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# Endothelial cell cultures as a tool in biomaterial research

C. J. KIRKPATRICK, M. OTTO, T. VAN KOOTEN, V. KRUMP, J. KRIEGSMANN, F. BITTINGER
*Institute of Pathology, Johannes Gutenberg University, Mainz, Germany*
*E-mail: kirkpatrick@pathologie.klinik.uni-mainz.de*

Progress in biocompatibility and tissue engineering would today be inconceivable without the aid of *in vitro* techniques. Endothelial cell cultures represent a valuable tool not just in haemocompatibility testing, but also in the concept of designing hybrid organs. In the past endothelial cells (EC) have frequently been used in cytotoxicity testing of materials, especially polymers, used in blood-contacting implants, as well as for investigating seeding technologies for vascular prostheses. At present the exponential development both in theory and practice of cell and molecular biology of the endothelium offers great promise in the biomaterial field. Up until now this EC research field has mostly been non-biomaterial orientated. Nevertheless, the relevance for biomaterial research is apparent. Four aspects will be concisely reviewed under the headings inflammation, with special reference to cell adhesion molecules (CAMs) and cytokines, angiogenesis, focusing on the healing response, signal transduction, presenting examples from cytokine- and metal ion-induced up-regulation of genes coding for CAMs, and, finally, endothelial functionality, with emphasis on the principal characteristics of the physiological endothelial phenotype. Finally, the application of these fields to three foci of biomaterial research will be discussed, emphasizing the role of EC culture techniques in controlling the host response to biomaterials (*microvascular* EC), controlling EC functionality ( promoting positive effects and down-regulating negative effects), and tissue engineering (integration of EC into hybrid organs/biosensors). The need for more co-culture and three-dimensional models will be stressed and data from the authors' laboratory presented to illustrate these principles.
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## 1. Introduction

The topics of hybrid organs and tissue engineering are very much in vogue in the vocabulary of modern biomaterials research and express the hope of being able to replace as fully as possible lost organ function and construct functional tissue structures by combining living cells and a tissue-like matrix. Whether or not this can be achieved for the numerous clinical situations in need of such replacement depends in large measure not only on further developments on the material side of biomaterials research but also on increasing our understanding of how cell function is controlled by biomaterials. This is a major challenge for the biomedical sciences, and in particular for cell and molecular biology. The ubiquity of the vascular system in the living organism and the central role of the cells lining the vessels (endothelial cells, EC) in the processes of inflammation and the healing response make endothelial cell pathobiology an important focus of biomaterial research. Since the original publication in 1973 on the technique of cultivating human endothelial cells *in vitro* [1] the application of tissue culture methods to various types of EC has transformed our view of the endothelium from that of a passive barrier between the blood and the vessel wall to that of a highly dynamic tissue with pathogenetic relevance for regulating haemostasis, vascular tone, angiogenesis and the inflammatory response, to name but a few of these functions [2]. The aim of the present paper is to review how endothelial cell culture systems can be used as a tool in biomaterials research. As well as a retrospective view of the developments until now, fields of endeavor for the present and the future will be discussed and in doing so, examples from the authors' own research activity will be presented.

## 2. Human ECs *in vitro*

As a result of the availability of umbilical cords, Jaffe's original model has remained the most widely used human EC type in endothelial research. However, it should be borne in mind that human umbilical vein EC (HUVEC) represent EC of embryonic origin and of *macrovascular* nature. A further source of human EC comes from the saphenous vein portions remaining after coronary bypass surgery. These human adult saphenous

vein EC (HASVEC) cultures have been employed to study the adhesion and spreading of EC on modified synthetic polymers [3]. The human femoral artery can also be employed as a source of arterial EC [4]. As a result of the intensive work-load involved in isolation and primary culture of these cells, various groups have attempted to develop permanent cell lines of human EC. An example of this approach is the EA hy926 cell line, derived by fusion of HUVEC with the human epithelial cell line A549 [5]. Human bone marrow EC (HBMEC) were used to establish a cloned cell line by microinjection of a recombinant plasmid expressing simian virus 40 early genes under the control of a deletion mutant of the human vimentin promoter [6]. The important caveat in the application of transformed cell lines is the requirement that the phenotypic characteristics of the endothelium are preserved, especially with respect to those functional parameters being studied.

Of greater relevance to biomaterials is the *microvascular* endothelium, as this is a central element of the tissue response to implantation. The requirement to employ such microvascular endothelial cells (MEC) will be discussed below.

## 3. EC in biomaterial research: an historical view

Up until now two principal fields of application of EC culture to biomaterials have been established, namely those of cytotoxicity testing and seeding technologies. Both fields have contributed to our understanding of EC interactions with biomaterials, but have generally tended to focus on the relatively simple question of whether or not EC will adhere and grow on a given biomaterial surface.

### 3.1. Cytotoxicity testing

Whereas most cytotoxicity testing methods employ permanent cell lines, such as HeLa cells, derived from a human cervical carcinoma [7], human EC also provide a useful cell type to study possible negative effects of biomaterials on cellular metabolism [8]. Furthermore, this cell type is biologically relevant, as toxic components leaching out of implanted biomaterials can be absorbed and transported by the cardiovascular system and thus can come into direct contact with the endothelium.

### 3.2. Seeding technologies

One method to improve the biofunctionality of vascular prostheses is to pre-seed them with the patient's own EC, isolated from a vein. Due to the fact that the vascular prosthetic materials, such as expanded polytetrafluoroethylene (ePTFE) or polyurethanes (PUR), are relatively inert (hydrophobic surface), this goal can only be achieved if some form of surface modification step is included. This topic has been reviewed elsewhere [9, 10]. Briefly, three principal techniques have been employed to promote cell adhesion. Firstly, there is the simple adsorption of adhesion-promoting molecules on to the luminal surface of the prosthesis. Examples are molecules such as fibronectin, which contain the tripeptide cell binding domain, RGD [11], as well as other proteins, such as collagen IV and laminin. Secondly, functional or reactive groups can be created on the luminal surface and include hydroxyl, carbonyl and carboxylic groups [12]. Among the technologies available to achieve this are plasma polymerization and radiation-induced grafting [13]. Thirdly, bioactive or ''signal'' molecules can be covalently coupled via spacer molecules on to the implant lumen. Cell-binding oligopeptides, recognized by integrins in the plasma membrane of the EC, are excellent candidates for such signal molecules [14, 15].

An alternative approach to the problem of colonization of the lumen of vascular prostheses with EC is to pose the question of why porous prostheses do not endothelialize to any degree *in vivo* in the human. Normally, ingrowth *per continuitatem* at the anastomotic sites occurs up to a maximum of 10–15 mm. This is in marked contrast to other mammalian species, including even the related primate, the baboon, in which complete endothelialization is reached within the period of a few weeks after implantation. This raises the issue of the vascular response to the prosthetic material, one hypothesis being that in the human the ingrowth of capillary blood vessels from the adventitial side (outer part) of the prosthesis is refractory. This response of capillary ingrowth is also termed ''angiogenesis'' and will be discussed below.

## 4. Endothelial research: the *status quo*

Massive developments have taken place in the cell and molecular biology of EC in the course of the past two decades. However, it must be stressed that the vast majority of this research has concerned non-biomaterial questions, although, as we shall see, a considerable body of data gathered from other fields is highly relevant to biomaterial applications. In this section the following spheres of research activity will be emphasized: inflammation, angiogenesis, signal transduction and the concept of endothelial functionality.

### 4.1. Inflammation

All implanted biomaterials will evoke some form of inflammatory response, the degree of which will depend on the type of biomaterial, the implantation site and the extent of tissue damage caused by the surgical procedure. This universal biological response is a very strong argument for advancing inflammation research within the sphere of biomaterials.

#### *4.1.1. Understanding the cytokine ''orchestra''*

The past 15 years have witnessed explosive growth in the cytokine field. The cytokines are an extremely complex family of low-molecular-weight proteins, produced by numerous cell types and responsible for regulating the immune response, inflammation, tissue remodeling and cell differentiation [16]. They tend to act in an autocrine or paracrine fashion and are subject to strict control under

physiological conditions. The fact that one cytokine can stimulate the synthesis and release of many cytokines results in the stimulation of a complex network of interacting molecules, which can act in an additive or potentiating way. A rational basis for tissue engineering is unthinkable without clear understanding of how the cytokine network is regulated. In this respect it is important to note that certain cytokines, such as interleukin-1 (IL-1β) and tumor necrosis factor-alpha (TNF-α) are principally pro-inflammatory, whereas other cytokines, such as IL-4 and IL-10 have marked anti-inflammatory functions, such as inhibition of further cytokine production [17].

#### 4.1.2. Understanding the endothelial "stage"

If we remain with the metaphor of a performing orchestra, in which cytokines are principal players, then the stage on which they perform is the micro-circulation, in which the inflammatory response take place. The regulatory function of the endothelium in the microcirculation is achieved by its production of inflammatory mediators, such as IL-1β, IL-6, IL-8 and platelet-activating factor (PAF), as well as by the expression of cell adhesion molecules (CAM), which control the interaction of EC and circulating blood cells. The central role of EC CAM expression in the regulation of inflammation has made it a focus of many research groups involved in inflammation research. By the same token this should be a research focus in the biomaterial field.

In our own investigative work on inflammation and biomaterials we have concentrated on the pathomechanisms of inflammation induction by metal ions, which can diffuse into peri-implant tissues following corrosion of metallic implants. We were able to demonstrate that metal ions, such as nickel and cobalt, are able even at micromolar concentrations to induce and upregulate the expression of endothelial CAM, such as intercellular adhesion molecule-1 (ICAM-1) and E-selectin [18]. EC cultures also offer the possibility to use blocking antibodies against CAM to investigate the functional significance of the upregulated CAM. Thus, using cocultures of peripheral blood polymorphonuclear granulocytes we were able to show that E-selectin is of central importance in mediating the metal ion-induced increase in granulocyte adherence to the endothelium [10].

#### 4.1.3. Defining the border between the physiological and pathological

Inflammation is, in the first instance, a physiological process, without which life in a hostile environment would be inconceivable. As such it is a desirable reaction which should not be tampered with. Nevertheless, the inflammatory response can become amplified, as is the case in sepsis and the multiple organ dysfunction syndrome [19], in which case it is clearly pathological. The definition of the border between the physiological and pathological response is essential to any therapeutic interventional stategy. The clinical relevance of these considerations is seen by the problems arising from the corticosteroid treatment of life-threatening inflammatory processes. This therapy has the advantage of suppressing the amplified inflammatory reaction, but has the severe disadvantage of simulataneously inhibiting essential functions in the entire immune response, a situation which makes the patient vulnerable to infections of various types, which themselves can cause death.

The question of the *optimal level* of inflammation following biomaterial implantation is still far from answered. Undoubtedly, this level will differ from application type to type. EC culture techniques present an excellent adjunct method in association with *in vivo* assays to study these phenomena.

### 4.2. Angiogenesis

Tissue remodeling in soft tissues is characterized by complex interactions involving various cell types, including inflammatory cells, such as monocytes, granulocytes, mast cells and lymphocytes, as well as fibroblasts, responsible for the fibrotic reaction and EC. The latter cell type manifests itself in the form of sprouting of capillaries as a component of granulation tissue. This process of angiogenesis has been intensively investigated in the past few years, with the result that a number of pro- and anti-angiogeneic factors have been discovered [20, 21]. Thus, it becomes apparent that the relevant EC type for *in vitro* studies must ultimately be from the microvasculature. In the application to biomaterials there are still many fundamental questions which are unanswered. Thus, for example, it is completely unknown what the optimal level of angiogenesis for a given implant is. Furthermore, the optimal degree of inflammatory cell interaction, which accompanies the angiogenetic response, is also an unknown quantity. Answers to these essential questions form the basis for the ability to control the host reaction to an implanted biomaterial.

In our laboratory we have established cultures of human pulmonary microvascular EC (HPMEC) by a complex combination of mechanical, enzymatic and immunomagnetic techniques, which permit the isolation of the numerous MEC from the mixture of different cell types which are integral components of the pulmonary tissue. In two-dimensional culture these cells show a biphasic pattern of morphology, which is especially prominent in the subconfluent state and consists of polygonal cells as well as bipolar EC, which form sprouts. This sprouting phenomenon becomes even more marked, if the HPMEC are embedded as individual cells in a three-dimensional (3-D) collagen type I gel. The current strategy is to use this 3-D model to investigate the angiogenic response of various biomaterials. It is hoped that new insights will be gained into structure-function relationships of biomaterials.

### 4.3. Signal transduction

Signal transduction research is a field of endeavor which is crucial to the understanding of how biomaterials regulate cellular responses. This topic concerns how signals received at the plasma membrane of a cell are

transduced within the cell to result in a particular functional response, which generally involves the transcription of specific genes in the nuclear DNA and subsequent translation of mRNA in the cytoplasm to yield specific proteins [22]. In the field of biomaterials this involves elucidating the intracellular pathways regulating the expression of important genes such as cytokines, growth factors (GFs), enzymes and cell adhesion molecules (CAMs).

Signal transduction involves a series of intracellular pathways, which, although interrelated, are also distinct from each other and represent clearly defined biochemical reactions, concerning phosphorylation and dephosphorylation steps. In general the pathways are initiated by specific receptor-ligand reactions at the plasma membrane. For genes involved in inflammation and the immune response one of the final common pathways leading to gene transcription is the activation of nuclear factor-κB (NF-κB) [23]. We were able to demonstrate that when metal ions induce cytokines and CAM in HUVEC the transcription factors NF-κB and AP-1 are upregulated [24]. In further studies on metal-ion-induced upregulation of CAM we were surprised to find that inhibitor substances blocking certain signal transduction pathways (via activation of protein kinases) and leading to inhibition of CAM induction (ICAM-1 and E-selectin) did not inhibit NF-κB but even resulted in its increase [25]. This finding provides evidence for the existence of NF-κB-independent signal transduction pathways for the upregulation for CAM in EC, although the exact nature of these pathways still needs to be elucidated. The significance of these results is that there appear to be ''pathological'' signal transduction pathways *separate* from the ''physiological'' ones, the latter being a *conditio sine qua non* for normal defence mechanisms in the entire organism. This has relevance for therapeutic intervention, as it opens up the theoretical possibility of using pharmacological methods to specifically block the pathological pathways without totally repressing the patient's inflammatory response. Whether this can be achieved is still speculative at the moment.

### 4.4. The concept of endothelial functionality

The past two decades have witnessed a transformation of our view of the endothelium from a passive barrier between flowing blood and the vessel wall to a highly active tissue with fundamental regulatory roles in numerous physiological processes. This topic has been reviewed elsewhere [2, 26], although a brief resumé here is instructive. There are four principal areas of biological function in which the endothelium is intrinsically involved. First, haemostatic control is achieved by maintaining a delicate balance between pro- and antithrombogenic signals, whereby the antithrombogenic function predominates under physiological conditions. Among the important antithrombogenic products are prostacyclin, nitric oxide, heparan sulphate proteoglycans, as well as thrombomodulin. Second, the endothelium is involved in growth control, for example, by producing growth factors (GF), such as platelet-derived growth factor (PDGF) and basic fibroblast growth factor (bFGF) in response to cytokine stimulation. Third, the endothelium exerts a vital controlling function in vascular tone, principally by the synthesis of nitric oxide and prostacyclin as potent vasodilators, antagonistic actions being achieved by production of, for example endothelin-1. Fourth, as has been discussed above (under the heading ''inflammation''), the endothelium is a central regulator of the inflammatory response.

The essential message from these cell biological considerations for the biomaterial research field is that attempted endothelization of biomaterial surfaces for blood contacting devices must result not only in the formation of an intact EC monolayer but also in maintenance of endothelial functionality, that is, the delicate physiological balance must be achieved. The corollary of this is that the demonstration by morphological means that an EC covering is present on a biomaterial is in no way scientific proof of its functionality.

## 5. Combining endothelial and biomaterial research: *Quo vadis?*

Major advances in hybrid organs and tissue engineering are inconceivable without considering the role of the endothelium. This statement finds its validity in the biological fact that the endothelium is practically ubiquitous, with the exceptions of cartilage and certain compartments of the eye. Furthermore, as a result of this, there is the possibility of developing therapeutic stategies for EC as a vehicle for genetic engineering, that is, the endothelium could be used as a target to alter gene function and thereby up- and down-regulate gene expression in a hybrid organ. This will increasingly become a focus of research activity in the next years. This review will conclude by briefly considering three aspects of the future application of endothelial research to the biomaterials field, namely attempts to control the host response to implanted biomaterials, regulating endothelial functionality and finally the practicality of furthering tissue engineering by developing relevant *in vitro* models employing EC.

### 5.1. Control of host response to biomaterials

The promotion of endothelialization will continue to be an important theme in blood-contacting devices, whether they be heart valves, vascular prostheses or stents. In this endeavor EC cultures using human tissue will be an important adjunct to *in vivo* studies. In addition, as has been alluded to under the heading of angiogenesis (above), the microvascular response to implants is one of the determining factors in the entire process of optimal wound healing. Future *in vitro* assays to investigate the individual pathomechanisms must involve microvascular, and not macrovascular EC. We have begun comparative studies between the most commonly used endothelial type, HUVEC, and our newly developed culture of endothelium from the lung microvasculature, HPMEC. The preliminary results indicate that there are significant variations in their response to relevant simuli. Because of the central role of CAM in the inflammatory response we have compared the expression of ICAM-1,

VCAM-1 and E-selectin in HUVEC and HPMEC under the influence of relevant pro-inflammatory stimuli (endotoxin, TNF-α and IL-1β), applied to confluent monolayers for 4 h, followed by time-related determination of CAM expression using cell-EIA (cell enzyme immunoassay), which measures the cell surface expression of these molecules. The results indicate that upregulation of vascular cell adhesion molecule-1 (VCAM-1) in HPMEC was much slower and less intense than in HUVEC. In addition, the induced expression of E-selectin decreased much more rapidly in HUVEC than in HPMEC. These data indicate that the inflammatory response in the microvasculature, which is the region of the vascular system in which inflammation is regulated, is not identical with that in EC derived from macrovascular regions. In summary, the results underline the necessity to concentrate on *in vitro* models using microvascular EC.

## 5.2. Control of endothelial functionality

The regulatory functions of the endothelium have already been alluded to above. The fact that the endothelium is capable of upregulating functions opposed to the normal physiological state make it necessary to ensure that in a biomaterial application the positive effects are enhanced and the negative effects suppressed.

### *5.2.1. Promoting positive effects*

In studying how biomaterials interact with EC it is important to strive for two principal goals. First, EC structural integrity must be maintained. A useful parameter to study this is the expression of a family of CAM responsible for endothelial–endothelial interactions, namely the cadherins. Second, up-regulating antithrombogenic activity must rank high on the list of priorities for a biomaterial with contained EC.

### *5.2.2. Down-regulating negative effects*

The reverse side of the coin is equally important for a functional endothelium. As corollary to the promotion of antithrombogenic activity there is the pre-requisite to down-regulate procoagulant activity, as the switching on of such functions as the production of tissue factor or the expression of coagulation factors V and VIII would be disastrous for the implanted device. Furthermore, the expression of pro-inflammatory CAM must be avoided, as this would result in the recruitment of inflammatory cells, such as granulocytes and monocytes which could damage not only the colonized EC, but also severely affect the structural integrity of the biomaterial, especially in the case of a bioresorbable material.

## 5.3. Tissue engineering aspects

Finally, it is important to make some brief considerations about how *in vitro* methods using EC can be improved so that they gain more relevance for tissue engineering application. Three areas should be stressed. First, with respect to the fact that endothelium *in vivo* is under constant shear stress, which of course influences endothelial function [27] and moreover varies from one vascular region to the other, it is unacceptable to perform EC cultures only under static conditions. The next rational step is to adopt flow systems *in vitro*, which permit the study of EC functionality under varying shear stress. We have developed a parallel plate flow chamber, which allows dynamic conditions to be so modified that a broad spectrum of flow types, from venous to arterial, and from laminar to turbulent, can be simulated. With the use of real-time imaging via a video camera in conjunction with a computer it is possible to quantitate the adhesion of blood cells to EC monolayers growing on different biomaterial surfaces [28–30].

Second, there is a need to develop more coculture models, in which cell–cell interactions can be more closely studied. An important example with respect to hybrid organs for the vascular system is the coculture of EC with smooth muscle cells (SMC) [31]. This higher level of cellular organization is essential to simulating the final medical device for application *in vivo*.

Third, it has become apparent that the endothelium could be a useful tissue to use as a target for genes to be incorporated into the body [32], with a carrier biomaterial as scaffolding. The possibilities opened up by such a technology have recently been underlined by Kader *et al*. [33], who have employed gene transfection techniques to overexpress the eNOS gene (endothelial nitric oxide synthase) in EC destined for synthetic small diameter vascular grafts. The rationale behind this is that more nitric oxide could be produced by these EC with the beneficial effects of reducing platelet activation and aggregation, as well as inhibition of SMC proliferation.

## 6. Conclusion

There can be no doubt that the future development of biomaterials for implantation in the human body will be influenced by advances in endothelial biology, especially in the fields of tissue engineering and hybrid organ construction. Already to be found in the endothelial literature are data pertinent to the biomaterials field but which have not yet been fully realized by the scientific community involved in biomaterial research. This will continue to be a challenge in the years to come and will hopefully lead to marked improvements in our attempts to adequately replace the function of diseased tissues and organs.

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