

In vitro methods to study the vascularization of natural and synthetic biomedical polymers

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Introduction

In the field of Regenerative Medicine and Tissue Engineering (TE) sophisticated strategies are being developed to combine a suitable, preferably biodegradable biomaterial system with relevant biomolecular cues with a view to promoting the growth and differentiation of autologous cells typical of the physiological and anatomical situation. In most cases success will also depend on the ability of the implanted system to rapidly become vascularized, especially in cases where a pre-seeding *ex vivo* has taken place.

Experimental

Due to our interest in vascularization, much of our experimentation involves establishing relevant assay systems using primary isolated endothelial cells (EC) from microvascular sources as well as endothelial progenitor cells (EPC) from human peripheral blood (Fig. 1). Use is also made of established human EC lines, such as the HPMEC-ST1 line developed in this laboratory [1]. Among the biopolymers of interest are the silk protein, fibroin, in combination with collagen type I and, in addition, chitosan- and starch-based

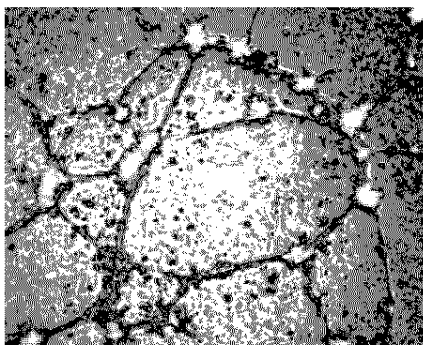


Figure 1. Human EPC in the form of mature outgrowth EC (mOEC) with the ability to form capillary-like vascular structures on Matrigel®. Cells are viable as shown by the positive green staining with the vital fluorochrome calcein-AM.

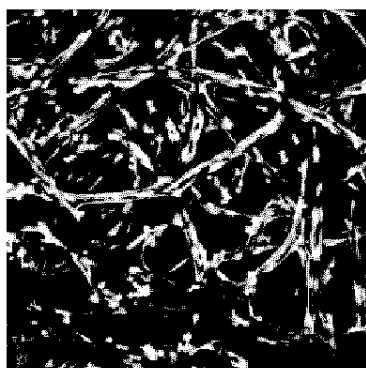


Figure 2. HUVEC growing on a silk fibroin microfibre mesh. Fluorescence microscopy showing viable cells staining positively with the vital fluorochrome calcein-AM.

scaffolds for bone TE. In the latter our focus of research concerns blends of starch with a synthetic polymer, poly(epsilon-caprolactone). In all of these polymers it has been observed that adhesion of EC to the biomaterial requires pre-adsorption of adhesion macromolecules, such as fibronectin.

Assays have been developed in which the colonization of polymer scaffolds is monitored by fluorescence microscopy, especially CLSM, using antibodies to essential gene products of the endothelial phenotype, such as cell adhesion molecules (CAMs) like E-selectin, ICAM-1 and VCAM-1 [2]. This is demonstrated in Fig. 2, in which human umbilical vein EC (HUVEC) are seen colonising a 3D-microfibre mesh of the purified silk protein fibroin, pre-coated with fibronectin. A further relevant variation of this type of three-dimensional assay is to seed the biomaterial, for example, the microfibre fibroin mesh, with EC and after about 2 days to overlay the culture with a hydrogel of collagen type I and culture for a further 5 days. This results in filling of the interstices of the mesh with a hydrogel, such as would be found in many tissues *in vivo*. Interestingly, the EC which were adherent to the fibroin surface in many cases left the biomaterial and began to invade and proliferate in the collagen, eventually forming capillary-like structures. Although this is an artificially created *in vitro* situation we regard it as an important form of assay, as it demonstrates whether adherent EC on a biomaterial have the ability to detach from the biomaterial surface in favour of the formation of vascular networks, this being the situation desirable *in vivo*. This raises many questions which at present do not have adequate answers, such as how firmly adherent should EC be to a biomaterial surface in order to be still able to detach and adopt the angiogenic phenotype. In addition, molecular biological methods such as RT-PCR enable gene expression at nucleic acid level to be studied and compared with that at protein level [3].

Using such experimental systems we hope to unravel the mechanisms of adhesion, proliferation and differentiation, including angiogenic functionality of various types of EC. With respect to EPC such knowledge could be used to enable polymer scaffolds to be modified in such a way that progenitor cells could home to the scaffold after implantation. The authors regard this approach as being one of the most attractive for biomaterial application. This goal could be achieved by chemical functionalization of the biomaterial surface using a variety of modern material science techniques to couple bioactive signal molecules to surfaces. In such a scenario pre-seeding of a biomaterial scaffold or matrix and/or an *ex vivo* culture period would not be necessary and might be more compatible with the usual surgical approach to implantation, where additional tedious laboratory steps are difficult to establish routinely and to be accepted by surgical staff. It is evident that such a surface functionalization strategy would depend on detailed knowledge of which molecules are essential to the recruitment of EPC from the peripheral blood in situations in which vascularization is required. It is as yet unknown if the cell adhesion molecule(s) (CAM) responsible are the same as those involved in various inflammatory processes or whether they are a completely new group of CAM. In order to investigate this it is necessary to be able to study EPC at various stages of maturity. To date our EPC studies indicate that the mature endothelial phenotype of so-called outgrowth endothelial cells (OEC), which can be isolated and cultivated from precursors in human peripheral blood, is much more stable in long-term culture than that of human EC, isolated from mature blood vessels, whether from the macro- or microvasculature [4]. This stability in culture, allowing cultivation for at least 3 months (longest period tested so far) and approximately 14 passages, is regarded as an essential criterion for meaningful experiments *in vitro* to study the molecular expression profile involved in EPC homing and recruitment.

Future Developments

A further strategy could be to use biodegradable polymer nanoparticles (NPs) to target the stem cell niche in the tissue where regeneration is required. Advances in stem cell biology have made it very clear that numerous adult tissues contain stem cells and that cell fate decisions are controlled within a so-called stem cell niche [5]. Currently we are using a co-culture model of the human air-blood (alveolo-capillary) barrier to study how inhaled engineered NPs interact with this important "portal of entry" into the systemic circulation [6]. Such strategies could be used for NP aerosol delivery of specific drugs or genes. Briefly, the barrier consists of an

alveolocyte (epithelial) monolayer in co-culture with a monolayer of human pulmonary microvascular EC (HPMEC), grown on both sides of a 0.4 μ m pore-sized poly(carbonate) filter membrane. The barrier has been established with the primary cell types in the human lung, but is equally successful with HPMEC combined with a pulmonary epithelial cell line, NCI-H441, having the phenotype of type II alveolocytes, this being the cell type responsible for regenerating the alveolar epithelial lining. After 10-12 days in co-culture trans-bilayer electrical resistance (TER) values of up to 600 ohm cm² can be achieved. Moreover, the barrier function, as monitored by TER values and the expression of important cell adhesion molecules in both epithelial and endothelial layers (ZO-1 and VE-cadherin respectively)(Fig. 3), can be reversibly modulated (e.g. using pro-inflammatory cytokines). Thus, a dynamic model is now available with which the mechanisms of uptake and transcellular transport of engineered polymer NPs with drug or gene cargoes can be studied. Preliminary experiments are currently being carried out. The cell biological data to be gained from such models are essential in developing a rational approach to drug and gene delivery via the lung. Thus, for example, if such a strategy is being conceived to treat pulmonary diseases, then the delivery system must be taken up by the cells in the barrier but not transported through the endothelial layer into the lung circulation. On the other hand, if the lung is being used as a portal of entry into the systemic circulation, the polymer drug- or gene-delivery system must traverse both the epithelial and endothelial layers without being sequestered, that is, a trans-bilayer transport must be achieved.

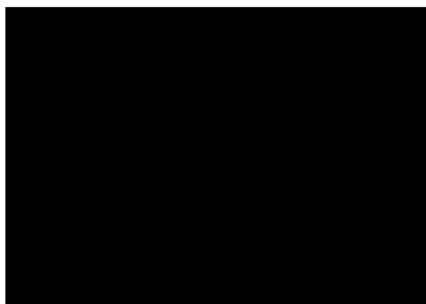


Figure 3. Intact monolayer of NCI-H441 pulmonary epithelial cells in the co-culture model of the air-blood barrier. Fluorescence microscopy shows the nuclei in blue. The barrier function depends on a physiological expression of the cell adhesion molecule ZO-1, shown as the red line outlining the individual cells.

A final caveat needs to be articulated, namely that all forms of *in vitro* experimentation represent model systems developed to dissect complex biological phenomena into systems of lesser complexity. The advantage of this is that parameters influencing these phenomena can be scientifically controlled in meaningful experiments. The disadvantage of this approach is that the extrapolatory jump from the *in vitro* experiment to the *in vivo* situation in the human organism remains precarious. This usually means that the mechanisms discovered from cell culture experiments must be tested in a (limited) number of animal experiments to ensure that the described phenomena are not artefacts of *in vitro* methodology. However, problems of extrapolation also apply to interpreting the significance of data gathered from animal experimentation.

Conclusions

In vitro methodology is regarded as an excellent possibility to test the suitability of biomaterials of all material classes for their support of endothelial cell (EC) growth and angiogenic potential. In this context it is important to use human EC and especially those cells involved in vascularization *in vivo*, namely EC of the microvasculature and endothelial progenitor cells (EPC). In addition, culture of EPC will hopefully help unravel the mechanisms by which circulating EPC are recruited to foci of vascularization. Knowledge of EPC homing, proliferation and differentiation towards the angiogenic phenotype could be applied to specifically functionalize biomaterial

scaffold surfaces to direct the vascularization process after implantation. Furthermore, *in vitro* techniques employing culture models of barrier systems as portals of entry into the body could assist in developing targeting systems using engineered nanoparticles to specifically target the stem cell niche in tissues where induction of regeneration is required.

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