Heart vascular endothelial cells in immune reactivity : an experimental model in the rat

Citation for published version (APA):

Derhaag, J. (1998). *Heart vascular endothelial cells in immune reactivity : an experimental model in the rat*. [Doctoral Thesis, Maastricht University]. Universiteit Maastricht. <https://doi.org/10.26481/dis.19980703jd>

Document status and date:

Published: 01/01/1998

DOI:

[10.26481/dis.19980703jd](https://doi.org/10.26481/dis.19980703jd)

Document Version:

Publisher's PDF, also known as Version of record

Please check the document version of this publication:

- A submitted manuscript is the version of the article upon submission and before peer-review. There can be important differences between the submitted version and the official published version of record. People interested in the research are advised to contact the author for the final version of the publication, or visit the DOI to the publisher's website.
- The final author version and the galley proof are versions of the publication after peer review.
- The final published version features the final layout of the paper including the volume, issue and page numbers.

[Link to publication](#)

General rights

Copyright and moral rights for the publications made accessible in the public portal are retained by the authors and/or other copyright owners and it is a condition of accessing publications that users recognise and abide by the legal requirements associated with these rights.

- Users may download and print one copy of any publication from the public portal for the purpose of private study or research.
- You may not further distribute the material or use it for any profit-making activity or commercial gain
- You may freely distribute the URL identifying the publication in the public portal.

If the publication is distributed under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license above, please follow below link for the End User Agreement:

www.umlib.nl/taverne-license

Take down policy

If you believe that this document breaches copyright please contact us at:

repository@maastrichtuniversity.nl

providing details and we will investigate your claim.

Heart vascular endothelial cells in immune reactivity

An experimental model in the rat

Josien Derhaag

ISBN 90-5681-037-5

Publication of this thesis was financially supported by:
Parke Davis B.V. the Netherlands.

Printed by:
Unigraphic, Maastricht, The Netherlands.

Cover illustration:
Part of the primary clone of endothelial cells subsequently expanded to RHEC 3.

Heart vascular endothelial cells in immune reactivity

An experimental model in the rat

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof. Dr. A. C. Nieuwenhuijzen Kruseman
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen
op vrijdag 3 juli 1998 om 14.00 uur

door

Josien Gerarda Derhaag

geboren te Sittard op 25 maart 1967

Promotor:

Prof. Dr. P. J. C. van Breda Vriesman

Co-promotor:

Dr. A. M. Duijvestijn

Beoordelingscommissie:

Prof. Dr. R. F. A. Zwaal (voorzitter)

Prof. Dr. J. W. Arends

Prof. Dr. M. R. Daha (Rijksuniversiteit Leiden)

Prof. Dr. G. J. van der Vusse

A man will turn over half a library to make one book

S. Johnson

Contents

Abbreviations	9
Introduction to the Chapters	11
Chapter 1: General introduction	13
Chapter 2: Production and characterization of spontaneous rat heart endothelial cell lines	35
Chapter 3: Heart EC respond heterogeneous on cytokine stimulation in ICAM-1 and VCAM-1, but not in MHC expression. A study with 3 rat heart endothelial cell (RHEC) lines	69
Chapter 4: A dual role for endothelial cells in cytomegalovirus infection ? A study of cytomegalovirus infection in a series of rat endothelial cell lines	95
Chapter 5: Effects of antibody reactivity to MHC and non-MHC alloantigens on graft endothelial cells in heart allograft rejection	115
Summary & Discussion	141
Samenvatting & Discussie	147
Publications	153
Dankwoord	155
Curriculum Vitae	157

Abbreviations

Ab	Antibody
AA	Arachidonic acid
ACE	Angiotensin-converting-enzyme
APC	Antigen presenting cell
BN	Brown norway
CCP	Complement control proteins
CD	Cluster of differentiation
CMV	Cytomegalovirus
EDRF	Endothelium-derived relaxing factor
EC	Endothelial cells
ET	Endothelin
FACS	Fluorescence activated cell sorter
GlyCAM-1	Glycosilation-dependent cell adhesion molecule-1
GP	Guinea pig
HUVEC	Human umbilical vein endothelial cell
ICAM-1	Intercellular adhesion molecule-11
IFN γ	Interferon γ
IL	Interleukin
LEW	Lewis
LEW.1N	Congeneic rats containing the BN MHC on a Lewis background
LFA-1	Lymphocyte-function associated antigen-1
LNC	Lymph node cells
MAdCAM-1	Mucosal addressin cell adhesion molecule-1
MHC	Major histocompatibility complex
mAb	Monoclonal antibody
MAC	Membrane attack complex
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
m.o.i.	Multiplicity of infection
NO	Nitric oxide
PAI-1	Plasminogen activator inhibitor -1
PAF	Platelet-activating-factor
PBS	Phosphate buffered saline
PFU	Plaque forming unit
PG	Prostaglandin
PGI ₂	Prostacyclin

PMA	Phorbol-meristate-acetate
RCMV	Rat cytomegalovirus
RECA-1	Rat endothelial cell antigen-1
REF	Rat embryonal fibroblast
RHEC	Rat heart endothelial cells
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNF α	Tumor necrosis factor α
t-PA	Tissue type plasminogen activator
TX	Tromboxane
Tx	Transplantation
VCAM-1	Vascular cell adhesion molecule
VLA-4	Very late antigen-4
vWF	von Willebrand Factor
uPA	Urokinase type plasminogen activator

Introduction to the Chapters

Endothelial cells (EC) are relevant for almost all physiological and pathological processes, due to their location between the circulating blood and the tissues surrounding the blood vessels. The advances in endothelial research over the past decade have given us insight in the role of EC in processes such as the control of blood flow, blood pressure and clotting as well as in pathological processes such as vasculitis, several autoimmune diseases and transplant rejection. The endothelium can be classified morphologically as one tissue, but because of its heterogeneity between and within organs and within the vascular tree, it is mandatory to use, for in vitro studies, the relevant EC type. In the present thesis an in vitro EC model was developed and used in studies addressing various aspects of endothelial physiology. Next to this, studies were executed to unravel the role of EC in immunological processes, we therefore choose a model for heart transplant rejection. The use of rat EC of the proper tissue, in this case from the heart of the used rat strain, was necessary. Since the isolation of EC from rat cardiac tissue is laborious and results in low yield, we developed a method to obtain endothelial cell lines out of rat hearts.

In **Chapter 1**, the general introduction, the concepts of the structure and physiological functions of the EC, and the current knowledge of the role of EC during immune reactivity are reviewed.

In **Chapter 2** the technique to develop endothelial cell lines and the phenotypical and functional characterization of the obtained 3 EC lines, called rat heart endothelial cell line (RHEC) 3, RHEC 10 and RHEC 11, are presented. In addition possible tumorigenicity of the spontaneously immortalized RHEC lines is studied.

During transplant rejection, a process in which inflammation and immune reactivity occur, interaction between EC and blood leukocytes is important for pathophysiological processes. In **Chapter 3** the RHEC lines are studied for cytokine-

induced expression of adhesion molecules and MHC antigens, both structures that are important for cell-cell interactions.

As cytokines the pro-inflammatory cytokine $\text{TNF}\alpha$ and the immune regulatory cytokine $\text{IFN}\gamma$ are used, individually or in combination, for periods from 4 to 64 hour. Observed heterogeneity in the induction of these molecules is further studied to gain more insight in the possible intrinsic regulation mechanisms in the EC monolayers.

To define whether the observed heterogeneity of the RHEC lines is of relevance for infection of the EC by CMV, the RHEC lines and other EC lines are studied for susceptibility for infection with rat CMV in **Chapter 4**. In addition the effects of CMV infection on the EC monolayer and the production of infectious virus are examined.

During transplant rejection the graft vasculature is considered to be a major target for immune reactants of the recipient. In **Chapter 5** the role of alloantibodies to endothelial MHC and non-MHC alloantigens in graft vasculopathy is investigated. The effects of the allosera reactive with MHC or non-MHC alloantigens on graft EC are studied with respect to complement activation, complement-mediated cytotoxicity and adhesion molecule induction. Furthermore the isotype distribution of alloantibodies reactive with MHC and non-MHC alloantigens is studied.

Finally the data obtained in the various studies described in these chapters are summarized and discussed in the **Summary & Discussion**.

1

General introduction

Josien G. Derhaag

Department of Immunology, Maastricht University, Maastricht, The Netherlands

The word endothelium is used in a general sense for the cells lining the luminal side of the vessel wall. The endothelium, which was considered for a long time to be just an inert barrier, had been barely explored until three decades ago. Since then, there has been an upsurge of interest in the fine structure and function of endothelial cells (EC). *In vivo* and *in vitro* investigations have revealed that the endothelium is a key element in a wide variety of normal activities and diseases and is far from being metabolically inert. The EC crucially contributes to the regulation of the entire vascular system and to the systemic homeostasis as well. It is now widely recognized that EC are morphologically and physiologically highly differentiated along the vascular bed as a biological adaptation to local needs.

1.1 Endothelial cell structure

EC are of mesenchymal origin. Electron microscopy has provided information about various micro-organelles and structures in the EC. *In situ* EC are elongated with the long axis aligned with the blood stream. The monolayer of EC has a cobble stone pattern in which the nucleus of the cell bulges into the vascular lumen. In addition mitochondria (M), a well developed rough endoplasmatic reticulum (rER) and a golgi complex (G) are present (1), see figure 1. Like other eukaryotic cells, EC contain several subclasses of intermediate sized filaments, predominantly of the vimentin subclass. Microfilaments (mf), decorated with the actin binding unit, and myosin-like filaments have also been

identified. Microtubules (mt) are only sporadically mentioned, although their occurrence in EC is an established fact (2). Furthermore EC contain specific transcytotic vesicles (V), which organize barrier and transport functions of the EC monolayer, as described below (Chapter 1.3.1). Quite specific for EC is the presence of structures known as Weibel Palade bodies. These rod-shaped granules which represent the intracellular storage sites of the EC borne von Willebrand Factor (vWF) and P-selectin have a typical tube-like internal structure. The Weibel Palade bodies are less numerous in venules and normally absent in capillary EC.

Ultrastructure of EC

Figure 1: Electron microscopy and scheme of rat heart EC, magnification 44000 X. L: lumen, V: transcytotic vesicles, N: nucleus, M: mitochondria, G: golgi complex, rER: rough endoplasmatic reticulum, mf: microfilaments, mt: microtubules.

1.2 Models for studying EC

Already between 1884 and 1950 papers were published dealing with various cultivation techniques for EC. Again in 1973-1975, a new era started with refined culture methods and subcultivation as well as identification of EC and smooth muscle cells by Jaffe et al. (1) and Gimbrone et al. (3,4). Since then a variety of systems have been used to study EC and their interactions. The first widely used successful method of isolating EC for culture involved infusion of a solution containing proteolytic enzymes to detach the endothelium from the intima. Of course it is possible that with this method structures like receptors will be destroyed, and the cells can not be used for studies involving these molecules. Methods for culturing EC from other organs and from different vasculature are all based on this enzymatic procedure. Specific markers used for identification of EC are the presence of angiotensin converting enzyme (ACE), and vWF, the binding of the lectin GS-I-B4, and the internalization of Dil-ac-LDL. In human systems, umbilical vein EC (HUVEC), which are relatively easy to obtain, are usually used. EC, obtained as described above, are from macrovascular origin. When this method is used for isolation of EC of microvascular origin, the procedure is hampered by low yield of EC. This disadvantage made the development of EC lines required. The successful production of EC lines has been described for human and murine species, mostly using SV-40 and adenovirus infections (5, 6, 7, 8), or transfection with SV-40 and polyoma virus oncogene constructs coding for viral T antigens (9, 10, 11). Unfortunately, the viral inserts may lead to interference with specific EC functions. In addition after long term culturing the EC can lose EC specific characteristics which makes it necessary to examine the transfected EC line herefor during the experiments. Next to cultured cell monolayers, small pieces of the vessel wall, mostly rat aorta, with the intact EC still present as a monolayer are used for *in vitro* studies. In this method the interaction of the EC with its matrix and the possible effects of substances produced by EC on the underlying cells can also be studied. Because of the heterogeneity of the EC between species, and within a species among organs and even within the vascular bed of a single organ, it is important to use the most proper EC for every examination.

1.3. EC functions

1.3.1 Transport and regulation of permeability

The endothelium consists of a single layer of cells and appears to play an important role for vessel wall structure and function. In this monolayer the endothelial plasma membrane exhibits luminal-abluminal polarity. Transport pathways are different in distinct vascular beds. Cooperative transport through the microvascular endothelial monolayer is accomplished by different pathways (Figure 2): (1) diffusion through the cell membrane; (2) via vesicular pathways; (3a) a cell surface layer pathway through the junctions; (3b) pore pathways localized at the intercellular junctions (small and large); (4) a non junctional channel variant formed by fusion of two or more vesicles; (5) open fenestrea in the endothelial monolayer of some organs (e.g. intestine); (6) fenestrae closed by a diaphragm (12, 13). Exchange of water and solutes across microvascular endothelium takes place through several intra- and intercellular pathways. In steady state, water and small lipid-soluble compounds diffuse through the entire endothelial surface, while the bulk flow of water and diffusion and convection of small lipid insoluble molecules can take place through intercellular channels. Transport of plasma proteins and other large molecules occurs via convection and diffusion through large pores or via vesicular exchange. Fenestrae, not present in all EC have a high permeability for water and ions as well as for small molecules (14). Although the EC is capable herein vascular permeability by receptor regulation of transcytosis the intercellular junctions seem to play the most important role for regulation of permeability. For instance, receptor activation (e.g. by bradikinin or thrombin) induces endothelial contraction by disconnecting the interaction between junction and actin filaments, which results in increased permeability between the EC (15). It should be noticed that the mechanisms involving transport and permeability are reflected in the distinct structural features that characterize EC in their various locations. These differences between local EC are associated with the specific functions of the tissues, e.g. the EC of the brain capillaries are connected by tight junctions to form a tight blood brain barriere protecting the connective brain tissue, and

the EC of the intestinal mucosal and the renal glomerular capillaries form a fenestrated layer allowing transport of macromolecules.

Transport pathways in EC

Figure 2: Scheme of transport pathways in microvascular endothelium. Transcytosis can occur via different pathways: 1) the cell membrane pathway consists of three barriers; apical plasma membrane, cytoplasm and basolateral plasma membrane. 2) the vesicular pathway, which tend to equilibrate luminal and abluminal fluids. 3a) The cell surface layer pathway through the junctions. 3b and 4) the small and large pore pathway which allow convective flow of water and permeating solutes as well as diffusion exchange of water and solutes. 5) open fenestrae, allowing transport of macromolecules and 6) closed fenestrae (not present in all endothelium), characterized by the high permeability for water, ions and small molecules. (Based on Renkin, E. M. 1977, *Circulation Research* 41:735-743).

1.3.2 EC in hemostasis

EC form the luminal vascular surface and are well situated to play an important role in the regulation of the hemostasis. Normally multiple anticoagulant mechanisms are operative on the EC (Fig. 3), whereas the balance between anticoagulant and procoagulant activity is compromised during trauma by induction of procoagulant and suppression of anticoagulant mechanisms.

Under normal circumstances the EC form an anticoagulant layer that counteracts blood coagulation and prevents platelet activation, while stimulating fibrinolysis.

Activation of the coagulation cascade results in the formation of thrombin which converts fibrinogen into a fibrin gel. EC can interrupt the coagulation cascade by inactivation of activated coagulation factors. Heparan sulphates on the EC surface interact with

antithrombin III, and this complex inhibits the activated coagulation factors Xa and thrombin (16). EC express thrombomodulin (TM), which promotes the activation of protein C by thrombin resulting in inactivation of the coagulation factors Va and VIIIa (17, 18). In addition, EC prevent coagulation by production of tissue factor pathway inhibitor (TFPI) which inhibits the activation of the extrinsic coagulation pathway induced by tissue factor (TF)(19). EC can prevent thrombus formation by exhibiting anti-platelet activity. Platelet adhesion to the EC is prevented by surface-bound proteoglycans. Platelet activation is counter acted by PGI₂ and PGE₂, released by EC after stimulation with vasoactive substances like thrombin (20). The effect of PGI₂ is enhanced by the production of nitric oxide (NO) by the EC. EC continuously produce tissue type plasminogen activator (tPA), which together with the constant presence of plasminogen activator inhibitor-1 (PAI-1) results in a physiological balance of pro- and anti-coagulant processes (21). If coagulation occurs, EC provide regulatory components for fibrinolysis, like tPA via both constitutive secretion and release from a storage pool. This storage pool, especially effective in preventing local thrombus formation, can be released by vasoactive substances like platelet activating factor (PAF), bradikinin and thrombin (22).

The anticoagulant phenotype of the vessel wall can be modulated to procoagulant in response to environmental stimuli of immune reactivity like in transplant rejection. Under challenging conditions, e.g. in the presence of endotoxin, tumor necrosis factor (TNF)- α and interleukin (IL)-1, a shift occurs in the balance of anti- and procoagulant properties favoring activation of coagulation. The EC start to express TF, promote thrombin generation, reduce their TM activity and enhance their production of PAI-1 (21, 23). After damage of the endothelial wall, EC also release vWF which promotes platelet adhesion to the EC leading to hemostatic plug formation (24). Furthermore, activated EC induce the expression of PAF on their membrane. Besides platelet activation, PAF also activates EC, and cooperates with adhesion molecules in leukocyte transmigration (25). Activation of the coagulation response appears to be non-specific, but local production of the mediators provides the system with specificity and flexibility in response to

environmental stimuli. Furthermore, differences exist in the production of tPA, NO, PGI₂ and PGE₂ between venous, arterial and microvascular EC, resulting in different procoagulant/anti-coagulant properties of the different vascular beds.

Endothelial mediators in hemostasis

Figure 3: Anticoagulant and procoagulant properties of EC. On the left: inhibition of the platelet function by prostacyclin (PGI₂), prostaglandin E₂ (PGE₂) and nitric oxide (NO), and inhibition of the coagulation. Thrombomodulin (TM) binds thrombin, promoting the activation of PC (Protein C) to aPC, which degrades factor Va and VIIIa. The complex of heparin like molecules and anti thrombin III inhibits thrombin (IIa) and Xa. Tissue factor pathway inhibitor (TFPI) inhibits the tissue factor (TF)-induced activation of the extrinsic coagulation pathway. On the right: von Willebrand factor (vWF) released from stimulated EC promotes platelet adhesion. Expression of platelet activating factor (PAF) on the membrane of the stimulated EC activates platelets. TF on the stimulated EC serves as co-factor (factor VIIa) to activate several coagulation factors. Generated thrombin activates platelets, coagulation and fibrinolysis. In addition continuously produced tissue plasminogen activator (tPA) activates fibrinolysis, a process which is inhibited by plasminogen activator inhibitor-1 (PAI-1), providing a balance of pro- and anti-coagulant processes. (Based on Wu, K. K. 1996 Annu. Rev. Med. 47:315-31).

1.3.3. Regulation of the vascular tone by EC

EC are an important cell type in the local regulation of the vascular tone. Hereto, the EC are able to release substances that result in vasodilatation or vasoconstriction (Table 1) .

Endothelium-dependent relaxation in vascular tissue is mainly mediated by endothelium-derived relaxing factor (EDRF) which in later studies turned out to be NO (26). NO increases the amount of cyclic guanosine monophosphate (cGMP) within the smooth muscle cell resulting in vasodilatation (27). In addition NO inhibits aggregation and adhesion of platelets (see above). Another vasodilator produced by EC is prostacyclin (PGI₂). EC can be stimulated to synthesize PGI₂ which results in induction of the release of adenosine tri phosphate (ATP) which is a potent vasodilator (28). For producing its vasorelaxant effects, arachidonic acid (AA) is dependent on an endothelium dependent component, cytochrome P450 dependent mono-oxygenase (29). Agonists like acetylcholine, bradykinin or histamin can evoke production of relaxation factors effecting the endothelial layer.

In addition to the production of substances resulting in vasodilatation, endothelium can release vasoconstricting substances. Angiotensin converting enzyme (ACE), produced by EC, is responsible for the generation of the vasoconstrictor angiotensin II. Furthermore, EC produce endothelin (ET-1) which is the most active vasoconstricting substance yet discovered, with a potency some 10 times higher than angiotensin II. The main effects of ET-1 release, enhanced by for instance thrombin, are generated on the smooth muscle cell. This results in enhanced phosphorylation of the regulatory light chain of myosin, resulting in constriction (30). The specific, high-affinity binding of ET-1 to the smooth muscle cell results in a characteristic long lasting response. *Ex vivo* experiments show that vasoconstricting factor ET-1 induces the release of PGI₂, PGE₂ and sometimes thromboxane A₂, which are vasodilatation factors. This suggests that ET-1 is a local factor, released in high concentrations, inducing the release of NO and prostacyclin, probably to overcome local intense vasoconstriction (31).

Endothelial mediators in the regulation of the vascular tone

vasodilatation	vasoconstriction
NO PG I ₂	ET-1 ACE

Table 1: NO: nitric oxide, PGI₂: prostacyclin, ET-1: endothelin, ACE: angiotensin converting enzyme.

1.3.4 EC and leukocyte migration

The endothelium plays a regulatory role in leukocyte traffic. Adhesion molecules on EC can interact with counterpart adhesion molecules on leukocytes, thus allowing extravasation. This process is also influenced by the migratory capacity of the leukocytes and by chemoattractants and chemokines. Which adhesion molecules are present on the endothelial membrane is related to the anatomical site of the EC and to activation of EC by cytokines.

Leukocyte extravasation occurs via a multi-step mechanism (32, 33). The first step is the tethering and rolling of the leukocyte along the endothelium. During this step cell-cell interactions are mediated by selectins, e.g. L-selectin on circulating leukocytes and P- and E-selectin on vascular endothelium. All selectins appear to recognize a sialylated carbohydrate determinant on their counterpart receptors. Receptors for L selectin are CD34 (34), glycosylation-dependent cell adhesion molecule-1 (GlyCAM-1) (35) and also mucosal addressin cell adhesion molecule-1 (MAdCAM-1) expressed on EC. Differential expression of adhesion molecules may direct different subsets of T cells to specific sites. CD44 also contributes to the adhesive interactions of lymphocytes and seems to play a role in the differential migration of activated lymphocyte subsets (32). For firm adhesion to the endothelium, adhesion molecules of the integrin family play an important role. Lymphocyte-function-associated antigen-1 (LFA-1, CD11a/CD18) (36) and MAC-1 (CR3, CD11b/CD18), present on leukocytes, mediate binding to EC by interaction with intercellular cell adhesion molecule-1 (ICAM-1, CD54). Before the

integrins LFA-1 and MAC-1 can increase adhesiveness they require activation by chemokines like for instance IL-8 (37). Integrin molecules bind to adhesion molecules of the Ig superfamily on the EC. Induction of ICAM-1 by cytokines may increase cell-cell interactions and leukocyte extravasation, whereas constitutive expression of ICAM-2 may be important in particular for adhesion of lymphocytes (32). Vascular cell adhesion molecule-1 (VCAM-1) inducible on EC by cytokines, interact with very late antigen 4 (VLA-4, CD49d) (33, 38). These interactions of leukocytes with EC results in firm adhesion and flattening of the leukocytes, and to transendothelial migration into the extracellular matrix along a concentration gradient of chemoattractants (Figure 4).

Transendothelial leukocyte migration

Figure 4: Transendothelial leukocyte migration, a multistep model. The first step involves attachment and rolling of the leukocyte along the endothelium through interactions between selectins and their ligands. This results in induction of the integrins on EC and activation of their ligands on the leukocyte. Tight binding between these molecules arrests the rolling and results in adhesion. Finally the leukocyte migrates along a concentration gradient of chemoattractants secreted by the underlying cells. (Based on: Springer, T. A. 1994, *Cell* 76:301-314).

1.4. Cytokine regulation of EC function

The vascular EC are both target and source of cytokines. Cytokines serve to communicate with leukocytes as well as with cells in other tissues and organs. The variety of EC responses elicited by cytokines during immunity, inflammation and thrombosis is wide, sometimes with different cytokines activating the same EC function. Next to responding upon cytokines the EC produce cytokines and chemokines. The latter are relatively novel mediators which also have effects on EC and circulating cells (Table 2).

1.4.1 Production of cytokines

The production of cytokines and chemokines by EC is essential in the recruitment of leukocytes in inflammatory reactions, immunity and thrombosis. EC are an important source of and target for hematopoietic growth factors, such as stem cell factor (SCF) and several colony-stimulating factors (CSF). These factors affect migration and proliferation, and amplify EC responsiveness to other signals (39). During immune reactivity EC are induced to produce IL-1, IL-6, IL-8 as well as PAF, and produce EC chemokines of the CXC and CC family. IL-8 and monocyte chemoattractant protein-1 (MCP-1) are the best studied chemokines in relation to EC (39). IL-8, a chemoattractant in leukocyte extravasation, also seems to have angiogenic properties, while MCP-1 induces EC activities similar to IL-8 and IFN γ .

1.4.2 Response upon cytokines

During immune reactivity EC are activated by cytokines to alter their physiological properties and to acquire new pathophysiological functions (40, 41).

IL-1 and TNF induce production of PGI $_2$, PAF and NO by EC. In the human system IL-1, TNF and IFN γ stimulation of EC results in NO production, while for instance in the bovine system, IFN γ has inhibitory effects on the NO production by EC (42). IFN γ amplifies responses to TNF and induces the chemokine MCP-1 and interferon inducible protein 10 (IP-10) which has angiostatic properties. TNF α , IL-1 and IFN γ

induce the expression of MHC class II and augment the expression of MHC class I, CD40 and ICAM-1 on EC (43, 44, 45, 46). Activation of EC by $\text{TNF}\alpha$ and $\text{IFN}\gamma$ occurs through different intracellular signaling pathways. Consequently, effects of EC stimulation with a combination of these cytokines may strongly differ from those of individual cytokine stimulation in expression levels of induced molecules and also the kinetics of induction. *In vitro* studies predominantly with HUVEC have shown overlapping as well as more specific effects of stimulation with individual cytokines. For induction of the adhesion molecules E-selectin and VCAM-1, $\text{TNF}\alpha$ appears a potent cytokine (47-51), whereas for the induction of MHC class II molecules $\text{IFN}\gamma$ plays a dominant role. With respect to induction or enhancement of ICAM-1, both cytokines have more or less similar induction capacities (52, 53). $\text{IFN}\gamma$ prolongs the effect of $\text{TNF}\alpha$ on E-selectin expression (46) and IL-4 prolongs the effect of $\text{TNF}\alpha$ on VCAM-1 expression (54). IL-1 and TNF also induce the production of cytokines by EC, including chemokine IL-6 and IL-1 itself (39, 51). The procoagulant activity of EC is enhanced by down regulation of proteoglycans (55) and TM (56) on the EC surface, an effect induced by $\text{IFN}\gamma$ and also TNF.

Data about the effects of IL-6 on EC, mainly based on HUVEC studies, are not conclusive. Experiments suggest that IL-6 plays a role in amplifying leukocyte recruitment and, in combination with the soluble IL-6 receptor, activates a unique functional program in EC (57). IL-10, also known as cytokine synthesis inhibitory factor, augments the action of IL-1 and TNF on EC. Furthermore IL-10 inhibits the antigen presentation function by human microvascular dermal EC (58). IL-4 induces urokinase-type plasminogen activator (uPA) in microvascular but not in macrovascular endothelium. IL-4 selectively induces VCAM-1 and inhibits expression of ICAM-1 and E-selectin. IL-4 is a weak inducer of IL-6 and MCP-1 production by EC but amplifies production of these mediators in concert with other stimuli (39). IL-13 and IL-4, which in general show structural and functional similarities, also have similar activities on EC which may be the result of sharing receptor binding domains by these cytokines (59). IL-

12 has anti-angiogenic activity, but this is probably not a direct effect on the EC but is mediated through effects of IL-12-induced IFN γ production (60).

It has been shown that not all EC respond similarly to cytokine stimulation and that differences such as species, strains, tissue and blood vessel type are important in this respect (61, 62, 63, 64).

Effects of cytokines on EC

Cytokine	Increased production by EC of:	Effect on circulation
IL-1 and TNF	PGI ₂ PAF NO IL-1 IL-6 adhesion molecule expression: E-selectin: ▲ ICAM-1: ▲ VCAM-1: ▲ expression: MHC I: ▲ MHC II: ▲ CD40: ▲	anti thrombotic vasodilatation platelet activation vasodilatation (1) T cell / Mφ activation T and B cell growth and differentiation interaction with leukocytes interaction with T cells
IL-6		not well defined
IL-8		angiogenic
IL-4, IL-13	uPA(2) adhesion molecule expression: VCAM-1: ▲ ICAM-1: ▼ E-selectin: ▼ IL-6 MCP-1	antithrombotic regulation interaction with leukocytes (3) IL-8 / IFNγ like effects
IFNγ (4)	expression: MHC I: ▲ MHCII: ▲ ICAM-1: ▲ CD40: ▲ IP10 MCP-1	interaction with T cells angiostatic IL-8 and IFNγ like

Table 2:

(1): in the human system IFNγ is also required

(2): only macrovascular EC produce uPA after IL-4 stimulation

(3): see IL-6 production after TNF and IL-1 stimulation of EC

(4): synergistic with TNF

▲: induced or upregulated

▼: down regulated

1.5 EC in immune reactivity

1.5.1 General aspects

When vascular endothelium is involved in immune reactivity it undergoes a number of functional and morphological alterations. Upon activation the EC alter their anticoagulant surface phenotype into one that promotes thrombosis and coagulation. In inflammation, activated EC induce vasodilatation by production of PGI₂ and NO(65).

Transendothelial leukocyte migration is increased during immune reactivity. The molecular mechanisms mediating physiological migration can be upregulated during immune reactivity. Neutrophil migration occurs mainly by cytokine-induced expression of E-selectin on the endothelium (66). Accumulation of lymphocytes and monocytes is a result of induction or upregulation of a series of adhesion molecules by cytokines in combination with production of chemoattractants by EC. In time, infiltrates change from cell types. These changes correlate with alterations in the adhesion molecules expressed by EC. These differences in adhesion molecules on the EC, and the production of chemoattractants and chemokines, allow fine tuning of leukocyte migration, allowing infiltration of different leukocyte populations in immune reactive lesions (65).

Besides induction of adhesion molecules by cytokines during immune reactivity the cytokines also enhance MHC class I and induce MHC class II expression in the EC. These MHC molecules are necessary for presenting antigenic peptides to T-lymphocytes, in the process of T cell activation. MHC binds peptides in the groove of the MHC molecule, allowing T lymphocytes to recognize the MHC/peptide complex with their T-cell receptor (TCR)(67). For T cell activation the interaction between the T cell, and the antigen presenting cell (APC) requires costimulatory signals, i.e. receptor/ligand interactions such as CD2/LFA-3, LFA-1/ICAM-1 and CD28/B7-1 or B7-2 and CD40/CD40 ligand (68). Without these costimulatory signals, antigen presentation may result in T cell anergy and abrogation of the immune response (69). Since EC can express most of the molecules involved in costimulatory signaling, they may function as APC.

1.5.2 EC in organ allograft rejection

The stimulus of rejection is based on the recognition by T cells of cells bearing foreign histocompatibility antigens. The exact nature of T cell allo-responses to (non-self) MHC is controversial to this day. T cells may recognize polymorphic MHC directly, or they may recognize non-self MHC peptides that are presented by self MHC proteins, or both. During rejection donor interstitial dendritic cells, which are the passenger leukocytes in the graft, or recipient dendritic cells can present the donor derived antigens. In the allograft, besides the prementioned cells, EC may contribute in antigen presentation processes (70, 71). Antigen presentation by EC may result in antigen-specific T cell activation, but more likely induces a state of allospecific unresponsiveness (72). Circumstantial evidence for such APC functions of EC is the finding that MHC class II expression is upregulated in EC in human renal, cardiac and liver allografts undergoing rejection (73, 74, 75). In the rat, however, no induction of MHC class II in the vasculature was seen in rejected kidney allografts(76).

During allograft rejection EC become activated by cytokines. The most important lymphokines involved are probably IFN γ , IL-1 and TNF. Activated CD4 and CD8 lymphocytes produce IFN γ , and monocytes are the main producers of TNF. These cytokines induce adhesion molecule expression in EC resulting in increased transmigration of leukocytes through the endothelium of the graft, and by differential expression of the various adhesion molecules, infiltration of relevant leukocyte subsets is controlled (45, 77). In human heart allograft rejection there is a relation between rejection and increased ICAM-1 expression. The role played by other cellular adhesion molecules such as VCAM-1 and especially E-selectin is less certain (78), although animal transplantation studies suggest that also the induction of VCAM-1 on the vasculature plays an important role in the rejection process (79). Applications of antibodies against VCAM-1, LFA-1 and ICAM-1 can inhibit the graft rejection phenomena in rat cardiac allografts (80, 81, 82). Thus, adhesion molecules that are upregulated on EC in settings of inflammation to support recruitment of immunological cells play an important role in rejection of transplanted organs (83, 84).

In addition, the cytokine-activated EC also promote inflammatory reactions during organ allograft rejection by secretion of a number of cytokines like IL-1, IL-6 and IL-8 as well as PAF and PAI-1. These mediators activate blood cells (65) and affect the balance of anticoagulation and procoagulation (56).

Transplantation in combination with cytomegalovirus (CMV) infection results in more often and earlier induction of the rejection process than without CMV infection. CMV infection induces an inflammatory type of response in the EC, because CMV is, in contrast to Herpes Simplex Virus, only able to infect but not to kill the endothelial cell (85). This inflammatory response results in an increase in leukocyte-EC interactions by adhesion molecules and MHC class II molecules on the EC (86, 87).

1.5.3 EC as target in the allograft rejection process

In organ allograft rejection the endothelium of the graft is of donor origin and therefore can be considered a target structure for the immunological rejection process generated by the recipient. As such the EC can be threatened by alloantibodies and cytotoxic T cells (88, 89), and additionally by Fc-receptor-expressing killer cells through antibody-dependent cell cytotoxicity (ADCC) mechanisms (90, 91, 92). The most dominant EC antigens involved in the rejection process are the constitutively expressed MHC class I antigens, but also the inducible MHC class II antigens. In addition to these MHC antigens EC may express other alloantigens, sometimes classified as 'minor histocompatibility antigens' or as non-MHC alloantigens (93, 94, 95, 96). Cell mediated immune responses against EC may affect lysis and subsequently vascular insufficiency. With respect to anti EC alloantibodies it is not clear yet which effect they may have on EC. Although complement-mediated cytotoxicity by anti EC antibodies has been demonstrated, in general, EC are rather resistant to this process. However, it has been shown that antibody reactivity with EC effects various processes in the graft vasculature. Antibody-induced sublytic complement activation triggers processes that lead to alterations in endothelial integrity. In addition complement activation on EC by antibodies results in production of IL-1, TF and PAF (97, 98, 99) and induction of the

adhesion molecules for leukocytes (100, 101). The production of C3a and C5a on the EC during complement activation influences recruitment and effector functions of platelets and leukocytes (102). The fact that in vascular rejection the progress of the rejection process is associated with the presence and the amount of alloantibodies reactive with endothelial MHC or non-MHC antigens suggests a role for these antibodies in the rejection process (92, 103).

1.6 References

1. Jaffe EA. Cell biology of endothelial cells. *Human Pathology* 1987; **18**: 234.
2. Simionescu M, Simionescu N. Ultrastructure of the microvascular wall: functional correlations. In: *Handbook of Physiology* Sect 2, Vol IV, 42-101.
3. Gimbrone MA, Cotran RS. Human vascular smooth muscle in culture. Growth and ultrastructure. *Laboratory Investigation* 1975; **33**: 16.
4. Gimbrone MA, Cotran RS, Folkman J. Human vascular endothelial cells in culture. Growth and DNA synthesis. *Journal of Cell Biology* 1974; **60**: 673.
5. O'Connel KA, Edidin M. A mouse lymphoid endothelial cell line immortalized by simian virus 40 binds lymphocytes and retains functional characteristics of normal endothelial cells. *Journal of Immunology* 1990; **144**: 521.
6. Harder R, Uhlig H, Kathan A, et al. Dissection of murine lymphocyte-endothelial cell interaction mechanisms by SV-40-transformed mouse endothelial cell lines: novel mechanisms mediating basal binding, and $\alpha 4$ -integrin-dependent cytokine-induced adhesion. *Exp Cell Res* 1991; **197**: 259.
7. Tatsuta T, Naiot M, Oh-hara T, Sugawara I, Tsuruo T. Functional involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. *Int Immunol* 1992; **2**: 165.
8. Laulajainen T, Julkunen I, Haltia A, Knuutila S, Miettinen A, Holthofer H. Establishment and characterisation of a rat glomerular endothelial cell line. *Lab Invest* 1993; **69**: 183.
9. Ades EW, Hierholzer C, George V, Black J, Candal F. Viral susceptibility of an immortalized human microvascular endothelial cell line. *Journal of Virological Methods* 1992; **39**: 83.
10. Fickling SA, Tootz JA, J. WGS. Characterization of human umbilical vein endothelial cell lines produced by transfection with early region of SV40. *Exp Cell Res* 1992; **201**: 517.
11. Juillerat-Jeanneret L, Aguzzi A, Wiestler D, Darekar P, Janzer R-C. Dexamethasone selectively regulates the activity of enzymatic markers of cerebral endothelial cell lines. *In Vitro Cell Dev Biol* 1992; **28A**: 537.
12. Renkin EM. Multiple pathways of capillary permeability. *Circulation Research* 1977; **41**: 735.
13. Renkin EM. Cellular aspects of transvascular exchange: A 40 year perspective. *Microcirculation* 1994; **1**: 157.
14. Ghitescu L, Bendayan M. Transendothelial transport of serum albumin: a quantitative immunocytochemical study. *Journal of Cell Biology* 1992; **117**: 745.
15. Haselton FR, et al. Modulation of endothelial paracellular permeability. In: N. Simionescu eds. *Endothelial cell dysfunctions*. New York: Plenum Press, 1992: 103.
16. Wu KK, Thiagarajan P. Role of endothelium in thrombosis and hemostasis. *Ann Rev Med* 1996; **47**: 315.
17. Esmon CT. Molecular events that control the protein C anticoagulant pathway. *Thrombosis and Haemostasis* 1993; **70**: 29.
18. Rozenberg RD, Bauer KA. The heparin-antithrombin system, a natural anticoagulant mechanism. In: R. W. H. Coleman J. Marder, E. W. Salmon, E. W. eds. *Hemostasis and Thrombosis*. Philadelphia, PA: Lippincott, 1994: 837.
19. Rappaport SI, Rao LV. Initiation and regulation of tissue factor-dependent blood coagulation. *Arteriosclerosis and Thrombosis* 1991; **12**: 1111.
10. Moncada S, Vane JR. Pharmacology and endogenous roles of prostaglandin endoperoxides, thromboxane A2 and prostacyclin. *Pharmacological Reviews* 1979; **30**: 293.
21. Kluff C. Constitutive synthesis of tissue type plasminogen activator (tPA) and plasminogen activator inhibitor type 1 (PAI-1): conditions and therapeutic targets. *Fibrinolysis* 1994; **8**: 1.

22. Hinsberg van VWM, Kooistra T, Emeis JJ, Koolwijk P. Regulation of plasminogen activator production by endothelial cells: role in fibrinolysis and local proteolysis. *International Journal Radiation Biology* 1991; **60**: 246.
23. Hinsberg van VWM, Sprengers ED, Kooistra T. Effect of thrombin on the production of plasminogen activators and PA inhibitor-1 by human forskin microvascular endothelial cells. *Thromb Haemostas* 1987; **57**: 148.
24. Moake JL, Turner NA, Stathopoulos NA. Involvement of large plasma vWF multimers in shear-stress induced platelet aggregation. *Journal of Clinical Investigation* 1986; **78**: 1456.
25. Bussolino F, Camussi G. Platelet-activating factor produced by EC. A molecule with autocrine and paracrine properties. *European Journal of Biochemistry* 1995; **229**: 327.
26. Palmer RMJ, Ferrige AG, Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; **327**: 524.
27. Griffith TM, Edwards DH, Lewis MJ, Newby AC, Henderson AH. The nature of endothelium-derived vascular relaxant factor. *Nature* 1984; **308**: 645.
28. Wu KK, Kulmacz RJ, Wang L-H, et al. Molecular biology of prostacyclin biosynthesis. In: G. M. Rubanyi and J. Vane eds. *Prostacyclin: New Perspectives for Basic and Novel Therapeutic Indications*. New York: Elsevier Sci., 1992: 11
29. Mullane KM, Pinto A. Endothelium, arachidonic acid and coronary vascular tone. *FASEB* 1987; **46**: 54.
30. Yanagisawa M, Kurihara H, Kimsura S, al e. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411.
31. De Nucci G, Thomas R, D'Orleans-Juste P. Pressor effects of circulating endothelin are limited by its removal in the pulmonary circulation by the release of prostacyclin and endothelium derived growth factor. *Proc Natl Acad Sci USA* 1988; **85**: 9797.
32. Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multi step paradigm. *Cell* 1994; **76**: 301.
33. Carlos TM, Harlan JM. Leukocyte endothelial adhesion molecules. *Blood* 1994; **84**: 2068.
34. Baumheuter S, Singer MS, Henzel W, et al. Binding of L-selectin to the vascular sialomucin, CD34. *Science* 1993; **262**: 436.
35. Rosen SD, Bertozzi CR. The selectins and their ligands. *Current Opinion in Cell Biology* 1994; **6**: 663.
36. Butcher EC. Cellular and molecular mechanisms that direct leukocyte traffic. *American Journal of Pathology* 1990; **136**: 1.
37. Zimmerman GA, Prescott SM, McIntyre TM. Leukocyte-endothelial cell interactions. *Immunology Today* 1992; **13**:
38. Rothlein R, Marlin SD, Rothlein R, Lawrence MB, McIntyre LV, Anderson DC. Leukocyte adhesion molecules, structure, function and regulation. In: T. A. Springer Anderson, D. C. Rosenthal, A. S. and Rothlein, R. eds. New York: 1990: 170
39. Mantovani A, Bussolino F, Dejana E. Cytokine regulation of endothelial cell function. *FASEB Journal* 1992; **6**: 2591.
40. Cotran RS, Pober JS. Effects of cytokines on vascular endothelium: their role in immune injury. *Kidney International* 1989; **35**: 969.
41. Mantovani A, Bussolino F, Introna M. Cytokine regulation of endothelial cell function: from molecular level to the bedside. *Immunology Today* 1997; **18**: 231.
42. Rozenkranz-Weiss P, Sessa WC, Milstien S, Kaufman S, Watson CA, Pober JS. Regulation of nitric oxide synthesis by proinflammatory cytokines in human umbilical vein endothelial cells. *Journal of Clinical Investigation* 1994; **93**: 2236.
43. Hollenbaugh D, Mischel-Petty N, Edwards CP. Expression of functional CD40 by vascular endothelial cells. *Journal of Experimental Medicine* 1995; **182**: 33.
44. Yellin MJ, Brett J, Baum D. Functional interactions of T cells with endothelial cells. The role of CD40L-CD40 mediated signals. *Journal of Experimental Medicine* 1995; **182**: 1857.
45. Sluiter W, Pietersma A, Lamers JMJ, J.F. K. Leukocyte adhesion molecules on the vascular endothelium: Their role in the pathogenesis of cardiovascular disease and the mechanism underlying their expression. *Journal of Cardiovascular Pharmacology* 1993; **22**: s37.
46. Leeuwenberg JFM, Damme van J, Maeger T, Jeunhomme TMMA, Buurman WA. Effects of tumor necrosis factor on the interferon- γ -induced major histocompatibility complex class II antigen expression by human endothelial cells. *European Journal of Immunology* 1988; **18**: 1469.
47. Bevilacqua MP, Nelson RM. Selectins. *Journal of Clinical Investigation* 1993; **91**: 379.
48. Smith CW, Anderson DC, Taylor, Rossen RD, Entman ML. Leucocyte adhesion molecules and myocardial ischemia. *Trends in Cardiovascular Medicine* 1991; **1**: 167.
49. Palluy O, Morliere L, Gris J, Bonne C, Modat G. Hypoxia/reoxygenation stimulates endothelium to promote neutrophil adhesion. *Free Radic Biol Med* 1992; **13**: 21.

50. Osborn L, Hession C, Tizard R, et al. Direct expression cloning of vascular cell adhesion molecule 1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* 1989; **59**: 1203.
51. Pober JS, Cotran RS. Cytokines and endothelial cell biology. *Physiol Rev* 1990; **70**: 427.
52. Fougerolles de AR, Stacker SA, Schwarting R, Springer A. Characterization of ICAM-2 and evidence for a third counter receptor for LFA-1. *Journal of Experimental Medicine* 1991; **174**: 253.
53. Carlos TM, Harlan JM. Membrane proteins involved in phagocyte adherence to endothelium. *Immunological Review* 1990; **114**.
54. Iademarco MF, Barks JL, Dean DC. Regulation of vascular cell adhesion molecule-1 expression by IL-4 and TNF α in cultured endothelial cells. *Journal of Clinical Investigation* 1995; **95**: 264.
55. Ihrcke NS, Wrenshall LE, Platt JL. Role of heparan sulphate in immune system-blood vessel interactions. *Immunology Today* 1993; **14**: 500.
56. Esmon CT. Cell mediated events that control blood coagulation and vascular injury. *Rev Cell Biol* 1993; **9**: 1.
57. Romano M, Sironi M, Toniatti C, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. *Immunity* 1997; **6**: 315.
58. Vora M, Yssel H, Vries de J E., Karasek MA. Antigen presentation by human dermal microvascular endothelial cells. Immune regulatory effect of IFN γ and IL-10. *Journal of Immunology* 1994; **152**: 5734.
59. Palmer-Crocker RL, Hughes CCW, Pober JS. IL-4 and IL-13 activate the LAK-2 tyrosine kinase and stat-6 in cultured human vascular EC through a common pathway that does not involve the gamma c chain. *Journal of Clinical Investigations* 1996; **98**: 604.
60. Voest EE, Kenyon BM, O'Reilly MS, Truitt G, D'Amato RJ, Folkman J. Inhibition of angiogenesis in vivo by interleukin 12. *Journal of the National Cancer Institute* 1995; **87**: 581.
61. Linke AT, Male DK. Strain specific variation in constitutive and inducible expression of MHC class II, class I and ICAM-1 on rat cerebral endothelium. *Immunology* 1994; **82**: 88.
62. Gumkowski F, Kaminska G, Kaminski M, L.W. M, Auerbach R. Heterogeneity of mouse vascular endothelium. *Blood Vessels* 1987; **24**: 11.
63. Petzelbauer P, Bender JR, Wilson J, Pober JS. Heterogeneity of dermal microvascular endothelial cell antigen expression and cytokine responsiveness in situ and in cell culture. *the Journal of Immunology* 1993; **151**: 5062.
64. Wang JM, Kumar S, Pye D, Vanagthoven AJ, Krupinski J, Hunter RD. A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. *Int J Cancer* 1993; **54**: 363.
65. Pober JS, Cotran RS. The role of endothelial cells in inflammation. *Transplantation* 1990; **50**: 537.
66. Geng GJ, Bevilacqua MP, Moore KL, et al. Rapid neutrophil adhesion to activated endothelium mediated by GMP-140. *Nature* 1990; **343**: 757.
67. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: A dynamic protein ensemble. *Ann Rev Immunol* 1988; **6**: 629.
68. Feldmann M. Cell cooperation in the antibody response. In: I. Roitt, J. Brostoff and D. Male eds. *Immunology*. Barcelona: Times Mirror International publishers limited, 1997: 8.
69. Steinman RM, Young JW. Signals arising from antigen presenting cells. *Current Opinion in Immunology* 1991; **3**: 361.
70. Hughes CCW, Savage COS, Pober JS. The endothelial cells as a regulator of T-cell function. *Immunol Rev* 1990; **117**: 85.
71. Savage CO, Hughes CW, McIntyre BW, Picard JK, Pober JS. human CD4+ T cells proliferate to HLA-DR+ allogeneic vascular endothelium. *Transplantation* 1993; **56**: 128.
72. Marelli-Berg FM, Hargreaves REG, Carmichael P, Dorling A, Lombardi G, Lechler RI. Major histocompatibility complex class II-expressing endothelial cells induce allospecific nonresponsiveness in naive T cells. *Journal of Experimental Medicine* 1996; **183**: 1603.
73. Fuggle SV, McWhinnie DL, Chapman RM, Taylor HM, Morris PJ. Sequential analysis of HLA-class II antigen expression in human renal allografts. *Transplantation* 1986; **42**: 144.
74. Rose ML, Coles MI, Griffin RJ, Pomerance A, Yacoub MH. Expression of class I and class II major histocompatibility antigens in normal and transplanted human heart. *Transplantation* 1986; **41**: 776.
75. Gouw ASH, Huitema S, Grond J, et al. Early induction of MHC antigens in human liver grafts. *American Journal of Pathology* 1988; **133**: 82.
76. Duijvestijn AM, Breda Vriesman van PJC. Chronic renal allograft rejection. *Transplantation* 1991; **52**: 2.
77. Bevilacqua MP. Endothelial-Leukocyte Adhesion Molecules. *Ann Rev Immunol* 1993; **11**: 767.
78. Tanio JW, Basu CB, Albelda SM, Eisen HJ. Differential expression of the cell adhesion molecules ICAM-1, VCAM-1 and E-selectin in normal and post-transplantation myocardium. *Circulation* 1994; **489**: 4.

79. Pelletier RP, Morgan CJ, Sedmak DD, et al. Analysis of inflammatory endothelial changes, including VCAM-1 expression, in murine cardiac grafts. *Transplantation* 1993; **55**: 315.
80. Pelletier R, Ohye R, Kincade P, Ferguson R, Orosz C. Monoclonal antibody to anti VCAM-1 interferes with murine cardiac allograft rejection. *Transplantation Proceedings* 1993; **25**: 839.
81. Paul LC, Davidoff A, Benediktsson H, Issekutz TB. The efficacy of LFA-1 and VLA-4 antibody treatment in rat vascularized cardiac allograft rejection. *Transplantation* 1992; **55**: 1196.
82. Paul LC, Davidoff A, Paul DW, Benediktsson H, Issekutz TB. Monoclonal antibodies against LFA-1 and VLA-4 inhibit graft vasculitis in rat cardiac allografts. *Transplantation Proceedings* 1993; **25**: 813.
83. Sedmak DD, Orosz CG. The role of vascular endothelial cells in transplantation. *Arch Pathol Lab Med* 1991; **115**: 260.
84. Robson SC, Candinas D, Hancock WW, Wrighton C, Winkler H, Bach FH. Role of endothelial cells in transplantation. *International archives allergy Immunology* 1995; **106**: 305.
85. Span AHM, Boven van CPA, Bruggeman CA. The effect of cytomegalovirus infection on the adherence of polymorphonuclear leukocytes to endothelial cells. *European Journal of Clinical Investigation* 1989; **19**: 542.
86. Roberts WH, Sneddon JM, Waldman J, Stephens RE. Cytomegalovirus infection of gastrointestinal endothelium demonstrated by simultaneous nuclei acid hybridization and immunohistochemistry. *Arch Pathol Lab Med* 1989; **113**: 461.
87. Waldman WJ, Knight DA, Adams PW, Orosz CG, Sedmak DD. In vitro induction of endothelial adhesion molecule and MHC antigen expression by cytomegalovirus-activated CD4+ T cells. *Transplantation Proceedings* 1995; **27**: 1269.
88. Bolton EM, Gracie JA, Briggs JD, Kampinga J, Bradley JA. Cellular requirements for renal allograft rejection in the athymic nude rat. *Journal of Experimental Medicine* 1989; **169**: 1931.
89. Ouwehand AJ, Baan CC, Vaessen LM, et al. Characteristics of graft infiltrating lymphocytes after human heart transplantation. HLA mismatches and the cellular immune response within the transplanted heart. *Hum Immunology* 1994; **39**: 233.
90. Yard BA, Hollander den D, Reterink T, et al. Involvement of minor histocompatibility antigens in the rejection of an HLA identical renal transplant from a living related donor. *Transplantation* 1993;
91. Bradley JA, Mowat AM, Bolton EM. Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. *Immunology Today* 1992; **13**: 434.
92. Dunn MJ, Crisp SJ, Rose ML, Taylor PM, Yacoub MH. Anti-endothelial antibodies and coronary artery disease after cardiac transplantation. *The Lancet* 1992; **339**: 1566.
93. Adams H, Tilney NL, Collins JJ, Karnovsky MJ. Experimental graft arteriosclerosis. *Transplantation* 1992; **53**: 1115.
94. Crisp SJ, Dunn MJ, Rose ML, Barbir M, Yacoub MH. Anti-endothelial antibodies after heart transplantation: the accelerating factor in transplant-associated coronary artery disease. *Journal of Heart and Lung Transplantation* 1994; **13**: 81.
95. Joyce S, Flye MW, Mohanakumar T. Characterization of kidney cell-specific, non-major histocompatibility complex alloantigen using antibodies from rejected human renal allografts. *Transplantation* 1988; **46**: 362.
96. Gilks WR, Gore SM, Bradley BA. Renal transplant rejection. Transient immunodominance of HLA mismatches. *Transplantation* 1990; **50**: 141.
97. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MAJ. Interleukin 1(IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *Journal of Experimental Medicine* 1984; **160**: 618.
98. Saadi S, Platt J. Transient perturbation of endothelial integrity induced by natural antibodies and complement. *Journal of Experimental Medicine* 1995; **181**: 21.
99. Camussi G, Bussolino F, Salvidio G, Baglioni C. Tumor necrosis factor/cachetin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *Journal of Experimental Medicine* 1987; **166**: 1390.
100. Vaporciyan AA, Mulligan MS, Warren JS, Barton PA, Miyasaka M, Ward PA. Up-regulation of lung vascular ICAM-1 in rats is complement dependent. *The Journal of Immunology* 1995; **155**: 1442.
101. Kilgore KS, Shen JP, Miller BF, Ward PA, Warren JS. Enhancement by the complement membrane attack complex of tumor necrosis factor-induced endothelial cell expression of E-selectin and ICAM-1. *The Journal of Immunology* 1995; **155**: 1434.
102. Brauer RB, Baldwin III WM, Ibrahim S, Sanfilippo F. The contribution of the terminal complement components to acute and hyperacute allograft rejection in the rat. *Transplantation* 1995; **59**: 288.

103. Baldwin WM, Souillou JP, Claas FHJ, Peyrat MA, Es van LA, Rood van JJ. Antibodies to endothelial antigens in eluates of 88 human kidneys; correlation with graft survival and presence of T- and B-cell antibodies. *Transplantation Proceedings* 1981; **13**: 1547.

2

Production and characterization of spontaneous rat-heart-endothelial-cell lines

Josien G. Derhaag, Adrian M. Duijvestijn, Jef J. Emeis*, Wim Engels#,
Peter J. C. van Breda Vriesman.

Department of Immunology, and Institute of Cardiovascular Diseases#, CARIM,
University of Limburg, Maastricht, The Netherlands, and Gaubius Laboratory
TNO-PG*, Leiden, The Netherlands

Published in *Laboratory Investigation*. 1996, Vol.74, No2, p. 437-451.

Abstract

Endothelial cells (EC) are important regulatory cells in physiology and pathology. *In vitro* studies with rat EC from heart tissue are hampered by laborious isolation and purification procedures, low yield and, limited life span of the cells. Therefore, it is essential to obtain long-term heart endothelial cell lines offering a more adequate *in vitro* system to study rat heart EC. An ex-vivo perfusion model was used to isolate EC from rat heart. Isolation and culture conditions were modified to allow spontaneous development of immortalized rat-heart-endothelial-cell (RHEC) lines. Produced cell lines were tested for endothelial nature using a panel of markers. The selected RHEC lines were subsequently tested for a series of phenotypic and functional properties representative to EC in the context of physiological and inflammatory functions *in vivo*. A series of 3 spontaneous (RHEC) lines were produced out of 13 isolations from Lewis rat hearts: RHEC-3, RHEC-10 and RHEC-11. These lines were stable for more than 30 passages (RHEC-3 even for more than 100). The cell lines were tumorigenic and developed hemangiomas on *in vivo* injection. All 3 lines expressed major-histocompatibility-complex (MHC) class I, but no MHC class II. Intercellular adhesion molecule 1 was only expressed by RHEC-3. Cytokine stimulation induced Vascular cell adhesion molecule 1 in RHEC-3 and RHEC-11, and MHC class II in all 3 lines in different quantities. The phenotypic characteristics of the different RHEC lines resembled the myocardial microvascular endothelium *in situ*. The three lines expressed angiotensin-converting enzyme, and responded to histamine and ATP, but not to thrombin and bradykinin. They constitutively produced small amounts of endothelin, and high levels of tissue plasminogen activator they produced little (after stimulation with phorbol ester PMA) or no von Willebrand factor. The RHEC lines produced thromboxane A₂, but no prostacyclin, on stimulation with arachidonic acid and A23187, they produced prostaglandin E₂. Therefore we conclude the following: 1) The described isolation and culture technique is successful for production of spontaneous stable EC lines from rat heart. 2) RHEC-3, 10, and 11 can be considered a series of different lines representative of the heterogeneity of heart microvascular endothelium *in vivo*. 3) The RHEC lines offer a series of valuable tools to study various heart EC functions and mechanisms in physiology and pathology.

2.1 Introduction

Endothelial cells (EC) are important regulatory cells in various physiological and pathological processes. For example, they are involved in the regulation of vascular permeability and vascular tone through reactivity with vasoactive amines such as histamine and serotonin, and production of endothelin (ET), angiotensin-converting enzyme (ACE), nitric oxide, and prostacyclin respectively (1, 2, 3, 4). They act in hemostatic processes by releasing mediators such as von Willebrand Factor (vWF), tissue-factor, tissue-plasminogen activator (t-PA), and arachidonic acid (AA) products (5, 6, 7, 8). EC also play a role in inflammation and settings of immune reactivity by cell-cell interactions such as recruitment of blood leukocytes through expression of EC adhesion molecules (9, 10, 11, 12) and antigen presentation to T cells through expression of major histocompatibility complex (MHC) class II antigens (13, 14, 15). In addition EC release inflammatory mediators and cytokines, such as platelet-activating-factor, IL-1, and IL-8 (16, 17, 18). As such vascular EC are multipotential cells forming a flexible and regulatory interphase between blood and tissues. Clinical and experimental animal studies *in vivo* have contributed significantly to information on the role of vascular endothelium in physiology and pathophysiology. To extend and precisely dissect the complex mechanisms and potential functions of EC *in vivo*, access to *in vitro* endothelial cell culture systems is required. This led us, in the context of studies in experimental heart inflammation, transplantation and autoimmune models in Lewis rats, to develop endothelial cell cultures from Lewis heart tissue. However, culturing isolated EC from rat tissues and organs including heart is hampered by laborious isolation and purification techniques, low yield, and limiting life span of cell cultures. Therefore, long-term rat endothelial cell lines are extremely desirable. The successful production of endothelial cell lines has been described in human and in murine species, mostly using SV-40 and adenovirus infections (19, 20, 21, 22) or transfections with SV-40 and polyoma virus oncogene-constructs coding for viral T-antigens (23, 24, 25). However, viral inserts in EC may lead to interference with specific cell functions by alteration of intracellular processes. Previously we noticed

(26) that endothelial cell lines occasionally (2 out of 60 isolates) developed spontaneously from senescent cultures of rat heart endothelial cells (RHEC). Herein we describe how a modification of this technique allowed the development of EC lines from Lewis rat heart in a much higher frequency. A series of three EC lines was produced that expressed rat endothelial cell-specific antigen RECA-1 (27), ACE, and VWF, bound lectin GS-I-B4, and internalized DiI-ac-LDL. The RHEC lines were further studied for tumorigenicity, and a set of phenotypic and functional properties relevant to physiological and pathological processes. Phenotypic studies included expression of MHC antigens, as well as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). Functional properties that were tested included the responsiveness to cytokines, and other endothelial cell-activating agents, the production of AA products (prostaglandins (PG), thromboxane (TX)), t-PA, vWF, and ET. The data show that the 3 RHEC lines are heterogeneous in their phenotypic and functional characterization profiles, and can be considered representative for a series of aspects of microvascular heart EC *in vivo*. As such, the RHEC lines can be considered unique tools for further *in vitro* studies regarding various functions and mechanisms of cardiac EC

2.2 Materials & methods

Animals

Lewis rats were obtained from the Central Animal Facility of the University of Limburg. Male rats (8-12 weeks old) were used. Animals were bred under specified pathogen free conditions and had free access to food and water.

Culture media

EC work medium: contained: 90 % M199 (Gibco BRL, Life Technologies B.V., Breda, the Netherlands), 10% (v/v) inactivated fetal calve serum (FCS; Integro, Belgium), 100 units/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamin and 25 mM Hepes.

EC culture medium: contained: 40% RPMI 1640 (Gibco), 40% M199 (Gibco), 20%(v/v) inactivated FCS (Integro), 2mM L-glutamin, 13 MM NaHCO₃, 10 mM Hepes, 20 U/ml heparin, and 0.1 %(v/v) bovine brain extract (14.93 mg/ml) prepared as described by Maciag (28), 50 units/ml penicillin and 50 µg/ml streptomycin and 2 µg/ml Fungizone.

Modified-Krebs-Ringer Solution (MKR): contained 115 mM NaCl, 2.6 mM KCl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄. H₂O, 10 mM NaHCO₃, 10 mM Hepes, 1.1mM Glucose, pH 7.3.

Endothelial cell isolation

EC isolations occurred as previously described (26) with modifications. Briefly, hearts were extirpated under ether anesthesia and rinsed in cold MKR. Hearts were connected with the aorta to the nozzle of a sterile *in vitro* perfusion system after which the right atrium was opened by incision. Hearts were retrogradely perfused with carbogen (95% O₂ plus 5% CO₂)-gassed MKR plus 50 U/ml penicillin, 50 µg/ml streptomycin and 100 mM CaCl₂ (MKR-plus) via the aorta at 37°C for 10 minutes, followed by recirculation perfusion with 50 ml MKR-plus solution containing 163 U/ml collagenase type-II (Wortington Biochemical Corp., New Jersey, USA), for 30

minutes by 37 °C, at a flow rate of 5 ml/minute. The perfusate was collected and centrifuged (200g, 10 minutes, 20 °C). Freshly isolated cells were washed twice with EC work medium, resuspended in EC culture medium and suspensions were seeded on fibronectin precoated, 6 well tissue culture dishes (Costar, Cambridge, USA) and cultured in a humidified, 5% CO₂ incubator at 37°C.

Cell culturing

EC were cultured in a CO₂ incubator at 37°C in EC culture medium. Confluent monolayers were passaged routinely in 1-3 dilution after treatment with 0.25% trypsin/0.2% EDTA in PBS (150 mM NaCl, 8mM Na₂HPO₄.2H₂O, 1.6 mM KH₂PO₄, pH 7.3). All experiments were performed between passages 20 and 30.

EC were tested for uptake of low density lipoprotein (LDL) by incubation of EC monolayers in 96 well culture dishes with 40 µg/ml 1,1'-dioctadecyl-1-3,3',3'-tetramethyl-indo-carbo cyanine-perchlorate-acetylated LDL (DiI-ac-LDL) for 4 hrs at 37 °C. Subsequently, monolayers were washed 3 times with PBS, fixed with 1% paraformaldehyde in PBS, and examined with a fluorescence reverse microscope.

Antibodies and lectins

The following Ab and lectins were used for staining. Mouse mAb RECA-1 (pan endothelial cell specific; 27), OX18 (anti-MHC class I), OX6 (anti-MHC class II), OX8 (NK and T cells, used as a non-relevant isotype-matched control) obtained through the European Collection of Animal Cell Cultures (Salisbury, UK), 1A29 (anti-ICAM-1, donated by Dr. M. Miyasaka (29)), 5F10 (anti-VCAM-I, donated by Drs. R. Lobb and P. Chisholm (30)), and 9B9 (anti-human ACE, crossreactive with rat ACE (Biotrack Incorp. USA). Polyclonal Ab used were rabbit Ab against vWF (DAKO, Denmark), and goat Ab against ACE (kindly donated by Dr. R.L. Soffer, (31)). The lectins used were biotin-labelled BS-I and isolectin BS-I-B4 (Sigma, Belgium). These lectins are derived from *Bandeiraea* or *Griffonia simplicifolia* and are specific for terminal α-galactosyl and for N-acetyl-α-galactosaminyl, as well as for α-galactosyl residues

respectively. Especially isolectin BS-I-B4 is used as an endothelial cell marker. Ab and lectins were used in pretested saturating concentrations.

(Imuno)peroxidase staining

Immunoperoxidase staining was performed on 6 μm frozen heart tissue sections, and on cytocentrifuge preparations of heart EC, using a 2-step immunoperoxidase technique as previously described (27). Cytocentrifuge preparations were made from suspensions of routinely trypsinized confluent EC cultures. EC were either fixed with 1% paraformaldehyde (PFA) in PBS for 30 minutes at 4°C, washed 3 times with PBS and resuspended in EC working medium, or resuspended unfixed. Preparations were made in a cytocentrifuge (Cytospin3, Shandon, England) used at 680 rpm for 10 minutes. Frozen-tissue sections and cytocentrifuge preparations of unfixed cells were acetone-fixed for 10 min, and air-dried. Cytocentrifuge preparations and tissue-section preparations were incubated with the specific Ab or biotin-labelled lectins diluted in 0.05% BSA in PBS for 60 minutes in a humidified chamber at room temperature. The preparations were washed 3 times with PBS for 5 minutes and incubated with either peroxidase-conjugated second-step Ab ((rabbit F(ab)2 anti-mouse IgG, 100 x diluted, Swine F(ab)2 anti-rabbit IgG, 100 x diluted, (Dako, Denmark), or rabbit anti-goat IgG, 100 x diluted (Nordic, The Netherlands)) supplemented with 3% normal rat serum (NRS), or peroxidase-conjugated streptavidin (100 x diluted; Amersham, U.K.) for 1 hour. Subsequently preparations were washed 3 times with PBS for 5 minutes and incubated for 10 minutes with a 0.05 mg/ml diaminobenzidine solution in PBS containing 0.5% H_2O_2 , washed 3 times in tap water and counter-stained with haematoxylin. Preparations were routinely dehydrated and cover slipped using Entellan.

Quantification of percentages of positive cells occurred microscopically on cytocentrifuge preparations by counting a minimum total of 200 cells in at least two fields, or in case of low numbers of positive cells, a minimum of 10 positive cells.

Immunofluorescence staining

Fluorescent staining was performed on viable confluent monolayers of heart EC in 96 well tissue culture plates. After a wash step with M199 containing 10% new born calf serum (NBCS), the cells were incubated with saturating concentrations of specific or isotype-matched control Ab diluted in M199 with 10% NBCS, for 60 minutes at room temperature. The cells were carefully washed 3 times in M199 medium with 10% NBCS, and incubated with a FITC-conjugated second step Ab (goat anti-mouse IgG, rat absorbed, 100 x diluted; Cappel), for 30 minutes. After 3x washing with M199 with 10% NBCS, monolayers were fixed in 1% PFA in PBS and examined under a Zeiss fluorescence reverse microscope.

Flow-cytometry analysis

For flow-cytometry immunofluorescent staining of EC surface antigens was performed in suspensions of routinely trypsinized confluent EC cultures. Cells showed more than 99% viability as tested by trypan blue exclusion. After a wash step with PBS with 0.5% BSA and 1% azide (PBSAA) the cells were incubated with the specific Ab diluted in PBSAA for 30 minutes at 0°C. The cells were washed 3 times in PBSAA and incubated with a FITC conjugated second-step Ab (goat anti-mouse IgG, rat absorbed, 100 x diluted, Cappel), for 30 minutes. After washing 3 times with PBSAA the cells were examined in flow-cytometry using a FACS-Sort (Becton Dickinson, Mountain View, CA, USA).

When immunofluorescent cell surface staining was combined with fluorescent DNA labelling, the cells were first immunostained as mentioned before. Subsequently they were fixed with 1% PFA for 30 minutes, washed 3 times and resuspended in a 10 µg/ml propidium iodid (Calbiochem, La Jolla California. 537059) solution in PBS containing 10 µg/ml RNase for 15-30 minutes at 0°C, and examined by two color flow cytometry in a FACS-Sort (Becton Dickinson).

Cytokine stimulation

For cytokine stimulation experiments the RHEC lines were stimulated with either 400 U/ml human tumor-necrosis-factor (TNF)- α (kindly donated by Prof Dr W. Fiers) or 200 U/ml rat interferon (IFN)- γ (kindly donated by Dr P van de Meide). Cytokines were diluted in EC culture medium and incubated on confluent monolayers of RHEC cultures for 32 hr. Experiments were performed in triplicate.

Tissue plasminogen activator and von willebrand factor measurements

tPA and vWF production were measured in the culture supernatant of confluent EC monolayers. Confluent monolayers had been incubated with EC culture medium alone (5 ml/25 cm²) or culture medium containing histamine (3 μ M) or phorbol-meristate-acetate (PMA; 30 μ g/ml). After 4, 8, 16, and 32 hrs supernatants were collected and stored at -70°C. tPA and vWF were measured with ELISA as previously described (32, 33). Data for tPA were expressed in ng/ml, and for vWF in U/ml, 100 U being equivalent to the amount of vWF present in 1 ml of pooled rat plasma.

For each time point, tPA values were compared for the three EC lines, using the Kruskal-Wallis test. Values of non-stimulated and stimulated cultures were compared using the Mann Whitney U-test. Differences were considered significant (two tailed) for $p < 0.05$.

PG and TX measurements

PG production was measured in culture supernatants of confluent monolayers. Confluent monolayers were incubated with fresh EC culture medium (without FCS and ECGF) alone (5 ml/25 cm²) or with AA (80 μ M), or with both AA and calcium ionophore A23187 (10 μ M) for 30 minutes. Supernatants were collected and stored at -70°C. PG I₂ (determined as the stable metabolite 6-keto F_{1a}) PGF_{2a}, PGE₂ and TX A₂ (TXA₂, determined as the stable metabolite TXB₂) were measured as previously described (34). In brief, supernatants were purified using C18 reversed-phase chromatography. The eluted PG/TX fractions were methoxymated, esterified with panaxyl bromide, and analyzed by high-performance liquid chromatography with fluorimetric detection.

Values of non-stimulated and stimulated cultures were compared using the Mann-Whitney U-test. Differences were significant (two-tailed) for $p < 0.05$

Calcium mobilization studies

To test whether the RHEC lines were responsive to various endothelial cell-stimulating agents, cells were stimulated with either thrombin, histamine, bradykinin, or ATP, and cellular mobilization of calcium ([Ca⁺⁺]_i) was measured using the calcium-sensitive fluorescence probe fura-2 acetoxymethyl ester, according to Van Haaster et al (35) with minor modifications. In brief: confluent monolayers of RHEC lines were washed with buffer A (10 mM HEPES, 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1mM MgCl₂, 1mg/ml glucose, 4 μ M L-glutamin) containing 0.25% BSA, and incubated with 1 μ M fura-2 in buffer A with 1% BSA for 45 min at 37 °C. Subsequently monolayers were washed and incubated with EC culture medium for 15 min. Cultures were incubated with 0.05% EDTA until detachment of cells and resuspended in buffer A containing 0.25% BSA. Cellular mobilization of Ca⁺⁺ in EC suspensions after stimulation with different concentrations of either thrombin, histamine, bradykinin, or ATP was measured in cuvetts using a spectrofluorometer (SLM Aminco) at 500 nm emission wave length. The change in ratio of fluorescence at 340 and 380 nm excitation

before and after stimulation was used to calculate the $[Ca^{++}]_i$. Calibration values for the calculation of $[Ca^{++}]_i$ were collected after addition to the suspensions of 1% (w/v) Triton X-100, followed by 1 mM EGTA according to the procedure of Grynkiewicz et al (36).

ET measurements

ET production was measured in culture supernatant of confluent EC monolayers after 4 and 16 hr using a radioimmunoassay-kit (Nichols Institute Diagnostics B.V., Wycken, The Netherlands). In brief: after acidification and extraction over C18 columns endothelin was measured in a sensitive and specific competitive protein-binding radioimmunoassay (37).

Tumorigenicity studies

EC from the three different cell lines were routinely trypsinized, washed in PBS and resuspended in PBS to 8×10^7 cells/ml. The three EC lines were individually injected in volumina of 80 and 160 μ l intra cutaneously in the ventral side of the body of syngeneic male Lewis rats. Tumor growth was examined every 3 days for 1 month. Between 2 and 3 weeks the tumors from some of the animals were collected and snap frozen in isopentane or fixed in 4% PFA and routinely paraffin-embedded.

Electron microscopy

EC suspensions from confluent monolayers were pelleted by centrifugation, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer for 30 min. Fixed cells were resuspended in warm 4% agarose in PBS and cooled on ice for 20 minutes. Coagulated agarose with EC was fixed in 2.5 % glutaraldehyde in 0.1M phosphate buffer for 20 min, washed in 0.1M phosphate buffer, fixed with 1% OsO₄ for 1 hour and embedded in Epon. Ultrathin electron microscopy sections were counterstained with uranyl acetate and lead citrate, and examined in a CM10 Philips electron microscope.

2.3 Results

2.3.1 Production of EC Lines

We used an *ex vivo* perfusion model to isolate heart EC. In an initial set of experiments we noticed that retrograde collagenase perfusion with an increased flow/pressure gave a higher cell yield (probably as a result of increased damage of the vascular integrity and thus better access for collagenase to digest matrix structures). However, this also led to increased contamination with non-endothelial cells (fibroblasts) easily leading to overgrowth of EC by these cells. An essential step in the production of cell lines was the low seeding of the rather pure EC isolates, which allowed establishment of EC lines from the surviving clones in the primary cultures. We therefore chose for a low perfusion flow rate of 5 ml/min, half of that previously used (26). A set of 13 isolation experiments was done to produce lines. Cell isolates were seeded and formed small foci of proliferating cells in 1 week. After 2-3 weeks in more than 50% of the experiments some of the foci had grown to distinct cell clones, whereas other foci were senescent. Clones mostly had endothelial (cobble-stoned) morphology and, because of the low numbers of clones per surface area, they expanded mostly as individual and separate clones. Contaminating spindle-shaped fibroblast-like cells were frequently present. If distinct fibroblast clones appeared in primary cultures, they were removed using a cell scraper. EC clones were allowed to grow further for another 1-2 weeks and then the primary cultures were passaged for expansion. During the first passages, uptake of DiI-ac-LDL was measured to identify the EC nature of growing clones (Fig. 1A & B). When fibroblast contamination remained after passaging, these cells were seen to grow adjacent to EC clones, but mostly remained separated from EC clones. During trypsinization fibroblasts detached differently from EC clones (in general more quickly), and thus could be separated from EC by a wash step. Five out of the thirteen isolation experiments led to development of lines. Three out of these five lines met the defined EC criteria by being positive in DiI-ac-LDL uptake, and by expressing endothelial cell-specific antigen RECA-1 and vWF, as shown by immunostaining of the cells on cytocentrifuge preparations (Fig. 1C&D).

Two other lines did not show distinct RECA-1 expression and were excluded. The 3 selected lines were designated RHEC-3, RHEC-10, and RHEC-11. RHEC-3 was cultured for more than 100 passages, and RHEC-10 and -11 for more than 30 passages. The RHEC lines were free of mycoplasma and when studied ultra structurally, particularly to check for presence of virus, no cellular abnormalities or indications for the presence of virus were observed.

Figure 1: A and B show a primary culture of isolated rat heart EC. A) Clone of EC marked by uptake of DiI-acetylated LDL. Note the granular cytoplasmic staining pattern. B) Phase contrast microscopy showing the same EC clone surrounded by contaminating non-endothelial cells. C and D are cytocentrifuge preparations of PFA-fixed suspensions of RHEC lines stained by immunoperoxidase. C) RECA-1 staining of RHEC-3 showing all cells to express the antigen in variable intensity. D) Staining for vWF in RHEC-10. Only part of the cells in the RHEC lines express intracellular vWF in a granular staining pattern. Note the presence of strongly (single arrow) and weakly (double arrow) positive cells. E and F are cytocentrifuge preparations of fresh suspensions of RHEC lines. Preparations were acetone-fixed and stained by immuno- or lectinperoxidase. E) Staining of RHEC-11 with lectin GS-I-B4. All cells show diffuse staining. F) Staining of RHEC-3 with mAb 9B9 directed against ACE. All cells are stained in a fine spicular pattern. x 625.

2.3.2 Tumorigenicity of the RHEC Lines

The mechanism of spontaneous immortalization of EC in primary cultures is unknown. To test whether the immortalized RHEC lines had tumorigenic potentials, as described for viral-transformed EC lines, the 3 lines were inoculated intracutaneously in syngeneic Lewis rats. Tumor development was checked by palpation every 3 days over a period of 1 month. All three RHEC lines developed distinct tumors in a dose-dependent way at the site of inoculation. The size of the tumors varied per cell line, and reached a maximum of about 0.5-1 cm in diameter in approximately 3 weeks. The tumors subsequently developed signs of necrosis. Histologic examination of tumor tissues showed that all three RHEC lines developed vascular tumors that could be classified as capillary to cavernous hemangiomas. In the hemangiomas clumps, or large lobular structures of poorly differentiated EC, sometimes with mitotic figures, were seen (Fig. 2 A&B). Ab and lectin staining studies of frozen tumor material showed similar results for the 3 lines. The lobular tumor fields were diffusely positive for GS-I-B4, whereas RECA-1 staining was seen in the tumor structures only in EC of intranodular blood vessels and along the peripheral cell layer lining blood sinuses and cavernosa (Fig. 2 B&C). Expression of vWF was scarce or absent and, if seen, present in blood-lining EC. VCAM-1 and MHC class II were not expressed by EC in the hemangiomas, whereas ICAM-1 staining was occasionally observed locally in blood-lining endothelium.

Figure 2: Haemangioma development of RHEC-3 at day 17 after intracutaneous inoculation. A and B are paraffin sections stained with H&E. A) In the subcutaneous tissue an endothelial cell tumor is seen with a lobular architecture. In and around the tumor cell fields small and large blood vessels are seen with a lining of plump EC along which adherent leukocytes can be seen (arrows). x 250 B) In the dense tumor cell mass poorly differentiated EC with moderate cellular and nuclear polymorphism are observed. A few mitotic figures are seen (single arrows), and locally capillary formation is shown (double arrow).x 625. C and D are sections from frozen material, peroxidase-stained. C) RECA-1 staining, showing that in the haemangioma only the EC lining intranodal blood spaces and vessels are positive. EC in dense tumor fields (asterisk) are negative. D) Lectin GS-I-B4 staining shows tumor cells are diffusely positive. Notice the absence of staining in non-endothelial vascular tissue (arrow). x 250.

2.3.3 Phenotypic Characterization of the RHEC Lines

Endothelial nature of the lines**Phenotypic characterization of Rat-Heart-EC lines**

Structure	Cell distribution	% positive cells ¹ ($\pm$ S.E.)		
		RHEC-3	RHEC-10	RHEC-11
RECA-1	Surface ²	>99	>99	>99
vWF	Intracellular	14.7 ($\pm$ 7.6)	8.1 ($\pm$ 6.5)	12.9 ($\pm$ 7.7)
GS-I	N.D.	>99	>99	>99
GS-I-B4	N.D.	>99	>95	>99
ACE ³	N.D.	>99	>99	>99
LCA	N.D.	<1	<1	<1
ICAM-1	Surface	11.5 ($\pm$ 3.5)	<1	<1
VCAM-1	N.A.	<1	<1	<1
MHC-I	Surface	>99	>99	>99
MHC-II	N.A.	<1	<1	<1

Table 1:

¹ Percentages of positive cells were measured in immunoperoxidase-stained cytocentrifuge preparations. Negative-control staining, performed with second step only and with non-relevant isotype-matched mAb OX-8, was negative.

² Cell surface expression was detected by immunofluorescence staining of viable EC monolayers.

³ As tested with mAb 9B9 against ACE.

N.A., not applicable; N.D., not done; RECA-1, Rat Endothelial Cell-specific Antigen; LCA, leukocyte-common-antigen; ICAM-1, Intracellular Adhesion Molecule-1; VCAM-1, Vascular Cell Adhesion Molecule-1; MHC-I/II, Major Histocompatibility Complex antigen-I/II; vWF, von Willebrand Factor.

The RHEC lines were screened for expression of various EC markers. In addition to the uptake of DiI-ac-LDL, lines were tested for staining with Ab to EC-specific RECA-1, vWF, ACE, and with lectin GS-I and specific isolectin GS-I-B4 (table 1). RECA-1 was expressed by all cells in the RHEC cultures (indicated as > 99% positive). Granular intracellular expression of vWF was seen in some of the cells (10-15%) in the three lines. Expression of ACE was detected using mAb and polyclonal Ab

against human ACE that cross-reacted with rat ACE. Staining with mAB 9B9 showed fine spicular positivity of all cells in the RHEC lines (Fig. 1E). Polyclonal anti-ACE showed >95% positive cells in the 3 lines, with staining intensity varying from spicular to intense diffuse. All cells in the RHEC lines were positive for binding lectin GS-I and isolectin GS-I-B4 (a marker for EC; fig. 1F). Only in RHEC-10 the intensity of cells stained with GS-I-B4 varied, and a small percentage negative cells was observed. When RHEC cultures were stained with mAb OX-1, directed to a rat leukocyte common antigen, <1% positive cells was found, demonstrating the non-leukocyte nature of the lines.

Expression of adhesion molecules and MHC antigens.

In addition to phenotypic markers defining EC nature of the cell lines, RHEC lines were tested for expression of ICAM-1 and VCAM-1 as well as for MHC class I and class II antigens. These molecules, which are important structures in the interaction between EC and leukocytes, and in particular lymphocytes, can be expressed by EC in various inflammatory and immunological settings. To test cell-surface expression of these molecules, viable EC monolayers were stained by immunofluorescence using specific monoclonal Ab (table 1 and fig. 3). Under standard culture conditions the RHEC lines showed surface positivity for MHC class I, but MHC class II and VCAM-1 were not expressed. ICAM-1 could be detected only on part of the cells in RHEC-3. The percentages of positive cells calculated from immunoperoxidase-stained cytocentrifuge preparations are presented in table I. It shows that ICAM-1 is expressed only by RHEC-3 in approximately 12% of the cells.

To test whether adhesion molecule and MHC class II antigen expression were inducible in the RHEC lines by cytokine stimulation, confluent cultures were stimulated with either 400U/ml TNF α or 200 U/ml IFN γ . ICAM-1, VCAM-1, and MHC class II expression were measured in cytocentrifuge preparations of cultures stimulated for 32 hr, a time point at which (as indicated by initial studies) the rather rapidly induced adhesion molecules as well as the more slowly induced MHC class II antigens could be

detected. As shown in table 2, cytokine stimulation of RHEC-3 led to increased numbers of cells expressing ICAM-1, and it induced VCAM-1 and MHC class II in some of the cells. $\text{TNF}\alpha$ was most effective in adhesion molecule induction, whereas $\text{IFN}\gamma$ was more potent in MHC class II induction. RHEC-10 stimulation resulted only in a minor expression of MHC class II. RHEC-11 showed a distinct induction of VCAM-1 and MHC class II after stimulation. MHC class I was expressed by all cells in the lines in these experiments.

Cytokine stimulation of RHEC lines

Structure	Stimulation	Expression ¹ at 32 hr		
		RHEC-3	RHEC-10	RHEC-11
ICAM-1	none	+	-	-
	$\text{TNF}\alpha$	++	-	-
	$\text{IFN}\gamma$	+	-	-
VCAM-1	none	-	-	-
	$\text{TNF}\alpha$	+	-	+
	$\text{IFN}\gamma$	±	-	+
MHC class II	none	-	-	-
	$\text{TNF}\alpha$	±	-	-
	$\text{IFN}\gamma$	++	±	+
MHC class I	none	++++	++++	++++
	$\text{TNF}\alpha$	++++	++++	++++
	$\text{IFN}\gamma$	++++	++++	++++

Table 2: Confluent monolayers of RHEC cultures were stimulated with either 400 U/ml $\text{TNF}\alpha$ or 200 U/ml $\text{IFN}\gamma$ for 32 hr (n=3). Expression was measured by immunostaining of cytocentrifuge preparations of paraformaldehyde-fixed cells.

¹ expression categories: -, <1% positive cells; ±, 1-10%; +, 10-30%; ++, 30-60%; +++, 60-99%; +++++, >99%.

Figure 3: Monolayers of confluent viable RHEC lines stained by immunofluorescence. A) RHEC-11 stained with RECA-1. All cells show variable intensity of cell-surface expression. B) Staining for MHC class I antigens on RHEC-10. All cells are positive in a staining pattern comparable to that of cells stained with RECA-1. C) ICAM-1 staining on RHEC-3. Only part of the cells express ICAM-1. D) Phase-contrast microscopy of a monolayer of RHEC-3 cells, x 625.

ICAM-1 expression by RHEC-3 cells is not related to cell cycle stage.

Although confluent RHEC monolayers were used for phenotypic studies, we noticed that some cells with mitotic figures were present, and thus that cell cycling occurred in confluent cell cultures. The fact that ICAM-1 expression under normal culture conditions was seen in only part of the RHEC-3 cells (Fig. 4A) led us to investigate whether expression was related to cell cycle stage. FACS analysis of RHEC-3 cells that had been DNA-labelled with propidium-iodid showed that the total cell population consisted of G1, S, and G2 cells. When RHEC-3 cells were double-stained for ICAM-1 and DNA, the gated population of ICAM-1 positive cells showed a cell cycle profile comparable with that of the total population (Fig. 4B) Apparently, ICAM-1 expression in RHEC-3 cells is not related to a particular cell cycle stage of the cells.

Figure 4: Flow-cytometry analysis of RHEC-3 cells. A) ICAM-1 immunofluorescence analysis showing that RHEC-3 consists predominantly of ICAM-1 negative cells (peak 1) and a small population of ICAM-1 positive cells (peak 2). B) The DNA profile of the total RHEC-3 population (upper black curve) and of the ICAM-1 positive RHEC-3 population (lower grey curve) was measured. The peaks 1 represent cells in the G₁ phase, the peaks 2 cells in the G₂ and M phases. Cells between peak 1 and 2 are in the S phase. As noticed, comparable DNA profiles are found for the total and for the ICAM-1 positive population. This indicates that ICAM-1 expression is random over the RHEC-3 cells in the various cell cycle stages.

Phenotype of RHEC lines in comparison to heart vascular EC in situ.

To relate the phenotypic profiles of the RHEC lines to that of vascular EC in heart tissue, we did staining on normal heart-tissue sections for ICAM-1, VCAM-1, MHC class I and II molecules, and vWF. RECA-1 and lectin GS-I-B4 staining were used as a reference pattern identifying all vascular endothelium (Fig. 5A). MHC class I staining showed a similar pattern as that found for RECA-1 and GS-I-B4, indicating distinct endothelial MHC-I expression in the entire blood vasculature. MHC class-II molecules were not detected on vascular EC in normal heart. Class II positivity was noticed on a population of scattered cells of non-endothelial origin, most likely macrophages or dendritic cells (Fig. 5B). ICAM-1 was expressed by endothelium of

some, predominantly small blood vessels in heart sections (Fig. 5C), whereas VCAM-1 was only occasionally seen on some EC of large vessels. Endothelium of all arteriolar and large venular vessels showed granular expression of vWF. This in contrast to the myocardial small vessels, only some of which showed vWF expression in section staining as described previously (27)(Fig. 5D). The data show that the phenotypic profiles of the three RHEC lines resemble the heterogenous heart endothelium in situ, and particularly the microvascular myocardial endothelium.

Figure 5: Immunoperoxidase staining on heart tissue-sections. A) RECA-1 staining marks all endothelium including that of the myocardial microvasculature as shown here. B) Staining for vWF indicates that (in comparison with RECA-1 staining) only part of the microvascular EC express vWF. C) Staining for MHC class II antigens reveals that class II molecules are absent on heart endothelium. Notice class II staining of a population of scattered non-endothelial cells (most likely macrophages or dendritic cells). D) ICAM-1 staining shows that only a few of the myocardial blood vessels have ICAM-1 positive EC, x 250.

2.3.4 Functional Characterization of the RHEC Lines

To address functional capacities of RHEC-3, 10, and 11, the lines were studied for production of a selected set of endothelial products, and for response to activating agents relevant to processes such as hemostasis, inflammation and vascular tone .

Production of t-PA and vWF.

Monolayers of RHEC lines were tested for production of t-PA and vWF in culture supernatants at 4, 8, 16, and 32 hrs. Although all three the RHEC lines had granular vWF storage, as shown by immunostaining, no vWF could be detected in the cell supernatants under normal culturing conditions. The RHEC lines constitutively produced distinct levels of t-PA (Table 3). To test whether activation could increase vWF and t-PA production, the RHEC lines were stimulated with 3 μ M histamine and 30 μ g/ml PMA. Only PMA stimulation gave a minor production of vWF (0.2-0.4 U/ml) by RHEC-3 and RHEC-11 but not RHEC-10 over 32 hrs (data not shown). PMA also significantly increased t-PA production in all three RHEC lines, whereas histamine stimulation had no measurable effect on t-PA production (Table 3).

PG and TX production.

RHEC lines were tested for production of eicosanoids of the cyclooxygenase pathway, such as prostacyclin (PGI_2), PG F_{2a} , PG E_2 , and TXA_2 . In a previous set of dose-response experiments the production of eicosanoids by primary rat heart EC cultures upon stimulation with AA and calcium ionophore A23187 were measured (38). Already with low micromolar concentrations of AA an increase in eicosanoid production was measured, reaching a maximum between 40-80 μ M. A combination of AA with A23187 in general gave similar but earlier production levels. Based on these results we tested the RHEC lines for production of prostanoids. To ensure maximal production, confluent EC cultures were stimulated for 30 min with 80 μ M AA, followed by analysis of PG and TX production in culture supernatants. As shown in Table 4, RHEC lines produced only low amounts of PGF_{2a} and TXA_2 under standard

culture conditions, whereas incubation with AA induced PGE₂ production and significantly increased PGF_{2a} and TXA₂ production. Production of PGI₂ could not be demonstrated. Incubation with AA in combination with calcium ionophore A23187 raised production levels of some of the eicosanoids, but no production of PGI₂ by the RHEC lines was observed.

Tissue-Plasminogen Activator production by RHEC lines

Exp	n	Incubation time in hrs.	Stimulation	t-PA production in ng/ml ($\pm$ S.E.)			note
				RHEC-3	RHEC-10	RHEC-11	
I	3	4	-	5.4 ($\pm$ 0.8)	5.4 ($\pm$ 0.2)	4.5 ($\pm$ 0.3)	2
		8	-	9.9 ($\pm$ 2.0)	10.2 ($\pm$ 0.2)	8.7 ($\pm$ 1.6)	
		16	-	15.9 ($\pm$ 3.2)	17.8 ($\pm$ 1.3)	14.0 ($\pm$ 1.3)	
		32	-	21.8 ($\pm$ 5.7)	22.3 ($\pm$ 5.9)	23.0 ($\pm$ 2.7)	1
II	1	4	Histamine ³	3.9	4.6	4.2	4
		8	Histamine	8.2	9.8	9.0	
		16	Histamine	15.6	19.8	18.2	
		32	Histamine	24.0	30.2	29.0	
III	1	4	PMA ⁵	3.9	7.8	5.1	6
		8	PMA	11.4	25.8	16.6	
		16	PMA	33.0	36.4	33.0	
		32	PMA	40.4	56.6	47.6	

Table 3: Culture supernatants were collected at 4, 8, 16 and 32 hrs from RHEC lines non-stimulated (Exp 1), or stimulated with histamine (Exp 2) or PMA (Exp 3). t-PA in supernatants was measured by ELISA. Each t-PA value in the table is the average of a triplicate.

1) $n=2$.

2) Differences in t-PA production per time point between the RHEC lines were not significant (Kruskal Wallis test)

3) Histamine concentration was 3 mM.

4) No significant differences (Mann-Whitney U-test) were found per time point between the group of combined values of t-PA production from the 3 RHEC lines after histamine stimulation and the group of combined values from the non-stimulated 3 RHEC lines (Exp 1)

5) PMA concentration was 30 ng/ml.

6) From $t=8$, differences were significant ($p < 0.01$) between the group of t-PA values from the combined 3 RHEC lines after PMA stimulation and the control group of combined t-PA values from non-stimulated RHEC lines. Since histamine did not stimulate t-PA production, these data were included in the control group.

Prostaglandin and thromboxane production by RHEC lines

RHEC line	Stimulation	Eicosanoid production in ng/ml ($\pm$ SD)			
		PGI ₂	PGF _{2a}	TxA ₂	PGE ₂
RHEC-3	none	0	1.6 ($\pm$ 0.3)	1.9 ($\pm$ 0.1)	0
	AA	0	3.8 ($\pm$ 0)	24.7 ($\pm$ 1.3)	7.3 ($\pm$ 1.4)
	AA + A23187	0	5.5 ($\pm$ 0.3)	31.5 ($\pm$ 0.7)	10.0 ($\pm$ 0.5)
RHEC-10	none	0	0.8 ($\pm$ 0.4)	2.4 ($\pm$ 0.9)	0
	AA	0	3.5 ($\pm$ 0.1)	33.9 ($\pm$ 4.7)	9.6 ($\pm$ 0.1)
	AA + A23187	0	5.2 ($\pm$ 0.2)	27.6 ($\pm$ 6.2)	11.1 ($\pm$ 1.7)
RHEC-11	none	0	0.8 ($\pm$ 0.1)	2.4 ($\pm$ 0.5)	0
	AA	0	5.0 ($\pm$ 0.1)	35.6 ($\pm$ 1.9)	15.3 ($\pm$ 0.3)
	AA + A23187	0	5.4 ($\pm$ 0.1)	32.8 ($\pm$ 4.0)	15.9 ($\pm$ 3.2)

Table 4: Eicosanoids were measured in culture supernatant of confluent endothelial monolayers cultured for 30 min with : medium alone (-), medium with 80 μ M arachidonic acid (AA), or medium with 80 μ M AA and 10 μ M calcium ionophore A23187 (AA + A23187). Data are corrected for background levels in medium without cells, and are given in means from duplo experiments. Per eicosanoid the combined values of AA-stimulated cells from the 3 RHEC lines were compared with those from non-stimulated cells using the Mann-Whitney U-test. The same was done for comparing values of (AA + A23187)-stimulation with those of AA-stimulation. Production of PGF_{2a}, TxA₂ and PGE₂ was significantly increased ($p < 0.01$) after stimulation with AA or (AA + A23187). Additional effects of A23187 on AA stimulation were significant only for PG-F_{2a} ($p < 0.01$).

ET production

To test whether RHEC lines produced the endothelial vasoconstricting agent ET, supernatants of confluent cultures were collected at 4, 8, and 16 hr and measured for ET quantities using a radioimmuno-assay. As shown in Table 5, the three RHEC lines constitutively produced small levels of ET. Over the time period of 16 hours, RHEC-3 was the highest producer of ET.

Endothelin production by RHEC lines

RHEC line	Endothelin in pg/ml culture supernatant		
	t=4 hr	t=8 hr	t=16 hr
RHEC-3	6.4	3.8	21.9
RHEC-10	4.9	5.4	14.8
RHEC-11	4.4	5.4	11.9

Table 5: Endothelin was measured by radio immunoassay in culture supernatant of RHEC lines in single experiments. Values were corrected for background levels in fresh culture medium (< 0.1 pg/ml).

Activation of RHEC lines by endothelial cell-stimulating agents

The RHEC lines were studied for responsiveness to agents with endothelial cell stimulating potentials. Therefore EC cultures were stimulated with thrombin, histamine, bradykinin, and ATP at physiological and higher concentrations, followed by fluorometric measurement of cellular mobilization of calcium ($[Ca^{++}]_i$ in nM). The 3 RHEC lines showed a dose-dependent response to stimulation with histamine in concentrations of 3-100 μ M. The lines also responded to stimulation with ATP concentrations of 10 and 100 μ M, but no response was observed to thrombin and bradykin, even not to rather high concentrations of 10 nM and 20 μ M respectively. In table 6 an experiment is presented illustrating the dose-response effects of histamine and

ATP stimulation for the three RHEC lines. Apparently the RHEC lines express functional receptors for histamine and ATP, but not for thrombin and bradykinin.

Calcium mobilization in RHEC lines by activating agents

Activating agent	[Ca ⁺⁺] _i (nM)		
	RHEC-3	RHEC-10	RHEC-11
Thrombin			
5 nM	0	0	0
10 nM	0	0	0
Histamine			
3 μ M	125	39	139
50 μ M	424	288	367
100 μ M	569	467	632
Bradykinin			
2 μ M	0	0	0
20 μ M	0	0	0
ATP			
10 μ M	320	265	477
100 μ M	474	492	553

Table 6: Cellular CA⁺⁺ mobilization ([Ca⁺⁺]_i) was measured in EC suspensions by fluorometry. Data are results from one experiment.

2.4 Discussion

We have produced and characterized three spontaneous endothelial cell lines from Lewis rat heart tissue. A modified isolation and culture technique was used to favor EC line establishment. In previous studies (26) only few isolations led to EC cell line production (2 out of 60). In the experiments presented here a significant increase in the frequency of successful production of spontaneous EC lines was obtained, e.g. 3 out of 13 isolations (23 %) led to an established RHEC line. The high EC purity of isolated cells in combination with the low seeding in tissue culture plates appears an essential improvement for development of spontaneous EC lines. Apparently, it allows the few spontaneously immortalized EC in senescent primary cultures to expand and give rise to a permanent cell line.

The molecular mechanisms of spontaneous immortalization of EC are, are currently unknown. There is evidence that immortalization may result from genetic mutations with karyotypic alterations, such as chromosomal trisomy (26)(39). No ultrastructural evidence was found proving that EC immortalization was induced by virus infection. The fact that all three RHEC lines were tumorigenic and developed hemangiomas on *in vivo* injection, as do EC lines induced by viral transfection (40), may indicate that both mechanisms of immortalization have comparable cellular effects.

All EC in the RHEC lines expressed RECA-1, ACE, bound lectin GS-I-B4, and they were positive for DiI-ac-LDL uptake, demonstrating their EC nature. The facts that 1) within the lines only a small part of the cells showed vWF staining, and 2) that variable numbers of cells expressed adhesion molecules and MHC class II antigens after cytokine stimulation indicate the heterogeneity of the heart EC. It is not clear yet whether heterogeneity is a result of the presence of distinct EC sub-populations or of intrinsic differentiation in an EC culture during growth to confluency. Long-term culturing of RHEC-3 for more than 100 passages, which allows growth competition between different populations, and thus selects for a particular subpopulation, did not alter percentages of cells expressing vWF. This suggests that heterogeneity within a line is the result of intrinsic differentiation within a RHEC culture. Heterogeneity, at least

for ICAM-1 expression in RHEC-3, is not explained by a difference in cell cycle stage of EC within a culture, because FACS analysis of the subpopulation of RHEC-3 cells expressing ICAM-1 showed a cell cycle profile that was similar to that of the whole population. With respect to the phenotypic profiles found for the 3 RHEC lines, it is important that a close resemblance with heart endothelium *in situ* was noticed. All endothelia were RECA-1 and MHC-I positive, but MHC-II negative, and bound GS-I-B4. The vWF was expressed by all large vessel endothelia but only by part of the myocardial microvascular endothelia. In previous studies (41) we described the absence or scarce presence of vWF in microvascular vessels of various rat organs including heart. For vWF, data from *in vitro* studies show that indeed microvascular EC have, in contrast to macrovascular EC, scant or absent vWF granules (42). VCAM-1 expression is barely seen and ICAM-1 only by some microvascular and sometimes also macrovascular endothelium in the rat heart *in situ*. In this respect RHEC-3 is an interesting line because it is the only one that showed constitutive ICAM-1 expression by part of the cells. Another study demonstrated that in rat cerebro-microvascular EC lines, only a subpopulation of cells (about 30 %) constitutively expressed ICAM-1 (43). The RHEC lines responded to cytokine stimulation with rather different effects on induction of ICAM-1, VCAM-1, and MHC class II. In contrast to RHEC-3, which showed induction of ICAM-1, VCAM-1, and MHC class II, RHEC-10 was rather insensitive to cytokine stimulation (i.e., only minor expression of MHC class II was observed). RHEC-11 expressed VCAM-1 and MHC class II on cytokine stimulation. Our phenotypic data suggest that the three RHEC lines form a set of EC lines representative for the heterogeneity of heart endothelium *in vivo*, and in particular for microvascular heart endothelium. We cannot exclude the possibility of an endocardial origin of EC in the RHEC lines, however, this is unlikely, because the initial heart perfusion route to isolate heart EC does not directly include endocardial endothelium.

The data on calcium mobilization by the RHEC lines upon stimulation show that the lines are functional in responsiveness to histamine and ATP, although for histamine a rather low effect was measured at the lowest (physiologic) concentration of 3 μ M. As

such, the observed Ca^{++} mobilization demonstrates maintenance of histamine and ATP receptors by the lines, and suggests that effector mechanisms through these receptors are operable. The absence of response to thrombin and bradykinin suggests the absence of receptors. It is unlikely that detachment of EC cultures with EDTA, to obtain cell suspensions for measurements, is the cause of thrombin or bradykinin receptor loss. After similar de-attachment of HUVEC cultures, the EC suspensions still were responsive to stimulation (data not shown). Thus, most likely specific receptor loss resulted from long-term culturing and passaging of the lines. All the RHEC lines constitutively produced ET, albeit in low quantities compared with macrovascular HUVEC which produce about 10 times more ET *in vitro* (44). Studies comparing endothelin production between macrovascular and microvascular bovine EC showed a 10 to 30 times higher ET production by macrovascular cells (45, 46). The low level ET found for the RHEC lines thus supports their microvascular nature. Also, the high t-PA quantities produced by the RHEC lines indicate a microvascular nature of the cells. High t-PA, production is a property ascribed to microvascular EC (7, 47). Macrovascular EC, such as HUVEC, produce much lower quantities of t-PA (7). Whereas for HUVEC, both histamine and PMA have been described to increased t-PA production (24, 48), the RHEC lines did not respond to 3 μM histamine stimulation. Apparently this is not a result of the absence of histamine receptors, because histamine stimulation was shown to lead to Ca^{++} mobilization in all RHEC lines. Most likely as indicated by the low effect in the Ca^{++} mobilization studies, the 3 μM histamine concentration used in t-PA studies was not sufficient. However, PMA stimulation significantly increased in t-PA production in the RHEC lines, demonstrating a functional cellular regulation mechanism for t-PA. The lines did not secrete vWF under standard culture conditions, and PMA stimulation induced only low vWF production in RHEC-3 and 11. In this respect RHEC lines differ from primary HUVEC cultures which do respond to PMA stimulation with significant production of vWF (5). Whether the minor or absent production of vWF by the RHEC lines is physiologic a result of *in vitro* long-term culturing remains unknown. Studies with hybrid EC lines indicate that

vWF production by EC lines can be easily lost during long-term culturing (49). Eicosanoids of the cyclooxygenase pathway were produced by all RHEC lines in similar profiles. Under standard culture conditions they produced only low amounts of TXA₂ and PGF_{2α}, but no PGE₂ and PGI₂. Stimulation of the lines with AA or a combination of AA and A23187 significantly increased TXA₂ and PGF_{2α}, and induced PGE₂ production, as also observed with freshly isolated heart EC cultures (38), but PGI₂ production could not be measured. Other investigators (38) showed that primary EC cultures from rat heart produced PGI₂ upon incubation with arachidonic acid, but in relatively low amounts compared with TXA₂. Apparently the RHEC lines, because of immortalization or long-term culture conditions, lost the capacity to produce PGI₂. In fact, the high TXA₂ and low PGI₂ production by primary cultures of heart EC after stimulation, as described by Linssen et al. (38), contrasts to data obtained with HUVEC, which on stimulation easily produce high amounts of PGI₂ but no or low amounts of TXA₂ (50). Whether these differences are related to organ specificity, micro or macrovascular origin of the cells, or culturing conditions is unknown and remains to be elucidated.

In conclusion, the applied isolation and culturing technique has been shown to be successful in the production of spontaneously immortalized EC lines from rat heart. The produced lines RHEC-3, 10, and 11 share a series of phenotypic and functional EC characteristics; however, each line also has a unique characterization profile. As such, the lines may be considered representative of endothelial heterogeneity as observed in heart endothelium *in situ*. In general, the RHEC lines offer a series of unique tools for *in vitro* studies of various heart endothelial cell functions and mechanisms relevant to processes in inflammation, hemostasis, and immunology.

Acknowledgments: The authors thank A. Gijsen for technical assistance, P. Lemmens for prostaglandin analysis, C. M. van den Hoogen for t-PA and vWF analysis, H. van Rie for electron microscopy assistance, and P. Schiffrers for endothelin measurements. We thank Dr. T. Tiebosch for pathological assistance in the tumorigenicity studies, Dr. J. G. M. C. Damoiseaux for assistance in FACS studies, Dr. P. Frederik for electron microscopy expertise, H van Rie for assistance in electron microscopical studies. Drs. J. Heemskerk and R. van Gorp for assistance in calcium mobilization studies, Dr M. Miyasaka for donation of 1A29, and Drs R. Lobb and P. Chisholm for donation of 5F10.

2.5 References

1. Majno G, Palade GE. The effect of histamine and serotonin on vascular permeability; an electron microscopic study. *Journal of Biophys Biochem* 1961; **11**: 571.
2. Yanagisawa M, Kurihara H, Kimsura S, et al. A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* 1988; **332**: 411.
3. Rees DD, Palmer RMJ, Moncada s. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci USA* 1989; **86**: 3375.
4. Auerbach R, Alby L, Grieves J, et al. Monoclonal antibody against angiotensin-converting enzyme: its use as a marker for murine, bovine and human endothelial cells. *Proc Natl Acad Sci USA* 1982; **79**: 7891.
5. Reinders JH, Groot de PG, Sixma JJ, Mourik van JA. Storage and secretion of von Willebrand factor by endothelial cells. *Haemostasis* 1988; **18**: 246.
6. Lindhout T, Blezer R, Schoen P, Nordfang O, Reutelingsperger C, Hemker HC. Activation of factor X and its regulation by tissue factor pathway inhibitor in small-diameter capillaries lined with human endothelial cells. *Blood* 1992; **79**: 2909.
7. Hinsberg van VWM, Kooistra T, Emeis JJ, Koolwijk P. Regulation of plasminogen activator production by endothelial cells: role in fibrinolysis and local proteolysis. *International Journal Radiation Biology* 1991; **60**: 246.
8. Whorton AR, Young SI, Data JL, et al. Mechanism of bradykinin-stimulated prostacyclin synthesis in porcine aortic endothelial cells. *Biochim Biophys Acta* 1982; **721**: 79.
9. Duijvestijn AM, Hamann A. Mechanisms and regulation of lymphocyte migration. *Immunology Today* 1989; **10**: 23.
10. Butcher EC. Cellular and molecular mechanisms that direct leukocyte traffic. *American Journal of Pathology* 1990; **136**: 1.
11. Cotran RS, Pober JS. Effects of cytokines on vascular endothelium: their role in immune injury. *Kidney International* 1989; **35**: 969.
12. Zimmerman GA, Prescott SM, McIntyre TM. Leukocyte-endothelial cell interactions. *Immunology Today* 1992; **13**:
13. Roska AK, Geppert TD, Lipsky PE. Immunoregulation by vascular endothelial cells. *Immunobiol* 1984; **168**: 470.
14. Savage CO, Hughes CW, McIntyre BW, Picard JK, Pober JS. human CD4+ T cells proliferate to HLA-DR+ allogeneic vascular endothelium. *Transplantation* 1993; **56**: 128.
15. Hughes CCW, Savage COS, Pober JS. The endothelial cells as a regulator of T-cell function. *Immunol Rev* 1990; **117**: 85.
16. Camussi G, Bussolino F, Salvidio G, Baglioni C. Tumor necrosis factor/cachetin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *Journal of Experimental Medicine* 1987; **166**: 1390.
17. Hebert CA, Luscinskas FW, Kiely JM, et al. Endothelial and leukocyte forms of IL-8. *Journal of Immunology* 1990; **145**:

18. Miossec P, Cavender D, Ziff M. Production of interleukin-1 by human endothelial cells. *Journal of Immunology* 1986; **136**: 2486.
19. O'Connell KA, Edidin M. A mouse lymphoid endothelial cell line immortalized by simian virus 40 binds lymphocytes and retains functional characteristics of normal endothelial cells. *Journal of Immunology* 1990; **144**: 521.
20. Harder R, Uhlig H, Kashan A, et al. Dissection of murine lymphocyte-endothelial cell interaction mechanisms by SV-40-transformed mouse endothelial cell lines: novel mechanisms mediating basal binding, and $\alpha 4$ -integrin-dependent cytokine-induced adhesion. *Exp Cell Res* 1991; **197**: 259.
21. Tatsuta T, Naiot M, Oh-hara T, Sugawara I, Tsuruo T. Functional involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. *Int Immunol* 1992; **2**: 165.
22. Laulajainen T, Julkunen I, Haltia A, Knuutila S, Miettinen A, Holthofer H. Establishment and characterization of a rat glomerular endothelial cell line. *Lab Invest* 1993; **69**: 183.
23. Ades EW, Hierholzer C, George V, Black J, Candal F. Viral susceptibility of an immortalized human microvascular endothelial cell line. *Journal of Virological Methods* 1992; **39**: 83.
24. Fickling SA, Tooze JA, J. WGS. Characterization of human umbilical vein endothelial cell lines produced by infection with early region of SV40. *Exp Cell Res* 1992; **201**: 517.
25. Juillerat-Jeanneret L, Aguzzi A, Wiestler D, Darekar P, Janzer R-C. Dexamethasone selectively regulates the activity of enzymatic markers of cerebral endothelial cell lines. *In Vitro Cell Dev Biol* 1992; **28A**: 537.
26. Linssen MCJG, Nieuwenhoven van FA, Duijvestijn AM, Glatz JFC, Vusse van der GJ. Continuous endothelial cells from adult rat heart. *In Vitro cell Dev Biol* 1993; **29a**: 611.
27. Duijvestijn AM, Goor van H, Klatter F, Majoor GD, Bussel van E, Breda Vriesman van PJC. Antibodies defining rat endothelial cells: RECA-1 a pan endothelial specific monoclonal antibody. *Laboratory Investigation* 1992; **62**: 459.
28. Maciag T, Cerendulo J, Isley S, Kellu PR, Forand R. An endothelial growth factor from bovine hypothalamus: identification and partial characterization. *Proc Natl Acad Sci USA* 1979; **76**: 5674.
29. Tamatani T, Miyasaka M. Identification of monoclonal antibodies reactive with the rat homolog of ICAM-1 and evidence for a differential involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. *International Immunology* 1990; **2**: 165.
30. May MJ, Entwistle G, Humphries MJ, Ager A. VCAM-1 is a CS-1 peptide-inhibitable adhesion molecule expressed by lymph node high endothelium. *Journal of Cell Science* 1993; **106**: 109.
31. Conroy JM, Hoffman H, Kirk ES, Hirzel HO, Sonnenblick EH, Soffer RL. Pulmonary angiotensin-converting enzyme. *Journal of Biol Chem* 1976; 4828.
32. Emeis JJ, Hoekzema R, Vos de A.F. Inhibiting interleukin-1 and tumour necrosis factor- α does not reduce the induction of plasminogen activator type-1 by endotoxin in rats in vivo. *Blood* 1995; **85**: 115.
33. Tranquille N, Emeis JJ. The simultaneous acute release of tissue type plasminogen activator and von Willebrand factor in the perfused rat hindleg region. *Thromb Haemostas* 1990; **63**: 454.
34. Engels W, Kamps M, Lemmens PJMR, Vusse vd GJ, Reneman RS. Determination of prostaglandins and thromboxane in whole blood by high performance liquid chromatography with fluorimetric detection. *Journal of Chromatography* 1988; **427**: 209.
35. Haaster MCJ, Engels W, Lemmens PJMR, Hornstra G, Vusse GJ van der, Heemskerk JWM. Differential release of histamine and prostaglandin D2 in rat peritoneal mast cells: Roles of cytosolic calcium and protein tyrosine kinases. *Biochim Biophys Acta* 1995; **1265**: 79.
36. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biol Chem* 1985; **260**: 3440.
37. Schrey G, Es van PN, Schiffers PMH, Leeuw de P. Renal arterial venous endothelin in hypertensive patients with or without renal artery stenosis. *Blood Pressure* 1994; **3**: 370.
38. Linssen MCJG, Engels W, Lemmens PJMR, et al. Production of arachidonic acid metabolites in adult rat cardiac myocytes, endothelial cells and fibroblast-like cells. *American Journal of Physiology* 1993; **264**: h973.
39. Cockerill GW, Meyer G, Noack L, vadas MA, Gamble JR. Characterization of a spontaneously transformed human endothelial cell line. *Laboratory Investigation* 1994; **71**: 497.
40. Garlanda C, Parravicini C, Sironi M, et al. Progressive growth in immunodeficient mice and host cell recruitment by mouse endothelial cells transformed by polyoma middle-sized T antigen: implications for the pathogenesis of opportunistic vascular tumors. *Proc Natl Acad Sci USA* 1994; **91**: 7291.
41. Duijvestijn A, Vlek L, Duistermaat L, Rie van H, Breda Vriesman P. Chronic renal allograft rejection: the significance of non-MHC alloantigens. *Transplantation International* 1992; **5 suppl 1**: s639.

42. Kern PA, Knedler A, Eckel RH. Isolation and culture of the microvascular endothelium from adipose tissue. *Journal of Clinical Investigation* 1983; **71**: 1822.
43. Carron Mac RM, Wang L, Siren AL, Spatz M, Hallenbeck JM. Adhesion molecules on normotensive and hypertensive rat brain endothelial cells. *PSEBM* 1994; **205**: 257.
44. Metsarinne K, Saijonmaa O, Yki-Jarvinen H, Fyhrquist F. Insulin increases the release of endothelin in endothelial cell cultures in vitro but not in vivo. *Metabolism* 1994; **7**: 878.
45. Wiebke jL, Montrose-Rafizadeh C, Zeitlin PL, Guggino WB. Effect of hypoxia on endothelin-1 production by pulmonary vascular endothelial cells. *Biochem Biophys Acta* 1992; **1134**: 105.
46. Glassberg MK, Nolop KB, Jackowski JT, W.M. A, Wanner A, Ryan US. Microvascular and macrovascular endothelial cells produce different constrictor substances. *Journal of Appl Physiol* 1992; **72**: 1681.
47. Levin EG, Del Zoppo GJ. Localization of tissue plasminogen activator in the endothelium of a limited number of vessels. *American Journal of Pathology* 1994; **144**: 855.
48. Hanss M, Collen D. Secretion of tissue-type plasminogen activator and plasminogen activator inhibitor by cultured human endothelial cells; modulation by thrombin, endotoxin and histamin. *Journal of Lab Clin Med* 1987; **109**: 97.
49. Edgell C-J, Reisner HM, Graham JB. Endothelial cell hybrids and the supression of factor VIII related antigen expression. *Britisch Journal of Haematology* 1980; **46**: 613.
50. Vossen RCRM, Dam Mieras van MCE, Lemmens PJMR, Hornstra G, Zwaal RFA. Membrane fatty acids composition and endothelial cell functional properties. *Biochem Biophys Acta* 1991; **1083**: 243.

3

Heart EC respond heterogeneously on cytokine stimulation in ICAM-1 and VCAM-1, but not in MHC expression

A study with 3 rat heart endothelial cell (RHEC) lines

Josien G. Derhaag, Adrian M. Duijvestijn, and Peter J. C. van Breda Vriesman

Department of Immunology, Cardiovascular Research Institute Maastricht,
Maastricht University, P. O. box 616, 6200 MD, Maastricht, The Netherlands

Published in *Endothelium*. 1997, vol. 5, p 307-319.

Abstract

Cytokine-induced expression of ICAM-1, VCAM-1, and MHC class I and II was studied at different time points in microvascular endothelial cells (EC) of heart origin, using three different rat endothelial cell (RHEC) lines that were stimulated with TNF α and/or IFN γ .

Each of the three RHEC lines responded to TNF α as well as to IFN γ ; stimulation with combined cytokines led to increased or even synergistic effects. TNF α was most potent in inducing ICAM-1 and VCAM-1, whereas MHC class II was most effectively induced by IFN γ . The 3 RHEC lines responded similarly regarding induction of MHC class II and upregulation of constitutively expressed MHC class I on the cells. However, the RHEC lines showed remarkable differences with respect to ICAM-1 and VCAM-1 induction, with each line having a unique expression profile. In RHEC-3, both ICAM-1 and VCAM-1 were well inducible, whereas in RHEC-10, no ICAM-1 and only some VCAM-1 could be induced. RHEC-11 showed minimal induction of ICAM-1, but strong induction of VCAM-1. For P-selectin induction, no such differences were found between the RHEC lines. These heterogeneous effects of cytokine stimulation could neither be explained by differences in mobilization of calcium nor by ultra-structural differences between the lines. Stimulation of the RHEC lines for ICAM-1 and VCAM-1 or MHC class II molecule induction resulted in expressing and non-expressing EC. Experiments with selected and subsequently cultured expressing and non-expressing cell populations for either ICAM-1, VCAM-1 or MHC class II, indicated that this selective induction most likely results from intrinsic regulation mechanisms in the cell cultures, and not from the presence of particular EC subpopulations within the lines.

We conclude that microvascular heart endothelial cells, as represented by the 3 RHEC lines, demonstrate a selective heterogeneity in expression of ICAM-1 and VCAM-1, but not of MHC class I and II, upon cytokine stimulation. The consequences of this heterogeneity for leukocyte-endothelial cell interactions in heart inflammation and immune reactivity is discussed.

3.1 Introduction

Vascular endothelial cells form the interphase between blood and tissue, and as such they play a major role in processes of arrest and extravasation of blood leukocytes in inflamed and immune reactive tissues. The interaction of leukocytes with endothelial cells is mediated through cell adhesion molecules expressed on both cell types. A series of relevant endothelial cell adhesion molecules has been described, such as P- and E-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). The endothelial P- and E- selectins are predominantly involved in the extravasation of neutrophils and monocytes through binding to a sialylated derivative of the Lewis X blood group determinants (Sle^x ligands) on these cells (1, 2, 3). ICAM-1 and VCAM-1 are both members of the immunoglobulin supergene family and play a major role in the adherence of lymphocytes and monocytes to endothelial cells (4, 5, 6, 7). The best known leukocyte ligands for ICAM-1 and VCAM-1 are the adhesion molecules LFA-1 (lymphocyte-function-associated-antigen 1) and VLA-4 (very-late-antigen 4) respectively, both members of the integrin family. By differential expression of the various adhesion molecules, vascular endothelial cells may regulate selective adhesion and extravasation of relevant leukocyte subsets in inflamed tissues (8, 9). In addition, in inflammatory processes with immune reactivity, vascular endothelial cells can be induced to *de novo* expression of MHC class II molecules, and as such may function as antigen presenting cells in interaction with blood T cells. In this process of antigen presentation by EC, adhesion molecules such as ICAM-1 and VCAM-1 are important for delivery of co-stimulatory signals (10, 11, 12, 13).

It has been shown that particular cytokines, such as TNF α , IL-1, and IFN γ are potent stimulatory agents for induction and enhancement of adhesion and MHC molecules in endothelial cells (8, 14). *In vitro* studies, predominantly with human umbilical vein EC (HUVEC) cultures, have shown overlapping as well as more specific effects of stimulation with individual cytokines. For induction of the adhesion molecules E-selectin and VCAM-1, TNF α appears a potent cytokine (3, 15,

16, 17, 18), whereas for the induction of MHC class II molecules IFN γ plays a dominant role. With respect to induction or enhancement of ICAM-1, both cytokines have more or less similar induction capacities (19, 20). Interestingly, activation of EC by cytokines such as TNF α and IFN γ occurs through different intracellular signalling pathways. Consequently, effects of EC stimulation with a combination of these cytokines may strongly differ from those of individual cytokine stimulation in expression levels of induced molecules and also in the kinetics of induction (14). It has been shown that not all EC respond similarly to cytokine stimulation and that differences such as species, strain, tissue, and blood vessel type are important in this respect (21, 22, 23, 24).

Our interest in inflammation and immune reactivity in heart diseases related to transplantation and autoimmunity, led us to emphasize the role of microvascular endothelium in these processes, using *in vitro* studies with rat EC cultures. Because endothelial cells express, as mentioned, tissue-specific characteristics, we wished to use EC of heart origin, and therefore developed a series of three rat heart endothelial cell (RHEC) lines. These lines were characterized previously and found representative for microvascular endothelial cells *in vivo* (25). In seeking the role of microvascular heart endothelial cells in blood leukocyte-endothelial cell interaction, RHEC lines were studied for induction of adhesion molecules and MHC antigens by cytokines. ICAM-1, VCAM-1, MHC class I and class II expression was measured at various time points after stimulation with TNF α and IFN γ individually or in combination. Our results show that the three RHEC lines respond different upon cytokine stimulation in the expression of ICAM-1 and VCAM-1, but similar in the expression of MHC molecules and P-selectin. It was further studied whether these differences were related to different Ca²⁺ mobilization potentials or ultrastructure. What the consequences of these heterogeneous responses of heart EC upon cytokine stimulation may be for processes of inflammation and local immune reactivity in heart tissue is discussed.

3.2 Materials & methods

Heart Endothelial Cell Lines

We used the EC lines RHEC 3, RHEC 10 and RHEC 11. These lines were produced as previous described (25, 26). They are derived from Lewis rats, and express the endothelial cell markers RECA-1, Angiotensin Converting Enzyme and von Willebrand Factor (vWF). In addition they bind EC specific isolectin GS-I-B4 and take up acetylated low-density-lipoprotein (25).

RHEC lines were routinely cultured in a CO₂ incubator at 37°C in EC culture medium (40% RPMI 1640 (Gibco BRL, Life Technologies B.V., the Netherlands), 40% M199 (Gibco), 20% inactivated FCS (Integro, Belgium), bovine brain extract (14.93 µg/ml) prepared as described by Maciag (27), 13 mM NaHCO₃, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mg/ml Fungizone, 10 mM Hepes, 20 U/ml heparin and 2mM L-glutamin). For passaging the cells, confluent monolayers were treated with trypsin (0.25%)/ EDTA(0.2%) in Phosphate Buffered Saline (PBS, 150 mM NaCl, 8 mM Na₂H₂PO₄, 1.6 mM KH₂PO₄, pH 7.3) for 1 minute. Trypsin was neutralized with EC Work medium (90% M199, 10% inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-glutamin and 25 mM Hepes). Cell viability was always >95%, as assessed by Trypan blue exclusion.

Stimulation of Endothelial Cell Monolayers

ICAM-1, VCAM-1 and MHC Induction

Incubation of the endothelial cells with cytokines was performed on confluent monolayers with 2.5 ml of culture medium in 6 well culture dishes for the time periods: 0, 4, 8, 16, 32, and 64 hours. Recombinant human TNFα (a gift from Prof. Dr. W. Fiers, Gent, Belgium) was used in a 400 U/ml concentration. Recombinant rat IFNγ (a gift from Dr. P. van de Meide, TNO Rijswijk, The Netherlands), was used in a 200 U/ml concentration. In these stimulation experiments the cell lines were treated with either control culture medium, culture medium with TNFα, culture medium with IFNγ or culture medium with combined TNFα and IFNγ. In 64 hours stimulation

experiments, the culture medium with cytokines was refreshed after 32 hours. Long-term incubation (32-64 hr) of the endothelial cell monolayers with the cytokines did not result in induction of proliferation, as shown by the unchanged total cell numbers of stimulated cultures. Reorganization in endothelial cells was studied morphologically in EC monolayers. After IFN γ stimulation the EC of the intact monolayer appeared more elongated in all cell lines.

At the end of the stimulation, endothelial cells were harvested using trypsin/EDTA (see above) and cell suspensions were used for cytocentrifuge preparations or FACS analysis. Experiments were performed in triplo, with cells of passage 20 to 30.

P-selectin Induction

Confluent monolayers of the endothelial cells were incubated with PMA (30 μ g/ml), TNF α (400U/ml), histamine (50 μ M) or the Ca $^{2+}$ ionophore A23187 (10 μ M) dissolved in 2.5 ml of culture medium in 6 well culture dishes for the time periods: 15 min, 30 min, 1 hr and 4 hr. Control incubations were carried out with culture medium in the absence of agonists. At the end of the incubation period the cells were harvested by treatment with 0.05% (w/v) EDTA and cell suspensions were used for cytocentrifuge preparations. Induction of E-selectin was not studied because antibodies against rat E-selectin were not available.

Cytocentrifuge Preparations

Cytocentrifuge preparations were made from EC fixed with 1% paraformaldehyde (PFA) in PBS for 30 min at 4°C and washed 3 times with PBS and resuspended in EC culture medium, or unfixed endothelial cell suspensions. Preparations were made in a Cytocentrifuge (Cytospin 3, Shandon, England) in a 10 min run at 690 RPM. Preparations were stored in an exsiccator at room temperature for maximally 10 days.

Antibodies and Immunostaining

The following murine monoclonal antibodies (Mab) were used: OX18 directed against MHC class I antigen, OX6 directed against MHC class II antigen, OX8 used as non-relevant control Mab (obtained through the ECACC, Salisbury, UK), 1A29 an anti ICAM-1 Mab, donated by Dr. Miyasaka (28), 5F10 an anti VCAM-1 Mab, donated by Drs. R. Lobb and P. Chisholm (29). Detection of P-selectin occurred with a polyclonal rabbit antibody against human CD62P (P-selectin) cross reactive with rat P-selectin (Pharmingen, Germany). For detection of vWF a rabbit polyclonal antibody A082 (DAKO, Denmark) directed to vWF was used (DAKO, Denmark).

Immunoperoxidase staining was performed on cytocentrifuge preparations of either 1 % PFA fixed or unfixed endothelial cells. Preparations of fresh EC were acetone-fixed for 10 minutes, and air dried before use. A 2-step immunoperoxidase technique was used, as described previously (30). Briefly, the cytocentrifuge preparations were incubated with the above mentioned antibodies or control antibodies, at pretested saturating concentrations, for 60 minutes in a humidified chamber at RT. Dilutions were made in PBS containing 0.05% BSA (0.05 % PBSA). The preparations were washed 3 times with PBS and incubated with peroxidase-conjugated second step antibodies (rabbit F(ab)₂ anti-mouse IgG, or swine F(ab)₂ anti-rabbit IgG, (Dako, Denmark), supplemented with 3% normal rat serum (NRS)), for 30 minutes. Subsequently preparations were washed 3 times with PBS for 5 minutes and incubated with a 0.05 mg/ml solution of diaminobenzidin (DAB) in PBS containing 0.05 % H₂O₂ for 10 minutes, and subsequently washed 3 times in water and counter-stained with haematoxylin. Preparations were routinely dehydrated in ethanol, and cover slipped with Entellan, (Merck, Germany). Quantification of percentages of positive cells occurred microscopically on cytocentrifuge preparations by counting a minimum total of 200 cells over at least 2 fields, or in case of low numbers of positive cells, a minimum of 10 positive cells.

Immunofluorescence staining was performed on viable confluent monolayers of heart endothelial cells in 96 well tissue culture plates as earlier described (25). Briefly, the cells were incubated with saturating concentrations of specific or non-relevant control antibodies in M199 containing 10% NBCS, for 60 minutes at room temperature. Subsequently the cells were washed and incubated with FITC-conjugated second step antibody (goat anti mouse IgG, rat absorbed, Cappel, Organon Teknika, the Netherlands) for 30 minutes. After washing the cells were fixed in 1% PFA and examined under a Zeiss fluorescence reverse microscope.

Flow Cytometer Analysis

Immunofluorescent staining was performed in suspensions of routinely trypsinized confluent endothelial monolayers of either non-stimulated or stimulated cultures. 5×10^5 cells were incubated for 30 minutes on ice with saturating amounts of monoclonal mouse antibodies OX8, OX18 or RECA-1, then washed three times in 0.5% PBSA and incubated for 30 minutes with a 100 X diluted FITC-conjugated goat anti-mouse IgG antibody (rat absorbed, Cappel, Organon Teknika, the Netherlands). Flow cytometry analysis was performed with a FACS-Sort (Becton Dickinson, Mountain View, CA, USA). Data were collected from 1×10^5 cells.

Electron Microscopy

EC suspensions from confluent monolayers were fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 30 min. Fixed cells were resuspended in warm 4% agarose in PBS and cooled on ice for 20 minutes. EC in coagulated agarose were post-fixed in 2.5% glutaraldehyde in 0.1M phosphate buffer for 20 minutes, washed in 0.1M phosphate buffer, fixed with 1% OsO₄ for 1 hour, and embedded in Epon. Ultrathin sections for electron microscopy were counter-stained with uranyl acetate and lead citrate and examined with a CM10 Philips electron microscope.

Calcium mobilization studies

Cellular mobilization of calcium was measured using the calcium sensitive fluorescent fura-2 acetoxymethyl ester (fura-2) according to van Haaster (31) with minor modifications. In brief: confluent monolayers of RHEC were washed with buffer A (10mM HEPES, 150 mM NaCl, 5mM KCl, 1.8 mM CaCl_2 , 1mM MgCl_2 , 1mg/ml glucose (w/v) and 4 μM L-glutamine), containing 0.25% (w/v) BSA and incubated in buffer supplemented with 1 μM fura-2 with 1% BSA, 37 °C, for 45 minutes. Subsequently monolayers were washed and incubated with EC culture medium for 15 minutes. Cells were detached by incubation with 0.05% EDTA, and resuspended in buffer A. Mobilization of Ca^{2+} in suspensions of EC was determined by stimulation with either 1-3 μM ionomycin, 25 μM histamine, or 30 $\mu\text{g/ml}$ PMA, using a spectrofluorometer (SLM Aminco) at a 500 nm emission wave length. Changes in ratio of fluorescence at 340 and 380 nm were used to calculate levels of $[\text{Ca}^{2+}]_i$, according to the procedure of Grynkiewicz et al (1985). Calibration values for the calculation of $[\text{Ca}^{2+}]_i$ were obtained by the addition of 1% (w/v) Triton X-100, followed by 1mM EGTA.

EC Selection

Selection of endothelial cells that were positive or negative for expression of either MHC class II, ICAM-1, VCAM-1 occurred with magnetic beads coated with sheep anti-mouse antibodies (Dynabeads M-450; Dynal, ITK Diagnostics BV, the Netherlands). Briefly, a monolayer of RHEC was stimulated with standard concentrations (see above) of $\text{TNF}\alpha$ and $\text{IFN}\gamma$ for 16 or 32 hours. The cells were routinely trypsinized and incubated in suspension with mouse mAb directed to rat MHC class II antigens (OX-6, 1:400 diluted), or against ICAM-1 (1A29, 1: 50 diluted), or against VCAM-1 (5F10, 1:500 diluted) for 30 minutes at 4°C. The cells were washed 3 times with PBS, and 1×10^7 cells were incubated with 1.4×10^8 magnetic beads in 0.2 ml 0.5% PBSA for 30 minutes on ice. The amount of beads used per incubation was based on an estimated 4- 20 beads per selected cell. During

the incubation, the suspensions were frequently rotated. Magnetic bead-labelled cells were separated from non-labelled cells, in a 4 ml volume, using a magnet for two minutes. The positively selected population consisted of more than 99% cells labelled with beads. In the negatively selected EC population no cells labelled with beads were observed, and staining for false negative cells (by incubation with second-step antibodies) showed less than 0.1% positive cells. Both positively and negatively selected populations were cultured for 10 passages. At passages 3 and 10, monolayers were stimulated with TNF α and IFN γ for 16 or 32 hr, and EC were subsequently tested for the selected phenotype by immunoperoxidase staining of either MHC class II, ICAM-1 or VCAM-1 on cytocentrifuge preparations.

3.3 Results

Basal Expression of ICAM-1, VCAM-1 and MHC Molecules in RHEC Lines.

In a first series of studies we tested the basal expression of ICAM-1, VCAM-1 and MHC class I and II molecules in the three EC lines RHEC-3, RHEC-10, and RHEC-11. Cytocentrifuge preparations of routinely cultured RHEC lines were stained with monoclonal antibodies to ICAM-1, VCAM-1, MHC class I and MHC class II using an immunoperoxidase technique. As shown in table 1, only RHEC-3 expressed ICAM-1, whereas MHC class I was expressed by all three RHEC lines. Interestingly, ICAM-1 expression in RHEC-3 was present on part (12.3%) of the cells only. In none of the 3 RHEC lines expression of VCAM-1 or MHC class II was observed during standard culture conditions.

**Adhesion molecule and MHC antigen expression
of the RHEC lines under standard culture conditions**

Structure	mean % positive cells $\pm$ SD.		
	RHEC-3	RHEC-10	RHEC-11
ICAM-1	12.3 $\pm$ 3.3	<1	<1
VCAM-1	<1	<1	<1
MHC class I	>99	>99	>99
MHC class II	<1	<1	<1

Table 1: Percentages of positive cells were measured on immunoperoxidase stained cytocentrifuge preparations of paraformaldehyde-fixed cells. ICAM-1, Intercellular Adhesion Molecule-1; VCAM-1, Vascular Cell Adhesion Molecule-1; MHC, Major Histocompatibility Complex antigen. Data are from 3 experiments. Absence of expression or less than 1% expression is categorized as <1%. Positivity of all cells, or less than 1% negative cells is categorized as >99%.

*Heterogeneous Induction of Adhesion Molecules of the Immunoglobulin Superfamily
on the RHEC Lines upon Cytokine Stimulation*

To investigate whether the RHEC lines could be induced to ICAM-1 and VCAM-1 expression by inflammatory cytokines, RHEC cultures were stimulated with $\text{TNF}\alpha$ and $\text{IFN}\gamma$ individually or in combination for periods from 4 to 64 hr. Stimulation occurred with saturating concentrations of the cytokines (400 U/ml for $\text{TNF}\alpha$ and 200 U/ml for $\text{IFN}\gamma$) as tested in initial experiments (data not shown).

ICAM-1 induction (fig. 1) was observed in RHEC-3 and RHEC-11, but not in RHEC-10. In RHEC-3 induction of ICAM-1 was obtained with $\text{TNF}\alpha$ as well as with $\text{IFN}\gamma$. Stimulation with combined $\text{TNF}\alpha$ and $\text{IFN}\gamma$ was more effective in ICAM-1 induction (e.g., higher percentages of positive cells were measured) than the individual cytokines, but effects were not additive (see also fig. 2c). The kinetics of ICAM-1 induction were different for the two cytokines, e.g., $\text{TNF}\alpha$ -induced expression was already maximal at 16 hr, whereas $\text{IFN}\gamma$ expression slowly inclined during the 64 hr time period. In RHEC-11, ICAM-1 induction was minimal, and only seen after stimulation with combined $\text{TNF}\alpha$ and $\text{IFN}\gamma$.

RHEC lines respond heterogeneously upon cytokine stimulation in ICAM-1 and VCAM-1 but not in MHC expression

Figure 1: Expression of ICAM-1, VCAM-1 and MHC class II in the RHEC lines after cytokine stimulation. RHEC-3, RHEC 10 and RHEC 11 cells were stimulated with either $\text{TNF}\alpha$, $\text{IFN}\gamma$, or a combination of both ($\text{TNF}\alpha + \text{IFN}\gamma$). Control incubations were executed with culture medium without cytokines. Expression was measured at 4, 8, 16, 32, and 64 hr of stimulation, by immunoperoxidase staining on cytocentrifuge preparations. One representative experiment out of 3 is presented.

Figure 2 -> Cell surface staining of RECA-1, ICAM-1 and MHC class II in RHEC cultures. Monolayers of endothelial cells were stimulated for 32 hours with the combined cytokines $\text{TNF}\alpha$ and $\text{IFN}\gamma$. Viable, non-fixed, monolayers were stained by immunofluorescence. A: Phase contrast microscopy showing a confluent monolayer of RHEC 10. B: RECA-1 staining of RHEC 11 showing expression of this EC-specific antigen by all cells. C: Staining for ICAM-1 showing ICAM-1 cell surface expression in RHEC 3. D: MHC class II induction in RHEC 11. In C and D only part of the cells are stained. Bars 16 μm .

Induction of **VCAM-1** (fig. 1) by cytokine stimulation was observed in all three RHEC lines with a maximum at 16 hr. But, as also shown for ICAM-1 induction, each cell line responded differently. RHEC-10 was the poorest responder in this respect, it showed a minor expression after IFN γ stimulation, whereas stimulation with the combined TNF α and IFN γ gave an increased, but still rather low expression. VCAM-1 was well inducible in RHEC-3 and RHEC-11 by either TNF α or IFN γ . RHEC-3 responded best to TNF α , whereas for RHEC-11 effects of TNF α and IFN γ were rather similar. VCAM-1 induction by TNF α was observed at an earlier time point than with IFN γ (4 versus 8 hr). The strongest induction was observed after combined TNF α and IFN γ stimulation (see also fig. 3a), with even synergistic effects in RHEC-3.

To define whether the heterogeneity among the RHEC lines in ICAM-1 and VCAM-1 induction also existed for acutely inducible **P-selectin**, RHEC cultures were stimulated with either PMA, TNF α , histamin or calcium ionophore A23187 for 15 min to 4 hr. In none of the RHEC lines increased P-selectin levels could be observed after stimulation.

Figure 3: Immunoperoxidase staining for VCAM-1 and MHC class II on cytocentrifuge preparations of RHEC lines after stimulation with a combination of $\text{TNF}\alpha$ and $\text{IFN}\gamma$ for 32 hr. A: VCAM-1 staining is seen on part of RHEC 3 cells (arrows). B: MHC class II staining of RHEC 3 showing part of the cells express the antigen. Staining of cells varied from weakly positive (single arrows) to strongly positive (double arrows). Bars 40 μm .

Similar Enhancement of MHC class I and II Expression in the RHEC Lines upon Cytokine Stimulation

In addition to adhesion molecule expression we investigated the induction of MHC class I and MHC class II antigens in the RHEC lines. For MHC class I studies the RHEC lines were stimulated with combined $\text{TNF}\alpha$ and $\text{IFN}\gamma$ for 32 hours, and FACS analysis was used to detect changes in constitutively expressed MHC class I antigens. All three cell lines showed similar enhanced MHC class I expression after cytokine stimulation. MHC class I enhancement was a specific effect, since no enhancement of expression was observed for pan endothelial cell-specific antigen RECA-1. In fig. 4 the RHEC-10 data, which are representative for the three RHEC lines, are shown.

In all three RHEC lines MHC class II expression, which was absent without stimulation, was induced by $\text{IFN}\gamma$. In most experiments expression was observed from 16 hr of stimulation and inclined to maximal levels at 32 or 64 hr. $\text{TNF}\alpha$ stimulation did not, or in very low levels, induce MHC class II expression in the RHEC lines. However, stimulation with combined $\text{TNF}\alpha$ and $\text{IFN}\gamma$ gave a strong synergistic effect in all three lines (see also fig. 2d and 3b). This clearly demonstrate that although $\text{TNF}\alpha$ stimulation alone has only a minor effect on MHC class II

expression, it has a strong costimulatory effect with IFN γ . In contrast with the heterogeneous induction of ICAM-1 and VCAM-1 by cytokines, MHC class I and class II induction was similar for all 3 RHEC lines.

MHC class I upregulation after TNF α /IFN γ stimulation

Figure 4: Expression of MHC class I antigens by RHEC-10 after 32 hr of stimulation with combined TNF α and IFN γ was measured by FACS analysis (one out of two similar experiments is shown). White histograms: expression profile under non-stimulation conditions. Grey histograms: expression profile after 32 hr of cytokine stimulation.

- Left panel: Control staining with non-reactive class-matched antibody (OX 8) under stimulation and non-stimulation conditions. Profiles are identical.
- Middle panel: Constitutive MHC class I expression under non-stimulated and stimulated conditions. Expression is enhanced by cytokine stimulation.
- Right panel: Constitutive RECA-1 expression under non-stimulation and stimulation condition. Curves have an identical profile, showing the absence of upregulation of RECA-1 by cytokine stimulation.

RHEC Lines Respond Similarly in Calcium Mobilization and Protein kinase C activation

Since in cell activation calcium mobilization and/or protein kinase C activation are important processes in signal transduction, we studied whether the RHEC lines differed in this respect. Calcium mobilization was studied by stimulation with the calcium ionophore ionomycin and additionally with histamin. Ionomycin stimulation elicited rather similar increase in intracellular calcium ($[Ca^{2+}]_i$) in the

three cell lines (range 1892 to 2086 nM). Comparable results were obtained with the 3 cell lines upon histamin stimulation (one experiment, $[Ca^{2+}]_i$ range: 1176 to 1982 nM). PKC activation was studied indirectly, by measuring the inhibitory effect of PKC activation on Ca^{2+} mobilization by histamin. As such, PKC activation obtained by preincubation of RHEC cultures with PMA, led to decreased Ca^{2+} mobilization by histamin, with similar results for the three RHEC lines ($[Ca^{2+}]_i$ range: 486 to 1177 nM). The data show that with respect to Ca^{2+} mobilization and PKC activation the 3 RHEC lines have rather similar potentials.

The RHEC Lines Show no Defined Ultrastructural Differences

To study whether the heterogeneous phenotype of EC in the RHEC lines was related to ultrastructural cellular differences, we studied suspensions of RHEC cultures by electron-microscopy (Fig. 5). Most cells of the RHEC lines showed a ruffled cell membrane with variable numbers of small processes. The nuclei showed an active state with present nuclei and a preponderance of euchromatin over heterochromatin. The cytoplasm of the cells frequently showed a cytocentre with an active Golgi apparatus. Cells contained many polyribosomes, endoplasmatic reticulum, and numerous mitochondria. Phagolysosomes were observed in variable numbers, sometimes with multi-laminar membranous material. Weible Palade bodies were not noticed (Fig. 5a). Heterogeneity in the EC of the RHEC lines was observed in the shape of the cells, and the numbers of cytoplasmatic vesicles. EC were variable in cell size, large cells showed extensive cytoplasmatic protrusions. Vesicles in EC varied in size and contained heterogeneous amorphous material. Sometimes numerous lipid droplets were observed (Fig. 5b). Occasionally in present small clusters of cells, contacts between cells with zonulae occludens could be noticed (Fig. 5c), indicating intensive contact between these EC. The data indicated that each of the RHEC lines consisted ultrastructurally of a rather heterogeneous population of EC, but no defined subpopulations of EC could be identified.

Figure 5: Electronmicroscopy of RHEC cells. A: Cells from RHEC 11. Notice the ruffled cell membranes and small processes, bar $0.2\mu\text{M}$. B: Cells from RHEC 10, vesicles with lipid droplets can be seen (arrow), bar $0.2\mu\text{M}$. C: Cells from RHEC 11, the arrows show 3 zonulae occludens between the cells, bar $0.9\mu\text{M}$.

Heterogeneous Expression of ICAM-1, VCAM-1 and MHC class II Seems not a Result of Specific Subpopulations of EC

As shown, cytokine stimulation gave a different pattern of ICAM-1, VCAM-1, and MHC class II expression in the cell lines. In addition stimulation did not lead to induction of these molecules on all cells of a RHEC culture. We wished to determine whether this was due to the presence of responsive and non-responsive EC subsets in the RHEC lines. Therefore, after combined $\text{TNF}\alpha$ and $\text{IFN}\gamma$ stimulation, positive and negative EC for either ICAM-1, VCAM-1, or MHC class II were selected, using magnetic beads to sort out specific antibody-labelled EC. The selected expressing and non-expressing populations were subcultured and tested at passage 3-4 and 10 for induction of ICAM-1, VCAM-1 and MHC class II respectively on stimulation with combined $\text{TNF}\alpha$ and $\text{IFN}\gamma$.

Figure 6: Immunoperoxidase staining on cytocentrifuge preparations of selected RHEC-11 cells after stimulation with a combination of $\text{TNF}\alpha$ and $\text{IFN}\gamma$ for 32 hr. A: MHC class II staining of RHEC 11 cells that had been selected 3 passages earlier for MHC class II expression (see text). Most cells express MHC class II after cytokine stimulation. Small black dots are remaining magnetic beads from the isolation procedure. B: MHC class II staining of RHEC 11 cells that had been selected 3 passages earlier for absence of MHC class II expression after cytokine stimulation. A low but distinct % of cells express MHC class II. Staining of cells varied from weakly positive (single arrows) to strongly positive (double arrows). Bars 40 μm .

**Effect of RHEC pre-selection on cytokine induced expression of
ICAM-1, VCAM-1, and MHC class II**

Passage after selection	Cytokine stimulation ¹	RHEC 3 experiment				RHEC 11 experiment	
		% ICAM-1 + cells		% VCAM-1 + cells		% MHC-II + cells	
		ICAM-1 + population	ICAM-1 - population	VCAM-1 + population	VCAM-1 - population	MHC-II + population	MHC-II - population
p 0 ²	yes	>99%	<1%	>99%	<1%	>99%	<1%
p 3-4	no ³	55%	12%	<0.1%	<0.1%	<0.1%	<0.1%
	yes	96%	38%	75%	32%	95%	13%
p 10	no	13%	15%	<0.1%	<0.1%	<0.1%	<0.1%
	yes	97%	57%	45%	41%	79%	51%

Table 2: Data are from a representative experiment.

¹Cytokine stimulation occurred with combined TNF α and IFN γ for 16 hr (ICAM-1 and VCAM-1 experiments) or 32 hr (MHC class II experiments).

²p 0 values were measured directly after the magnetic bead selection step. In the negative selected EC populations these values were calculated from cytocentrifuge preparations immunoperoxidase-stained with second step antibodies only. In the positive selected EC populations the values were calculated from the numbers of free, non bead-rozatted, EC.

³RHEC 3 shows already low ICAM-1 expression (see table 1) under normal (non-stimulated) conditions.

The selection procedure yielded ICAM-1, VCAM-1, and MHC class II positive and negative populations with a purity of >99%. Upon subsequent culturing of these populations without cytokines, VCAM-1 and MHC class II expression completely disappeared from the positively selected populations, and ICAM-1 expression diminished to basal levels (about 10-15% positive cells). Re-stimulation of the populations with cytokines at passage 3-4 and subsequently at passage 10 showed that the selection phenotype of VCAM-1 or MHC class II positive and negative populations gradually diminished during culturing, and at passage 10 had returned to

rather pre-selection expression levels. (Table 2 and Fig. 6) Similar results were obtained with the ICAM-1 negative population, but not with the ICAM-1 positive population, which at passage 10 after selection still showed very high ICAM-1 expression upon cytokine stimulation. When the expression levels of vWF were compared between MHC class II positive, MHC class II negative, and non-selected EC, no differences were found (about 13% positive cells, data not shown). Apparently vWF expression did not co-select and remained randomly distributed over the selected populations. In another approach to define the presence of particular subpopulations in the RHEC lines, EC of RHEC 3 were cloned by limiting dilution. Two clones selected from the 0.5 cell/well plate were tested for response on stimulation with combined IFN γ and TNF α for 16 and 32 hr. These clones 1D4 (and 1C5 expressed RECA-1 and MHC class I but no ICAM-1, VCAM-1 or MHC class II under standard culture conditions. After 16 and 32 hr of cytokine stimulation both clones expressed VCAM-1 (highest at 16 hr) and MHC class II (highest at 32 hr). But, expression levels were comparable with RHEC 3 levels before cloning. The same was found for ICAM-1 expression in clone 1D4, but not for 1C5. Cytokine stimulation of 1C5 led to expression of ICAM-1 on >99% of the cells. These results indicate that at least for VCAM-1 and MHC class II the heterogeneous induction in the RHEC lines is likely not due to cytokine-responding and non-responding subpopulations.

3.4 Discussion

To obtain insight in the role of vascular endothelial cells in leukocyte-EC interactions in heart inflammatory and immune reactive processes, we studied the induction of cell adhesion molecules of the immunoglobulin superfamily, and that of MHC antigens by cytokines in heart EC cultures *in vitro*. For these studies, a series of 3 rat heart endothelial cell (RHEC) lines were selected, that were representative for heterogeneous heart microvascular endothelium *in vivo* (25). Our data show that all three heart EC lines respond to the cytokines TNF α and IFN γ . With respect to induction of the adhesion molecules ICAM-1 and VCAM-1, the strongest effects were obtained with TNF α , e.g. peak expression was higher and reached earlier than with IFN γ . This was not due to suboptimal cytokine concentrations, because 2 and 4 times (800 U/ml) higher IFN γ concentrations did not increase expression (data not shown). The effectiveness of pro-inflammatory cytokines such as TNF α on induction of cell adhesion molecules in the microvascular heart EC has also been described for macrovascular EC *in vitro*, such as HUVEC (8). Apparently, pro-inflammatory cytokines can be considered most relevant for induction of cell adhesion molecules on microvascular EC, as shown in this study, as well as on macrovascular EC in local acute inflammation.

In none of the RHEC lines adhesion molecule induction was found in the whole EC population. Such heterogeneity was also observed by others in microvascular EC of mouse heart and human dermal origin (22, 23). In this respect microvascular heart EC respond differently than macrovascular EC, such as HUVEC, in which cytokine stimulation has been described to induce adhesion molecule expression in the whole cell population (32). Our stimulation experiments using combinations of TNF α and IFN γ clearly demonstrated the importance of IFN γ as a co-stimulatory cytokine for adhesion molecule induction. This immune regulatory cytokine, which is produced by activated T cells and natural killer (NK) cells, increased the induction of ICAM-1 by TNF α and in two of the three RHEC lines had a synergistic effect on TNF α -induced VCAM-1 expression.

Interestingly, the 3 RHEC lines differed from each other in their specific ICAM-1 and VCAM-1 expression profiles upon cytokine stimulation. RHEC-3, which had a basal ICAM-1 expression, was inducible to relatively high levels of both ICAM-1 and VCAM-1, whereas RHEC-11 showed very poor ICAM-1 but high VCAM-1 induction. Surprisingly, RHEC-10 was not at all inducible to ICAM-1 expression, and only minorly to VCAM-1 expression. The latter was not due to a non responsiveness of RHEC 10 to cytokine stimulation (e.g. due to absence of cytokine receptors on the EC), as demonstrated by the fact that IFN γ stimulation induced MHC class II expression. Also, TNF α synergistically increased this expression in this cell line. The different responses of the 3 endothelial cell lines in ICAM-1 and VCAM-1 induction upon cytokine stimulation, contrasted with the responses of the 3 lines in MHC molecule induction. MHC class I molecules, constitutively expressed by EC, were similarly upregulated in all 3 RHEC lines. Also MHC class II expression profiles after cytokine stimulation did not differ between the 3 RHEC lines. A synergistic effect of TNF α on IFN γ -induced MHC class II expression was noticed. This indicates that different, e.g. opposite synergistic effects are obtained with IFN γ versus TNF α in ICAM-1/VCAM-1 and MHC class II induction respectively. Such specific effects of cytokine combinations in stimulation of EC, and its implication for inflammatory conditions have also been described by others. For example, Leeuwenberg et al., showed that IFN γ prolonged the effect of TNF α on E-selectin expression (14), whereas a similar effect was shown for IL-4 in combination with TNF α for VCAM-1 expression (33). We showed that the different responses of the RHEC lines could not be explained by differences in Ca²⁺ mobilization or PKC activity. Also ultrastructurally no indications were found pointing at differences between the cell lines. In fact the data together, including the similar responses of the cell lines in P-selectin expression, suggest that the heterogeneity in ICAM-1 and VCAM-1 is a specific characteristic of the heart EC population. The fact that the induction of adhesion molecules and MHC class II antigens was shown in only part of the EC population, and the observation that vWF and also ICAM-1, in RHEC 3 (25)

was never seen in all EC in standard cultures, demonstrate a phenotypical heterogeneity of EC within a RHEC line. Previously we have shown that this heterogeneity is probably not related to differences in cell cycle stage of the EC in confluent cultures (25). The experiment with selected either ICAM-1, VCAM-1 or MHC class II positive and negative populations showed that, except for the selected ICAM-1 positive EC population, the selected profile was slowly lost during subsequent culturing. Also the cloned cell population from RHEC 3 still showed heterogeneity in adhesion molecule and MHC molecule expression upon cytokine stimulation. Apparently this heterogeneity within a RHEC line results from line-specific intrinsic regulation mechanisms, and not from the presence of particular subpopulations of EC in the RHEC lines. Such programmed heterogeneity among EC *in vitro* cultures has also been described by others (22, 23, 24), and was also noticed in microvascular EC in the normal rat heart *in vivo* (25). We noticed in immunofluorescence microscopy studies of confluent monolayer cultures of RHEC lines that the expression of ICAM-1, VCAM-1, or MHC class II was mostly seen in foci or strands of cells. In fact, this may be relevant for understanding how *in vivo* such reactivity of EC in the microvascular endothelium occurs. A possible explanation for the remarkable differences in expression profiles between the 3 lines could be their origin from different hearts. However, this is not likely because the rats used were from the Lewis inbred strain. We think it is more likely that such heterogeneity is related to the primary cultures representing endothelial isolates from various locations in the heart micro-vasculature.

One may expect that in settings of inflammation and local immune reactivity in heart tissue the selective differences of vascular EC as demonstrated here, has an impact on leukocyte-endothelial cell interactions in local inflammatory and immune processes. For example, if upon cytokine stimulation microvascular heart EC develop different phenotypes regarding expression profiles of MHC and adhesion molecules, this may imply different functions of EC in processes such as leukocyte infiltration and antigen presentation to blood T cells. With respect to leukocyte infiltration,

differential expression of ICAM-1 or VCAM-1 may regulate preferential local adhesion and infiltration of specific leukocyte subpopulations. MHC class II expression in combination with costimulatory molecules such as ICAM-1 and/or VCAM-1 is required for antigen presentation to T helper cells (12). However, antigen presentation by MHC class II in the absence of such costimulatory adhesion may render T cells anergic, or apoptotic. In general our data suggest that heart microvascular EC *in vivo* display heterogeneous differentiation profiles, which in inflammation and immune reactivity leads to different phenotypes with respect to adhesion and MHC molecule expression. As such this knowledge contributes to understanding how local EC can control different types and effects of leukocyte-EC interactions in heart tissue.

Acknowledgements: The authors would like to thank A. Gijssen and M. Maes for technical assistance. We thank Dr. J. Damoiseaux for assistance in FACS studies, Dr. J. Heemskerk and M. Feijge for assistance in calcium mobilization studies, P. Bomans for electron microscopy assistance, Drs. R. Lobb and P. Chisholm for donation of 5F10, Dr. M. Miyasaka for donation of 1A29, Prof. Dr. W. Fiers and Dr. P. van de Meide for donation of TNF α and IFN γ respectively.

3.5 References

- Hakkert BC, Kuijpers T, Leeuwenberg JFM, Mourik van JA, Roos D. Neutrophil and monocyte adherence to and migration across monolayers of cytokine activated endothelial cells: contribution of CD18, ELAM-1 and VLA-4. *Blood* 1991; **78**: 2721.
- Lawrence MB, Springer TA. Neutrophils roll on E-selectin. *The Journal of Immunology* 1993; **151**: 6338.
- Bevilaqua MP, Nelson RM. Selectins. *Journal of Clinical Investigation* 1993; **91**: 379.
- Elices MJ, Osborne L, Takada Y, et al. VCAM-1 on activated endothelium interacts with leukocyte integrin VLA-4 at a distinct of the VLA-4/fibronectin binding site. *Cell* 1990; **60**: 577.
- Springer TA. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multi step paradigm. *Cell* 1994; **76**: 301.
- Kuyper TW, Harlan JM. Monocyte endothelial interactions insights and questions. *Journal of Lab Clin Med* 1993; **122**: 641.
- Carlos TM, Harlan JM. Leukocyte endothelial adhesion molecules. *Blood* 1994; **84**: 2068.
- Sluiter W, Pietersma A, Lamers JMJ, J.F. K. Leukocyte adhesion molecules on the vascular endothelium: Their role in the pathogenesis of cardiovascular disease and the mechanism underlying their expression. *Journal of Cardiovascular Pharmacology* 1993; **22**: s37.
- Bevilaqua MP. Endothelial-leukocyte adhesion molecules. *Annu Rev Immunol* 1993; **11**: 767.
- Dougherty GJ, Murdoch S, Hogg N. The function of human intercellular adhesion molecule 1 (ICAM-1) in the generation of an immune response. *European Journal of Immunology* 1988; **18**: 35.
- Makgoba MW, Sanders ME, Ginther Luce GE, et al. Functional evidence that intercellular adhesion molecule 1 (ICAM-1) is a ligand for LFA-1 dependent adhesion in T cell mediated cytotoxicity. *European Journal of Immunology* 1988; **18**: 637.
- Steinman RM, Young JW. Signals arising from antigen presenting cells. *Current Opinion in Immunology* 1991; **3**: 361.
- Damle NK, Klussman K, Leytze G, et al. Costimulation of T lymphocytes with integrin ligands intercellular adhesion molecule-1 or vascular adhesion molecule-1 induces functional expression of CTLA-4, a second receptor for B7. *Journal of Immunology* 1994; **152**: 2686.
- Leeuwenberg JFM, Damme van J, Maeger T, Jeunhomme TMM, Buurman WA. Effects of tumor necrosis factor on the interferon- γ -induced major histocompatibility complex class II antigen expression by human endothelial cells. *European Journal of Immunology* 1988; **18**: 1469.
- Smith CW, Anderson DC, Taylor, Rossen RD, Entman ML. Leucocyte adhesion molecules and myocardial ischemia. *Trends in Cardiovascular Medicine* 1991; **1**: 167.
- Palluy O, Morliere L, Gris J, Bonne C, Modat G. Hypoxia/reoxygenation stimulates endothelium to promote neutrophil adhesion. *Free Radic Biol Med* 1992; **13**: 21.
- Osborn L, Hession C, Tizard R, et al. Direct expression cloning of vascular cell adhesion molecule-1, a cytokine-induced endothelial protein that binds to lymphocytes. *Cell* 1989; **59**: 1203.
- Pober JS, Cotran RS. Cytokines and endothelial cell biology. *Physiol Rev* 1990; **70**: 427.
- Carlos TM, Harlan JM. Membrane proteins involved in phagocyte adherence to endothelium. *Immunological Review* 1990; **114**:
- Fougerolles de AR, Stacker SA, Schwarting R, Springer A. Characterization of ICAM-2 and evidence for a third counter receptor for LFA-1. *Journal of Experimental Medicine* 1991; **174**: 253.
- Linke AT, Male DK. Strain specific variation in constitutive and inducible expression of MHC class II, class I and ICAM-1 on rat cerebral endothelium. *Immunology* 1994; **82**: 88.
- Gumkowski F, Kaminska G, Kaminski M, L.W. M, Auerbach R. Heterogeneity of mouse vascular endothelium. *Blood Vessels* 1987; **24**: 11.
- Petzelbauer P, Bender JR, Wilson J, Pober JS. Heterogeneity of dermal microvascular endothelial cell antigen expression and cytokine responsiveness in situ and in cell culture. *The Journal of Immunology* 1993; **151**: 5062.
- Wang JM, Kumar S, Pye D, Vanagthoven AJ, Krupinski J, Hunter RD. A monoclonal antibody detects heterogeneity in vascular endothelium of tumours and normal tissues. *Int J Cancer* 1993; **54**: 363.
- Derhaag J, Duijvestijn A, Emeis J, Engels W, Breda Vriesman van P. Production and characterization of spontaneous rat-heart endothelial cell lines. *Laboratory Investigations* 1996; **74**: 437.
- Linssen MCJG, Nieuwenhoven van FA, Duijvestijn AM, Glatz JFC, Vusse van der GJ. Continuous endothelial cells from adult rat heart. *In Vitro cell Dev Biol* 1993; **29a**: 611.
- Maciag T, Cerendulo J, Isley S, Kellu PR, Forand R. An endothelial growth factor from bovine hypothalamus: identification and partial characterization. *Proc Natl Acad Sci USA* 1979; **76**: 5674.

28. Tamatani T, Miyasaka M. Identification of monoclonal antibodies reactive with the rat homolog of ICAM-1 and evidence for a differential involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. *International Immunology* 1990; **2**: 165.
29. May MJ, Entwistle G, Humphries MJ, Ager A. VCAM-1 is a CS-1 peptide-inhibitable adhesion molecule expressed by lymph node high endothelium. *Journal of Cell Science* 1993; **106**: 109.
30. Duijvestijn AM, Goor van H, Klatter F, Majoor GD, Bussel van E, Breda Vriesman van PJC. Antibodies defining rat endothelial cells: RECA-1 a pan endothelial specific monoclonal antibody. *Laboratory Investigation* 1992; **62**: 459.
31. Haaster van MCJ, Engels W, Lemmens PJMR, Hornstra G, J. VvdG, Heemskerk JWM. Differential release of histamin and prostaglandin D2 in rat peritoneal mast cells: roles of cytosolic calcium and protein tyrosine kinases. *Biochem Biophys Acta* 1995; **1265**: 79.
32. Wedgwood JF, Hatam L, Bonagura VR. Effect of interferon- γ and tumor necrosis factor on the expression of class I and class II major histocompatibility molecules by cultured umbilical vein endothelial cells. *Cellular Immunology* 1988; **111**: 1.
33. Iademarco MF, Barks JL, Dean DC. Regulation of vascular cell adhesion molecule-1 expression by IL-4 and TNF α in cultured endothelial cells. *Journal of Clinical Investigation* 1995; **95**: 264.

4

A dual role for EC in cytomegalovirus infection?

A study of cytomegalovirus infection in a series of rat endothelial cell lines

Renée C. R. M. Vossen*, Josien G. Derhaag#, Marlea E. P. Slobbe van Drunen*,
Adrian M. Duijvestijn# Maria C. E. van Dam Mieras§ and Cathrien A. Bruggeman*.

*Department of Medical Microbiology, Maastricht University, P. O. Box 5800,
6202 AZ Maastricht, and #Department of Immunology, Maastricht University,
Maastricht, The Netherlands, and §Department of Natural Science, Open University,
Heerlen, The Netherlands.

Published in: Virus Research 1996, Vol. 46, p. 64-74.

Abstract

Several clinical findings point to the involvement of microvascular endothelial cells (EC) in cytomegalovirus-related pathology. In this study the interactions of cytomegalovirus (CMV) with microvascular EC was investigated in an *in vitro* rat model. A series of rat endothelial cell lines, considered representative for the heterogeneity of heart microvascular endothelium *in vivo*, were infected with rat CMV (RCMV). The course of infection and production of infectious virus were examined using immunofluorescence staining and plaque titration assays, and was compared with the infection of fully permissive rat fibroblasts. These endothelial cell lines displayed differences in susceptibility to CMV infection. Two endothelial cell lines (RHEC 50 and 191) were practically non-permissive, while four endothelial cell lines (RHEC 3, 10, 11 and 116) were partly permissive for CMV infection. In contrast to CMV infection on fibroblasts, only limited infection of the permissive endothelial cell lines was observed without spreading of CMV infection through the monolayer, although infectious virus was produced. Detachment of infected EC and recovery of the monolayer with time was observed. The detached EC were able to transmit CMV infection to fibroblast monolayers, but not to endothelial cell monolayers. Our *in vitro* results demonstrate differences in permissiveness for RCMV between the series of rat endothelial cell lines, which is suggestive for endothelial heterogeneity to CMV infection *in vivo*. Our findings indicate that EC are relatively resistant to CMV infection and that, upon infection, the endothelial monolayer may dispose of the virus via detachment of the infected cells. This points to a dual role for the endothelium in CMV infection *in vivo*: a barrier for CMV infection (by the endothelial monolayer) on the one hand and spreading of CMV infection (by detached infected cells) on the other hand.

4.1 Introduction

Infection of healthy individuals by cytomegalovirus (CMV) is usually asymptomatic. However, in immunocompromised individuals, like AIDS patients and transplant recipients, CMV infection causes life-threatening complications. A variety of symptoms like retinitis, vasculitis and gastrointestinal ulcers is observed in these patients with acute CMV infection. Furthermore, chronic CMV infection has been associated with atherosclerosis and transplant associated atherosclerosis (1, 2, 3).

During the acute phase of the infection, especially in the severely immunocompromised host, microvascular endothelial cells (EC) have been described to be infected in several organs (4, 5). Furthermore, circulating CMV infected cells of endothelial origin are found in the peripheral blood of organ transplant recipients during acute CMV infection (6). These circulating EC harbor infectious virus and it is speculated that they may be involved in dissemination of CMV throughout the body when they become trapped in capillaries (6).

The clinical observations listed above can be simulated and studied in an *in vivo* rat model. Infection of immunocompromised rats with rat CMV (RCMV) leads to a generalised infection with virus in most organs and to comparable symptoms as described in severe immunocompromised HCMV-infected patients (7). A recent study of acutely infected immunocompromised rats (8) describes the appearance of gastrointestinal bleedings (as observed in AIDS patients) and direct endothelial damage in the microvasculature. Furthermore, by using a rat model to study the pathogenesis of interstitial lung disease after bone marrow transplantation, endothelial damage is found to be one of the multiple factors in the development of clinical symptoms (9).

The *in vivo* data described above suggest a role for EC from the microvasculature in CMV-associated pathology. To establish the link with the *in vivo* system it is attractive to study the interactions of CMV with EC in more detail in an *in vitro* rat model. Recently, six continuous microvascular endothelial cell lines, derived from the rat heart microvasculature, have been isolated and characterized (10, 11). These rat heart endothelial cell lines (RHEC) exhibit common endothelial markers but show differential

phenotypic and functional characteristics; differential expression of activation markers such as VCAM-1, ICAM-1 and MHC-II, differences in the production of prostaglandins, tissue plasminogen activator and its inhibitor. This series of cell lines have been shown to be representative for the heterogeneity of heart microvascular endothelium *in vivo* (10, 11).

In the present study, the interaction of rat CMV with these rat endothelial cell lines was investigated. The results indicated that these cell lines display heterogeneity with respect to RCMV permissiveness. In addition, limited CMV infection without spreading of the virus through the endothelial cell monolayer as well as detachment of infected EC from the monolayer was observed. Our results in combination with *in vivo* findings suggest a barrier function of the endothelial monolayer for CMV infection *in vivo*. On the other hand, detached infected EC may be involved in CMV dissemination.

4.2 Materials & methods

Virus and cell culture

Rat cytomegalovirus (RCMV, Maastricht strain) was cultured in rat embryonal fibroblasts (REF) in minimum essential medium (EMEM, ICN Biomedicals, Zoetermeer, the Netherlands), containing 2% fetal calf serum (FCS) (Biowithacker, Verviers, Belgium), 200 mM L-glutamine (Serva, Heidelberg, Germany) and non-essential aminoacids (ICN Biomedicals, Zoetermeer, The Netherlands). At 100% cytopathic effect, the culture supernatant was clarified by centrifugation for 10 min at 500 x g. RCMV preparations were stored at -70°C and infectivity titers were determined by plaque assay on REF monolayers as described below.

Spontaneously transformed endothelial cell lines (originally isolated from rat heart) were cultured in RPMI 1640/M199 (1/1,v/v)(Gibco BRL, Breda, The Netherlands), containing 20% FCS (Integro, Zaandam, The Netherlands), 200 mM l-Glutamine, 10 U/ml heparin (ICN Biomedicals, Zoetermeer, The Netherlands), 2 ml/l endothelial growth supplement (12), 50 µg/ml gentamicin. The following cell lines were used between passage 20 and 35: RHEC 3, 10, 11 described by Derhaag (10) and RHEC

50, 116 and 191 described by Linssen (11). All cell lines have been characterized as EC by staining with von Willebrand Factor and internalization of Dil-ac-LDL as described (10, 11).

Infection of cells

RHEC or REF were cultured in 96 well culture plates (Costar, Badhoevedorp, The Netherlands) till 95% confluency. The cells were washed twice with phosphate buffered saline (PBS) and incubated in infection medium (Iscove's modified Dulbecco medium (IMDM, ICN Biomedicals, Zoetermeer, The Netherlands) containing 15% FCS for RHEC and EMEM containing 2% FCS for REF) for 4 h, and washed again with PBS. The cells were inoculated at m.o.i. of 1 or 0.2 or 0.02 with RCMV diluted in infection medium or with infection medium alone. A m.o.i. of 1 means that 1 plaque forming unit (PFU, viral titre as determined by plaque titration assay on rat fibroblasts) of CMV is applied per cell. The cells were either incubated with RCMV for 60 min at 37°C or centrifuged with RCMV for 45 min at 700 x g at 20°C, followed by washing and incubation with infection medium. Enhancement of HCMV infection of human fibroblasts by centrifugational infection was previously described (13). At the indicated times after infection (1, 3, 5, 7 or 13 days) viral antigens were detected by immunofluorescent staining as described below. Infected and uninfected cells were examined daily for appearance of cytopathic effect.

To investigate if soluble factors in culture supernatants of non-infected RHEC monolayers may influence the CMV permissiveness of RHEC, culture supernatants of every cell line were prepared by incubating the RHEC monolayers with infection medium for 24 h. The permissive cell lines (RHEC 3, 10, 11 and 116) were pre incubated for 4 h with the culture supernatants obtained from non-permissive cell lines (RHEC 50 and 191), followed by infection with RCMV and subsequent incubation for 24 h with the same culture supernatants. Concomitantly, non-permissive cell lines were incubated with the culture supernatants obtained from permissive cell lines. Infection with RCMV (m.o.i. = 1) and detection of viral antigens was performed as described above.

To investigate their susceptibility to second RCMV infection, RHEC monolayers were infected with RCMV (m.o.i. = 1) as described above and viral antigens were detected by immunofluorescence staining at the indicated times after infection (day 13, 15, 17 and 19 post infection (p.i.)). Non-infected RHEC monolayers were used as controls.

Detection of viral antigens by immunofluorescence

At the indicated times, culture supernatants were removed and cells were fixed with 3% formaldehyde in PBS for 15 min at room temperature. The cells were permeabilised in 0.05% Nonidet P40 in PBS for 10 min at RT. After blocking with PBS containing 1% bovine serum albumin (BSA), cells were incubated with a mixture (1:1) of mab 8 and 35 against nuclear (41, 46 and 91 kD) and cytoplasmic (29 kD) RCMV antigens (14) diluted 1 in 350 in PBS containing 0.1% BSA, followed by a second step incubation with FITC labeled rabbit anti mouse IgG (ITK, Uithoorn, The Netherlands) diluted 1 in 80. As a control for non-specific staining, infected and non-infected cells were incubated with an anti human CMV mab of the same subclass non reactive with RCMV. For total nuclei staining, 4',6-diamino-2-phenylindole (DAPI 0.5 ng/ml) in PBS containing 2.3% 1,4-diazo-bicyclo-(2,2,2)-octane (DAPCO) was added to the wells. Positive FITC fluorescent cells and total nuclei were counted in triplicate wells (3 microscopic fields per well) using a Zeiss axiovert 100 inverted microscope equipped for epifluorescence, and percentage infection was calculated.

Detection of detachment of infected cell

At indicated times after infection of RHEC the culture supernatant was recovered under gentle agitation and fixed with 3% formaldehyde. Cytospin preparations were made at 600 rpm for 8 min using a Shandon cytofuge. Infected cells were detected by immunofluorescence staining as described above. Only intact stained nuclei were counted, excluding cellular debris.

Detection of infectious virus production.

Infectious virus production by plaque titration assay is based on lytic viral infection in permissive REF. REF were grown to 95% confluency in 24 well plates (Greiner, Frickenhausen, Germany) and were incubated with serial dilutions of the RHEC or REF supernatants in infection medium. After 1 h at 37°C, the cells were refreshed with 0.6% agarose (Seakem, FMC, Rockland, ME, USA) containing basal eagle medium (BME, Gibco BRL, Breda, The Netherlands), 2% FCS and 50 µg/ml gentamicin. After 7 days incubation, the cells were fixed with 3.7% formaldehyde and stained with 0.5% cristal violet. The plaques were counted and expressed as infectious plaque forming units per milliliter (PFU/ML)

Detection of infectious virus transmission

Transmission of infectious virus is measured based on viral antigen detection. REF and RHEC were grown to 95% confluency in 96 well culture plates. At indicated times after infection, the culture supernatant of infected RHEC was harvested under gentle agitation and centrifuged at 400 x g for 10 min at room temperature. The cell fraction was obtained and resuspended in an equal amount of infection medium. The cell fractions were incubated with either REF monolayers or with new RHEC monolayers for detection of viral transmission from detached RHEC to REF or new RHEC monolayer, respectively. After 24 h incubation, REF and RHEC were fixed with 3% formaldehyde and infection was measured by immunofluorescence staining as described above.

4.3 Results

RCMV Infection of Rat Endothelial Cell Lines and Rat Fibroblasts.

Although *in vivo* data show that EC from the microvasculature are involved in CMV associated pathology, most *in vitro* results have been obtained using EC from the macrovasculature. In this study we used a series of six microvascular endothelial cell lines, which have been shown to be representative for the heterogeneity of rat heart endothelium *in vivo* (10, 11). These rat endothelial cell lines (RHEC) were infected with RCMV at m.o.i. of 1 and 0.2 with and without centrifugation. Centrifugation is known to cause enhancement of CMV infection (13). After removal of the inoculum, the cell lines were supplied with fresh medium and fixed at day 1, 3, 5, 7, or 13 p.i. The cell lines showed distinct differences in viral antigen expression as determined by immunofluorescence staining (Fig. 1).

Figure 1: Infection of six rat heart endothelial cell lines (RHEC) with RCMV. The cell monolayers were centrifugational infected with RCMV at m.o.i. of 1. At the indicated time post infection, the amount of infected cells, expressed as percentage of the total cells in the monolayer, is shown as detected by immunofluorescence staining. Data are shown from one experiment (performed in triplicate) representative of six experiments performed.

Without centrifugation, a low level of viral antigen expression was found in RHEC 10 and 11 monolayers (<2% infection) while infection of RHEC 3, 50, 116 and 191 was below the detection limit (data not shown). After centrifugational infection (m.o.i. = 1), viral antigen expression at day 1 p.i. was detected in 30% of RHEC 10 and RHEC 11 monolayers, in 14% of RHEC 116 and in 9% of RHEC 3 (Fig. 1). Only a few infected cells were detected in RHEC 50 and RHEC 191 monolayers (0.8%). When using a lower virus titer (m.o.i. = 0.2) comparable differences between the cell lines were found with much lower levels of infection. These differences in CMV permissiveness did not correlate with the previously studied differential characteristics of these cell lines (10). Foci of infected EC developed starting at day 2 p.i. These infected cells were rounded and emerged from the monolayer while staying adhered (Fig. 2). Enlarged nuclei and clustering of infected cells were detected in RHEC 116, and more pronounced in RHEC 3, 10 and 11. Such morphological changes were not observed in RHEC 50 and 191 or in uninfected cells.

Figure 2: Morphological changes induced by RCMV infection of a rat endothelial cell line. The monolayer of RHEC 3 is shown at day 1 (A, B, C) and at day 3 (D, E, F) after centrifugational infection (m.o.i.=1): RCMV positive antigens are indicated (A, D), total nuclei (B, E) and the monolayer morphology (C, F). Notice the enlargement of the nuclei, and rounding up and clustering of the infected cells at day 3. Original magnification x 400.

The number of infected EC in the monolayer tended to decline, which is in contrast to infected rat embryonal fibroblasts (REF) monolayers in which the number of infected cells increases with time. At day 13 p.i. only a few infected cells were detectable within the monolayer of all endothelial cell lines. This may be due to detachment of infected cells from the monolayer, as infected cells were detected in cytospot preparations of the culture supernatants of RHEC 3, 10, 11 and 116. At days 1 and 3 post RCMV infection only a few detached cells were found in the culture supernatant, while detached infected cells (30-40%) were observed at days 5 and 7 (data not shown). It can not be ruled out that lysis of CMV infected cells may also contribute to a relative decrease of the number of infected cells in the endothelial monolayer.

RCMV infection of REF (infected at m.o.i. of 1, 0.2, 0.02) differed from that of RHEC in that the infection rate in REF was much higher (depending on viral input) and that almost 100% infection of the monolayer was reached in time (Table 1). Detached infected cells were observed in cytospin preparations from 1 and 0.2. detachment of the whole monolayer, due to complete infection, was observed at day 4 p.i. for m.o.i. of 1 and 0.2 and at day 5 for m.o.i. of 0.02.

Infection course of rat embryonal fibroblasts (REF)

	m.o.i. = 1	m.o.i. = 0.2	m.o.i. = 0.02
Day 1	74 (4)	52 (1)	19 (2)
Day 2	93 (3)	88 (3)	58 (3)
Day 3	95 (3)	94 (2)	66 (3)
Day 4	X	X	83 (4)
Day 5	X	X	X

Table 1: The infection level of the monolayer (expressed as percentage infection; number of infected cells per total cells) is shown at the indicated time after centrifugational infection with RCMV at m.o.i. of 1, 0.2 or 0.02, as detected by immunofluorescence staining. From day 4 post infection at m.o.i. of 1 and 0.2 and from day 5 at m.o.i. of 0.02 the whole REF monolayer was detached, indicated by an X, and a high level of RCMV infected cells were detected in the culture medium. Data are expressed as percentage infection (SED). Data are shown from one experiment (performed in triplicate) representative of two experiments performed.

Production of Infectious Virus by Rat Endothelial Cell Lines and Rat Fibroblasts.

To study whether infectious virus was produced by RHEC, the culture supernatant was obtained from infected RHEC monolayers at different time points post infection. Infected REF monolayers were used as control. Infectious virus production of RHEC was compared with that of REF using a plaque titration assay on REF monolayers. As shown in Fig. 3, infectious virus was produced by RHEC 3, 10, 11 and 116 monolayers, while only low amounts of virus (<2300 PFU/ml) was recovered from RHEC 50 or 191 monolayers. In comparison, extracellular infectious virus production of REF was much higher and started earlier than that of RHEC (Fig. 3).

Figure 3: Comparison of infectious virus production by RHEC and REF monolayers. At the indicated time after infection, the release of infectious virus in the culture supernatants of RHEC and REF was measured by plaque titration assay on REF monolayers. Data are shown from one experiment representative of three experiments performed. Infectious virus production by REF was detected earlier and with a higher virus titer compared to that of RHEC.

Investigation of the Differences in Susceptibility to CMV Infection of Rat Endothelial Cell Lines.

To investigate whether soluble factors released by the rat endothelial cell lines are responsible for the differences in permissiveness for RCMV, the influence on CMV infection of culture supernatants, obtained from the different RHEC monolayers, was investigated. For this purpose, permissive cell lines (RHEC 3, 10 11 and 116) were incubated with culture supernatants obtained from uninfected non-permissive cell lines (RHEC 50 and 191). Non-permissive cell lines were also incubated with supernatants from uninfected permissive cell lines. No influence of culture supernatants on the infection levels were observed in all experiments (data not shown) suggesting that factors constitutively released by RHEC are not involved in the observed differences in CMV permissiveness.

To investigate whether detached cells from the CMV infected RHEC monolayer were able to transmit CMV to other cells, the following experiments were performed. At different time points after infection, detached RHEC were incubated with REF and new RHEC monolayers and the infection of these REF and RHEC monolayers was monitored by immunofluorescence staining. As shown in Fig. 4, detached RHEC were able to infect REF monolayers, indicating that the detached EC contain infectious virus. However no infection of the RHEC monolayers was observed (data not shown). Also stimulation of RHEC monolayers by centrifugation did not have any effect. It is likely that the amount of infectious virus in detached RHEC is high enough to infect fully permissive REF monolayers, but too low for infection of new RHEC monolayers. These results suggest that, although detached EC can play a role as a transport vehicle of infectious virus, transmission of the infection to other cells may be limited depending on the cell type.

Contrary to fibroblast monolayers, which detached completely after CMV infection, we observed no spreading of CMV through the endothelial monolayer but detachment of the infected EC, which suggests recovery of the endothelial monolayer from CMV infection. We therefore investigated whether endothelial monolayers, that had

previously been infected by CMV, showed an altered susceptibility to CMV infection. For this purpose RHEC monolayers were tested 2 weeks after the first infection, when the amount of infected cells in the monolayer had decreased to less than 2% (see Fig. 1). RHEC at day 12 p.i. and previously uninfected RHEC at days 1 or 12 after confluency were infected with RCMV (m.o.i. = 1). We found that re-infection of previously infected RHEC monolayers resulted in a comparable infection level as in primary infected RHEC (data not shown). This indicates that the permissiveness for CMV infection of the endothelial monolayer is not influenced by a previous infection challenge, suggesting recuperation of the monolayer.

Figure 4: Transmission of CMV infection by detached infected RHEC. At the indicated time, virus transmission of detached infected RHEC to REF was measured by incubation of REF monolayers with the cell fraction of infected RHEC culture supernatants. REF infection was detected by immunofluorescence staining. Data are shown from one experiment representative of two experiments performed.

4.4 Discussion

Microvascular EC have been suggested to play a role in CMV-related pathology. Therefore, it was attractive to investigate the interactions of CMV with microvascular endothelium *in vitro*. We used a series of six rat heart endothelial cell lines, which are considered representative for heterogeneity of the heart endothelium *in vivo*. We observed that these cell lines displayed differential susceptibility to RCMV infection and that, contrary to monolayers of fibroblasts, endothelial monolayers did not reach a 100% infection level, despite the fact that infectious virus could be obtained from the culture supernatants. In addition, most of the infected EC detached from the monolayer. From these intriguing observations, we hypothesize that EC can resist a CMV infection to a certain extent and, in addition, infected endothelial monolayers dispose of the virus via detachment of the infected cells. This points to a barrier function of the inner lining of blood vessels *in vivo*. Such a defensive role for EC against CMV infection is also suggested by experimental results obtained in the *in vivo* rat model (15). In this model it was observed that removal of the endothelial lining of the vessel by balloon denudation resulted in CMV infection of the exposed smooth muscle cells, while infection of smooth muscle cells was not observed when these cells were covered with intact endothelium.

Previous *in vitro* studies learned that, irrespective of the source, EC only be infected to a limited extent. There is no satisfying explanation for the low permissiveness of EC for CMV, but several factors seem to be involved such as the virus strain used and the origin of the cells. Several laboratory strains of human CMV reach only 5-15% infection in human umbilical vein EC (HUVEC), Towne strain (16), Kerr strain (17), AD169 (18), and EC of neural origin appear more susceptible than large vessel HUVEC (19). In addition, HUVEC seems more permissive to low passage clinical CMV isolates and to endothelial propagated virus(20, 21).

Until now mostly large vessel EC originating from human umbilical cords were used to study the virus-endothelial cell interactions *in vitro*, but according to *in vivo* findings it would be appropriate to use EC from the microvasculature in such investigations. By using a series of six different endothelial cell lines, originating from

the rat heart microvasculature (10, 11), we observed distinct differences in susceptibility to RCMV infection. The rat endothelial cell lines RHEC 50 and 191 were practically non-permissive, while RHEC 3, 10, 11 and 116 were partly permissive for RCMV infection. Why the endothelial cell lines show such a variation in susceptibility to infection with RCMV is an intriguing question especially considering the common origin of the cells (heart microvasculature). In addition to the observed differences in phenotypic and functional characteristics (10, 11) these endothelial cell lines displayed also heterogeneity with respect to CMV permissiveness. Our observations are suggestive of endothelial heterogeneity for CMV infection in the heart microvasculature and possibly in other organs *in vivo*.

Several factors may contribute to the observed differences in endothelial permissiveness for RCMV: (i) limited entrance of CMV due to variable expression of endothelial receptors for binding or fusion of RCMV; (ii) differences in viral gene expression or viral replication; and (iii) a release of endothelial factors which may interfere with CMV infection.

The first option, differences in CMV binding or fusion, is suggested by several studies with human CMV showing that cells express various host cell receptors for CMV: class I HLA (22), CD13 (23), the gH receptor (24), and annexin II (25). The variability in permissiveness for RCMV found in the different rat endothelial cell lines could thus be due to differences in receptor expression.

The second option, interference with expression of viral DNA in EC, is suggested by observations in other cell types. Restriction of CMV replication *in vitro* by differentiation-specific negative regulators of the expression of CMV immediate early proteins (26) may point to this option.

Finally, the secretion by the endothelium of factors which may interfere with the infection process, is suggested as the third option. Although our experiments with culture supernatants suggested that constitutively released (stable) factors are not involved in the differential permissiveness observed, it can not be ruled out that inhibition of infection of neighbouring EC via cell-cell contact occurs. Such a phenomenon has been described

recently for herpes virus (HSV-1) infection in polarized MDCK cells, suggesting a role for tight junctions in HSV-1 infection (27).

Our results indicate only limited CMV infection of the rat endothelial cell lines and, in spite of production of infectious virus, no spreading of CMV infection in all endothelial monolayers comparable with that seen in fibroblasts. Furthermore, our observations suggest that the endothelial monolayer is able to recover from the CMV challenge (2 weeks after infection) after detachment of the infected cells. From this we conclude that in our *in vitro* system the endothelial monolayer may constitute a barrier to CMV infection.

The observed detachment of infected EC from the monolayer may be caused by an altered interaction of the cells with extracellular matrix proteins as is described for human CMV infection (28). Our *in vitro* finding is in agreement with the *in vivo* observation that circulating infected EC are found in immunocompromised patients with an active CMV infection (6). It has been speculated that these circulating infected EC may contribute to CMV dissemination throughout the body (6). Our *in vitro* results, however, indicate that transmission of CMV infection from the detached infected cells is limited to fully permissive cells like fibroblasts, whereas the endothelial monolayer seems resistant. This suggest that on the one hand limited permissiveness to CMV infection and detachment of infected cells would contribute to a barrier function of the EC, but on the other hand a role for circulating infected EC in CMV dissemination throughout the body can not be ruled out. To elicit a possible role in dissemination, further investigations of the interactions of these detached infected EC with other cell types are necessary.

Acknowledgements: The authors thank Dr. Marieke Dormans-Linssen for the RHEC 50, 116 and 191 cell lines, and Annemie Gijssen for technical assistance with culturing RHEC 3, 10 and 11.

4.5 References

1. Bruggeman CA, Dam-Mieras van MCE. The possible role of cytomegalovirus in atherogenesis. In: JL Melnick (Ed) *Progress in Medical Virology*, Karger, Basel 1991; 1.
2. Hayry PJ, Alatali S, Myllamiemi M, Raisanen-Sokolowski A, Lemstrom KB. Cellular and molecular biology of chronic rejection. *Transplantation Proceedings* 1995; **27**: 71.
3. Melnick JL, Adam E, Debakey ME. Cytomegalovirus and atherosclerosis. *European Heart Journal* 1993; **14**: 30.
4. Meyerson D, Hackman RC, Nelson JA, McDoughall JA. Widespread presence of histologically occult cytomegalovirus. *Hum Pathology* 1984; **15**: 430.
5. Roberts WH, Sneddon JM, Waldman J, Stephens RE. Cytomegalovirus infection of gastrointestinal endothelium demonstrated by simultaneous nucleic acid hybridization and immunohistochemistry. *Arch Pathol Lab Med* 1989; **113**: 461.
6. Grefte JMM, Giessen van der M, Son van WJ, The TH. Circulating human cytomegalovirus (HCMV)-infected endothelial cells in patients with an active HCMV infection. *Journal of Infection Diseases* 1993; **167**: 270.
7. Stals FS, Bosman F, Boven van CP, Bruggeman CA. An animal model for therapeutic intervention studies of CMV infection in the immuno-compromised host. *Arch Virol* 1990; **114**: 91.
8. Persoons MCJ, Stals FS, Dam-Mieras van MCE, A. BC. Multiple organ involvement during experimental cytomegalovirus infection is associated with disseminated vascular pathology. 1996; submitted.
9. Stals FS, Steinhof G, Wagenaar SS, et al. Cytomegalovirus induces interstitial lung diseases in allogeneic bone marrow transplant recipients rats independent of acute graft versus host response. *Laboratory Investigation* 1996; **74**: 452.
10. Derhaag J, Duijvestijn A, Emeis J, Engels W, Breda Vriesman van P. Production and characterization of spontaneous rat-heart endothelial cell lines. *Laboratory Investigations* 1996; **74**: 437.
11. Linssen MCJG, Nieuwenhoven van FA, Duijvestijn AM, Glatz JFC, Vusse van der GJ. Continuous endothelial cells from adult rat heart. *In Vitro cell Dev Biol* 1993; **29a**: 611.
12. Maciag T, Cerendulo J, Isley S, Kellu PR, Forand R. An endothelial growth factor from bovine hypothalamus: identification and partial characterization. *Proc Natl Acad Sci USA* 1979; **76**: 5674.
13. Hudson JB. Further studies on the mechanism of centrifugal enhancement of cytomegalovirus infectivity. *Journal of Virological Methods* 1988; **19**: 97.
14. Bruning JH, Bie de WHM, Dormans PHJ, Meijer H, Bruggeman CA. The development and characterization of monoclonal antibodies against rat cytomegalovirus induced antigens. *Arch Virol* 1987; **94**: 55.
15. Persoons MCJ, Daemen MJAP, Bruning JH, Bruggeman CA. Active cytomegalovirus infection of arterial smooth muscle cells in immunocompromised rats. A clue to herpes-associated atherogenesis. *Circulation Research* 1994; **75**: 214.
16. Smiley HL, Mar E. Cytomegalovirus infection and viral induced transformation of human endothelial cells. *Journal of Medical Virology* 1988; **25**: 213.
17. Span AHM, Boven van CPA, Bruggeman CA. The effect of cytomegalovirus infection on the adherence of polymorpho-nuclear leucocytes to endothelial cells. *European Journal of Clinical Investigation* 1989; **19**: 542.

18. Geelen van AGM, Slobbe-van Drunen MEP, Muller AD, Bruggeman CA, Dam-Mieras van MCE. Membrane related effects in endothelial cells induced by human cytomegalovirus. *Arch Virol* 1995; **140**: 1601.
19. Lathey JL, Wiley CA, Verity MA, Nelson JA. Cultured human brain capillary endothelial cells are permissive for infection by human cytomegalovirus. *Virology* 1990; **176**: 266.
20. Ho DD, Rota TR, Andrews CA, Hirsch MS. Replication of human cytomegalovirus in endothelial cells. *Journal of Infection diseases* 1984; **150**: 956.
21. Waldman WJ, Snaddon JM, Stephens RE, Roberts WH. Enhanced endothelial cytopathogenicity induced by a cytomegalovirus strain propagated in endothelial cells. *Journal of Medical Virology* 1989; **28**: 223.
22. Grundy JE, McKeating JA, Ward PJ, Sanderson HR, Griffiths PD. B2 microglobulin enhances the infectivity of cytomegalovirus and when bound to virus enables class I HLA molecules to be used as a virus receptor. *Journal Gen Virol* 1987; **68**: 793.
23. Soderberg C, Giugni TD, zaia JAL S., Wahlberg JM, Moller E. CD13 (Human aminopeptidase N) mediated human cytomegalovirus infection. *Journal of Virology* 1993; **67**: 6576.
24. Keay S, Merigan TC, Rasmussen L. Identification of cell surface receptors for the 86-kilodalton glycoprotein of human cytomegalovirus. *Proc Natl Acad Sci USA* 1994; **86**: 10100.
25. Wright JF, Kurosky A, Wasi S. An endothelial cell-surface form annexin II binds human cytomegalovirus. *Biochem Biophys Res Commun* 1994; **198**: 983.
26. Taylor-Wiedeman J, Sissons P, Sinclair J. Induction of endogenous human cytomegalovirus gene expression after differentiation of monocytes from healthy carriers. *Journal of Virology* 1994; **68**: 1597.
27. Hayashi K. Role of tight junctions of polarized epithelial MDCK cells in the replication of herpes simplex virus type I. *Journal of Med Virol* 1995; **47**: 323.
28. Pande H, Terramani T, Tressel T, Churchill MA, Hawkins GG, Zaia JA. Altered expression of fibronectin gene in cells infected with human cytomegalovirus. *Journal of Virology* 1990; **64**: 1366.

5

Effects of antibody reactivity to MHC and non-MHC alloantigens on graft endothelial cells in heart allograft rejection

Josien G. Derhaag, Adrian M. Duijvestijn, Jan G. M. C. Damoiseaux and
Peter J. C. van Breda Vriesman.

Department of Immunology, CARIM, Maastricht University, P. O. Box 616,
6200 MD, Maastricht, The Netherlands

Submitted for publication.

Abstract

To gain insight in the pathogenesis of vascular lesions in heart allograft rejection, we investigated effects of allosera reactive with MHC or non-MHC alloantigens on graft endothelial cells (EC) in a rat transplantation model.

Anti MHC and anti non-MHC allosera were obtained from BN (RT.1n) recipients of a Lewis (RT.1l) or congenic LEW.1N (RT.1n) heart allograft respectively. Reactivity with endothelial alloantigens was studied *in vitro* using a series of 3 rat-heart endothelial-cell (RHEC) lines of Lewis origin. Phenotypic studies of MHC and non-MHC alloantigen expression, complement activation, and adhesion molecule induction on EC were performed by immunostaining and FACS analysis. Complement-mediated cytotoxicity of allosera was studied using a ^{51}Cr release assay.

Both anti MHC allosera and anti non-MHC allosera showed reactivity with all 3 RHEC lines. EC stimulation with $\text{TNF}\alpha$ and $\text{IFN}\gamma$, resulted in upregulation and induction of MHC class I and II antigens respectively, but not of non-MHC alloantigens. Anti MHC allosera showed distinct complement-mediated cytotoxicity for EC which was increased after cytokine stimulation. With anti non-MHC allosera only low cytotoxicity was measured which was not increased by cytokine stimulation. In both anti MHC and anti non-MHC allosera allospecific IgG had similar subclass profiles, but allospecific IgM was only detected in anti MHC allosera. Complement-mediated cytotoxicity of anti MHC allosera appeared effected mainly by IgM but not IgG alloantibodies. Anti MHC allosera activated complement of both guinea pig (GP) and rat origin. Anti non-MHC allosera led only to C3 expression of GP origin. No indications were found that alloantibody reactivity or complement activation led to EC activation processes relevant to ICAM-1 or VCAM-1 induction.

Our data show that in heart allograft rejection MHC but also non-MHC alloantigens on EC are target structures in the alloantibody response. Complement-mediated cytotoxicity for graft EC is effected by IgM alloantibodies and appears to play a role in rejection of MHC but not of non-MHC mismatched heart allografts. Whether alloantibody reactivity with endothelial MHC or non-MHC alloantigens can provoke other endothelial effects relevant to graft vasculopathy requires further investigation.

5.1 Introduction

Successful heart transplantation (Tx) is hampered by graft malfunctioning and eventually graft loss as a result of the recipient's immune response against graft alloantigens. The endothelium of the graft vasculature is considered a major target tissue in the rejection process because of its direct and continuous contact with the circulating immune reactants of the recipient. Since adequate EC functioning is required for good graft performance and survival, interference by immune reactants with vascular homeostatic processes of EC contributes to the pathogenesis of graft rejection.

In allogeneic transplantation MHC class I and class II antigens are considered the most important target structures in the recipient's immune response directed to the graft. The immune reactants formed in this response are of cellular and humoral (antibody) nature (1, 2, 3, 4) and may readily interact with constitutively expressed MHC class I and, in settings of immune reactivity, induced MHC class II antigens on graft EC.

Most studies on immunological processes in allograft rejection are dealing with acute rejection, and emphasize cellular effector mechanisms and in particular the important role of T cells (5, 6, 7). Antibodies have been considered to play a minor role in acute allograft rejection and consequently their precise effects are poorly defined. However, increasing interest in vascular rejection processes, due to its significance in chronic rejection, has focussed on the role of anti-EC antibodies in development of graft vasculopathy. It appears that in this respect antibody reactivity with EC affects various processes in the graft vasculature. Recently it has been shown that antibodies can activate or perturb EC (8), triggering processes that lead to alterations in endothelial integrity. For instance complement activation on EC by Ab results in production of substances relevant for coagulation processes (9, 10, 11) and the induction of endothelial cell adhesion molecules for leukocytes (12, 13). This induction of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin appears to result from autocrine stimulation by newly formed endothelial IL-1 (13, 14).

Complement activation also leads to the formation of split products of early complement components such as C3a and C5a which influence recruitment and effector functions of platelets and leukocytes (15). This series of antibody effects provokes local inflammatory processes contributing to development of vasculopathological lesions.

Alloantibody reactivity with EC may also have other effects relevant for development of graft vasculopathy. When alloantibodies on EC activate complement, this may lead to formation of the membrane attack complex (MAC or C5b-9) and subsequent EC lysis. In fact, next to such complement-mediated cytotoxic effects of alloantibodies, anti-EC alloantibodies facilitate EC destruction through the processes of anti-body-dependent-cell-mediated-cytotoxicity (ADCC) (16, 17). Whereas MHC molecules can be considered the most dominant antigens in alloantibody reactivity with graft EC, a series of studies have demonstrated that also non-MHC alloantigens are involved in rejection processes (18). Such alloantibody reactivity with endothelial non-MHC alloantigens appears associated with vasculopathy, and in particular with graft arteriosclerotic lesions (19, 20).

To define whether in heart allograft rejection alloantibodies to endothelial MHC and non-MHC alloantigens play a role in the development of graft vasculopathy, we used a heart allograft model in the rat in combination with *in vitro* cultures of heart EC. Allosera reactive with graft MHC or non-MHC alloantigens were obtained from BN recipients of an allogeneic (LEW) or congenic (LEW.1N) heart respectively. These sera were tested for endothelial cell reactivity, complement activation, and complement-mediated cytotoxicity on non-stimulated and cytokine-stimulated cultures of 3 Rat-Heart-Endothelial-Cell (RHEC) lines from LEW origin. In addition, alloantibodies reactive with EC were isotyped and it was tested whether reactivity with EC led to induction of the adhesion molecules ICAM-1 and VCAM-1. Our data show that in heart allograft rejection graft EC express, in addition to constitutively expressed MHC class I and inducible class II antigens, non-MHC alloantigens that are targets in the alloantibody response. Both anti MHC and anti

non-MHC allosera show low complement activation and cytotoxic effects with non-stimulated EC, but a distinct increase of the effects is measured for anti MHC allosera with cytokine-stimulated (MHC class II expressing) EC. We demonstrated that these effects are dependent on the presence of alloantibodies of the IgM type, which appear to be absent in anti non-MHC allosera. No evidence was found that alloantibody reactivity leads to activation of EC mechanisms effecting adhesion molecule induction. The results are discussed in the context of development of graft vasculopathy in allograft rejection.

5.2 Materials & methods

Animals

Lewis, (LEW; RT.-1^l), Brown Norway (BN; RT.-1ⁿ) and congenic Lewis 1N (LEW.1N, RT.-1ⁿ) rats were obtained from the Central Animal Facility of Maastricht University. Male rats in the age of 8 to 12 weeks were used. Animals were bred under specified pathogen free conditions and had free access to food and water.

Heart transplantation and allosera collection

Hearts were routinely transplanted heterotopically in the abdomen. In short: the donor heart aorta and artery pulmonalis were end to side anastomized to the recipient aorta abdominalis and vena cava inferior respectively (21, 22). Transplantations were considered technically successful when heart beat was more than 250 beats/min on the second day after transplantation (Tx). Transplanted hearts were considered rejected when heart beat was absent or less than 100/min. The following heart Tx were executed for allosera production:

- 1: LEW -> BN, (MHC and non-MHC) -mismatched;
bloodsamples were collected at the day of rejection (7-8 days after Tx).
- 2: LEW.1N -> BN, non-MHC mismatched; bloodsamples were collected
at day 8, 16, 21 days after Tx, and the day of rejection (29-35 days after Tx).
- 3: BN -> BN, matched non rejected hearts; bloodsamples were collected at day 8
and 29 after Tx.

Bloodsamples were prepared for sera and stored at -70°C until use. Unless mentioned otherwise the sera used were from the day of rejection.

Endothelial cells

For *in vitro* EC studies 3 EC lines from LEW heart origin were used. These Rat Heart Endothelial Cell lines RHEC 3, RHEC 10, and RHEC 11 were prepared as previously described (23). EC were routinely cultured in EC culture medium (40% RPMI 1640 (Gibco BRL, Life Technologies B.V., Breda, the Netherlands, 40% M199

(Gibco BRL), 20 % inactivated FCS (Integro, Belgium), 0.5 µl/ml bovine brain extract (14.93 mg/ml) prepared as described by Maciag (24), 13 mM NaHCO₃, 50 U/ml penicillin, 50 µg/ml streptomycin, 2 mg/ml Fungizone, 10 mM Hepes, 20 U/ml heparin and 2mM L-Glutamin). Cells were cultured using 5% CO₂ in air, at 37°C. For passaging the cells, confluent monolayers were treated with trypsin (0.25%)/EDTA (0.2%) in phosphate buffered saline (PBS) for 1 minute. Trypsin was neutralized with EC Work medium (90% M199 (Gibco BRL), 10% inactivated FCS, 50 U/ml penicillin, 50 µg/ml streptomycin and 2 mM L-Glutamin and 25 mM Hepes).

Cytokine stimulation of RHEC cultures

Confluent EC monolayers were stimulated with a combination of the cytokines TNFα and IFNγ for 24 hours. Recombinant human TNFα (a gift from Prof. Dr. W. Fiers, Gent, Belgium) was used in a 400 U/ml concentration. Recombinant rat IFNγ (a gift from Dr. P. van de Meide, TNO Rijswijk, The Netherlands) was used in a 200 U/ml concentration.

Detection of allosera reactivity with EC

Suspensions of EC from trypsinized monolayers were used (see above). 5×10^5 cells were incubated with 5x diluted heat-inactivated allosera in PBS containing 0.5% bovine serum albumin (PBSA) for 1 hr, on ice. Cells were washed 3 times with PBSA and incubated with saturating amounts of a FITC conjugated second step swine F(ab)₂ anti rat Ab (DAKO, Denmark) 30 min, on ice. After 3 times washing cells were analyzed by flow cytometry, using a FACS-sort (Becton Dickinson, Mountain View, CA, USA). Data were collected from 1×10^4 cells. For data analysis the program Cell Quest was used (Becton Dickinson, Mountain View, CA, USA).

Quantitation of MHC class I and MHC class II antibody-binding-sites on EC

The numbers of MHC class I and II antibody-binding-sites per cell were quantitated using a Quifikit system (Dako, Denmark). As shown by Poncelet et al. (25) the thus quantitated numbers of antibody binding sites closely correlate with the actual numbers of specific antigen per cell. Briefly, EC suspensions, were incubated with saturating amounts of mouse mAb OX18 or OX6 (obtained through the ECACC, Salisbury, UK), recognizing rat MHC class I or MHC class II antigens respectively, for 1 hr on ice. Next, the cells were stained with fluorescence-labeled second step antibody (FITC-conjugated goat anti-mouse IgG (rat-adsorbed, Cappel, Organon Teknika, the Netherlands) diluted in PBSA. Cell suspensions were washed 3 times with PBSA, analyzed by flow cytometry (see above), and peak fluorescence intensity was determined. For calculation of numbers of binding sites per cell, a series of reference microbeads coated with known numbers of mAb binding sites was used. These reference microbeads were stained with the same fluorescence-labeled second step Ab as was used for the cell staining. Peak fluorescence intensities of the series of beads were measured to produce a reference line to relate peak fluorescence intensities of cell populations with numbers of specific antibody binding sites per cell.

Complement-mediated cytotoxicity of alloantisera

Cytotoxicity against EC was determined using a ^{51}Cr release assay. Endothelial cell suspensions were obtained as mentioned above. Suspensions of 5×10^7 EC/ml were incubated with $5\mu\text{Ci } ^{51}\text{Cr}/\text{ml}$ (Amersham International, England) in EC culture medium for 45 min at 37°C . During labelling the cells were permanently rotated. The labeled EC suspensions were washed twice with EC Work medium and per well 1×10^5 cells in $25\mu\text{l}$ were incubated with $25\mu\text{l } 5 \times$ diluted heat-inactivated allosera in EC culture medium, and $25\mu\text{l}$ agarose-adsorbed guinea pig (GP) or full rat serum as complement source, for 0.5 hr at 37°C . For some experiments IgG and non-IgG (IgM) allosera fractions, or mAb against MHC class I (OX 18) and MHC class II (OX 6) were examined for mediating cytotoxicity. In all experiments hyper immune

serum, made by immunization of BN rats with LEW LNC, was included as a standard for maximal biological release. Hyper immune serum was obtained after 3 immunizations with LNC, sera were collected 3 days after the last immunization injection. Negative controls included were medium or control Tx sera. All experiments were performed in triplicate with the 3 endothelial cell lines. ^{51}Cr release was measured in a gamma counter. Percentages specific lysis were calculated as follows: % specific release = $((\text{cpm alloserum} - \text{cpm control Tx serum}) / (\text{cpm immune serum} - \text{cpm control Tx serum})) \times 100$.

Isotyping of the anti-endothelial antibodies in allosera

EC (0.5×10^5), were incubated with allosera in a 5x dilution for 1 hr, 0°C . Cells were washed 3 times with PBSA and incubated with polyclonal rabbit anti-rat IgM, IgG1, IgG2a, IgG2b, IgG2c (a generous gift from Prof. H. Bazin, VCL, Louvain, Belgium), for 1 hour on ice. Cells were washed 3 times with PBSA and incubated with FITC labeled swine F(ab) $_2$ anti rabbit Ig (DAKO, Denmark) 30 min, on ice. Cells were washed 3 times with PBSA and analyzed by flow cytometry (see above).

Separation of the IgG and non-IgG fractions

IgG was purified from whole alloserum by affinity chromatography on protein G sepharose (Pharmacia LKB, Woerden, The Netherlands). After IgG purification the IgG fraction as well as the non-IgG (IgM) fractions were used for complement-mediated cytotoxicity measurements with EC. Antibody fractions were used in concentrations equivalent to allosera levels.

Detection of complement activation by the alloantisera

Cytokine stimulated EC (5×10^5) were incubated for 1 hr, 0°C with of the allosera from the different Tx models, 5x diluted in 0.5% PBSA. Rat or GP complement was added simultaneously (v/v/v: 1/1/1). Cells were washed 3 times with

PBSA and incubated with directly FITC labeled polyclonal rabbit anti rat C3 serum (1/100) prepared in our laboratory, monoclonal 2A1, a mAb against rat C5b-9 (a kind gift of W. G. Couser, Seattle, U.S.A.) or with polyclonal rabbit IgG anti GP C3 (1/100) (Kindly donated by M. Daha, Leiden, the Netherlands), 1 hr, 0°C. Cells were washed 3 times with PBSA and when a not directly labeled first step Ab was used, incubated for 30 min with FITC conjugated swine anti rabbit Ig, 1/200 in PBSA. Cells were washed 3 times with PBSA and analyzed by flow cytometry (see above).

ICAM-1 and VCAM-1 induction by the alloantisera

EC monolayers were incubated with 5x diluted allosera and 2x diluted rat or GP complement in EC culture medium for 8 or 16 hours at 37°C. Cells were washed and harvested using trypsin/EDTA (see above). EC suspensions were washed with PBSA and 0.5×10^5 cells were incubated for 1 hr on ice with the monoclonal antibodies: OX8, used as non-relevant control Ab, RECA-1 (26), a pan endothelial marker in the rat, 1A29 against ICAM-1 (27), and 5F10 against VCAM-1(28). Suspensions were washed three times with PBSA and incubated for 30 minutes with FITC-conjugated goat-anti mouse IgG (rat adsorbed, Cappel), diluted in PBSA. Cells were washed 3 times with PBSA and Propidium Iodide (10 mg/ml PBSA, Calbiochem, La Jolla, U.S.A.) was added to the cells for 15 minutes to stain non-viable cells. Data were collected by flow cytometry from 1×10^4 viable cells.

5.3 Results

Both anti MHC and anti non-MHC allosera react with EC

**Anti MHC and anti non-MHC allosera
are reactive with donor heart EC**

	non-stimulated EC	stimulated EC
Allosera:		
control Tx	-	-
anti MHC	+	+ ¹
anti non-MHC	+	+
Mab:		
OX 18	+	+ ¹
OX 6	-	+/-

-: all cells negative

+: all cells positive

+/-: part of the cells positive

¹: cytokine stimulation increased the staining intensity of the anti MHC allosera.

Table 1:

Reactivity of the allosera was measured by immunofluorescence staining and FACS analysis of EC from all three RHEC lines. Stimulation of EC was executed with the cytokines TNF α and IFN γ for 24 hours. OX 18: monoclonal Ab against MHC class I. OX 6: monoclonal Ab against MHC class II.

Whether anti MHC and anti non-MHC allosera recognize alloantigens on donor heart EC was studied by FACS analysis of the 3 RHEC lines. As shown in table 1, all cells of the 3 RHEC lines expressed MHC class I antigens, whereas MHC class II antigens were expressed by part of the cells (55.7 %) only after cytokine stimulation (see also Derhaag et al. (29)). Anti MHC allosera (from LEW->BN Tx) showed reactivity with all 3 RHEC lines (Table 1). Allosera reactivity was increased (higher mean fluorescence intensity) with cytokine stimulated EC. Anti non-MHC allosera (from LEW.1N->BN Tx), collected at days 8, 15, 21 and at the day of rejection, showed that reactivity with the EC of all 3 RHEC lines increased in time (Fig. 1). Cytokine stimulation of EC did not increase the reactivity of anti non-MHC

allosera. Control Tx sera did not react with neither non-stimulated nor cytokine-stimulated EC.

Figure 1: Anti non-MHC allosera reactivity with EC was detected by FACS analysis. Reactivity is given in mean fluorescence intensity (MFI). Day 8: n=4, day 15: n=3, day 21: n=2 and day 28: n=2.

To gain information about expression levels of MHC class I and class II antigens on non-stimulated and cytokine-stimulated EC we quantitated these antigens on EC using the Quifikit system (Fig.2). MHC class I antigens were expressed more on cytokine stimulated EC than on non-stimulated EC (n=3). While MHC class II antigens were absent on non-stimulated EC.

Figure 2:

MHC class I antigens on EC were detected with the mAb OX 18 and MHC class II with mAb OX6. The numbers of antibody binding sites (ABS) were determined using a Quifikit, see materials and methods. ABS were measured on EC under standard culture conditions and after cytokine stimulation. Data are of one experiment.

Figure 3:

⁵¹Cr cytotoxicity tests with allosera were executed with non-stimulated and TNF α + IFN γ (24 hr)-stimulated EC of the RHEC lines, in the presence of agarose adsorbed GP complement. Experiments were executed with a series of 4 anti non-MHC and 9 anti MHC allosera. Values are means with standard deviation of 3 experiments. Specific lysis by the allosera is given as a percentage of the lysis obtained with hyper immune serum collected from BN rats immunized with Lewis lymph node cells. N.D.: not done.

Anti MHC allosera are more cytotoxic for EC than anti non-MHC allosera

Whether observed reactivity with donor EC leads to complement-mediated cytotoxicity was examined with EC of the 3 RHEC lines. Figure 3 shows that incubation of EC with anti MHC allosera in the presence of complement of GP origin resulted in 25-30% lysis. When cytokine-stimulated EC were used, lysis increased to 65-75% in the RHEC lines. Anti non-MHC allosera showed much lower cytotoxic effects on EC (10-15% for the 3 RHEC lines), and cytotoxicity was not increased when cytokine-stimulated EC were used. When non-MHC allosera from the series of days 8, 16, 21 and 29 days after Tx were tested, an increasing cytotoxicity was noticed (5.7%, 12.1%, 14.1% and 21.0% respectively in one representative experiment). To study if cytotoxic effects of the allosera were different with

syngeneic complement instead of xenogeneic GP complement, cytotoxicity experiments with LEW rat serum as complement source were executed. The use of rat complement resulted in less cytotoxicity as compared to the use of GP complement (Fig. 4).

Figure 4:

^{51}Cr cytotoxicity tests with allosera were executed with $\text{TNF}\alpha + \text{IFN}\gamma$ (24 hr) -stimulated EC from RHEC 3 in the presence of either rat complement or GP complement. Experiments were executed with a series of 2 anti non-MHC and 2 anti MHC allosera. Values are means with standard deviation of 3 experiments. Specific lysis by the allosera is given as a percentage of the lysis obtained with hyper immune serum collected from BN rats immunized with Lewis LNC.

Mab OX 18 and OX 6 (both from the IgG1 isotype) directed against MHC class I and II antigens respectively showed only low complement (GP)-mediated cytotoxic effects on cytokine-stimulated EC in comparison with anti MHC allosera (11.6% for OX18 and 6.8% for OX6 versus 65-75% for anti MHC allosera). No blocking effect was found on the cytotoxicity of anti MHC allosera when EC were preincubated with OX18 and OX6 antibodies (data not shown).

It was thought that the lower cytotoxicity measured with syngeneic rat complement as compared to xenogeneic GP complement could be related to the

activity of syngeneic complement controlling proteins (CCP) in EC. To obtain information about this, both complement sources were tested for cytotoxic effects on sheep (xenogeneic) red blood cells (SRBC) coated with anti-SRBC Ab. GP complement showed in this system also an about five times higher activity than rat complement, indicating that low effects of rat complement in cytotoxicity of anti MHC and anti non-MHC allosera is likely not due to CCP activity in EC.

Allospecific IgM is only present in the anti MHC allosera and responsible for cytotoxicity

We investigated whether differences in cytotoxicity of anti MHC and anti non-MHC allosera was related to the class of alloantibodies in the sera. Isotyping of alloantibodies reactive with EC, performed by FACS analysis of allosera-incubated EC stained with anti-isotype specific Ab, showed a rather similar isotype subclass profile, with the presence of all 4 IgG subclasses, for allospecific IgG in anti MHC and anti non-MHC allosera (Fig. 5).

Allospecific IgM is only present in anti MHC allosera

Figure 5:

Isotype distribution of the anti EC antibody response by FACS analysis. Immunofluorescence staining for the different isotypes was executed on TNF α + IFN γ (24 hr)-stimulated EC from RHEC 3.

····: control Tx sera,

—: anti MHC allosera

---: anti non-MHC allosera.

MFI of the experimental sera is given in the upper right corner.

However, EC reactive alloantibodies of the IgM isotype were detected in anti MHC but not in anti non-MHC allosera. Even in anti non-MHC allosera collected earlier after Tx, i.e. at day 8, 16 and 21, no IgM alloantibody reactivity could be detected.

We tested if the differences in cytotoxicity against EC of anti MHC allosera versus anti non-MHC allosera was related to the different isotype profiles of alloantibodies in the sera, i.e. the presence and absence of IgM alloantibodies respectively. Therefore cytotoxicity tests were executed with purified IgG fractions and IgG-depleted (IgM containing) fractions of allosera (Fig.6). The purified IgG fraction of anti MHC allosera showed a dramatic decrease (87.8% reduction) in specific lysis of cytokine-stimulated EC. In contrast the IgG-depleted (IgM containing) fraction of these anti MHC allosera showed only a small decrease in cytotoxicity (22% reduction). Apparently the measured complement-mediated cytotoxicity of anti MHC allosera is predominantly an effect of IgM alloantibodies. The fact that purified IgG fractions prepared from anti non-MHC allosera showed rather similar cytotoxicity in comparison to whole sera, indicated that the low cytotoxicity of these sera is indeed effected by IgG and not IgM alloantibodies.

Figure 6:

^{51}Cr cytotoxicity tests with IgG or IgM containing allosera fractions were executed on $\text{TNF}\alpha + \text{IFN}\gamma$ (24 hr)-stimulated EC from RHEC 3, in the presence of GP complement. Values and standard deviations are of one experiment. Specific lysis by the allosera is given as a percentage of the lysis obtained with hyper immune serum from BN rats immunized with Lewis lymph node cells.

Complement activation on EC is effectuated by anti MHC but hardly by anti non-MHC allosera

Complement-mediated cytotoxicity experiments demonstrated that lytic effects of complement activation on EC was clearly effectuated by anti MHC but only slightly by non-MHC allosera. This indirectly indicates that formation of lytic amounts of C5b-9 complexes (MAC) were triggered by binding of alloantibodies (presumably IgM) from anti MHC allosera. However, sublytic amounts of MAC and also formation of the early complement activation components C3a, C3b and C5a on EC may contribute significantly to immunological processes relevant for vasculopathy. We therefore studied effects of anti MHC, anti non-MHC allosera and immune serum on the formation of C3 and C5b-9 on cytokine-stimulated EC. Binding of the complement factors by the tested sera was compared to those from

control Tx sera using the Kolmogorov-Smirnov test. FACS analysis (Fig. 7) showed that immune serum and MHC allosera, incubated either with GP or rat complement, induced C3 formation on EC. Anti non-MHC allosera showed C3 expression levels with GP complement but not with rat complement. Effects of allosera on formation of C5b-9 complexes on EC was only studied with rat complement due to lack of antibodies to the GP C5b-9 complex. Formation of C5b-9 was induced by all sera, i.e. immune serum, anti MHC allosera, and anti non-MHC allosera. Although C5b-9 expression was significantly increased, the relevance of this expression is a matter of debate since no correlation was observed with lytic activity and C3 binding using complement of rat origin to the same extend.

Figure 7: Immunofluorescence staining for C3 and C5b-9 (MAC) expression was performed with TNF α + IFN γ (24 hr)-stimulated EC from RHEC 3. For complement activation EC were incubated for 1 hour with either anti MHC, anti non-MHC allosera, or immune serum in the presence of rat or GP complement. Binding of the complement factors by the tested sera was compared with those from control Tx sera using the Kolmogorov-Smirnov test, $p < 0.01$. All sera significantly induce the binding of the complement factors ($p < 0.01$), except rat C3 induction by anti non-MHC alloserum.
 . . . : control Tx sera,
 — : test sera.

No induction of the adhesion molecules ICAM-1 and VCAM-1

We studied if alloantibody reactivity through direct interaction with MHC or non-MHC alloantigens or through complement activation triggers endothelial processes leading to ICAM-1 or VCAM-1 expression. EC were incubated with anti MHC or anti non-MHC allosera in the presence or absence of complement for 8 or 16 hr followed by ICAM-1 or VCAM-1 detection by FACS analysis. In the absence of a complement source none of the allosera alone showed induction of ICAM-1 or VCAM-1 on EC after 8 or 16 hr. Also in the presence of complement, irrespective whether from rat or GP origin, ICAM-1 or VCAM-1 expression could not be detected. Apparently alloantibodies do not react with putative signalling epitopes of alloantigens involved in activation processes relevant for induction of adhesion molecules. Also complement activation with MAC formation, as demonstrated for anti MHC allosera, appears not to trigger EC processes effecting induction of ICAM-1 and VCAM-1.

5.4 Discussion

Vascular EC can be considered a primary target for the alloantibody response generated in the rejection process of heart allografts. We therefore studied effects of alloantibody reactivity with EC in 2 models for rat heart allograft rejection. By using donor hearts from fully-mismatched and congenic (MHC matched but non-MHC mismatched) strains, allosera reactivity with endothelial MHC and non-MHC alloantigens respectively could be studied. Allosera reactivity was tested *in vitro* using cultures of well characterized rat-heart-endothelial-cell (RHEC) lines with the appropriate donor MHC and non-MHC haplotype (23). Our results show that in MHC mismatched and in non-MHC mismatched heart Tx, alloantibodies reactive with graft EC are generated. Also in clinical studies allosera reactivity with endothelial alloantigens in heart allograft rejection has been described, however in these studies discrimination between HLA and non-HLA alloantigens was not further elucidated (19, 30). Both the anti MHC and anti non-MHC allosera showed reactivity with normal, non-stimulated heart EC cultures. Since in immune reactive processes such as rejection EC become activated by inflammatory cytokines, the allosera were also tested for reactivity with TNF α and IFN γ -stimulated EC. Whereas for anti non-MHC allosera reactivity no differences between non-stimulated and cytokine-stimulated EC were found, anti MHC allosera reactivity was increased with cytokine-stimulated EC. From the FACS analysis studies with anti MHC class I and class II monoclonal antibodies it can be concluded that this increase in reactivity was due to cytokine-induced upregulation and induction of MHC class I and class II antigens respectively (see also (29)). Apparently expression of non-MHC alloantigens is not influenced by cytokine stimulation. We noticed that reactivity of allosera was similar for all 3 RHEC lines, and as such this demonstrates that the heterogeneity of heart EC, as previously described in studies using these lines (23, 29), seems not to apply to expression of alloantigens. The EC cytotoxicity studies with the allosera showed only minor complement-mediated cytotoxicity effects of anti non-MHC allosera. The observation that for cytokine-stimulated EC no increase in cytotoxicity was noticed is

consistent with our findings that cytokine stimulation does not affect expression levels of non-MHC alloantigens on EC. Anti MHC allosera, however demonstrated distinct cytotoxicity for EC, with a significant increase when cytokine-stimulated EC were used as target cells. Clinical heart Tx studies also describe complement-mediated cytotoxicity by anti-EC antibodies, but notify that EC are relatively resistant in this respect (31). Interestingly, the cytotoxicity we measured with anti MHC allosera was effected majorly by present IgM alloantibodies. Apparently rat IgG alloantibodies have only minor cytotoxic effects. Isotyping studies of anti endothelial alloantibodies showed that this was not due to the absence of complement fixing IgG subtypes, because all 4 rat IgG isotypes, including the complement-fixing IgG2b (32), were detected. Probably the contribution of IgG2b in the alloresponse is too low for mediating distinct cytotoxic effects. The fact that anti non-MHC allosera showed a similar IgG isotype profile of EC alloantibodies as anti MHC allosera, but in contrast were devoid of IgM alloantibodies, is consistent with the minor cytotoxic effects of these anti non-MHC allosera. These studies suggest that in rejection of rat heart allografts, destructive complement-mediated effects of alloantibodies for graft EC only play a role in the early alloantibody response, when relatively high titers of IgM alloantibodies can be expected. The fact that anti non-MHC allosera did hardly contain IgM alloantibodies even at 1 week after Tx indicates that production is low and apparently entirely absorbed by graft tissue. Whether non-MHC IgM alloantibodies present in the graft are sufficient for EC cytotoxicity remains to be elucidated. Also in renal allograft rejection, alloantibodies reactive with non-MHC alloantigens on graft EC have been described (33, 34, 35) and, consistent with our studies, these antibodies showed hardly any cytotoxicity for EC (35).

We noticed that lower cytotoxic effects of alloantibodies were obtained when rat instead of GP complement was used. Also in previous studies on cytotoxicity of antibodies in renal allograft rejection this was observed (36). This lower cytotoxicity could have been effected by endothelial complement control proteins (CCP) such as decay accelerating factor and CD 59, which are known to prevent complement

activation on homologous but not heterologous cell membranes (37, 38). However, no evidence was found that the lower cytotoxicity effects measured with rat complement were due to inhibition of complement activation by CCP. Since the GP serum used as complement source was agarose-adsorbed it is not likely that the relatively high cytotoxicity obtained with GP complement is a result of present natural (xeno)antibodies to rat.

Our FACS analysis studies on C3 and C5b-9 formation on EC showed that anti non-MHC allosera gave only C3 expression with GP and not with rat complement. C5b-9 formation, which could technically only be measured in the rat complement system, was hardly present. With the anti MHC allosera distinct C3 formation was observed with GP as well as with rat complement sources. Anti MHC allosera also showed a distinct rat C5b-9 formation. The data on C3 expression induced by the allosera were consistent with the cytotoxic effects observed with these sera, and illustrated the induction of the complement cascade necessary for cytotoxicity. Although the induction of C5b-9 could be detected, the observed differences found in the cytotoxicity tests with rat complement between immune serum and anti MHC/anti non-MHC allosera (Figure 4) were not observed in the rat C5b-9 induction experiments. The capability of the used antibody to detect rat C5b-9 in these experiments, therefor remains a matter of debate.

Although we demonstrated that complement activation with C5b-9 or MAC formation occurred, we could not demonstrate that this initiated EC perturbation or activation leading to expression of adhesion molecules as ICAM-1 and VCAM-1. In this respect our data do not support previous studies demonstrating that antibody alone or by formation of sublytic amounts of MAC on EC induces endothelial cell activation and adhesion molecule expression (14, 39, 40). In those studies it is suggested that adhesion molecule expression is effected by autocrine stimulation of induced IL-1 production. Although we did not find evidence for such activation pathway, it may be that MAC formation triggers other activation processes, such as

production of platelet activating factor or tissue factor, as described in other studies (9, 10, 11).

In clinical heart and also kidney transplantation studies, the presence of anti EC alloantibodies has been associated with chronic rejection, and in particular vascular rejection including graft arteriosclerosis (20, 30, 41). It is interesting that in chronic rejection, where predominantly IgG alloantibodies are present, complement-mediated cytotoxicity of endothelial antibodies apparently plays a minor role. To which other processes relevant for vascular rejection anti EC alloantibodies may contribute remains to be elucidated. In this respect, it may be expected that, in addition to complement activation effects, anti EC alloantibodies mediate interactions of Fc-receptor positive ADCC or inflammatory cytokine-producing cells with the endothelium of the blood vasculature.

Acknowledgements: The authors would like to thank A. P. Gijzen for technical assistance. H. A. M. D. van der Heijden for heart transplantation expertise. We thank Dr Chisholm for donation of 5F10, Dr. Miyasaka for donation of 1A29, Prof. H. Bazin for donation of the polyclonal anti rat isotype antibodies, Prof. W. G. Couser for donation of 2A1, Prof. M. Daha for donation of the anti GP C3 Ab, Prof. Dr. W. Fiers for donation of TNF α and Dr. P. van der Meide for donation of IFN γ .

5.5 References

1. Colvin RB. Cellular and molecular mechanism of allograft rejection. *Annual Reviews of Medicine* 1990; **41**: 361.
2. Colson YL, B.H. M, Zeevi A, Duquesnoy RJ. Interactions between endothelial cells and alloreactive T cells involved in allograft immunity. *Transplantation Proceedings* 1988; **47**.
3. Russell PS, Chase CM, Winn HJ, Colvin RB. Coronary atherosclerosis in transplanted mouse hearts. *Journal of Immunology* 1994; **152**: 5135.
4. Tixier D, Tusio P, Czer L, et al. Characterization of anti-endothelial cell and anti-heart antibodies following heart transplantation. *Transplantation Proceedings* 1993; **25**: 931.
5. Moller E. Cell interactions and cytokines in transplantation immunity. *Transplantation Proceedings* 1995; **27**: 24.
6. Lowry RP, Gurley KE, Forbes RDC. Immune mechanisms in organ allograft rejection. *Transplantation* 1983; **36**: 391.
7. Bolton EM, Gracie JA, Briggs JD, Kampinga J, Bradley JA. Cellular requirements for renal allograft rejection in the athymic nude rat. *Journal of Experimental Medicine* 1989; **169**: 1931.
8. Saadi S, Platt J. Transient perturbation of endothelial integrity induced by natural antibodies and complement. *Journal of Experimental Medicine* 1995; **181**: 21.
9. Bevilacqua MP, Pober JS, Majeau GR, Cotran RS, Gimbrone MAJ. Interleukin 1(IL-1) induces biosynthesis and cell surface expression of procoagulant activity in human vascular endothelial cells. *Journal of Experimental Medicine* 1984; **160**: 618.
10. Saadi S, Holzknecht RA, Patte CP, Stern DM, Platt JL. Complement mediated regulation of tissue factor activity in endothelium. *Journal of Experimental Medicine* 1995; **182**: 1807.
11. Camussi G, Bussolino F, Salvidio G, Baglioni C. Tumor necrosis factor/cachectin stimulates peritoneal macrophages, polymorphonuclear neutrophils, and vascular endothelial cells to synthesize and release platelet-activating factor. *Journal of Experimental Medicine* 1987; **166**: 1390.
12. Vaporciyan AA, Mulligan MS, Warren JS, Barton PA, Miyasaka M, Ward PA. Up-regulation of lung vascular ICAM-1 in rats is complement dependent. *The Journal of Immunology* 1995; **155**: 1442.
13. Kilgore KS, Shen JP, Miller BF, Ward PA, Warren JS. Enhancement by the complement membrane attack complex of tumor necrosis factor-induced endothelial cell expression of E-selectin and ICAM-1. *The Journal of Immunology* 1995; **155**: 1434.
14. Carvalho D, Savage COS, Black CM, Pearson JD. IgG anti-endothelial cell autoantibodies from scleroderma patients induce leukocyte adhesion to human vascular endothelial cells in vitro. *Journal of Clinical Investigation* 1996; **97**: 111.
15. Brauer RB, Baldwin III WM, Ibrahim S, Sanfilippo F. The contribution of the terminal complement components to acute and hyperacute allograft rejection in the rat. *Transplantation* 1995; **59**: 288.
16. Yard B, Spruyt-Gerritse M, Claas F, et al. The clinical significance of allospecific antibodies against endothelial cells detected with an antibody-dependent cellular cytotoxicity assay for vascular rejection and graft loss after renal transplantation. *Transplantation* 1993; **55**: 1287.
17. Bradley JA, Mowat AM, Bolton EM. Processed MHC class I alloantigen as the stimulus for CD4+ T-cell dependent antibody-mediated graft rejection. *Immunology Today* 1992; **13**: 434.
18. Adams H, Tilney NL, Collins JJ, Karnovsky MJ. Experimental graft arteriosclerosis. *Transplantation* 1992; **53**: 1115.
19. Dunn MJ, Crisp SJ, Rose ML, Taylor PM, Yacoub MH. Anti-endothelial antibodies and coronary artery disease after cardiac transplantation. *The Lancet* 1992; **339**: 1566.
20. Crisp SJ, Dunn MJ, Rose ML, Barbir M, Yacoub MH. Anti-endothelial antibodies after heart transplantation: the accelerating factor in transplant-associated coronary artery disease. *Journal of Heart and Lung Transplantation* 1994; **13**: 81.
21. Schilfgaarde van R, Hermans P, Terpstra JL, Breda Vriesman van PJC. Role of mobile passenger lymphocytes in the rejection of renal and cardiac allografts in the rat. A passenger lymphocyte mediated graft versus host reaction amplifies the host response. *Transplantation* 1980; **29**: 209.
22. Derhaag JG, Duijvestijn AM, Damoiseaux JGMC, van Breda Vriesman PJC. An in vitro model for studying the role of graft endothelial cells in rat heart allograft rejection: A study on the regulation and function of heart endothelial histocompatibility antigens and cell adhesion molecules. *Transplantation Proceedings* 1997; **29**: 1717.
23. Derhaag J, Duijvestijn A, Emeis J, Engels W, Breda Vriesman van P. Production and characterization of spontaneous rat-heart endothelial cell lines. *Laboratory Investigations* 1996; **74**: 437.

24. Maciag T, Cerendulo J, Isley S, Kellu PR, Forand R. An endothelial growth factor from bovine hypothalamus: identification and partial characterization. *Proc Natl Acad Sci USA* 1979; **76**: 5674.
25. Poncelet PG, Lavabre-Bertrand T. Immunological detection of membrane-bound antigens and receptors. *Methods of Immunological Analysis* 1993; 388.
26. Duijvestijn AM, Goor van H, Klatter F, Majoor GD, Bussel van E, Breda Vriesman van PJC. Antibodies defining rat endothelial cells: RECA-1 a pan endothelial specific monoclonal antibody. *Laboratory Investigation* 1992; **62**: 459.
27. Tamatani T, Miyasaka M. Identification of monoclonal antibodies reactive with the rat homolog of ICAM-1 and evidence for a differential involvement of ICAM-1 in the adherence of resting versus activated lymphocytes to high endothelial cells. *International Immunology* 1990; **2**: 165.
28. May MJ, Entwistle G, Humphries MJ, Ager A. VCAM-1 is a CS-1 peptide-inhibitable adhesion molecule expressed by lymph node high endothelium. *Journal of Cell Science* 1993; **106**: 109.
29. Derhaag JG, Duijvestijn AM, Breda Vriesman van PJC. Heart EC respond heterogeneous on cytokine stimulation in ICAM-1 and VCAM-1, but not in MHC expression; A study with 3 rat heart endothelial cell (RHEC) lines. *Endothelium* 1997; **5**:307.
30. Ferry BL, Welsh KI, Dunn MJ, et al. Anti cell surface endothelial antibodies in sera from cardiac and kidney transplant recipients. *Transplantation Immunology* 1997; **5**(1): 17.
31. Orosz CG. Endothelial activation and chronic allograft rejection. *Clinical Transplantation* 1994; **8**: 299.
32. Sekhavat M, Ravoet AM, Neirynck A, Sokal G. Rat monoclonal antibodies for complement mediated and cell mediated cytotoxicity.
33. Gilks WR, Gore SM, Bradley BA. Renal transplant rejection. Transient immunodominance of HLA mismatches. *Transplantation* 1990; **50**: 141.
34. Duijvestijn A, Vlek L, Duistermaat L, Rie van H, Breda Vriesman P. Chronic renal allograft rejection: the significance of non-MHC alloantigens. *Transplantation International* 1992; **5 suppl 1**: s639.
35. Joyce S, Flye MW, Mohanakumar T. Characterization of kidney cell-specific, non-major histocompatibility complex alloantigen using antibodies from rejected human renal allografts. *Transplantation* 1988; **46**: 362.
36. Breda Vriesman van PJC, Sanen-Sierag L, Vlek LFM. Cytotoxic and enhancing properties of early γ M alloantibodies elicited by first set renal allografts. *Transplantation* 1975; **20**:385.
37. Funabashi K, Okada N, Matsuo S, Yamamoto T, Morgan BP, Okada H. Tissue distribution of complement regulatory membrane proteins in rats. *Immunology* 1994; **81**: 444.
38. Dalmaso AP, Vercellotti GM, Platt JL, Bach FH. Inhibition of complement-mediated endothelial cell cytotoxicity by decay-accelerating factor. *Transplantation* 1991; **52**: 530.
39. Briscoe DM, Schoen FJ, Rice GE, Bevilacqua MP, Ganz P, Pober P. Induced expression of endothelial-leukocyte adhesion molecules in human cardiac allografts. *Transplantation* 1991; **51**: 537.
40. Pelletier RP, Morgan CJ, Sedmak DD, et al. Analysis of inflammatory endothelial changes, including VCAM-1 expression, in murine cardiac grafts. *Transplantation* 1993; **55**: 315.
41. Woude van der FJ, Deckers JG, Mallat MJ, Yard BA, Schrama E, Saae van JL, Daha MR. Tissue antigens in tubulointerstitial and vascular rejection. *Kidney International Suppl* 1995; **53**: 1119-1127.

Summary & Discussion

Endothelial cells (EC) form an organ which is very important in almost all physiological and pathological processes. The EC are important for the control of blood flow, blood pressure and clotting as well as for the regulation of the permeability of the vessel wall to molecules and cells. Pathogenic mechanisms lead to the loss of EC integrity. This results in malfunctioning of prementioned physiological processes in which the EC is involved. In this thesis the role of EC in immune reactivity, and especially in rat heart allograft rejection, is described. This was studied in an *in vitro* model using cultures of EC lines.

In **Chapter 1** the current facts about the structure and physiological functions of EC are reviewed. Changes of these functions during immune reactivity, and especially during transplant rejection are discussed. During normal physiological circumstances the EC coordinate the transport of molecules through the endothelial monolayer, regulate leukocyte migration, the vascular tone and control the coagulation.

During immune reactivity all these processes change by the intervention of cytokines, chemokines and cells involved in immunity. This results in increased transport of molecules through the EC monolayer, increased migration of specific leukocyte subpopulations, and in changes of the vascular tone by production of vasoconstriction or vasodilatation factors by the EC. Besides, immune reactivity may also lead to disturbance of the procoagulant/ anti coagulant balance, normally controlled by the EC.

During allograft rejection EC contribute to the rejection process by participating in the immune response. In addition to regulation of leukocyte migration, vascular endothelial cells may present antigens together with co-stimulatory signals to T-lymphocytes, initiating an immune response against the graft. Besides, EC become a target for cellular or antibody-mediated immune reactions.

In **Chapter 2** the development of an *in vitro* EC model is described, as well as the phenotypical and functional characterization of developed EC lines. The lack of EC of proper species and organ origin, the low yield of EC obtained with the isolation procedure, and the limited lifespan of the EC obtained from described isolation procedures, made it necessary to develop immortalized cell lines.

3 spontaneous rat heart endothelial cell (RHEC) lines of Lewis rat origin were produced using an *ex vivo* perfusion model to isolate EC from rat heart. It was shown that the cell lines, RHEC 3, RHEC 10 and RHEC 11, are of EC nature. The spontaneously immortalized EC lines are tumorigenic and develop hemangiomas on *in vivo* injection. These cellular effects of immortalization on EC are also shown by EC lines obtained via virus infection-induced immortalization. The RHEC lines respond upon cytokine stimulation in adhesion molecule and MHC expression. Functional characterization of the RHEC lines shows that the EC from the RHEC lines express angiotensin converting enzyme, and respond to histamine and ATP. They produce small amounts of endothelin and high amounts of t-PA, which supports their microvascular nature. The EC lines, apparently because of long-term culturing, lost the capacity to produce PGI₂, but they do produce TXA₂ and PGF₂ α , and upon stimulation also PGE₂.

The modified method to obtain EC cell lines increases the frequency of successful production of EC lines. The data suggest that the 3 RHEC lines are representative for rat heart microvascular endothelium of Lewis rat origin *in vivo*, and could be used to study graft EC in the Lewis to BN rat heart transplantation model.

To examine the role of microvascular EC in inflammation and immune reactivity in transplant rejection the response of EC upon stimulation with the inflammatory cytokines TNF α and IFN γ was studied as described in **Chapter 3**. RHEC lines were studied for the induction kinetics of adhesion molecules and MHC antigens over a time period of 64 hr.

The induction of the molecules by the cytokines $\text{TNF}\alpha$ and $\text{IFN}\gamma$ occurs not on all cells in the cultures. $\text{TNF}\alpha$ is most potent in inducing ICAM-1 and VCAM-1, whereas $\text{IFN}\gamma$ is most potent in MHC class II induction. Combined cytokine stimulation result in increased and even synergistic effects on the expression profiles. The cell lines show remarkable differences with respect to ICAM-1 and VCAM-1 induction, with each cell line having an unique expression profile. This in contrast with the induction profiles for MHC molecules which are similarly for all three the RHEC lines. The selective induction of the molecules on not all the EC within a cell line results from an intrinsic regulation mechanisms in the cell culture.

In heart tissue *in vivo*, the heterogeneity of vascular EC, as demonstrated here *in vitro*, may have an impact on leukocyte endothelial interactions especially during inflammatory and immune processes. Here, preferential local adhesion and infiltration of specific lymphocyte populations regulated by the stimulated EC may occur. In addition, by expression of MHC molecules EC can present antigens to T cells which in the presence or absence of the necessary costimulatory adhesion molecules may result in initiation of the immune response or the development of anergic or apoptotic T cells respectively.

In order to demonstrate effects of EC heterogeneity for cytomegalovirus (CMV) infection the obtained Lewis RHEC lines were used together with RHEC 50, RHEC 116 and RHEC 191 in CMV infection studies as described in **Chapter 4**. Since in the pathology of transplantation associated atherosclerosis, cytomegalovirus (CMV) infected EC are detected, a role for EC in this process is suggested.

The RHEC lines display differences in susceptibility to CMV infection. RHEC 50 and 191 are practically non-permissive, while RHEC 3, 10, 11 and 116 are differently permissive for CMV infection. CMV infection does not spread out through the monolayer of EC, although infectious virus is produced. The virus produced appears able to infect fibroblasts but not EC. From the monolayer detached CMV-infected EC are also only able to infect fibroblasts and not EC.

The heterogeneous response of EC in their permissiveness for CMV of the different EC lines suggest that *in vivo*, although the EC are relatively resistant to CMV infection, the infection of EC with CMV can be locally different. Detached EC can be responsible for spreading the virus through the vascular system and, at sites of endothelium damage, fibroblast or smooth muscle cells can be infected. As such detached EC may accelerate systemic CMV infection.

To define whether in heart allograft rejection alloantibodies to endothelial MHC and non-MHC play a role in the development of graft vasculopathy, the effects of these allo-antibodies are studied as described in **Chapter 5**.

Anti MHC as well as anti non-MHC allosera show specific reactivity with EC from the RHEC lines. Anti MHC allosera show distinct complement-mediated cytotoxicity for EC, whereas anti non-MHC allosera show only low cytotoxicity for EC. Cytokine stimulation of the EC, which resulted in upregulation and induction of MHC class I and II (see Chapter 3), increases the cytotoxicity of the anti MHC allosera. Non-MHC alloantigens are not upregulated by cytokine stimulation, and consequently cytotoxicity does not increase either. It is shown that complement-mediated cytotoxicity of anti MHC allosera is related to the IgM fraction of the allosera, which is only present in the anti MHC allosera. IgG subclass profiles of both the allosera are rather similar. Highest efficacy in antibody mediated cytotoxicity against EC is obtained when xenogeneic complement is used in comparison with syngeneic complement. Anti MHC allosera activate complement on EC, while anti non-MHC allosera hardly activate complement on EC.

The results of these experiments suggest that both MHC and non-MHC alloantigens on EC are target structures of the alloantibody response in heart allograft rejection. Complement mediated cytotoxicity is only present in allosera reactive with MHC alloantigens, and is effected by IgM alloantibodies. Whether the alloantibodies against MHC and non-MHC alloantigens on the EC do not destroy but activate the EC, and so influence the rejection process, needs further investigation.

Data from *in vitro* studies with RHEC lines indicate only a minor role of antibody-mediated cytotoxicity against EC during heart transplant rejection. This suggests that next to alloantibody mediated cytotoxicity other mechanisms may effect the graft, resulting in transplant rejection. The rejection process is characterized by cell infiltrates in the graft in which the endothelium appears to be the initial target. In preliminary cellular cytotoxicity test executed with different effector populations out of transplant rejecting recipients, no cytotoxicity against cytokine stimulated EC can be detected, even when very high effector target ratio's are used (data not shown).

In summary our study indicate that the RHEC lines which show to be representative for the microvascular EC of the Lewis heart, are heterogeneous in their response upon cytokine stimulation and also in their permissiveness for CMV infection. Observed heterogeneity accentuates the difficulties in studying EC processes in for instance only one cell line. Observed heterogeneity among the vascular bed *in vivo* together with the presented *in vitro* data suggests that local different events occur during immune reactivity. The obtained RHEC lines increase the possibility to study the heterogeneous EC *in vitro*. During the transplantation rejection EC are a target for the cellular as well as the humoral immune response. Our studies show that humoral immune reactivity against graft EC does not result in destruction of EC, but most likely results in EC activation, effecting the rejection process.

Samenvatting & Discussie

Endotheel cellen (EC) vormen een orgaan, belangrijk voor bijna alle fysiologische en pathologische processen in het lichaam. EC spelen een belangrijke rol bij de controle van de bloedstroom, de bloeddruk en de stolling, maar reguleren ook de permeabiliteit van de vaatwand. Pathogene mechanismen leiden tot het verlies van deze EC integriteit. Dit resulteert in een ontregeling van de voornoemde fysiologische processen waarbij EC betrokken zijn. In dit proefschrift is de rol van EC in immuun reactiviteit en meer specifiek in rat hart transplantaat afstoting beschreven. Dit is bestudeerd in een *in vitro* EC model dat gebruik maakt van rat hart EC.

Hoofdstuk 1 is een overzicht van de actuele feiten over structuur en fysiologische functies van het endotheel in het algemeen. Veranderingen in deze functies gedurende immuun reactiviteit, en in het bijzonder tijdens transplantaat afstoting, worden bediscussieerd. Tijdens fysiologische omstandigheden coördineren EC het transport door de endotheel monolayer, reguleren leukocyt migratie en de vaat tonus en controleren de stolling.

Tijdens immuun reactiviteit veranderen al deze processen door tussenkomst van cytokines, chemokines en cellen betrokken bij de immuun response. Dit resulteert in een toegenomen transport door de EC monolayer, toegenomen migratie van specifieke leukocyt subpopulaties, en in veranderingen van de vaattonus door productie door EC van stoffen die leiden tot vasoconstrictie of vasodilatatie. Daarnaast kan immuun reactiviteit de procoagulant/anti coagulant balans verstoren, welke onder fysiologische omstandigheden in stand wordt gehouden door EC.

Door deelname aan de immuun respons tijdens transplantaat afstoting hebben EC een rol binnen het afstotings proces. Naast regulatie van de leukocyt migratie, kunnen vasculaire EC samen met de benodigde co-stimulatoire signalen antigeen presenteren aan lymfocyten, en daarmee kunnen ze een immuun respons tegen het transplantaat initiëren.

Bovendien zijn EC het doelwit van de cellulaire en/of antilichaam gemedieerde immuun reactiviteit

In **Hoofdstuk 2** wordt de ontwikkeling van een *in vitro* EC model en de fenotypische en functionele karakterisering van de ontwikkelde rat hart EC lijnen beschreven. De afwezigheid van EC van het juiste species en het juiste orgaan, de lage opbrengst verkregen met de beschreven isolatie procedure en de gelimiteerde levensduur van de EC verkregen met eerder beschreven opzuiverings procedures maakte de ontwikkeling van de cellijnen noodzakelijk.

Drie rat hart endotheel cel (RHEC) lijnen van Lewis rat origine werden geproduceerd met behulp van een *ex vivo* perfusie model voor EC uit rat hart. De cel lijnen, RHEC 3, RHEC 10 en RHEC 11, zijn zuiver EC. De spontaan geïmmortaliseerde EC lijnen zijn tumorigeen en ontwikkelen hemangiomas wanneer zij *in vivo* worden geïnjecteerd. Deze cellulaire effecten van de immortalisatie op EC zijn ook aangetoond bij EC lijnen die geïmmortaliseerd zijn met behulp van virus infectie. De RHEC lijnen reageren op cytokine stimulatie door adhesie moleculen en MHC moleculen tot expressie te brengen. Functionele karakterisering van de RHEC lijnen toont aan dat EC van de RHEC lijnen angiotensin convertend enzyme, ACE expresseren op hun membraan en dat zij reageren op histamine en ATP. Ze produceren kleine hoeveelheden endotheline en grote hoeveelheden tPA, wat hun microvasculaire oorsprong benadrukt. De EC lijnen zijn hun capaciteit om PGI₂ and PGE₂ te produceren verloren, waarschijnlijk door de langdurige kweek periode, maar produceren een weinig TXA₂ en PGF₂α.

De gebruikte aangepaste methode om EC lijnen te verkrijgen uit EC isolatie heeft de frequentie van het succesvol produceren van deze lijnen verhoogd. De verkregen data omtrent deze 3 RHEC lijnen suggereren dat ze representatief zijn voor rat hart microvasculair endotheel van Lewis rat origine *in vivo* en dat ze gebruikt kunnen worden om transplantaat EC te bestuderen in het Lewis naar BN rat hart transplantatie model.

Om de rol van microvasculair EC in ontsteking en immuun reactiviteit tijdens transplantaat afstoting te bestuderen is de respons van EC op stimulatie met de ontsteking

cytokines TNF α and IFN γ bestudeerd zoals beschreven in **Hoofdstuk 3**. De inductie kinetiek voor adhesie moleculen en MHC antigenen van de RHEC lijnen is bestudeerd in een periode van 64 uur.

De inductie van deze moleculen door de cytokines TNF α and IFN γ vindt niet op 100% van de cellen plaats. TNF α is het meest potent in het induceren van ICAM-1 en VCAM-1, terwijl IFN γ het meest potent was in MHC class II inductie. Gecombineerde cytokine stimulatie resulteert in toegenomen en zelfs synergistische effecten op de expressie profielen. De cellijnen vertonen opmerkelijke verschillen met betrekking tot ICAM-1 en VCAM-1 inductie, waarin elke cellijn een uniek expressie patroon heeft. Dit in tegenstelling tot de inductie profielen voor de MHC moleculen welke voor alle drie de RHEC lijnen gelijk zijn. De selectieve inductie van de moleculen op niet alle EC binnen één cellijn is het resultaat van een intrinsiek regelmechanisme in de weefselkweek.

In hart weefsel *in vivo* heeft de heterogeniteit van vasculaire EC, zoals hier gedemonstreerd *in vitro*, mogelijk een impact op leukocyt/endotheel interacties, met name gedurende ontsteking en immuun processen. Er kan dan sprake zijn van preferentiële lokale adhesie en infiltratie van specifieke lymfocyt populaties gereguleerd door het gestimuleerde EC. Daarnaast kunnen MHC expresserende EC antigeen presenteren aan T cellen, wat in de aan of afwezigheid van de noodzakelijke costimulatoire adhesie moleculen kan resulteren in initiatie van respectievelijk de immuun response of de ontwikkeling van anergische of apoptotische T cellen.

Om de effecten van deze EC heterogeniteit voor cytomegalovirus (CMV) infectie te demonstreren zijn de verkregen Lewis RHEC lijnen gebruikt samen met RHEC 50, RHEC 116 en RHEC 191 in CMV infectie studies zoals beschreven in **Hoofdstuk 4**. Omdat tijdens de pathologie van transplantatie geassocieerde atherosclerose, CMV geïnfecteerde EC zijn waargenomen, wordt er een rol voor EC in dit proces gesuggereerd.

De RHEC lijnen vertonen verschillen in hun gevoeligheid voor CMV infectie. RHEC 50 en 191 zijn vrijwel niet toegankelijk, terwijl RHEC 3, 10, 11 en 116

differentieel toegankelijk zijn voor CMV infectie. CMV infectie verspreidt zich niet door de EC monolayer, alhoewel er wel infectieus virus wordt geproduceerd. Het geproduceerde virus blijkt fibroblasten te kunnen infecteren maar niet EC. De van de monolayer losgelaten CMV-geïnfecteerde EC zijn ook enkel in staat om fibroblasten te infecteren en niet EC.

De heterogene respons van EC in hun toegankelijkheid voor CMV van de verschillende EC lijnen suggereert dat *in vivo*, ondanks het feit dat de EC relatief resistent zijn voor CMV infectie, de infectie van EC met CMV lokaal heel verschillend kan zijn. Losgelaten EC kunnen verantwoordelijk zijn voor verspreiding van het virus door het vasculaire systeem en, op plekken waar het endotheel beschadigd is, fibroblasten of gladde spiercellen infecteren. Op deze manier kunnen de losgelaten geïnfecteerde EC systemische CMV infectie doen accelereren.

Om vast te stellen of gedurende hart transplantaat afstoting alloantilichamen tegen endotheliale MHC en non-MHC moleculen een rol spelen in de ontwikkeling van transplantaat vasculopathie zijn de effecten van deze alloantilichamen bestudeerd zoals beschreven in **Hoofdstuk 5**.

Anti MHC en ook anti non-MHC allosera vertonen specifieke reactiviteit met EC van de RHEC lijnen. Anti MHC allosera vertonen duidelijke complement-gemedieerde cytotoxiciteit voor EC, terwijl anti non-MHC allosera alleen lage cytotoxicity voor EC vertonen. Cytokine stimulatie van de EC, resulterend in opregulatie en inductie van MHC klasse I en II (zie Hoofdstuk 3), leidt tot verhoogde cytotoxiciteit van de anti MHC allosera. Non-MHC alloantigenen worden niet opgereguleerd door cytokine stimulatie en als een gevolg hiervan wordt de cytotoxiciteit ook niet verhoogd. In dit hoofdstuk wordt getoond dat de complement-gemedieerde cytotoxiciteit van de anti MHC allosera gerelateerd is aan de IgM fractie van de allosera, welke alleen aanwezig is in de anti MHC allosera. De IgG subklasse profielen van beide allosera zijn vrijwel gelijk. De hoogste efficiëntie van antilichaam gemedieerde cytotoxiciteit tegen EC wordt verkregen wanneer xenogeen complement wordt gebruikt. Anti MHC allosera activeren complement op de

membraan van EC. Anti non-MHC allosera zijn nauwelijks in staat om complement op EC te activeren.

De resultaten van deze experimenten suggereren dat zowel MHC als non-MHC alloantigenen op EC doelwit structuren zijn van de alloantilichaam respons tijdens hart transplantaat afstoting. Complement gemedieerde cytotoxiciteit is alleen aanwezig in de allosera die reactief zijn met MHC alloantigenen en wordt veroorzaakt door IgM alloantilichamen. Of de alloantilichamen tegen MHC en non-MHC alloantigenen op EC de cellen niet vernietigen maar wel activeren, en zo het afstotingsproces beïnvloeden, vraagt verder onderzoek.

De data van de *in vitro* studies met de RHEC lijnen impliceren een ondergeschikte rol voor de antilichaam-gemedieerde cytotoxiciteit tegen EC gedurende hart transplantaat afstoting. Dit suggereert dat er naast alloantilichaam gemedieerde cytotoxiciteit andere mechanismen het transplantaat beïnvloeden, wat uiteindelijk resulteert in transplantaat afstoting. Het afstotingsproces wordt gekarakteriseerd door cel infiltraten in het transplantaat waarin het endotheel het initiële doelwit lijkt te zijn. In preliminary cellulaire cytotoxiciteit testen, uitgevoerd met verschillende effector populaties uit transplantaat afstotende ontvangers, kan *in vitro* geen cytotoxiciteit tegen cytokine gestimuleerd EC waargenomen worden, zelfs niet wanneer er gebruik gemaakt wordt van zeer hoge effector target ratio's (data niet getoond).

Samenvattend laat onze studie zien dat de RHEC lijnen, welke representatief zijn voor microvasculaire EC van het Lewis hart, heterogeen zijn in hun respons op cytokine stimulatie en ook voor hun toegankelijkheid voor CMV infectie. Deze geobserveerde heterogeniteit accentueert de problemen die er ontstaan bij het bestuderen van EC met behulp van bijvoorbeeld een enkele cellijn. De geobserveerde heterogeniteit in het vasculaire stelsel *in vivo* samen met de gepresenteerde *in vitro* data suggereert dat er gedurende immuun reactiviteit lokaal verschillende gebeurtenissen plaatsvinden. De verkregen RHEC lijnen verbeteren de mogelijkheid om heterogene EC *in vitro* te bestuderen. Tijdens transplantaat afstoting zijn EC een doelwit voor de cellulaire maar

ook voor de humorale immuun respons. Onze studies laten zien dat de humorale immuun reactiviteit tegen transplantaat EC niet resulteert in vernietiging van EC, maar, naar alle waarschijnlijkheid, resulteert in EC activatie en zo het afstotingsproces beïnvloedt.

Publications

Wander SL, Ten-Kate J, Van der Linden E, Derhaag JG, Dinjens WN, Bosman FT. Does ex vivo labelling of proliferating cells in colonic and vaginal mucosa reflect the S-phase fraction in vivo. *Histochemistry*, 1992; **4**: 267.

Derhaag JG, Duijvestijn AM, Emeis JJ, Engels W, van Breda Vriesman PJC. Production and characterization of spontaneous rat heart endothelial cell lines. *Laboratory Investigation* 1996; **74**: 437.

Vossen RCRM, Derhaag JG, Slobbe-van Drunen MEP, Duijvestijn AM, van Dam-Mieras MCE, Bruggeman CA. A dual role for endothelial cells in cytomegalovirus infection? A study of cytomegalovirus infection in a series of rat endothelial cell lines. *Virus Research* 1996; **46**: 65.

Duijvestijn AM, Derhaag JG, Koornstra J, and Zierhut M. Lymphocyte homing to lymphoid and inflamed joint and eye tissues: the role and control of high endothelial venules and adhesion molecules. *Immunology of the joint and the eye*, M. Zierhut, and H.-J. Thiel, 1996; 35.

Derhaag JG, Duijvestijn AM, Damoiseaux JGMC, and van Breda Vriesman PJC. An in vitro model for studying the role of graft endothelial cells in rat allograft rejection, a study on the regulation and function of heart endothelial histocompatibility antigens and cell adhesion molecules. *Transplantation Proceedings* 1997; **29**: 1717.

Haaster van CMCI, Derhaag JG, Engels W, Lemmens PJMR, Gijsen AP, Hornstra G, Van der Vusse G, and Duijvestijn AM. Mast cell-mediated induction of ICAM-1, VCAM-1, and E-selectin in endothelial cells in vitro: constitutive release of inducing mediators but no effect of degranulation. *European Journal of Physiology*, 1997; **435**:137.

Derhaag JG, Duijvestijn AM, and van Breda Vriesman PJC. Heart EC respond heterogeneous on cytokine stimulation in ICAM-1 and VCAM-1, but not in MHC expression. *Endothelium* 1997; **5**: 307.

Duijvestijn AM, and Derhaag JG. Mechanisms controlling leukocyte migration to inflamed tissues, emphasizing lymphocyte homing to skin and eye. *Immunology of the skin and the eye*, M. Zierhut, and H.-J. Tiel, In press.

Derhaag JG, Duijvestijn AM, Damoiseaux JGMC, and van Breda Vriesman PJC. Effects of antibody reactivity to MHC and non-MHC alloantigens on graft endothelial cells in heart allograft rejection. Submitted for publication.

Dankwoord

To him that absent is, all things succeed amiss

Cervantes

Een "boekje" schrijf je nooit alleen.

Het leven van een AIO gaat niet over rozen !

Ik wil graag iedereen bedanken die, elk op zijn eigen wijze, heeft meegeholpen aan de tot standkoming van mijn proefschrift, of mij in enig ander opzicht tot steun is geweest gedurende mijn promotietijd.

Josien

Curriculum vitae

Josien Derhaag werd geboren op 25 maart 1967 te Sittard. Na het behalen van het Atheneum β diploma aan de R.K. Scholengemeenschap "Serviam" te Sittard begon zij in 1986 met de studie Gezondheidswetenschappen met als afstudeerrichting Biologische Gezondheidkunde aan de Universiteit Maastricht. In augustus 1991 werd het doctoraal diploma behaald. Na een jaar werkzaam te zijn geweest bij de vakgroep Moleculaire Celbiologie & Genetica van de Universiteit Maastricht begon zij in 1993 als assistent in opleiding in dienst van de Universiteit Maastricht bij de vakgroep Immunologie. Onder leiding van Prof. Dr. P.J.C. van Breda Vriesman en Dr. A.M. Duijvestijn werd gedurende deze periode het onderzoek verricht beschreven in dit proefschrift. Vanaf april 1998 is zij werkzaam als bioloog bij de afdeling Obstetrie & Gynaecologie van het Academisch Ziekenhuis Maastricht.