Biomaterials 29 (2008) 4306–4313

Contents lists available at ScienceDirect

Biomaterials

journal homepage: www.elsevier.com/locate/biomaterials

Endothelial cell colonization and angiogenic potential of combined nano- and micro-fibrous scaffolds for bone tissue engineering

Marina I. Santos [a,b,c], Kadriye Tuzlakoglu [a,b,d], Sabine Fuchs [c], Manuela E. Gomes [a,b], Kirsten Peters [e], Ronald E. Unger [c], Erhan Piskin [d], Rui L. Reis [a,b], C. James Kirkpatrick [c,*]

[a] 3B's Research Group – Biomaterials, Biodegradables and Biomimetics, Department of Polymer Engineering, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
[b] IBB – Institute for Biotechnology and Bioengineering, PT Government Associated Laboratory, Braga, Portugal
[c] Institute of Pathology, Johannes Gutenberg University Mainz, Langenbeckstrasse 1, Mainz 55101, Germany
[d] Hacettepe University, Chemical Engineering Department and Bioengineering Division and TÜBITAK-Biyomedtek: Center for Biomedical Technologies, Beytepe, 06532 Ankara, Turkey
[e] Department of Cell Biology, Junior Research Group, Medical Faculty, University of Rostock, Schillingallee 69, 18057 Rostock, Germany

ARTICLE INFO

Article history:
Received 17 July 2008
Accepted 23 July 2008
Available online 15 August 2008

Keywords:
Starch-based scaffolds
Vascularization
Nano-fibers
Endothelial cells
Bone tissue engineering

ABSTRACT

Presently the majority of tissue engineering approaches aimed at regenerating bone relies only on post-implantation vascularization. Strategies that include seeding endothelial cells (ECs) on biomaterials and promoting their adhesion, migration and functionality might be a solution for the formation of vascularized bone. Nano/micro-fiber-combined scaffolds have an innovative structure, inspired by extracellular matrix (ECM) that combines a nano-network, aimed to promote cell adhesion, with a micro-fiber mesh that provides the mechanical support. In this work we addressed the influence of this nano-network on growth pattern, morphology, inflammatory expression profile, expression of structural proteins, homotypic interactions and angiogenic potential of human EC cultured on a scaffold made of a blend of starch and poly(caprolactone). The nano-network allowed cells to span between individual micro-fibers and influenced cell morphology. Furthermore, on nano-fibers as well as on micro-fibers ECs maintained the physiological expression pattern of the structural protein vimentin and PECAM-1 between adjacent cells. In addition, ECs growing on the nano/micro-fiber-combined scaffold were sensitive to pro-inflammatory stimulus. Under pro-angiogenic conditions *in vitro*, the ECM-like nano-network provided the structural and organizational stability for ECs' migration and organization into capillary-like structures. The architecture of nano/micro-fiber-combined scaffolds elicited and guided the 3D distribution of ECs without compromising the structural requirements for bone regeneration.

1. Introduction

To become widely used in clinical practice tissue engineering products must overcome a series of major challenges, the vascularization of the biomaterial constructs being one of the major current limitations [1–3]. To date, most approaches in tissue engineering have relied on post-implantation neovascularization from the host, but for large and metabolically demanding organs, which rely on blood vessel ingrowth, this is clearly insufficient to meet the implant's demand for oxygen and nutrients [4–6].

In vascularized tissues/organs such as bone a complex network of blood vessels is more than just simple conduits that provide nutrients and oxygen and simultaneously remove by-products. They also have important metabolic and rheological functions which are organ-specific [7–9]. In bone, the intraosseous circulation allows traffic of minerals between the blood and bone tissue, and transmits the blood cells produced within the bone marrow into the systemic circulation [9,10]. New blood vessels are intimately involved in osteogenesis (intramembranous and endochondral) and, furthermore, cytokines and growth factors that regulate intraosseous angiogenesis also regulate bone remodelling [7,9]. In addition, vascularization is also vital for the survival of the implanted cells on the carrier material after implantation [6].

Many approaches have been proposed to increase vascularization in bone such as gene and/or protein delivery of angiogenic growth factors [11,12], provision of a vascularized bone flap [13,14] and *ex vivo* culturing of scaffolds with ECs alone or in combination with other cell types [6,15]. Recently the work of Levenberg et al. on skeletal muscle showed that pre-vascularization of constructs improved *in vivo* performance of the tissue construct, shedding light into *ex vivo* use of ECs to accelerate vascularization [16]. Thus, the scaffold design must not only take into consideration the

* Corresponding author. Tel.: +49 6131 177301; fax: +49 6131 17477301.
E-mail address: kirkpatrick@ukmainz.de (C.J. Kirkpatrick).

0142-9612/$ – see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biomaterials.2008.07.033

structural and mechanical properties of bone but also ECs' adhesion, migration and blood vessel formation and ingrowth. In blood vessels ECs are attached as a monolayer to a basement membrane composed of protein fibers in the nanoscale, such as type IV collagen and laminin fibers, embedded in heparin sulfate proteoglycan hydrogels [17,18]. This natural extracellular matrix (ECM) provides structural and organizational stability to ECs and during angiogenesis EC migration is dependent on the adhesion to this matrix [19].

In this present work we evaluate the interaction of ECs with a scaffold made from a blend of starch with poly(caprolactone) (SPCL) with an innovative structure, inspired by the ECM, and combining polymeric micro- and nano-fibers in the same construct. This architecture was designed for bone regeneration to simultaneously provide mechanical support and to mimic the physical structure of ECM. We hypothesized that the presence of a nano-network might favour the adhesion of ECs and increase the density of cell colonization between micro-fibers, and might thus accelerate vascularization of the implanted scaffold. Previous work demonstrated favourable activity and differentiation of bone-like cells on this nano/micro-fiber-combined scaffold [20]. In this paper we addressed several important biological questions, such as whether this nano-network favours the growth pattern of ECs on the scaffold, cell morphology, inflammatory gene expression profile, expression of structural proteins and finally the angiogenic potential.

2. Materials and methods

2.1. Scaffolds

The scaffolds used in this study were based on a blend of starch with poly (caprolactone) (SPCL, 30/70 wt%). Nano/micro-fiber-combined scaffolds resulted from a two-step methodology. First by a fiber bonding methodology an SPCL fiber-mesh scaffold composed of micro-fibers (∅160 μm) with 70% porosity was obtained and second, by electrospinning the scaffold was impregnated with nano-fibers (∅400 nm). SPCL fiber-mesh scaffold without the nano-network was used as control. Further details concerning scaffold production have been published elsewhere [20–22]. Samples were cut into discs of 8 mm diameter and 2 mm height and sterilized by ethylene oxide. Prior to cell seeding scaffolds were soaked overnight in medium without serum.

2.2. Cells, culture conditions and scaffold seeding

Primary cultures of human ECs isolated from umbilical cord (human umbilical vein EC/HUVEC) and from human dermis (human dermal microvascular EC/HDMEC) were used. HUVECs were isolated from umbilical vein by collagenase digestion according to a published method [23]. HDMECs were obtained from enzymatic digestion of juvenile foreskin as previously described [24]. HUVECs were cultured in M199 medium (Sigma–Aldrich, Germany) supplemented with 20% fetal calf serum (FCS; Gibco, Germany), 100 U/100 μg/mL Pen/Strep (Sigma–Aldrich, Germany), 2 mM glutamax I (Life Technologies, Germany), 25 μg/mL sodium heparin (Sigma–Aldrich, Germany) and 25 μg/mL endothelial cell growth supplement (ECGS, BD Biosciences, USA). HDMECs were cultivated in Endothelial Basal Medium MV (PromoCell, Germany) supplemented with 15% FCS (Invitrogen, Germany), 100 U/100 μg/mL Pen/Strep (Sigma–Aldrich, Germany), 2.5 ng/mL basic fibroblast growth factor (bFGF; Sigma–Aldrich, Germany), 10 μg/mL sodium heparin and 100 U/100 μg/mL Pen/Strep. In order to promote better cell adhesion, ECs were seeded into culture flasks previously coated with gelatine. All assays were conducted with cells until passage 4.

Prior to cell seeding scaffolds were coated with a fibronectin solution (10 μg/mL PBS, Roche, Germany) for 1 h at 37 °C. Confluent HUVECs and HDMECs were trypsinized and a suspension of 2×10^5 cells was added to each scaffold. The scaffolds were incubated under standard culture conditions (37 °C, 5% CO_2, humidified atmosphere).

2.3. ECs' imaging

The viability, phenotype and growth of ECs on nano/micro-fiber-combined scaffolds and on SPCL fiber-mesh scaffolds were analyzed by scanning electron microscopy [25] and by confocal laser scanning microscopy (CLSM) after 3 and 7 days. For viability assessment, the EC-seeded scaffolds were incubated for 10 min in medium supplemented with 0.1 μM calcein-AM. Viable cells convert the non-fluorescence and membrane permeable calcein-AM due to the presence of active intracellular esterases into the green fluorescent and impermeable calcein. Viable cells are identifiable by the green fluorescent cytoplasm when viewed with CLSM (Leica TCSN NT). For SEM analysis the samples were fixed for 30 min with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, dehydrated in increasing concentrations of acetone, dried with hexamethyldisilazane and sputter coated with gold prior to SEM observation (Leica Cambridge S360).

2.4. Gene analysis of pro-inflammatory genes

The gene analysis of two pro-inflammatory cell adhesion molecules E-selectin and intercellular adhesion molecule (ICAM-1) was carried out by Real-time PCR. The mRNA expression of cell adhesion molecules as well as the housekeeping gene GAPDH was analyzed in HUVECs growing for 7 days on SPCL fiber-mesh scaffold and on nano/micro-fiber-combined scaffold. As control HUVECs were grown on cell-culture plastic. Furthermore, as positive control, gene expression was analyzed when the samples were cultured in the presence of 1.0 μg/mL of lipopolysaccharide (LPS) for 4 h (Sigma–Aldrich, Germany). Total mRNA from HUVEC cells was extracted using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's protocol. Afterwards, total RNA (0.5 μg) was reverse transcribed using Omniscript RT Kit (Qiagen, Germany). Gene amplification was performed using Applied Biosystems 7300 Real-Time PCR System (Applera Deutschland GmbH, Germany). The number of cycles and annealing temperature were selected according to the manufacturer's instructions. Real-time PCR was performed with 2.5 ng cDNA and 12.5 μL of 2× master mix, primers (0.25 μL forward and 0.25 μL reverse primer) in a final volume of 25 μL. The following gene-specific primer sets were used: (1) E-selectin, sense 5′-CCCGTGTTTGGCACTGTGT-3′, antisense 5′-GCCATTGAGCGTCCATCCT-3′; (2) ICAM-1, sense 5′-CGGCTGACGTGTGCAGTAAT-3′, antisense 5′-CACCTCGGTCCCTTCTGAGA-3′; (3) GAPDH, sense 5′-ATGGGGAAGGTGAAGGTCG-3′, antisense 5′-TAAAAG-CAGCCCTGGTGACC-3′. Gene expression was normalized to the expression of the housekeeping gene GAPDH. Relative quantification of gene expression was calculated in stimulated samples (+LPS) compared to samples cultured in the absence of pro-inflammatory stimulus (−LPS).

2.5. Immunocytochemistry

The expression pattern of the structural protein vimentin and of the platelet endothelial cell adhesion molecule (PECAM-1, CD31) was examined by immunocytochemistry. After 7 days in culture, EC-confluent SPCL scaffolds were fixed with a solution of 2% paraformaldehyde for 30 min at room temperature (RT). Samples were rinsed in PBS and then treated with PBS-buffered 0.1% Triton X-100 for 5 min at RT to permeabilize the cell membranes for the antibody reactions. The samples were incubated for 45 min at RT with the primary antibodies: mouse anti-human PECAM-1 (1:50, Dako, Denmark) or mouse anti-human vimentin (1:200, Sigma–Aldrich, Germany). Following PBS washing, a second incubation was performed for 45 min at RT with the secondary antibody anti-mouse Alexa Fluor 488 (Invitrogen, Germany). The nuclei were counterstained with 1 μg/mL Hoechst in PBS for 5 min. SPCL fiber meshes were then washed with PBS, mounted with Gel/Mount (Natutec, Germany) and visualized by CLSM.

2.6. Induction of *angiogenesis* in vitro

The angiogenic potential of HDMEC growing on SPCL fiber-mesh scaffolds was assessed by observing the cell migration from the scaffold into a collagen type I gel that mimics the *in vivo* microenvironment. When HDMECs reached confluence on the scaffolds the scaffolds were transferred to a Petri dish and covered with a 1.5 mg/mL solution of collagen type I in M199 medium containing 2% sodium bicarbonate, 0.05 M NaOH and 200 nM HEPES. As soon as the solution solidified into a gel, culture medium supplemented with angiogenic growth factors 50 ng/mL vascular endothelial growth factor (VEGF; Biomol, Germany) and 10 ng/mL bFGF was added. After an additional 7 days in culture, materials were examined for the migration of ECs and organization into capillary-like structures after calcein-AM live-staining and visualization by CLSM. All the above-referred reagents were from Sigma–Aldrich, Germany.

In order to have a better perception of the spatial distribution of capillary-like structures and micro-fibers the confocal images were post-processed using the image processing software ITK-SNAP [26]. Individual confocal image stacks from nano/micro-fiber-combined scaffold composed of 99 sections were examined. Capillary-like structures were identified and labelled in green and red, respectively, using the manual segmentation tool and the segmented elements were processed into a final 3D image.

Aimed at the evaluation of EC ultrastructure, transmission electron microscopy (TEM) of collagen gel ultrathin sections was performed. Scaffolds plus collagen gel were fixed in 2.5% glutaraldehyde in cacodylate buffer, post-fixed in 1% osmium tetroxide for 2 h and dehydrated in increasing ethanol concentration. Samples were embedded in agar resin 100 (PLANO, Germany) with ethanol as solvent for transition state and subjected to polymerization at 60 °C for 48 h. Ultrathin sections were cut, placed onto copper grids and examined by transmission electron microscope (Philips EM 410).