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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 applica-tion. 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 diffficult 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^{++}) inhibits angiogenesis and induces EC apoptosis. Co^{++} and Ni^{++} also interfere with endothelial cell cycle kinetics and induce upregulation of the proinflammatory phenotype. Using fluorochrome labeling (the vital dye calcein-AM) of EC, coupled with confocal laser scanning microscopy (CLSM), we have studied the vascularization potential of novel biomaterials, both natural (silk protein fibroin) and synthetic (e.g. microporous 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.

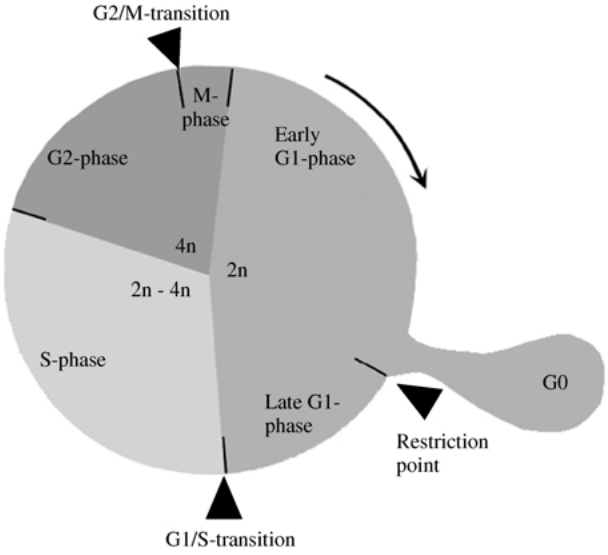

Figure 1 Schematic course of the cell cycle. G0 cells are not proliferating and thus outside the cell cycle. In G1 cells are preparing for DNA synthesis. During the S-phase DNA synthesis takes place (doubling of chromosomes). In G2 cell division is prepared. The cell cycle completes with the final M-phase (mitosis). Proliferation control checkpoints are in G1 and G2; in case of, for example, atypical cell environment or DNA replication errors it comes to a cell cycle arrest.

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^{++} and Ni^{++} (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^{++} 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^{++} 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^{++} and Ni^{++} 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^{++} 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^{++} and Ni^{++} [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, calcein-AM, with CLSM to visual cells at various time points after adding cells to the matrices. This cell-permeant esterase substrate serves as

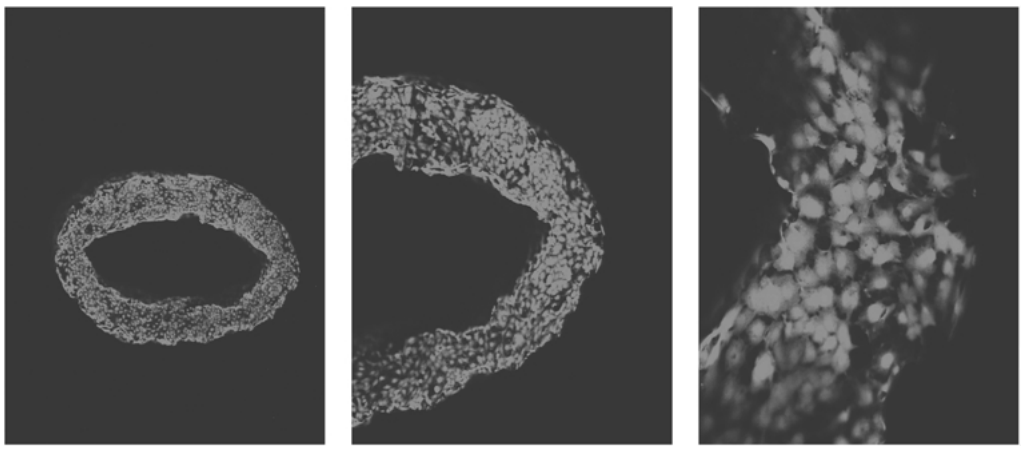

Figure 2 HUVEC growing on microporous polyethersulfone microfiber as observed by confocal microscopy after staining with calcein-AM.

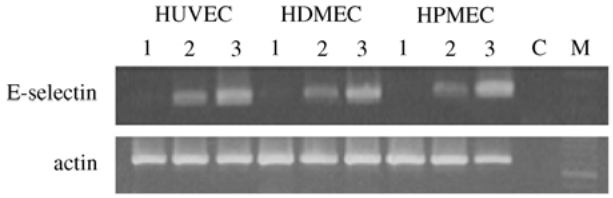

Figure 3 PCR results of cDNA isolated from HUVEC, HDMEC and HPMEC growing on silk protein fibroin nets (1) no LPS and (2) with LPS and on normal cell culture plastic in the presence of LPS.

a marker for the viability of the cells; enzyme activity is required to activate fluorescence and cell-membrane integrity is required for the intracellular retention of the fluorescent product (Molecular Probes). In addition, this compound does not exhibit a significant toxic effect until several hours after addition to the cells, thus providing a significant window of time to examine the cells in the CLSM (Fig. 2).

Analysis of endothelial cell gene regulation on 3D biomaterials

An understanding of the regulation of EC phenotype and function of cells growing on 3D matrices is important in the evaluation of a matrix for implant. A biomaterial-induced modulation of endothelial cell functions is generally not desired and cells should maintain normal phenotypes when growing on the matrices. However, endothelial cells should also retain the ability to react to stimuli as they do under normal conditions *in vivo*, for example, by exhibiting the induction of adhesion molecule expression when confronted with pro-inflammatory stimuli.

We are utilizing two approaches to analyze EC phenotypes growing on a biomaterial matrix. One method involves the use of reverse-transcriptase, polymerase chain reaction to examine the expression of specific genes at the mRNA level. RNA can be isolated from cells growing on a matrix, transcribed to cDNA and analyzed by PCR with gene-specific primers (Fig. 3).

In this way, a semi-quantitative analysis of the level of expression of many different genes can be rapidly carried out. The other method uses specific antibodies to analyze protein expression at the single cell level via immuno-fluorescence. Cells grown on biomaterials are fixed and permeabilized as needed, incubated with specific primary antibodies to proteins of interest and bound antibodies are detected with the aid of secondary antibodies labeled with a fluorescent marker. Furthermore, nuclear stains help to identify the location of all the cells and the biomaterials are either examined with a fluorescent microscope or confocal microscope (Fig. 4).

In this way, an entire population of cells can be examined for the expression or induction of a specific protein at the single cell level. By using multiple fluorescent antibodies it should be possible to examine the expression patterns of at least three different protein simultaneously.

In vitro models of angiogenesis

EC are the major cell type of blood vessel formation (called angiogenesis). During angiogenesis a directed migration of EC occurs and for the elongation of the developing blood vessel EC proliferation is necessary. The stabilization of the newly formed vessel is achieved by the recruitment of further cell types (e.g. smooth muscle cells, pericytes, fibroblasts) and the production of extracellular matrix (ECM) compounds. These different steps of angiogenesis are induced or influenced by soluble factors such as cytokines and growth factors. These combined factors work together in a highly complex regulated fashion [5].

Studies on angiogenesis *in vivo* are hindered by this complexity. To overcome these difficulties a number of different models of angiogenesis *in vitro* have been developed [6–8]. The *in vitro* model systems use ECM compounds to generate tissue-like conditions. Furthermore additional angiogenic stimuli (e.g. basic fibroblast growth factor – bFGF, vascular endothelial cell growth factor – VEGF) are necessary.

Successful wound healing after the insertion of an implant is dependent on an adequate vascularization of the developing granulation tissue. We have developed *in vitro* model systems to evaluate the effects of novel biomaterials or their extraction and corrosion products on angiogenesis. Since different *in vitro* models may reflect varying aspects of the *in vivo* process we utilize two different *in vitro* models of angiogenesis.

The first *in vitro* angiogenesis model is a 2D-system originally described by Montesano *et al*. [9]. In this model system a confluent HDMEC monolayer is covered by a gel of type I collagen and in a few hours the EC migrate establishing aggregates from which tube-like structures (TLS) evolve. The establishing TLS formed are mainly located in one plane and therefore the system is termed 2D. The induction of angiogenesis-like morphogenesis is thought to be a result of a changed EC polarity.

Due to the 2D character of this *in vitro* model it is easily quantified and we have established a method of computer-assisted evaluation of the angiogenic-degree *in vitro*. Using this method, we could show that Co^{++} inhibits angiogenesis *in vitro* in concentrations lower than detected in peri-implant tissues of CoCr alloys [10].

The second *in vitro* model of angiogenesis is a 3D-system and reflects the invasive aspect of angiogenesis *in vivo*. Here, detached HDMEC are suspended in a gel of type I collagen and fibrin (resembling the ECM of the developing granulation tissue). Through the addition of pro-angiogenic factors (bFGF/VEGF) a three-dimensional network of capillary-like structures is established within several days (Fig. 5). Computer assisted quantification of this 3D-system is more difficult in comparison to the 2D-system and we are currently developing methods for the determination of a reliable quantification.

Endothelial cell lines and primary cells

The use of primary cells in biomaterial research has certain limitations. Primary endothelial cells not only have a limited lifespan and reduced phenotype expression with each passage *in vitro*, but contaminating cells of other types may also be present and the quantities required for extensive analysis of multiple samples may

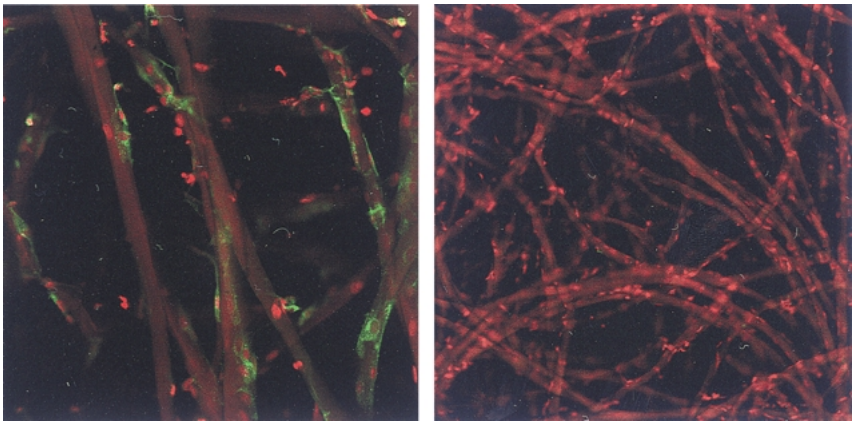

Figure 4 Expression of E-selectin in endothelial cells growing on fibroin nets in the presence (left) and in the absence of LPS (right). Cell nuclei were stained with propidium iodide (red) and E-selectin was detected by immunofluorescence (green).

TABLE I Comparison of EC phenotypes of the EC line HPMEC-ST1 to primary HDMEC, HPMEC and HUVEC in culture

Endothelial cell type	Constitutive expressed markers							Adhesion molecule induction by LPS/ TNFα/ IL-1β		
	vWF	CD31	CD34	DiI-Ac-LDL uptake	Cord formation on Matrigel	Flt-1	KDR	VCAM	ICAM	E-Selectin
HDMEC	+	+	+	+	+	+	+	+/+/+	+/+/+	+/+/+
HPMEC	+	+	+	+	+	+	+	+/+/+	+/+/+	+/+/+
HUVEC	+	+	+	+	+	+	+	+/+/+	+/+/+	+/+/+
HPMEC-ST1	+	+	+	+	+	+	+	+/+/+	+/+/+	+/+/+

Cells were cultured and identification of different phenotypes is described in Unger *et al*. [11] and Krump-Konvalinkova *et al*. [12]. VWF, CD31, CD34 were detected by immunocytology, Flt-1 and KDR by RT/PCR; induction of VCAM, ICAM and E-Selectin by LPS, TNFα or IL-1β, LPS/ TNFα/IL-1β, respectively as detected by immunoassay, + is positive induction.

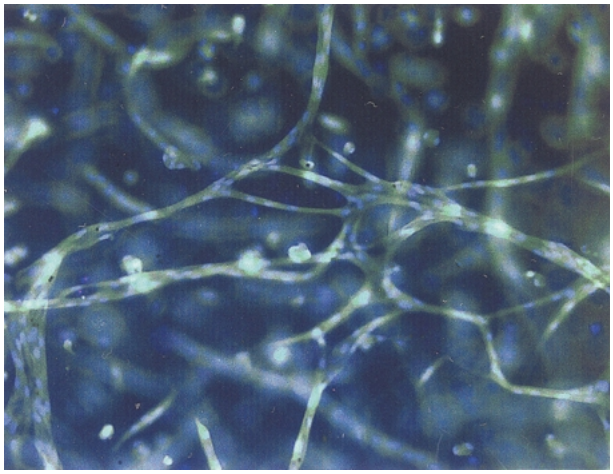

Figure 5 Capillary-like network of type I collagen/fibrin embedded HDMEC under the influence of pro-angiogenic factors. Incubation time 6 d; fluorescence microscopic image; digital overlay of a vital staining with Calcein-AM (Molecular Probes) and a nuclear staining with Hoechst 33342 (Sigma).

not be available. Furthermore, endothelial cells of different tissue origins are highly heterogeneous and microvascular endothelial cells are the relevant cells involved in vascularization and angiogenesis. We have described conditions for the isolation and culture of microvascular endothelial cells of the skin (HDMEC, human dermal microvascular endothelial cell) and the lung (HPMEC, human pulmonary), however, the isolation is time and labor intensive and dependent on donors. Thus use of these cells should be reserved for the final testing of materials and cell lines should be used for the initial experiments and screening.

A number of human EC lines have been described, however, extensive analysis of specific endothelial defining cell traits in the different cell lines compared to primary cells in culture had not been examined until recently. Few of the human endothelial cell lines exhibited significant numbers of primary endothelial cell phenotypes [11]. We have generated a permanent endothelial cell line derived from HPMEC, HPMEC-ST1, that is very similar to primary HPMEC and HDMEC in culture [12]. HPMEC-ST1 is proving of considerable value as the problems of biological variation observed with primary endothelials are avoided. This cloned cell line demonstrates the essential phenotypical characteristics of the endothelium (vWF, CD34 and CD31) and cell adhesion molecule expression relevant during inflammation (ICAM-1, VCAM-1 and E-selectin). In addition, these cells exhibit an angiogenic response when exposed to specific extracellular matrix components. This is essential for using these cells to study implant vascularization (Table I, summary of phenotypic traits of HPMEC-ST.1, see also Unger *et al*. [11] and Krump-Konvalinkova *et al*. [12]).

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