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Biological characterisation of vascular grafts cultured in a bioreactor

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Abstract

In this study, the development is described of a tissue-engineered construct mimicking the structure of a natural blood vessel. Smooth muscle cells (SMC) were cultured under pulsatile flow conditions in porous tubular scaffolds composed of crosslinked type I insoluble collagen and insoluble elastin. Under these dynamic culture conditions, average wall shear rate, systolic and diastolic pressures and pressure wave-forms comparable to conditions in the human carotid artery were obtained. Culturing of SMC in tubular scaffolds under dynamic conditions resulted in enhanced tissue formation compared to static conditions. Higher SMC numbers, a more homogeneous distribution of SMC throughout the scaffolds and higher collagen mRNA expression levels were found when cells were cultured under dynamic compared to static conditions. mRNA expression levels of markers of proliferation and apoptosis showed that the higher cell numbers in the scaffolds cultured under dynamic conditions can be explained by increased cell proliferation but not by decreased apoptosis. Glucose consumption and lactate formation by the cells showed that cell metabolism was more aerobic under dynamic compared to static conditions. Lining of the dynamically cultured constructs with a luminal monolayer of endothelial cells might result in vessels suitable for in vivo applications.

Keywords: Vascular tissue engineering; Smooth muscle cells; Collagen; Elastin; Cell proliferation; Apoptosis

1. Introduction

Approaches to prepare a tissue engineered (TE) small-diameter blood vessel include the use of synthetic [1–5] or natural materials [6,7] as scaffolds for autologous cell seeding. These scaffolds provide a temporary biomechanical structure until cells produce their own extracellular matrix (ECM) [8]. Shortcomings of these strategies include insufficient mechanical properties, a lack of elastin (ELN) deposition and long culture time periods. The development of bioreactors in which biological and/or biochemical processes develop under monitored and controlled environmental and operating conditions could further improve the potential of tissue engineering [9]. Several studies on culturing of vascular cells in tubular scaffolds in specific bioreactors demonstrate that an environment resembling in vivo conditions may promote both the development of TE constructs with sufficient mechanical strength to be implanted [2,3,10] and the modulation of appropriate cellular functions [7,11]. Continuous mechanical stress loading has at least three different effects on smooth muscle cells (SMC) present in TE constructs: (i) acceleration of the orientation of SMC, (ii) acceleration of the production of collagen fibre bundles and (iii) induction of the phenotypic modulation of SMC from a synthetic to a contractile state [11]. However, the optimal bioreactor design and culture conditions for the development of a functional arterial graft remain to be elucidated [9,12].

In this paper, the biological properties are described of human vascular SMC seeded in tubular scaffolds composed of type I insoluble collagen and insoluble ELN and cultured in a pulsatile flow bioreactor. The relatively low trans-wall pressure difference in this particular bioreactor

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and the presence of ELN fibres in the scaffolds, most probably prevented the scaffolds from bursting under cyclic mechanical stress [9–13]. In other studies, a silicon support in the lumen of the vessel is used to regulate the degree of deformation under these conditions [3,10,14]. The lack of any support inside the tubular scaffolds in our system enables us to simulate wall shear rate and arterial pressure comparable to the conditions in the human carotid artery simultaneously, in contrast to other systems where those parameters are investigated separately [14–20]. The effects of dynamic compared to static culture conditions on final cell numbers, distribution, proliferation, apoptosis and ECM production of the seeded SMC inside the TE constructs were evaluated in time. During culturing, changes of the culture medium composition due to active cell metabolism and processes mentioned above were followed.

2. Materials

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Gibco BRL (Breda, The Netherlands). Penicillin, streptomycin, fetal bovine serum and trypsin/ethylene diamine tetra-acetic acid (EDTA) were purchased from Biowhittaker (Verviers, Belgium). Gelatin type B from bovine skin, *N*-(3-dimetylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC), *N*-hydroxysuccinimide (NHS), poly(propylene glycol)-bis-(2-aminopropyl ether) (Jeffamine 230, J230), proteinase K and DNAse-free RNAse were obtained from Sigma and Aldrich (St Louis, MO, USA). Mouse monoclonal antibodies (mAbs) against human α-smooth muscle actin (α-SMA) and human vimentin and fluorescein isothiocyanate-labelled rabbit anti-mouse immunoglobulins (RAM-FITC) were purchased from DAKO (Glostrup, Denmark). Collagenase type 2 was purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA). Human serum was acquired by overnight coagulation of blood (collected from healthy volunteers), subsequently pooled and stored at −80 °C. QIAmp RNA Blood Mini Kit was from QIAgen (Hilden, Germany). Dithiothreitol (DTT) and Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase enzyme were obtained from Invitrogen (Paisley, UK). dNTPs were from Amersham Pharmacia Biotech (Cambridge, UK). RNAse inhibitor was from Roche (Basel, Switzerland). Primer and probe sequences of cyclin E, tissue transglutaminase (tTG) and type I collagen (COL1A1) and TaqMan® Gene Expression Assays for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ELN were from Applied Biosystems (Nieuwerkerk A/D IJssel, The Netherlands). Insoluble collagen (type I from bovine achilles tendons) and insoluble ELN (from equine ligamentum nuchae), purified as described in [21] were kindly donated by Dr. T.H. van Kuppevelt, Department of Biochemistry, University Medical Centre Nijmegen, The Netherlands.

3. Methods

3.1. Isolation of SMC

SMC were isolated from human umbilical veins by a collagenase digestion method according to the method of Heimli et al. [22] with some minor modifications as previously described [23]. Cells were cultured on gelatin-coated (0.5% w/v) tissue culture polystyrene (g-TCPS) using DMEM containing 10% (v/v) heat-inactivated (30 min, 56 °C) pooled human serum, 10% (v/v) heat-inactivated (30 min, 56 °C) fetal bovine serum, 50 units/ml penicillin and 50 μg/ml streptomycin [22]. Culture medium was filtered (0.20 μm) before use. During culturing, medium was refreshed every 2–3 d. Cells were cultured in a humidified atmosphere containing 5% CO_2 inside an incubator at 37 °C (CleanAir Techniek bv, Woerden, The Netherlands) [24]. When sub-confluent cultures were obtained, cells were detached from the support with 0.125% (w/v) trypsin/0.05% (w/v) EDTA and subcultured for several passages (split ratio 1:3) [25]. Sub-confluent cultures of SMC from passages 5 to 9 were used to seed tubular scaffolds for tissue engineering applications.

3.2. Identification of SMC

SMC were identified using mAbs against human α-SMA and human vimentin [22–24]. Fluorescence of the secondary antibody RAM-FITC was examined with immuno-fluorescent microscopy and with flow cytometry as described earlier [23]. Negative controls were obtained by omitting first antibodies and by staining human umbilical vein endothelial cells (HUVEC) and human dermal fibroblasts using the above-mentioned antibodies.

3.3. Scaffold properties

Porous tubular scaffolds with an inner diameter of 3 mm, an outer diameter of approximately 6 mm and a length of 4 cm, were produced by freeze-drying a suspension of type I insoluble collagen and insoluble ELN (1:1 w/w) at −18 °C as described by Buttafoco et al. [13]. Scaffolds were optimised in terms of pore size and crosslink density [23]. To improve the mechanical properties, crosslinking of the scaffolds was performed either with a water-soluble carbodiimide in combination with a succinimide (EDC/NHS) or with a diamine crosslink spacer (J230) in the presence of EDC/NHS [26,27]. Non-crosslinked scaffolds, EDC/NHS crosslinked scaffolds and J230/EDC/NHS crosslinked scaffolds had a porosity of 95%, 94%, and 93% and an average pore size of 143, 131, and 151 μm, respectively, as determined by Micro Computed Tomography (Micro-CT) [13]. Human aortic SMC in suspension at 37 °C have a length of 54.5 ± 1.5 μm and a diameter of 7.5 ± 0.3 μm [28]. The pores of all scaffolds were interconnected and after crosslinking more than 80% of the pore volume was accessible for SMC [13].

3.4. Seeding SMC in tubular scaffolds

Tubular scaffolds were cannulated and tied on both ends with sutures (Ethicon Mersilene, Johnsson & Johnsson Intl., St. Stevens-Woluwe, Belgium) to thin-walled stainless-steel tubes having an outside diameter of 3 mm matching the inside diameter of the vessels at physiological pressure. Scaffolds were then mounted in home-made glass flow chambers in which the outside and inside of the scaffolds were in contact with fluid. After disinfection of the scaffolds with 70% ethanol for 10 min and rinsing three times with PBS, scaffolds were incubated overnight with serum-containing culture medium to enhance cell attachment. SMC from sub-confluent cultures were detached from their g-TCPS support with 0.125% (w/v) trypsin/0.05% (w/v) EDTA. Cell concentrations were determined with a hemacytometer (Bürker) and 10^7 SMC suspended in 20 ml culture medium were seeded into each scaffold by a filtration seeding procedure. The technique of filtration seeding promotes a more uniform cell distribution inside three-dimensional porous scaffolds compared to static seeding procedures [29–31]. Two syringes were used to infuse the cell suspension in the lumen from both ends of the scaffold simultaneously. In this way, cells were filtered through the porous wall of the scaffold. Subsequently, the flow chamber was completely filled with culture medium, the top opening was closed and the seeded scaffolds were placed in an incubator (37 °C and 5% CO_2) and rotated 90° around the longitudinal axis every 30–60 min for the first 2.5 h to promote homogeneous cell adhesion in the scaffolds. Finally, cells were allowed to adhere statically in the scaffolds for an additional period of 24 h.

3.5. Culturing SMC under pulsatile flow conditions

After seeding, four flow chambers each containing one cell-seeded scaffold were mounted in a bioreactor in which pulsatile flow and pressure

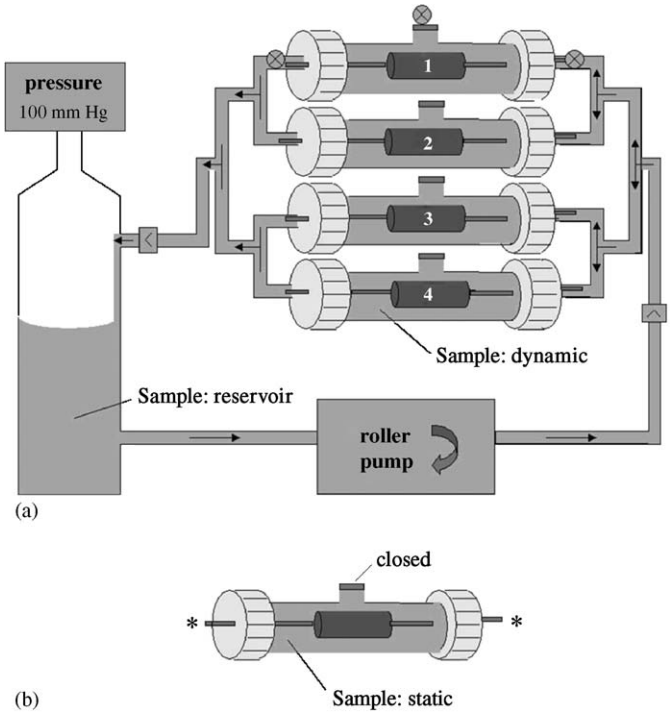

Fig. 1. Schematic representation of dynamic culturing in the pulsatile flow bioreactor used in this study (a) compared to static culturing (b). Places of culture medium sampling for evaluation of metabolic parameters are indicated: ⊗ represents a pressure sensor and ⊠ a valve. For static culturing, TE constructs were mounted in flow chambers filled with culture medium and placed inside an incubator but were not mounted in the bioreactor. * Indicates open mounting sides of the flow chambers during static culturing.

can be varied (Fig. 1a). In this system, a peristaltic roller-pump (Watson Marlow Sci-Q-323, Brussels, Belgium) placed proximal to the vessels, was used to pump culture medium from a home-made three-port glass fluid reservoir (60 ml) via highly distensible silicone rubber tubing (Watson Marlow, Brussels, Belgium, 3.2 mm ID × 6.4 mm OD) to the four flow chambers in parallel position. In this way, a pulsatile flow of culture medium was obtained through the lumen of the cell-seeded tubular scaffolds which were surrounded by culture medium in the flow chambers. A pressure of 100 mmHg (compressed air) was applied to the culture medium reservoir and regulated by means of an electronically controlled Venturi valve (T5200-50, Fairchild Company, Winston-Salem, NC, USA). The applied pressure was monitored by pressure sensors (Edwards Lifesciences LLC, Unterschleissheim, GmbH, Germany). Pressure signals were displayed during the entire culture period by means of a pressure transducer (Instrumentation Department, Academic Medical Centre, Amsterdam, The Netherlands) and a scope meter (Fluke 199BM scopemeter, Adquipment Medical B.V., Hellevoetsluis, The Netherlands). Two valves (DATEX, Helsinki, Finland) preventing back flow of the culture medium, were inserted at the proximal and distal position of the chambers, in order to obtain a more accurate reproduction of the pressure wave-forms experienced by blood vessels in vivo during ventricular systole and diastole. The culture medium reservoir and the flow chambers containing the cell-seeded scaffolds were placed in humidified atmosphere inside an incubator (37 °C and 5% CO_2). By increasing the rotational speed of the roller pump, pressure pulses were gradually increased from 30 to 120 beats/min during 3 d of culturing. After 3 d, a volumetric flow rate of 9.6 ml/min in each construct resulted in an average wall shear rate of 61 s^{-1}, which fits the lower range of shear rates found in the human carotid artery (60–775 s^{-1}) [32]. Pressure wave-forms with a mean pressure of 82 mmHg, a systolic pressure of 124 mmHg and a diastolic pressure of 61 mmHg, similar to the wave-forms in the human carotid artery were established in the constructs. The average Reynolds number was 96, which is indicative of laminar flow [33]. Laminar flow is necessary for efficient and homogeneous transfer of oxygen, nutrients and waste products to and from the three-dimensional constructs [30,34].

Cells seeded in tubular scaffolds were cultured under these dynamic conditions, or under static conditions as control, for 1, 3, 7 and 14 d. In the static environment, flow chambers containing cell-seeded scaffolds were placed inside an incubator (37 °C and 5% CO_2) but were not mounted in the bioreactor (Fig. 1b). Culture medium in the flow chambers used for dynamic and static culturing as well as medium inside the culture medium reservoir in case of dynamic culturing were refreshed every 2 d. At the predetermined time points, the TE constructs were disconnected from the bioreactor and characterised in terms of morphology and biological properties.

3.6. Cell numbers

Numbers of SMC present in the TE constructs were quantified by the CyQuant Cell Proliferation assay according to the manufacturer's instructions (Molecular Probes, Leiden, The Netherlands). Construct samples with a length of 7 mm were rinsed with PBS and digested with 200 µl proteinase K solution (1 mg/ml in PBS) for a minimum of 16 h at 56 °C. Samples were stored at −80 °C until further analyses. Various dilutions were prepared with cell-lysis buffer (Molecular Probes, Leiden, The Netherlands) supplemented with 180 mM NaCl, 1 mM EDTA and 1.35 Kunitz units/ml DNAse-free RNAse. Samples were incubated for 1 h at RT to remove the RNA and single stranded DNA. Finally, samples were mixed with CyQUANT® dye and after 2 min, fluorescence was measured in 96-well plates using a Viktor fluorescence analyser (PerkinElmer Life Sciences, Turku, Finland). Excitation and emission wavelengths were 480 and 520 nm, respectively. The measured fluorescence intensities were correlated to SMC numbers using a calibration curve made by means of dilutions with known concentrations of SMC from a sub-confluent culture on g-TCPS. Cells were detached from the support with 0.125% (w/v) trypsin/0.05% (w/v) EDTA, counted with a hemacytometer (Bürker) and then analysed in the same way as described above to obtain the calibration curve.

3.7. Histology

After culturing, 3-mm pieces of the TE constructs were rinsed with PBS and fixed with formalin (4% v/v) for at least 24 h. Samples were impregnated with paraffin, cut into transverse sections and stained by the hematoxylin and eosin procedure (HE) or by immuno-staining of α-SMA according to standard procedures.

3.8. RNA isolation and cDNA synthesis

Messenger RNA (mRNA) gene transcripts of the SMC present in the TE constructs after culturing were quantified by a semi-quantitative RT-PCR method on a real-time TaqMan analyser (7900 HT Sequence Detection System, Applied Biosystems, Nieuwerkerk A/D IJssel, The Netherlands) [35,36]. Construct samples after 7 and 14 d of culturing with a length of 7 mm were rinsed with PBS and total RNA contents were harvested by disrupting the cell membranes with 600 µl of cell lysis buffer (Buffer RLT® of QIAgen, Hilden, Germany) containing 1% (v/v) β-mercaptoethanol using a mechanical homogeniser. Cell numbers in the scaffolds were first determined with the CyQuant Cell Proliferation assay after which RNA was isolated from the same amount of cells either cultured under dynamic or static conditions. One volume of 70% ethanol was added to the lysates and total RNA was isolated from each sample using the QIAamp RNA Blood Mini columns and kit according to the manufacturer's instructions. A standard cDNA synthesis with Moloney Murine Leukaemia Virus (M-MLV) reverse transcriptase enzyme was performed as described by Volokhina et al. [37].

3.9. Real-time semi-quantitative PCR

mRNA expression levels were determined using the gene specific primer and probe sequences for cyclin E, tTG, COL1A1, and ELN. Fragments of the cyclin E sequence were amplified using the primer (300 nM) and probe (200 nM) set as described by Müller-Tidow et al. [38]. Fragments of the tTG sequence were amplified using the primer (900 nM) and probe (200 nM) set as described by Volokhina et al. [37]. Fragments of the COL1A1 sequence were amplified using the primer (300 nM) and probe (100 nM) set as described by Martin et al. [30,39]. Fragments of the ELN and GAPDH sequences were amplified using standard assays of Applied Biosystems (Nieuwerkerk A/D IJssel, The Netherlands). Primer and probe concentrations were optimised following the guidelines of the provider. mRNA expression levels of tTG, cyclin E, COL1A1 and ELN were normalised to expression of GAPDH mRNA [10,39], which was not influenced by static or dynamic culture conditions.

3.10. Metabolic parameters during culturing

To analyse the metabolic activity of SMC present in the TE constructs during culturing, several metabolic parameters of the culture medium were evaluated in time. Acidity (pH), partial oxygen (pO_2) and carbon dioxide pressures (pCO_2), and glucose and lactate concentrations of culture medium in the flow chambers and the culture medium reservoir were measured every 48 h prior to culture medium refreshment. Three millilitre samples of culture medium were measured within 15 min after sampling with a blood-gas analyser (Radiometer, ABL 700 series, Copenhagen, Denmark). Measurements of freshly prepared culture medium were performed to determine baseline data at time point zero. Because culture medium was refreshed every 2 d, actual values of the glucose and lactate concentrations did not represent total glucose consumption and total lactate formation. Cumulative glucose consumption and lactate formation were calculated and plotted as a function of time. In case of culturing under dynamic conditions, total glucose consumption and lactate formation per TE construct were calculated by taking the sum of the amount present in a flow chamber and a quarter of the amount present in the culture medium reservoir. In case of static conditions, the amount present in a flow chamber was taken.

3.11. Statistical analyses

Data are presented as the mean ± standard error of the mean (SEM) of three experiments performed in duplicate. Differences in cell numbers and relative mRNA expression levels were analysed using an unpaired two-tailed *t*-test. Results were considered significantly different at p values <0.05.

4. Results and discussion

SMC isolated from human umbilical vein were successfully cultured and expanded on g-TCPS. No microscopic abnormalities or changes in α-SMA or vimentin expression were observed during the expansion time [23]. After 28 d of culturing, approximately 10×10^6 cells were obtained, the amount required for seeding one scaffold of 4 cm length. About 36 d of culturing were necessary to obtain an appropriate amount of cells for the experiments described in this study. During dynamic culturing in the bioreactor, repeated observation of the cell-seeded scaffolds through the glass flow chamber walls showed a good stability of the scaffolds and no macroscopic evidence of a bacterial or fungal contamination.

Compared to non-crosslinked collagen/ELN tubular scaffolds, crosslinking of the scaffolds with EDC/NHS did not significantly influence cell numbers present inside the scaffolds after 7 d of dynamic culturing. In contrast, crosslinking of the scaffolds with J230/EDC/NHS resulted in significantly lower cell numbers compared to non-crosslinked and EDC/NHS crosslinked scaffolds after 7 d of dynamic culturing (Fig. 2). J230 functions as a spacer incorporated into the scaffolds. The chemical nature of J230 or the presence of unreacted amine groups as a consequence of crosslinking with J230/EDC/NHS may have altered the ability of SMC to adhere, proliferate and migrate in these scaffolds [13,40]. The presence of J230 may contribute to an increased hydrophilic character of the scaffolds, which decreases protein adsorption and cell adhesion and increases levels of apoptosis of adherent cells [41].

In addition to evaluation of the biological properties of the TE constructs, their mechanical properties were evaluated in our laboratories [13]. It was concluded that crosslinked scaffolds have a better ability to withstand load compared to non-crosslinked scaffolds. Considering both biological and mechanical properties of the TE constructs, EDC/NHS crosslinked scaffolds were selected for further studies.

A significant increase of the amount of SMC present in tubular EDC/NHS crosslinked scaffolds was found after 7 and 14 d of dynamic culturing, compared to days 1 and 3 (Fig. 3). In addition, significantly higher cell numbers were found after 7 and 14 d of dynamic compared to static culturing. The same trends were observed by histology (Fig. 4). Moreover, a difference in cell distribution in the scaffolds was observed after 7 and 14 d of culturing under dynamic compared to static conditions. Under dynamic

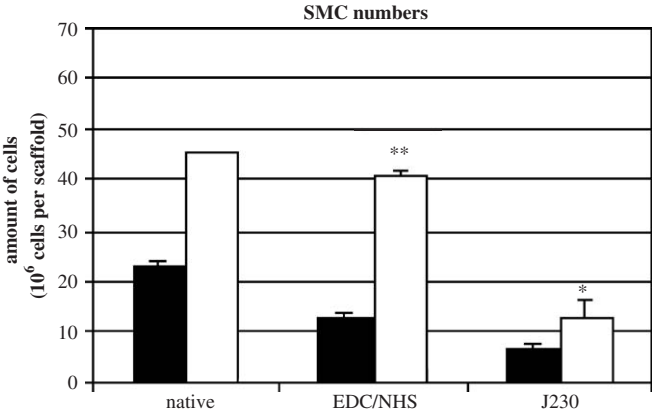

Fig. 2. Amount of SMC present in tubular scaffolds composed of collagen and elastin, uncrosslinked (native) or crosslinked with either EDC/NHS or J230/EDC/NHS. Constructs were cultured for 7 d under static (black bars) or dynamic conditions (white bars). Cell numbers of three experiments performed in duplicate (±SEM) are presented. * Indicates a significant difference compared to uncrosslinked (native) and EDC/NHS crosslinked scaffolds cultured under dynamic conditions ($p<0.05$). ** Indicates a significant difference compared to EDC/NHS crosslinked scaffolds cultured under static conditions ($p<0.05$).

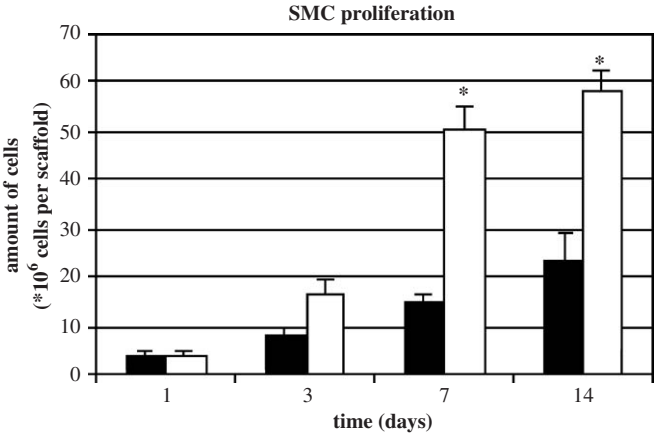

Fig. 3. Amount of SMC present in tubular scaffolds composed of collagen and elastin crosslinked with EDC/NHS after 1, 3, 7, and 14 d of culturing under static (black bars) or dynamic conditions (white bars). Cell numbers of three experiments performed in duplicate (±SEM) are presented. * Indicates a significant difference compared to 1 and 3 d of culturing under dynamic conditions and compared to 7 or 14 d of culturing under static conditions ($p<0.05$).

conditions, cells grew inside and on the ''adventitial'' side of the tubular scaffolds. In contrast, under static conditions, cell growth was observed predominantly on the ''adventitial'' side of the scaffolds. Immuno-histochemistry of the construct sections showed that cells present in and on the scaffolds stained positive for the presence of α-SMA after dynamic and static culturing (data not shown). According to the presence of α-SMA fibres in the cells, no fundamental differentiation or dedifferentiation processes took place during culturing [24,42].

By means of histology and scanning electron microscopy (SEM) we were not able to show that SMC orient in a circumferential way mimicking the in vivo situation. However, the question remains whether this orientation is strictly necessary to obtain a functional construct. One can speculate that after implantation, cells present in the graft will mature and orient according to the need of the specific tissue.

Significantly higher cyclin E mRNA expression levels of SMC were measured after 7 d of culturing in tubular EDC/NHS crosslinked scaffolds under dynamic compared to static conditions (data not shown). After 14 d of culturing, cyclin E mRNA expression levels were also higher under dynamic conditions but not significantly different compared to static conditions. No increase of cyclin E mRNA expression in time was found during dynamic culturing. No significant differences of tTG mRNA expression levels after 7 and 14 d of culturing were found under dynamic compared to static conditions (data not shown). Also, no significant difference in time of tTG mRNA expression was found during dynamic or static culturing. These results indicate that the higher cell numbers in the TE scaffolds cultured under dynamic conditions can be explained by increased cell proliferation in these constructs and not by decreased apoptosis.

Collagen mRNA expression levels of SMC in tubular EDC/NHS crosslinked scaffolds were significantly higher after 14 d of culturing under dynamic compared to static conditions. Moreover, expression levels were significantly higher after 14 d of dynamic culturing compared to 7 d of static and dynamic culturing (Fig. 5). Although ELN mRNA expression levels of SMC in tubular EDC/NHS crosslinked scaffolds were detected, levels were too low for quantitative analyses. As described by Buttafoco et al. [13], TE constructs with increased high strain stiffness were obtained after 14 d of static and especially dynamic culturing, compared to 7 d of culturing, confirming the production of collagen. One can speculate that the newly synthesised ECM present in the constructs and the dynamic culture conditions provide appropriate signals to the vascular cells for stimulation of ELN production. Longer culture time periods, culture medium supplements or co-culturing various vascular cell types in the present model are approaches that can be tested to improve the ELN production [14,43].

The consumption of O_2 and glucose and the formation of CO_2 and lactate in culture medium is an indication of active cell metabolism. Under aerobic conditions, CO_2 and water are formed out of glucose and O_2, whereas under anaerobic conditions, lactate is formed. Total glucose consumption and lactate formation were not significantly different between dynamic and static culture conditions during the first 8 d of culturing (Fig. 6). However, during this time period the actual pH and glucose concentrations were significantly higher and the actual pCO_2 and lactate concentrations significantly lower in the flow chambers used for dynamic compared to static culturing. This is indicative of mass transport between the culture media in the reservoir and the flow chambers. After 14 d of dynamic culturing, total glucose consumption and lactate formation were higher than under static conditions (Fig. 6). This was related to the higher cell numbers present under dynamic conditions. Again the actual glucose and lactate concentrations, pH and especially the lower pCO_2 values in the flow chambers used for dynamic culturing during days 10–14, were indicative of improved mass transport under dynamic compared to static conditions. This is in agreement with other studies showing that dynamic culture conditions may contribute to reduction of external mass-transfer limitations [30].

Assuming that the amount of cells present in the TE constructs did not change between 12 and 14 d of dynamic and static culturing, the glucose consumption and lactate formation in this time interval expressed per cell can be calculated. Glucose consumption amounted to 5 and 8 pmol/cell and lactate formation to 8 and 14 pmol/cell under dynamic and static conditions, respectively. The lower lactate formation per cell under dynamic culture conditions suggests that cell metabolism was more aerobic compared to static conditions. In addition, the higher glucose consumption per cell under static culture conditions shows the lower efficiency of anaerobic compared to aerobic cell metabolism.

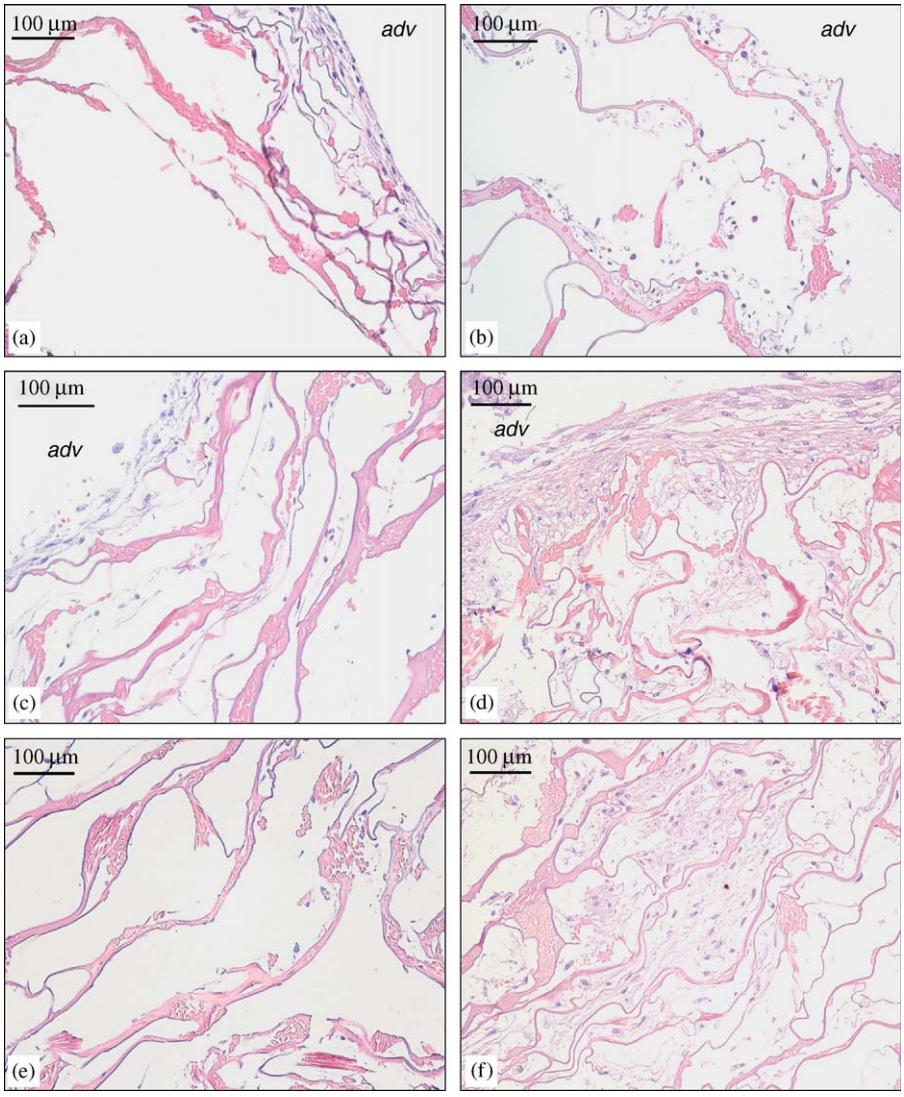

Fig. 4. Histology of SMC present in tubular scaffolds composed of collagen and elastin crosslinked with EDC/NHS after 7 d (a, b) or 14 d (c–f) of culturing under static (a, c, e) or dynamic conditions (b, d, f). Transverse sections of the constructs showing the inner and "adventitial" (adv) side (a–d) or only the inner side (e, f) were stained by the standard haematoxylin and eosin (HE) procedure.

For clinical applications, dynamically cultured constructs should be provided with a luminal endothelial lining. The culture period needed for graft production should be as short as possible [14]. Cell sources, culture medium components, cell seeding efficiency, cell culture conditions and bioreactor design are all elements that should be optimised to decrease culture and maturation periods.

5. Conclusions

Culturing of SMC in porous tubular scaffolds of EDC/NHS crosslinked insoluble type I collagen and insoluble elastin under dynamic conditions simulating the human carotid artery in terms of wall shear rate, pulsatile flow and arterial pressure, stimulates tissue formation. Higher SMC numbers, a more homogeneous distribution of SMC throughout the scaffolds and a higher collagen mRNA expression were found when cells were cultured under dynamic compared to static conditions. Under dynamic culture conditions, mass transport of nutrients and waste products to and from the cells present in the TE constructs was improved and cell metabolism was more aerobic compared to static conditions. Lining of the constructs with a luminal monolayer of endothelial cells might result in vessels suitable for in vivo applications. For clinical applications, however, optimisation of cell sources and culture conditions is required to decrease the time to produce a vascular graft using this procedure.

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H. Jahr and G.J.V.M. van Osch (Erasmus University, Medical Center, Department of Orthopaedics, Rotterdam,

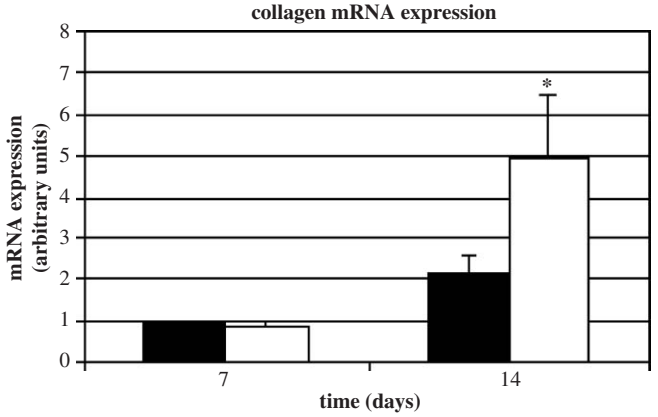

Fig. 5. Type I collagen mRNA expression levels of SMC present in tubular scaffolds composed of collagen and elastin crosslinked with EDC/NHS after 7 and 14 d of culturing under static (black bars) or dynamic conditions (white bars). mRNA expression levels of cells cultured for 7 d under static conditions were arbitrarily set at 1 unit. Relative mRNA expression levels were determined from three experiments performed in duplicate (±SEM). * Indicates a significant difference compared to 7 d of static and dynamic culturing and 14 d of static culturing ($p<0.05$).

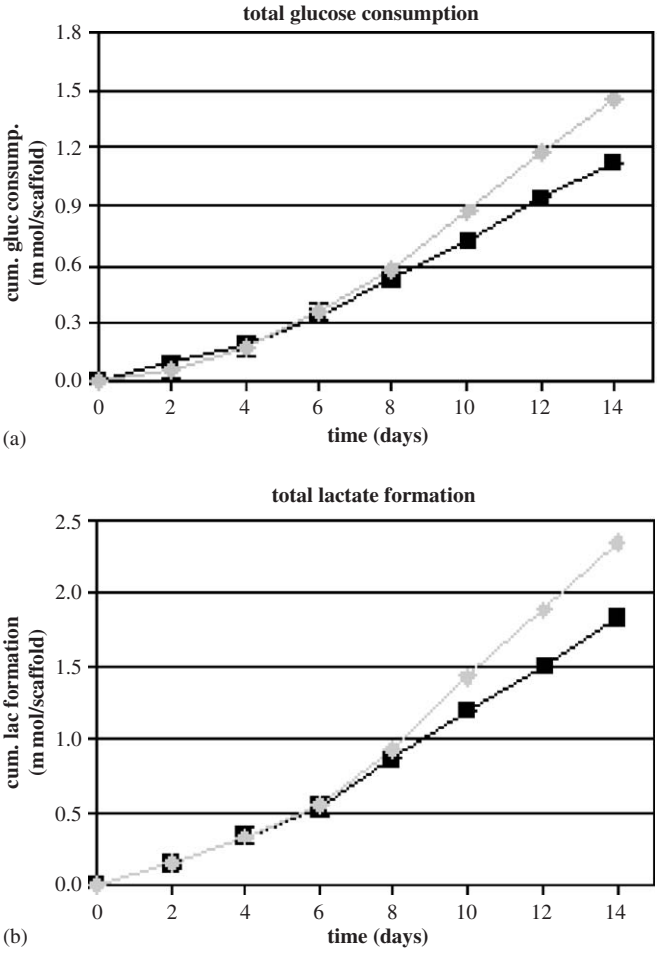

Fig. 6. Cumulative (cum) glucose consumption (a) and lactate formation (b) in case of dynamic (white line) and static (black line) culturing plotted as a function of time. For details see Section 3. Data represent four measurements (±SEM) during the first 6 d and a single measurement for days 8–14.

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