Experimental approaches to study vascularization in tissue engineering and biomaterial applications

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The success of tissue engineering and biomaterial applications is not only dependent on the growth and functioning of the organ- or tissue-specific cells on the biomaterial but is entirely dependent in most cases on a successful vascularization after implantation. The process of vascularization involves angiogenesis; the formation of new blood vessels which spread into the implant material and supply the existing cells with the nutrients to survive. We have established in vitro methods using human microvascular endothelial cells to evaluate novel biomaterials for endothelial cell attachment, cytotoxicity, growth, angiogenesis and the effects on gene regulation. These in vitro studies can be used to rapidly evaluate the potential success of a new biomaterial and for the development of matrix scaffolds which will promote a physiological vascularization response.

How the microvasculature reacts to biomaterials plays a central role in the success or failure of any implant application. Central elements of this reaction are the processes of inflammation and tissue regeneration. Angiogenesis (formation of new blood vessels from the preexisting vasculature) involves marked alterations in endothelial cell (EC) function and is vital in tissue engineering, especially in those cases in which matrices are previously colonized by cells with aerobic metabolism. Animal studies of vascularization are difficult to interpret, as angiogenesis in non-human species shows a different reaction. This has been most clearly demonstrated in the study of vascular prosthesis endothelialization.

We have established in vitro assays using human microvascular EC from the lung (HPMEC) and skin (HDMEC), including a novel permanent EC line (HPMEC-ST1), developed in our laboratory, to investigate how biomaterials modulate EC function. This can be studied both at the level of diffusible factors and in direct contact with the biomaterial. Metal ion release (Co\(^{2+}\)) inhibits angiogenesis and induces EC apoptosis. Co\(^{2+}\) and Ni\(^{2+}\) also interfere with endothelial cell cycle kinetics and induce upregulation of the proinflammatory phenotype. Using fluorochrome labeling (the vital dye calcine-AM) of EC, coupled with confocal laser scanning microscopy (CLSM), we have studied the vascularization potential of novel biomaterials, both natural (silk protein fibrin) and synthetic (e.g. micro-porous polyethersulfone microfibers) in the form of three-dimensional matrices. In both examples, a rapid EC colonization occurred. The use of RT–PCR, immunocytochemistry and other techniques of cell and molecular biology enables further prediction of biomaterial-induced modulation of EC function. Of great importance is the ability to study these cell functions both at gene transcript level (mRNA) and gene product level (protein).

The novel permanent EC line, HPMEC-ST1 is proving of considerable value in these studies, as problems of biological variation of endothelial response from one human donor to another are thus avoided. This cloned cell line demonstrates the essential phenotypical characteristics of the endothelium, including the expression of vWF, CD34 and CD31. In addition, cell adhesion molecules (CAMs) relevant for inflammation, such as ICAM-1, VCAM-1 and E-selectin, can be upregulated by pro-inflammatory stimuli. Importantly, on relevant extracellular matrix components a marked angiogenic response is found, this being essential for its application to the study of implant vascularization.

Thus, such in vitro methods with cultured human EC can be applied to the development of matrix scaffolds which will promote a physiological vascularization response.

Cytotoxicity
The evaluation of the cytotoxic potential of novel biomaterials is essential to determine effects, which could affect vascularization. This analysis allows a differentiation between a general cytotoxicity or specific effects on cellular functions. The term cytotoxicity is not clearly defined and may be attributed to an impaired cellular function (e.g. reduced energy metabolism, decreased proliferation) or cell death (necrosis/apoptosis), respectively.
Due to the variety of this field we are working with different cytotoxicity/viability assays. The choice of assay should depend on the tested compound. For example, using the so-called MTS-assay (Promega; indirect detection of a mitochondrial dehydrogenase activity) we showed that the corrosion products Co\(^{3+}\) and Ni\(^{3+}\) (1 mM) led to a nearly identical decrease in dehydrogenase activity in HDMEC. We have however found that the long-term exposure of HDMEC with Co\(^{3+}\) led to apoptosis (shown by the apoptosis detecting TUNEL assay − Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; Roche) and the long-term exposure with Ni\(^{3+}\) resulted in cell death most likely due to necrosis [1]. Therefore, although MTS assay yielded nearly identical results, the actual cause of the cytotoxic effect of the two compounds differed dramatically.

A further aspect of cytotoxicity is an impaired proliferation. The treatment of human umbilical vein EC (HUVEC) with Co\(^{3+}\) and Ni\(^{3+}\) ions led to a nearly comparable concentration-dependent reduction of cell number. By flowcytometric analysis of cell cycle phases we could show that both ions led to an arrest in G1, but only the treatment with Co\(^{3+}\) ions led to an additional arrest in G2 phase (Fig. 1).

**Inflammation**

EC are participants in inflammation since they are able to secrete cytokines and to express CAMs on their surfaces in the presence of pro-inflammatory stimuli (e.g. by tumor necrosis factor α or interleukin-1β). These CAMs participate in leukocyte adhesion and transmigration towards the trigger of inflammation. HUVEC exhibit a pro-inflammatory phenotype by expressing E-selectin, ICAM-1 (intercellular adhesion molecule-1) and VCAM-1 (vascular cell adhesion molecule-1) when treated with Co\(^{3+}\) and Ni\(^{3+}\) [2]. Since the degree of CAM-expression is an indicator of pro-inflammatory stimulation we are using this characteristic for the testing of novel biomaterials.

**Analysis of the growth of endothelial cells on 3D biomaterials**

Many novel materials for implant are being examined for their suitability to support the growth of cells. For such an implant material to be successful, it must not only support the growth of the cells making up the organ or structure it is to replace in vivo, it must also support the growth of endothelial cells and develop an effectively functioning vasculature to supply the cells with oxygen. Thus, the visualization of endothelial cells growing on 3D matrices and the analysis of their gene regulation and biological functioning are essential in determining the suitability of a material for implant use.

We have developed a number of methods to analyze the growth and biological properties of endothelial cells added to both natural silk protein fibroin [3] as well as synthetic microporous polyethersulfone microfiber [4] 3D matrices in vitro. One of the principal problems in establishing an effective in vitro assay for 3D matrices is the visualization of the growing cells over a period of time. We have solved this problem by combining a fluorescent vital dye, calcine-AM, with CLSM to visual cells at various time points after adding cells to the matrices. This cell-permeant esterase substrate serves as