of a polycaprolactone-polylactic acid copolymer reinforced with woven PGA, which were seeded with bone marrow cells. These TEVGs were successfully used to reconstruct the low-pressure pulmonary outflow tract in pediatric patients (Shin'oka *et al.*, 2005). In addition, human marrow stromal cells were used for the creation of TE heart valves (Hoerstrup *et al.*, 2002).

Kadner *et al*. compared cells isolated from the human umbilical cord artery, vein and whole umbilical cord to cells derived from the saphenous vein. The umbilical cord cells had similar growth properties as the saphenous vein cells. In addition, when these cells were seeded on PGA/P4HB scaffolds, the resulting TE constructs had equal mechanical properties and a similar biochemical composition (Kadner *et al.*, 2004).

Endothelial progenitor cells (EPCs) isolated from peripheral or umbilical cord blood also have been investigated for the use in TE applications (Kaushal *et al.*, 2001; Schmidt *et al.*, 2005; Schmidt *et al.*, 2006). Recently, EPCs have also been used for the endothelialization of decellularized grafts in animal models, which remained patent for 130 days (Kaushal *et al.*, 2001). Schmidt *et al*. demonstrated the fabrication of cardiovascular patches and TEVGs using human umbilical cord-derived MFs in combination with ECs derived from umbilical cord blood EPCs (Schmidt *et al.*, 2005; Schmidt *et al.*, 2006). A functional endothelium was created on these constructs, however some issues have to be addressed concerning the ECM composition as the collagen amount was only 1% of native tissue (Schmidt *et al.*, 2006). Thus, cell sources other than venous cells obtained by invasive techniques are clinically attractive and promising for vascular tissue engineering.

8.4 Future perspectives and recommendations

8.4.1 Mechanical behavior

It has been proposed that for implantation the burst pressure of a TEVG has to be similar to that of a saphenous vein, which is approximately 1700 mmHg (L'Heureux *et al.*, 2007a). However, it is questionable whether this is indeed necessary as the blood pressure never reaches that level. Stekelenburg *et al*. demonstrated that although the mechanical properties of TEVGs were not similar to native equivalents, the differences were minimal within the physiological range (0-300 mmHg)(Stekelenburg *et al.*, 2008). In addition, TEVGs were implanted in the jugular veins of lambs (Swartz *et al.*, 2005), and although this was the low pressure system it demonstrated the successful implantation of relatively weak TEVGs.

Another criterion for TEVGs is that they should display elastic recoil. In native arteries, this property contributes to their resistance to dilation and aneurysm formation over time. Elastin is the structural component which is responsible for the elastic properties of a blood vessel. Despite the great promise that TEVGs have to offer, elastin has been notably absent from most approaches utilized to engineer vascular grafts (Patel *et al.*, 2006), including ours. However, it is unclear whether this absence of elastin indeed results in aneurysm formation after implantation. To establish such a relation, an extended *in vitro* characterization of the mechanical properties could be performed. By subjecting the grafts to physiological pressures for a prolonged period of time, the long-term mechanical behavior of the TEVGs could thus be determined. There is evidence however, that the *in vivo* remodeling process is quite extensive and among other features involves the formation of elastin (L'Heureux *et al.*, 2006; Swartz *et al.*, 2005). L'Heureux *et al*. showed abundant elastin formation in the neomedia of human TEVGs implanted in nude mice (L'Heureux *et al.*, 2006). When fibrin based TEVGs were implanted in jugular veins of lambs, elastic fibers were formed *in vivo* (Swartz *et al.*, 2005). However, after 15 weeks of implantation the fibrillar organization of elastin was not as extensive as native tissue. Despite this lower elastin organization, no aneurysm formation was reported. In contrast, Opitz *et al*. demonstrated a significant dilation of engineered grafts 6 months after implantation in the aorta of juvenile sheep. They observed a lack of mature elastin structures which likely was the cause of the dilation (Opitz *et al.*, 2004). These results suggest that for the high pressure circulation an improved elastic structure is necessary. In contrast, sheet based TEVGs did not dilate after implantation in the abdominal aorta position of primates (L'Heureux *et al.*, 2006), which likely resulted from the high stiffness of the grafts.

If aneurysm formation indeed would occur in our TEVGs, future studies should focus on investigating substances to improve mature, cross-linked elastic fiber formation. It has been reported previously that the addition of TGF-β1 and insulin to the culture medium increases elastin synthesis *in vitro* (Long and Tranquillo, 2003). In addition, other substances, such as calcitriol (Tukaj *et al.*, 2000) and retinoic acid (Hayashi *et al.*, 1995; Ogle and Mooradian, 2002), have been shown to increase elastogenesis *in vitro*.

8.4.2 Endothelial cell functions

The TEVGs had a confluent layer of ECs, that were aligned in the direction of flow and could withstand 1.25 Pa of shear stress (Chapter 7). The shear stress was gradually increased, which has been shown to result in EC retention after implantation (Dardik *et al.*, 1999). By using a roller pump, an average physiological flow could be applied. However, the flow profile depended on the pump speed, which resulted in a higher pulse frequency compared to a physiological flow profile. Therefore, future studies should include an adapted shear stress conditioning method to reveal whether the demonstrated confluency persists.

The non-thrombogenic behavior of an EC layer was demonstrated on TE constructs (Chapter 5). It is expected that the confluent EC layer of the TEVGs also has this non-thrombogenic function, as this is a similar cell layer compared to the TE constructs. In addition, this EC layer is shear stress conditioned (Chapter 7). ECs under shear stress will upregulate several molecules, such as nitric oxide (NO) and prostacyclin, which inhibit platelet aggregation (Barakat and Lieu, 2003; Traub and Berk, 1998). Staining the EC layer of the TEVGs for endothelial nitric oxide synthase or measuring NO production would further add to the functional characterization of a TE EC layer and potentially helps to predict successful clinical outcome of TEVGs.

It was demonstrated that ECs have the capacity to influence the phenotype of MFs (Chapter 4). Whether this is also the case in the 3D co-culture can be studied by comparing the endothelialized TEVGs with non-endothelialized grafts and investigate any phenotypic changes in the MFs, such as proliferation or SMC characteristics. These *in vitro* measurements would give an indication whether intima hyperplasia would occur after implantation. It was demonstrated that the presence of a shear stress conditioned EC layer reduced neointimal thickness (Dardik *et al.*, 1999). Intima hyperplasia is a complex process which is difficult to investigate *in vitro*, therefore animal studies should also be conducted with our TEVGs, to investigate thrombogenicity, intimal hyperplasia and long term patency.

Whether the TEVGs possess any vasoactive properties was not yet determined. The system used for analyzing the possible vasoactive properties of xanthan gum (Chapter 6) could be used to measure whether the MFs have any vasoconstrictive properties. The vasoactive properties of vessels are also influenced by the ECs, as they sense changes in the blood flow. Therefore endothelium-dependent vasodilation should also be measured, further demonstrating a functional EC layer.

8.4.3 Animal models

In vivo implantation in animal models is needed to further elucidate the potential of the developed TEVGs and to gain mechanistic insight into the specific vascular biology of these grafts. In preclinical models, the degradation of the scaffold and the *in vivo* remodeling process can be studied. It is expected that the tissue remodeling process will continue after implantation, as this was demonstrated for similar TEVGs which were implanted in lambs as pulmonary artery replacements (Hoerstrup *et al.*, 2006). After 100 weeks echocardiography demonstrated a good functional performance. Growth of

the TEVGs was demonstrated by an increase in diameter by 30% and length by 45%. In addition, histology showed tissue formation reminiscent of native artery. Similar results were obtained with fibrin-based constructs that were implanted in jugular veins of lambs (Swartz *et al.*, 2005). At 15 weeks post-implantation, the TEVGs were patent and exhibited remarkable ECM remodeling with production of collagen and elastin fibers and orientation of SMCs perpendicular to the direction of blood flow.

Autologous cell approaches require the development of TEVGs with cells that correspond with the recipient preclinical model. Porcine grafts that were conditioned by pulsatile stretch in a bioreactor could be implanted in minature pigs and remained patent for 4 weeks (Niklason *et al.*, 1999). However, attempts to use this approach to create mechanically strong TEVGs using adult human cells initially failed (Niklason, 1999). Even after genetically modifying the human SMCs, the burst pressures of the TEVGs reached only 356 mmHg (McKee *et al.*, 2003). It appears that for each species one needs to redefine the optimal conditions to achieve sufficient mechanical strength.

Another point of caution is that spontaneously endothelialization in animals occurs (Vara *et al.*, 2005). Although high patency rates could be observed in several animal models after implantation of EC seeded TE grafts (Borschel *et al.*, 2005; Dardik *et al.*, 1999; Niklason *et al.*, 1999; Swartz *et al.*, 2005), this could have been the result of rapid host derived re-endothelialization in the face of early loss of graft endothelium (Swartz *et al.*, 2005). Although the evidence is rather slim, it is commonly accepted that re-endothelialization of vascular grafts in humans is slow and may not be complete (Berger *et al.*, 1972; Rahlf *et al.*, 1986). The observations that formed the basis for this notion were made in explanted grafts from deceased patients, which may have biased the interpretation. It is however likely that re-endothelialization is more variable in a clinical target population with conditions that are known to compromise endothelial function such as uremia, hypercholesterolemia or diabetes. To gain more insight into the fate of TE endothelium in preclinical models, it would be advised to label the engrafted ECs to distinguishing them from *in vivo* recruited endothelium. Another way to overcome many of the discussed translational issues is to use human TEVGs in immunocompromised animals or immunologically humanized animals (L'Heureux *et al.*, 2006). Such an approach adds appreciable complexity to the experimental set up and comes with its own limitations.

The second major role of preclinical models is to assess the safety of TEVGs before engaging into a clinical trial. The translational value should thus be considered on the basis of safety criteria such as patency, tendency to form aneurysms, medium term thrombogenicity and risk of a acute perforation. Once proven safe, efficacy testing of TEVGs should be performed in patients.

8.4.4 Towards clinical application

When the presented human TEVGs have demonstrated a long term patency and a stable diameter in an animal model, one might start the transition to the clinical setting. One of the postulated criteria of TEVGs is “off the shelf” availability (Campbell and Campbell, 2007). The TEVGs presented in this thesis require a culture period of 5

weeks. Although this is shorter than previous described TEVGs (L'Heureux *et al.*, 2006; Niklason *et al.*, 2001; Poh *et al.*, 2005), these TEVGs will not be available "off the shelf". TEVGs which are needed for emergency coronary artery bypass surgery procedures or critically ischemic limbs will indeed have to be readily available. However, this is not true for TEVGs necessary for many other coronary and distal vascular bypass procedures, as well as AV-shunt procedures, which can be predicted and delayed, allowing a longer TEVG production (L'Heureux *et al.*, 2007a). A promising approach for the creation of "off the shelf" available TEVGs, seems the implantation of a synthetic scaffold which will be repopulated with cells *in vivo*. Recently, Torikai *et al*. developed PGA/PLLA grafts with a diameter of 10 mm, which were implanted in a porcine model without seeded cells prior to implantation. These grafts showed morphologic evidence of good *in vivo* cellularization and could withstand arterial pressure for 12 postoperative months (Torikai *et al.*, 2008). The PLLA layers were still present in the explants and it was questioned whether the grafts would be affected in the long term by further time-dependent deterioration of the mechanical properties. Although this method seems promising, it remains to be seen whether this technique is also applicable for small diameter grafts (<5 mm) and for grafts in a low flow environment.

Initially, the grafts will have to be placed at so called low risk positions. This was done by L'Heureux *et al*. who implanted TEVGs cultured for 24 weeks as arteriovenous shunts in hemodialysis patients in a first clinical safety study. Graft failure in this model is unlikely to be life or limb-threatening. The results are promising as the TEVGs are functioning well for hemodialysis access with follow-ups of 12 months (L'Heureux *et al.*, 2007b). A different approach was the use of TEVGs, made of a polycaprolactone-polylactic acid copolymer reinforced with woven PGA seeded with bone marrow cells, which were successfully used to reconstruct the low-pressure pulmonary outflow tract in pediatric patients (Shin'oka *et al.*, 2005). These clinical trials clearly demonstrate that TEVGs can be used successfully in a clinical setting. The next challenge is to optimize TEVGs for broader indications such as peripheral and coronary artery bypass grafting. Widespread clinical use will come with its own tribulations, including but not limited to quality control of autologous cell culture, regulatory issues, and medical technology assessment.

8.5 General conclusion

In conclusion, a bioreactor system was developed and used to culture small diameter human TEVGs. After optimization of the culture conditions, a functional EC layer was created on these grafts, which was able to withstand a physiological shear stress. This functional EC layer is an important step towards the clinical use of these TEVGs.

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Samenvatting

Er zijn veel mensen die beschadigde of verstopte kransslagaderen of perifere vaten hebben. Bij deze patiënten is het noodzakelijk om de bloeddoorstroming te herstellen. Het uitvoeren van een bypass operatie is de voornaamste behandeling hiervoor. Een andere groep patiënten die ook afhankelijk kunnen zijn van vasculaire grafts zijn patiënten met een nierziekte die afhankelijk zijn van dialyse. Tegenwoordig worden voor bypass operaties vaak patiënt-eigen (slag)aders of synthetische grafts gebruikt. Sommige van deze grafts hebben een beperkte levensduur en functioneren niet optimaal. Wanneer bijvoorbeeld een ader uit het onderbeen wordt gebruikt om een kransslagader te vervangen zit na 10 jaar ongeveer 57% van deze bypasses dicht. Tevens is het zo dat voor vaatvervangingen grafts steeds vaker nodig zijn, aangezien een toenemend aantal patiënten een heroperatie moet ondergaan en geen geschikte vaten meer over heeft. Het kweken van bloedvaten in het laboratorium (tissue-engineering) zou een goed alternatief kunnen zijn om de beperkingen van de huidige grafts te verhelpen. Zulke bloedvaten bestaan uit levend patiënt-eigen weefsel en hebben daardoor de eigenschap om zich te herstellen en te remodelleren.

Recentelijk zijn er grote vooruitgangen geboekt in de ontwikkeling van sterke humane getissue-engineerde grafts met een kleine diameter. Er is echter minder onderzoek gedaan aan de ontwikkeling van een functionele endotheelcel (EC) laag op die grafts. De EC laag is een zeer actieve cellaag die betrokken is bij weefsel homeostase en de regulering van vaatwandspanning. Verder is deze cellaag ook betrokken bij de regulering van groei van andere celtypen. Daarnaast wordt trombose, wat een belangrijke oorzaak is van het falen van grafts, actief voorkomen door de aanwezigheid van een intacte EC laag. Daarom was het doel van dit proefschrift om een functionele EC laag te creëren op humane getissue-engineerde grafts. Deze grafts zijn gebaseerd op een scaffold van polyglycolic acid met een poly-4-hydroxybutyrate coating, die gezaaid wordt met humane myofibroblasten (MF) afkomstig van de vena saphena.

Om een getissue-engineerde graft te endothelialiseren is het noodzakelijk de twee celtypen (ECs en MFs) samen te kweken. Dit is niet triviaal, aangezien de twee celtypen normaal verschillende kweekomstandigheden vereisen. In dit proefschrift is een 3D co-cultuur ontwikkeld, waarin de omstandigheden om de MFs en ECs samen te kweken konden worden geoptimaliseerd. Er is aangetoond dat ECs niet overleven in standaard kweekmedium (DMEM), maar dat deze cellen een speciaal EC medium nodig hebben. Wanneer dit medium gebruikt wordt en de ECs pas na 3 of 4 weken kweken op de vasculaire constructen gezaaid worden, resulteert dit in mooie volle EC laag.

Het is bekend dat ECs het fenotype van de cellen in hun omgeving kunnen beïnvloeden wanneer deze cellen samen gekweekt worden. Het is in dit proefschrift aangetoond dat zowel de groei als de expressie van een gladde spiercel marker van MFs beïnvloed wordt door het samen kweken met ECs. Het is ook aangetoond dat een laag ECs de weefselcompositie van de vasculaire constructen beïnvloedt.

Een functionele EC laag dient ook niet trombogeen te zijn. Om dit te onderzoeken zijn de vasculaire constructen blootgesteld aan een bloedstroming en hiermee is aangetoond dat ECs inderdaad de trombogeniciteit van de constructen verlaagd.

Als laatste is er een bioreactor ontwikkeld om getissue-engineerde bloedvaten met een kleine diameter te kweken en waarmee ook een EC laag aangebracht kan worden. Eén dag na het zaaien van de ECs is de cellaag bijna vol en hebben de cellen een ronde vorm. De gezaaide ECs worden vervolgens geconditioneerd door kweekmedium door de grafts te laten stromen, wat een afschuifspannig op de cellen veroorzaakt. De viscositeit van het kweekmedium is verhoogd tot de waarde van bloed door middel van xanthaan gom. Xanthaan gom is een stabiel verdikkingsmiddel en zorgt al bij lage concentraties voor hoge viscositeiten. Het gebruik van xanthaan gom zorgt ervoor dat, wanneer we een fysiologische stroming aanbrengen in het vat, ook een fysiologische afschuifspanning ontstaat. Het is in dit proefschrift aangetoond dat xantaan gom geen invloed heeft op de groei van ECs, hun oriëntatie in de richting van de vloeistofstroming en hun bloedvatverwijdende eigenschappen. De afschuifspanning op de ECs van de getissue-engineerde vaten zorgt ervoor dat de ECs wel een volle laag vormen. Tevens oriënteren de ECs zich in de richting van de stroming en krijgen ze een langgerekte vorm, in tegenstelling tot de ongeconditioneerde ECs, die geen volle cellaag vormen.

Samenvattend kan gesteld worden dat in dit proefschrift verschillende EC functies zijn onderzocht met behulp van verschillende modelsystemen. Er is tevens een bioreactor systeem ontwikkeld waarmee bloedvaten met een kleine diameter gekweekt zijn. Na optimalisatie van de kweekomstandigheden is op deze vaten een functionele EC laag gecreëerd, die fysiologische afschuifspanningen kan weerstaan. Deze functionele EC laag is een belangrijke stap voor de klinische toepasbaarheid van deze vaten.

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Rolf,
December 2008

Curriculum vitae

Rolf Pullens is geboren op 14 september 1980 te Waalwijk. In 1998 behaalde hij zijn VWO diploma aan het Dr. Mollercollege te Waalwijk. Aansluitend studeerde hij Biomedische Technologie aan de Technische Universiteit Eindhoven. Als onderdeel van deze studie heeft hij stage gelopen aan de University of Oxford, Verenigd Koninkrijk. Hier deed hij onderzoek naar de invloed van pH op het metabolisme van kraakbeencellen. Zijn afstudeerwerk richtte zich op het bestuderen van de invloed van verschillende mechanische belastingsprotocollen op het metabolisme van kraakbeencellen. Na zijn afstuderen in 2004, besloot hij te beginnen aan zijn promotie-onderzoek aan de faculteit Biomedische Technologie van de Technische Universiteit Eindhoven, resulterend in dit proefschrift.