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

Rolf A. A. Pullens1*, Maria Stekelenburg1, Frank P. T. Baaijens1 and Mark J. Post1,2
1Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands
2Department of Physiology, CARIM, Maastricht University, Maastricht, The Netherlands

Abstract

Tissue engineering of small diameter (<5 mm) blood vessels is a promising approach to develop viable alternatives for autologous vascular grafts. Development of a functional, adherent, shear resisting endothelial cell (EC) layer is one of the major issues limiting the successful application of these tissue engineered grafts. The goal of the present study was to create a confluent EC layer on a rectangular 3D cardiovascular construct using human venous cells and to determine the influence of this layer on the extracellular matrix composition and mechanical properties of the constructs. Rectangular cardiovascular constructs were created by seeding myofibroblasts (MFs) on poly(glycolic acid) poly-4-hydroxybutyrate scaffolds using fibrin gel. After 3 or 4 weeks, ECs were seeded and co-cultured using EGM-2 medium for 2 or 1 week, respectively. A confluent EC layer could be created and maintained for up to 2 weeks. The EGM-2 medium lowered the collagen production by MFs, resulting in weaker constructs, especially in the 2 week cultured constructs. Co-culturing with ECs slightly reduced the collagen content, but had no additional affect on the mechanical performance. A confluent endothelial layer was created on 3D human cardiovascular constructs. The layer was co-cultured for 1 and 2 weeks. Although, the collagen production of the MFs was slightly lowered, co-culturing ECs for 1 week results in constructs with good mechanical properties and a confluent EC layer. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords tissue engineering; endothelial cells; 3D co-culture; myofibroblasts; extracellular matrix; vascular constructs

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 lifetime (Raja et al., 2005), as is shown by a patency of 57% after 10 years (Sabik 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 tissue-engineered grafts should provide sufficient mechanical support and should also contain a functional EC layer (Mitchell and Niklason, 2003). The endothelium

*Correspondence to: Rolf A. A. Pullens, Eindhoven University of Technology, Department of Biomedical Engineering, Den Dolech 2/PO Box 513, 5600 MB Eindhoven, The Netherlands. E-mail: r.a.a.pullens@tue.nl

# This work was performed at the Department of Biomedical Engineering, Eindhoven University of Technology, Eindhoven, The Netherlands.
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 favourable 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 then the commonly used 1–3 days (Borschel et al., 2005; Niklason et al., 1999).

However, in co-culture with ECs, smooth muscle cells (SMCs) 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 two-dimensional (2D) co-culture system, SMC proliferation was stimulated by bovine ECs but was downregulated in a three-dimensional (3D) culture system (Williams and Wick, 2005).

Recently, strong small-diameter TE vascular grafts were cultured in our laboratory 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 (MFs) 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 analysed.

2. Materials and methods

2.1. Cell isolation and culture

Endothelial cells (ECs) and myofibroblasts (MF), were harvested from fresh discarded vein segments of the human saphenous vein obtained from patients 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 min in an antibiotics solution containing PBS supplemented with 2.5 µg/ml amphotericin B and 200 µg/ml 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 min. After incubation, the cell suspension was collected and pelleted by centrifugation at 250 × g for 5 min. The cell pellet was re-suspended 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%), R2-insulin-like growth factor 1 (0.1%), ascorbic acid (0.1%), human epidermal growth factor (0.1%), gentamycin sulphate amphotericin-B (0.1%) and heparin (0.1%).

Cells from the vessel wall were isolated using an explant technique and expanded using regular cell-culture methods (Schnell et al., 2001), and characterized as a mixture of V (vimentin) and VA (vimentin/actin) type myofibroblasts (MFs) (Mol et al., 2006).

The culture medium of MFs consisted of DMEM Advanced (Gibco, USA), 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, contained DMEM Advanced supplemented with 10% FBS, 1% GlutaMax, 0.3% gentamycin and L-ascorbic acid 2-phosphate (0.25 mg/ml; Sigma).

2.2. Scaffold preparation and seeding

Rectangular scaffolds (30 × 9 mm; n = 30) composed of rapid degrading non-woven polyglycolic acid (PGA; thickness, 1.0 mm; specific gravity, 70 mg/cm²; Cellon, Bereldange, Luxembourg) were coated with poly-4-hydroxybutyrate (P4HB; provided by Symetis Inc., Zürich, Switzerland). The scaffolds were placed in six-well plates.
and the outer 5 mm of the long axis of each strip was glued to the well using a 20% solution of polyurethane (PU; DSM, The Netherlands) in tetrahydrofuran, leaving a 20 \times 9 \text{ mm} area for cell seeding (Figure 1A). The cell-seeded rectangular scaffold strips are referred to hereafter as TE constructs. The solvent was allowed to evaporate overnight. The constructs were sterilized by placing them in 70% ethanol for 4 h. Afterwards, the constructs were washed with PBS and placed in culture medium overnight. Seeding of the MFs was performed at a density of \( \pm 2.5 \times 10^6 \) cells (passage 7)/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 onto the constructs, resulting in a density of 1 \times 10^4 \text{ cells/cm}^2.

### 2.3. Tissue culture

After seeding of the MFs, the six-well plates were placed on a shaker (50 r.p.m.) in an incubator to allow mixing of the TE medium (T). The constructs were divided into five groups (\( n = 6 \); Figure 1B) and cultured for 5 weeks. The culture medium was changed to EC medium (E) at the moment of EC seeding, as pilot studies showed that ECs did not survive in TE medium. 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 (T5) 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 T3E2 and T4E4. 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 T3E2 + ECs and T4E4 + ECs.

### 2.4. Qualitative tissue analyses

After the 5 week culture period, the EC seeded constructs were stained for 45 min with FITC UEA-1 lectin (5 \( \mu \text{M}; \) Sigma), for visualization of the endothelial cells (Hormia et al., 1983), and Cell Tracker Orange (10 \( \mu \text{M}; \) CTO, Invitrogen, USA), for visualization of viable cells. Afterwards, the constructs were detached from the wells and analysed 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 formalin and embedded in paraffin; 10 \( \mu \text{m} \) sections were stained with haematoxylin and eosin (H&E) for general tissue morphology and Masson trichrome (MTC) for collagen visualization. To analyse 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 colour definition.

### 2.5. Quantitative tissue formation analyses

Quantitative tissue formation was determined by measuring the amount of DNA as an indicator of the number of cells, sulphated glycosaminoglycans (GAGs), and hydroxyproline as an indicator of collagen content (per mg dry weight of tissue). For the analyses, lyophilized tissue samples were digested in papain solution (100 \( \text{mM} \) phosphate buffer, 5 \( \text{mM} \) L-cysteine, 5 \( \text{mM} \) ethylenediaminetetraacetic acid (EDTA), and 125–140 \( \mu \text{g} \) papain/ml) at 60°C for 16 h. The DNA content was determined using the Hoechst dye method (Cesarone et al., 1979) combined...
with a standard curve from calf thymus DNA (Sigma). It is assumed that the measured DNA content represents the DNA of the MFs, as the number of ECs is negligibly small compared to the number of MFs. The sulphated GAG content was determined using a modification of the assay described by Farndale et al. (1986) and a standard curve from chondroitin sulphate from shark cartilage (Sigma). The hydroxyproline content was determined using a modification of the assay described by Huszar et al. (1980) and a standard curve from trans-4-hydroxyproline (Sigma). A 1:7.6 ratio of hydroxyproline to collagen was assumed. To obtain a measure for the amount of the matrix components produced per cell, the collagen and GAG content were normalized for the amount of DNA.

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

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 20 N 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.

2.7. Statistics

Quantitative data were averaged per group, and represented as average ± standard error of the mean (SEM). Using 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. Results

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 (T4E1 + ECs), a continuous, connecting monolayer of UEA-1- (Figure 2A), CD31- (Figure 2B,C) and vWF (Figure 2D,E)-positive ECs was found. Similar results were found in the T3E2 + ECs group, indicating a stable EC layer for 1–2 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 [T3E2 + ECs (Figure 3) and T3E2] compared to the ones cultured in TE medium, suggesting a negative influence of the EC medium on tissue development. 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 T3 control group and the T4E1 + ECs group (Figure 3).

3.2. Quantitative tissue formation analyses

The cells produced large amounts of collagen. The average collagen content was 190 µg/mg, equalling 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 1; *p* < 0.05 and *p* < 0.001). The DNA content of the T4E1 group and the GAG content of the T3E2 and T4E1 groups were higher than the control group T5 (Figure 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 (T3E2 and T4E1; Figure 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 4D). In contrast, the collagen production per cell was decreased in the T3E2 group (Figure 4D; *p* < 0.005).

Culturing the constructs in EC medium has a stimulating effect on the number of HP crosslinks per triple helix in the TE constructs (Table 1; *p* < 0.005). The highest amount was found in the T3E2 group (Figure 5). This result suggests that EC medium alters the balance between collagen production and collagen maturation.

Co-culture of ECs reduced the collagen content compared to respective controls (Table 1; *p* < 0.001). The collagen amount was significantly lower (*p* < 0.05) in the T4E1 + ECs group, compared to T4E1 – ECs. However, when normalized for the amount of DNA, this difference was not present (Figure 4D), suggesting that...
ECM composition of 3D engineered cardiovascular constructs

Figure 2. Characterization of the EC monolayer after 1 week of culture (T4E1 + 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. H&E (A–C) and Masson trichrome (MTC) staining (D–F) of control group T5 (A,D) and EC-seeded groups T4E1 + ECs (B,E) and T3E2 + ECs (C,F). H&E staining shows homogeneous tissue formation. Collagen, stained blue in MTC staining, was abundantly present in the tissues. The T3E2 + ECs group had a less homogeneous ECM structure. Scale bar represents 200 µm.

3.3. Mechanical properties

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 HP crosslinks per triple helix (Figure 4A, B; Figure 5, Table 1).

The constructs were relatively strong and stiff, as indicated by an average UTS and Young’s modulus of 2 MPa and...
Figure 4. The amounts of DNA, GAG and collagen per dry weight (A–C) and the amount of collagen and GAG per DNA (D) of all groups. Culturing in EC medium for 1 week increased the amount of DNA in the constructs (A). EC medium also increased the amounts of GAG in the T4E1 and T3E2 groups (B). When EC medium was used for 2 weeks, the collagen amount decreased (C). Co-culturing with ECs did not influence the amounts of DNA and GAG (A, B) but decreased the amount of collagen. Culturing for 2 weeks in EC medium lowered the collagen production per cell (D). *p < 0.05; **p < 0.005

Table 1. F and p values of two-way ANOVA analysis

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<tr>
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<th>EC medium</th>
<th>Endothelial cells</th>
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<tr>
<td></td>
<td>F</td>
<td>Significance</td>
</tr>
<tr>
<td>DNA (µg/mg)</td>
<td>4.44</td>
<td>p &lt; 0.05</td>
</tr>
<tr>
<td>GAG (µg/mg)</td>
<td>14.23</td>
<td>p &lt; 0.001</td>
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<tr>
<td>Collagen (µg/mg)</td>
<td>10.54</td>
<td>p &lt; 0.005</td>
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<tr>
<td>GAG/DNA (-)</td>
<td>1.77</td>
<td>NS</td>
</tr>
<tr>
<td>Collagen/DNA (-)</td>
<td>11.45</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Crosslinks per triple helix</td>
<td>4.34</td>
<td>p &lt; 0.005</td>
</tr>
<tr>
<td>Young’s modulus</td>
<td>12.97</td>
<td>p &lt; 0.001</td>
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<tr>
<td>UTS</td>
<td>7.49</td>
<td>p &lt; 0.005</td>
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The presence of EC medium has a significant influence on the ECM composition, except for the GAG/DNA, and the mechanical properties. The presence of ECs has an influence on the collagen amount per dry weight only. NS, not significant.

11 MPa, respectively (Figure 6). The constructs cultured in EC medium for 2 weeks (T3E2) had lower Young’s moduli than the control constructs (T5; p < 0.005), suggesting that a shorter culture period in EC medium is advised. Seeding and co-culturing of ECs (T3E2 + ECs and T4E1 + ECs) did not have an additional effect on the mechanical properties (Table 1).

Figure 5. The amounts of HP crosslinks per triple helix of all groups. EC medium increased the amount of HP crosslinks per triple helix. **p < 0.01

4. Discussion

The development of a functional, adherent, shear-resisting endothelial layer is one of the major issues
Co-culturing ECs on the tissue-engineered 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, the EC-seeded groups did have a lower 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 crosslinks are also important for mechanical properties, but they were not influenced by the ECs. It is expected that the small decrease in collagen amount is not enough to cause a reduction in 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 in which animal ECs were directly cultured on 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 tissue development in these constructs in an endothelium-dependent manner (Nackman et al., 1998). Second, 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 reduced the collagen content even more but there was no additional effect on the mechanical properties. The 1 week co-cultured constructs had a confluent EC layer and good mechanical properties. This set-up can be used for the development of a shear-resistant EC layer in human tissue-engineered vascular grafts.
Acknowledgements

The authors thank Kang Yuen Rosaria Chak from Eindhoven University of Technology and Jessica Snael from TNO Leiden for performing the biochemical assays. This research was performed in the Dutch Programme for Tissue Engineering from the Dutch Technology Foundation (STW) and Applied Science Division of NWO.

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DOI: 10.1002/term