Endothelial Dysfunctions after Transplantation: Adverse Effects of anti-HLA I Antibodies and Immunosuppression

Graduate School for Cellular and Biomedical Sciences

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PhD Thesis

Submitted by

Michael Bieri

from Schangnau, BE

Thesis advisor

Prof. Dr. med. P. Mohacsi
Department of Clinical Research
Medical Faculty of the University of Bern

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Bern, Dean of the Faculty of Medicine

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2 ABBREVIATIONS

Abbreviation	
AJ	Adherens junction
AMR	Antibody mediated rejection
APC	Antigen presenting cell
AT_1	Angiotensin II type 1 receptor
BCR	B cell receptor
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
CMR	Cellular mediated rejection
CsA	Cyclosporin A
Csk	C-terminal Src kinase
DC	Dendritic cell
DMSO	Dimethyl sulfoxyde
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial NO synthase
ERL	Everolimus
EtOH	Ethanol
FK-506	Tacrolimus
HHpred	Homology detection & structure prediction by HMM-HMM comparison
HLA I	Human leukocyte antigen class I
HLA II	Human leukocyte antigen class II
HUVEC	Human umbilical vein endothelial cell
Ig	Immunoglobulin
IL	Interleukin
kD	kilo Dalton
LC-MS/MS	High pressure liquid chromatography coupled to tandem mass spectrometry
L-NAME	N-Ω-nitro-L-arginine methyl ester
MAC	Membrane attack complex
MHC I	Major histocompatibility complex class I

MHC II	Major histocompatibility complex class II
MICA	MHC polypeptide-related sequence A
MRLC2	Myosin regulatory light chain 2
mTOR	Mammalian target of rapamycin
NAC	N-acetyl-cystein
NFAT	Nuclear factor of activated T cells
PI3K	Phosphoinositidyl-3 kinase
PRA	Panel reactive antibody
PSI	Proliferation signal inhibitor
qRT-PCR	Quantitative reverse transcript polymerase chain reaction
RNOS	Reactive nitrogen and oxygen species
RT	Room temperature
siRNA	Short interference RNA
SRL	Sirolimus / Rapamycin
TCR	T cell receptor
TiO_2	Titanium dioxide
TJ	Tight junction
TMR	T cell mediated rejection
VE-cadherin	Vascular endothelial cadherin
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
VE-PTP	Vascular endothelial-protein tyrosine phosphatase

3 SUMMARY

Organ transplantation is often the last life-saving treatment for patients with severe organ failure. Edema formation within the graft after transplantation is a criterion for the occurrence of antibody mediated graft rejection. Edema are also observed as side effects in immunosuppressed patients after mammalian target of rapamycin (mTOR) inhibitor treatment.

The present thesis focuses on the effects of anti-human leukocyte antigen class I (HLA I) antibodies and mammalian target of rapamycin (mTOR) inhibitors on endothelial cells (EC). Anti-HLA I antibodies were shown to increase the paracellular permeability of cultured ECs isolated from umbilical veins. Vascular endothelial (VE)-cadherin expression was investigated as a possibility for the development of increased endothelial permeability. Anti-HLA I antibodies dose-dependently decreased the expression of VE-cadherin already after 30 min exposure, which was inhibited by blocking vascular endothelial growth factor receptor 2 (VEGFR2) or Src. Only the entire antibodies had an effect on the VE-cadherin expression. Antigen binding or crystallizable fragments of the same antibody, generated by papain digestion, did not influence the expression of VE-cadherin after 24 h stimulation. The mRNA level of VE-cadherin was not affected by anti-HLA I antibodies. Posttranslational tyrosine phosphorylation of VE-cadherin was detected after 30 min and 24 h anti-HLA I stimulation, which was inhibited by bloking VEGFR2 and Src. VEGFR2 was activated by vascular endothelial growth factor (VEGF) produced by the ECs. Besides increased endothelial permeability, VEGF also stimulated ECs to proliferate in an autocrine manner.

Furthermore, mTOR inhibition alone led to increased paracellular permeability and decreased VE-cadherin protein and mRNA expression. Calcineurin inhibitors, another class of immunosuppressive drugs, had no influence on endothelial permeability or VE-cadherin expression. By inhibition of PI3K, an upstream kinase of mTOR, the expression of VE-cadherin decreased comparable to mTOR inhibition. ECs produced more reactive nitrogen and/or oxygen species (RNOS) in the presence of mTOR inhibitors. The production of RNOS and the increased permeability of ECs were prevented by the addition of antioxidants during treatment with mTOR inhibitors.

Taken together, we were able to demonstrate that anti-HLA I antibodies and mTOR inhibitors increase endothelial permeability by down-regulation of VE-cadherin. Anti-HLA I antibodies reduced VE-cadherin expression by the production of endothelial VEGF, which led to phosphorylation of VE-cadherin after autocrine activation of VEGFR2. On the other hand, mTOR inhibitors directly down-regulated the biosynthesis of VE-cadherin.

4 SCIENTIFIC BACKGROUND

4.1 Antibodies in Human Allograft Rejection

4.1.1 The Basic Science of Alloantibody Production

Both, the humoral and the cellular immune system are activated in an immune response against the allograft. Current immunosuppressive drugs mostly inhibit the activation of recipients' CD4+ and CD8+ T cells. Therefore T cell mediated rejection (TMR) and antibody mediated rejection (AMR) can be controlled adequately. However, in patients with preexisting anti-graft antibodies, immunosuppressants may fail due to the presence of long living plasma cells.

The alloimmune response of AMR and TMR can be triggered by any foreign antigens, such as polymorphic surface molecules. Naturally, every individual has preexisting antibodies against the ABO blood group antigens, which develop after birth as cross-reactivity to microbes in the intestine. Antibodies against major histocompatibility antigens (MHC) are produced after the contact to another blood product (transfusions, pregnancies, surgery or previous transplants). These antibodies are directed against donor MHCs class I and class II and are present as high-affinity immunoglobulins (Ig). The primary target of anti-MHC antibodies is the vascular endothelium of the graft, where antibodies recruit all available effector systems including complement system, leukocytes, natural killer cells and macrophages.

Antigen Presentation and T Cell Activation

The immune response to allografts is initiated by the recognition of foreign antigens in the graft by recipient's T cells. There are three possibilities, how recipient T cells can recognize alloantigens: by direct or indirect presentation (*1, 2*) or by a new discovered semidirect presentation that combines the direct and indirect presentation (*3, 4*).

The indirect presentation is the usual and common way in which the immune system recognizes foreign antigens. Host antigen presenting cells (APCs), such as dendritic cells, can take up graft-derived cells or donor-released antigens (Figure 4.1-1). After processing the alloantigens in intracellular compartments, the APCs present the foreign peptides on MHC class II molecules to CD4+ T cells (*5*). Only a few alloantigens enter the cytosolic processing for MHC class I presentation (cross-presentation) and are encountered by CD8+ T cells or cytolytic T cells (*6*). Therefore, most of the immune responses after indirect presentation of alloantigens are CD4+ dependent.

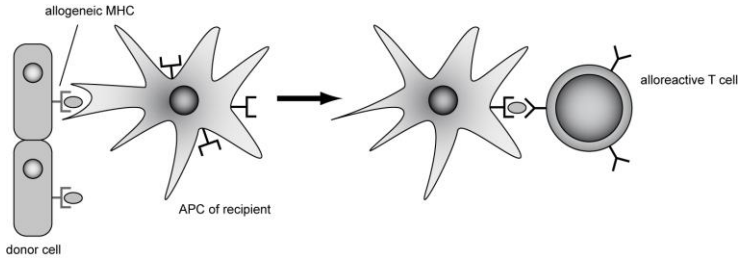


Figure 4.1-1: The indirect presentation. Donor antigen is processed by host APC and presented to host T cell

The direct presentation requires donor APCs derived from the graft (Figure 4.1-2). MHCs of donor APCs, which migrate from the graft to the lymphoid organs, were recognized by host T cells. In the direct presentation, CD4+, CD8+ or cytolytic T cells can get activated depending on which MHC class was recognized (*5*). Activation of T cells by direct presentation fades by time, as the donor APCs die off.

Another possibility of direct recognition occurs if host T cells enter the graft and encounter allogenic antigens in the graft (*1*).

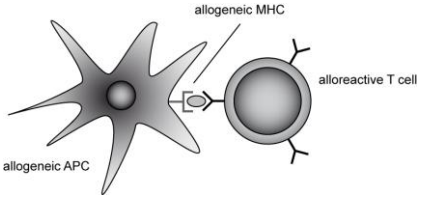


Figure 4.1-2: Direct Presentation. An allogeneic APC activates directly a host T cell.

The third way of presentation (semidirect presentation) combines the direct and indirect pathway (*3, 4*). Host APCs are able to incorporate membrane fragments containing intact allo-MHCs into their own membrane after cell-to-cell contact to donor cells. The acquired donor MHC might be presented to the recipient's immune system and generate an immune response. The semidirect presentation allows the APCs to prime cytolytic T cells against intact MHCs that are present on endothelial cells in the graft. This is an alternative

mechanism to the cross-presentation of donor MHC peptides by host APCs in the indirect presentation.

As a result of activation, T cells start to proliferate and migrate to the secondary lymphoid organs (lymph nodes, spleen) and form a T cell rich germinal center, where they are able to activate B cells.

B Cell Activation and Alloantibody Production

B cell response against donor antigens starts with the binding of the B cell receptor (BCR) of an alloreactive B cell to foreign antigen. BCRs recognize the native form of the antigen and are able to bind antigens on donor cells, secreted antigens or antigens on membrane vesicles. The antigen recognition of B cells occurs in secondary lymphoid organs or within the graft. Activated B cells start to proliferate and process the encountered alloantigen in an APC like manner. Resulting peptides are then displayed on MHC II.

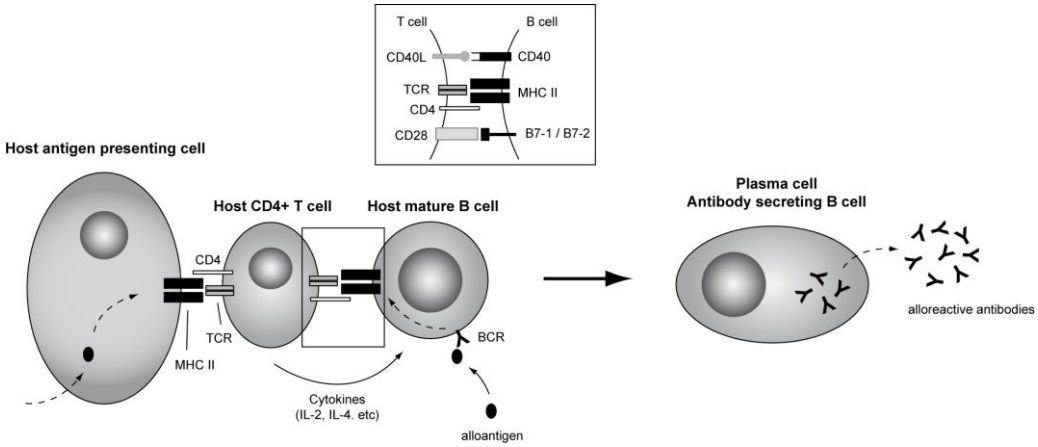


Figure 4.1-3: An external antigen is processed by a host APC and indirectly presented on MHC II to a helper T cell. Contemporary, a B cell that encountered the same antigen, presents this antigen to the helper T cell. As a consequence of the dual activation, the helper T cell secrets cytokines to activate the B cell, which transforms to an antibody producing plasma cell.

Mature allogeneic B cells take up donor MHC either in the secondary lymphoid organs or in the circulation (*7*). Activated B cells are located in primary follicles in the cortex of the secondary lymphoid organs and increase the expression of chemokine receptor CCR7, which responses to the T cell zone CCL19 and CCL21 chemokines (*8*). Upon activation primary follicles become secondary follicles by forming a ring of B cells around the germinal center.

At the same time activated T cells in the germinal center increase the expression of chemokine receptor CXCR5 (*9*). Activated T cells migrate to the B cell rich follicles around

the germinal center. This ensures an interaction of activated B and T cells and finally the production of antigen specific antibodies.

The antibody response is triggered by the binding of a peptide – MHC II complex of a specific B cell to an activated CD4+ T cell (Figure 4.1-3). Subsequently, the B cell increases the expression of costimulators B7-1 and B7-2 that interact with CD28 on T cells (*10*). CD4 on the CD4+ T cell interacts with a non polymorphic region of the MHC II of the B cell and to the costimulators, which upregulates the CD40 ligand (CD40L) expression of the T cell (*11*). CD40L binds to CD40 of the B cell and initiates proliferation and differentiation into antigen producing (plasma-) or memory B cells (Figure 4.1-3) (*12*). Subsequently, the T cell secrets cytokines that leads to the final B cell activation. Different cytokines are responsible for the specific B cell response (Table 4.1-1).

Table 4.1-1: Cytokines involved in specific B cell response

Cytokine	Effect
IL-2	Growth factor for T (autocrine) and B cells, increases production of IL-4 and IFN-γ
IL-4	Major stimulus for IgE and IgG4 isotype switch, Reciprocal antagonist of IFN-γ, inhibits cell-mediated immune response
IL-5	IL-5 enhances function of IL-4, stimulation of B cell proliferation, IgA and IgE isotype switch
IFN-γ	IgG isotype switch, inhibits IL-4 dependent isotypes, increases expression of MHC II
TGF-β	Inhibits proliferation and activation of T cells, stimulates production of IgA

B cells activated by chemokines undergo clonal expansion and differentiation into antibody producing plasma cells. Plasma cells that left the germinal center start to produce and secret low affinity IgM antibodies, which were present as membrane bound antibodies (as BCR) prior to activation (*13*). The remaining proliferating B cells start to rearrange the polymorphic region of their receptor / antibody by somatic hypermutation, leading to the production of high affinity IgG antibodies and memory B cells. During somatic hypermutation (affinity maturation), B cells rearrange their BCR (mainly membrane bound IgM) and get in contact with follicular dendritic cells (DC). Only high affinity B cells gain a competitive advantage and continue to proliferate and to produce high affinity IgG antibodies (*14*). After about 14 days, the primary antibody response reaches peak antibody serum level (*15*).

Memory B cells and long living plasma cells migrate to the bone marrow, where they maintain a persisting antibody response (*16*).

In a secondary B cell response, alloantigens activate high affinity memory B cells. These B cells undergo rapid clonal expansion and antibody production. More high affinity IgG and less

IgM antibodies are produced compared to the primary B cell response. Additionally, lower levels of antigens are needed to initiate a B cell response.

The Major Histocompatibility Complex

The major histocompatibility complex (MHC) is a highly polymorphic cell surface protein, which plays an important role in immunity. In humans, the MHC was first described in the 1950ies when serum antibodies reacted to certain, but not all, leukocytes of a mixed population (*17*). Antigens that were identified by these sera were called human leukocyte antigens (HLA), which is the human analogue of MHC.
Later on, MHC or HLA was identified as a protein produced from 6 linked genetic regions on the short arm of chromosome 6 (*18*). HLA loci are grouped in 3 classes (Figure 4.1-4) as follows:

Class I: HLA-A, HLA-B and HLA-C

Class II: HLA-DR, HLA-DQ and HLA-DP; the peptide transporter genes TAP-1 and TAP-2; and LMP1 and 2, the peptide-producing proteasomes

Class III: complement component C2 and C4 and properdin factor B, tumor necrosis factor (TNF) α and β, heat shock protein 70 and 21 hydroxylase α and β as the most important genes

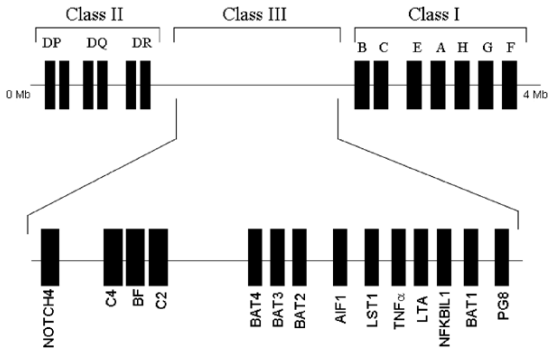


Figure 4.1-4: Schematic HLA loci on chromosome 6. The HLA complex is classified into 3 groups: class I (HLA-A, HLA-B and HLA-C), class II (HLA-DP, HLA-DQ and HLA-R) and class II (C2, C4 and properdin factor B (BF, among others) (*19*).

Each HLA locus on the DNA of all classes encodes a specific protein. The HLA loci are very polymorphic, but only one allelic form of every group can be found on one chromosome. The nomenclature is composed of the locus and an identifying number: e.g. HLA-A2 is the second allele of HLA-A. By serological identification, 25 distinct alleles were found for HLA-A and 43 alleles for HLA-B. Much more polymorphism was found by molecular biological methods, where an even bigger diversity was determined on the genetic level. The HLA

polymorphism is an important aspect in the susceptibility of common diseases or autoimmunity, as HLAs present antigens to immune cells (*20-22*).

Each person carries two HLA haplotypes: one paternal and one maternal. If a father has hypothetically a haplotype set of AB and a mother a set of CD, their children might have one of 4 potential sets of haplotype: AC, AD, BC or BD. Therefore, siblings have a 25% chance of carrying the same set of HLA haplotypes among each other. As expected, each HLA locus should be present with equal frequency in the population. However, there are some loci, which are more common in the population than others. This phenomenon is called linkage disequilibrium and can be explained by geographic isolation or migrations (*23*). For example HLA-A2 is found in approximately 50% of all Orientals and Caucasians.

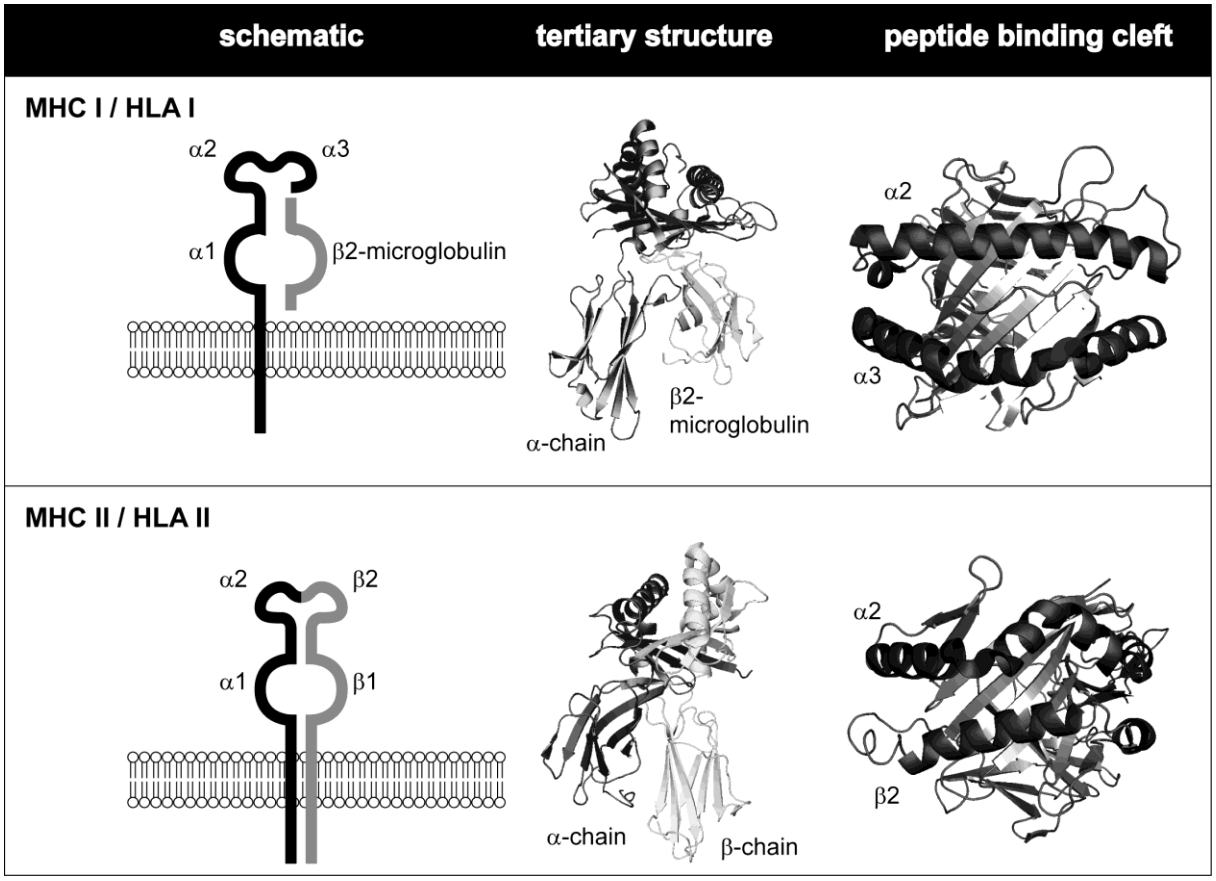


Figure 4.1-5: Major histocompatibility complex (in human: human leukocyte antigen (HLA)). (3d modeling of HLA I (HLA-A0201) and HLA II (HLA-DM) by PyMOL (*24*) using structure files obtained from www.pdb.org).

HLA I:

The structure of HLA I is depicted in Figure 4.1-5. The protein is composed of two chains (*25*): The polymorphic glycosilated polypeptide α-chain is non-covalently linked to a conserved polypeptide, the β2-microglobulin (encoded on chromosome 15). The N-terminal part of this transmembrane heterodimer forms 3 extracellular domains (α1-3). The α3 domain forms, as β2-microglobulin, an immunoglobulin (Ig) like domain, which is conserved and

binds to CD8 on T cells during immune response. Only the α-chain is anchored in the plasma membrane.
The function of HLA I is to present cytosolic peptides to CD8+ or to cytotoxic T cells. The peptide binding pocket is formed by 8 anti-parallel β-strands and located between the α2 and α3 domain. It is able to bind 8 – 10 amino acid long polypeptides. HLA I molecules are found on almost every nucleated cell.

HLA II:
HLA II molecules are heterodimers (*26*) composed of a glycosilated α-chain and a non-covalently assembled β-chain (Figure 4.1-5). Unlike class I HLAs, both chains are located on chromosome 6. Both, the α- and β-chains are anchored in the cell membrane. After an Ig-like domain (α1 and β1), a polymorphic domain (α2 and β2) is followed at the N-terminal end.
HLA II heterodimers are also involved in the immune response. HLA II is only expressed on APCs and presents endocytosed antigens to CD4+ T cells. The peptide-binding cleft is formed at the interface of the α2 and β2 domains and is able to carry 10 – 30 amino acid long polypeptides.

4.1.2 Effector Functions of Antibodies

Antibodies are proteins that are mainly produced and involved in the adaptive immunity. All antibodies consist of two heavy chains connected to a light chain. They are able to bind to a broad range of antigens, such as lipids, polysaccharides, proteins and chemicals by their variable region. The effector functions of antibodies include several mechanisms, such as the oppsonization of pathogenic cells, which mark them for endocytosis by macrophages or for killing by activated natural killer cells, the neutralization of toxins or the activation of the complement system.

The effector functions of immunoglobulins are mediated by the Fc part of the antibody. The most important antibody effector function in graft rejection is the activation of the complement system, which leads to hyperacute or acute rejection by tissue injury or chronic rejection by arteriosclerosis (*27*). Nevertheless, antibodies may also act as agonistic antibodies to polymorphic surface receptors, such as the angiotensin II receptor type 1, which activates the renin angiotensin system that may lead to vasoconstriction and vasculopathy (*28*).

Structure of Antibodies

All antibodies share a common structure, which is depicted in Figure 4.1-6. The Y-like structure is composed of two juxtaposed heterodimers of a light and heavy chain (*29*). The heavy chain folds into four Ig domains and is linked at the N-terminal part via disulfide bonds to the light chain, which is folded into 2 Ig domains. The two highly polymorphic antigen binding sites are formed by the variable regions of the heavy (V_H) and light chain (V_L). The variable region of the heavy chain is followed by three constant Ig domains (C_H). The second constant region of the heavy chain (C_H2) is the binding site for Fc receptors and the complement. A flexible hinge is located between C_H1 and C_H2. The schematic and tertiary structure of IgG is depicted in Figure 4.1-6.

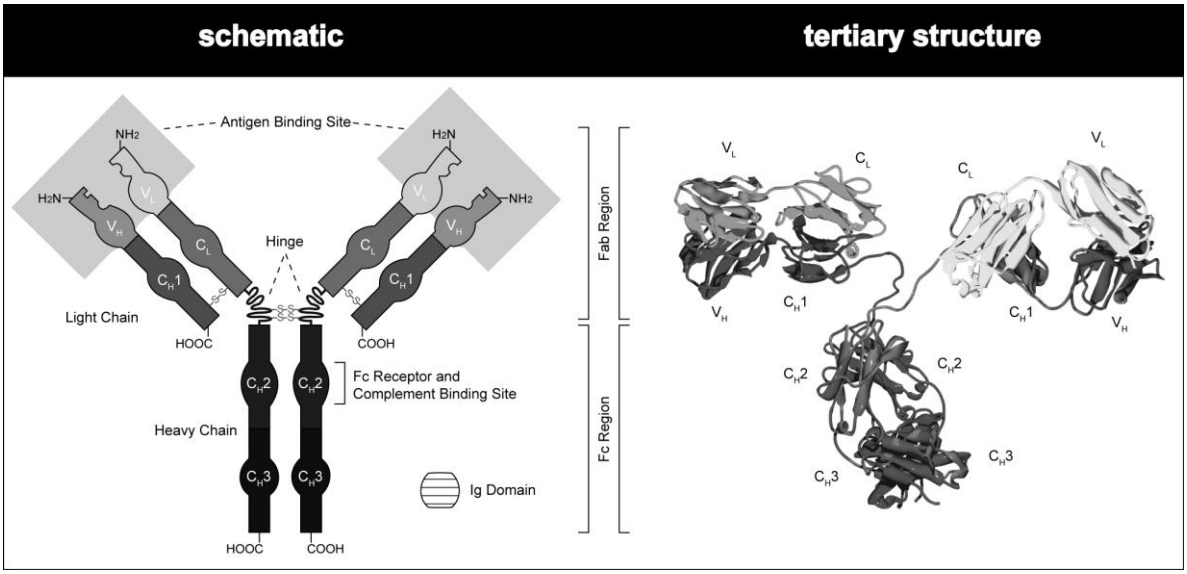


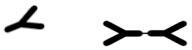
Figure 4.1-6: The structure of an IgG antibody consists of two juxtaposed heterodimers of a light and heavy chain. Each heavy chain is folded into four Ig domains and each light chain into two Ig domains. The antigen binding sites are formed by the variable N-terminal part of the heavy chain (V_H) and light chain (V_L). Between the first and second constant region (C_H) of the heavy chain is the flexible hinge that separates the Fab (antigen binding fragment after proteolytic cleavage by pepsin) from the Fc (crystallizable fragment after pepsin cleavage) part. The second constant region of the heavy chain (C_H2) forms the binding site for Fc receptors and complement.

Proteolytic digestion of an antibody by pepsin results in a cleavage into two parts: an antigen binding fragment (Fab2) that consist of the two light chains and the heavy chains below the hinge and a crystallizable part (Fc), which is made by the two heavy chain parts under the hinge (*29*). Papain, another proteolytic enzyme, cleaves the antibody into three pieces: two Fab fragments and one Fc fragment by cleaving right above the hinge.

There are different classes of antibodies, which serve different functions (Table 4.1-2). In mammals five isotypes exist, which are named IgA, IgD, IgE, IgG and IgM (*29*). The

structural difference is located at the C-terminal heavy chains. Heavy chains of IgAs, IgDs and IgMs are composed of three constant Ig regions, whereas IgMs consist of four constant Ig domains. All isotypes are secreted as monomers except of IgA, which is only stable as a dimer and IgM, which only occurs as a pentamer.

Table 4.1-2: Antibody Isotypes and their function

Isotype	Subclasses	Function	Secretory Form
IgA	2	In mucosal areas (gut, respiratory and urogenital tract) and saliva, tears and milk, prevents colonization by pathogens, occurs as a monomer or dimer	
IgD	1	Found as antigen receptors on naive B cells	
IgE	1	Binds to allergens and induces histamine release from mast cells and basophils, causes allergy, protects against helmints	
IgG	4	Major antibody of adaptive immunity, only antibody that crosses placenta and provides passive immunity, activates complement, oppsonisation of pathogens	
IgM	1	Pentamer with high avidity, early stage of humoral immune response, antigen receptor on naive B cells (BCR), activates complement	

Antibodies initiate the effector functions via their Fc domain. IgG and IgM are able to oppsonize viruses. The Fc parts of IgG or IgM bind to Fc receptors on macrophages, which eliminate the virus by phagocytosis. Fc region of IgE bind to mature mast cells and eosinophils, where they serve as receptor to recognize antigens (*30*).

Role of Complement System in Graft Injury in Transplants

The predominant way how antibodies damage the endothelium is the activation of the complement system. Complement activating IgG or IgM bind to the vasculature of the graft and activate the complement leading to hyperacute or acute rejection. IgM antibodies have the highest ability to activate the complement system, followed by IgG3 and IgG1 subclasses, whereas IgG2 acts only weakly complement activating. IgG4, IgA and IgE are not able to activate the complement system (*31*).

After ischemia/reperfusion injury of the graft, the endothelium releases its proteoclycans (e.g. heparan sulfate) from the extracellular matrix, which exposes new antigens (*32, 33*) to the

blood stream, which might activate the complement system. The most important activation of the complement cascade after transplantation is the classical pathway.

The classical complement pathway is activated after binding of the C1 complex (composed of C1q, C1r, and C1s) to two or more antibody-antigen complexes in the graft (Figure 4.1-7). The binding of C1q to antibody–antigen complexes induces a conformational change in C1q, which activates C1r (*30*). Activated C1r cleaves and activates C1s, which is now able to cleave the next complement proteins, C4 and C2. After the cleavage of C4, C4a is released and the bigger fragment C4b binds to the endothelium. Upon inactivation of C4b by factor I, C4b becomes C4d, which remains covalently bound on the vasculature and is a useful marker of complement activation in the clinics (*34*). On the other hand, C2 is cleaved by C1s and the activated fragment (C2a) forms together with C4b the classical pathway C3 convertase C4b2a.

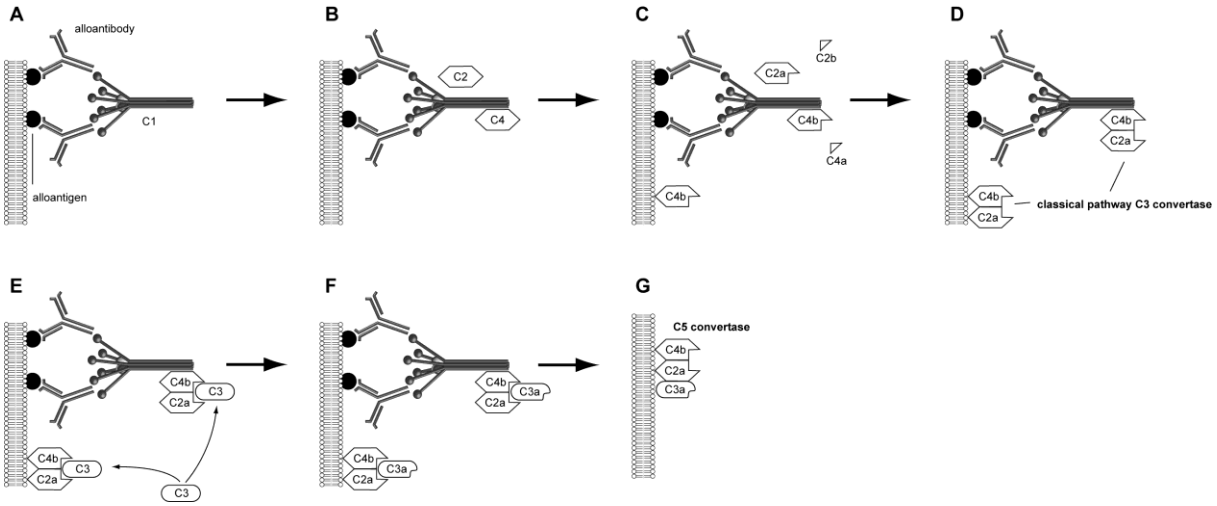


Figure 4.1-7: The formation of the C5 convertase starts with the binding of the C1q/C1r/C1s complex to the antibody–antigen complex (A). The activated C1 complex binds C4 and cleaves it to C4a and C4b (B + C) and cleaves the soluble C2 into C2a and C2b. The split products C2a and C4b bind covalently to the antibody or the lipid bilayer and form the classical pathway C3 convertase (D). The C3 convertase cleaves C3 and the C3b fragment (E + F) that forms together with C2a and C4b the covalently bound C5 convertase (G).

The C3 convertase proteolytically cleaves C3 into a small C3a and a bigger C3b fragment, which binds covalently to cell surfaces or the antibody and forms the C5 convertase C4b2a3b. This cleaves C5 into C5a and C5b, which initiates the formation of the membrane attack complex (MAC). After the addition of C6 and C7, the resulting complex C5b67 inserts into the plasma membrane followed by the insertion of C8 (Figure 4.1-8). Subsequent addition of up to 15 C9 molecules polymerize around the complex forming pores in the lipid bilayer. The completely formed MAC causes lysis of endothelial cells in the graft, which may lead to graft rejection.

The complement system also triggers other effects of the adaptive immune system (*33, 35*) that may favor graft rejection. The released complement split products C2a, C3a and C5a act as chemoattractants for macrophages and neutrophils. Furthermore, C3a induces the release of prostaglandin E2 from macrophages and C5a induces the release of histamine from mast cells, which may lead to the formation of interstitial edema (*36*). The biologically active C3a and C5a also activate endothelial cells to increase the adhesion molecule expression and the cytokine production (*37-39*). Endothelial adhesion molecules, such as ICAM-1, VCAM-1 and E-selectin, also increase after stimulation of ECs by the soluble C5b-C9 complex (*40*). C5a and C5-C9 induces biosynthesis of tissue factor that might be responsible for the thrombotic injury in severe AMR (*41*).

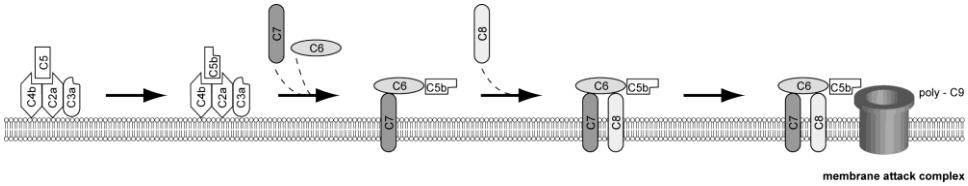


Figure 4.1-8: C5 convertase cleaves C5 into C5a and C5b, which forms together with C6 and C7 a complex that is integrated in the lipid bilayer. After addition of C8 in the lipid bilayer, up to 15 C9 molecules polymerize around the complex and generate pores into the plasma membrane. The C5b-C9 complex is called the membrane attack complex (MAC).

Agonistic and Antagonistic Antibody Effects

Next to the activation of several immune components, antibodies are also able to act agonistic or antagonistic to receptors. If the anti-receptor antibody recognizes the receptor binding site, which is responsible for the action of the receptor, the antibody can either act as an activating or inhibitory signal.

Agonistic antibodies against angiotensin II type 1 receptor (AT_1) were found in kidney transplanted patients (*28*). These IgG1 and IgG3 antibody subtypes bound to two different epitopes on the second extracellular loop of AT_1 and activated the renin angiotensin system, This might lead to vasoconstriction and may contribute to refractory vascular rejection.

Anti-HLA I antibodies are able to activate several protein kinases in ECs (*42-45*). Two distinct physiological consequences were found after anti-HLA I activation of ECs: a mammalian target of rapamycin (mTOR) dependent increase of endothelial proliferation (*46, 47*) and cell survival (*42, 48*).

Receptor antibodies can also play a role in autoimmune diseases. In Sjögren's Syndrome, antagonistic antibodies against the muscarinic receptors M1R and M3R inhibit the saliva production of salivary glands (*49*).
In Chagas disease, autoantibodies against the secondary extracellular loop of the muscarinic receptor M2 were claimed to contribute to heart problems and to alterations in colon function (*50, 51*).

4.1.3 Diagnostic Tools in Antibody Mediated Rejection

The first methods to detect alloantibodies in recipients' serum were lymphocytotoxicity assays, which were first described by Patel and Terasaki in 1969 and are still in use (*17*). Novel high-throughput methods increased specificity (anti-HLA antibodies) and sensitivity (low serum titers). These methods facilitate investigation of antibody mediated syndromes, such as AMR. Antibody detection systems used in transplantation research and diagnostic are summarized in Figure 4.1-9. To interpret antibody studies as well as clinical and pathological outcomes, the difference in the sensitivity of these tests has to be considered.

Complement Dependent Cytotoxicity Tests

The first tests to investigate anti-donor antibodies in recipient's serum were lymphocytotoxicity tests (complement dependent cytotoxicity test (CDC)). Either lymphocytes from a single donor (cross-match test) or a panel of selected donor cells representing common HLA alleles of a population (panel reactive antibody (PRA) test) are mixed with serum of a recipient (*52*). Antibodies of recipient's serum, if present, bind to donor or selected lymphocytes. Unbound antibodies are removed by washing. Complement is added subsequently, which leads to complement-mediated lysis of cells following formation of MAC. After addition of a vital dye, dead cells are stained red and can be detected in the microscope.
The simple cross-match variant is used to monitor a possible alloreaction of recipient's antibodies to the graft. A positive T cell cross-match (recipient's antibodies react against donor T cells) indicates anti-HLA class I reactivity of donor antibodies to the graft. On the other hand, positive B cell cross-match (recipient's antibodies react against donor B cells) suggests the presence of antibodies against donor HLA class II. Positive cross-matches are contraindicatory for successful transplantations.

The extended version of the cross-match is the PRA test; in which pooled lymphocytes with known HLA phenotype are diluted in multi-well plates (*52*). The percentage of PRA is formed by the fraction of wells containing a majority of dead cells compared to the total number of wells. When known phenotypes of HLAs are plated out, even the specificity of anti-HLA antibodies can be determined by this method.

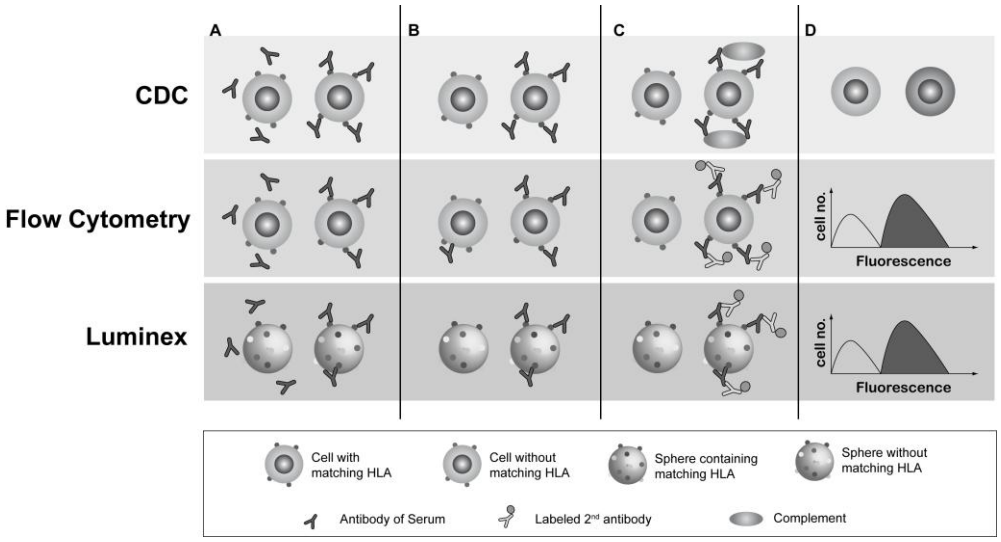


Figure 4.1-9: Comparison of the different antibody detection systems used in transplantation. Complement dependent cytotoxicity test (CDC), flow cytometry and the Luminex test share all the same procedures: First, recipients' serum is incubated with either donor or known lymphocytes or microspheres (A). After washing (B) bound antibodies are detected either with complement (which causes necrosis of cells with bound recipient's antibodies) or a secondary labeled antibody (can be detected).

Flow Cytometry

The problem of above described CDC assays is their low sensitivity. Advances were made by the development of flow cytometric approaches to detect serum antibodies (*53-57*). This technique allows monitoring even low titer antibodies or those that are not complement activating.

In a first step, lymphocytes either from the donor or with known phenotype (similar to CDC) are mixed with recipients' serum. Unbound, unspecific antibodies are removed by washing as it is done in CDC tests. In CDC, bound antibodies are detected by the addition of complement and by the subsequent lysis of cells. In flow cytometry, fluorescent anti-human IgG antibodies are added and detected. This secondary antibody will bind to the previously bound specific antibodies on the lymphocytes. Stained antibodies can now be detected by flow cytometry. After reaching a certain fluorescent threshold, the test can be considered as positive (comparing to a suitable negative control).

This method can also be used for a simple cross-match (mixing of donor lymphocytes with recipient's serum) or to determine the antibody-specificity (mixing of lymphocytes with known HLA phenotype with patient's serum). Another advantage of flow cytometry is the use of a secondary anti-human IgG antibody, which avoids false positivity deriving from IgM antibodies.

Solid Phase Assay

Detection of serum antibodies by Luminex is very similar to the flow cytometry method. However, there is no need for living cells. Instead, microspheres, which are packed with a set of different recombinant HLAs and carrying different fluorescent dyes, are mixed with recipient's serum (*58*). Specifically bound antibodies are detected by fluorescent anti-human IgG antibodies. Therewith, the specificity and amount of anti-HLA antibodies can be read out by the fluorescent value and the set of positive microspheres.
Alternatively, this test can also be performed as an enzyme-linked immunosorbent assay (ELISA). Recombinant HLAs are linked to multi-well plates instead of microspheres (*52*).
However, there is a need for clinical interpretation, as this test, as well as the flow cytometry method, does not show the functional properties of the detected antibodies.

4.1.4 Antibody Mediated Rejection in Clinical Practice

In the process of an organ rejection, the graft activates the immune system of the recipient. Depending on the elapsed time since the onset of rejection it can be classified into hyperacute, acute and chronic rejection (*59-61*). Both, the cellular and the humoral immune system participate in rejection, but the exact mechanisms and significance of humoral immunity is not completely investigated and understood yet.

Hyperacute Rejection

Antibody mediated rejection in modern clinical transplantation was first described by Terasaki and Patel in 1968 (*17*). In this study 32 of 225 kidney transplant patients had primary non-function of the graft. Out of these 32 patients 24 (75%) were CDC positive. From the remaining 193 healthy patients only 6 (3%) were also CDC positive. Nowadays, this primary

non-function of the graft is classified as hyperacute rejection, which occurs shortly after reperfusion and comes along with tremendous intravascular thrombosis and necrosis. The reasons for hyperacute rejection are preformed anti-graft antibodies, in most cases anti-ABO blood group antigens or anti-HLA antibodies.

In the past 40 years the CDC test, as used in this study (*17*), was only slightly modified and formed the basis for the cross-match. Due to the CDC and the knowledge of antibody mediated syndrome, hyperacute rejection could almost be eliminated in clinical transplantation. Nevertheless, hyperacute rejection still appears in recent years, as depicted in Figure 4.1-10:

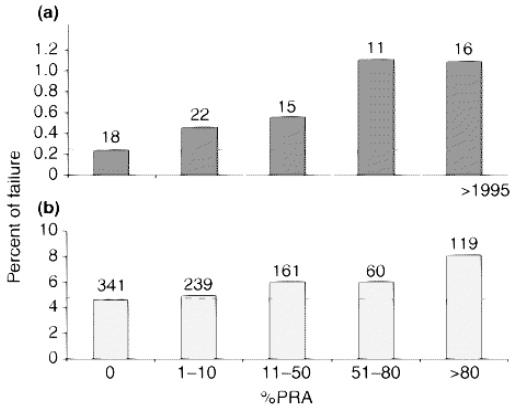


Figure 4.1-10: Correlation of panel reactive antibodies (PRA) and hyperacute rejection (a) or primary graft dysfunction (b). Percent of graft loss were plotted against PRA groups. The number above the bars represents the number of patients in the particular group. Increased primary heart dysfunction and hyperacute rejection are proportional to the appearance of higher PRAs. (Graphic reprinted from (*62*)).

The importance of antibodies in other phases of rejection was not clear for a long time. The development of C4d staining of biopsies was a break-through as a proof for complement activity (*63, 64*). This technique renewed the interest in humoral rejection.

Acute Rejection

Next to the well-documented cellular cytotoxicity in acute rejection (*59, 60*), Halloran and colleagues reported in 1990 that antibodies may play a role in the pathogenesis of acute rejection (*65, 66*). In a study with 64 patients with acute rejection, each patient (100%) displayed anti-HLA I antibodies in the serum accompanied by vascular lesions in peritubular capillaries. Only 41% of the patients without similar histology also displayed anti-HLA I antibodies in their serum.

Collins et al. compared biopsies of 10 patients with acute AMR (anti-HLA antibodies in the serum) to 14 patients with acute cellular mediated rejection (CMR) for the presence of C4d

(*63*). C4d staining was present in the peritubular capillaries in all of the acute AMR patients. In acute CMR, only traces of C4d were detected.

Positive C4d staining correlates with the presence of circulating antibodies (either preformed or newly formed) and became an important tool for the prediction of acute AMR (*63, 64, 67-72*). The correlation of many single- and multi-center studies of the presence of anti-HLA antibodies, C4d staining and adverse clinical outcome led to a formal definition of acute AMR, next to cellular rejection (*73*). The updated criteria for acute AMR are summarized in Table 4.1-3:

Table 4.1-3: Criteria for acute AMR (from (*61*)):

1. Clinical evidence of acute graft dysfunction
2. Histological evidence of acute capillary injury (*a* and *b* are required)
   a. Capillary endothelial changes: swelling or denudation with congestion
   b. Macrophages in capillaries
   c. Neutrophils in capillaries (more severe cases)
   d. Interstitial edema and/or hemorrhage (more severe cases)
3. Immunopathologic evidence for antibody mediated injury (in the absence of OKT 3 induction) *a* or *b* or *c* are required
   a. Ig (G,M, and/or A) + C3d and/or C4d or C1q (equivalent staining diffusely in capillaries, 2–3+), demonstrated by immunofluorescence
   b. CD68 positivity for macrophages in capillaries (identified using CD31 or CD34), and/or C4d staining of capillaries with 2–3+ intensity by paraffin immunohistochemistry
   c. Fibrin in vessels (optional; if present, process is reported as more severe)
4. Serologic evidence of anti-HLA class I and/or class II antibodies or other anti-donor antibody (e.g., non-HLA antibody, ABO) at time of biopsy (supports clinical and/or morphologic findings)

Chronic Rejection

Chronic rejection remains one of the biggest challenges in clinical transplantation, although major improvements and rising knowledge in the diagnosis and treatment of acute CMR were worked out. Evidence that antibodies may contribute to the pathogenesis of transplant glomerulopathy and the classical defined chronic rejection rose by time (*64, 73-83*).

Lee and coworkers investigated the development of anti-HLA antibodies in 139 kidney transplanted patients (*79*). Chronic rejection was diagnosed by CDC tests. All of the 29 patients who developed chronic rejection displayed anti-HLA antibodies in their serum. Out of these 29 patients, 14 patients developed anti-HLA antibodies after transplantation. In contrast, only 27% of the remaining 110 patients developed antibodies post-transplant.

In a large collaborative study 2231 kidney transplanted patients with functional kidneys were tested for anti-HLA antibodies within 2 years (*84*). Thereby, 478 patients with anti-HLA antibodies were detected. Among these patients 15% lost their grafts compared to 7% of the remaining 1753 without anti-HLA antibodies.
In another study of Terasaki, 14 patients with chronic kidney rejection without pre-transplant anti-HLA antibodies were tested on the development of anti-HLA before rejection (*79*). The results are summarized in Figure 4.1-11:

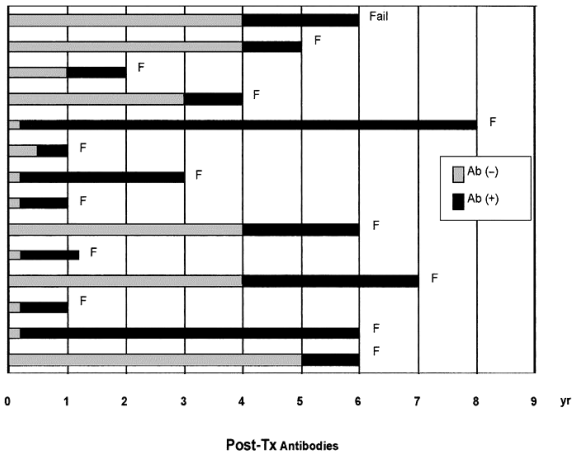


Figure 4.1-11: Patients with chronic kidney rejection (n = 14) without preformed anti-HLA antibodies were tested for the presence of anti-HLA antibodies (Ab) before rejection. Each patient developed anti-HLA antibodies before rejection (reprinted from (*79*)).

All patients developed de novo anti-HLA antibodies prior to chronic rejection. The time span for the presence of anti-HLA antibodies persisted in the patients' serum ranged from 6 month to 8 years before rejection.

4.1.5 Antigens in Antibody Mediated Allograft Rejection

For the generation of alloantibodies, which mediated AMR, the host's immune system has to encounter and recognize efficiently non-self antigens in the graft. ECs are considered the primary target of antibody-mediated graft rejection. Target antigens are all kind of polymorphic surface antigens on ECs, such as blood group antigens and HLAs, among others. Only very few antigens different from HLAs have been shown to contribute to AMR and alloantibody production. The most important antigens relevant in clinical transplantation are discussed in the following sections.

ABO Blood Group Antigens

Already in the early days of transplantation, the ABO blood group system was considered to be important in organ transplantation (*85*). Blood group antigens, as the name indicates, are expressed on the surface of red blood cells. The type of ABO antigen present on the red blood cells is inherited by the parents. In renal transplantation, anti-A and anti-B antibodies target A (including subgroup A1 and A2) and B antigens on ECs, interstitium and the glomeruli. Mesangial and tubular cells do not act as target for anti-A or anti-B antibodies (*86*). ABO-antibodies are typically IgM, mostly against A1 and B, although A2 was also reported the be involved in AMR (*87*).

ABO incompatible kidney transplantation has shown some success. In 1987, twenty-four kidneys from living-related donors were transplanted across the ABO barrier (*88*). Out of the 24 transplantations, eighteen recipients had kidney graft function for more than one year. This good outcome after ABO incompatible kidney transplantation encouraged to increase transplantations across the ABO barrier. Between 1989 and 2001, more than 300 ABO-incompatible renal transplantations were reported (*89*). The 1 year graft survival was 83% in these studies.

Transplantations across the ABO barrier of heart and lung grafts are very rare. In most cases, these transplantations were done accidentally as the outcome is rather bad (*90*). In 1988, a successful heart transplantation from a blood group B donor into a blood group O recipient was reported (*91*). In 2000, another blood group O patient received accidentally a blood group B cardiac allograft (*92*). This patient suffered from transplant vasculopathy after 5 years. In this patient, the expression of the ABO antigens was monitored for 44 month after transplantation. Surprisingly, it was found that the blood group type of the graft's endothelium began to change after 1 year from type B to the recipient's type O. The underlying mechanism of the changed ABO blood group on ECs is yet not understood.

The ABO incompatible liver transplantation do not manifest in strong AMR (*93*), although cases were reported (*94*). Nevertheless, ABO incompatible liver transplantation is only performed in life-thretening emergency cases.

The effects of antibodies directed against ABO blood group antigens occur mainly early after transplantation but do not have late effects.

Human Leukocyte Antigens

Mismatch of histocompatibility antigens between donor organ and recipient stimulates the recipient's immune system to induce rejection. In several studies, anti-HLA antibodies were associated with increased acute and chronic rejection and decreased graft survival of heart,

kidney, liver and lung transplanted patients (*62, 74, 77, 79, 80, 95-97*). In a study, 113 adults and 31 children, who received cardiac transplants between 1996 and 2003, were determined (*98*). Patients with donor specific anti-HLA antibodies (measured by Luminex) suffered more frequently of acute rejection, developed transplant-related coronary artery disease and decreased graft survival. On the other hand, the outcome of patients with anti-HLA antibodies, which are not directed against the graft, and patients without any anti-HLA antibodies was identical.

Antibodies against both HLA classes (I and II) cause graft injury. Nevertheless, anti-HLA I antibodies seem to be more injurious than anti-HLA II antibodies. A possible explanation is that HLA I is highly expressed in the vasculature. On the other hand, HLA II is only expressed in capillary ECs and, surprisingly, the only HLA class in coronary artery (*99*). Only a few studies demonstrated a correlation of anti-HLA II antibodies to AMR (*97, 100*).

Both, IgG and IgM anti-HLA antibodies are clinically relevant, as the effects may occur early and late after transplantation.

Other Antigens

Beyond ABO blood group antigens or HLAs, other polymorphic surface molecules have to be considered as targets for AMR. However, only few other antigens were described up to now.

Activating antibodies against the polymorphic AT_1 were found in serum of 16 kidney-transplanted patients who were suffering from malignant hypertension and who had no anti-HLA I antibodies (*28*). These antibodies were shown to bind to two different epitopes on the secondary extracellular loops of AT_1. Activation of AT_1 might lead to vasoconstriction following activation of the renin-angiotensin system.

Another polymorphic protein expressed on ECs is MICA (MHC polypeptide-related sequence A). Because of the similarity to MHC proteins, it is thought that MICA binds peptides or short ligands. In a study of 44 cardiac transplanted patients, the occurrence of MICA antibodies was determined (*101*). MICA antibodies were found in significantly higher amount in patients with severe acute rejection than in those without rejection (60.7% vs. 14.3%). The production of MICA antibodies proceeded after acute rejection. Two other studies demonstrated a worse graft survival in patients having HLA and/or MICA antibodies in the sera (*102, 103*).

As it was hypothesized that only polymorphic antigens contribute to AMR, it was surprising that antibodies against autologous proteins may be also involved in AMR. The intermediate filament protein vimentin was described as a possible contributor of AMR. Under normal conditions, vimentin is located intracellularly in ECs and is therefore hidden from the immune system. Following surgery or EC injury following chronic rejection, vimentin can become exposed to the immune system (*104*). This newly exposed self-antigen may evoke the

development of anti-vimentin antibodies, which might contribute to further graft damage. Azimzadeh and colleagues investigated humoral immunity to autologous vimentin following cardiac transplantation in nonhuman primates (*105*). Anti-vimentin antibodies (IgM and IgG) were detected in sera of primates after heart transplantation, and complement deposition on the endothelium was detected in rejected hearts.

4.2 Immunosuppression

After successful organ transplantation, the immune system starts to react against the graft. To inhibit immune responses and to protect the graft from rejection, immunosuppressive drugs are used. These drugs can be divided into 5 groups according to their mechanisms: Calcineurin inhibitors, mTOR inhibitors (proliferation signal inhibitors (PSI)), metabolic toxins, antibodies or anti-inflammatory agents (Table 4.2-1).

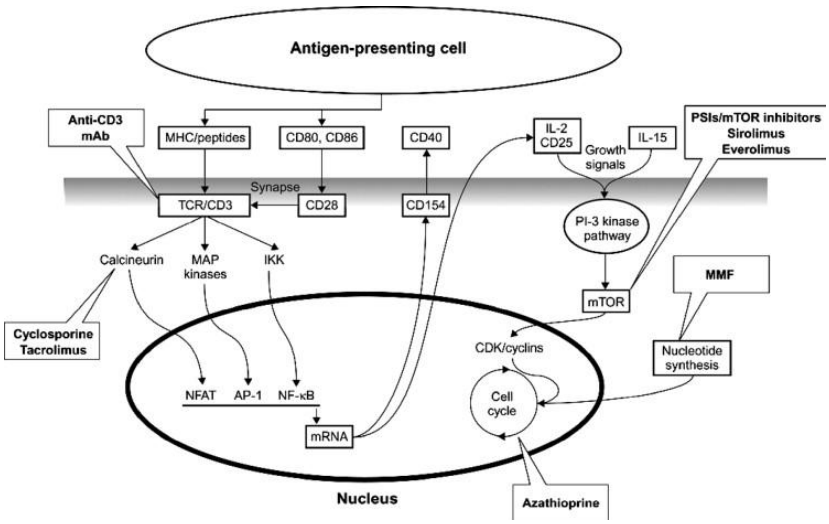


Figure 4.2-1: Schematic mode of action of the different immunosuppressive drugs (reprinted from (*106*)). MHC, major histocompatibility complex; TCR, T-cell antigen receptor; MAP, mitogen-activated protein; IKK, Ikkinase; NFAT, nuclear factor of activated T cells; AP-1, activated protein 1; NF-κB, nuclear factor κB; PI-3, phosphatidylinositol 3-phosphate; mTOR, mammalian target of rapamycin; PSI, proliferation signal inhibitor; MMF, mycophenolate mofetil.

The main function of all immunosuppressive drugs is to prevent the activation of the immune system (Figure 4.2-1). Antibodies eliminate immune cells (*107-110*).The metabolic toxins, calcineurin inhibitors and mTOR inhibitors (PSI) block the activation of T cell proliferation (*111, 112*, *113-115*). In contrast to calcineurin and mTOR inhibitors, metabolic toxins circumvent the production of purin, which is necessary for cell proliferation and metabolism. Mostly a mixture of different immunosuppressive drugs with different mechanisms is

administered to the patients (*106*). This enables to control the immune response at different stages and minimizes side effects, as single drugs can be replaced with others.

Table 4.2-1: Immunosuppressive Drugs

Drug	Target	Literature
Calcineurin Inhibitors		
Cyclosporin	Inhibits calcineurin, which activates nuclear factor of activated T cells (NFAT)	(*111*)
Tacrolimus (FK-506)	Inhibits calcineurin, which activates nuclear factor of activated T cells (NFAT)	(*111, 112*)
mTOR Inhibitors (Proliferation Signal Inhibitors)		
Sirolimus / Rapamycin	Binds to FK binding protein 12 (FKBP12) and inhibits mTOR, which controls cell cycles; mTOR becomes activated by the growth factor IL-2 that is secreted by activated T cells.	(*106, 111, 112, 116*)
Everolimus	Binds to FKBP12 and inhibits mTOR; acts similar to sirolimus	(*106, 116*)
Metabolic Toxins		
Azathioprine	Purin synthesis inhibitor, inhibits proliferation of T cells	(*117*)
Mycophenolate Mofetil	Inhibits inosine monophosphate dehydrogenase, which controls synthesis of guanine monophosphate in the purin synthesis pathway	(*113*)
Antibodies		
ATG (Anti-thymocyte globulins)		(*107*)
Anti-IL 2 Receptor		(*118*)
Anti-CTLA4		(*109*)
Anti-CD40L		(*108*)
Anti-Inflammatory agents		
Corticosteroids	Class of steroid hormones, involved in immune response and regulation of inflammation	(*119*)

Most common immunosuppressive drugs are the calcineurin inhibitors cyclosporin (CsA) and tacrolimus (FK-506) or the mTOR inhibitors sirolimus (SRL) and everolimus (ERL). Surprisingly, FK-506, SRL and ERL are all macrolides and have similar chemical structure. However, the effect of FK-506 differs from the effects of SRL and ERL (Figure 4.2-2). All three drugs first bind to the FK binding protein 12 (FKBP12). SRL and ERL in complex with FKBP12 inhibit the biological action of mTOR, whereas the FK-506 – FKBP12 complex inhibits calcineurin (*111, 112*).

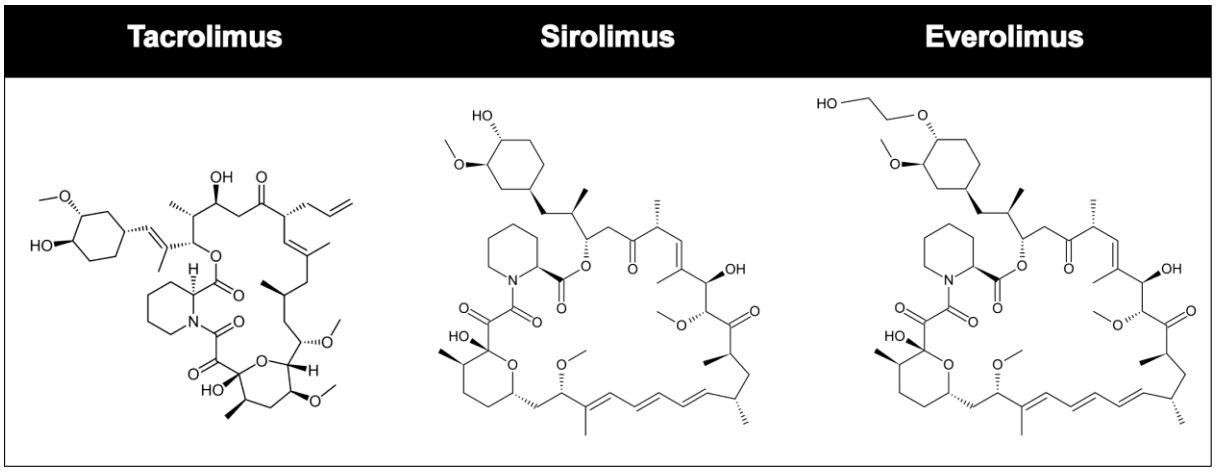


Figure 4.2-2: Chemical structure of tacrolimus, sirolimus and everolimus. All three chemicals are macrolides. Everolimus and sirolimus only differ by one hydroxyl group at C(40) of sirolimus (*112*).

4.3 Endothelial Cells – The Barrier Function

The whole vasculature is delimited with ECs forming the endothelium. ECs function as gatekeepers that control the infiltration of blood borne cells or proteins from blood vessels to underlying tissues. The controlled filter capacity of ECs is regulated by a coordinated opening and closing of cell-to-cell contacts (*120-125*).

4.3.1 Cell-to-Cell Junctions

Specialized junctional regions can be observed at the interface between single ECs: the adherens junctions (AJs) and the tight junctions (TJs) (Figure 4.3-1). Generally TJs are located at the more apical side of the cell-to-cell junctions. Desmosome like structure can be found in certain types of ECs, such as the lymphatic system or veins(*126*).

Adhesion proteins bind via their cytoplasmic domain to the cytoskeleton and certain signaling proteins (*124, 127*). Beside the possibility to decrease the expression of adhesion molecules, ECs are able to control the strength of the intercellular adhesion by dynamic alteration of the cytoskeleton (*128*). On the other hand, adhesion molecules are able to act as sensors to translate extracellular signals into the cells or to react to certain extracellular signals (*127, 129, 130*).

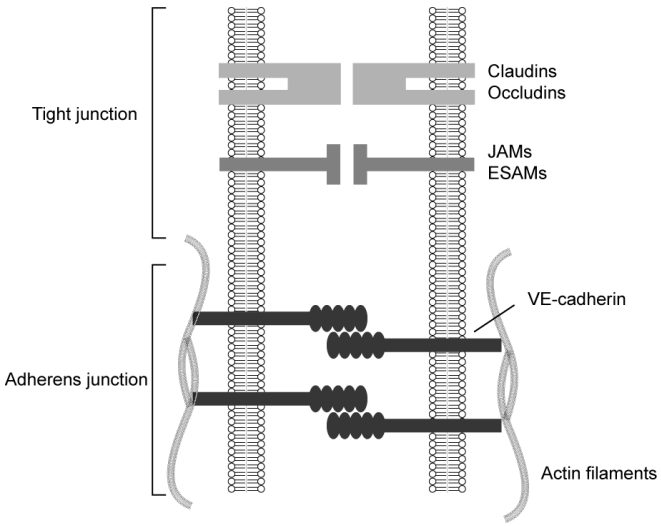


Figure 4.3-1: Cell-to-cell junctions of endothelial cells. Tight junctional adhesion is mediated by occluding, claudins, junctional adhesion molecules (JAM) and endothelial cell selective adhesion molecules (ESAM). AJs are mainly composed of VE-cadherin. Both kinds of junctions are built up by different molecules. Nevertheless, adhesion is formed in a similar way at TJs and AJs: Transmembrane proteins promote homophilic interactions and form a pericellular structure along the border of the ECs (*123*). In contrast to other cell types, ECs express cell specific transmembrane molecules, such as VE-cadherin at AJs (*131*) and claudin-5 at TJs (*132*).

The two types of adhesion junctions are connected to each other, allowing a certain cross-talk between the molecules of the different adhesion junctions. VE-cadherin is able to upregulate the gene activity of the AJ molecule claudin-5 (*129*). This effect is due to a release of the inhibiting forkead box factor FoxO1 and the Tcf-4-β-catenin transcriptional complex by a VE-cadherin dependent phosphorylation of FoxO1.

4.3.2 Endothelial Permeability

The most important function of ECs is the regulation of endothelial permeability. ECs control the passage of blood cells and plasma components to the underlying tissues. Therefore, ECs play an important role in many physiological processes, such as sepsis, inflammation, diabetes and ischemia (*127*). Impaired barrier functions of ECs may lead to severe and even fatal organ dysfunction.

Extracellular factors, such as shear forces or circulating mediators are able to influence endothelial integrity (*133, 134*). On the other hand, ECs are able to actively control its barrier function by intracellular changes. In the following section, only effects that are actively controlled by ECs are described.

Myosin Mediated Cell Contraction

One possibility for the development of endothelial permeability is the contraction of ECs, followed by disruption of AJs (*135*). The whole cell is spanned by an actin cytoskeleton of

actin filaments. The actin filaments are connected to the motor protein myosin. The contraction of myosin results in a contraction of the cells and a disruption of AJs (*136-138*) (Figure 4.3-2):

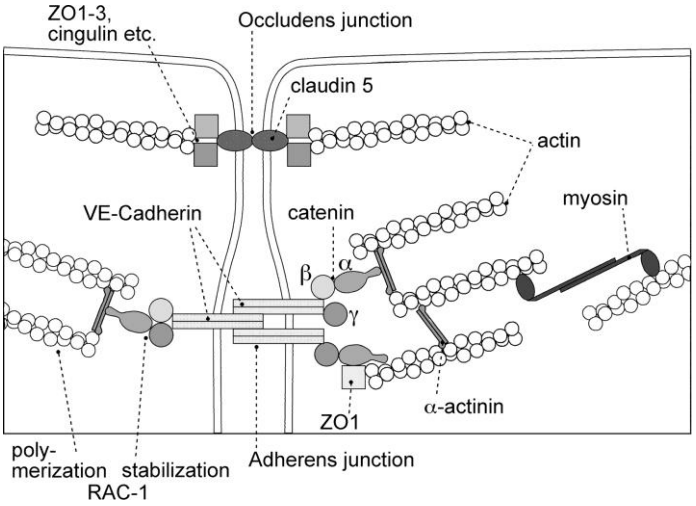


Figure 4.3-2: Contraction of the myosin filament causes contraction of the actin cytoskeleton and the whole cell. Therewith, the adherens junctions are disrupted and ECs loose their integrity (reproduced from (*135*)).

Myosin is responsible for cell contraction of muscle and non-muscle cells and makes the major protein of smooth muscle cells. The myosin complex is composed of one or two heavy chains and several light chains (*139*). Among the different myosin light chains, the most important molecule for myosin mediated cell contraction is the myosin regulatory light chain 2 (MLRC2), which contracts after Ca^{2+} dependent phosphorylation of serine 19 and threonine 18 (*140, 141*). The two amino acids serine 19 and threonine 18 are located in an EF hand motif (helix – turn – helix). After phosphorylation, a structural change in the EF hand occurs that leads to contraction of MLRC2 (*142*).
Myosin dependent endothelial contraction is one possible mechanism responsible for for the formation of paracellular endothelial permeability.

Role of Adherens Junctions

Endothelial permeability can be regulated by a controlled opening and closing of adherens junctions (AJ). The major molecule of adherens junctions is VE-cadherin, which is exclusively expressed in ECs. Many studies demonstrated a strong correlation between VE-cadherin expression and endothelial permeability (*143-151*). The breakthrough of endothelial integrity is often a consequence of reduced AJs, which occurs after intracellular phosphorylation of VE-cadherin and its endocytosis (*146*). Many stimuli are reported to induce phosphorylation of VE-cadherin. These stimuli are summarized in Table 4.3-1:

Table 4.3-1: Stimuli, which affect adhesive function of VE-cadherin

Signal	Mechanism	Reference
Histamine	Increase phosphorylation of VE-cadherin and enhance endothelial permeability	(*152*)
Shear force	Increase phosphorylation of VE-cadherin/catenin complex and increase paracellular electrical resistance of ECs	(*153*)
Thrombin	Enhance endothelial permeability by dissociation of β-catenin, followed by increased VE-cadherin phosphorylation; downregulation of β-catenin	(*154*)
VEGF	Enhanced endothelial permeability by phosphorylation and endocytosis of VE-cadherin	(*146, 147*)

In this thesis, the expression of VE-cadherin was determined as a marker for endothelial permeability. Therefore, the regulation and functions of VE-cadherin are discussed in the next section.

4.4 VE-cadherin

4.4.1 Structure

VE-cadherin (cadherin-5) is a 784 amino acids (135 kD) long glycosilated molecule located at adherens junctions of ECs and a member of the cadherin type II superfamily. In the presence of Ca^{2+}, VE-cadherin mediates a homotypic binding to a neighboring EC (Figure 4.4-1B) by forming a trans-dimer (*155*). The N-terminal extracellular part consists of five domains that are responsible for its binding properties. The transmembrane region is followed by a cytosolic part, which is connected over catenins or plakoglobin to the cytoskeletal protein actin and over desmoplakin to vimentin (Figure 4.4-1C).
VE-cadherin is expressed in high amount along cell borders of confluent ECs at AJs. There are 9 tyrosines at the intracellular part, which are potential phosphorylation sites of tyrosine kinases.

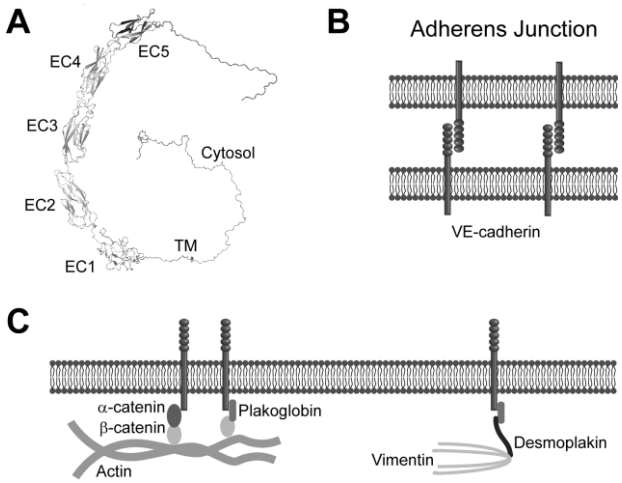


Figure 4.4-1: The predicted structure of VE-cadherin (generated by HHpred; http://toolkit.tuebingen.mpg.de/hhpred) consists of 5 extracellular domains (EC1-5), a transmembrane region (TM) and a cytosolic part (A). The extracellular domains of VE-cadherin bind homotypic to another VE-cadherin of a neighboring cell and form a trans-dimer (B). The cytosolic part is connected to actin over α- and β-catenin or plakoglobin and to vimentin over desmoplakin (C).

4.4.2 Function and Regulation

Cell-to-cell junctions are important in many biological processes, such as cell migration (*156, 157*), angiogenesis (*120, 158*) and tumor metastasis (*159, 160*). As AJs are the first formed cell-to-cell junctions, VE-cadherin becomes a key player in all of these processes (*129*). The expression of VE-cadherin is important to maintain the cell-to-cell junctions and to retain the paracellular permeability (*123*). Several mechanisms have been described how the protein expression of VE-cadherin is regulated. The expression decreases by cytoskeleton-mediated alterations of the VE-cadherin - catenin complex (*144, 161*), hydrostatic pressure (*133*), in the presence of glycation end products (*162*) as well as oxidized low density lipoproteins (oxLDL) (*163*). Intracellular phosphorylation of VE-cadherin after vascular endothelial growth factor (VEGF) or H_2O_2 (*164*) stimulation promotes a release of the VE-cadherin and β-catenin complex (*147, 165*), which initiates the internalization of VE-cadherin into endosomes and its degradation (*146*). Three intracellular tyrosines were identified to be important for the endothelial barrier function. Tyrosine-to-glutamic acid point mutations Y658E and Y731E inhibited the cellular barrier function (*147*) of transfected Chinese hamster ovary cells (CHO). It was demonstrated that purified Src is able to directly phosphorylate a synthetic peptide of the intracellular part of VE-cadherin containing Y685 in an in vitro experiment (*130*).

The extra- and intracellular part of VE-cadherin is connected to several proteins with signaling activity (*166*), which are listed in Table 4.4-1.

Table 4.4-1: VE-cadherin associated signaling molecules

Molecule	Function	Reference
Extracellular binding:		
Fibrin	The natural fragment of fibrin NDSK-II and the fibrin derived peptide (β 15 – 42) binds to VE-cadherin at EC3 and EC4 and affects leukocyte extravasation	(*167, 168*)
Fragment of tRNA-synthase	The antiangiogenic fragment of tryptophanyl tRNA synthetase binds to VE-cadherin	(*169*)
VE-PTP	This receptor type phosphatase associates with VE-cadherin and reduces tyrosine phosphorylation and endothelial permeability	(*170*)
Intracellular binding:		
Csk	Binds to phosphorylated tyrosine 685 and inhibits Src activity	(*143, 171*)
PAR-3	The polarity protein binds by its third PDZ domain to the C-terminus of VE-cadherin	(*172*)
PAR-6	Associates with the C-terminal region of VE-cadherin	(*172*)
RPTP-μ	Binds to VE-cadherin at the cytoplasmic region	
SHP-2	Binds to β-catenin, recruits to VE-cadherin after VEGFR2 activation	(*143, 150*)
VEGFR2	After VEGF stimulation, VEGFR2 associates with VE-cadherin	

An example of VE-cadherin signaling activity is the VE-cadherin dependent activation of endothelial NO synthase (eNOS) after VEGF stimulation of ECs: In the basal state, VE-cadherin is clustered to Src and in a Y685 dependent interaction to C-terminal Src kinase (Csk), which is a negative regulator of Src (*143*). After VEGF stimulation, Csk is released from the complex by the recruitment of protein tyrosine phosphatase SHP2, leading to an increased Src activation (Figure 4.4-2). This activated signaling complex signals further to phosphatidylinositol-3-kinase (PI3K), Akt and eNOS (*173*).

A physiological important signaling function of VE-cadherin is the contact inhibition of cell growth. ECs proliferate in the presence of VEGF (*174*), until they reach a cobblestone-like confluent monolayer. At this point, VE-cadherin clusters together with VEGF receptor type 2 (VEGFR-2) and induces survival instead of proliferation signals (*175*) to the ECs.

VE-cadherin is an important protein of ECs. It is not only responsible for the maintenance of cell-to-cell contacts and the endothelial barrier function; it is also able to transfer extracellular signals into the cytoplasm and providing the possibility to ECs to react on different conditions, such as the previously mentioned contact inhibition of cell growth (*175*).

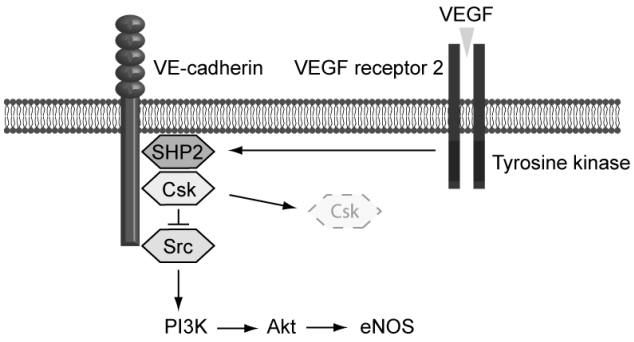


Figure 4.4-2: VE-cadherin dependent signaling. VEGF activates VEGFR2, which recruits SHP2 to VE-cadherin. SHP2 releases Csk, a negative Src regulator, from VE-cadherin, allowing Src to activate several signaling protein, such as PI3K, Akt and eNOS. This VEGF induced activation promotes several cellular functions. (Picture adapted from Ha et al. (*143*)).

4.5 Aim of the Thesis

After organ transplantation, the immune system is activated to react against the graft. Current immunosuppressive drugs, such as mTOR or calcineurin inhibitors, are used to inhibit the activation of the cellular arm of the immune system. Nevertheless, humoral rejection may still occur. Not much is known about effects of anti-graft antibodies after transplantation. Criteria for the diagnostic of humoral rejection were published recently (*59*). One criterion is the formation of interstitial edema. Edema were also reported to occur in patients after mTOR inhibitor administration (*176*).

Several mechanisms may lead to the formation of edema in patients. In this thesis, we investigated the effects of anti-HLA I antibodies and mTOR inhibitors on endothelial permeability. The expression of VE-cadherin was examined as a possible reason for increased endothelial permeability in vitro.

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5 RESULTS

Articles

5.1 Anti-HLA I Antibodies Induce VEGF Production by Endothelial Cells, which Increases Proliferation and Paracellular Permeability

Michael Bieri[1], Melinda Oroszlan[1], Nathalie Ligeti[1], Jürg Bieri[2], Paul Mohacsi[1]*

[1]Laboratory of Heart Transplantation Immunology, Swiss Cardiovascular Center, Inselspital, University of Bern, 3010 Bern, Switzerland, [2]Department of Gynecology, Lindenhof Hospital, 3001 Bern, Switzerland

ABSTRACT

Anti-human leukocyte antigen class I (HLA I) antibodies were shown to activate several protein kinases in endothelial cells (EC), which induces proliferation and cell survival. An important phenomenon in antibody mediated rejection is the occurrence of interstitial edema. We investigated the effect of anti-HLA I antibodies on endothelial proliferation and permeability, as one possible underlying mechanism of edema formation. HLA I antibodies increased the permeability of ECs. Anti-HLA I antibodies induced the production of vascular endothelial growth factor (VEGF) by ECs, which activated VEGF receptor 2 (VEGFR2) in an autocrine manner. Activated VEGFR2 led to a c-Src dependent phosphorylation of vascular endothelial (VE) -cadherin and its degradation. Aberrant VE-cadherin expression resulted in impaired adherens junctions, which might lead to increased endothelial permeability. This effect was only observed after cross-linking of HLA I molecules by intact antibodies. Furthermore, our results suggest that increased endothelial proliferation following anti-HLA I treatment occurs via autocrine VEGFR2 activation. Our data indicate the ability of anti-HLA I to induce VEGF production in ECs. Transactivation of VEGFR2 leads to increased EC proliferation and paracellular permeability. The autocrine effect of VEGF on endothelial permeability might be an explanation for the formation of interstitial edema after transplantation.

Submitted

INTRODUCTION

The process of cellular rejection is well studied and can be controlled adequately by current immunosuppressive drugs. Not much is known about the mechanisms of preformed or newly formed anti-graft antibodies. One of the biggest tasks in the research of antibody-mediated rejection (AMR) is the sensibility of methods to detect antibodies in serum (1, 2).

Several clinical studies demonstrated the importance of preformed ABO antibodies in ABO incompatible transplantations (3-6), which activate the complement system and immediately cause tissue injury. Other studies correlated the presence of antibodies against the human leukocyte antigens (HLA) in cardiac and renal transplantation to bad long-term survival of the grafts (7-10). These effects were mostly mediated by the deposition of complement split products to the grafts' endothelium with the consequences such as graft arteriosclerosis (11). Nevertheless, antibodies are also able to act agonistic or antagonistic to polymorphic surface receptors and initiate different biochemical and pathophysiological functions in endothelial cells (EC). Antibodies against the angiotensin II type 1 receptor were linked with chronic AMR in the absence of anti-HLA antibodies (12). These antibodies led to an increase of graft vasculopathy by the activation of the renin-angiotensin system. Antibodies against HLA I were shown to activate several protein kinases in ECs, such as phosphatidylinositol 3-kinase (PI3K), Akt and Src (13-17). This effect leads to increased EC proliferation and cell survival as possible causes of vasculopathy and/or chronic rejection.

New criteria for AMR were published in a companion paper (18) to the revised working formulation for the standardization of the nomenclature in the diagnosis of heart rejection (19). Endothelial swelling and interstitial edema are mentioned as characteristics for AMR.

In the present study, we focused on complement independent effects of anti-HLA I on endothelial permeability as a possible underlying mechanism of edema formation. VE-cadherin is expressed at adherens junctions on ECs and performs homophilic binding to neighboring ECs. This molecule plays an important role in maintaining the endothelial barrier function. Several studies demonstrated the correlation of VE-cadherin expression with endothelial permeability (20-24). Here, we demonstrate that ECs treated with anti-HLA I initiate the production of VEGF. Autocrine VEGF receptor type 2 (VEGFR2) activation induces endothelial proliferation and paracellular permeability by VE-cadherin phosphorylation.

EXPERIMENTAL PROCEDURE

Reagents

Anti-VE-cadherin (C19), anti-HLA I (W6/32), p-Tyr (PY99) antibodies and Protein G PLUS-agarose Immunoprecipitation Reagent were purchased from Santa Cruz Biotechnology Inc., Switzerland; anti-phospho-(tyr) p85 PI3K and anti-phospho-Akt were purchased from Cell Signaling Technology (LabForce AG, Switzerland); rabbit anti-total actin (C11) antibody was purchased from Sigma and horseradish peroxidase (HRP) labeled secondary antibodies, goat anti-rabbit-HRP, goat anti-mouse-HRP and donkey anti-goat-HRP, were purchased from Pierce Biotechnology Inc., USA. The pharmacological inhibitors sirolimus, LY429002, SU6676 and SU4312 and the VEGF neutralizing antibody (clone 26503) were purchased from Sigma-Aldrich. Primers were obtained from TIB Molbiol, Germany.

Cell Culture

All experiments were performed on freshly isolated human umbilical vein endothelial cells (HUVEC) obtained from a regional hospital. Patients signed an informed-consent letter which was approved by the ethical commission of Bern (KEK).
Cells were isolated following the protocol of Jaffe *et al.*(25). Briefly, cells were released from the umbilical veins by collagenase (Sigma Chemical Co., St. Louis, USA), seeded on 1% gelatin- (Sigma) coated flasks and cultured in medium M199 (Gibco/Life Technologies Inc., Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS, BioConcept, Allschwil, Switzerland), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 7.5 IU/ml heparin (Sigma), 2 ng/ml epidermal growth factor (EGF) (Sigma) and 250 pg/ml β-endothelial cell growth factor (β-ECGF) (Sigma), referred to complete medium. Cells were only used at passage 2 to 4.

Wound healing assay

HUVECs were cultured to confluency in starving medium (M199 supplemented with 2% heat inactivated FCS) on a 6 well culture plate. Three scratches using a 200 µl pipette tip were performed in each well. Medium was refreshed and the anti-HLA I antibody was added in different concentration as indicated in the respective experiments. Inhibitors were added 2 h before treatment. Six pictures were taken from each well after 0, 1, 3, 6, 12 and 24 h using an optical microscope (Leica Diavert) and a Nikon coolpix digital camera. Cell growth was measured by the relative scratch area, which was quantified by software (ImageJ, www.nih.org).

Endothelial permeability

ECs were cultured on 1% gelatin-coated transwell filters (Becton Dickinson, 0.4 µm pore size) inserted on 12-well plates to confluency and cultured overnight in starving medium (M199 supplemented with 2% heat inactivated FCS). Afterwards, cells were cultured for 24 h in the presence of different dilutions of anti-HLA I antibody. Subsequently, 20 µg/ml FITC-dextran (4 kDa, Sigma) was added to the culture medium in the upper chamber and the flux of FITC-dextran was measured after 30 min in the lower chamber by a TECAN microplate reader (excitation: 495 nm, emission: 520 nm).

Immunofluorescent staining

HUVECs were cultured on 1% gelatin-coated chamber slides in the presence of 10 µg/ml anti-HLA I for either 30 min or 24 h. Afterwards, the cells were fixed with an 1:1 mixture (v/v) of acetone-methanol and permeabilized (100 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 200 mM sucrose, 10 mM Hepes and 0.2% Triton-X, pH 7.1). Cells were incubated with the primary antibody anti-VE-cadherin (1:200 in PBS containing 1% bovine serum albumin (BSA)) overnight at 4°C. For visualization, Alexa 488 (Molecular Probes) conjugated to polyclonal donkey anti-goat antibody was used. For nuclear counterstaining, the fixed cells were incubated for 15 min with Hoechst 33342 (Invitrogen) diluted in PBS (1:10000). Pictures were taken by fluorescence microscopy (Leica DM-RB and Olympus DP10 camera).

Fab preparation

The anti-HLA I antibody was digested by immobilized papain (ImmunoPure Fab Preparation kit, Pierce, Switzerland), which was previously equilibrated in digestion buffer (42 mg cystein-HCl in 12 ml Phosphate Buffer, both from Pierce) overnight at 37°C. The crude digest was separated from the immobilized papain and filled into a new tube. In order to optimize fragment recovery, immobilized papain was washed with binding buffer (ImmunoPure IgG Binding Buffer, Pierce) and collected. The pooled supernatants were loaded on an AffinityPeak Protein A Column (Pierce) and antigen binding fragments (Fab) were eluted by binding buffer. Crystallizable fragments (Fc) were released from the column by elution buffer. The Fab and Fc collections were concentrated on 10 kD filters (Vivaspin 6, Milian) to 150 µl. The concentration of the Fab and Fc solutions were determined by Nanodrop (ND-1000, Witec AG, Switzerland).

Immunoprecipitation

Cells were cultured overnight in starving medium and treated for a defined period with the anti-HLA I antibody. ECs were washed 2x with cold TBS (150 mM NaCl, 20 mM Tris-HCl in H_2O, 2 mM $CaCl_2$, pH 7.5) and lysed in a mild lysis buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8, 2 mM $CaCl_2$, 1% Triton-X-100, 1% Protease Inhibitor Cocktail and 1% Phosphatase Inhibitor Cocktail 2, both from Sigma) for 10 min on ice. Supernatants were precleared with empty protein G coated agarose beads (20 µl, Protein G PLUS-Agarose Immunoprecipitation Reagent, Santa Cruz, Switzerland) for 2 h at room temperature (RT). Afterwards, the supernatants were incubated with anti-VE-cadherin antibody (2 µg) for 4 h at 4°C, followed by the addition of protein G coated agarose beads (20 µl) and incubated overnight at 4°C. Beads were washed 3x with TBS completed with 2 mM $CaCl_2$. To release the proteins from the agarose beads, samples were boiled in the presence of 20 µl 2x Laemmli Buffer (Biorad, Switzerland) at 95°C for 5 min.

Immunoblot

Cells were washed 2x with cold PBS and lysed for 10 min on ice as mentioned above (Immunoprecipitation). Cell debris were spun down for 1 min at 10'000 x g. Total protein concentration was measured by the Bradford assay (Biorad, Switzerland) and 20 µg of protein was loaded on a PAGEr® GOLD Precast gradient gel (4 – 20%, Cambrex, Rockland, ME USA). After gel electrophoresis, proteins were blotted onto a nitrocellulose membrane (Protran®, Schleicher & Schuell, Germany, BA85) and the membrane was blocked for 30 min in 5% TopBlock (VWR International AG, Switzerland). Immunoblot was performed for VE-cadherin, phospho-tyrosine and actin as the loading control. Signals were detected using HRP labeled antibodies and photoreaction was started using the Super Signal Dura West kit (Pierce) and captured by Chemidoc (Biorad). The pictures were analyzed by densitometry (QuantityOne, Biorad) and actin was used as the loading control.

RNA isolation and quantitative RT-PCR

RNA was isolated using the RNeasy mini kit (Quiagen, Switzerland) according to the manufacturer's instructions. Briefly, confluent cells in 12 well culture plates were lysed by 350 µl Buffer RLT and vortexed briefly. After addition of 350 µl 70% EtOH, mixture was placed into the spin columns and washed with Buffer RW1 and 2 x with Buffer RLT. RNA was eluted with 30 µl RNase free H_2O and yield was quantified by Nanodrop. cDNA was synthesized using the SuperScript III First-Strand Synthesis Super Mix for qRT-PCR kit (Invitrogen, Switzerland) according to the standard protocol. Briefly, 150 ng of RNA were mixed together

with 10 µl of 2x reaction and 2 µl RT enzyme mix and brought to a final volume of 20 µl with diethylpyrocarbonate (DEPC) treated water. The reverse transcription was performed at 50°C for 30 min and terminated for 5 min at 85°C. The RNA was digested by 1 µl *E. coli* RNase H for 20 min at 37°C.

Real time PCR was performed in an iCycler (Biorad) using the SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), including hot-start Taq DNA polymerase and uracil DNA glycosylase (UDG). Primers were designed of sequences obtained from the National Centre for Biotechnology Information (NCBI) nucleotide database for VE-cadherin (**NM_001795**, forward: 5'-CCTACCAGCCCAAAGTGTGT-3', backward: 5'- GACTTGGCATCCCATTGTCT-3'), gamma actin (**NM_001614**, forward: 5'-TCTGTGGCTTGGTGAGTCTG-3', backward: 5'-AGTAACAGCCCACGGTGTTC-3') and VEGF (**M32977**, forward: 5'-CCCACTGAGGAGTCCAACAT-3', backward: 5'-AAATGCTTTCTCCGCTCTGA-3'). After hot-start, 40 cycles at 62°C for 1 min (annealing and elongation) and 95°C for 15 sec (denaturation) were performed. Primers were used in 200 nM concentrations in a total reaction volume of 30 µl. Relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method against the actin gene.

Statistical Analysis

All experiments were performed in triplicates (from 3 different donors). Statistical significance was calculated using Prism 4 (GraphPad) by the two-way ANOVA or the Man-Whitney U-Test, if indicated. $P < 0.05$ was considered as statistically significant. Non-parametric tests were used as normal distribution was not assumed. Means are indicated together with the standard error of the mean (SEM)

RESULTS

Increased endothelial permeability

The occurrence of interstitial edema is a criterion for humoral rejection. Therefore, we examined the paracellular permeability of endothelial cells in the presence of anti-HLA I antibodies without complement. Human umbilical vein endothelial cells (HUVEC) were cultured on transwell filters to confluency and treated with anti-HLA I antibody for 24 h in different concentrations. Subsequently, the flux of 4 kD FITC-dextran within 30 min was measured as a marker of paracellular permeability. Endothelial permeability was shown to be increased dose-dependently in the presence of anti-HLA I antibodies (**Figure 1**). A concentration of 1 µg/ml of anti-HLA I antibodies increased the permeability about 30% (P = 0.04) and 10 µg/ml increased about 60% (P = 0.004), compared to the control.

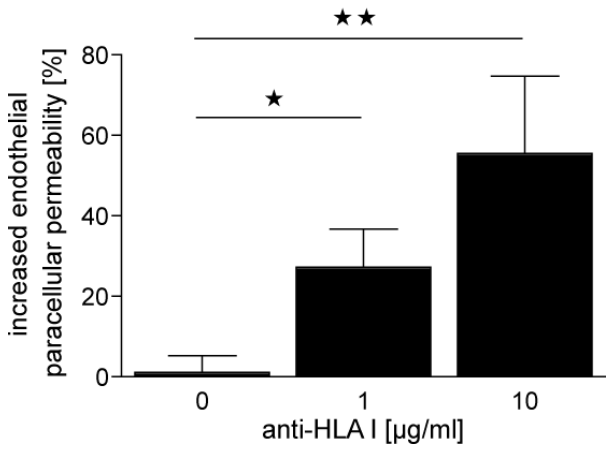

Figure 1: Anti-HLA I antibodies increase endothelial permeability. HUVECs were cultured to confluency on transwell filters. After 24 h incubation with anti-HLA I antibodies in different concentrations, the flux of 4 kD FITC-labeled dextran from the upper to the lower chamber was measured. Anti-HLA I antibodies dose-dependently increased paracellular permeability of endothelial cells. In a concentration of 1 µg/ml anti-HLA I antibody, the permeability increased 27% (P = 0.04) and in a concentration of 10 µg/ml 55% (P = 0.004). Statistical significance was calculated by the Man-Whitney U-Test. Data are represented as mean +/- SEM.

Decreased VE-cadherin expression

We determined the expression level of VE-cadherin, which is expressed at adherens junctions of ECs and shows strong correlation with endothelial permeability. Stimulation of confluent ECs with anti-HLA I antibodies for 30 min already resulted in a dose-dependent decrease of VE-cadherin expression (**Figure 2A**). A concentration of 0.1 µg/ml of anti-HLA I antibodies decreased the expression to 90% (P = 0.04), 1 µg/ml to 80% (P = 0.02) and 10

µg/ml to 75% (P = 0.01) compared to the control. A prolonged incubation period of 24 h further decreased the VE-cadherin expression (to 70% after 1 µg/ml and to 55% after 10 µg/ml anti-HLA I stimulation, **Figure 2B**). To investigate which part of the antibody was responsible for these effects, anti-HLA I antibodies were digested by Papain to generate an Fc and two Fab fragments. Binding properties of anti-HLA I Fab fragments to HLA I on HUVECs were confirmed by dot blots (data not shown). Neither anti-HLA I Fc nor anti-HLA I Fab fragments showed any effect on the expression of VE-cadherin after 24 h incubation (**Figures 2C+D**). Only entire anti-HLA I antibodies were able to decrease the expression of VE-cadherin. A recent study demonstrated the involvement of c-Src in VE-cadherin expression and endothelial permeability (26). By blocking c-Src with the pharmacological inhibitor SU6656 (10 µM), the anti-HLA I induced decrease of VE-cadherin after 24 h incubation time was completely inhibited in our study (**Figure 2E**). Blocking VEGFR2 by the pharmacological inhibitor SU4312 prevented anti-HLA I mediated decrease of VE-cadherin expression after 24 h stimulation (**Figure 2F**).

In immunofluorescent pictures, VE-cadherin is expressed continuously at the borders of ECs in untreated cells. In the presence of 10 µg/ml anti-HLA I antibodies for 30 min, the expression of VE-cadherin decreased and the expression pattern changed to a dotted structure (**Figure 3A**). This effect further progressed after 24 h of anti-HLA I stimulation, where VE-cadherin expression was strongly decreased and expressed discontinuously along the cell borders. Analysis of the green particles (pixels) in the immunofluorescent pictures revealed a shift to darker green after treatment, which qualitatively represents the decreased VE-cadherin expression (**Figures 3B+C**).

To understand more about the mechanism of how VE-cadherin expression is regulated, RT-PCR experiments were performed. After 24 h incubation of the ECs with anti-HLA I antibody in different concentrations, only the mRNA expression of the ECs treated with 10 µg/ml anti-HLA I was slightly decreased (data not shown). This decrease is not proportional to the decrease of the protein expression, which may indicate the involvement of posttranslational modifications of the VE-cadherin protein.

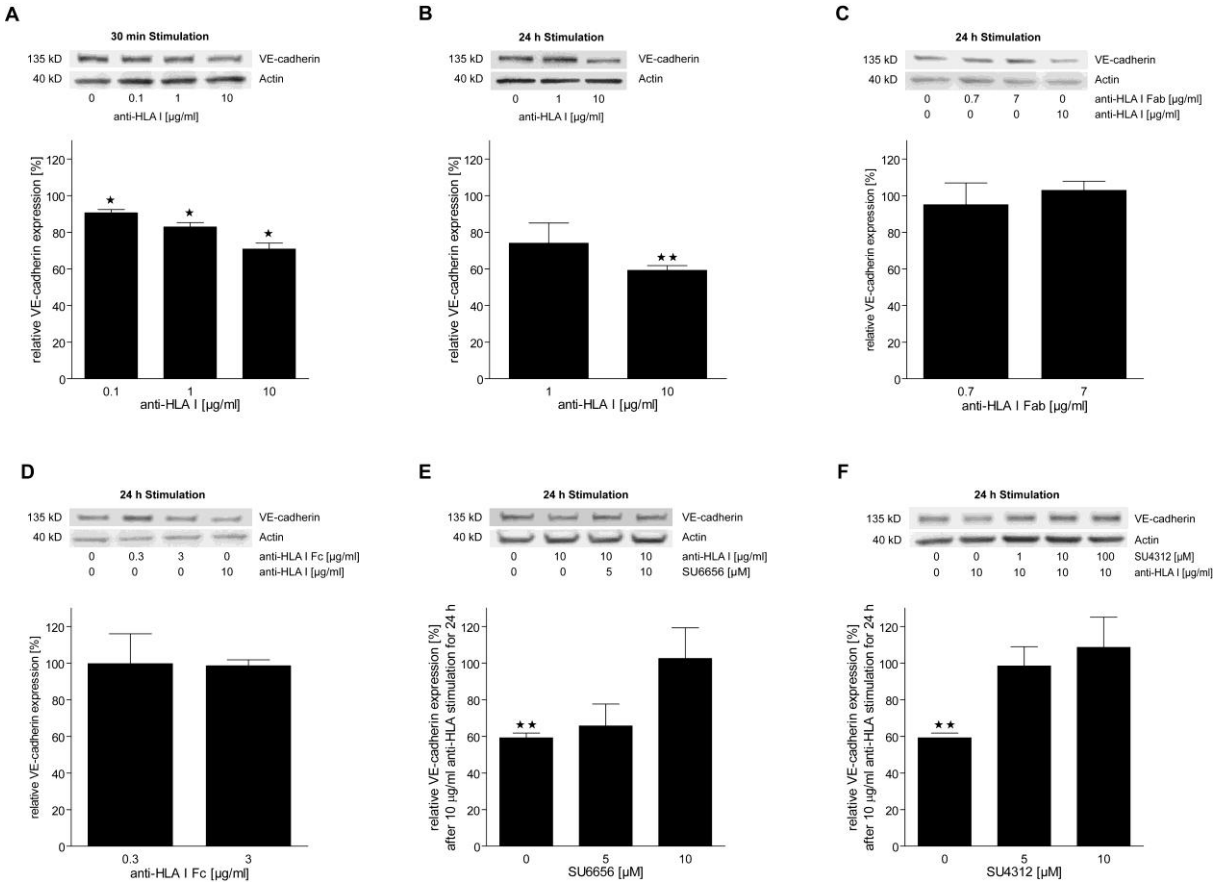

Figure 2: Protein expression of VE-cadherin after anti-HLA I stimulation. ECs were cultured to confluency and starved overnight. The protein expression was determined by Western blot after anti-HLA I treatment for different time-points and conditions. Anti-HLA I antibodies decreased the expression of VE-cadherin after 30 min stimulation to 91% (P = 0.04) in a concentration of 0.1 µg/ml, to 83% (P = 0.02) in 1 µg/ml and to 71 (P = 0.01) in 10 µg/ml anti-HLA I concentration compared to untreated cells (**A**). A prolonged incubation period for 24 h decreased the VE-cadherin expression further (**B**). In a concentration of 1 µg/ml anti-HLA I, the VE-cadherin expression decreased to 74% and to 59% (P = 0.004) after 10 µg/ml anti-HLA I stimulation for 24 h compared to the controls. The anti-HLA I antibody was digested by papain to generate two Fab and an Fc fragment. Neither the Fc nor the Fab fragments had any effect on the VE-cadherin expression after 24 h stimulation (**C**+**D**). By blocking Src by SU6656, the effect of the anti-HLA I antibody on VE-cadherin after 24 h was inhibited dose-dependently. SU6656 in a concentration of 10 µM neutralized the effect of anti-HLA I completely (**E**). After inhibition of VEGFR by the pharmacological inhibitor SU4312, the decrease of VE-cadherin after anti-HLA I stimulation for 24 h was blocked (**F**). Data are represented as mean +/- SEM.

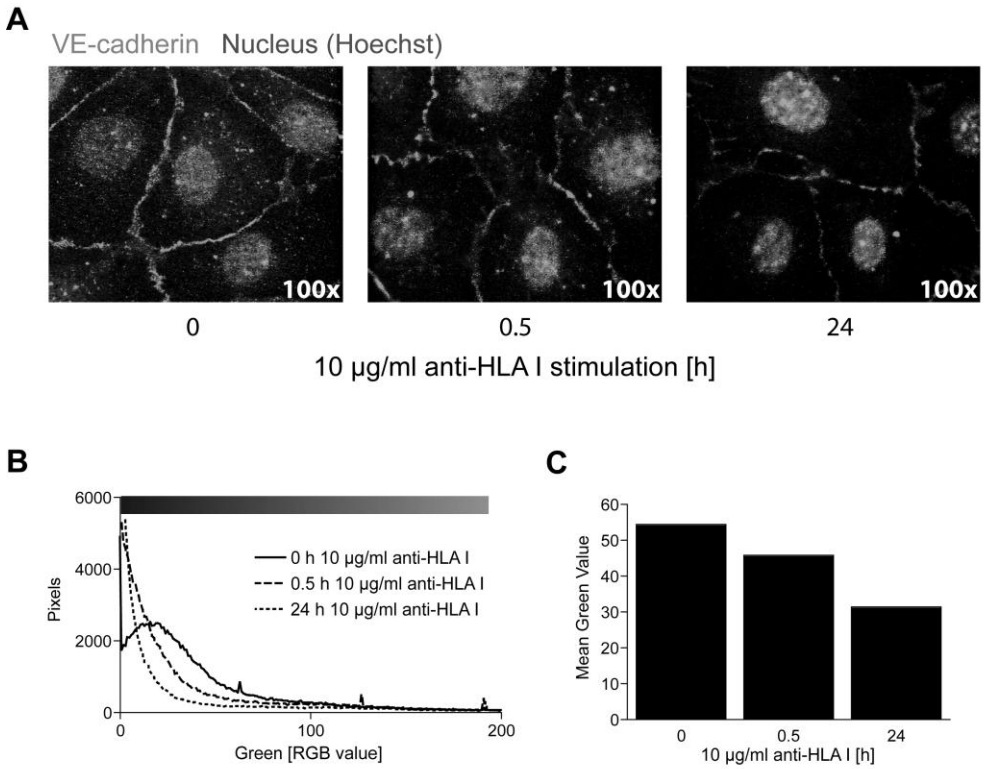

Figure 3: Immunofluorescent staining for VE-cadherin. HUVECs were cultured to confluency on chamber slides. VE-cadherin (green) and the nucleus (blue) were stained. The expression of VE-cadherin decreased after 30 min anti-HLA I stimulation (**A**). After 24 h anti-HLA I stimulation, VE-cadherin was only stained slightly and in a dotted pattern. Green pixels of the representative picture (**A**) were analyzed by the software ImageJ (**B**+**C**). The values of green decreased in a time dependent manner.

Phosphorylation of VE-cadherin after anti-HLA I stimulation

A possible posttranslational modification of VE-cadherin that initiates its degradation is the phosphorylation of intracellular tyrosines of the molecule (21). Therefore, VE-cadherin was immunoprecipitated and immunoblotted against phospho-tyrosine. ECs cultured in the presence of 10 µg/ml anti-HLA I for 30 min and 24 h demonstrated a higher tyrosine phosphorylation grade compared to the untreated cells, as visualized by Western blots (**Figures 4A+B**). The phosphorylation of VE-cadherin by anti-HLA I stimulation was inhibited after blocking VEGFR2 with 100 µM SU4312.

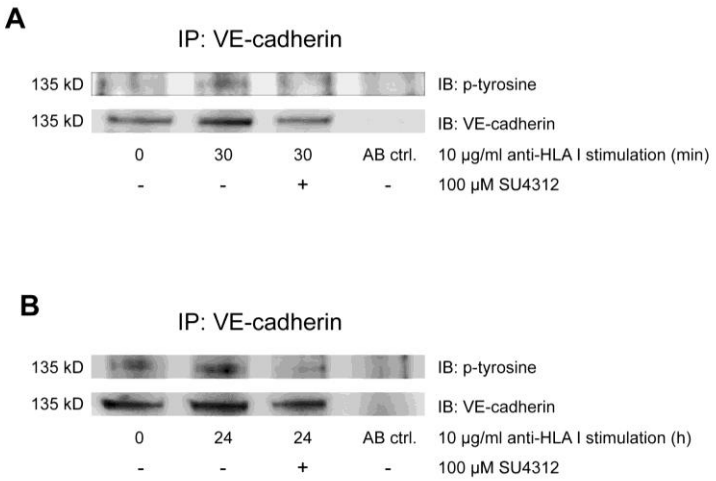

Figure 4: Phosphorylation of VE-cadherin after anti-HLA I stimulation. HUVECs were cultured to confluency and starved overnight. After anti-HLA I treatment, cell lysates were incubated with anti-VE-cadherin antibodies and captured with Protein G beads. After gel-electrophoresis, immunoprecipitated (IP) VE-cadherin was immunoblotted (IB) for phospho-tyrosine and VE-cadherin. VE-cadherin was higher phosphorylated after 30 min (**A**) and 24 h (**B**) anti-HLA I stimulation. The phosphorylation was inhibited by blocking VEGFR2 with 100 µM SU4312.

Anti-HLA I antibodies induce the production of VEGF

To examine the correlation of HLA I activation with the activity of VEGFR2, the supernatant of the cell cultures was tested for the presence of VEGF by ELISA. No VEGF was measured in the supernatant, perhaps as a consequence of an autocrine activation/absorption by the ECs (data not shown). Therefore, the production of VEGF after anti-HLA I stimulation was measured by RT-PCR experiments (**Figure 5A**). The mRNA level of VEGF increased up to 5-fold after 24 h anti-HLA I stimulation (10 µg/ml). Blocking PI3K by 20 µM LY294002 or mTOR by 100 nM Sirolimus (SRL, rapamycin) inhibited the anti-HLA I induced (stimulated for 24 h) VEGF production, whereas blocking of c-Src (by 10 µM SU6656) did not show an inhibition of VEGF production (**Figure 5B**). Nevertheless, the autocrine action of VEGF on ECs and its secretion was still not confirmed. To inhibit the biological function of VEGF, VEGF neutralizing antibodies were added to the culture medium to absorb newly synthesized VEGF. Neutralizing VEGF antibodies were able to prevent the anti-HLA I induced decrease of VE-cadherin after 24 h stimulation in a dose-dependent manner (**Figure 5C**).

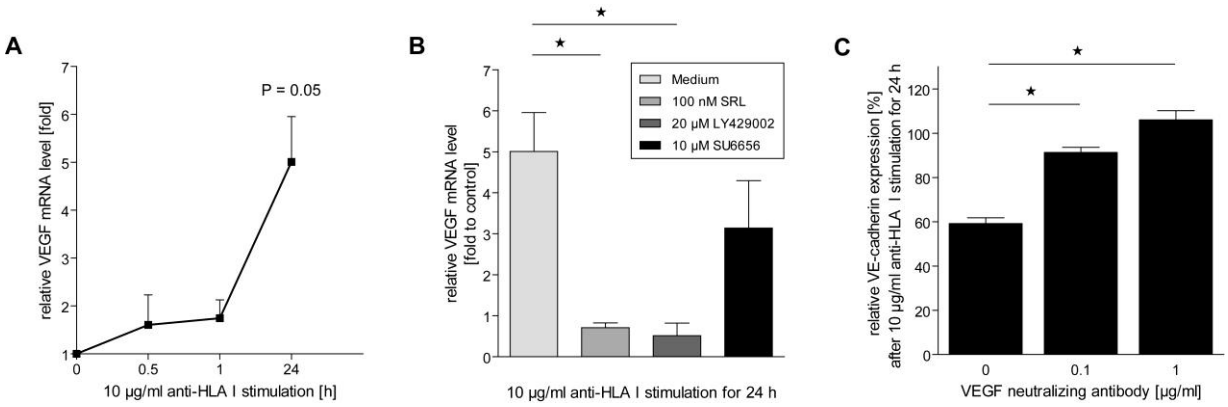

Figure 5: VEGF production and action on ECs after anti-HLA I stimulation. Confluent HUVECs were stimulated by anti-HLA I antibodies at different time points and in different conditions. Afterwards, cDNA was synthesized of isolated RNA and VEGF cDNA was amplified. The relative expression level of VEGF mRNA was evaluated by the $2^{-\Delta\Delta CT}$ method. The VEGF mRNA level increased time-dependently after anti-HLA I stimulation (**A**). This effect was inhibited by blocking PI3K (20 µM LY429002) and mTOR (100 nM sirolimus) but not after Src (10 µM SU6656) inhibition (**B**). To assess an extracellular action of VEGF, VEGF was neutralized by anti-VEGF antibodies added to the culture medium. HUVEC cell lysates were immunoblotted for VE-cadherin after 10 µg/ml anti-HLA I stimulation for 24 h. The decrease of VE-cadherin after anti-HLA I antibodies was inhibited by the addition of VEGF neutralizing antibodies to the culture medium in a dose-dependent manner (**C**). Statistical significance for VEGF mRNA levels was calculated by the Man-Whitney U-Test. Data are represented as mean +/- SEM.

Increased endothelial proliferation after anti-HLA I stimulation

Chronic antibody mediated rejection can be caused by increased EC proliferation, which may lead to transplant vasculopathy. HUVECs were cultured to confluency in starving medium and scratches were performed by a 200 µl pipette tip. The scratch area was observed under light microscope and quantified by the ImageJ software. Stimulation of HLA I by the anti-HLA I antibody W6/32 dose-dependently increased endothelial proliferation (**Figures 6A+E**). To determine, whether this anti-HLA I induced proliferation is dependent on mTOR, Src or VEGFR2, confluent ECs were preincubated with the inhibitors SRL, SU6656 (inhibits Src) or SU4312 (inhibits VEGFR2), respectively. All experiments using inhibitors were quantified against the inhibitor treated samples without antibodies (controls). Anti-HLA I induced proliferation could be inhibited by 100 nM SRL and 10 µM SU4312 (**Figures 6B+C**), whereas the Src inhibitor SU6656 (10 µM) had no influence (**Figure 6D**).

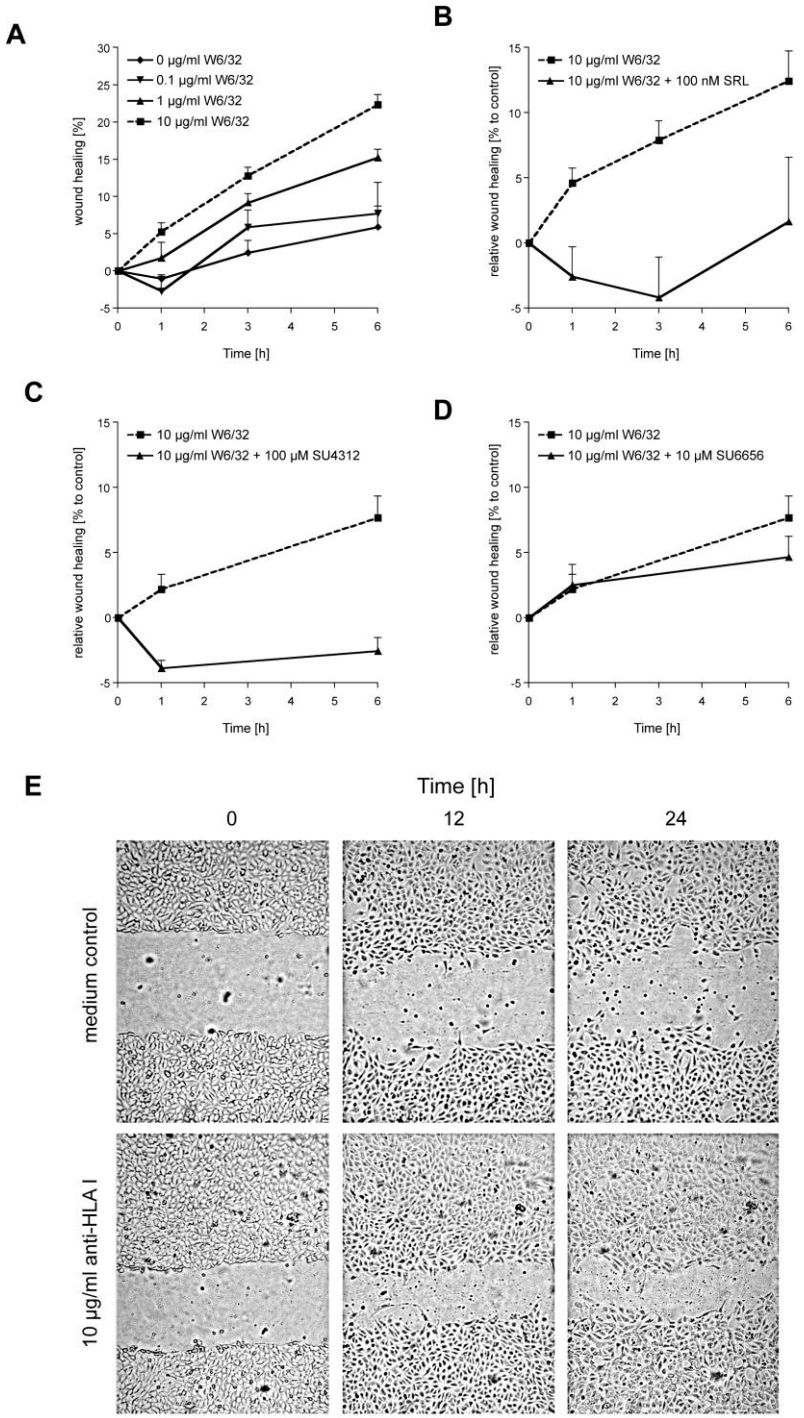

Figure 6: Endothelial proliferation after anti-HLA I stimulation. The proliferation of ECs after anti-HLA I stimulation was determined by the wound healing assay. Into confluent HUVEC monolayers, scratches using a 200 µl pipette tip were performed. The scratch area was measured by the software ImageJ. Anti-HLA I antibodies increased the endothelial proliferation dose-dependently (**A**). The light microscope pictures of the HUVEC monolayer show the time dependent cell growth of anti-HLA I stimulated ECs (**E**). Unstimulated ECs did not proliferate and more dead cells were observed in the supernatant. The anti-HLA I induced proliferation was inhibited by blocking mTOR (100 nM sirolimus, **B**) and VEGFR2 (100 µM SU4312, **C**). Inhibition of Src by 10 µM SU6656 had no effect on anti-HLA I induced proliferation of ECs (**D**). Data are represented as mean +/- SEM.

DISCUSSION

The impact of anti-HLA I antibodies in clinical transplantation is not clarified yet as there is not much known about their effects after transplantation. An essential problem in this issue is the missing clinical data about humoral rejection in patients, as it was not routinely screened for the presence of anti-HLA I serum antibodies or the presence of C4d in biopsies. The main reasons of this lack are the difficulties, how to measure anti-HLA I antibody concentrations in the sera and how to interpret the occurrence of C4d in biopsy specimens. This work attempted to give an insight into the possibility of anti-HLA I antibodies to induce endothelial permeability without the contribution of complement. Therefore, a model anti-HLA I antibody (W6/32) was used, which recognizes the constant α3 region of HLA I (27).

The anti-HLA I antibody W6/32 increased the paracellular permeability of confluent HUVECs (**Figure 1**). The expression of VE-cadherin was investigated as a possible reason for endothelial permeability (20-24). The protein expression of VE-cadherin decreases after HLA I stimulation. (**Figures 2+3**). Only the entire anti-HLA I antibody was able to reduce VE-cadherin expression. Both, the Fab and Fc fragments had no effect on VE-cadherin. Therefore, we assume a need of cross-linking HLA I molecules to initiate a signaling cascade, which is responsible for the VE-cadherin down-regulation.

The mRNA expression of VE-cadherin was not affected after anti-HLA I stimulation of ECs for 24 h. Only slight decrease of the mRNA level was observed in a concentration of 10 µg/ml W6/32 after 24 h stimulation, which was not congruent to the protein expression. This may indicate a post-translational modification of VE-cadherin. A well documented post-translational modification is the phosphorylation of VE-cadherin, which initiates its degradation (20, 21, 24, 26). VE-cadherin phosphorylation was detected by Western blot after treatment with 10 µg/ml anti-HLA I for 30 min (**Figure 4**).

From literature it is known, that phosphorylation may occur after VEGF stimulation of ECs (20, 26). VEGFR2 becomes activated by VEGF and induces the phosphorylation of VE-cadherin in a Src dependent process. In this study, anti-HLA I mediated decrease of VE-cadherin expression was inhibited after blocking VEGFR2 and Src (**Figure 2**). Therefore, an activation of VEGFR2 after anti-HLA I stimulation was assumed. ECs are able to produce VEGF, which may act in an autocrine manner (28). In our experiments, an elevated VEGF mRNA level was measured in cultured ECs after anti-HLA I antibody stimulation (**Figure 5**). The production of VEGF was prevented after inhibition of mTOR and PI3K, but not after Src blocking. This supposes that the signaling responsible for VEGF production is PI3K and mTOR dependent, whereas Src occurs later in the mechanism that reduces VE-cadherin expression. So far, we were only able to show that anti-HLA I antibodies had an effect on the production of VEGF. To prove that newly synthesized VEGF is secreted by the ECs and acts autocrine on VEGFR2, secreted VEGF was captured by VEGF neutralizing antibodies added

to the cell supernatant. By addition of VEGF neutralizing antibodies, the anti-HLA I induced decrease of VE-cadherin was inhibited (**Figure 5C**). An extracellular antibody was able to interrupt the effect of the anti-HLA I antibody on VE-cadherin expression. This finding strengthens the hypothesis that ECs start to produce VEGF in the presence of anti-HLA I antibodies and secret them into the supernatant. There, it activates VEGFR2 and induces phosphorylation and degradation of VE-cadherin.

Similar results were obtained for the anti-HLA I induced cell proliferation. Anti-HLA I antibody was able to stimulate the proliferation of cultured ECs (**Figure 6**). This effect was reverted by blocking mTOR and VEGFR2, whereas Src had no influence on the effect of anti-HLA I antibodies on endothelial proliferation. Proliferation experiments were performed using the wound healing assay, which does not clearly distinguish between cell proliferation and migration. Therefore, we measured the uptake of BrDU (bromodeoxyuridine; synthetic analogue of thymidine) of subconfluent ECs as an alternative proliferation assay (data not shown). Anti-HLA I antibodies increased the uptake of BrDU.

These findings presume the involvement of VEGF and VEGFR2 in the effects described above. PI3K and mTOR are involved in the signaling that initiated the VEGF production. VEGFR2 controls the endothelial proliferation and permeability, whereas Src signaling is only important for paracellular permeability. The anti-HLA I induced endothelial proliferation was only slightly affected by blocking Src, which was similar for the VEGF mRNA production. Blocking mTOR inhibited the elevated VEGF mRNA level and the increase of endothelial proliferation. Therefore, mTOR and PI3K appear earlier in anti-HLA I signaling than VEGFR2. On the other hand, Src had no influence on the VEGF mRNA production after anti-HLA I stimulation, but inhibited VE-cadherin phosphorylation. This indicates that Src signaling occurs after VEGFR2 activation.

Taken together, we hypothesize the following mechanism, which is depicted in **Figure 7**: Cross-linking of HLA I by anti-HLA I antibody triggers the activation of PI3K and mTOR, which initiates the production of VEGF. VEGF might immediately activate VEGFR2 in an autocrine manner. Subsequently, activated VEGFR2 diverges at least into two distinct physiological functions. On the one hand, VEGFR2 initiates the Src-dependent phosphorylation of VE-cadherin that leads to an increase of endothelial permeability. On the other hand, VEGFR2 is supposed to stimulate endothelial proliferation.

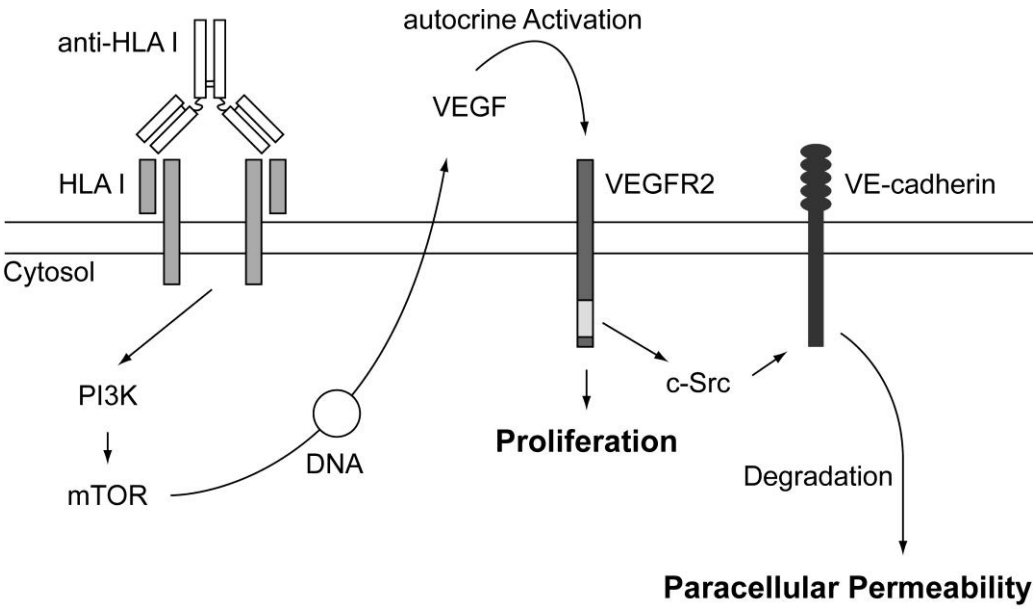

Figure 7: Hypothesized mechanism. We propose that anti-HLA I antibodies cross-link two HLA I molecules. This activates PI3K and mTOR and the production of VEGF, subsequently. ECs secrete the newly synthesized VEGF witch activates VEGR2. Two distinct mechanisms are activated by VEGFR2: On one hand, an Src dependent phosphorylation of VE-cadherin initiates its degradation that might lead to increased endothelial permeability. On the other hand, the proliferation of ECs is stimulated by VEGFR2 in an Src independent manner.

Limitations of the study:

Permeability assays using inhibitors for PI3K, mTOR, VEGFR2 or Src could not be performed, as all these kinases are essential for cell homeostasis. ECs were not able to build a tight monolayer in the presence of the inhibitors used in other experiments. The effects measured in the permeability assay were mediated by the kinase inhibitors (even the controls demonstrated gaps in the EC-monolayer) and are therefore not shown.

Only primary ECs isolated from umbilical veins (HUVEC) were used. Limitations to extend our results to pathophysiologic effects of anti-HLA I antibodies to whole organs are acknowledged. Nevertheless, the results obtained in this study are valid and established in this model. Further studies will demonstrate whether similar mechanisms also occur in other ECs and in vivo.

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5.2 Biosynthesis and Expression of VE-cadherin is Regulated by the PI3K / mTOR Signaling Pathway

Michael Bieri[1], Melinda Oroszlan[1], Christian Zuppinger[2], Paul J. Mohacsi[1]*

[1]Laboratory for Heart Transplantation Immunology and [2]Cardiomyocyte Biology Laboratory, Cardiology, Swiss Cardiovascular Center, University Hospital, 3010 Bern, Switzerland

ABSTRACT

Vascular endothelial (VE)-cadherin is an essential protein of adherens junctions of endothelial cells and plays a pivotal role in vascular homeostasis. Mammalian target of rapamycin complex 2 (mTORC2) deficient mice display defects in fetal vascular development. Blocking mTOR or the upstream kinase phosphoinositide 3-kinase (PI3K) leed to a dose-dependently decrease of the VE-cadherin mRNA and protein expression. Immunofluorescent staining showed a strongly decreased expression of VE-cadherin of confluent human umbilical endothelial cell (HUVECs) cultures. We conclude that VE-cadherin seems to be dependent on mTOR and PI3K signaling.

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INTRODUCTION

Vascular endothelial cadherin (VE-cadherin) is a calcium (Ca^{2+}) dependent adhesion molecule located at adherens junctions (AJ) of vascular endothelial cells (EC) and a member of the cadherin type II superfamily. There are two types of cell-to-cell junctions on ECs: AJs and tight junctions (TJ). AJs are cytogenetically developed earlier than TJs, which also supports the central role of VE-cadherin in endothelial integrity (*1*). VE-cadherin consists of an extracellular part, which is composed of five extracellular domains, a transmembrane region and an intracellular domain, which binds via α-catenin to actin or via desmoplakin to the intermediate filament vimentin (*2*). The extracellular domains mediate a homotypic interaction between ECs by forming a trans-dimer.

Cadherins, briefly, are connected to numerous signaling proteins. An important feedback signaling of VE-cadherin is the contact inhibition of cell growth (*1, 3*). In sub-confluent cells, vascular endothelial growth factor (VEGF) stimulates cells to proliferate until the cells form a cobblestone-like confluent monolayer. In this state, VE-cadherin is clustered together with VEGF receptor type 2 (VEGFR-2). This complex induces survival signals instead of proliferation. The proliferation of ECs is promoted by the activation of the phosphoinositide 3-kinase (PI3K) via the mTOR signaling pathway. VEGF stimulation of confluent ECs leads to endocytosis of the VE-cadherin – VEGFR-2 complex into intracellular vesicles, where VEGFR-2 remains able to signal and stimulate the cells to proliferate (*4*).

The known changes of VE-cadherin expression is mediated by post-translational modifications, including reorganization of the cadherin-catenin complex (*4*), cytoskeleton-mediated alterations (*5*) in this complex or VEGF-induced tyrosine phosphorylation of VE-cadherin (*6*). The latter is thought to control the integrity of AJs.

mTOR complex 2 (mTORC2) deficient mice display defects in fetal vascular development (*7, 8*) and VE-cadherin is able to activate the PI3K / mTOR signaling pathway after stimulation of VEGFR2 (*9*) or by forming a cadherin-cadherin trans-dimer (*10*). These findings indicate a link between the mTOR signaling pathway and VE-cadherin.

The present study focused on biochemical signals involved in the regulation of VE-cadherin expression. By blocking mTOR and PI3K, we observed a decrease in VE-cadherin synthesis on the gene and protein level.

MATERIALS AND METHODS

Reagents

Sirolimus (SRL, Sigma, R0395), Tacrolimus (FK-506, Sigma, F4679) and Wortmannin (Calbiochem, 681675) were dissolved in dimethyl sulfoxide (DMSO, Sigma). Primary antibodies, goat anti-human VE-cadherin (C-19) were purchased from Santa Cruz Biotechnology Inc., while the rabbit anti-total actin (C11) antibody was from Sigma. The horseradish peroxidase (HRP) labeled secondary antibodies, goat anti-rabbit-HRP (1858415) and donkey anti-goat-HRP (31402), were purchased from Pierce Biotechnology Inc., USA. For knockdown experiments, siPort Amine (Applied Biosystems, 4502) and short interference RNA (siRNA, SignalSilence mTOR siRNA, Bioconcept, Switzerland, 6381) were used.

Cell culture

Human umbilical vein endothelial cells (HUVECs) were freshly isolated from umbilical cords obtained from a regional hospital. Patients signed an informed-consent letter, which was approved by the ethical commission of Bern (KEK 102/06).
Cells were isolated following the protocol of Jaffe et al. (*11*). Briefly, cells were digested from the umbilical veins by collagenase (Sigma Chemical Co., St. Louis, USA), seeded on 1% gelatine- (Sigma) coated flasks and cultured in medium M199 (Gibco/Life Technologies Inc., Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS, BioConcept, Allschwil, Switzerland), 100 IU/ml penicillin (Gibco), 100 µg/ml streptomycin (Gibco), 7.5 IU/ml heparin (Sigma), 2 ng/ml epidermal growth factor (EGF) (Sigma) and 250 pg/ml β-endothelial cell growth factor (β-ECGF) (Sigma), referred as complete medium. Cells were only used at passage 2 to 4. In the experimental settings, confluent HUVECs were incubated for 24 h at 37°C and 5% CO_2 in the presence of 10 or 100 nM SRL and FK-506, 1 or 10 nM Wortmannin and properly diluted vehicle, DMSO, as control.

Immunofluorescent staining

HUVECs were cultured on 1% gelatine-coated chamber slides in the presence of 100 nM SRL, 100 nM FK-506, 10 nM Wortmannin or properly diluted DMSO as vehicle controls. After 24 h, cells were fixed with 1:1 mixture (v/v) of acetone-methanol and permeabilized with a buffer containing 100 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 200 mM sucrose, 10 mM HEPES and 0.2% Triton-X (pH 7.1). The primary antibody, anti-VE-cadherin (1:200 in PBS containing 1% bovine serum albumin (BSA)), was incubated overnight at 4°C. For visualization, Alexa

488 (Molecular Probes) conjugated polyclonal donkey anti-goat antibody was used. Pictures were taken by fluorescence microscopy (Leica DM-RB and Olympus DP10 camera).

Transfection and mTOR silencing

To knock down the expression of mTOR, siRNA specific against mTOR were transfected via liposomes using siPort Amine (Applied Biosystems, Switzerland) into HUVECs according to the standard protocol. Briefly, serum free medium for endothelial cells (SFM, Gibco) was mixed with siPort Amine for 10 min at room temperature (RT) on a polystyrene plate. Concomitant, SFM was mixed with siRNA in a final concentration of either 10 or 100 nM for 5 min and combined with the diluted liposomes. Afterwards, the newly formed transfection complexes were added to the cells. The medium was replaced with fresh SFM and the culture continued for 24 h. The gene activity was assayed after 48 hrs. Transfection was performed on subconfluent cells in SFM. For control samples, cells were transfected with empty liposomes (without siRNA).

Western blot analysis

HUVECs were washed twice with ice-cold PBS and lysed with lysis buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl pH 8, 1% Triton-X) on ice for 10 min. The protein concentrations were measured using the Bradford Assay (Biorad, Switzerland, 500-0201). A total of 8 µg of proteins in 20 µl Laemmli Buffer (Biorad) was loaded on a PAGEr® Duramid® Precast gradient gel (4 – 20%, Cambrex, Rockland, ME USA).
After electrophoresis, the gel was blotted to a Protran® nitrocellulose membrane (Schleicher & Schuell, Germany, BA85) and blocked for 30 min by 5% Top-Block solution (VWR International AG, Switzerland). Subsequently, the membrane was incubated with the primary antibody anti-VE-cadherin (1:1000 in 5% Top-Block) overnight at 4°C. The secondary antibody (donkey anti-goat-HRP, 1:2000 in 5% Top-Block solution) was added for 1 h at RT. To normalize the VE-cadherin signal, actin staining was performed on the same membrane according to the following protocol: The membrane was incubated with anti-actin antibody (1:1000, 5% Top-Block solution) for 1 h at RT followed by goat anti-rabbit-HRP antibody for 1 h at RT. Chemiluminescence was developed by Super Signal Dura West kit (Pierce) and detected using a Chemidoc (Biorad) system. The pictures were analyzed by densitometry (Quantity One®, Biorad) to evaluate the relative amount of detected proteins.

Primer selection

According to the sequences of gamma actin (**NM_001614**, forward: 5'-TCTGTGGCTTGGTGAGTCTG-3', backward: 5'-AGTAACAGCCCACGGTGTTC-3') and VE-cadherin (**NM_001795**, 5'-CCTACCAGCCCAAAGTGTGT-3', backward: 5'-GACTTGGCATCCCATTGTCT-3') obtained from the National Centre for Biotechnology Information (NCBI) Nucleotide database, 20 nucleotide long primers were designed by the use of Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). To avoid self-annealing, the sequences were tested by T-Coffee (http://www.expasy.ch). Primers were obtained from TIB Molbiol (Germany). The primer-sequences are listed in table 1.

RNA isolation and quantitative real time RT-PCR

RNA was isolated using the RNeasy mini kit (Qiagen, Switzerland) and quality (260/280 nm ratio) and quantity were determined using a Nanodrop® ND-1000 system (Witec AG, Switzerland). cDNA was synthesized using the SuperScript III First-Strand Synthesis Super Mix for qRT-PCR kit (Invitrogen, Switzerland) according to the standard protocol. Briefly, 150 ng of isolated RNA were added to 10µl of 2x reaction mix and 2 µl RT-enzyme mix and brought to a final volume of 20 µl with diethylpyrocarbonate (DEPC) – treated water. The reaction mixture was incubated for 30 min at 50°C and for 5 min at 85°C to terminate the reverse transcription. To digest the RNA, 1 µl E. coli RNase H was added for 20 min at 37°C. For the real time PCR reaction, an iCycler (Biorad) and SYBR GreenER qPCR SuperMix for iCycler (Invitrogen), including hot-start Taq DNA polymerase and uracil DNA glycosylase (UDG), were used. The reactions were performed in a volume of 30 µl. The denaturation temperature was 95°C for 15 sec, the annealing and elongation temperature was 60°C for 60 sec. Primer concentrations were 200 nM for the forward and the reverse primers. Relative gene expression was calculated according to the $2^{-\Delta\Delta CT}$ method against the actin gene.

Statistical analysis

All experiments were performed in triplicate from 3 different isolations. Two-way ANOVA was calculated using Prism 4.00 (GraphPhad Software, USA). $P < 0.05$ was considered significant (★: $P < 0.05$, ★★: $P < 0.005$). Means are indicated together with the standard error of the mean (s.e.m.).

RESULTS

Expression of VE-cadherin after mTOR inhibition

Sirolimus (SRL), an indirect mTOR inhibitor, was used to block mTOR of human umbilical vein endothelial cells (HUVECs). It binds first to the FK binding protein-12 (FKBP12) and this complex inhibits the activity of mTOR. Short-term treatment (5-30 min) of SRL blocks selectively the mTOR complex 1 (mTORC1) whereas long-term SRL-treatment (> 30 min) also inhibits the activity of mTOR complex 2 (mTORC2) (*12*). HUVECs treated with SRL for 24 h in different concentrations showed a significant and dose-dependent decrease in the expression of VE-cadherin measured by Western blot and densitometry (Figure 1A+B).

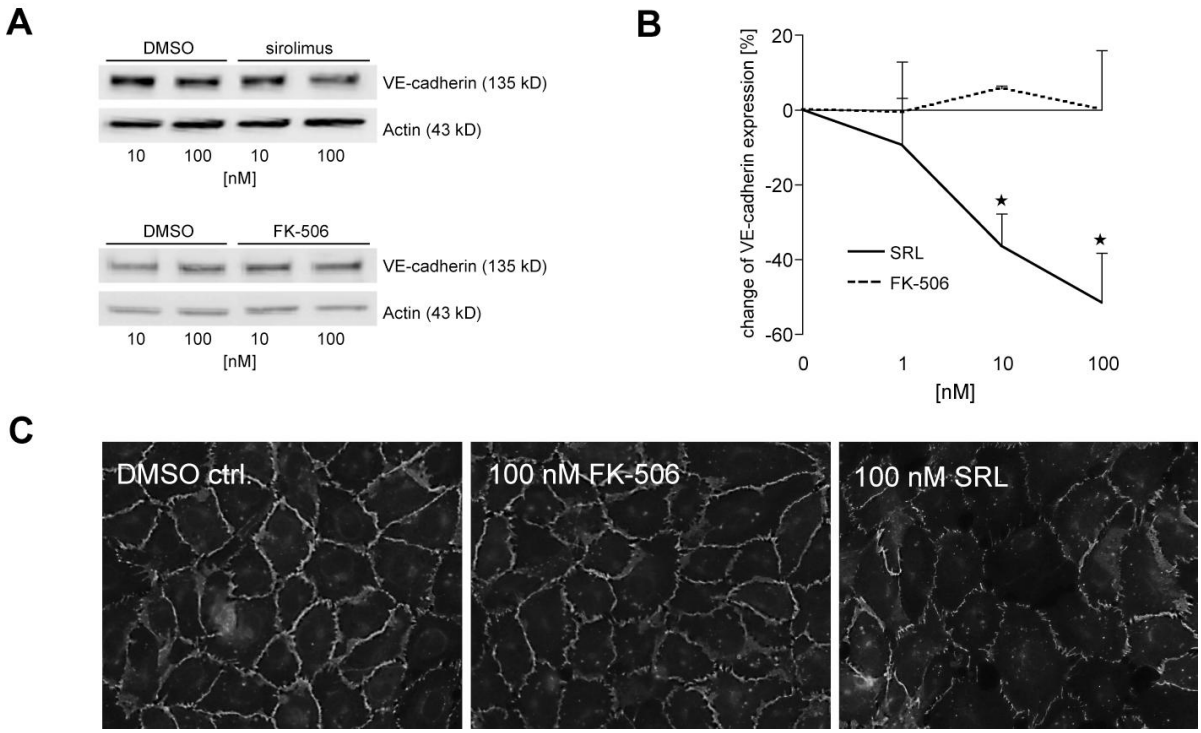

Figure 1. VE-cadherin protein expression after mTOR inhibition. Confluent HUVECs were incubated for 24 h with SRL, FK-506 (1, 10, 100 nM) and DMSO as vehicle control. (A+B) Cells were lysed, proteins separated by gel electrophoresis and immunoblotted with anti-VE-cadherin and anti-actin antibodies. Relative VE-cadherin protein expression of treated cells was determined against the vehicle control and quantified by densitometry. Significant decrease was observed in VE-cadherin expression following 10 nM (36.5 ± 5.1 %, $P = 0.007$) and 100 nM (51.7 ± 7.7 %, $P = 0.02$) of SRL treatment, whereas no differences were detected in FK-506 treated cells. (C) Cells were cultured in 8 well chamber slides, fixed and immunostained by an Alexa 488 conjugated anti-VE-cadherin antibody (green). On DMSO control and FK-506 treated cells, VE-cadherin is expressed continuously along the cell borders. SRL treated cells showed decreased VE-cadherin expression.

The expression of VE-cadherin decreased 9.5 ± 13 % after 1 nM, 36.5 ± 5.1 % (*P* = 0.007) after 10 nM and 51.7 ± 7.7 % (*P* = 0.02) after 100 nM SRL treatment. To exclude the involvement of FKBP12, tacrolimus (FK-506) was investigated as well. FK-506 has a similar chemical structure as SRL (*13*) and also binds to FKBP12. However, the FK-506 - FKBP12 complex inhibits calcineurin (*14*). The relative protein expression of VE-cadherin was not affected by FK-506 at equivalent concentrations (Figure 1A+B). Immunofluorescent staining was performed to visualize any morphological changes in the VE-cadherin expression of HUVECs. In control cells, VE-cadherin was expressed homogeneously along the cell borders (Figure 1C). SRL treated cells showed a decrease in VE-cadherin expression accompanied by intercellular gap formation. A similar phenomenon was not observed in FK-506 treated ECs.

Protein expression of VE-cadherin following specific mTOR silencing

Because SRL and FK-506 have anti proliferative effects, which may influence VE-cadherin expression via an unknown mechanism, siRNA experiments to specifically knock down mTOR expression were performed. After silencing mTOR in siRNA treated cells, the expression of mTOR and VE-cadherin decreased (Figure 2A). mTOR and VE-cadherin signals on the membrane were quantified by densitometry to calculate relative protein expression (Figure 2B). A concentration of 10 nM siRNA decreased VE-cadherin expression 17.9 ± 10.7 % (*P* = 0.24, and mTOR expression 18.7 ± 8.7 %) while 100 nM siRNA reduced it 31.6 ± 2.4 % (*P* = 0.006, mTOR expression: 30.2 ± 9.6 %).

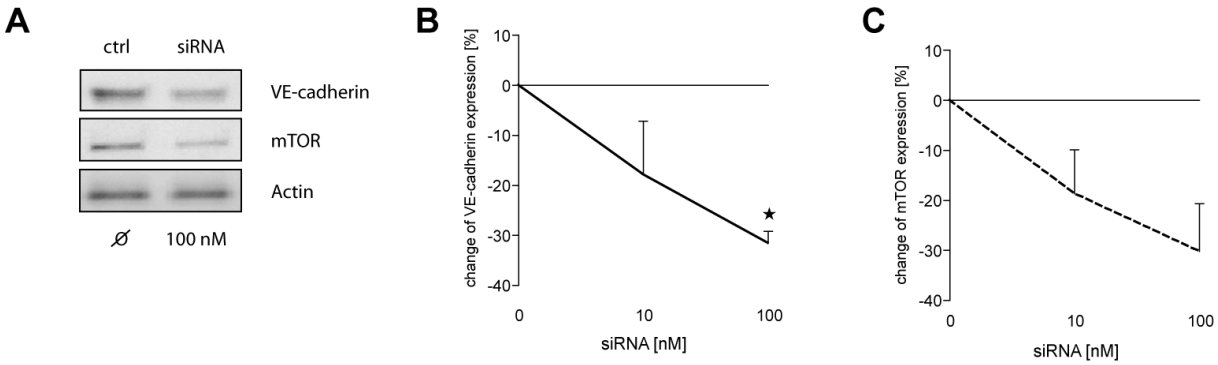

Figure 2. VE-cadherin protein expression after mTOR silencing. HUVECs were transfected with siRNA specific against mTOR for 24 h in serum free medium and cultured for another 24 h after replacement of the medium. (A) Cells were lysed and proteins were separated by SDS-PAGE, followed by immunoblotting with specific antibodies for VE-cadherin, mTOR and actin as the loading control. (B) Relative VE-cadherin protein expression was analyzed by densitometry. Knocking down mTOR by 100 nM siRNA (decrease of mTOR expression: 30.2 ± 9.6 %) resulted in a down regulation of VE-cadherin of 31.6 ± 2.4 % (P = 0.006) and 10 nM siRNA down regulated the relative expression 17.9 ± 10 % (mTOR: 18.7 ± 8.7 %). No statistical significance was calculated for the mTOR expression because of inconstant transfection success of the siRNA (data not shown).

These data indicate that mTOR plays a role in VE-cadherin protein expression. mTOR is known to control mRNA translation by regulating the initiation and elongation stages (*15*). To demonstrate a direct influence of mTOR on VE-cadherin gene activity and to exclude lower ribosomal activity or enhanced protein degradation, RT-PCR experiments were performed.

Gene expression of VE-cadherin after mTOR inhibition

cDNA was synthesized from isolated total RNA of treated (SRL or FK-506) and control HUVECs. The relative gene (mRNA) expression of VE-cadherin was quantified by the $2^{-\Delta\Delta CT}$ method using γ–actin as the housekeeping gene. SRL treated cells showed a dose dependent decrease in VE-cadherin gene expression (Figure 3A). The relative mRNA expression of VE-cadherin was down-regulated 21.9 ± 13.6 % (*P* = 0.24) following treatment with SRL in a concentration of 10 nM and 39.1 ± 3.7 % (*P* = 0.009) on cells treated with SRL in a concentration of 100 nM. No significant changes were observed after FK-506 administration.

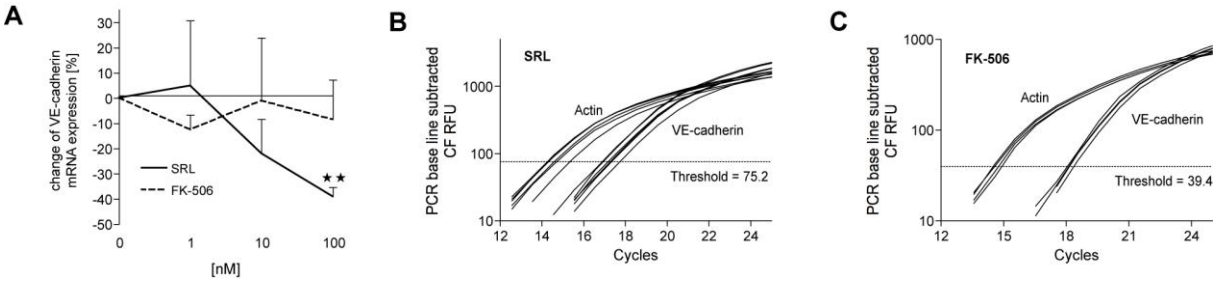

Figure 3. VE-cadherin gene expression after mTOR inhibition. RNA was isolated from SRL treated HUVECs using the RNeasy mini kit. Quantitative real time RT-PCR was performed with primers specific for VE-cadherin and □ □ actin as the housekeeping gene. Relative gene expressions were quantified using the $2^{\Delta\Delta CT}$ method. (A) Down-regulated gene expression in cells treated with SRL in 10 nM (21.9 ± 13.6 %, *P* = 0.24) and in 100 nM (39.1 ± 3.7 %, *P* = 0.009) was measured. Amplification curves demonstrate the variations of the VE-cadherin cDNA amplicon in SRL treated cells (B) and a constant amplification in FK-506 treated cells (C).

Role of PI3K in the expression of VE-cadherin

As shown above, mTOR plays a role in protein and gene expression of VE-cadherin. To understand more about the signals regulating VE-cadherin, the role of PI3K, which signals downstream to mTOR (*9*), was determined. Wortmannin, a metabolite of Penicilium wortmannii, is a specific inhibitor of PI3K. The IC 50 concentration is 5 nM and higher concentrations than 200 nM result in a non specific inhibition of other kinases (according to the product description). HUVECs were exposed to 1 and 10 nM Wortmannin for 24 h and VE-cadherin protein expression was quantified using Western blot.

Wortmannin decreased the VE-cadherin expression 20.2 ± 7.0 % (*P* = 0.0077) in the case of 10 nM (Figure 4A+B), whereas 1 nM Wortmannin had no influence on the protein expression. Using immunofluorescent staining, we examined whether Wortmannin in 10 nM concentration led to changes in the expression or distribution of VE-cadherin. The DMSO vehicle control showed homogenous distribution of VE-cadherin along the cell borders of the cobblestone-like monolayer (Figure 4C). In Wortmannin treated cells, expression of VE-cadherin was reduced and the expression pattern changed to a discontinuous distribution between the cells.

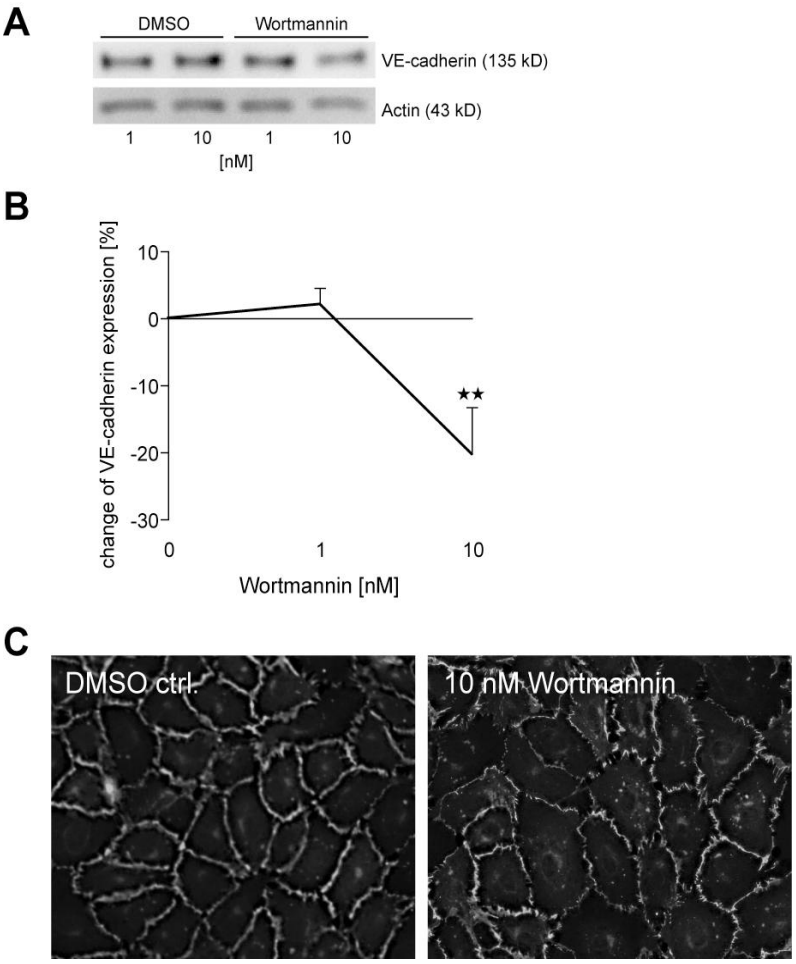

Figure 4. Protein expression of VE-cadherin after PI3K inhibition. Lysates of Wortmannin and DMSO (1 and 10 nM) treated HUVECs were analyzed by SDS-PAGE and immunoblotted using anti-VE-cadherin and anti-actin antibodies. (A+B) The relative protein expression was quantified by densitometry of the actin-normalized VE-cadherin signals (Wortmannin / DMSO vehicle control). A significant decrease in protein expression was observed at 10 nM Wortmannin (20.2 ± 7.0 %, *P* = 0.0077). (C) Immunofluorescent staining for VE-cadherin (green) was performed on Wortmannin-treated (10 nM) HUVECs. Decreased VE-cadherin expression was observed along the cell borders.

Gene expression after Wortmannin treatment

PI3K is a central molecule involved in many different signaling cascades. To demonstrate that PI3K influences the gene transcription of VE-cadherin, RT-PCR experiments were performed. The effect of Wortmannin on VE-cadherin mRNA expression was analyzed by

quantitative real time PCR. Isolated RNA of HUVECs treated with 1 and 10 nM Wortmannin or DMSO was reverse transcribed into cDNA.

The relative VE-cadherin gene expression was analyzed with the $2^{-\Delta\Delta CT}$ method against γ–actin. Wortmannin in a concentration of 1 nM had, as we showed earlier for protein expression (Figure 5), no decrease on gene expression (1.1 ± 21 %), but 10 nM Wortmannin treatment decreased VE-cadherin mRNA expression significantly for 38.0 ± 5.0 % (*P* = 0.017) (Figure 5A+B).

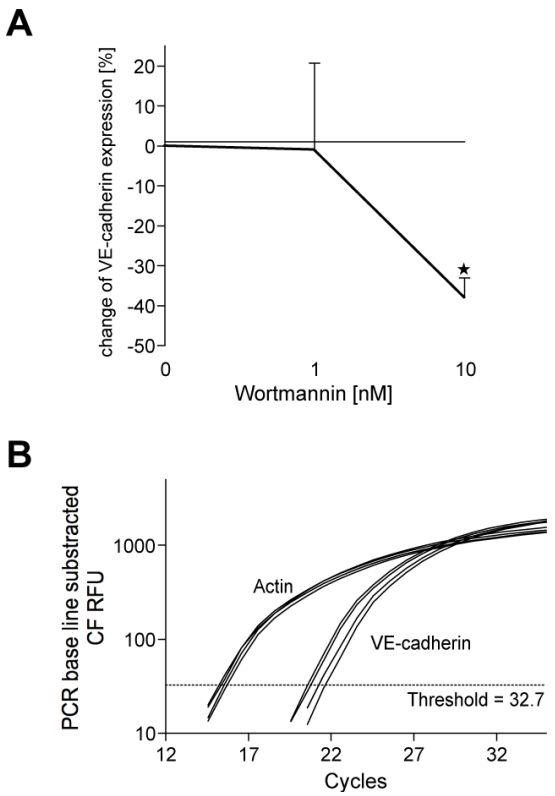

Figure 5. Gene expression of VE-cadherin after PI3K inhibition. HUVECs were cultured for 24 h in the presence of Wortmannin and DMSO (1 and 10 nM). RNA was isolated and quantitative real time RT-PCR with primers for the VE-cadherin- and γ–actin-genes was performed. VE-cadherin mRNA expression decreased 38.0 ± 5.0 % (*P* = 0.017) after treatment with 10 nM Wortmannin. Wortmannin in a concentration of 1 nM did not lead to decreased expression. VE-cadherin expression was quantified using the $2^{-\Delta\Delta CT}$ method by using actin as the housekeeping gene. The PCR amplification curves of Wortmannin treated cells show variations of the VE-cadherin amplicon, which demonstrate the decreased VE-cadherin mRNA expression (B).

To summarize, VE-cadherin was demonstrated to be under the control of PI3K and mTOR at both, the transcription and translation level.

DISCUSSION

We investigated the involvement of PI3K and mTOR in the regulation of VE-cadherin synthesis. mTOR was blocked by siRNA and SRL. On the other hand, PI3K was blocked by Wortmannin, used in concentrations of 1 and 10 nM, because its IC50 is 5 nM. Wortmannin causes non-specific blocking of other kinases, including mTOR, in concentrations higher than 200 nM. SRL and FK-506 were used in concentrations of 10 and 100 nM. Higher concentrations have been reported to induce cytotoxicity (*16*). FK-506 has been taken as a control for SRL because it has a similar chemical structure and also binds to the FKBP12. Other than SRL, the FK-506 - FKBP12 complex inhibits calcineurin instead of mTOR. To avoid unexpected side effects of SRL that might influence the VE-cadherin expression, HUVECs were transfected with siRNA specific for mTOR. ECs were transfected with labeled unspecific siRNA for monitoring transfection success (data not shown). A wide variation in transfection efficiency was observed.

By blocking mTOR with SRL and siRNA as well as PI3K with Wortmannin, we demonstrated a significant down-regulation in the protein and gene expression of VE-cadherin. Blocking PI3K resulted in less down regulation of VE-cadherin than in mTOR-blocked samples. The mRNA level of the PI3K inhibited samples decreased stronger (namely 38%) than the protein level (20.2%), which supports that PI3K signaling happens earlier than mTOR in the VE-cadherin biosynthesis pathway. In the immunofluorescent pictures of Wortmannin treated cells, the VE-cadherin expression decreased less than in sirolimus treated cells, but the expression pattern changed to a discontinuous distribution at the intercellular contacts.

Taken together, our results suggest the involvement of mTOR and PI3K in the regulation of VE-cadherin expression on the protein and mRNA level. Our proposed signaling pathway for VE-cadherin production is depicted in Figure 6. An extracellular signal stimulates mTOR via PI3K phosphorylation. Subsequently, mTOR activates the transcription of the VE-cadherin gene. The transcription factor linked with this VE-cadherin pathway is still unknown. After synthesis of the VE-cadherin mRNA, it is translated into the protein (Figure 1A+B, 3 and 4A+B) and expressed on the surface of ECs (Figure 1C and 4C). It has been reported that VE-cadherin is able to activate PI3K (*10, 17*) after contacting another VE-cadherin molecule from a neighbouring cell. This signal could be interpreted as a feedback signal to maintain VE-cadherin expression and to retain the barrier function of the endothelium. This feedback signal is necessary for the maintenance of the cell-to-cell junction because the half life of cadherins is only 5 hours (*18*)

In conclusion, we were able to demonstrate for the first time that mTOR and PI3K are involved in homeostasis of cell-to-cell contacts by regulating the VE-cadherin biosynthesis.

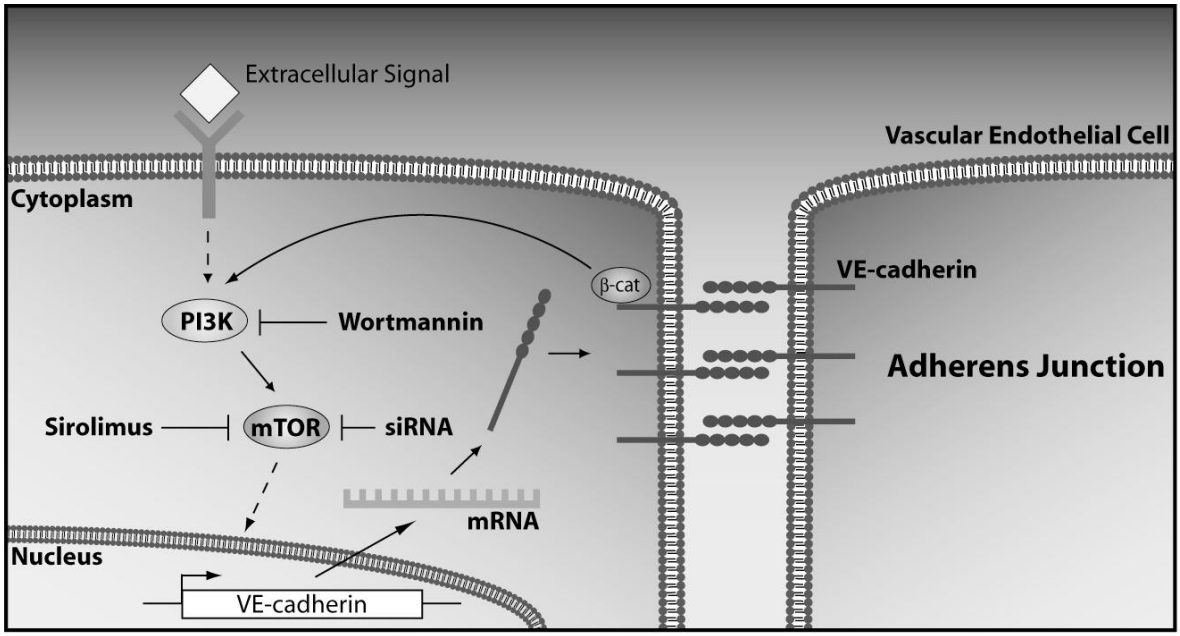

Figure 6. Schematic presentation of our proposed mechanism for the expression of VE-cadherin. An external signal activates the phosphoinositide 3-kinase (PI3K), which activates the downstream signaling molecule mammalian target of Rapamycin (mTOR). These signals initiate the transcription of the VE-cadherin gene into mRNA, which will be translated to the protein and expressed on the cell membrane. VE-cadherins, which bind to another molecule, send a feedback signal to the PI3K via the associated □-catenin (*10, 17*) to maintain its own protein production.

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5.3 Proliferation Signal Inhibitors-Induced Decrease of VE-cadherin and Increase of Endothelial Permeability on vitro are Prevented by an Antioxidant

Oroszlán M, Bieri M, Ligeti N, Farkas A, Koestner SC, Meier B, Mohacsi PJ

Department of Cardiology, Heart transplant (HTx) - Research Laboratory, Swiss Cardiovascular Center Bern, University Hospital Bern (Inselspital), Switzerland

ABSTRACT

Background: Rapamycins (sirolimus, SRL or everolimus, ERL) are proliferation signal inhibitors (PSIs). PSI therapy often leads to edema. We hypothesized that increased oxidative stress in response to PSIs may modulate the expression of VE-cadherin on endothelial cells (ECs) and subsequently vascular permeability, which in turn might be involved in the development of edema. **Methods:** Experiments were performed on human umbilical vein ECs (HUVECs). Oxidative stress was measured by dichlorofluorescein-diacetate. The expression of VE-cadherin was evaluated by immunofluorescent staining and Western blot analysis. Endothelial "permeability" was assessed using a transwell model.
Results: SRL and ERL in concentration of 1, 10 and 100 nM enhanced the oxidative stress ($24 \pm 12\%$, $29 \pm 9\%$, $41 \pm 13\%$ ($p < 0.05$, in all three cases of SRL) and $13 \pm 10\%$, $27 \pm 2\%$, $40 \pm 12\%$, respectively ($p < 0.05$, in the latter two cases of ERL) on HUVECs, which was inhibited by the antioxidant, N-acetyl-cysteine (NAC) and in a smaller degree by the specific inhibitor of nitric oxide synthase, N-Ω-nitro-L-arginine methyl ester. By the use of NAC, the VE-cadherin expression remained comparable to the control, both in immunocytochemistry and Western blot analysis. Permeability was significantly increased by 100 nM of SRL and ERL ($29.5 \pm 6.4\%$ and $33.8 \pm 4.2\%$, respectively), however, co-treatment with NAC abrogated the increased permeability. **Conclusions**: EC homeostasis, indicated by VE-cadherin expression, might be damaged by SRL and ERL and be resolved by the antioxidant NAC.

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INTRODUCTION

Proliferation signal inhibitors (PSIs), sirolimus (SRL) and everolimus (ERL) interfere with the mammalian target of rapamycin (mTOR). mTOR regulates cell growth, metabolism and proliferation (*1-3*). PSIs are very effective immunosuppressants and also used in heart transplantation (*4-6*). Several reports are available in the literature, indicating the importance of edema occurrence during treatment with PSIs (*4, 7-10*). Edema leads, as reported by patients, to significant discomfort, malcompliance and subsequently even to PSI-withdrawal. The underlying pathophysiological mechanism is unknown. Edema occurrence is seldom in patients treated with calcineurin inhibitors (CNIs), tacrolimus (FK-506) or cyclosporin A (CsA).

From the clinical point of view, edema may be a consequence of elevated hydrostatic, respectively decreased oncotic pressure or increased endothelial permeability. Other underlying mechanisms have to be considered, such as i) decreased glomerular filtration with subsequent salt and H_2O retention, ii) any causes of endocrinological imbalance, the administration of glucocorticoids, or iii) electrolyte alterations.

Vascular homeostasis is dependent on endothelial cells (ECs). The barrier function of the endothelium is largely determined by the integrity of the tight and adherens junctions (AJs) (*11*). Vascular endothelial cadherin (VE-cadherin) is exclusively expressed on ECs, located in the AJ region (*12*). Normal expression of VE-cadherin is required for the maintenance of proper endothelial barrier function (*13*). Earlier studies demonstrated that decreased expression of VE-cadherin is associated with increased endothelial permeability in response to several stimuli (*14-20*). The importance of the reactive nitrogen and/or oxygen species (RNOS) as biological messengers has been increasingly recognized during the last decade in the pathogenesis of several vascular diseases (*21, 22*) including edema. The studies of Zhang *et al*. show that the dysfunctional endothelial filter mechanism is prevented by the use superoxide-dismutase or MnTMPyP, as a peroxynitrite scavenger during platelet-activating factor treatment or preeclampsia. Their observations suggest the role of oxidative stress in increased endothelial permeability (*18, 19*). Trapp *et al*. demonstrate that immunosuppressants including SRL induce increased oxidative stress on human microvascular ECs (*23*).

The present study examined *in vitro* the effect of PSIs on endothelial barrier function and compared their effect with them of CNIs. We investigated whether PSIs may induce oxidative stress and influence VE-cadherin expression, as well as permeability. Furthermore we investigated the effect of an antioxidant on PSI-regulated VE-cadherin expression and permeability (**Figure 1**).

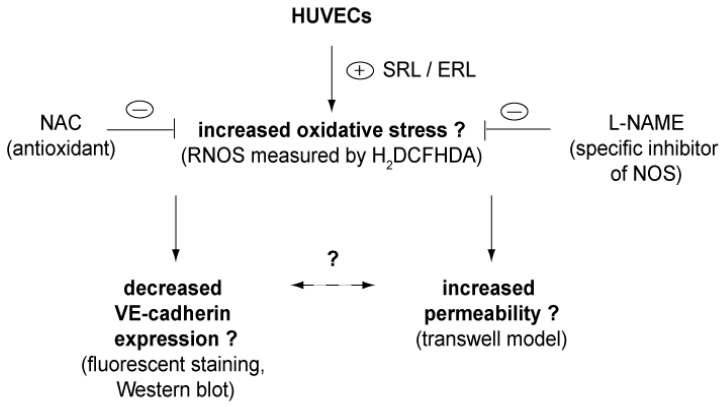

Figure 1: Presumed effects of PSIs on VE-cadherin expression, in vitro permeability and their relation to oxidative stress. Abbreviations: NAC = N-acetyl-cysteine, L-NAME = N-nitro-L-arginine methyl ester, H_2DCFHDA = 2`,7`-dihydrofluorescein diacetate, RNOS = reactive nitrogen and/or oxygen species, NOS = nitrogen oxide synthase

MATERIALS AND METHODS

Culture of human umbilical vein ECs (HUVECs)

Human umbilical cords were obtained from a regional hospital. The local ethical committee approved the study and information consent was obtained. ECs were isolated according to Jaffe *et al* (*24*) and cultured according to Oroszlan *et al* (*25*). Culture medium is referred to as complete medium. ECs were used at passages 2 to 4.

Reagents

SRL, CsA and FK-506 were purchased from Sigma. ERL was kindly provided by Novartis (Basel, Switzerland). SRL and FK-506 were dissolved in dimethyl-sulfoxide (DMSO). ERL and CsA were dissolved in 99% ethanol (EtOH). Properly diluted vehicles, either DMSO or 99% EtOH were used as controls.

Cytotoxicity assay

The cytotoxic effect of the drugs was measured by Toxilight® bioassay kit (Cambrex Bio Science Rockland, Inc., USA). Cells were cultured until confluency on gelatin-coated 96-well plates and treated for 24 hrs. Afterwards, the assay was performed according to the standard protocol provided by the company.

Measurement of intracellular RNOS

Cells were cultured on gelatin-coated 96-well plates until confluency. Cells were loaded with 2`,7`-dihydrofluorescein diacetate (H_2DCFHDA, Sigma) at a concentration of 5 μM, diluted in PBS (37°C). After 30 minutes of incubation (37°C/5% CO_2), cells were washed with PBS and cultured for 24 hrs in the presence of the drugs in complete medium, however containing 2% of FCS. The fluorescence signal was obtained after 24 hrs by using the TECAN microplate reader with filters for excitation (492 nm)/emission (520 nm) (**Figure 2A**).

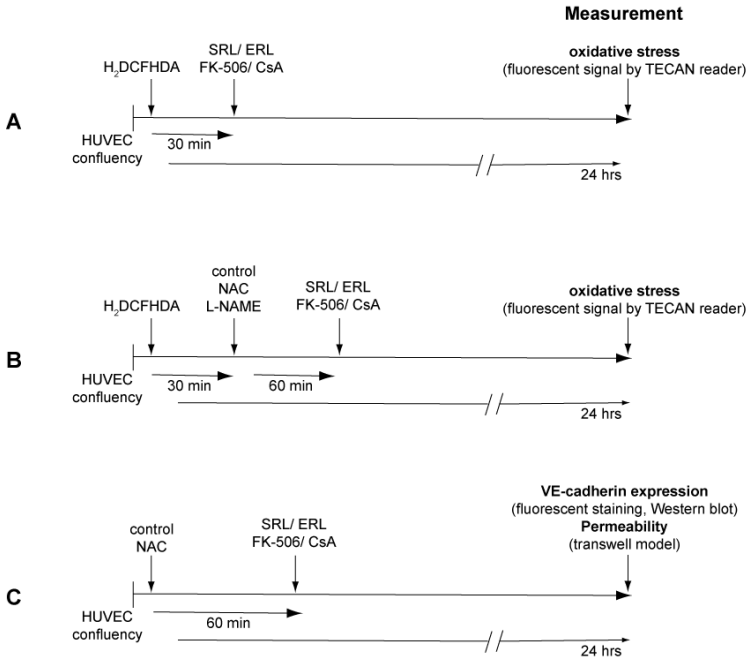

Figure 2: Design of experiments

N-acetyl-cysteine (NAC, Sigma) in 3 mM or N-Ω-nitro-L-arginine methyl ester (L-NAME, Sigma; dilutions of 4, 20, 100 and 500 μM) were added for 60 minutes at 37°C/5% CO_2 to the cells after loading with H_2DCFHDA and prior to the drugs (**Figure 2B**).

Relative RNOS production (RNOS signal per cell number) was assessed by using two steps: measurement of RNOS production and second the nucleus staining (see below) in order to achieve the cell numbers: Briefly, cells were fixed with a 1:1 mixture (v/v) of acetone-methanol. Afterwards, cells were incubated for 30 minutes in the presence of Hoechst-33342 diluted in PBS (1/10000). The plate was read by TECAN microplate reader with filters for excitation (355 nm)/emission (460 nm). The cellular response after drug treatment was then referred to respective vehicle control.

Immunofluorescent staining of VE-cadherin

HUVECs were seeded onto 1% gelatin-coated chamber slides. Cells were cultured for 24 hrs in medium supplemented with the drugs, or vehicles. Cells were pretreated for 60 minutes with NAC (3 mM) or complete medium as control, prior to the addition of the drugs. Following fixation, cells were permeabilized with a buffer containing 100 mM KCl, 3 mM $MgCl_2$, 2 mM $CaCl_2$, 200 mM sucrose, 10 mM HEPES and 0.2% Triton-X (pH 7.1). As primary antibody, goat anti-human VE-cadherin (Santa Cruz Biotechnology, Inc.) was used at a dilution of 1/200 in PBS with 1% bovine serum albumin and incubated overnight at 4°C. The cell-bound antibodies were detected with secondary antibody, polyclonal donkey anti-goat, which was conjugated to the fluorescent dye, Alexa 488 (Molecular Probes, Inc). A qualitative analysis was performed by fluorescence microscopy (Leica DM-RB and Olympus DP10 camera) (**Figure 2C**).

Western blot analysis

Confluent ECs on 1% gelatin-coated plates were cultured for 24 hrs in the presence of the drugs, or vehicles. In case, where ECs were pretreated for 60 min with NAC (3 mM) or complete medium as control, the supernatant was completed with SRL or ERL and cells were cultured for an additional 24 hrs.

Cells were lysed with a buffer (150 mM NaCl, 2 mM EDTA, 20 mM Tris-HCl and 1% Triton-X, pH 8). Twenty-five μg protein was loaded on PAGEr Duramide Precast Gel (4-20%, Cambrex, Rockland, USA), and transferred to a nitrocellulose membrane. The membranes were blocked by 5% Topblock (VWR International AG, Dietikon, Switzerland) and incubated overnight at 4°C with the following primary antibodies: goat anti-human VE-cadherin and rabbit anti-actin (Sigma). Secondary antibodies (HRP-conjugated anti-goat and anti-rabbit antibodies, respectively (Pierce Biotechnology, Inc., Rockford, USA) were added for 1 h. The immunoreactive proteins were detected by a chemiluminescence system. Results were analyzed with Quantity One® 4.5.2 1-D Analysis Software (BioRad). Actin signal was used as a reference to normalize the expression of VE-cadherin (**Figure 2C**).

In vitro permeability assay (transwell model)

Confluent HUVECs were seeded on 1% gelatin-coated transwell inserts (Becton Dickinson, 0.4 μm pore size) in 12-well plates and cultured with 400 μl complete medium in the upper- and 1400 μl medium in the lower chamber. At the start of the experiment, the culture medium was supplemented with the drugs, or vehicles and the culture continued for 24 hrs.

HUVECs were pretreated with NAC (3 mM) or complete medium as control, for 1 h at 37°C.

Next, the supernatant in the upper chamber was completed with SRL or ERL to the final concentration of 100 nM. Afterwards, 20 μg/ml FITC-dextran (relative molecular mass: 4 kDa, Sigma) was added to the upper chamber. After 1 h of incubation at 37°C, 100 μl of the medium was taken from the lower compartment and measured by TECAN microplate reader with filters for excitation (495 nm)/emission (520 nm) (**Figure 2C**).

Statistical analysis

Mann-Whitney test was performed using GraphPad Prism version 4.00 (GraphPad Software, San Diego California USA). Values are presented as mean ± SEM (standard error of the mean). $P \leq 0.05$ was considered significant. All experiments were performed three times unless otherwise indicated.

RESULTS

Cytotoxic analysis

In our experimental setting, the HUVECs monolayer represents a model of the vascular barrier. Therefore, all experiments were performed in confluent state.
A loss of cell integrity leads to cell leakage of adenylate kinase (AK) into the culture medium. The Toxilight® bioassay is based on the detection of AK. Serial dilutions of SRL, ERL, FK-506 or CsA induced no cytotoxicity compared to vehicles at 24 hrs (**Figure 3**).

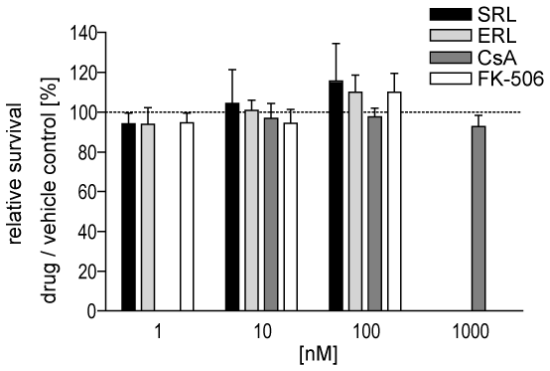

Figure 3: Cytotoxicity of SRL, ERL, FK-506 and CsA. Cells were cultured for 24 hrs in the presence of 1-100 nM SRL, ERL, FK-506 or 10-1000 nM CsA and their respective vehicles. Cytotoxicity assay was performed on the basis of AK release into the culture medium. Results represent the percentage of relative survival compared to the respective vehicle.

PSIs, but not CNIs induce increased RNOS production

Exposure of ECs to SRL or ERL for 24 hrs resulted in a dose-dependently enhanced oxidative stress. The treatment with SRL in concentrations of 1, 10 and 100 nM led to 24 ± 12% (p = 0.03), 29 ± 9% (p = 0.0087) and 41 ± 13% (p = 0.0091) increase, respectively. ERL in 1, 10 and 100 nM enhanced the oxidative stress with 13 ± 10%, 27 ± 2% (p = 0.0017) and 40 ± 12% (p = 0.03), respectively. No effect was detected of FK-506 and CsA (**Figure 4A**). NAC in the concentration of 3 mM abrogated completely the increased RNOS production in response to SRL or ERL in concentration of 100 nM (**Figure 4B**).

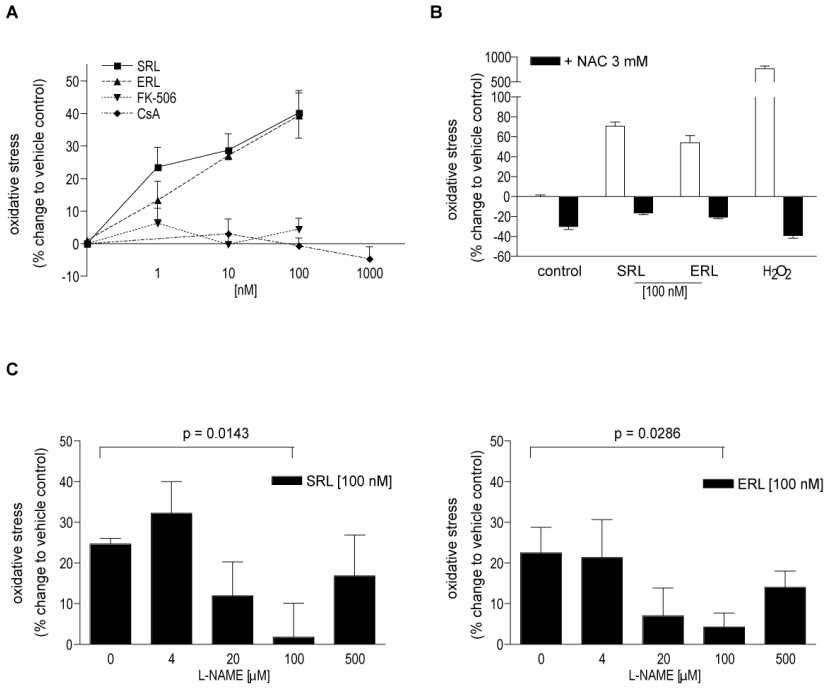

Figure 4: Production of RNOS following SRL, ERL, FK-506 or CsA treatment. **(A)** After administration of the above-mentioned drugs for 24 hrs to the HUVECs, RNOS were measured by H_2DCFHDA, (*n* = 4). (see also **Figure 2A**). **(B)** ECs were exposed to NAC (3 mM) for 1 hr prior to addition of SRL or ERL in the final concentration of 100 nM. The consecutive RNOS production was measured after 24 hrs. Cells stimulated with H_2O_2 in concentration of 20 µM were used as positive controls. The picture is representative of 3 independent experiments. (see also **Figure 2B**). **(C)** ECs were pretreated for 1 hr with the specific NOS inhibitor, L-NAME used in concentrations as in the graph indicated. Afterwards, the supernatant was completed with SRL or ERL to final concentration of 100 nM. The production of RNOS was determined after 24 hrs treatments (n = 4). (see also **Figure 2B**)

Since H_2DCFHDA is not specific, measuring the oxidative activity is complicated by the possibility of detecting multiple forms of reactive species. L-NAME inhibits nitric oxide synthase (NOS) and the consequent nitric oxide (NO) production. As **Figure 4C** shows, the increased production of RNOS during SRL or ERL treatment was dose-dependently inhibited by the use of L-NAME (inhibition with 100 µM L-NAME reached a significant difference, (p =

0.0143 and p = 0.0286, respectively). The increase of oxidative stress in the higher concentration of L-NAME is not surprising, since NO has a dichotomous effect between pro- and antioxidative state.

NAC prevents the loss of VE-cadherin on HUVECs during treatment with PSIs

Relative expression of VE-cadherin was investigated by Western blot. ECs treated with SRL or ERL in concentration of 1, 10 and 100 nM showed a dose-dependent decrease in VE-cadherin expression to 90.48 ± 12.2%, 63.5 ± 5.1%, 40.6 ± 0.006% and 99.8 ± 9.8%, 83.2 ± 5.2%, 37 ± 3.35%, respectively. Neither FK-506 nor CsA were able to impair the relative expression of VE-cadherin (**Figure 5A**).

We examined the expression of VE-cadherin following SRL, ERL, FK506 (100 nM) or CsA (1 μM) treatment in the presence or absence of NAC (3 mM). **Figure 5B** summarizes the fluorescent staining. The distribution of VE-cadherin is continuous and circumscribing the cells (see control). In contrast, VE-cadherin has a point-like pattern after 24 hrs of treatment with PSIs. Treatment with FK-506 or CsA did not lead to change of VE-cadherin pattern, compared to the control.

The Western blot analysis (**Figure 5C**) showed a similar phenomenon. PSIs–treated ECs showed in the presence of NAC a VE-cadherin expression comparable to the vehicle controls (20 ± 10.9% and 2 ± 5.89% changes, respectively).

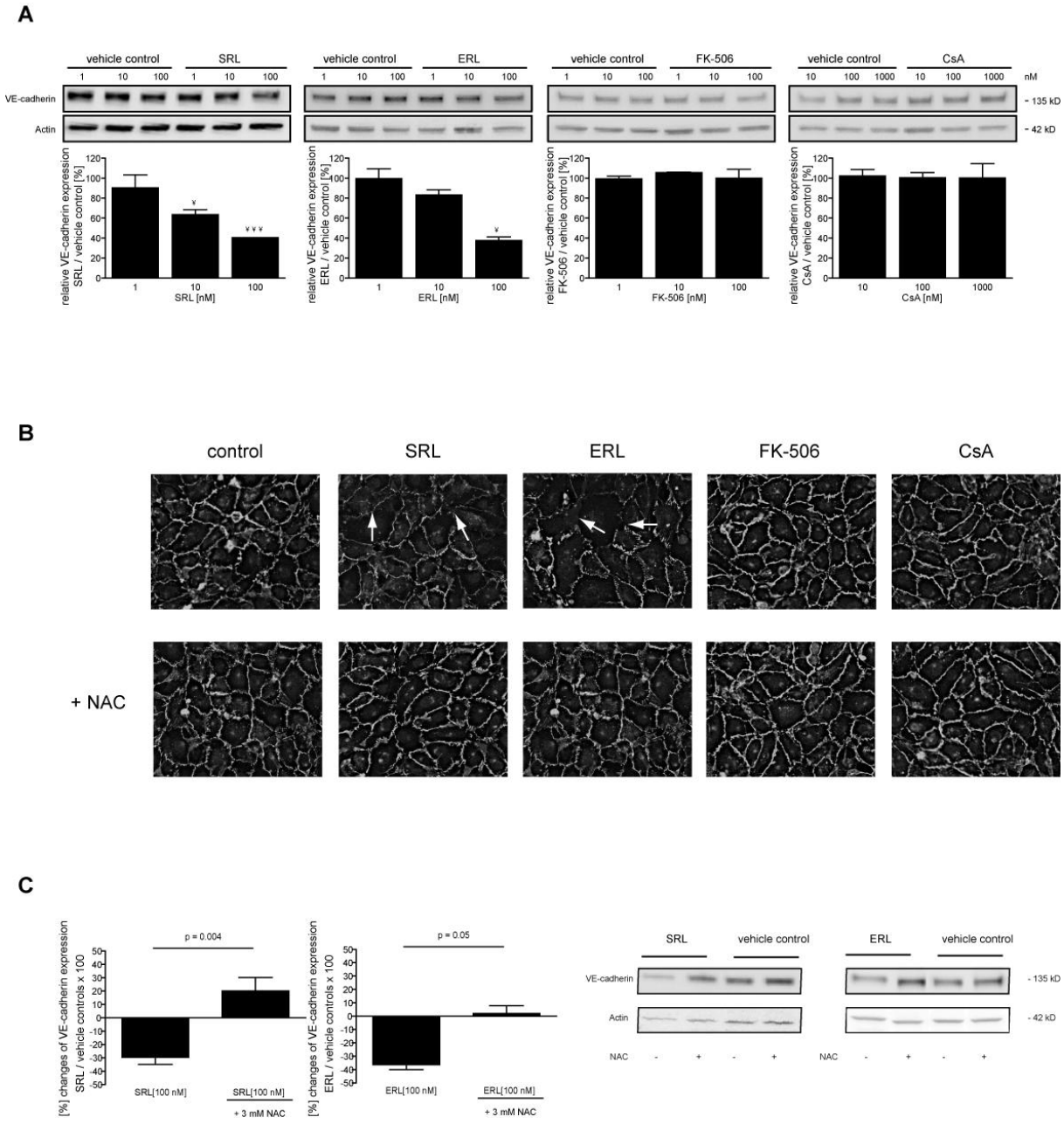

Figure 5: VE-Cadherin expression after SRL, ERL, FK-506 or CsA treatment (see also **Figure 2C**). **(A)** Western blot analysis of VE-cadherin expression was determined by following 24 hrs treatments with SRL, ERL, FK-506 or CsA in the concentrations as in the graph indicated. The graphs show the relative expression of VE-cadherin with respect to properly diluted vehicles. **(B)** Expression of VE-cadherin following SRL, ERL, FK-506 or CsA treatment in the presence or absence of NAC. Confluent ECs were preconditioned for 1 hr with NAC in the concentration of 3 mM. Afterwards, the culture continued for 24 hrs in the presence of SRL, ERL, FK-506 in 100 nM or CsA in 1 μM. The picture is representative of three independent experiments. **(C)** Western blot analysis of NAC conditioned HUVECs after SRL or ERL treatment. Confluent ECs were pretreated with NAC (3 mM) for 1 hr. Afterwards, SRL or ERL was administered to the ECs in the concentration of 100 nM for 24 hrs. The graphs show the relative expression of VE-cadherin with respect to properly diluted vehicles.

NAC prevents the increased permeability of ECs treated with PSIs

The endothelial permeability was determined by measuring the FITC-dextran passage through the endothelial monolayers, which were grown on cell culture inserts. FITC-dextran content, representing endothelial permeability increased dose-dependently in response to SRL. Significant increase was detected in concentration of 100 nM (29.5 ± 6.4%, p = 0.0002, n = 5). ERL did not show a change in HUVECs permeability at 10 nM, but a significant increase of FITC-dextran in the lower compartment at concentration of 100 nM (33.8 ± 4.2%, $p < 0.0001$, n = 5). Neither FK-506 nor CsA did show this effect (**Figure 6A).**

Figure 6B depicts the FITC-dextran passage in ECs treated with 3 mM NAC prior to the addition of PSIs (100 nM). Following a treatment period of 24 hrs, the SRL- or ERL-dependent increased passage of FITC-dextran was significantly prevented by the use of NAC (-1 ± 8% and –1 ± 3%, respectively) and comparable to vehicle controls.

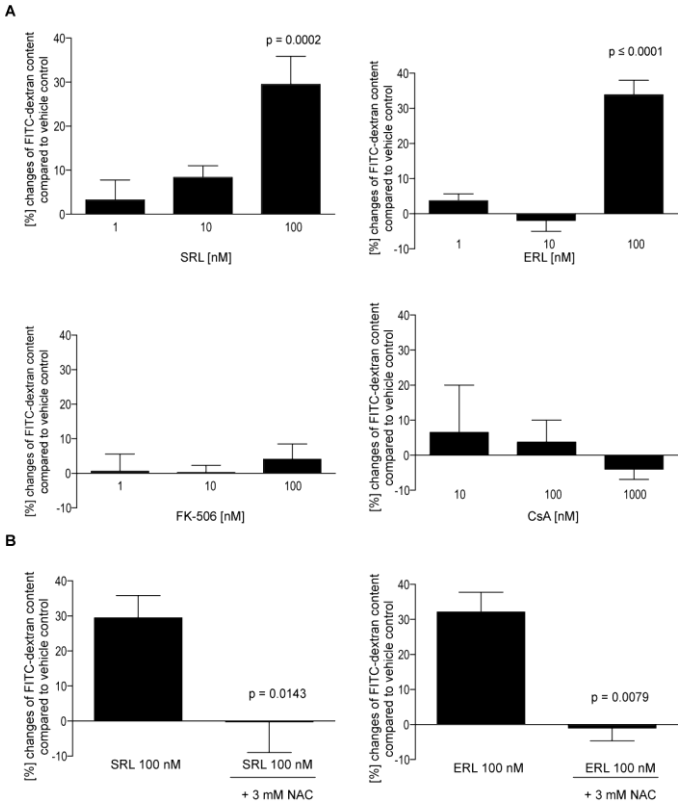

Figure 6: Endothelial permeability following SRL, ERL, FK-506 or CsA treatment (see also Figure 2C). **(A)** ECs were cultured on transwell membranes and exposed to SRL, ERL, FK-506 or CsA for 24 hrs in the concentrations as in the graphs indicated. Afterwards, the leakage of FITC-dextran across HUVECs monolayer was measured from the lower compartment. **(B)** Permeability of HUVECs was measured in the presence of NAC (3 mM) in response to SRL or ERL. Cells were exposed to NAC for 1 hr, Afterwards the medium was completed with SRL or ERL to final concentration of 100 nM. The permeability is expressed as % change with respect to properly diluted vehicles as controls.

DISCUSSION

The present study investigated and compared *in vitro* the effect of PSIs (SRL and ERL) and CNIs (FK-506 and CsA), on one known regulatory mechanism of the endothelial barrier, namely the expression of VE-cadherin. We found that PSIs induce endothelial barrier dysfunction shown by increased FITC-dextran leakage in a transwell model in association with decreased expression of VE-cadherin. The antioxidant NAC prevented the loss of VE-cadherin due to PSI administration and normalized the barrier function of the endothelium. Our results suggest that RNOS modulate vascular permeability following treatment with PSIs by downregulation of VE-cadherin expression.

Normal cellular homeostasis balances between formation and elimination of oxidants. Oxidative stress can be understood as an overproduction of oxidants or the result of impaired antioxidant capacity. The vascular endothelium is not only the target for exogenous oxidants, but ECs itself can directly contribute in RNOS formation. Potential enzymatic sources of RNOS include the mitochondrial electron transport chain, the arachidonic acid metabolizing lipoxygenase and cycloxygenase, the cytochrome P450s, xanthine oxidase, NAD(P)H oxidases, uncoupled NOS, peroxidases and other hemoproteins (*26*).

We now provide data showing that PSIs induce oxidative stress on HUVECs, which has an unfavorable effect for VE-cadherin expression and endothelial permeability. We demonstrated a relationship between PSIs evoked oxidative stress and dysfunctional barrier mechanism of ECs. These effects were prevented by the use of an antioxidant. Furthermore, oxidative stress was partially preventable by the use of L-NAME, indicating the involvement of NO. It is known that besides its beneficial effect, NO may interact also with H_2O_2 or superoxide, which further contributes to oxidative stress (*27, 28*). Oxidative stress following treatment with immunosuppressive drugs is not limited to PSIs. Elevated ROS formation was also observed after CNI administration (*23, 29*). The discrepancy to our results might be due to the origin of ECs or the culture conditions.

Edema during PSIs therapy has not been investigated, yet. To date, only one speculation for this phenomenon exists: it has been shown that prostacyclin release was stimulated by SRL on human aortic ECs (*30*). Pascual *et al.* speculates that prostacyclin-induced vasodilatation might be responsible for capillary leakage in SRL-treated patients (*8*). Increased prostacyclin release was found following treatment with CsA and FK-506 (*30*). Although, we have to handle critically the extrapolation of results on ECs from different origins –against the role of prostacyclin- only PSIs resulted in increased permeability of HUVECs in our study.

We tested the various concentrations of the drugs ranging from subtherapeutic to supratherapeutic levels on the barrier function of ECs. Our data showed an enhanced permeability of HUVEC-monolayers, only after administration of either SRL or ERL.

Although, significantly increased permeability was measured following treatment with 100 nM SRL or ERL, in case of SRL, a dose-dependent increase was observed.

SRL or ERL are highly hydrophobic with a high binding and retaining capacity to vascular tissue. Levin *et al.* report that rapamycin binds to the artery at 30–40 times bulk concentration (*31*). Therefore, tissue concentration of rapamycin can exceed the applied concentration several fold. This phenomenon might have serious clinical consequences. In fact, the in vitro concentration of 10 nM, investigated, represents the usually targeted trough levels of SRL or ERL in cardiac transplant patients. We speculate that impaired endothelial permeability is a result of decreased VE-cadherin expression. In accordance with maintained VE-cadherin expression, neither FK-506 nor CsA induced increased endothelial permeability. Although SRL, ERL and FK-506 are chemically related macrolides, they show different biological characteristics (*32-34*). All three molecules bind to the intracellular protein, FK-binding protein 12, however, SRL and ERL inhibit mTOR. It is tempting to speculate that expression of VE-cadherin is under the regulation of mTOR signal pathway.

In summary, our results demonstrate that oxidative stress modulates vascular permeability following treatment with PSIs via downregulation of VE-cadherin expression. These data indicate that the use of antioxidants during rapamycin therapy might have a beneficial effect.

LIMITATIONS OF THE STUDY

Barrier function of the endothelium can be measured *in vivo*, *ex vivo* or on cell culture systems. The chosen model is determined by the question being addressed. We considered HUVECs as a suitable cell model, since ECs –independently from their origin- express abundantly VE-cadherin. We are aware of the fact that - among others - disturbed barrier function of endothelium is only one possible cause of edema. However, ECs in the vasculature are the first candidates exposed to drugs and our study investigated the involvement of disturbed endothelial integrity. Cell culture systems allow only limited period of observation, therefore our experiments were investigated only during 24 hrs of drug administration. Of course, side effects of immunosuppressants might further accelerate during long term.

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6 DISCUSSION

For some patients with severe organ failure, the only life saving treatment is the replacement of whole organs. Big success was done over the past years to improve the outcome and survival rate of these patients. There is still a lot, which is not understood. Due to improvements in cross-matching or phenotyping of donors and recipients, hyperacute rejection occurs only rarely nowadays. Furthermore, current immunosuppressive drugs are able to prevent acute rejection by controlling cellular immunity. These advances allowed the implementation of transplantation in clinical daily routine. Nevertheless, it is not possible to fully control chronic rejection, which still occurs due to many unkown causes.

After successful organ transplantation, the primary target for the immune system is the vasculature of the graft. Newly encountered antigens on the surface of graft ECs that activate the immune system or induce pathophysiological alterations of ECs due to immunosuppression may change the physiological function of the endothelium. The main task of the endothelium is to separate the blood from the underlying tissue. This barrier is controlled by ECs, which form the border of vessels. Dysfunctions of ECs often lead to a break in endothelial barrier function. In the present study we investigated effects of anti-HLA I antibodies and immunosuppressive drugs (mTOR inhibitors and calcineurin inhibitors) on ECs.

6.1 Effect of anti-HLA I Antibodies on ECs

The formation of interstitial edema was selected as one criterion for humoral rejection (*1*). Effector functions of anti-graft antibodies can be mediated by the activation of the complement system. The formation of the MAC complex leads to lysis of ECs in the vasculature. The consequences are impaired barrier function of the endothelium and the occurrence of interstitial edema. This classical model builds on the activation of the complete complement system.

As ECs have several possibilities to protect themselves against complement (*2*), we investigated whether there is another mechanism that may lead to increased endothelial permeability. In a first step, we demonstrated that endothelial permeability increased in the presence of anti-HLA I antibodies in vitro. We could exclude the involvement of complement,

as these experiments were performed without complement products in vitro. As a marker for endothelial permeability, we further investigated the expression of VE-cadherin, which was shown to correlate with endothelial permeability (*3, 4*). The protein expression of VE-cadherin decreased on ECs stimulated with anti-HLA I antibodies. This effect was already observed after 30 min stimulation. After 24 h anti-HLA I antibody stimulation, VE-cadherin expression further decreased. The mRNA expression of VE-cadherin was not affected by anti-HLA I antibodies, which was not very surprising, as the effect of protein decrease was observed already after 30 min. Therefore, we assumed the involvement of post-translational modifications. In literature, phosphorylation of VE-cadherin that induces its degradation is well documented (*5*). Our hypothesis was that anti-HLA I antibodies induce VE-cadherin phosphorylation, which we could confirm experimentally. By Western blot analysis, VE-cadherin phosphorylation was already measured after 30 min of anti-HLA I antibody incubation; higher phosphorylation rate of VE-cadherin was detected after 24 h anti-HLA I administration.

From literature it was known that phosphorylation of VE-cadherin might be mediated by Src (*6, 7*). By blocking Src with the pharmacological inhibitor SU6656, the anti-HLA I induced decrease of VE-cadherin was inhibited. As Src is activated by VEGFR2 after VEGF stimulation (*7*), VEGFR2 was blocked with another pharmacological inhibitor (SU4312). The inhibition of the tyrosine kinase domain of VEGFR2 avoided decrease and phosphorylation of VE-cadherin after anti-HLA I stimulation of ECs. These two observations suggested the involvement of VEGFR2 and Src in the anti-HLA I mediated down-regulation of VE-cadherin. The link between HLA I and VEGFR2 signaling was still missing. Two potential connections between HLA I and VEGFR2 migth exist: an unknown intracellular pathway may signal from HLA I to VEGFR2 or ECs produce VEGF, which activates VEGFR2 extracellularly. Therefore, we determined the presence of VEGF in the EC supernatant. No VEGF could be measured maybe due to low titer or its immediate absorbance on ECs. To increase the sensibility of the VEGF measurement, mRNA levels of VEGF were determined by PCR. Indeed, elevated VEGF mRNA levels were detected after anti-HLA I antibody stimulation. This effect could be inhibited by blocking PI3K and mTOR. Blocking Src had no effect on the VEGF mRNA expression after antibody stimulation. Therefore, we conclude that Src signaling occurs after the production of VEGF. Using VEGF neutralizing antibodies in the EC supernatants, the extracellular action of VEGF on VEGFR2, which leads to decreased VE-cadherin expression, was demonstrated. Neutralizing VEGF antibodies captured VEGF secreted from ECs, which inhibited the activation of VEGFR2.

Increased endothelial proliferation after anti-HLA I administration is known to be mTOR dependent (*8*). To link this knowledge with our findings, we performed in vitro proliferation assay of ECs in the presence of anti-HLA I antibodies. Thereby we were able to demonstrate that endothelial proliferation increased dose-dependently in the presence of anti-HLA I

antibodies. This effect was inhibited by blocking mTOR and VEGFR2, but not by blocking Src. This indicates that the increased endothelial proliferation is also mediated by the production of VEGF. In contrast to the increased endothelial permeability, the increase of endothelial proliferation was not Src dependent. After activation of VEGFR2 by VEGF, two distinct pathways are activated: on one hand, endothelial permeability increases in a Src dependent manner; on the other hand, VEGFR2 induces endothelial proliferation in a Src independent manner.

Both phenomena described above might be involved in graft rejection. The formation of interstitial edema occurs due to increased endothelial permeability, which might be followed after VE-cadherin degradation. Increased endothelial proliferation could contribute to vasculopathy and cause chronic rejection.

Taken together, we describe a complement independent mechanism that can favor graft rejection. The clinical importance of these in vitro findings has to be further investigated in vivo. However, this study has several limitations. First of all, an antibody against the constant α3 domain of HLA I was used. This antibody will not occur in patients, as it would cause autoimmunity. For in vitro investigations, an antibody against a constant region of HLA I had to be selected, as HLA I is very polymorphic. Furthermore, all experiments were only performed on ECs isolated from umbilical veins. In the human body, there are many different ECs, which differ in their phenotypes. For example macrovascular ECs behave differently than microvascular ECs in some aspects.

To strengthen the clinical significance of the findings mentioned above, we are performing animal experiments to investigate the effect of anti-MHC I antibodies on the vasculature of mice. The same parameters as in the in vitro study will be determined in order to compare the effects of anti-HLA I antibodies on HUVECs to anti-MHC I antibodies on mice. Furthermore, the determination of VEGF-levels in blood of patients that have signs of humoral rejection is a further interesting aspect that should be adressed. VEGF in transplanted patients' blood could be introduced as a new marker for humoral rejection, which could be diagnostically applied in additionally to the C4d staining of biopsies.

6.2 Effect of mTOR Inhibitors on ECs

To prevent graft rejection, the immune system is suppressed by current immunosuppressive drugs. These drugs do not specifically suppress graft rejection; they inhibit certain immune cells to proliferate or to differentiate. As a consequence, patients having a suppressed immune system are more successible for any infections.
A clinical observation after immunosuppression using mTOR inhibitors is the occurrence of edema (Figure 6-1) (*9*).

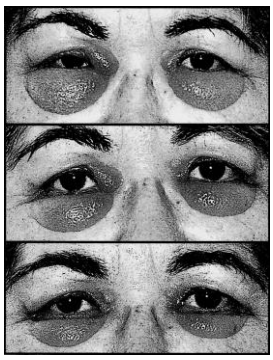

Figure 6-1: Patient showing eyelid edema. Upper panel: sirolimus administration of 5 month. Middle panel: 3 weeks after sirolimus withdrawal. Lower panel: 2 months after sirolimus withdrawal (reprinted from (*9*)).

There are many mechanisms, which could cause edema in patients (*10, 11*). We investigated endothelial permeability after mTOR inhibitor exposure as a possible reason for the development of edema in patients. SRL and ERL are both mTOR inhibitors, that differ only in the C(40) position of the molecule (Figure 4.2-2). As we demonstrated, mTOR inhibitors increased endothelial permeability in vitro. To compare this effect with other currently used immunosuppressive drugs, we further tested the influence of FK-506, which shares structural and binding properties with SRL and ERL, but which inhibits calcineurin and not mTOR (*12*). CsA, another calcineurin inhibitor, was also exposed to ECs. Both of these calcineurin inhibitors had no influence on endothelial permeability. As a marker for increased permeability, we investigated the expression of VE-cadherin after mTOR and calcineurin inhibition (*4*). VE-cadherin protein and mRNA expression decreased in SRL and ERL stimulated ECs, whereas FK-506 and CsA had no influence. The influence of mTOR in the biosynthesis of VE-cadherin was confirmed by specific knock-down of mTOR by sort interference RNA (siRNA) experiments.
To understand more about the mechanism of VE-cadherin biosynthesis, PI3K, an upstream protein kinase of mTOR, was blocked by the pharmacological inhibitor Wortmannin. Similar effects than on mTOR inhibitor stimulated ECs were measured. Wortmannin inhibition of PI3K in ECs decreased the protein and mRNA expression of VE-cadherin. Therefore, we concluded that PI3K and mTOR signaling is important in biosynthesis of VE-cadherin.

Furthermore, effects of mTOR and calcineurin inhibitors on endothelial reactive nitrogen and oxygen species (RNOS) production were determined. Only mTOR inhibitors increased the production of RNOS in ECs. The antioxidative reagent N-Ω-nitro-L-arginine methyl ester (L-NAME) and N-acetyl-cystein (NAC) inhibited the mTOR inhibitor induced RNOS production. The decrease of VE-cadherin and increase of endothelial permeability was also inhibited by NAC. Therefore, we conclude that oxidative stress is involved in the down-redulation of VE-cadherin and in increased endothelial permeability.

Herewith, we demonstrated that mTOR inhibitors decreased VE-cadherin expression on the protein and mRNA level. Furthermore, mTOR inhibitor induced RNOS production in ECs. Both findings could be linked theoretically: Inhibition of mTOR leads to decreased biosynthesis of VE-cadherin. VE-cadherin is able to activate eNOS, which is involved in RNOS production. This could boost the physiological effect of mTOR inhibition. However, this hypotetical mechanism has to be proven.

The results of the present study indicate a potential application of antioxidants to prevent dysfunctional endothelial prermeability during mTOR inhibition due to immunosuppressive drugs. If antioxidant drugs might prevent the occurrence of edema in mTOR inhibitor treated patients has to be proven as well.

6.3 Complexicity of Signaling

Many different signaling molecules are involved in innumerable cellular processes. Individual kinases are even involved in different pathays. In this work, we observed the converse effects of PI3K and mTOR signaling on the expression of VE-cadherin (Figure 6-2): Activation of PI3K and mTOR by agonistic anti-HLA I antibodies led to decreased VE-cadherin expression. On the other hand, inhibition of PI3K and mTOR decreased VE-cadherin expression too. The read out of VE-cadherin protein expression is completely different in these two cases. To understand the different actions of mTOR and PI3K, the exact underlying mechanism has to be examined. After anti-HLA I stimulation of ECs, the mRNA level of VE-cadherin does not change, but ECs start to produce VEGF, which induces the decrease of VE-cadherin. Other than in anti-HLA I stimulated ECs, mTOR inhibited ECs decrease the biosynthesis of VE-cadherin. mTOR inhibition results in the down-regulation of VE-cadherin mRNA. To conclude, mTOR inhibition directly regulates VE-cadherin biosynthesis, whereas anti-HLA I antibodies induce VEGF production and decrease of VE-cadherin after post-translational modification.

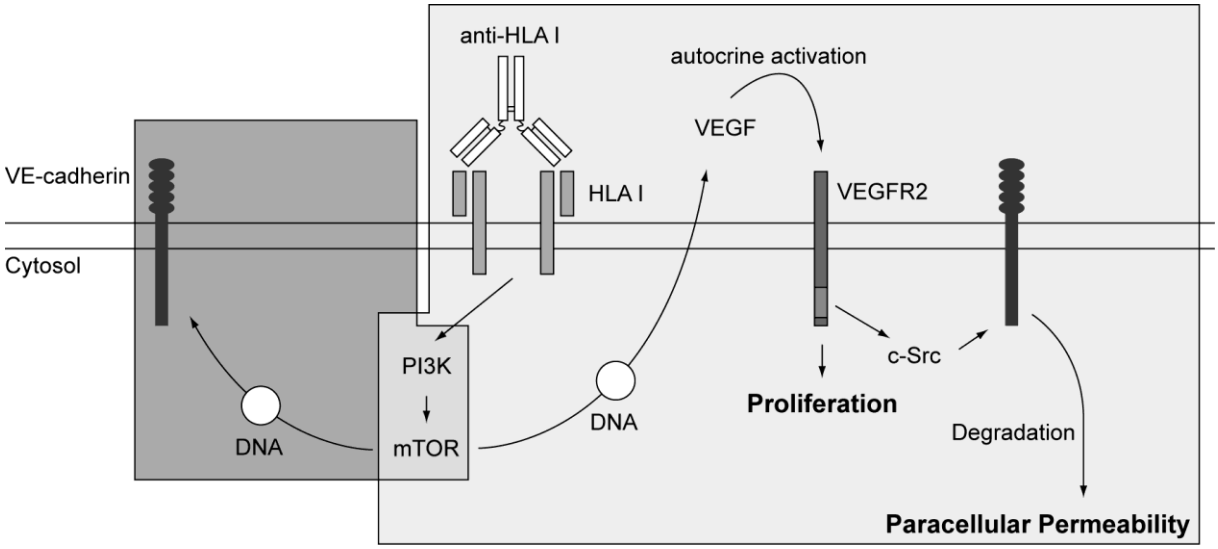

Figure 6-2: Signaling complexicity. Anti-HLA I antibodies are able to decrease the expression of VE-cadherin. This occurs by activation of PI3K and mTOR, which induces the production of VEGF. VEGF activates VEGFR2 in an autocrine manner that results in the phosphorylation of VE-cadherin. On the other hand, inhibition of PI3K and mTOR also leads to decreased VE-cadherin expression. Other than after anti-HLA I stimulation, not a post-tranlational process is involved. Inhibition of PI3K and mTOR controls and decreases mRNA level of VE-cadherin.

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8 CURRICULUM VITAE

Name: Michael Damian Bieri
Academic Title: MSc Chemistry, dipl. chem.
Date of Birth: 20.11.1979
Citizenship: Swiss
Private Address: Stürlerstr. 2, 3006 Bern, Switzerland
Work Address: Department of Clinical Research, Cardiology HTx
Patho L621, Murtenstr. 31, 3010 Bern, Switzerland
Tel.: +41 31 632 13 82, Fax: +41 31 632 88 37
E-mail: michael.bieri@students.unibe.ch
Web: http://www.cvrc.dkf.unibe.ch
Marital Status: unmarried

Education:

2005 to present **PhD** in *Biochemistry (Graduate School for Cellular and Biomedical Sciences),* Department of Clinical Research, Laboratory of Heart Transplantation Immunology, University of Bern, Prof. P. Mohacsi, *"Humoral Immune Responses in Cardiac Graft Rejection"*

2003 **Master** in *Bioorganic Chemistry*, Department of Chemistry and Biochemistry, University of Bern, Prof. Ch. Leumann, *"Development of an Assay for cAMP specific Phosphodiesterases" (in german)*

1999 – 2003 University of Bern, Studies in *Chemistry*, Department of Chemistry and Biochemistry

1994 – 1999 Freies Gymnasium Bern, Matura Typ C (Grammar School)

Specializations:

Biochemistry, Chemical Synthesis, Immunology, Molecular Biology, Protein Chemistry, Spectroscopy / Spectrometry, Vascular Biology

Languages:

German (mother tongue), English (good oral and written), French (oral and written), Italian (basics)

Publications:

P-1 BIOSYNTHESIS AND EXPRESSION OF VE-CADHERIN IS REGULATED BY THE PI3K / mTOR SIGNALING PATHWAY*;* **Michael Bieri**, Melinda Oroszlan, Christian Zuppinger, Paul Mohacsi; *Mol Immunol*, accepted 2008

P-2 PROLIFERATION SIGNAL INHIBITORS-INDUCED DECREASE OF VE-CADHERIN EXPRESSION AND INCREASE OF ENDOTHELIAL PERMEABILITY *IN VITRO* ARE PREVENTED BY AN ANTIOXIDANT; Melinda Oroszlan, **Michael Bieri**, N. Ligeti, A. Farkas, Simon C. Koestner, Bernhard Meier, Paul Mohacsi; *J Heart Lung Transplant*, accepted 2008

P-3 ANTI-HLA CLASS I ANTIBODIES INCREASE ENDOTHELIAL PROLIFERATION AND PERMEABILITY BY AUTOCRINE VEGF ACTIVATION, **Michael Bieri**, Melinda Oroszlan, Nathalie Liegti, Jürg Bieri, Paul J. Mohacsi; submitted

Abstracts:

A-1 *Rapamycin downregulates VE-cadherin expression and enhances permeability of cultured human endothelial cells*, M. Oroszlan, **M. Bieri**, S. Koestner, P. Mohacsi, 12th Cardio-vascular and Clinical Implications Meeting, Muntelier, 5.-6.10.06

A-2 *Proliferation signal inhibitors downregulate VE-cadherin expression and enhancepermeability of cultured human endothelial cells*, M. Oroszlan, **M. Bieri**, S. Koestner, P.Mohasci, Tag der klinischen Forschung, 15.11.2006

A-3 *Der Stellenwert des Seattle Heart Failure Models zur Risikoratifizierung bei Patienten mit Schwerer Herzinsuffizienz*, R. Frank, M. Martinelli, **M. Bieri**, T. Carell, P. Mohacsi,Schweizerische Gesellschaft für Kardiologie

A-4 *Proliferation signal inhibitors downregulate cadherin expression and enhance permeability of cultured human endothelial cells: a possible explanation for development of edema in PSI treated patients?,* M. Oroszlan, **M. Bieri**, S. Koestner, B. Meier, P. Mohacsi, Sweizerische Gesellschaft für Kardiologie

A-5 *mTOR-signaling is involved in VE-cadherin expression in human umbilical vein endothelial cells*, **M. Bieri**, M. Oroszlan, S. Koestner, P. Mohacsi, 1st Graduate School Students' Symposium, 16.3.2007

A-6 *mTOR-signaling controls VE-cadherin expression in human umbilical vein endothelial cells*, **M. Bieri**, M. Oroszlan, S. Koestner, P. Mohacsi, Wolfsberg Meeting 2007, 26. - 28.3.2007

A-7 *Proliferation signal inhibitors regulate VE-cadherin expression in human umbilical vein endothelial cells*, **M. Bieri**, M. Oroszlan, S. Koestner, P. Mohacsi, ESC Congress 07, Vienna, 1. – 5.9.2007

A-8 *Proliferation Signal Inhibitors (PSI) Downregulate Cadherin Expression and Enhance Permeability of Cultured Human Endothelial Cells: A Possible Explanation fro Development of Edema in PSI Treated Patients?*, M. Oroszlan, **M. Bieri**. S. Koestner, P. Mohacsi, American Transplant Congress, San Francisco, USA, 5.-9.5.2007

A-8 *Expression of vascular endothelial cadherin is regulated by the PI3K / mTOR signaling pathway*, **M. Bieri**, M. Oroszlan, S. Koestner, C. Zuppinger, P. Mohacsi, Basic Science Symposium 2007, Halifax, Canada, 5. – 8.9.2007

A-9 *anti-HLA I antibodies induce proliferation and decrease VE-cadherin expression in endothelial cells,* **M. Bieri**, N. Ligeti, M. Oroszlan, J. Bieri, P. Mohacsi, 13th Cardiovascular Biology and Clinical Implications Meeting, 4.-5.10.2007

A-10 *Effects of anti-HLA I antibodies on endothelial cell proliferation and VE-cadherin expression in vitro*, **M. Bieri**, N. Ligeti, M. Oroszlan, J. Bieri, P. Mohacsi, Tag der klinischen Forschung 2007, 7.11.2007

A-11 *Increased Proliferation and Permeability of Cultured Endothelial Cells after anti-HLA I Stimulation*, **M. Bieri**, N. Ligeti, M. Oroszlan, D. Stalder, J. Bieri, P. Mohacsi, 2nd Graduate School Students' Symposium, 31.1.2008

A-12 *Increased Endothelial Permeability and Proliferation induced by anti-HLA I antibodies*, **M. Bieri**, N. Ligeti, M. Oroszlan, D. Stalder, J. Bieri, P. Mohacsi, 28.-30.5.2008, Jahrestagung der Schweizerischen Gesellschaft für Kardiologie (SGK)

A-13 *Molecular Effects of anti-HLA I Antibodies on Endothelial Proliferation and Permeability*, **M. Bieri**, M. Oroszlan, N. Ligeti, D. Stalder, M. Heller, J. Bieri, P. Mohacsi, 14th Cardiovascular Biology and Clinical Implications Meeting, 2.-3.10.2007

A-14 *Complement Independent Antibody Mediated Rejection by Autocrine VEGF Activation*, **M. Bieri**, M. Oroszlan, N. Ligeti, D. Stalder, M. Heller, J. Bieri, P. Mohacsi, Tag der Klinischen Forschung, 2008, 5.11.2008

Presentations:

T-1 *Expression of vascular endothelial cadherin is regulated by the PI3K / mTOR signaling pathway*, Basic Science Symposium 2007, Halifax, Canada, 5. – 8.9.2007

T-2 *HLA I antibodies induce endothelial proliferation and decrease VE-cadherin*

expression: a complement independent role of humoral rejection?, Cardiovascular Research Conference, University Hospital Bern, 18.12.2007

T-3 *Increased Proliferation and Permeability of Cultured Endothelial Cells after anti-HLA I Stimulation*, 2nd Graduate School Students' Symposium, Department of Chemistry and Biochemistry, University of Bern, 31.1.2008

T-4 *Endothelial Dysfunctions Mediated by anti-HLA I antibodies*, Research Presentation of the Laboratory of Heart Transplantation Immunology, Inselspital, Bern, 5.2.2008

T-5 *Endothelial Dysfunctions Mediated by HLA I Antibodies, 2nd Year Exam of Graduate School of Cellular and Biomedical Sciences*, Inselspital, Bern, 15.4.2008

T-6 *Increased endothelial permeability and proliferation induced by anti-HLA class I antibodies*, Jahrestagung der Schweizerischen Gesellschaft für Kardiologie und der Schweizerischen Gesellschaft für Angiologie, BEA Expo, Bern, 28. – 30.5.2008

T-7 *Biochemical Effects of HLA I Antibodies on Endothelial Cells*, HTx Research Presentation,Inselspital, Bern, 3.7.2008

T-8 *Molecular Effects of anti-HLA I Antibodies on Endothelial Permeability and Proliferation*, 14th Cardiovascular Biology and Clinical Implications Meeting, Muntelier, 2.-3.10.08

9 DECLARATION OF ORIGINALITY

Last name, first name: **Bieri, Michael**

Matriculation number: **99-105-421**

I hereby declare that this thesis represents my original work and that I have used no other sources except as noted by citations.
All data, tables, figures and text citations which have been reproduced from any other source, including the internet, have been explicitly acknowledged as such.
I am aware that in case of non-compliance, the Senate is entitled to divest me of the doctorate degree awarded to me on the basis of the present thesis, in accordance with the "Statut der Universität Bern (Universitätsstatut; UniSt)", Art. 20, of 17 December 1997.

Place, date Signature

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