The Clinical Importance Of Anti-Endothelial Cell Antibodies In Chronic Kidney Dysfunction And Organ Transplantation

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ABSTRACT

The endothelium is the thin layer of cells that lines the interior surface of blood vessels and is the first site of contact between immune cells and tissues. Therefore, alterations at the level of the endothelium are crucial to understand the nature of vascular diseases and organ damage. The presence of anti-endothelial cell antibodies (AECAs) has been associated with such conditions. However, their immunomodulatory and clinical relevance is unclear.

This work focused on two clinical groups, patients with chronic kidney disease and heart- and vein-transplanted patients. In chronic kidney disease patients, some biological mechanisms by which AECAs may affect the kidney microvascular permeability were explored. In transplanted patients, special emphasis on the clinical contribution of these antibodies to monitor graft outcome pre- and post-transplantation was analyzed. Detection of AECAs using different tissue endothelial cells as targets and experimental *in vitro* assays were utilized to evaluate immune interactions of AECAs.

In patients with end-stage renal disease, AECAs were specifically reactive with kidney endothelial cells. *In vitro*, these antibodies decreased the expression of intercellular junction assembly proteins altering endothelial permeability. In heart-transplanted patients, the presence of autologous AECAs post-transplantation was associated with the presence of donor-specific AECAs and early rejection episodes. In the context of a first case report of a bioengineered transplanted vein, AECAs appeared after nearly a year post-transplantation.

In summary, AECAs in patients with chronic kidney diseases may be novel biomarkers of kidney endothelial dysfunction, and may be used as indicators of renal diseases. Additionally, AECAs have important implications in organ transplantation regarding the development of techniques for improved donor organ selection and monitoring of recipients. Results may also be extended to new approaches in tissue engineering.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Förändringar i det innersta cellagret av blodkärl – endotelet - har visat sig ha stor betydelse för utveckling inom djurriket. Förändringar är särskilt viktiga i samband med uppkomst av många sjukdomar som drabbar våra organ. Förekomst av antikroppar riktade mot endotelet (AECAs) har påvisats mot en rad sjukdomar, men den exakta betydelsen och mekanismen av AECAs är däremot dåligt känd.

Avhandlingen fokuserar på förekomst av AECA vid njursjukdom, vid hjärttransplantation samt vid regenerering av vener använda för att ersätta en icke fungerande ven till levern från tarmen hos barn.

Hos njursjuka patienter påvisades AECAs kopplade till förbindelse proteiner i kärlväggen. Den föreslagna verkningsmekanismen är att genomsläppligheten av äggviteämnen ökar, med skada av njurfunktionen som följd.

Hos hjärttransplanterade patienter medförde förekomst av AECAs riktade mot den egna vävnaden att risken för uppkomst av skadliga vävnads antikroppar mot givaren (DSA) ökade. Detta kan innebära en ökad risk för både akut och kronisk avstötning av hjärtat efter transplantation.

Vid regenerering av ven från ett acellulärt extra cellulärt (ECM) matrix från givarblodkärl kunde AECAs riktade mot egen vävnad upptäckas efter ett år, men det är osäkert om mängden är av signifikans eftersom materialet är litet och det är svårigheter att veta hur stor förändring från kontroll individer som ska anses vara signifikant.

Sammanfattningsvis kan AECAs få en potentiell roll som markör vid vissa njursjukdomar. Vidare förekomst av AECAs vid hjärttransplantation vara en markör för akut eller kronisk avstötning och motiverar därför ytterligare studier. Vid regenerering av vävnader och organ – en ny teknik som börjat användas på patienter – är det oklart om AECAs innebär en risk för patienten. Ytterligare studier är därför motiverat även här.

LIST OF PUBLICATIONS

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II. Sigurdardottir V, Kolsrud O, **Hernandez N**, Dellgren G. Endothelial cell antibody-mediated rejection and successful retransplantation in a heart transplanted patient. Eur J Cardiothorac Surg, May 2012; 42(6):1044-1046.

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IV. Olausson M, Patil PB, Kuna VK, Chougule P, **Hernandez N**, Methe K, Kullberg-Lindh C, Borg H, Ejnell H and Holgersson-Sumitran S. Transplantation of an allogeneic vein bioengineered with autologous stem cells: a proof-of-concept study. Lancet, Jun 2012; 380(9838):230-237.

ABBREVIATIONS

AECAs	Anti-Endothelial Cell Antibodies
AMR	Antibody-Mediated Rejection
CKD	Chronic Kidney Disease
CDC	Complement Dependent Cytotoxicity
CVD	Cardio Vascular Disease
DSA	Donor Specific Antibodies
ECs	Endothelial Cells
EPCs	Endothelial Progenitor Cells
EMB	Endo Myocardial Biopsy
ESRD	End-Stage Renal Disease
FC	Flow Cytometry
GCM	Giant Cell Myocarditis
HLA	Human Leukocyte Antigen
HAECs	Human Aortic Endothelial Cells
HKECs	Human Kidney Endothelial Cells
HLECs	Human Lung Endothelial Cells
HCMECs	Human Cardiac Micro Vascular Endothelial Cells
HUVECs	Human Umbilical Vein Endothelial Cells
HBSS	Hank's Balanced Salt Solution
ICAM	Intercellular Adhesion Molecule
ICC	Immunocytochemistry
IHC	Immunohistochemistry
IF	Immunofluorescence
INR	International normalized ratio
Ig	Immunoglobulin
ITP	Idiopathic thrombocytopenic purpura
MCDB	Molecular Cellular and Developmental Biology
MHC	Major Histocompability Complex
MICA	MHC class I–related chain A
NK	Natural Killer
PBMCs	Peripheral Blood Mononuclear Cells
PBS	Phosphate Buffered Saline
RPMI	Roswell Park Memorial Institute
SLE	Systemic Lupus Erythemathosus
TA	Takayasu's Arteritis
VEGF	Vascular Endothelial Growth Factor
VCAM	Vascular Cell Adhesion Molecule
WG	Wegener´s Granulomatosis

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INTRODUCTION

Cardio Vascular Disease (CVD) is a group of diseases recognized as the number one cause of death worldwide. They are categorized within a pathological-conglomerate that affects primarily the heart and blood vessels. Although, there is no precise consensus as to the origin of CVD, several risk factors such as age, ethnicity, family history, high cholesterol, obesity, physical inactivity, unhealthy diets, diabetes, hypertension, tobacco exposure and immoderate use of alcohol have been attributed (1).

In physiological terms, a vascular network of arteries, veins and capillaries supports every organ in the body. Dysfunction of the vascular tree at different levels (e.g. macro and/or micro) leads to a chain of inflammatory events and improper blood supply resulting in organ damage (2). From this standpoint, a reciprocal relationship exists between the vascular health and organ health.

Vascular dysfunction is a complex process characterized by an imbalance of cellular components and vasoactive substances that alter the broad functionality of the endothelium. With more knowledge about the nature of the endothelial cells, vascular dysfunction is now viewed from a wider perspective, including the constant changes of damage/repair that the endothelium undergoes in response to biological, chemical and mechanical injuries (2, 3). For instance, an increasing number of reports suggest the importance of anti-endothelial cell antibodies (AECAs) in endothelial dysfunction, especially in autoimmune and inflammatory disorders, as biomarkers of disease activity (4). AECAs have also been regarded as important in organ transplantation. In particular, AECAs have been associated with allograft rejections of HLA-matched individuals (5, 6). Even though the presence of AECAs has been documented in conjunction with such conditions, there are controversial opinions and contradictory data about their clinical significance (7, 8). One of the fundamental reasons behind these inconsistencies is the lack of reliable methods for AECAs detection (8-10). Owing to the heterogeneity of endothelial cells, it is important to study the functionality of these antibodies using appropriate endothelial targets. In this work, the clinical relevance of AECAs in CKD and heart-transplanted patients was investigated. AECAs were also explored in the context of a bioengineered vein. Selective AECAs were detected by flow cytometry using specific endothelial cell targets from the kidney, heart and peripheral endothelial progenitor cells.

Endothelium

The endothelium (from the modern Latin endo "within" and the Greek thēlē "nipple", which together suggest “deep within our breast”) constitutes the monolayer of cells that cover the inner surface of blood vessels and has direct contact with the bloodstream. The endothelium consists of approximately 60 trillion endothelial cells and has a mass and equivalent surface area of approximately 1kg and more than 4000m^2 respectively (11). Historically, it was conceived as a mere homogeneous and inert membrane of cells. This notion changed when electron microscopy studies revealed that the endothelium from various organs and different vascular sites within the same organ was physically distinct (12). Currently, it is considered an integrated organ system because it is actively involved in many physiological processes as a driving force controlling homeostasis, permeability selection, vasomotor tone, leukocyte trafficking, immunity and angiogenesis (13). Therefore, endothelial cells (ECs) are not a simple barrier between blood fluid and underlying tissue. They also act as an endocrine and paracrine organ. These fundamental characteristics explain the reason why the ECs display remarkable heterogeneity in space, time, structure, health and disease.

Endothelial heterogeneity

Endothelial cell heterogeneity is the core of the endothelial system. Although the precise properties of how ECs acquire heterogeneity are unknown, it is believed that the dynamic and adaptive behaviour of ECs are determined by interactions of the endothelium with the surrounding tissue environment, soluble factors, and via cell–cell interactions (14). However, a genetic predisposition as phenotypic endothelial heterogeneity has also been ascribed (15). Evolutionary approaches also seek to explain the endothelial discrepancy. The endothelium throughout the body must provide a broad menu of functions that are adapted to the diverse needs of the underlying tissues. Site-specific differences in function will, in turn, be reflected by structural and molecular heterogeneity. Thus, ECs must adapt to different environments simply to survive. For example, ECs in the vasa recta of the inner medulla of the kidney are exposed to profoundly low levels of oxygen, in addition to high potassium (K^+) levels (16). These unique adaptations to “harsh” microenvironments render them phenotypically distinct from other endothelial types.

Endothelial cells may be classified by size, thickness, shape, and nuclear orientation. Depending on where they are located the morphology changes according to their functional need (17). Usually, a uniform or continuous layer of cells represents the endothelium of large vessels where adjacent ECs are held

together by tight junctions to limit fluid exchange between the plasma and the tissue fluid. Clear examples are found in most of vascular arteries, veins and capillaries from the central nervous system, dermis, skeletal tissue, cardiac smooth muscle and ovary tissues. ECs may also be lined as discontinuous layers to allow cellular trafficking between intercellular gaps such as in liver, spleen and bone marrow sinusoids. Other intercellular gaps common in ECs include the presence of fenestrae, as found in the intestinal mucosa and the renal glomeruli, which facilitates the selective permeability required for efficient absorption, secretion, and filtering. The only difference between discontinuous and fenestrated ECs is the diameter of the pore and the presence or absence of a diaphragm, Image 1 (18, 19).

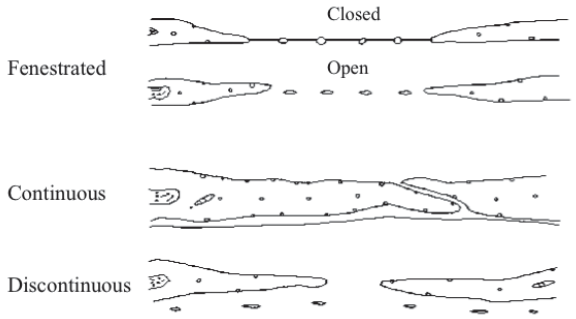

Image 1. Disposition of endothelial cell layers (Columbia University, Kung-ming, 2005)

Other functional ECs properties are also regulated by the expression of receptor proteins on the surface of the ECs membrane (20). The mechanisms on how ECs control this “opening and closing” of intercellular junctions are not well understood. It is accepted that trans-membrane adhesive molecules (gap junctions, tight junctions, adherens junctions and desmosomes) are linked to a network of cytoplasmic/cytoskeletal proteins, which work in a coordinated fashion to control endothelial permeability (21-23). When circulating inflammatory agents are present, this permeability increases by binding ECs receptors and generating intracellular signals, which in turn cause cytoskeletal reorganization and opening of inter-endothelial cell gaps (24).

Endothelial progenitor cells (EPCs)

Despite initial research focused on the endothelial cells of vascular tissues, more recent studies on endothelial damage suggest the existence of a subpopulation of

circulating vascular repair cells such as EPCs. Their number correlates inversely with the degree of disease suggesting that EPCs may serve as a surrogate biomarker of vascular function and cardiovascular risk (25-27).

Most publications define EPCs as endothelial cells, which express hematopoietic cell markers such as VEGFR+, CD34+, CD133+ or Tie-2. These cells may have multiple sources of origin such as hemangioblasts, non-hematopoietic mesenchymal precursors in bone marrow, monocytes and tissue resident stem cells (28-31). Accepted theories suggest that the number of adult EPCs increases in the blood in conjunction with the manifestation of vascular emergency where they arrive to the site of vascular damage to propagate the formation of a new blood vessel (30-32). Although these cells have been reported in healthy individuals and different diseases and conditions, their mode of action is still not clear.

The endothelium in diseases

Extrinsic or intrinsic alterations of the endothelium may result in a dysfunctional state affecting the vascular network in a local or systemic fashion. These irregularities were previously associated with a limited number of diseases related to arteriosclerotic disorders. Recent work suggests an expanded view regarding the role of the endothelium. Given that the endothelium is multifunctional and highly distributed in space, it is now thought to play an important role in most if not all diseases, either as a primary target of pathophysiology or as a victim of collateral endothelial cell injury (33).

Antibodies

Antibodies are a group of globular proteins called immunoglobulins (Ig) produced by plasma cells and found in blood, secretions and other fluidic tissues. They are formed of many various amino acid sequences and are the most diverse proteins known (34). The function of an antibody is to bind to an antigen, which is normally a foreign or non-self molecule. This binding capacity is determined by their chemical structure conferring them distinctive features of binding versatility, binding specificity and biological activity (34, 35).

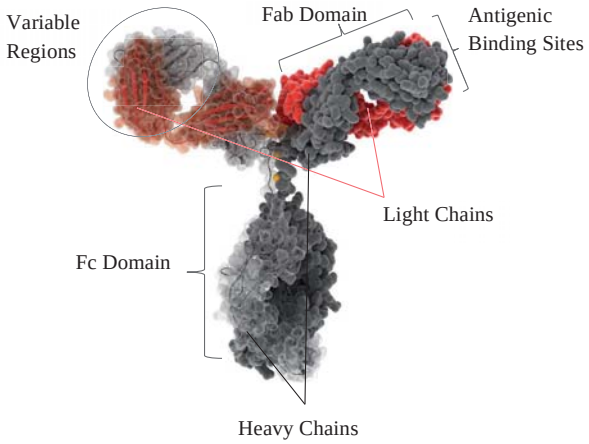

Image 2. Three-dimensional structure of a typical IgG antibody (Visual Science, 2014)

Generally, antibodies are Y-shaped consisting of two heavy and two light chains linked by disulfide bonds and non-covalent interactions (Image 2). In the two arm regions (for both, heavy and light chain), they have an antigenic-binding region (Fab fragment) that functions as an antigenic binding site. Each chain has a variable region at the end of both arms (circle). The rest of the chain is considered as a constant region. The tail-region of the heavy chain, which decides the biological activity of the antibody, is known as the fragment crystallizable (Fc) region. The Fc region defines whether the response against a particular antigen will lead to complement-mediated lysis, enhanced phagocytosis, or allergy (36). Because the amino acid sequence differs in the arms of various antibody molecules, each antibody can bind specifically to one unique antigenic determinant. Thus, the arms of an antibody molecule confer the versatility and specificity of responses that a host can mount against antigens (37).

Based on differences in the amino acid sequences in the constant region of the heavy chains, there are five major types of antibodies known as IgG, IgM, IgA, IgE and IgD (37).

IgG has a monomeric configuration and is the most common and versatile of all the antibodies in the body (around 70-75% in serum). They are the only type of antibodies that pass to a fetus through a mother's placenta and are particularly important to fight off infections. IgG fix complement (a group of circulating proteins of the immune system), promotes neutralization, agglutination, and opsonization of the antigen (37).

IgM is the larger antibody and usually has a pentameric structure. It is found in serum (10-15%) and lymph fluid and is the first antibody to appear during an immune response. Because of its many antigen-binding sites, IgM can quickly clump antigen and efficiently activate complement. IgM acts as one of the main receptors on the surface of mature B cells, along with IgD. When IgM is a surface receptor, it is in its monomeric form (35, 36). IgM also activates macrophages to phagocytize pathogens.

IgA antibodies (10-15% in serum) are dimers, predominantly present in secretions from the lung and intestine as well as mucus, saliva, breast milk and tears. Normally IgA does not fix complement, unless aggregated. They protect body surfaces that are exposed to outside foreign substances (mucous membranes) (38).

The monomer IgD constitutes less than 1% in human serum and they have been found in tissue lining of the chest and stomach. IgD is an antibody whose function remains unknown, even though it is one of the main receptors on mature B cells. As B cells mature IgD is replaced by other antibodies. IgD may be a regulator of immune responses through its role in antigen internalization (36, 39)

IgE makes up less than 0.003% of the antibody in serum. IgE is a monomer and binds through its Fc part to mast cells or basophils (a type of white blood cells). On later exposure to the same antigen, mast cells and basophils bind antigen with membrane-bound IgE and trigger allergic reactions. For that reason IgE levels are often high in people with allergies (e g. pollen, fungus spores, animal dander, some food, medicines and poisons). IgE protects against parasites by releasing mediators that attract eosinophils (36, 39).

Common aspects of Anti-endothelial cell antibodies (AECAs)

AECAs were first reported in the 1970's in studies with tissues of kidney biopsies and sera of patients with rheumatic diseases and Systemic Lupus Erythematosus (SLE) (40, 41). In addition, they have been reported predominantly in systematic autoimmune and vascular disorders such as Rheumatoid Arthritis, Wegner Granulomatosis, Kawasaki Disease, Behcet's Disease, Takayasus Arteritis, Microscopic Polyangiitis, SLE and Inflammatory Bowel Diseases (4, 42-46), and their presence has been also described in organ transplantation in association to rejections and in normal individuals as a set of natural antibodies (47, 48).

AECAs belong to a heterogenic group of immunoglobulins that covers a large spectrum of target antigens that may be constitutively expressed or induced by cytokines and adherent molecules on ECs (7). Different isotypes of IgG, IgM and IgA have been mostly documented and several studies suggest that AECAs from

different sources recognize multiple types of ECs target molecules (7, 46, 47, 49, 50), see Table 1. These isotypes bind mainly through the $F(ab)_2$ portion of the Ig and might be tissue-specific and correlated to the disease origin (51-53).

Table 1. Antigenic specificities of AECAs in healthy individuals and different diseases.

Condition	Target antigen	Pathogenicity
Normal Individuals	β-Actin, α-Tubulin, Vimentin Prolyl-4-hydroxylase β-Glyceraldehyde-3-phosphate-deshydrogenase α-Enolase	
Systemic lupus	DNA-DNA-histone	
Erythematosus	Ribosomal P protein PO Ribosomal protein L6 Elongation factor 1-alpha Adenylyl cyclase-associated protein Profilin 2 Plasminogen activator inhibitor Fibronectin Heparan sulfate β2-glycoprotein I Heat-shock protein 60 (Hsp 60) Heat-shock protein 70 (Hsp 70) Fibronectin leucine-rich transmembrane protein 2 (FLRT2)	Apoptosis Complement dependent cytotoxicity
Mixed connective tissue disease	Voltage-dependent anion-selective channel 1 (VDAC-1)	
Systemic sclerosis	Topoisomerase I Centromere protein B (CENP-B)	
Vasculitis	Proteinase 3 Myeloperoxidase Peroxiredoxin 2 Adenosine triphosphate (ATP) synthase	Cytokine secretion Intracellular acidification
Microscopic polyangiitis	Human lysosomal-associated membrane protein 2	
Behçet's disease	Alpha-enolase C-terminus of Ral-binding protein 1 (RLIP76)	Apoptosis
Kawasaki disease	Tropomyosin T-plastin	
Thrombotic thrombocytopenic purpura	Glycoprotein CD36	
Heparin-induced thrombocytopenia	Platelet factor 4 (PF4) Heparin sulfate	

Whether AECAs exert a pathophysiological effect is poorly understood. Investigations have shown that in pathological conditions by concurrent inflammation of different types of blood vessels, AECAs recognize a large panel of antigens on ECs (47). They mediate their effects via increasing leukocyte adhesiveness, apoptosis, cytotoxicity, coagulation, and thrombosis (54-57). This is in contrast to the case of healthy individuals in which AECAs react with a limited number of self-antigens (47, 58).

Observations in patients with Takayasus Arteritis (chronic inflammation of the large blood vessels that distribute blood from the heart, including the aorta and its main branches) have shown that AECAs may cause vascular dysfunction by inducing expression of adhesion molecules E-selectine, VCAM-1 on aortic endothelial cells as well as production of interleukins IL-4, -6 and -8, and apoptosis (59). However, these AECAs have also been detected on other endothelial targets, where they may bind and activate macrovascular ECs from umbilical cord and not microvascular immortalized ECs from bone marrow. This occurs through elevation of adhesion molecule expression associated with NF-kB activation and adhesion of monocytes, and may therefore play a pathogenic role in the development of vasculopathies (60).

Other instances of the etiological role of AECAs in SLE and Wegner Granulomatosis patients include up-regulation of proinflammatory cytokines and chemokines with leukocytic accumulation and display of proadhesive endothelial proteins (53). Different data also suggest that AECAs might mediate endothelial changes through complement fixation or cytotoxic effector cells causing ECs lysis (61).

AECAs in Chronic Kidney Disease

Chronic kidney disease (CKD) describes the gradual loss of kidney function over a period of time. The primary function of the kidneys involves the regulation of blood composition by removing wastes from the body (e.g. urea, ammonia, drugs, toxic substances), keeping constant acid/base concentrations, and regulating calcium levels and water volume (62, 63). Disruption of this integrity is thought to be result of ongoing endothelial damage in the capillary system (blood-urine interface, glomeruli) of the renal medulla, accompanied by small vessel disappearance or reduced blood flow leading to microalbuminuria, a clinical sign of “kidney leakage” (64-66). However, the molecular mechanisms by which the kidney loses the microvascular capacity for controlling the glomerular permeability are unclear.

During the course of CKD, a large proportion of patients develop cardiovascular disease (67). Although diverse factors are implicated, it has been considered that endothelial dysfunction represents a key modulator mechanism by which the endothelium of the vascular system is perturbed accompanying the progressive deterioration of the renal function (68, 69).

In previous publications about endothelial cell dysfunction, a high presence of AECAs in patients with CKD has been reported. In a study model, using patients with Wegner Granulomatosis disease (a vascular disorder with inflammation of the respiratory tract and necrotizing of small blood vessels resulting in pulmonary hemorrhage and renal failure), it was found that AECAs were associated with tissue specific endothelial cells from kidney, nose and lung but not umbilical cord (70). These results suggest that AECAs in Wegner Granulomatosis appear to have a correlation with organ distribution. In addition, important observations regarding the possible role of stimulated ECs with inflammatory cytokines IFN- γ and TNF- α, show alterations in the accessibility of ECs surface molecules, resulting in a significant decrease of AECAs binding (70, 71).

Patients with end-stage of renal disease (ESRD) are prone to develop severe CVD. This group of patients represents the worst-case scenario of kidney damage and these patients are good candidates for studying the mechanisms of localized microvascular dysfunction and the poorly understood way that tissue-specific AECAs affect the renal microvascular circulation. It is therefore clinically relevant to explore the functional effects of AECAs as a mediator of increased ECs permeability on a controlled group of kidney ECs.

AECAs in Transplantation

In organ transplantation it is accepted that the presence of antibodies to human leucocyte antigens (HLA) are detrimental to patient survival and function. Furthermore, the presence of HLA is known to increase the probability of rejection of the transplanted organ (72).

HLA - or the Major Histocompatibility Complex (MHC) - is encoded by a group of genes on chromosome 6 giving rise to most of the polymorphic proteins residing on the surface of almost every cell in the body (73). There are two major types of HLA antigens, HLA class-I (HLA-A, -B and -C) and HLA class-II (HLA-DR, -DQ and -DP). The prime function of these antigens is to serve as recognition molecules in the initiation of an immune response where they present peptides from foreign

substances to effector cells. These peptides are responsible for driving both the cellular and the humoral immunity (74).

Traditional tests of immune histocompatibility between donors and recipients include HLA typing (to match genetically similar individuals), blood group (ABO) and a lymphocytic crossmatch test (75). Patient serum is tested for their reactivity with donor T- and/or B-lymphocytes, which is detected by the presence of Donor Specific anti-HLA Antibodies (DSA). In patients awaiting a kidney transplant, the presence of DSA is strongly associated with acute Antibody Mediated Rejection (AMR) (76, 77). Therefore, the immune histocompatibility match plays an important role in the success of kidney transplantation. For liver and pancreas transplants, an exact match is less influential. Heart and lung patients generally profit from matching as well, although urgency is the major criterion for allocating these organs (78).

Naturally occurring HLA antibodies may be present in some individuals (79). Indeed, natural anti-HLA-A2 and anti-B8 have been identified by in some patients where they belong to the IgM class (79-82). Apart from naturally occurring HLA antibodies, these antibodies may arise in individuals as a result of pregnancy, blood transfusion, or previous transplantation (83, 84).

However, knowing that pre-transplant HLA crossmatches are performed to identify patients at risk of losing their grafts and post-transplant immunosuppressive regimens are combined as part of the management to reduce rejections; in the absence of HLA antibodies or even in identical HLA-matches, rejections are still known to occur (85, 86). This suggests that other antigenic systems different to the traditional lymphocytic HLA or non-HLA are implicit in the etiology of organ rejection (5, 6, 87).

Since the endothelium of a donor organ is the first tissue that the recipient's blood encounters, the ECs are the most immediate targets for the host's immune system during allograft rejection. This is supported by the fact that ECs have been considered as important carriers of non-HLA antigens (88, 89). Investigations on organ injury and manifestations of rejections have suggested that AECAs, grouped as part of the non-HLA antibodies, are clinically important (48, 90, 91). AECAs do not react with lymphocytes; therefore, they are not detected by conventional crossmatch tests. Furthermore, currently their presence is not investigated in routine clinical procedure due to the lack of reproducible methods of detection. Nonetheless, characterization of some putative antigens recognized by AECAs has been reported in transplantation, Table 2, reviewed by Sumitran-Holgersson in (5).

Table 2. AECAs associated with graft rejection

Endothelial specificity	Type of transplantation	Type of rejection
Vimetin, Desmin	Heart	AR
Endothelial specific antibodies against a **100 kD** antigen	Kidney	HAR, AR
Endothelial-monocyte specific antibodies (HMA-1 and HMA-2)	Kidney	HAR, AR
MICA	Kidney	EGL, AR
Angiotensin II type-1 Receptor	Kidney	VR
α-Galactosidase	Xeno	HAR

AR=Acute rejections; **HAR**=Hyperacute rejections; **EGL**=Early graft loss; **VR**=Vascular rejection
MICA= MHC class I–related chain A

Mechanisms by which AECAs may cause endothelial dysfunction

Induction of proinflammatory phenotypes

AECAs may induce an EC phenotype that will support leukocyte extravasation through upregulated expression of adhesion molecules, e.g. E- and P-selectin (CD62E/P), VCAM-1 (CD106) and ICAM-1 (CD54), inflammatory cytokines (IL-1β, TNF-α) and chemokines such as IL-8 (92).

By inducing expression of co-stimulatory molecules (CD86), antibodies may facilitate direct T-lymphocyte activation by the endothelium (93). Further, AECAs have been shown to facilitate migration of human NK cells across porcine endothelium (94).

Augmentation of cellular immunity

Transforming growth factor beta (TGF-β) is a cytokine constitutively produced by several endothelial cells and is well known for suppressing T cell proliferation. It has been reported that binding of AECAs to Liver ECs in liver transplant patients down-regulates TGF-β levels, thereby enhancing cellular immune responses (93). Thus, increased T cell proliferation during liver allograft rejections could be a result of combined antigen presentation by liver ECs with decreased TGF-β levels. It can be speculated that AECA binding to an antigen expressed on the ECs of the

transplanted liver allograft may facilitate uptake of the antigen by antigen-presenting cells and presentation to allo-specific host T cells.

Induction of Prothrombotic phenotypes

In kidney transplant patients, MICA antibody-containing sera from patients may activate kidney ECs and induce a pro-thrombotic phenotype leading to vascular thrombosis and graft loss (95). Similarly, induced non-α-Galactosidade antibodies from baboons transplanted with porcine kidneys have been shown to induce tissue factor expression on pig ECs (96).

Induction of Morphological changes

In liver disease patients, it is reported that AECA binding to liver ECs can capillarize the sinusoidal endothelial cells by inducing production of a basement membrane, loss of fenestrae and formation of tight junctions (97). The ensuing consequences may result in the development of hepatocellular failure. Thus, morphological transformation of liver EC to vascular-type endothelial cells in patients with AECAs may have important clinical consequences. Capillarization of liver ECs in liver allografts have been reported during rejections, thus AECAs to liver ECs may mediate liver allograft rejections (98). Furthermore, AECAs may induce cell shape changes, motility, cytoskeletal redistribution and EC permeability. The clinical consequences of these changes are less well-known.

Apoptosis

AECAs have been reported to exert pro-apoptotic activity on ECs. It has been has shown that AECAs from pre-transplant kidney recipients may induce arterial EC death in vitro through apoptosis (91). However, whether AECAs promote or not endothelial apoptosis has to be further studied.

AIMS

The general aims of this work were:

1) To study the frequency and presence of AECA in patients with CKD and some transplanted patients.
2) To study the clinical relevance of these antibodies in endothelial dysfunction.

The specific aims of the study were:

- To explore mechanisms of endothelial dysfunction in CKD patients caused by AECAs reactive to renal microvascular endothelial cells (Paper I)

- To study the relationship between AECAs and early rejection events in heart-transplanted patients (Paper II and III)

- To screen for the presence of AECAs in a patient transplanted with a bioengineered vein (Paper IV).

METHODOLOGICAL CONSIDERATIONS

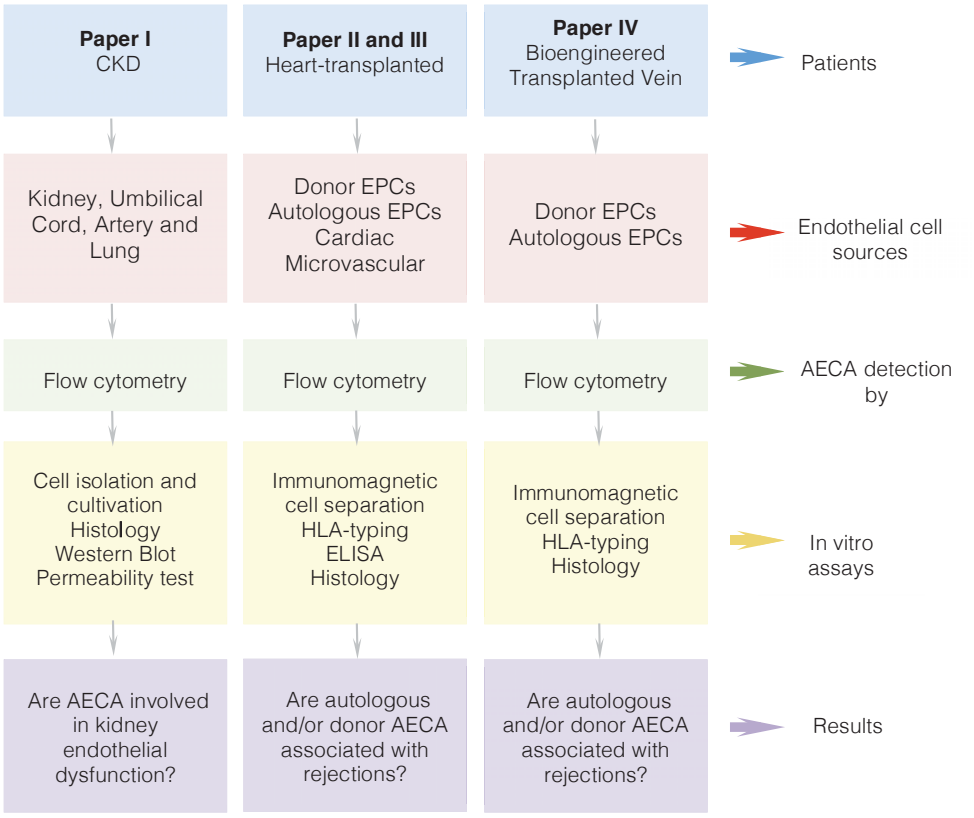

Diagram 1. Methodological overview to study AECAs

This section outlines how AECAs were studied (Diagram 1) and other general methodological considerations. For more details, please refer to the respective paper.

Patients

Paper I

Forty-five ESRD patients at the Karolinska University Hospital, Huddinge, Stockholm were included. The patients were enrolled at a time point close to the start of renal replacement therapy with a median glomerular filtration rate (GFR) of 5.7. The causes of CKD were: chronic glomerulonephritis in 10 patients, diabetic nephropathy in 14 patients, polycystic kidney disease in 4 patients, nephrosclerosis in 2 patients, and unknown etiologies in 15 patients. The presence of clinical CVD was defined by medical history, clinical symptoms, and/or findings of cardiac, cerebrovascular (stroke), and/or peripheral vascular disease. Other clinical characteristics are shown in Table 1 of this study.

Paper II

A 42-year-old male patient with ankylosing spondylitis (a form of arthritis causing chronic inflammation of the spine) and ulcerative colitis in remission was diagnosed with giant cell myocarditis (GCM - a rare cardiovascular disease where patients develop abnormal heartbeats, chest pain and even heart failure) at the Sahlgrenska University Hospital. According to endomyocardial biopsies (EMBs), the GCM regressed on cyclosporine, azathioprine and corticosteroids. He was stable for four years, but deteriorated thereafter with advanced heart failure.

Paper III

Twenty-five consecutive heart recipients were selected from the Sahlgrenska Transplant Institute´s Biobank for the isolation of PBMCs. Eleven patients were excluded due to lack of donor PBMCs or pre-transplant serum from the recipients leaving fourteen patients included in the study. The immunosuppressive regimen protocol consisted of mycophenolate mofetil, steroids and tacrolimus or cyclosporine (see Table 1 of this paper).

Paper IV

A 10-year-old girl developed thrombocytopenia (disorder with abnormally low amount of platelets) and splenomegaly (enlargement of the spleen) and was diagnosed with idiopathic thrombocytopenic purpura (ITP). Her case was handled for several years at a local hospital. When the patient was 9 years old she was reassessed and referred to the Sahlgrenska University Hospital. Oesophageal varicose veins and splenomegaly were confirmed. The patient's international normalized ratio (INR) was slightly elevated (1.4), concentrations of protein S and

protein C were normal, and activated protein C resistance was excluded. The patient was given β-blockers to reduce portal hypertension.

Serum Samples

In the present studies, AECAs were obtained from serum samples. Peripheral blood from the patients were collected, centrifuged, aliquoted and stored at -80°C for further experiments. In patients that underwent a transplant (Papers II, III and IV) the same procedure was repeated. Blood samples were collected before and after surgery. Before surgery, samples were taken between five months to one month prior to and on the day of intervention. After transplantation, samples were taken at different time intervals of one and two weeks during immediate recovery and subsequently at one, three, and six months into recovery. Only samples from the patient in Paper IV were taken during more than one year. Blood from healthy male individuals (controls) were also included for generation of serum.

Biopsies

Paper I

Kidney biopsies from ESRD patients and normal individuals were used for immunohistochemical (IHC) staining of cell junction proteins.

Paper II and III

Cardiac biopsies from heart-transplanted patients were included for immunohistochemistry (IHC) in papers II and III and immunofluorescence (IF) staining of antibody deposition and rejection markers in paper III.

Paper IV

Biopsy samples from the graft were taken for immunobiological assessment by IHC and IF before (native tissue), during (decellularization) and after (recellularization) the creation of the new vein.

Isolation of PBMCs from donors and patients (Paper II, III and IV)

Peripheral Blood Mononuclear Cells (PBMCs) were isolated from blood of 16 deceased heart organ donors and two cadaveric vein graft donor via the density gradient media, Lymphoprep ™ (Axis-Shield PoC AS, Oslo, Norway). A lymphoprep-blood suspension was prepared and centrifuged at 1500 rpm for 20 min at room temperature. After centrifugation, a white interface layer (mononuclear cells) was collected, washed and frozen in liquid N_2 for subsequent use. The same procedure

was used for the isolation of PBMCs from the patients at the same time intervals as indicated above for the isolation of sera.

Immunomagnetic cell separation (Paper II, III and IV)

Immunomagnetic cell separation is a technique based on the attachment of small magnetic particles to cells via antibodies or lectins. When a mixed population of cells in suspension is placed in a magnetic field, those cells that have magnetic beads attached will be attracted to the magnet and may thus be separated from the unlabeled cells (99). This method is quick, easy to perform and generally gives a good yield of cell isolation. It should be noted, however, that only positive and negative cell populations can be separated. However, the limitation is that selection cannot be made based on level/density of expression of various molecules. Moreover, only cell surface molecules can be used as markers for magnetic separation of live cells and not by other means such as the expression of green fluorescent protein in transfected cells (100, 101).

Papers II, III and IV

Immunomagnetic cell separation was utilized in studies II, III and IV for the isolation of EPC/Tie-2 positive cells from blood using a Dynal™ magnet that is suitable for working with large samples and where the degree of cell purity is not a crucial factor (since the cells were not intended for cultivation). In study IV, a more robust separation magnet using magnetic columns from MACS® was used for the isolation of CD133 positive cells from bone marrow and subsequently, cell cultivation, differentiation and repopulation of a new bioengineered-vein graft.

Isolation and cultivation of endothelial cell lines (Paper I, III and IV)

Paper I

Human kidney endothelial cells (HKECs) were isolated from a donated kidney, considered non-suitable for transplantation, using an enzymatic method described by Daneker, GW. et al. 1998 (102). The cells were cultured in MCDB 131 endothelial medium, supplemented with growth factors EGM-2 single-quots (Lonza, Clonetics®). To maintain fenestrae in HKEC, vascular endothelial growth factor (VEGF) was also added and scanning electron microscopy was used to detect endothelial fenestrae. Capillary formation in Matrigel® and morphological characterization of HKECs were also performed by optical microscopy. Other ECs lines used in this study were human ECs from umbilical vein (HUVECs), artery

(HAECs) and lung (HLECs) which were purchased from Clonetics® and cultured in same conditions as HKECs in MCDB 131 medium.

Paper III

Human Cardiac Microvascular Endothelial Cells (HCMECs, Clonetics®) were included in this study as base-line cells. The procedure for cell culturing was the same as the mentioned with HKECs.

Paper IV

Autologous ECs from the bone marrow of the patient were isolated using CD133-coated mini MACS beads, following manufacture's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). To differentiate the endothelial lineage, CD133 cells were cultured with MCDB 131 specifically enriched endothelial growth medium.

CD133 positive cells, smooth muscle cells were also isolated from the same bone marrow. The cells were grown and differentiated in a commercially available medium for smooth muscle cells (Cascade Biologics medium 231 plus growth factors and differentiation supplements, Invitrogen Corporation Portland, USA).

Isolation of circulating EPCs (Paper II, III and IV)

Papers II, III and IV

In these studies, the patients underwent either a heart transplant (Papers II and III) or a bioengineered-vein transplant (Paper IV). In parallel to the standard assays performed in clinical routine to detect the degree of compatibility between a donor and a recipient (HLA-typing) at Sahlgrenska Hospital, a recently introduced endothelial precursor cell crossmatch test (EPC-XM, XM-ONE® AbSorber AB, Stockholm, Sweden) was also performed. These additional tests were carried out in order to assess AECAs in a clinical setting.

PBMCs isolated from donors and patients were thawed and washed with RPMI medium. Cell viability was examined using optical microscopy with trypan blue staining. The cells were then incubated for 30 min with immunomagnetic nanobeads coated with the anti-Tie-2 antibody. Finally, the cells were then placed in a Dynal™ magnet for 10 min at room temperature. Tie-2 negative cells not adhering to the magnet were pipetted and transferred to another tube. The remaining Tie-2+ positive cells (EPCs) were recovered by removing the tubes from the magnet and resuspending the cells in PBS. After each separation, aliquots of cells were set aside for incubation with pre and post transplantation serum samples.

Flow cytometry (Paper I - IV)

Flow cytometry is used for the analysis and sorting of a mixed population of cells or particles in suspension that are labeled with particular fluorochromes. By observing scattered light from the interaction between labeled cells and the laser/flourochrome, it is possible to measure multiple parameters simultaneously (e.g. size, granularity, cell cycle, debris, cell death and expression of proteins) on each cell. However, since it requires a suspension of single cells or other particles, with minimum clumps and debris, the tissue architecture and any information about the spatial relationship between different cells are lost when single cells or nuclei are prepared (103). Flow cytometry was particularly useful in these experiments as it offered a rapid quantitative method for phenotyping ECs and sera samples.

Endothelial phenotyping (Paper I)

Isolated ECs from a kidney were phenotyped by flow cytometry with a panel of different fluorescent markers to detect endothelial cell identities.

Screening of sera samples for detection of AECA (Paper I - IV)

Stimulation of ECs with cytokines in culture induces changes in ECs functions. A set of HKECs/HUVECs, unstimulated and stimulated with IFN-γ and TNF-α (16 h), were used for the screening of AECAs in sera of 45 ESRD patients and 20 healthy controls (Paper I). Sera from healthy non-transfused blood group AB men known not to have any antibodies served as negative controls. A pool of sera from patients who had formed alloantibodies as a result of multiple blood transfusions or organ transplantations was used as a positive control. For the assay, HKECs cells were incubated with patient's serum for 1h and then washed with PBS. Fluoresceinated goat anti-human IgG and IgM antibodies were then added and incubated. After a washing step, the cells were resuspended in PBS and immediately analyzed by flow cytometry. Fluorescence signals from 10,000 cells were counted, and the percentage of FITC-positive cells was recorded. A shift in the mean fluorescence of 20 channels in the test sample indicated the presence of AECAs when compared with control samples (104). All sera giving positive reactions were further diluted to determine titers of AECAs.

In papers II and III cardiac microvascular ECs were used, and in papers II, III, and IV a subpopulation of peripheral EPC/Tie-2 positive cells was isolated using the novel EPC-XM kit. EPCs share tissue endothelial characteristics (105) and one of the advantages of this EPC-XM method is that it is possible to perform a lymphocyte crossmatch test simultaneously. Donor and autologous PBMCs were used for the

assays. Donors' Tie- 2+ cells were distributed in tubes and incubated with serum from their respective recipients and stained with anti-IgG and anti-IgM (FITC), anti-CD3 PE (T cells) and anti-CD19 PerCP (B cells) fluorescent secondary antibodies. The samples were run on Becton Dickinson flow cytometer (FACSorter, Becton Dickinson, USA) using CELLQUEST PRO analysis software (Paper II and III) and a Guava Millipore flow cytometer (Paper IV). Negative and positive control sera were included and fluorescence signals of 7000 cells were counted. Positive and negative donor-AECAs test values were established for each immunoglobulin isotype following parameters from a kidney EPC-XM multicenter study (106) and positive lymphocyte crossmatch tests according to a protocol developed by Alheim, M. et al. 2010 (107). Autologous IgM AECA values were determined by testing AECAs in a set of 20 healthy individuals matched in age and sex using the EPC-XM. Shifts in the mean fluorescence channel of more than 52 channels, in the test sample as compared to the negative control, were considered as positive.

Standard HLA typing and screening for HLA-panel reactive antibodies (PRA) of patient and donor samples were assessed by personnel from the Transfusion and Medicine Department at Sahlgrenska University Hospital, using Luminex, flow cytometry and complement-dependent cytotoxicity assays (CDC). Detection of donor specific anti-HLA antibodies (DSA) by flow cytometry and CDC crossmatch tests was also included.

Isolation of IgG fractions from AECAs in ESRD patients (Paper I)

When accurate concentrations of antibody are required, purified antibody fractions must be used (108). It is known that the multifunctional IgG is the most abundant in human serum (35).

To assess the concentration and reactivity of AECAs on HKECs, purified IgG fractions were isolated by immunoprecipitation of pooled sera from ESRD patients with (positive) and without (negative) AECA, using goat anti-human IgG (Fc-chain specific) agarose beads 127 (Sigma-Aldrich Sweden, Stockholm, Sweden). The concentration of IgG was then determined by the standard Mancini method. Normal IgG (11 mg/ml, ChromPure Human IgG) was also used in the tests as a control sample.

Western blot (Paper I)

The western blot or immunoblotting is a technique used for separation and detection of individual proteins in a protein mixture (e.g. a cell or tissue lysates, fluids). The protein mixture sample is applied to a gel electrophoresis in a carrier matrix (SDS-

PAGE, native PAGE, isoelectric focusing, 2D gel electrophoresis, etc.) to sort the proteins by size, charge and/or conformation in individual protein bands. The separated protein bands are then transferred to a carrier membrane (e.g. nitrocellulose, nylon or polyvinylidene difluoride PVDF) where they adhere to the membrane in the same pattern as they have been separated due to interactions of charges. Finally, this immunoblot is then accessible for antibody binding detection (109). The advantages of this technique are that it can determine the size of the target protein, it is possible to semi-quantify the protein of interest by running an internal quantity standard in parallel with the samples in the gel and different samples can be compared simultaneously. The main drawbacks are that it is time-consuming and has a high demand in terms of optimizing the experimental conditions (i.e. protein isolation, buffers, type of separation, gel concentration, etc.).

A western blot analysis was performed in Study I to check phosphorylation of ECs adherens proteins in kidney ECs after stimulation with different IgG fractions.

The presence of tyrosine phosphorylation of VE-cadherin, a cell-cell adherence junction protein, has been reported in conjunction with increased ECs permeability (110, 111). Cell lysates of stimulated HKECs with IgG from AECA-positive, AECA-negative and normal individuals were prepared as described by Sumitran et al, 1999 (112) and immunoblotted with anti-phospho VE-cadherin using standard SDS-PAGE and Western blot analysis.

Measurement of endothelial cell barrier function (Paper I)

In culture, ECs grow attached one to each other in monolayers creating a mesh-like structure with certain electrical resistance. Under experimental conditions, (e.g., when influenced by external stimulus), the functionality of this resistance is measurable and provides explanatory information about cellular disturbances (113). This approach was employed in Study I.

ECs were cultured into confluent monolayers on detachable cell culture inserts to a cell density of $2x10^5$ cells/membrane. The cells were then stimulated with AECAs positive and normal IgG solutions in order to assess the induced permeability of ECs in the presence of AECAs via electrophysiology-based Ussing Chamber System. This method estimates the epithelial electrical resistance (114). ECs membranes were detached from the inserts and mounted in chambers using Krebs buffer solution. The cultures were maintained at 37°C, oxygenated with 95% O_2, 5% CO_2, and stirred by gas flow. Green FITC-dextran of different molecular weights (4,000, 70,000 and 150,000 Sigma) was added to the lumen of the half-chamber.

Serosa samples of 0.2 ml were then collected at different time points during 2h and analyzed. The luminal to serosa permeability of the probes was measured by fluorescence at wavelengths of 480 and 535 nm for excitation and emission, respectively. All data sampling and pulse inductions were computer controlled using specially constructed hardware and software developed in LabView.

Measurement of intracellular Ca2+ on endothelial cells (Paper I)

Intracellular Ca2+ modulates numerous physiological cellular phenomena as an intracellular signaling molecule inducing cell proliferation, differentiation, migration and survival as well as triggering pathological events such as cell injury and death (115). For this reason, measurements of Ca2+ concentrations yield substantial insight into both normal and abnormal cell function. The principle of this method is that stimulated cells stained with fluorescents probes generate an excitation shift upon intracellular Ca2+ binding. However, this characterization technique is restricted because intracellular signaling pathways cannot be identified. In Study I, intracellular measurements of Ca2+ were recorded in kidney and umbilical ECs after stimulation with different AECAs IgG fractions from CKD patients.

Calcium flux is a primary feature of cell activity. The ability of IgG to mobilize Calcium flux was carried out as described in (116). Cultured HKECs and HUVECs cells were incubated with the fluorescent indicator Fura 2-AM (Calbiochem, La Jolla, CA) and the surfactant Pluronic F-127 (Sigma- Aldrich, Sweden) in HBSS solution (saline solution) with HEPES buffer for 30 min at 37°C and the ratio of fluorescence between 340 and 380 nm was recorded with commercially available software (Miracal, Life Science Resources). After 10 s of recording, 50 ml of IgG from ESRD patients with or without AECAs or normal IgG were added to the cells in different concentrations. The total recording time was 200s.

ELISA (Enzyme-linked immunosorbent assay) (Paper III)

ELISA is a qualitative or quantitative method to measure concentrations of a particular antigen (e.g. proteins, peptides, hormones or antibodies) present in a given fluid sample. ELISA begins by fixing an antibody to a plate. Next, non-specific analytes are blocked and the sample to be analyzed is applied to the plate. If the sample contains antigens corresponding to the fixed antibody binding will occur. Then, the sample is washed to remove all unbound components from the substrate. Then a secondary antibody which is coupled to a flouro or chromophore-containing enzyme is applied which will only bind to the antigens (if any) remaining from the sample. Finally, a substrate is added allowing the linked enzymes to demonstrate a chromographic or flourescent signal (117). ELISA is a useful technique because it is

quick, simple to carry out, and relatively economical when working with uniform cell populations or multiple samples. Although ELISA provides information on the presence of a given analyte, it lends no information on its biochemical properties such as molecular weight or its spatial distribution in a tissue. Furthermore, the immobilization or fixation of cells might mask true antigens making them undetectable or giving false positive results (118).

Detection for anti-vimentin IgG antibodies (Paper III)

Vimentin is a cytoskeletal intermediate filament exhibited in many type of cells including ECs. In transplantation, antibodies to this protein are grouped along with non-HLA antibodies. Anti-vimentin IgG antibodies in sera samples were semi quantified by ELISA.

Creating a bioengineered-vein (Paper IV)

Vein Decellularization

Decellularization describes the process where cells are removed from an original tissue in order to maintain a natural matrix on which to create a new tissue. In this study, a 9 cm vein segment from a deceased organ donor (30 years) was washed and cleaned with distilled water and surfactant solutions containing antibacterial and antifungal agents to avoid microbial contamination. First, the vein was rinsed in distilled water for 72 h followed by incubation in detergent 1% triton X (for 3 h), extracting solvent 1% tri-n-butyl phosphate (for 3 h) and 4 mg/L deoxyribonuclease I, an enzyme to break residues of DNA (Sigma, Gothenburg, Sweden) in 1 M sodium chloride (for 3 h). One end of the graft was kept open while the other was clamped and the lumen was filled with 1% Triton X. The other end was then clamped and placed on an agitator at 37°C for 3 h with gentle shaking. At the end of the incubation time, one end of the specimen was opened, the contents of the lumen were emptied and the specimen was washed with phosphate buffered saline. The procedure was followed for treatment with tri-n-butyl phosphate and DNAse. Finally, the specimen was washed with distilled water overnight to remove cell debris. Seven cycles were run from triton to water. At the end of the decellularization process, the graft was washed continuously for 48 h with PBS, which was changed every 6 h.

Recellularization of a new vein

After decellularization, endothelial and smooth muscle cells (around $7{\cdot}5\times10^{4}$cell/cm^{2}) differentiated from the patient's bone marrow cells were seeded in the new clean matrix. First, one end of the graft was clamped and endothelial cells

were applied longitudinally to the internal surface of the matrix with a micro syringe. Then, the open end was clamped and the matrix was placed on a laboratory shaker system (Rock n Roller Mixer 820, Boule Medical, Stockholm, Sweden) at 37°C with 5% CO_2. After 3 days, the internal surface was seeded with smooth muscle cells suspended for 3 days. The matrix was then placed within a bioreactor. Endothelial serum-free medium was added internally (25 mL) and serum-free smooth muscle cell differentiation medium externally (25 mL) then rotation started at 1.5 revolutions per min (at 37°C with 5% CO_2). The external and internal medium was changed every 72 h. The extracted medium was tested for microbial colonization with a commercially available kit (Invitrogen, Sweden, category number C-7028). The bioreactor culture lasted 2 weeks in total.

Histology (Paper I - IV)

Histology is a technique that is commonly used to examine and diagnose tissues and cells microscopically by staining samples with different antibody markers. In cell cultures or cell suspensions, it is called immunocytochemistry and in tissues, immunohistochemistry (119). Once tissues are removed from the body, they undergo a process of autolysis, which is initiated soon after cell death by the action of intracellular enzymes causing the breakdown of protein and eventual liquefaction of the cell (120). The main objective with the histology is to preserve cells and tissue constituents in as close a life-like state as possible and to allow them to undergo further preparative procedures without change. Different methods have been developed and different settings are used in the laboratories depending of the tissue/cells and characteristics of the antibodies used. In this work, immunofluorescence and immunohistochemistry staining were used according to local standard procedures.

Immunocytochemistry (Paper I and IV)

In Paper I immunofluorescence staining of adherens and tight junction proteins on HKECs was performed. $3x10^5$ HKECs were grown on eight-chamber cell culture slides and then stimulated with either AECAs-positive IgG fractions from ESRD patients or healthy controls overnight. After washing these cells, primary antibodies to vascular endothelial cadherin (VE-cadherin, BD Pharmigen) and tight junction proteins ZO-1, Claudin-1 and Ocludin-1 (Zymed Laboratories, San Francisco, CA) were stained on HKECs using secondary fluorescent antibodies CY3 goat anti-mouse (Jackson ImmunoResearch) and Alexa Fluor 488 donkey anti-mouse (Invitrogen). Nuclei staining with DAPI were also included. Specimens were then

mounted and dried for microscopic analysis under a confocal fluorescence microscope.

Staining of actin filaments was also applied on monolayers of HKECs, which were grown and stimulated with different IgG fractions from AECAs positive, negative and normal. Then, these HKECs monolayers were fixed with formaldehyde and permeabilized with Triton X-100 for staining of actin filaments with FITC-conjugated phalloidin (Sigma-Aldrich Sweden). After additional washes, the HKECs were viewed in a fluorescence microscope.

In Paper IV differentiated endothelial cells from bone marrow of the patient were stained, with dual-colour immunofluorescence for vascular endothelial cadherin, acetylated LDL, and von Willebrand factor, and counterstained with DAPI to confirm the endothelial phenotype. In the same way, smooth muscle cells were stained with α-actin and vimentin to confirm the smooth muscle cell phenotype.

Immunohistochemistry (Paper I – IV)

Paper I

Paraffin-embedded kidney biopsy sections from normal individuals and ESRD patients were incubated with sera AECA-positive, negative and normal and stained with the same panel of adherens and tight junction antibodies used in immunocytochemistry; claudine-1 and ocludin-1 by biotin-peroxidase complex and VE-cadherin and ZO-1 by proteinase K method.

Paper II

Paraffin-embedded cardiac biopsies sections from a twice-heart-transplanted patient were analyzed for cellular and antibody mediated-rejection with CD68 (monocyte/macrophage marker) and C4d (an AMR marker) antibodies, under protocols from the department of pathology at Sahlgrenska Hospital.

Paper III

Immunofluorescence deposition of IgG and IgM on capillary endothelium in heart frozen sections from transplanted patients with and without rejection episodes were analyzed. Consecutive biopsy sections were stained with endothelial adhesion anti-human CD31 (Sino Biological Inc., Beijing, China) and incubated at 4°C overnight. On the following day, goat anti-mouse Alexa fluor 594 (Invitrogen, Stockholm, Sweden), goat anti-human IgG and IgM (Jackson ImmunoResearch Laboratories West Grove, PA, USA) secondary antibodies were added and incubated. After three washing steps with cold PBS, blue fluorescent nuclei stain with DAPI was performed and specimens were mounted and dried for microscopy analysis. Immunoglobulin

deposition was quantified with a specific protocol, established by the Centre for Cellular Imagining (core facility) at Gothenburg University.

Paper IV

After each decellularization cycle of the vein, a small piece of tissue was also screened for the presence of nuclei (DAPI staining), HLA class I, and II antigens, and was verified histologically with standard procedures (121).

Statistics

Paper I

All values are expressed as means SD (standard deviation) unless otherwise indicated. A P value <0.05 was considered significant. Differences among groups were analyzed by ANOVA using a Kruskal-Wallis test. The statistical analysis was performed using SAS software (version 9.1.3, SAS, Cary, NC). Relative changes in electrical resistance as well as cumulative permeability of probes were performed by Student's T-test for paired and unpaired values using SPSS software (SPSS, Chicago, IL).

Paper III

Data is presented as mean SD for continuous variables and number of subjects. Percentages are used for categorical variables. Fisher's two-sided exact test was used for the comparison of categorical variables. In calculations comparing presence of AECAs in serum and respective EMB outcome, generalized estimating equations (GEE) were used. Differences between EMB images from AECAs positive patients and AECAs negative (controls) were analyzed using Mann-Whitney-U test. A probability value of $p \leq 0.05$ was considered to indicate statistical significance.

Ethical permits

The consent obtained from each individual and all the protocols for the studies were approved by the different Ethics Committees of the Karolinska University Hospital Huddinge in Stockholm (Paper I) and the Sahlgrenska University Hospital in Gothenburg, Sweden (Papers II, III and IV).

REVIEW OF RESULTS

Tables and Figures of each study are only mentioned in the text. To facilitate interpretation of the results, please refer to respective paper.

AECAs in ESRD patients (Paper I)

Characterization of ECs from kidney

Investigations for detection of endothelial injury caused by AECAs have traditionally used ECs from easily access tissue from large vessels such as umbilical cord, which may be not clinically relevant. Herein, tissue-specific ECs lines of HUVECs were used as a control, and HKECs were used to target specific AECAs in ESRD patients. The isolated HKECs displayed their characteristic cobblestone morphology in culture and a tubular network formation in Matrigel. Electron microscopy revealed the presence of fenestrae and phenotypic expression of various endothelial markers, which are distinctive features of glomerular ECs.

AECAs IgG fractions bind to tissue-specific ECs from the kidney

Among the 45 ESDR patients in the study, more than half of them had positive flow cytometric AECAs that bound to HKECs (P<0,0001) and not to HUVECs (56% vs 4%) compared to controls (Table 3). Furthermore, to confirm the retention capacity of binding in these antibodies, IgG fractions from the sera of patients with AECAs were isolated and tested against different ECs including aortic and lung. Once again, a strongly binding capacity was found only to HKECs (Figure 1E). These results allowed for the discrimination of a pool of kidney-reactive sera samples specific to AECAs positive and negative cases. The presence of AECAs was associated with females, systolic blood pressure, and peripheral TNF-α but not with any other clinical parameter.

AECAs IgG fractions induce alterations on HKEC

In order to investigate the effect of AECAs on HKECs permeability, in vitro staining with structural and cytoskeletal proteins on HKECs and HUVECs were performed in conjunction with penetrability assays. In culture, monolayers of HKECs stimulated with IgG fractions from AECAs positive patients demonstrated a decreased expression of tight junctions ZO-1 and Claudin-1 and adherens VE-cadherin proteins. Identical treatments to AECAs negative, AECAs normal and HUVECs cells showed no such effect (Figure 2 and 5A). A similar pattern was observed on kidney biopsies from patients with AECAs. In these patients, tight and adherens junction proteins were almost absent. This is in contrast to normal tissues, which generally

maintain the expression of tight junctions ZO-1 and occludin along tubular and glomerular regions. Normal tissues also exhibit Claudin-1 around the periphery of glomeruli and VE-cadherin in capillary and other vessel areas (Figure 6). Furthermore, these antibody fractions were also able to phosphorylate VE-cadherin, an endothelial adherent protein associated to vascular permeability (Figure 5B).
A comparable reactive influence was seen in HKECs treated with AECAs positive. Upon binding, these antibodies triggered the activation of HKECs as detected by a rapid calcium flux response and an increase in the number and density of F-actin stress fibers (Figure 4). Macromolecular flux and electrical resistance measurements were taken via the diffusion of different FITC-dextrans and the electrical resistance of monolayers of ECs stimulated with IgG positive fractions and IgGs from controls. AECAs positive facilitated a significant passage of dextrans and a correlated decrease in the measured electrical resistance (P=0.002). This demonstrates the permeability of the kidney endothelial cell barrier is affected negatively when AECAs are present (Figure 3).

Successful heart retransplantation with endothelial cell AMR (Paper II)

First transplant - clinical facts

Prior transplantation, the patient had no HLA antibodies and the crossmatch test for donor T and B cells was negative. After his first transplant, the patient received induction therapy with different immunosuppressant and corticosteroid medications (a detailed regimen is described in the paper). However, his status deteriorated progressively within the first month showing clinical signs of rejection as corroborated on his EMB. For this reason, he was accepted for urgent retransplantation. In conjunction with treatment, an analysis on autologous AECAs and donor AECAs was performed. Before first transplantation, both tests were negative for the presence of AECAs. During the second week following his operation, the patient presented a mild cellular rejection (ISHLT-1R), and negative Cd4 deposition. Additionally, tests for donor AECAs (type IgM) returned a positive result, and test results for autologous AECAs were negative. After three weeks, he showed severe macrophage infiltration (CD68+), diffuse eosinophilic and lymphocytic infiltration, and negative C4d. Tests for both donor AECAs IgM and autoreactive AECAs IgG and IgM were positive. During the next several days, the patient started to develop cardiogenic shock. Extracorporeal membrane oxygen was initiated and later he was put on a biventricular assist device (BVAD). His EMB

showed myocyte necrosis, severe antibody, cellular-mediated rejection (ISHLT-3R, AMR) and C4d capillary deposits (Figure 2A). At this point, a severe AMR caused by non-HLA AECAs was assumed, and an AMR protocol consisting of plasmapheresis, intravenous immunoglobulin and rituximab was initiated.

Second transplant - clinical facts

Due to his poor prognosis, the patient was accepted for urgent retransplantation and a donor organ was available within 1 day. Lymphocytic crossmatch tests and tests for donor and autoreactive AECAs were negative. One week after retransplanation, his EMB showed normal myocardium, negative C4d, low lymphocytic infiltration, a slightly increased amount of macrophages (CD68+, Figure 2B) and negative donor and autologous AECAs. Although the patient was clinically stable, extended plasmapheresis treatment and immunosuppressive therapy were administered. During the next weeks, patient's EMB showed regression of macrophage infiltration along with C4d deposition. Tests for donor and auto AECAs were also negative. One month after retransplantation, his EMB showed antibody cellular rejection (ISHLT-2R), but no AMR. Seven weeks after, a normal myocardium, without macrophages (ISHLT-0R) and negative C4d were seen in his EMB. Thirteen weeks after the first transplantation, the patient was discharged home. Thirty months later, the patient is still doing well. A summary of all the major events is shown in Figure 1 of this paper.

Relevance of de Novo allo and auto reactive AECAs in heart-transplanted patients (Paper III)

EPC/Tie-2 cells isolated from PBMCs

EPC/Tie-2 cells from donors and patients were isolated from frozen PBMCs by flow cytometry with the EPC-XM kit. Two major cell populations were displayed, lymphocytes and monocytes-granulocytes. The monocyte region was tested for AECAs. PBMCs viability at the time of EPC/Tie-2 isolation for both donor and patients ranged between 70-95% with a Tie-2 yield of $1,2\pm4x10^6$ cell/25-50 mL blood and $1,9\pm7x10^6$ cells, respectively.

Donor and autologous AECAs

All the patients were transplanted in the absence of DSA HLA prior transplantation. They also had negative standard lymphocytic crossmatch test, negative

lymphocytic EPC-XM and negative autologous AECAs. However, three patients presented positive donor AECAs. A significant association between the use of a ventricular assist device (VAD) and the presence of IgG AECAs was found (P=0.027).

Donor AECAs vs. rejection events

Post transplantation eight out of 14 patients had donor AECAs (four with IgG AECAs, one with IgM and three with both) and all the available sera samples after transplantation were correlated with their respective EMB outcome (ISHLT 0R-3R). Among 42 samples, 10 had positive IgM AECAs and six were associated to ongoing rejection episodes (P=0.012, ISHLT-1R-3R) (Figure 2A, Table 3 of this paper). Most of the patients with IgG AECAs (12/42) had no rejections. Meanwhile, nearly half of the patients with IgM AECAs demonstrated mild (1R) to moderate (2R) rejections.

Autoreactive AECAs vs. rejection events

Autologous IgM AECAs were positive in four patients. Their sera samples were significantly associated with verified allograft rejection in EMB (P=0,008 Figure 3). Three patients were positive for autologous AECAs with ongoing rejection and two tested positive for donor AECAs. Furthermore, one patient had positive autologous and donor AECAs but no ongoing rejection.

AECAs detected with EPC-XM vs. cardiac microvascular ECs

To confirm that the AECAs detected by EPC-XM test were also reactive to tissue-specific EC, flow cytometry was performed to assess their binding capacity to HCMECs. 5/14 patients had positive AECAs against HCMECs. These patients were also positive AECAs reactive by the EPC-XM test.

AECAs detected with EPC-XM vs. other non-HLA antibodies

Rejections and organ failure have also been associated to two other important non-HLA antigenic determinants called MICA and vimentin. Screenings of these proteins showed that only one patient was AECAs and anti-vimentin positive. None of the patients had anti-MICA antibodies.

Deposition of IgG/IgM on EMB

Given the correlation between the presence of donor and auto AECAs (type IgM) with rejection episodes, deposition tests of IgG and IgM in the capillary endothelium were performed on the EMB of patients with and without AECAs. Tests indicated that there was no significant difference in the distribution of IgG in patients with and without AECAs. On the contrary, the tests of the distribution of IgM in the biopsies of

patients with AECAs were markedly higher when compared with controls (P=0.0001) (Figure 4).

AECAs in a bioengineered-transplanted vein (Paper IV)

A patient diagnosed with splenomegaly, portal hypertension and advanced extrahepatic portal vein obstruction was accepted for a Meso-Rex bypass procedure with an autologous stem cell derived vein graft as a rescue alternative.

The new created vein graft

The donor's iliac vein was biopsied for histological staining. The tests revealed an intact nucleated tissue with a defined endothelial layer expressing HLA-I but not HLA-II since ECs and smooth muscle cells do not express these antigens constitutively. During seven cycles of decellularization, a translucent tissue was obtained. This tissue showed the architecture of an acellular preserved matrix and did not contain HLA-I or HLA-II, Figure 3A. The patient's CD133-bone-marrow-derived-positive cells were successfully grown and differentiated into mature endothelial and smooth muscle cells. These differentiated cells were then taken to recellularize a new blood vessel in a bioreactor over the course of two weeks. Phenotypic endothelial characterization was confirmed by the positive IF expression of VE-cadherin, acetylated-low density lipoprotein and positive von Willebrand factor. Positive tests for α-smooth actin and vimentin denoted the distinctive line of smooth muscle cells (Figure 4). Tissue sections of the newly recellularized vessel displayed a widespread re-endothelialization to the lumen and valves of the vein, and a homogeneous smooth muscle attachment in the tunica media (Figure 5). All tests for autologous and alloreactive AECAs prior to the surgical procedure were negative.

Transplantation of the bioengineered-graft

A section with suitable length of the new vein was placed in cold storage solution immediately before surgery. An illustrative image of the surgical correction is shown in Image 3.

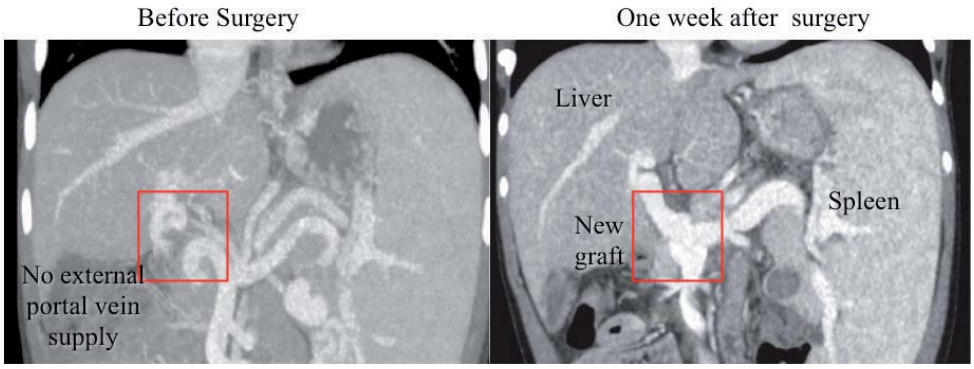

Image 3. Surgical replacement of the new blood vessel with autologous stem cells

Once the graft was implanted, blood flow was measured presenting normal flow rates of 25–30 cm/s in the portal vein and 40 cm/s in the artery. One week after surgery, the vein graft was visualized by computed tomography (CT) angiography. The vein exhibited good attachment around the surrounded vasculature (Image 3). During the first six months of recovery the patient received daily doses of 75 mg of aspirin, and 10 mg omeprazole. No immunosuppressive medication was administered, and the patient's routine laboratory tests were normal. However, nine months into recovery, the patient had a decreased platelet count low blood flow from the portal artery, and a reduced lumen in the implanted vein (8 mm to 4–6 mm). At one-year follow-up, a CT angiography showed a narrowed lumen. To correct the defect of the graft, a second operation was undertaken. Two alternatives were considered. First was the transplantation of an autologous vein from the left internal jugular, and second was the creation of a new graft using the patient's stem cells. Intra-operatively, the affected graft showed a compressed segment with a blocked passage of blood through the mesocolon, and the affected vein graft was replaced, using a new bioengineered-vein. The alternative use of internal jugular vein was discarded because they were too short. After surgery, blood flow in the new graft and intrahepatic portal vein was restored (Figure 2). The patient was put on intravenous heparin for two weeks and was prescribed anticoagulants for almost a year. Despite two surgical procedures, the quality of life of the patient showed an evident physical and mental improvement manifested in increased height, weight, physical activity and power of concentration during school activities along with improvement in her speech articulation.

Follow-up measurements for AECAs were taken at one, three, six, nine, and 12 months. AECAs tests taken after the first transplant were negative for AECAs.

However, autologous AECAs IgM started to appear with slightly positive fluctuations during the first six months after the second transplantation as indicated in (Image 4).

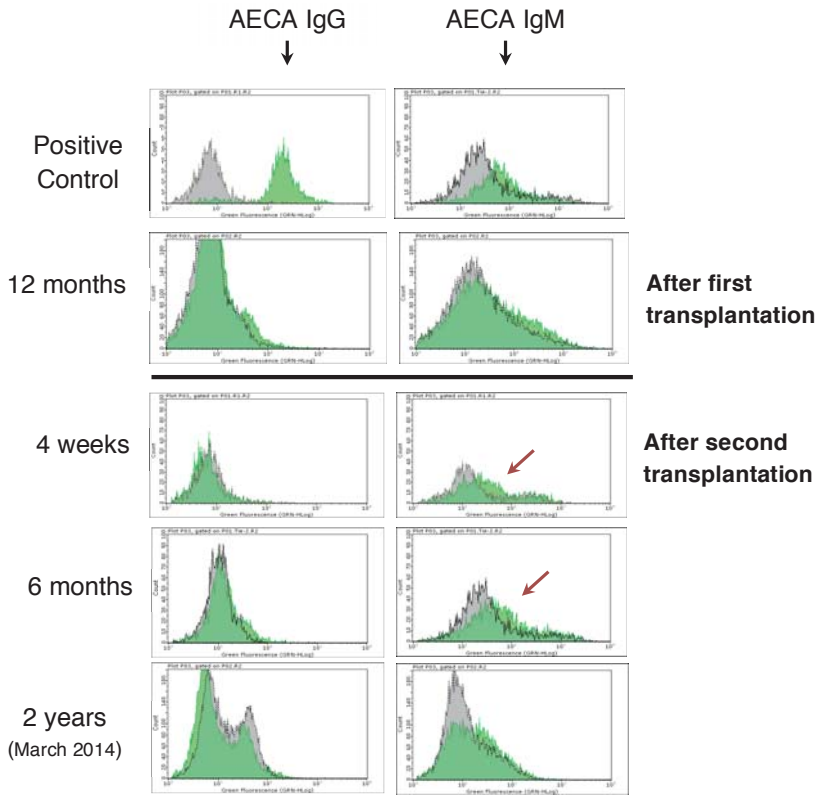

Image 4. Flow cytometric histograms showing negative controls (grey) and positive controls along with patient's sera samples AECAs (green)

GENERAL DISCUSSION

In paper I, the obtained results suggest that AECAs alter the expression of important tight and adherens proteins that maintain the renal microvascular permeability. By working with in vitro monolayers of cultivated HKECs and within vivo kidney biopsies, a decreased expression of regulator permeability proteins, tight junctions (ZO-1), Claudin-1 and adherens (VE-cadherin) were observed upon stimulation with immunoreactive AECAs. The main function of tight and adherens junction proteins is to seal adjacent cells thus providing a strong mechanical attachment (122). However, they also serve to regulate the passage of molecules and ions trough the space between cells (123). In some animal models, it has been demonstrated that the expression of specific claudins can alter transepithelial electrical resistance and charge selectivity (124-126).

In HKEC-monolayers stimulated with AECAs, the diffusion of enhanced dextran, the falling of endothelial electrical resistance and the rapid mobilization of cytosolic Ca2+, may indicate a reorganization of cytoskeletal actin filaments, essential for changes in cell shape and formation of F-actin (127). Endothelial cell permeability has been associated with calcium-dependent conformational changes in the endothelial cell cytoskeleton, leading to cell contraction and intercellular gap formation (116, 128). It was showed that stimulation of HKECs through AECAs activation resulted in loss of peripheral actin bands and increased stress fiber density, which in turn, is a sign of membrane disturbance and loss of endothelial permeability.

Many pathologic processes target junctional proteins, resulting in the disassembly of junctions and breakdown of tissue integrity (129). Reactive AECAs also induced phosphorylation of VE-cadherin, an almost exclusive endothelial adherent protein in endothelial cells and reported as regulatory of vascular permeability (130, 131). This demonstrates that these antibodies might have some specific functional effects on kidney endothelial cells.

Cytokines and ischemic injury has been regarded to endothelial dysfunction where they increase permeability, and intercellular gap formation (15, 132, 133). In vitro studies have shown how the permeability of kidney, umbilical epithelial cells/ECs and liver sinusoidal ECs may be provoked or impeded transcellularly through the formation of caveolae and fenestrations (134, 135). However, the mechanisms that interrupt the endothelial regulatory balance within the kidney microvascular bed are still not understood.

Kidney endothelial dysfunction in ESRD patients is recognized as a critical risk factor for atherosclerotic CVD (136). Since CVD is a manifestation of vascular dysfunction, it is valid to assume that biological alterations are occurring within the kidney endothelium in CKD patients. In general, ESRD patients represent a group with severe disease and it is possible that the correlation between increased levels of AECAs reactive with specific HKECs is an epiphenomenon. However, damage to the ECs during the progression of ESRD might lead to the exposure of antigens, which engender the development of AECAs. Therefore, it is more reasonable to hypothesize that these antibodies possess functional properties, which induce alterations contributing to the impairment of the kidney glomerular barrier.

In paper II a clinical case of endothelial cell AMR and successful treatment in a heart-transplanted patient was presented. Traditionally, AMR is relatively minor compared to cellular-mediated rejection in heart transplantation (137). However, cases of hyper and acute rejection due the presence of HLA antibodies have been reported (138, 139). Study II is unique insofar as the patient was monitored to control the prevalence of AECAs in parallel with regular clinical assessments.

By monitoring presence of donor-specific AECAs type IgM post-transplant, during cardiac rejection, and graft failure, it was possible to implement an adequate regiment of desensitization making the patient eligible for retransplantation. Thus, this study suggests that in heart-transplanted patients, early heart failure might be avoidable if negative HLA and positive AECAs are observed shortly after transplantation.

In paper III, preliminary data about the prevalence of de novo allo and autologous AECAs was presented in association with early rejection events by testing sequential sera samples pre- and post-transplantation within six months follow-up. As in paper II, the new EPC-XM assay and cardiac microvascular ECs to detect AECAs was used.

The significant correlation found between the presence of AECAs prior to transplantation and the use of VAD was not surprising. VAD is often necessary to maintain circulation in patients with heart failure and its use has been related with the development of HLA antibodies (140-142). It is also likely that VAD might induce an immune response leading to the formation of AECAs.

Good HLA matches do not guarantee rejection-free transplantation. Apart of AECAs, the presence of other non-HLA antibodies such as the MICA (MHC class I–related chain A) has been associated with an increased frequency of graft loss, especially in kidney transplants (143, 144). However, these antibodies are also present in heart

transplants (145) but in the group of our heart-transplanted patients, none of the individuals had these antibodies. Similar results were seen for anti-vimentin antibodies, where only one patient had anti-vimentin positive reaction. This fact suggests that in heart-transplantation, AECAs are clinically relevant (146).

Many of the donor and/or autologous AECAs positive patients suffered from inflammatory disease (29%) and other non-related pathologies to inflammation (36%). In paper II, the retransplanted patient also had ulcerative colitis and in this study, three patients that had autoreactive AECAs also had autoimmune diseases as well, giving the possibility that AECAs were a triggering cause. Nevertheless, autoantibodies in most cases appear to represent de novo autoantibody development rather than recurrence of preexisting autoimmune condition (147-150). Furthermore, the sera samples from patients tested positive for AECAs by the EPC-XM kit, were also tested against cardiac microvascular ECs showing comparable results. This indicates that the EPC-XM kit can be clinically reliable test for AECAs.

In paper IV the successful transplantation of a bioengineered blood vessel was reported. An acellular human blood vessel was recellularized with autologous stem cells. Using a bypass procedure, this technology might serve as an alternative treatment for patients suffering venous insufficiency, obstructed veins or inadequate autologous veins without the need of an immunosuppressive regimen.

In the transplantation scenario, the shortage of organs is a reality (151). Advancements in tissue engineering utilizing natural or artificial tissue scaffolds repopulated with stem cells may be a viable alternative to contemporary techniques.

The vein graft in Study IV was recellularized with autologous stem cells and tissue rejection after transplantation was not expected. The patient was closely monitored for more than a year for the presence of HLA and AECAs antibodies. Donor HLA and AECAs were not present. Although, the patient tested marginally positive for AECAs IgM after his second transplantation, it is premature to suppose that their presence was directly related to the procedure. It might believe that these antibodies developed as a result other phenomena.

In general, IgM are usually considered as irrelevant natural autoantibodies (152). However in organ transplantation, they can be directed against HLA. It has been found HLA IgM antibodies against donor HLA antigens before transplantation and after both early and late post-transplant course (153, 154). Nonetheless, the effect of the IgM antibodies is still a matter of debate. Some authors show that they have a protective effect on the graft, but other reports show that they can negatively influence graft outcome (155-157).

Representation of possible mechanisms by which AECAs included in this thesis might contribute in endothelial dysfunction.

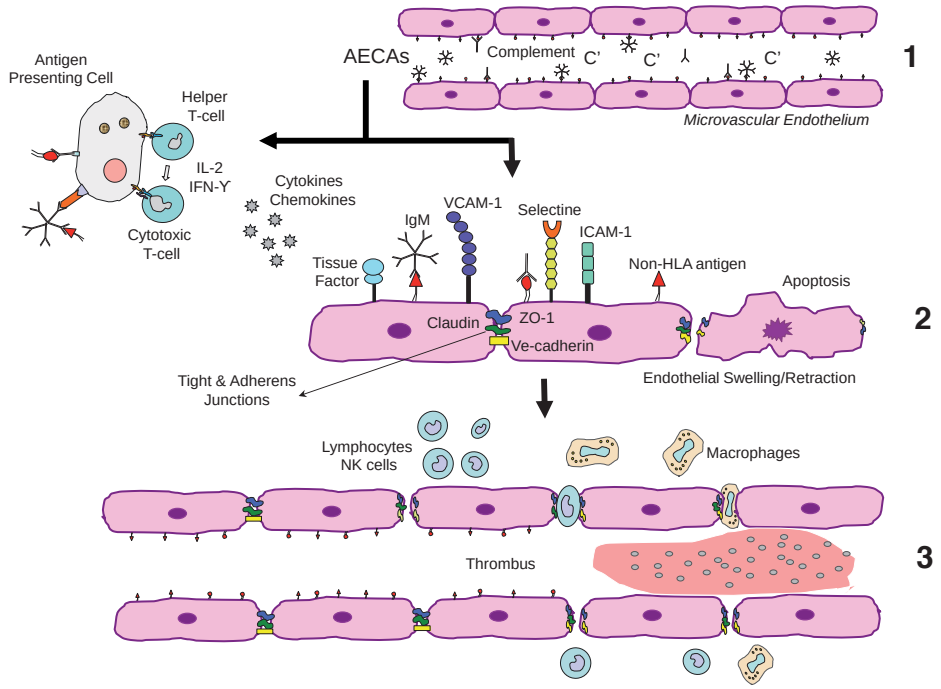

Image 5. Possible mechanisms of endothelial dysfunction mediated by anti-endothelial cell antibodies.

1. AECAs IgG/IgM bind endothelial cell antigens and may activate complement (C') leading to cytotoxicity. Alternatively: **2.** AECA-antigen complex are taken up by antigen presenting cells via complement or Fc receptors and proinflammatory events are initiated, where signaling processes within the microvascular endothelium stimulate the secretion of cytokines, chemokines and generate the activation of different surface endothelial adhesion molecules such as VCAM-1, ICAM-1 and E- and P-selectines. In parallel, upregulation of tissue factor and other clotting agents as trombodulin, trigger a procoagulative activity. Apoptosis and cell shrinkage may also be initiated by disrupting tight and adherens junction proteins (Claudin, ZO-1 and Ve-cadherin) on kidney microvascular endothelial cells leading to a "leaky" endothelium. Cytokines/Chemokines production attracts the migration of inflammatory cells (NK cells, lymphocytes, monocytes and macrophages) to the endothelium leading to enhanced cell-mediated immunity. **3.** As immature monocytes enter the tissue, they become macrophages which modulate activation of T cells by secreting more cytokines/chemokines leading to adaptive immune responses. AECA may also bind antigens on the endothelium via Fc receptors leading to activation of NK cells and antibody-dependent cell-mediated cytotoxicity (ADCC). As result of all this cascade of events, AECAs may amplify inflammatory responses or cause microvascular damage leading to pathological changes resulting in organ disorders or allograft rejections.

Important considerations about AECAs results

It is important to take into account that additional microvascular ECs lines must be investigated in conjunction with AECAs and CKD. In these studies, ECs from a single donor were used. It would be informative to see how AECAs react to other human glomerular ECs lines.

In heart-transplanted patients, donor-specific and autologous AECAs of the non-HLA type detected post-transplant were significantly associated with cardiac allograft rejections. Even though the sample size of this study was small, the results were preliminary in essence. Further work will take into account a larger number of patients in order to improve the statistics of these findings. Furthermore, it is necessary to gather more data from heart-transplanted patients to establish reliable cutoff points for the detection of positive donor and autologous AECAs by the EPC-XM kit. In studies II and III, the criteria for a positive AECA reaction against donor were the same used in the only kidney multicenter EPC-XM study by Breimer et al, 2009, where a shift of more than or equal to 50 and 80 channels for IgG and IgM respectively above the negative control serum was considered positive (106). Given that the EPC-XM system is relatively new, it is still necessary to evaluate its efficacy in a clinical setting for heart-transplanted patients.

Comparably, to consider positive autologous AECAs by flow cytometry in bioengineered vein grafts, consistent cutoff points have to be determined by using proper control groups. Based on a set of 20 healthy individuals (the same as in paper II and III), autoantibodies IgM were established where a shift of more than 52 channels, was assumed as significant. The presented data is from one patient, which enormously limits the condition of our AECA report. However, it is important to mention that this kind of transplants with bioengineered veins has been carried out in two more children, and they are in focus for monitoring AECAs at our center.

CONCLUSIONS

The studies covered under this thesis support the following conclusions:

- The presence of AECAs is a critical problem in chronic kidney diseases and organ transplantation.
- AECAs binding to glomerular endothelial cells are present in high prevalence in ESRD patients. These antibodies may cause dysfunction of renal endothelium leading to pathological changes of clinical relevance.
- AECAs are present in heart-transplanted patients, especially in the absence of HLA-antibodies prior-transplantation. These antibodies may be associated with poor heart graft outcome.
- The presence of donor and/or autologous AECAs detected by the EPC-XM test may increase the risk of early rejection events in heart-transplanted patients.
- In transplants of autologous bioengineered tissues, autologous-reactive AECA may be produced. However, the exact significance of the presence of these antibodies in such transplants needs to be further studied.

PERSPECTIVES

Endothelial dysfunction caused by AECAs is becoming increasingly recognized as critical in the pathogenesis of various organ disorders as well as acute and chronic organ allograft outcomes. Clinically important unknown AECA immune targets and the consecutive lack of detection methods currently make AECA mediated destruction particularly difficult to diagnose and treat.

The existence of polymorphism or presence of antibodies with different specificities in the endothelial-reactive/specific antigenic systems justifies the need for detection and specificity determinations of non-HLA antibodies in organ transplantation. Continued efforts to define the AECA antigens using relevant or donor-specific endothelial cells as targets is absolutely essential to clarify the pathogenic mechanisms by which they contribute to rejection or other forms of tissue injury. Furthermore, the clinical importance of AECAs in organ transplants other than kidney has to be studied and established. The extension of EPC-XM studies pre- and post- transplantation of different organs will add important value to further improvement in donor organ selection and transplant outcome. A better recipient selection based on a negative lymphocyte and endothelial cell crossmatch may probably decrease the incidence of acute rejections and early graft loss in heart transplanted patients.

In CKD patients, determining and quantifying the presence of AECAs at different stages of renal disease will elucidate the clinical prevalence of AECAs in this group of patients.

Conclusively, to define the antigens recognized by AECAs will provide greater understanding of the nature of these antibodies, which can be potentially harmful. This will continue to be an area of importance in terms of fully understanding the role of AECAs as mediators of endothelial destruction in organ and cell transplantation as well as other disease with vascular involvement with the potential for clinical intervention.

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