Maturation of capillary-like structures in a tube-like construct in perfusion and rotation culture

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Abstract.   In an effort to engineer capillary-like networks *in vitro*, different cultivation methods were compared. Five small-diameter tube-like constructs ('vessel equivalents') were fabricated from porcine gelatin scaffolds and seeded with human adipose tissue stromal cells and umbilical vein endothelial cells. After initial growth, the vessel equivalents were divided. One segment was exposed to pulsatile perfusion and the other was kept in rotating culture. Specimens harvested at the start of the experiments and after 16 days of rotation or perfusion were compared histomorphometrically with respect to capillary-like network formation in the vessel wall. Most capillary-like structures were found in the luminal portion of perfused vessel equivalents. Maturation of these capillary-like structures ascertained by recruitment of α-actin-positive cells also reached the highest degree in the luminal portion of perfused specimens. Perfused specimens showed significantly less apoptosis. Pulsatile perfusion promotes the development and maturation of a capillary-like network in this *in-vitro* approach.

In many instances of tissue engineering, vascularization may develop via the secondary ingrowth of blood vessels *in vivo*, but the engineering of metabolically active and voluminous tissues demands an immediately functioning vasculature. Recent literature has increasingly focused on the issue of nutrition and oxygenation of larger tissue equivalents in tissue engineering[1,3,6]. Supply by diffusion does not exceed 100–300 μm both *in vivo* and *in vitro*. As a consequence, there is an obvious need for a vascular network or alternative equivalent. The implementation of a capillary-like network in a stromal tissue model was recently presented in which the set up followed the principles of tissue engineering[3,4,5].

There is convincing evidence that mechanical stimuli such as strain and fluid shear stress are key factors in the regulation of vascular growth and remodelling[10,13]. Fluid shear stress has been shown to be an important regulator of vascular structure and function through its effect on the endothelial cell. There are also direct effects on smooth muscle cells: pulsatile pressure increases cell growth[15] and migration and cytokine expression[12]. It can therefore be assumed that these factors also play a role *in vitro* and that hydrodynamic forces improve the maturation of artificial microvessels.

The present study deals with the development of a capillary-like network in a tissue-engineered vessel equivalent *in vitro*. The hypothesis to be tested is that the directed perfusion in a perfusion culture system resembling the pressure changes in natural vessels is superior for the maturation of a vascular network than a non-directed flow, as it takes place in a rotating culture container. A perfusion system based on these considerations was established and compared with a commercially available rotating culture system.

Materials and methods

Cells

Adipose tissue stromal cells

Small pieces of adipose tissue (<0.5 cm^3) were collected from routine operations. Informed consent was obtained from and signed by all patients. The adipose tissue was minced with sterile scissors and subjected to collagenase digestion (collagenase type II, Boehringer, Mannheim, Germany). The suspension was filtrated over a 100-µm nylon mesh to remove tissue remnants, centrifuged and plated on tissue-culture flasks (Greiner, Frickenhausen, Germany). Cells were cultured at 37 °C in a 5% humidified CO$_2$ atmosphere in an incubator (Kendro, Hanau, Germany). The culture medium (Iscove's modified Dulbecco's medium (IMDM)/Ham F-12 1:1 with 10% newborn calf serum (NCS), all from Life Technologies, Paisley, UK) was changed weekly. The cells were passaged in a 1:4 ratio; 3rd passage cells were used in the experiments. In flow cytometry, $22 \pm 5\%$ of 3rd passage cells expressed α-actin, $83 \pm 19\%$ expressed CD90 (AS02), $44 \pm 35\%$ expressed CD105 and $92 \pm 22\%$ expressed SH3 (CD73); the latter are known to be positive in mesenchymal multilineage cells. Less than 0.5% ($0.33 \pm 0.23\%$, mean $\pm$ SD) expressed CD31. The stem-cell character also was proved by the ability to undergo adipogenic, osteogenic and smooth muscle differentiation[16].

Human umbilical vein endothelial cells (HUVEC)

Umbilical cords (Department of Gynaecology and Obstetrics, University of Leipzig) were clamped immediately and stored at 4 °C in buffered saline until further processing. The umbilical vein was rinsed and filled with collagenase 0.1% (collagenase type II, Boehringer, Mannheim,

Germany). A serum-supplemented medium was added (IMDM/Ham F-12 1:1 with 10% NCS) and the resulting cell suspension centrifuged (300 g, 10 min). The pellet was seeded on tissue-culture flasks and cultivated in the incubator with IMDM/Ham F-12 1:1 supplemented with 10% NCS, insulin 10 µg/ml, transferrin 4 µg/ml and hydrocortisone 1 µg/ml (endothelial cell culture medium). Passages 3–4 were used for the experiments. The purity of the HUVEC was checked through phase-contrast morphology, DiI-Ac-LDL uptake (uptake of acetylated low-density lipoprotein labeled with dioctadecyltetramethyl-indocarbo-cyaninperchlorate is typical for endothelial cells) and von Willebrand antigen staining.

Fabrication of the vessel equivalents

Commercially available, stiff porcine gelatin sponge material (Spongostan, Johnson & Johnson, Norderstedt, Germany) was carved into a tube and an inner lumen was excavated mechanically. The tubes had an outer diameter of 5–8 mm; the inner luminal diameter was 1–2 mm with a length of 50 mm. They were gas-sterilized and subsequently soaked in phosphate-buffered saline (PBS). After having been rinsed three times, the tubes were placed in a rotating culture module (In Vitro Systems and Services, Göttingen, Germany). The modules were filled with endothelial cell culture medium. Culture modules were placed on a roller unit (Heraeus Instruments, Osterode, Germany) and set on rotation at 10 rpm. Following an adjustment phase, these were each inoculated with a densely grown 75-cm^2 tissue-culture flask of adipose tissue stromal cells (ca. 10^7 cells) and HUVEC (ca. 10^6 cells). During a culture period of 3–4 weeks, inoculation was repeated three times at weekly intervals in order to reach high cell densities and generate matrix formation. Two days prior to the commencement of the experiments, the inner lumen was lined with endothelial cells again as follows: HUVEC (ca. 10^6 cells) were trypsinized and re-suspended in 0.5 ml fibrin solution 7.5 mg/ml (preparation described in FRERICH et al. 2001[3]). The suspension was added to the tubes and a silicon tube (diameter 1 mm) placed like a stylet into the lumen. The tubes were placed back in the culture module. By this procedure, the outer surface also was seeded with endothelial cells again. After 6 h, the silicon tube was removed. The following day, the tubes were ready for use in experiments.

Perfusion experiments

The prepared tubes were divided into three sections. Firstly, a 1-mm segment was cut off and harvested as an initial specimen ('start group'). The remaining tube was cut into two pieces, one measuring a third and the other two thirds of the length. The smaller part (a third of the length, ca. 1–1.5 cm) was placed back into the rotation culture container ('control group'). The serum-supplemented medium was removed and the container filled with a serum-free medium formulation (endothelial cell culture medium without serum, supplemented with bovine serum albumin 1%, and Dextran 40 (Sigma, Deisenhofen, Germany) 2% in order to adjust viscosity to near physiological levels. The reason for serum-free cultivation was that it is desirable from the viewpoint of clinical application. The longer part of the tube ('perfusion group', two thirds of the length, ca. 2.5 cm) was placed in a commercially available double perfusion chamber (gradient container, minucells and minutissue, Bad Abbach, Germany) which was modified for the experiments. This consisted of a 47-mm-diameter chamber with two pairs of outlets located opposite each other. The lower pair was used to lead small silicon tubes into the chamber, serving as connections to the artificial vessels. Both ends of the vessel equivalent were fixed with surgical sutures between these silicon tubes. The loss in length through suturing was the reason for the unequal length distribution between the perfused and the rotated specimens. Through this tubing, the vessel equivalent was perfused with a medium using a roller pump. The upper pair of outlets was used for pressure compensation and controlled medium drainage with a second roller pump. Fig. 1 is a diagram of the perfusion system. The experiments were conducted over a period of 16 days. During this time, the perfusion rate was raised from 100 to 500 µl/min, the pulse rate altered from 6 to 16/min and the pulse length reduced from 1 to 0.6 s in 8 steps (every 2 days). The idea was to start with lower shear forces and to increase them during the experiment to represent conditioning of the vessel wall. Finally, the specimens were harvested, fixed in formaldehyde 2%/paraformaldehyde 2% in PBS for 24 h and cut in 2-mm cross-sectional slices. Five experiments were performed in this way.

Immunohistochemical staining

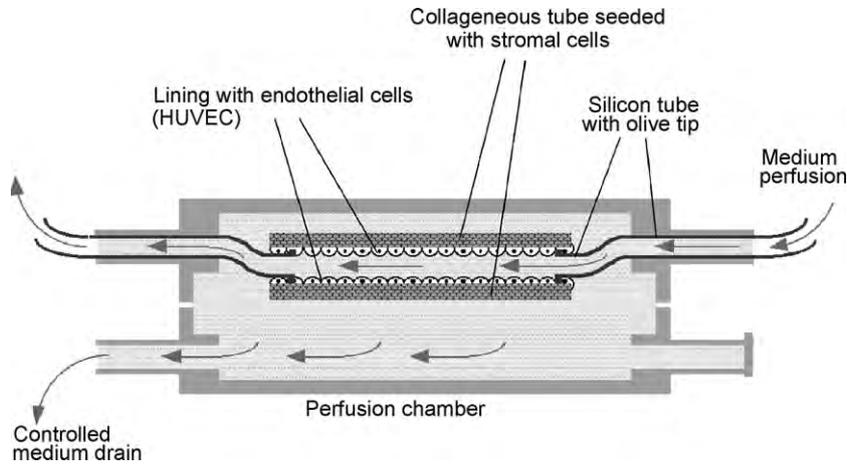The morphology of the lumen, the development of capillary-like structures and the

Fig. 1. Diagram of the perfusion chamber with the vessel equivalent.

recruitment of perivascular cells to these structures were evaluated on histological cross sections (2.5 μm) which were double-labelled with anti-CD31 and anti-α-actin. All reagents for the immunohistochemical staining were obtained from DAKO (Hamburg, Germany). After deparaffination and rehydration, endogene peroxidase activity was blocked (0.03% H_2O_2). The specimens were incubated with a mouse-anti-human CD31 antibody diluted 1:200 in PBS with 0.5% bovine serum albumin and subsequently with an alkaline phosphatase-labelled goat-anti-mouse-polymer conjugate (EnVision AP, DAKO). After having been covered with diluted mouse serum, the specimens were incubated with mouse-anti-human α-actin-EPOS/horseradish peroxidase (HRP) conjugate (HRP-labelled polymer conjugated with anti-α-actin). Visualization was performed with BCIP/NBT substrate for the alkaline phosphatase - labelled structures (CD31 positivity) and DAB solution for the HRP-conjugated α-actin-positive structures. Finally, the sections were counterstained with methyl green or nuclear fast red and embedded in DePeX (Serva, Heidelberg, Germany).

Confocal laser scanning microscopy

Specimens were additionally imaged by laser scanning microscopy. Fixed specimens were cut into 1-mm slices and labelled *en bloc* first with rhodamin-labelled UEA-I-lectin (Sigma-Aldrich GmbH, Steinheim, Germany). After rinsing they were additionally incubated with a monoclonal anti-α-actin antibody (DAKO, Hamburg, Germany) and subsequently labelled with fluorescein isothiocyanate-coupled goat anti-mouse Fab'2 fragment. The tissue blocks were embedded in gelatine and the label visua-

lized with a confocal laser scanning microscope (Leica TCS 4D, Leica, Germany).

Transmission electron microscopy

Specimens for transmission electron microscopy were fixed in glutaraldehyde 2.5% in buffered saline for 3 days and rinsed in Cacodylat buffer for a further 3 days. Thereafter they were fixed in osmium and embedded in Araldite. Ultrathin sections were observed in a transmission electron microscope and photographed.

Histomorphometric examination of capillary density and recruitment of mural cells

In order to evaluate the extent to which the deeper tissue layers were also involved in the development of a capillary-like network, it was necessary to view both the surface and the middle portion of the vessel wall separately. The density of capillary-like structures and the recruitment of α-actin-positive cells were therefore judged in three regions of the vessel wall: the inner luminal layer, the outer layer (towards the ''extra-luminal'' compartment) and the enclosed central portion of the wall (Fig. 2). Annular, oval or more irregular but lumen-containing sections of CD31-positive cell strains were defined as capillary-like structures (examples, Fig. 3), counted on eight defined positions of each of the three portions of the wall with the aid of a counting grid and the values summarized. Given the area of 0.4 mm × 0.4 mm of the counting grid, the results were expressed as numbers of capillary-like sections per 1.28 mm^2 (=8 × 0.16 mm^2). Eight histological sections from each specimen were evaluated

according to this method and then averaged ('capillary density').

The recruitment of perivascular, i.e. α-actin-positive mural cells, was evaluated semi-quantitatively at the same capillary-like structures on the eight histological sections of each vessel equivalent. According to the extent of the covering with α-actin-positive cells, four categories were defined for capillary-like structures: no covering, <50% covering, >50% covering and full covering with α-actin-positive cells. This allowed four values to be obtained for each of the three portions of the wall; these were then averaged for the whole group of five experiments and expressed as a percentage of the total number of vessels counted in the respective portions of the vessel equivalent.

Statistical analysis

A SPSS statistical software package was utilized for statistical analysis. For the problems of the number of capillary-like structures, pericytal recruitment and rate of apoptosis, all experimental groups were compared with each other using the Mann–Whitney U-test (2-tailed). The level of significance was set at $P < 0.05$.

Results

The perfused specimens ('perfusion group') were compared to the control specimens from the rotating cultures ('control group') and the specimens which had been harvested prior to the experiments ('start group'); each group contained five specimens except the start group ($n = 4$), in which one specimen could not be evaluated. Also in one control specimen the inner and the middle portion could not be evaluated, because the lumen had collapsed. The histomorphometric evaluation of cross sections of the vessel equivalents included the following parameters: (1) the morphology of the inner lumen, (2) the development of capillary-like structures in the wall of the vessel equivalents, and (3) the recruitment of α-actin-positive cells to these capillary-like structures.

Morphology of the inner lumen of the construct

In the specimens of the start group which represented the common starting point of the perfusion and rotation groups, the wall was hypocellular and appeared to be weak and instable. The lumen was shaped irregularly. There were only scattered endothelial cells, and a complete lining
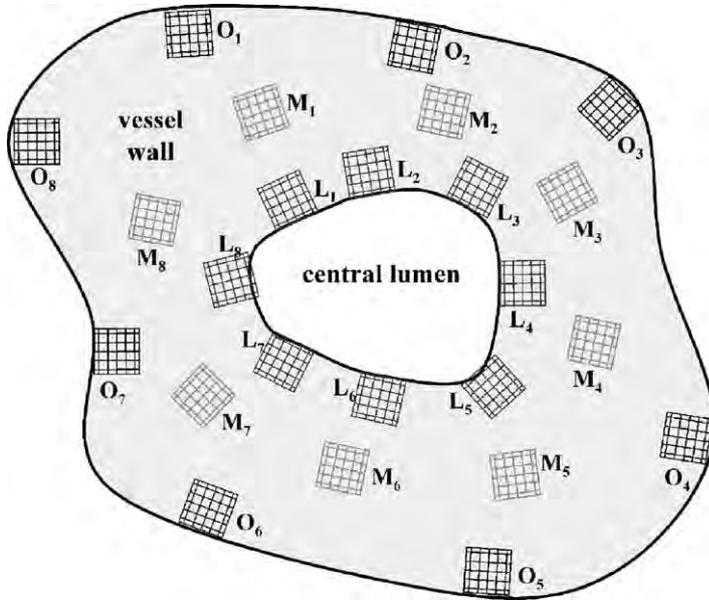
Fig. 2. Example of the positioning of the counting grids on a cross section of the vessel equivalent for measurement of the density of capillary-like structures and recruitment of mural cells in the inner luminal portion (L_{1-8}), the central wall portion (M_{1-8}) and the outer ''extra-luminal'' portion (O_{1-8}) of the vessel equivalent's wall.

with CD31-positive endothelial cells was still absent. An underlying cell layer and α-actin-positive cells were mostly absent. In the specimens of the control group which had been subjected to rotating culture, the lumen was also irregularly shaped. The originally tubular lumen appeared to have collapsed. A complete luminal monolayer arrangement of endothelial cells was occasionally observed. In one of the control specimens the lumen had completely collapsed, so could not be included in the histomorphometric evaluation. A regular, almost round cross-sectional shape of the lumen with a complete endothelial covering of the luminal surface could only be observed in the specimens of the perfusion group (Fig. 4).

Development of capillary-like structures in the tube wall

The densities of capillary-like structures are depicted in Fig. 5. Generally, most capillary-like structures were found near the luminal and the outer surface of the vessel equivalent wall. The highest capil-
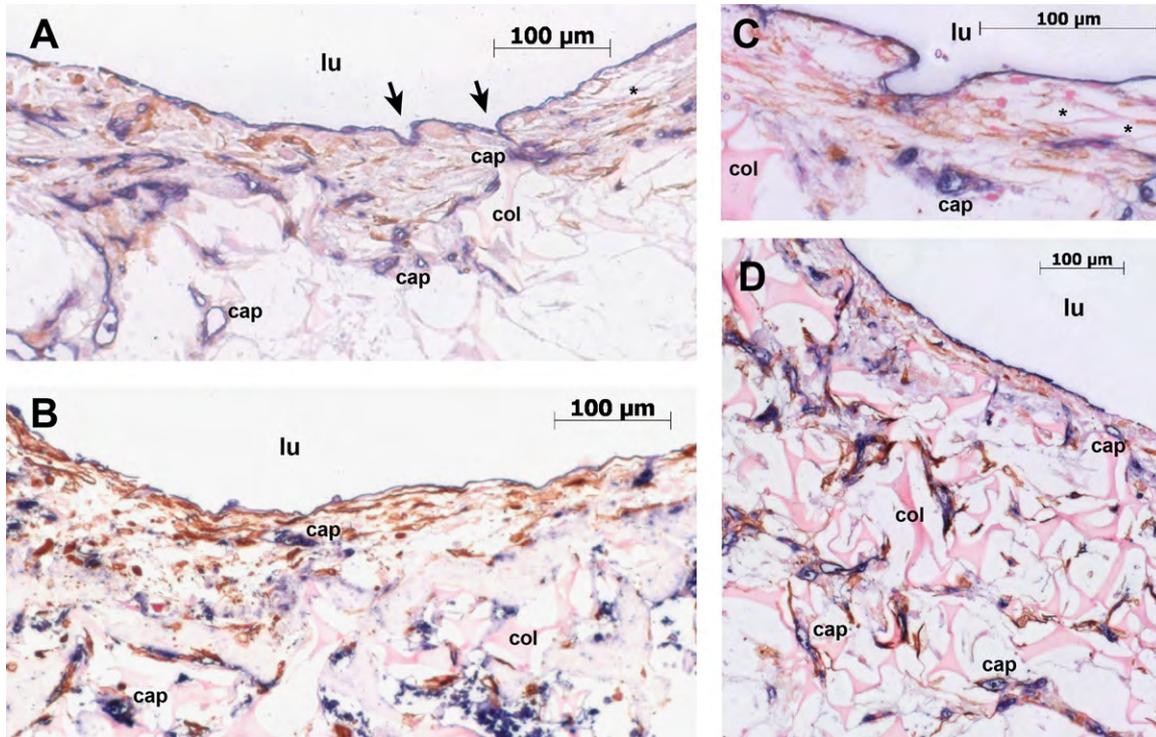

Fig. 3. Microgaphs of the inner luminal region of perfused and rotated specimens (double-labelling of CD31-positive cells with tetrazolium (blue), α-actin-positive cells with DAB (brown)). Arrows indicate branches emerging from the central lumen. (A) Perfused specimen, (B) control specimen. The control specimen also exhibits an endothelial lining of the central lumen, but not as smooth as the perfused one. Although there are a great number of α-actin-positive cells, these are rounded in the deeper layers in comparison to the perfused counterparts. (C) Detail of a perfused specimen with a branch emerging from the central lumen. (D) View to the deeper layers of the vessel wall demonstrating that almost all of the α-actin-positive cells are recruited to capillary-like structures. Note the filling of the capillaries with apoptotic cells in B (control), whereas the capillaries in A and D (perfused) are almost free. lu = central lumen of the vessel equivalent; col = collagenous scaffold; cap = capillary-like structure. Asteriks in (A) and (C) mark adipocytic differentiated cells.
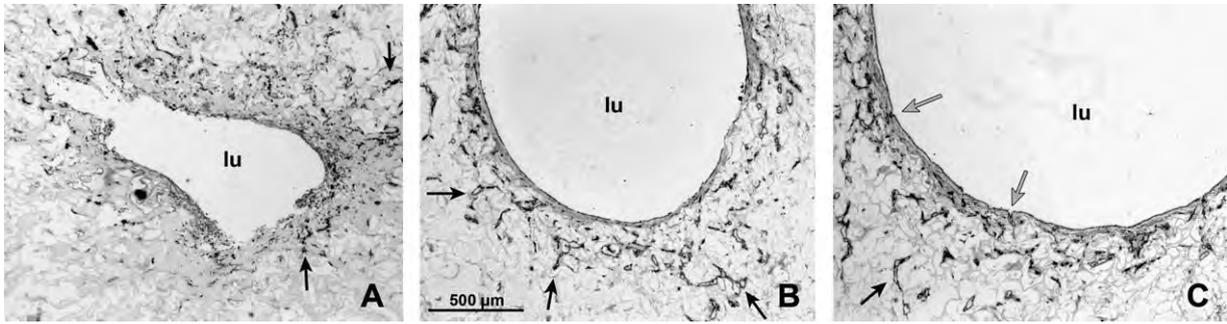
Fig. 4. Low-power magnification micrographs of specimens from rotation (A) and perfusion group (B, C); black arrows indicate capillary-like structures. (C) Shows details with 'entrances' emerging from the central lumen to the subendothelial capillary-like network (grey arrows). CD31-positive cells are labelled with tetrazolium, counterstaining with methyl green. lu = central lumen of the vessel.

lary density (mean: 167 per 1.28 mm^2) was found in the luminal region of the perfused constructs, whereas less than half of this number was counted in the luminal region of constructs of the start and rotation groups ($P < 0.05$ for perfusion vs control; see Fig. 5). There was almost no difference between the luminal and the extraluminal surface at the commencement of the experiments (start group, 50 and 58 respectively per 1.28 mm^2). After continued rotation (control group), the number of capillary-like structures only increased extraluminally. During perfusion the vascular density increased in both regions and was markedly higher on the luminal side than on the extraluminal side. In all three groups almost no capillary-like structures were found in the middle portion of the tube wall. Although the perfusion conditions had a positive effect on the formation of a capillary-like network, they were not yet able to induce a complete capillary branching system running from the inner lumen to the extraluminal region. High-power magnifications and transmission electron microscopic images (Figs. 6 and 7) revealed that capillary-like structures in places had complete endothelial boundaries and coverage with smooth muscle cells. In the older perfused and rotated tubes often sections with adipocytic differentiation were found by haematoxylin & eosin staining as shown in Fig. 7B; therefore these regions were empty in the double-labelled specimens stained only with nuclear fast red (Fig. 3C and D).

Recruitment of perivascular cells

Evaluation of the recruitment of α-actin-positive cells as a parameter of vessel maturation was carried out through classification of the capillary-like structures counted above into four categories: no, <50%, >50%, full covering of the section of a capillary-like lumen with α-actin-

positive cells. This allowed the degree of maturation to be expressed by the percentage distribution of these four categories. The results are included in Fig. 5 and are shown as percentage values of the respective mean number of capillary-like structures. Generally, it can be stated that the degree of maturation correlated positively with the number of capillary-like

structures. Consequently, the greatest extent of capillary-like structures covered with α-actin-positive cells was found in the luminal region of the perfused vessel equivalents (see also examples, Fig. 3). In this group, only 13% of the capillary-like structures remained completely uncovered and the differences as compared to the luminal region of the start and control
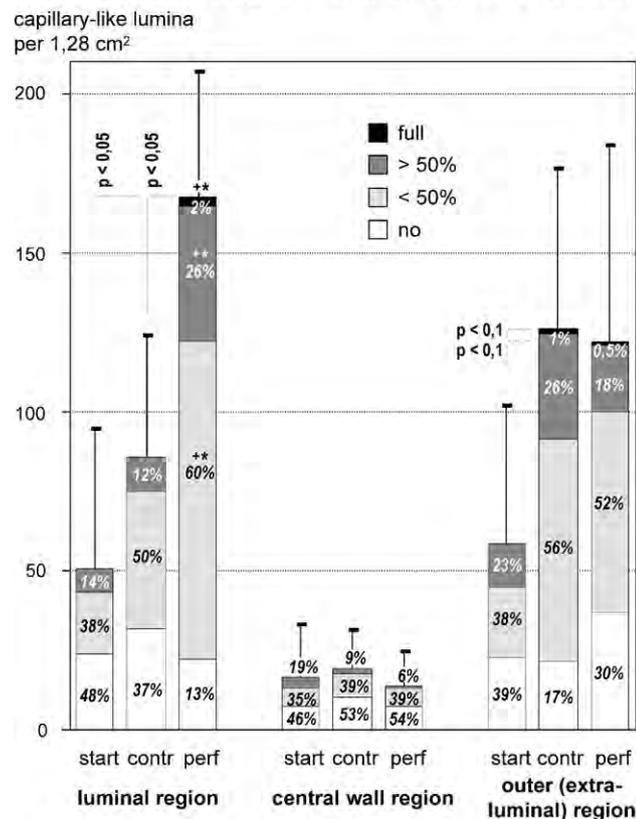

Fig. 5. Columns show the mean number of capillary-like sections in the luminal and central wall portion of the vessel equivalent's wall of the three experimental groups. The grey shaded areas represent the percentage portion of the different recruitment categories and the percentage values are given additionally. start = start group, contr = rotation group, perf = perfusion group, $N = 4$–5 at each point, *$P < 0.05$ vs control, +$P < 0.05$ vs start group concerning recruitment categories. *P*-values of differences in capillary density (represented by the height of the columns) are indicated in the diagram. Bars represent standard deviation of capillary densities.
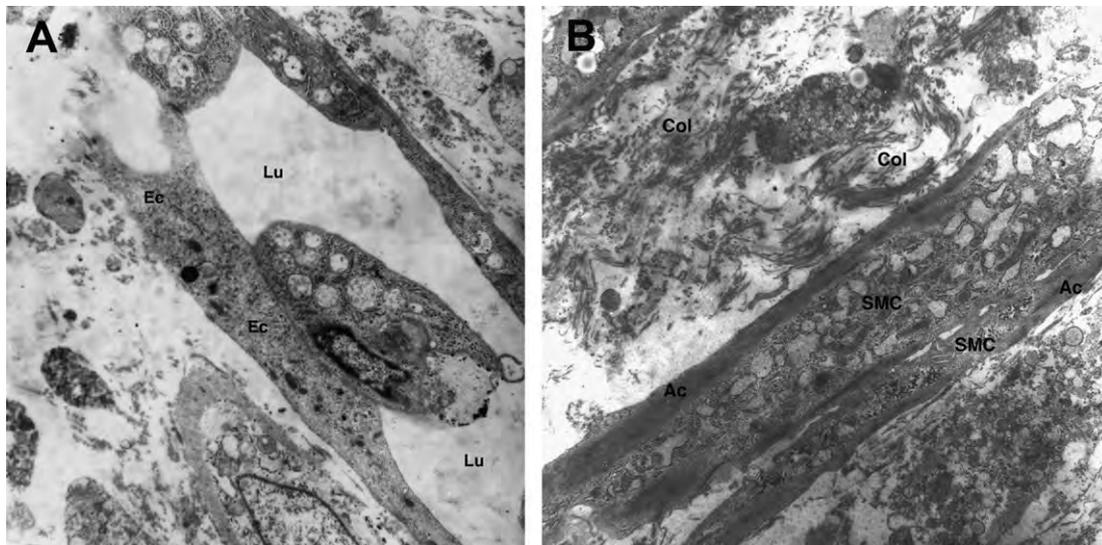
Fig. 6. Transmission electron micrographs of capillary-like structure (A) and cells with α-actin-positive border resembling smooth muscle cells (B). Magnification 7800× (A) and 6400× (B). Ec = endothelial cells, Lu = capillary-like lumina, Col = collagen, Ac = actin, SMC = smooth muscle cell.

specimens were significant ($P < 0.05$, Fig. 5). The maturation of capillary-like structures in the central region of the vessel equivalent wall was equally poor in all three experimental groups. In consideration of the fact that – compared to the start group – the specimens of the control group represent the continuation of rotating cultivation (whereas the perfusion group represents a change to perfusion culture), it can be stated that the maturation in the luminal region improved only under perfusion conditions.

Discussion

Adequate nutrition and oxygenation are an essential prerequisite for the engineering of larger tissue equivalents and form the basis for the concept of 'microvascular' engineering. Prior to the demonstration of a functional capacity, the main problems of microvascular tissue engineering are related to the stabilization of vascular structures. In static culture, newly formed capillary-like structures undergo rapid apoptotic decay, even when supplemented with vascular endothelial growth factor (FRERICH et al.[3] and unpublished data). While the early initiation of a vascular network does not require mural cells[14], its stabilization is dependent on the existence of perivascular mural cells, namely pericytes and smooth muscle cells. Immature capillaries are still in a state of plasticity and will only be arrested and stabilized in a mature state by the recruitment of perivascular cells[2]. Platelet-derived growth factor (PDGF)-receptor-B-positive pericytes are recruited from arterial walls or vascular plexus by PDGF-B, and these migrate along these PDGF-expressing endothelial sprouts[8,9]. Pericytes associated with newly formed vessels express vascular endothelial growth factor, perhaps providing one mechanism through which vessel stability could be accomplished[11]. Hence, the recruitment of perivascular α-actin-positive cells is a significant sign of vessel maturation and could serve as a suitable parameter for the present study.

The results show that perfusion conditions significantly improved the maturation of capillary-like structures in a tissue-like
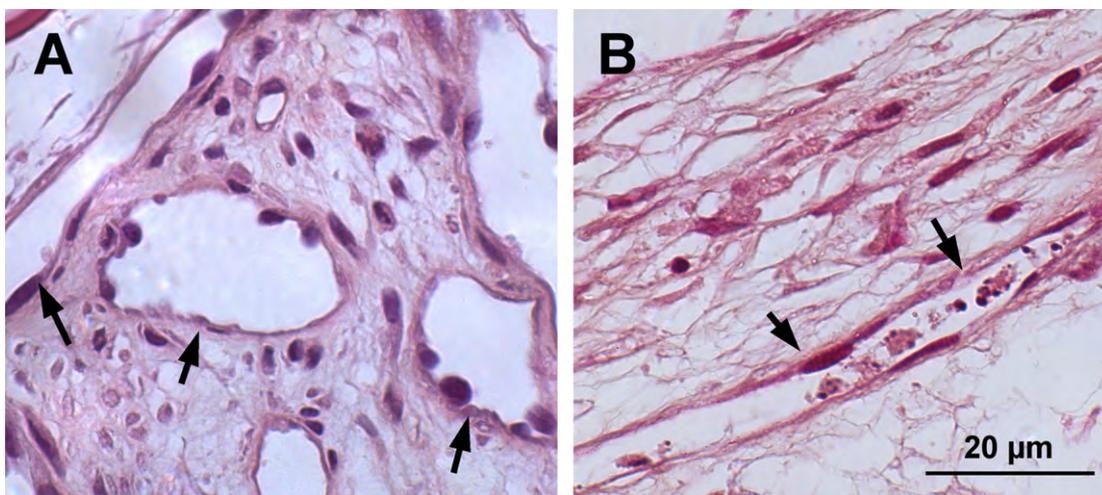

Fig. 7. High-power magnification of capillary-like lumina (arrows) in the start group (A) and longitudinal section of a capillary-like structure in a perfused specimen, surrounded by adipocytic differentiated cells (B). (Colour images are available in the electronic version under www.ijoms.com.)
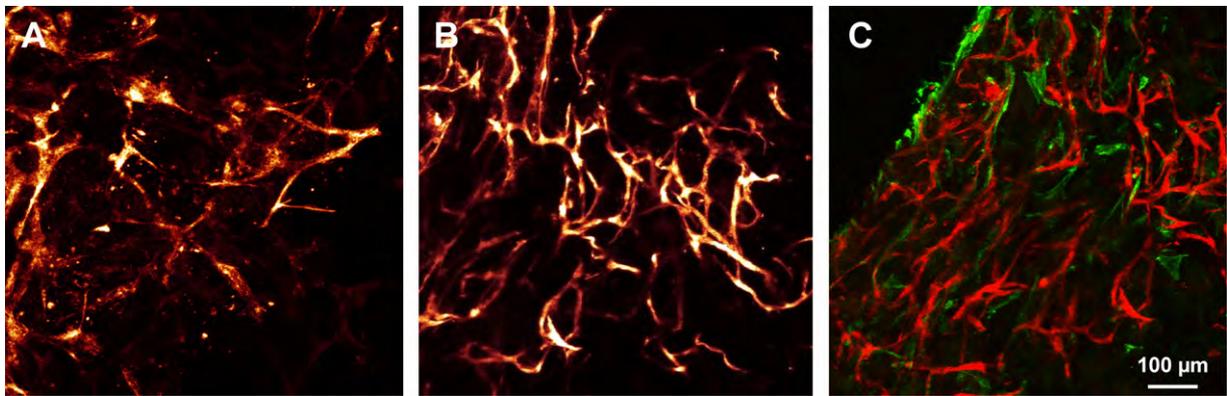
Fig. 8. Three-dimensional visualization of network formation by LSM. (A) Control specimen with shorter and interrupted capillary-like structures. (B and C) Perfused specimens with significant network formation, in (C) with simultaneous visualization of smooth muscle cells (green; capillary-like structures red). (A and B) UEA-TRITC, (C) UEA-TRITC (red) and α-actin (green). (Colour images are available in the electronic version under www.ijoms.com.)

compound, as reflected by the extent of mural cell recruitment. It must be stated that the central region of the vessel wall remained hypocellular in all experimental groups, including the perfusion group. The number of capillary-like structures and the degree of maturation remained equally low, indicating that the artificial microvasculature was not able to nourish this region by perfusion. Consequently, it has to be conceded that a real perfusion of the artificial microvessels is not demonstrable by these experiments. It has been demonstrated that perfusion conditions do improve the formation of connections or 'entrances' from the central lumen of the artificial vessel into the microvascular network of the tube wall[5], and thus it may be presumed that a limited perfusion of the artificial microvasculature is feasible by perfusion culture. Three-dimensional imaging of the capillary-like network by laser scanning microscopy (LSM, Fig. 8) also showed that the structure of the network is influenced by culture conditions and that pulsatile perfusion produces a more coherent and arborescent network, which has been proven by image analysis of LSM data sets[17].

The source of the mural cells was a population of stromal cells from adipose tissue which are known to have the potential of differentiating into various mesenchymal cell types[18]. It is also known that the contact between endothelial cells and mural cells promotes the expression of transforming growth factor-β1 which induces the differentiation of precursor cells to pericytes or smooth muscle cells[7]. Whether the α-actin-positive cells were derived from pre-existing pericytes in the stromal cell population or were the result of a differentiation process arising from undifferentiated mesenchymal stem cells can not be concluded from this study. The total amount of α-actin-positive mural

cells in the vessel wall did not meet the requirements of an applicable small-diameter vessel substitute. The results do indicate an association between the culture mode and microvessel maturation in the wall of this small-diameter vessel equivalent. Further experiments are required to demonstrate the degree to which these features can be utilized in the engineering of a fully functional vessel substitute.

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