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Mechanisms of endothelial cell dysfunction in Wegener´s granulomatosis

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To my parents and grandmother

ORIGINAL PAPERS

This thesis is based on the four papers below, referred to by their Roman numerals (I-IV):

I. Wegener's granulomatosis is associated with organ-specific anti-endothelial cell antibodies.
Holmén C, Christensson M, Pettersson E, Bratt J, Stjärne P, Karrar A, and Sumitran-Holgersson S.
Kidney International. 2004; 66(3): 1049-1060.

II. Heterogeneity of human nasal vascular and sinusoidal endothelial cells from the inferior turbinate.
Holmén C, Stjärne P, and Sumitran-Holgersson S.
American Journal of Respiratory Cell and Molecular Biology. 2005; 32(1): 18-27.

III. Circulating inflammatory endothelial cells contribute to endothelial progenitor cell dysfunction in vasculitis patients with kidney involvement.
Holmén C, Elsheikh E, Stenvinkel P, Rashid Qureshi A, Pettersson E, Jalkanen S, and Sumitran-Holgersson S.
Accepted in *Journal of the American Society of Nephrology*, July 2005.

IV. Binding of IgG AECA to kidney endothelial cells induces expression of MICA and production of neutrophil activating chemokines in Wegener's granulomatosis.
Holmén C, Christensson M, Jaksch M, Johansson AS, Jalkanen S, Sundström K, and Sumitran-Holgersson S.
Manuscript.

LIST OF ABBREVIATIONS

Ab	Antibody
Ac-LDL	Acetylated low-density lipoprotein
AECA	Anti-endothelial cell antibody
Alpha-actin	Smooth muscle
ANCA	Anti-neutrophil cytoplasmic antibody
CD31	Platelet/endothelial cell adhesion molecule (PECAM)
CD62E	E-selectin
CD105	Endoglin
CD106	Vascular cell adhesion molecule 1 (VCAM-1)
CD141	Thrombomodulin
CD142	Tissue factor
CD144	Vascular endothelial cadherin
CK18	Cytokeratin 18
EC	Endothelial cell
EPCAM	Epithelial cell adhesion molecule
FITC	Fluorescein isothiocyanate
GCP-2	Granulocyte chemotactic protein-2
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GRO-α	Growth-regulated oncogene-alpha
HLMEC	Human lung microvascular endothelial cell
HRE	Human renal epithelial primary cell line
HKMEC	Human kidney microvascular endothelial cell
HUVEC	Human umbilical vein endothelial cell
HNEC	Human nasal endothelial cell
HLSEC	Human liver sinusoidal endothelial cell
IL	Interleukin
Ig	Immunoglobulin
IFN-γ	Interferon gamma
L-SIGN	Liver/lymphnode-specific ICAM-3 grabbing nonintegrin
LWG	Limited Wegener´s granulomatosis
MCP-1	Monocyte chemotactic protein-1
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein 1-alpha
MMP-1	Matrix metalloproteinase-1
MPA	Microscopic polyangiitis
MPO	Myeloperoxidase
NK cell	Natural killer cell
PE	Phycoerythrin
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PR3	Proteinase 3

RA	Rheumatoid arthritis
RLD	Renal limited disease
SEM	Scanning electron microscopy
SLE	Systemic lupus erythematosus
TEM	Transmission electron microscopy
TNF-α	Tumor necrosis factor alpha
VAP-1	Vascular adhesion protein 1
WG	Wegener´s granulomatosis
vWF	von Willebrand factor

TABLE OF CONTENTS

ABSTRACT

"Vasculitides" are a heterogenous group of disorders that share a common feature of blood vessel inflammation. A major cell type of the blood vessel affected is the endothelial cell (EC). Wegener's Granulomatosis (WG) is one type of vasculitis of unknown etiology, involving granulomatous inflammation and necrosis that most frequently targets the small and medium-sized vessels of the upper respiratory tract, lower respiratory tract and kidneys. A crucial event in the initiation, localization and propagation of EC injury in WG involves activation of the EC by various stimuli, one of which is anti-endothelial cell antibodies (AECA). However, the exact mechanisms by which EC in WG are damaged are not known.

Important criteria for distinguishing the various vasculitides are the size of the vessels involved and the organs supported by the inflammed vessels. Thus, use of the relevant EC as target cells for studies involving AECA carries implications for understanding the clinically important mechanisms underlying the pathogenesis/progression of WG. However such studies are lacking.

Therefore, in order to understand some of the mechanisms by which EC in WG are damaged we performed the following studies:
In paper I, we studied the frequency and interaction of AECA with EC isolated from the clinically relevant small blood vessels of the nose, lung and the kidneys. We demonstrated, as compared with other patient groups, that WG was significantly associated with non-cytotoxic AECA that selectively bind surface antigens on unstimulated nasal, kidney, and lung EC. However AECA binding was lost/decreased when cytokine activated EC were used. We also demonstrated that EC from various organs are characterized by heterogeneity in morphological/functional aspects, marker proteins of cell activation, and responsiveness to cytokines.

In paper II, we report a novel finding that demonstrates the occurrence of two heterogenous populations of EC within the nasal microvasculature. One EC population exhibited classic vascular endothelial markers with cobblestone-like morphology, while the other was sinusoidal in nature, possessing fenestrae. We also established novel protocols for the isolation and culture of these EC.

In paper III, we suggest a novel mechanism by which EC dysfunction in WG may perpetuate vasculitis. Our findings suggest that inflammatory EC (IEC) may detach from the inflamed organ and enter the circulation, and via production of soluble factors may have an inhibitory effect on the repair function of EC progenitors (EPC). We demonstrated that during active WG disease the number of circulating IEC was significantly higher as compared to WG patients in remission and normal controls. Furthermore, IECs but not EPC expressed two novel EC inflammatory markers; vascular adhesion protein 1 (VAP-1) and MHC class-I related chain A (MICA). These markers were also highly expressed in kidney biopsies of WG patients during active disease.

In paper IV, we studied the functional role of WG AECA on kidney EC. We report that isolated IgG fractions from WG patients induced a rapid calcium flux, up-regulation of MICA, and production of neutrophil/monocyte activating chemokines. Furthermore, western blot analysis of immunoprecipitated kidney EC proteins with WG IgG revealed three bands of: 190-200 kDa, 70-73 kDa and 50-53 kDa.

Our data suggest that AECA may play an important role in the dysfunction of EC in WG. AECA *per se* may not be cytotoxic, but may act as "modulators" of the immune responses. Thus, a pro-inflammatory loop may exist between the binding of AECA to EC and the possible recruitment of inflammatory cells via stress/adhesion molecules and production of chemokines resulting in inflammation and EC dysfunction. Furthermore, circulating IECs may parallely contribute to the pathogenesis/progression of WG by interfering with the functional capacity for vessel wall repair by EPC.

Autoimmunity

The immune system is an extraordinarily adaptive defence system that has evolved to protect us from invading pathogenic microorganisms and cancer. It has the capacity to distinguish between foreign molecules and the body´s own cells and proteins, but in some individuals, the immune system loses its sense of self and non-self, resulting in an immune attack against the host/self. This failure of self-tolerance is the fundamental cause of autoimmunity and the symptoms differ depending on which tissues or organs that are affected. In rheumatoid arthritis [1] the joints are affected, while in multiple sclerosis the brain and cental nervous system are assaulted [2, 3].

Self-tolerance can be divided into central tolerance and peripheral tolerance. In central tolerance, immature lymphocytes that recognize self-antigens in generative lymphoid organs (the bone marrow for B-cells and the thymus for T-cells) die by apoptosis [4]. In peripheral tolerance, when mature self-reactive lymphocytes encounter self-antigens in the peripheral tissues they are shut off or killed [4, 5]. Normal healthy individuals also possess mature, recirculating, self-reactive lymphocytes. Since the presence of these self-reactive lymphocytes does not result in autoimmune reactions, their activity is regulated by mechanisms of the peripheral tolerance, namely anergy (functional unresponsiveness), deletion (apoptotic cell death), and/or suppression by regulatory T-cells [2-4].

Breakdown of these peripheral regulations can lead to activation of self-reactive clones of B- and T-cells, generating humoral or cell-mediated responses against self-antigens. These reactions can cause serious damage to cells and organs, sometimes with fatal consequences. Autoimmune hemolytic anemia [6, 7] is an example of an antibody-mediated disease, where antigens on red blood cells are recognized by autoantibodies, leading to destruction of the blood cells resulting in anemia. In rheumatoid arthritis [1] self-reactive T-cells attack the tissue in the joints, causing an inflammatory response that result in swelling and tissue destruction.

The mechanisms for the induction of autoimmunity are various and not fully identified, and it is unlikely that autoimmunity develops from one single event but rather from a number of different events. Some of the proposed mechanisms are; thymic defects, release of sequestered antigens, molecular mimicry, inappropriate expression of MHC class II molecules, non-specific polyclonal B cell activation and hormonal differences in combination with genetic variants, acquired environmental triggers and other random events [2, 3, 8, 9].
Autoimmune diseases affect 5-7 % of the human population and there are no existing curative treatments. Current therapies are aimed at reducing the symptoms to provide the patient with an acceptable quality of life. Mostly, these treatments offer non-specific suppression not leaving the rest of the immune system undamaged. Immunosuppressive drugs are often given with the purpose to slow down the proliferation of lymphocytes. This treatment might help to diminish the severity of the autoimmune symptoms, but may augment the risk of infection [10, 11].
Here, special reference is made to a presumed autoimmune disease with unknown aetiology named Wegener´s granulomatosis. This thesis includes studies concentrating at understanding some of the mechanisms involved in endothelial cell dysfunction occurring in Wegener's granulomatosis.

Wegener's granulomatosis – a vasculitis disease

Wegener's granulomatosis (WG) is a rare disease affecting 1 in 20 000 to 30 000 people. WG belongs to a group of diseases named vasculitis - simply defined as inflammation of the blood vessels [12, 13]. How the vasculitis is defined and develops clinically depends both by the size of the vessel involved and what organ the inflammed vessels support [12]. The classification of vasculitides was founded upon the 1993 "Chapell Hill consensus" in which the vasculitides are distinguished by the smallest vessel involved [14]. WG belongs to the small-medium vessel vasculitides (Figure 1) [14]. There is no known cause for WG, it is not contagious or hereditary. It affects equal numbers of men and women and can occur at any age, but most often it occurs in the 4th and 5th decade of life. It appears that Caucasians are more frequently affected than other racial groups [12, 13, 15-17].

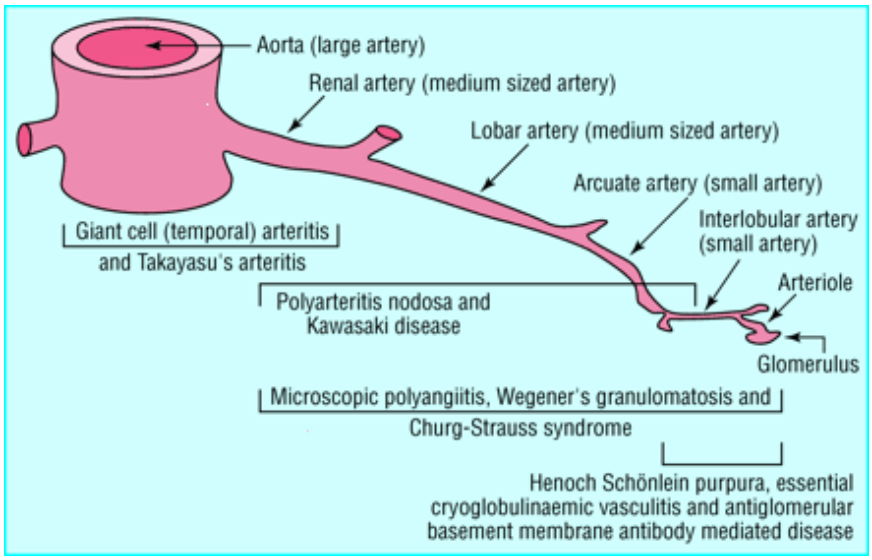

Figure 1. Spectrum of systemic vasculitides organised according to predominant size of vessels affected. Adapted from Jennette et al, *Arthritis Rheum* 1994; 37:187-192.

WG can affect the whole body (systemic), but the main target organs are the upper (sinuses, nose, ears, windpipe) and lower (lungs) respiratory tract and kidneys. Characteristic features are granulomatous inflammation of the respiratory tract and necrotizing vasculitis of the small arteries, capillaries and veins resulting in pulmonary haemorrhage and renal failure [18].

Signs and symptoms

The onset of WG may be gradual or more rapid and severe. About 90% of patients have cold like symptoms with a runny nose that fail to respond to usual treatment and last far longer than the common upper respiratory tract infection [17, 19, 20]. Other non-specific symptoms may include cough, shortness of breath, fatigue, skin rash, fever, loss of appetite, weight loss, joint pain, night sweats, protein and blood in the urine. More specific symptoms are inflammation of the ear and hearing problems, inflammation of the eye and sight problems, narrowing of the trachea and nasal membrane ulcerations/crustings resulting in a "saddle-nose" deformity [12, 13, 15, 18, 21-28].

At the cellular level, the endothelial cells are the target of initial injury resulting in swelling, necrosis and deadherance of the endothelial cells. Lysed neutrophils are found within affected vessel. In the lung, vessels are infiltrated by polymorphonuclear leukocytes and the microvascular vasculitis is the cause of the pulmonary haemorrhage. In the kidney, the basement membrane subsequently ruptures due to the neutrophil degranulation giving rise to glomerular capillary thrombosis and later focal crescentic glomerulonephritis [14, 17]. WG is called "pauci-immune" since there are no immunoglobulin and/or complement deposits detected in glomerular lesions or at other sites [17].

Diagnosis

Both clinical and laboratory findings such as the ANCA blood test, other blood tests, urine tests, x-rays, and tissue biopsy are needed to establish a diagnosis. ANCA is found in the majority (90-95%) of patients who have active generalized WG and can be useful for diagnosis and the recommendation for further diagnostic tests [12, 29]. C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) are common blood tests that measure the increase in inflammatory generated proteins. CRP is one acute phase protein that provides a general indication of acute inflammation. ESR measures the distance that red blood cells settle in a tube of blood in one hour; increased rates are often associated with anemia or inflammatory states. Urine tests for hematuria and proteinuria (red blood cells and protein in urine) indicate kidney involvement and a rise in serum creatinine levels are seen in progressive glomerulonephritis. Chest X-ray is used to evaluate lung infiltrates and a biopsy commonly taken from the nose or kidney is examined to verify the diagnosis of WG [13, 14].

Treatment

The mean survival of untreated WG patients is approximately five months with over 80% dying within one year of disease onset. In the 1970s, the introduction of cyclophosphamide (cytotoxic agent) together with cortisone (a glucocorticoid) dramatically improved the prognosis with over 80% five-year survival [14, 20, 30].
For patients suffering from a more severe disease the goal is to quickly control the inflammation in order to reduce the tissue damage. Plasma exchange [31] that removes immunoglobulins and other inflammatory mediators from the circulation is then performed. When the patient reaches remission, the maintainance therapy has to be balanced against the risk of a new relapse. Most widely used drugs for maintainance are methotrexate and azathioprine (cytostatics) [12, 13, 18, 31-33].

Table 1 shows the most commonly used drugs in the treatment of WG.

Table 1. Drugs used in the treatment of WG.

Drug	Drug name	Effects	Side effects
Cytostatics	Cyclophosphamide	Inhibit DNA replication Leads to cell death	Carcinogenic, hairloss, vomiting
	Methotrexate	Inhibit synthesis of DNA, RNA, proteins and affect mainly the proliferation of leukocytes and lymphocytes	Anemia, neutropenia, nausea, bruising, pulmonary fibrosis
	Azathioprine	Inhibit DNA synthesis - affects both the cell and the humoral immunity	Bone marrow suppression
Glucocorticoid	Prednisone	Diminish both the cellular and humoral immunity	High blood glucose levels, weight gain, osteoporosis
	Methylprednisolone	Anti-inflammatory	Weight gain, glaucoma, osteoporosis

ENDOTHELIAL CELLS

Endothelial cells (EC) consist of a heterogeneous population covering the inner surface of blood vessels comprising the interface between the cellular and non-cellular components of the blood and the extravascular tissues. It is a highly specialized and active monolayer composed of approximately 1-6 x 10^{13} cells and covers a surface area of approximately 1-7 m^2. A typical EC is elongated with a size of 12-30 μm [34, 35]. A normal endothelial cell function is vital for all parts of vascular homeostasis [36]. The EC properties to maintain normal hemostatic properties of the endothelium are listed in Table 2. When the endothelium is injured, these regulatory functions become altered and the endothelium loses its specialized properties, resulting in "endothelial cell dysfunction", which is a hallmark of vascular diseases [35, 37, 38].

Table 2. Endothelial cell properties regulating vascular homeostasis.

Vessel wall development, growth and development
Vascular tone; blood flow and blood pressure
Leukocyte traffic to extravascular tissues
Fluid permeability across the vessel wall
Platelet adhesion and aggregation
Blood coagulation
Fibrinolysis

Heterogeneity of endothelial cells

There are differences between the endothelium of diverse species, between large and small vessels, and between EC derived from different microvascular endothelial beds [39]. In addition, endothelial cell heterogeniety exist in different organs, within the vascular loop of a given organ, and even between neigboring EC of a single blood vessel where EC from different sites of the vascular tree may differ in size, shape, thickness and nuclear orientation (Table 3) [34, 35, 37-44]. In different organs this heterogeneity can contribute to special functions within the organ. For example, fenestrated and discontinous EC are found in the nose [41], liver, spleen, bone marrow sinusoids, kidney glomeruli and lung alveoli that facilitates selective permeability required for efficient absorption, secretion, and filtering, while continous and tight junctions are present between EC in the brain [39, 43, 45-48].

How EC acquire heterogeniety is not resolved. The genetic (intrinsic hypothesis) predicts that an organ is predetermined with a specific phenotype before they migrate from the mesoderm to the specific vascular bed [40, 43].
The environmental (extrinsic hypotheis) suggest that site-specific properties of EC are governed by environmental signals present within the local tissue. Interaction between the microenvironment and EC may involve soluble mediators, cell-cell communication and organization of matrix proteins on which the endothelium grows and adheres. Probably the phenotypic heterogeneity of the endothelium is determined by a combination of both genetic and environmental factors [40, 42, 43].

Table 3. Levels of EC heterogeneity.

Structure	Expression patterns	Function
Size and shape	Protein	Hemostasis
Thickness	mRNA	Vasomotor tone
Filaments	Transcription networks	Barrier function
Vesicles	Signaling pathways	Leucocyte trafficking
Junctions		Cell survival
Microvilli		Cell migration/proliferation
Nuclear orientation		Antigen presentation

Role of the endothelium in vasculitis

EC have a significant and complex contribution to the vasculitis pathogenesis, both as an active partner and as a target of injury in the inflammatory response [51]. The interaction of EC with components of the cellular immune system involves specific receptor ligand pairs, inflammatory interactions with neutrophils, lymphocytes and monocytes and also the humoral immune system - all of importance, which may be both damaging and beneficial. These phenomena are mediated by cytokines and inflammatory mediators as well as products of the coagulation and fibrinolytic pathways [34, 49-51].

The endothelium as a mediator of vasculitis

The endothelium is closely involved with the recirculation of leukocytes and with the recruitment of leukocytes to the site of inflammation. Both processes are mediated by specific receptor-ligand interactions between the leukocytes and the endothelium. Inflammatory molecules and cytokines alter the cell surface expression of many of these molecules [52]. Some adhesion molecules expressed on the surface of EC and their respective ligands on immunocompetent cells are listed in Table 4.

Table 4. Endothelial cell adhesion molecules and ligands.

Endothelial cell receptor	CD nomenclature	Structure	Leukocyte ligand
P-selectin	CD62P	Selectin	Sialyl Lewis X
E-selectin	CD62E	Selectin	Sialyl Lewis X
ICAM-1	CD54	Immunoglobulin	LFA-1, Mac-1
ICAM-2	CD102	Immunoglobulin	LFA-1
VCAM-1	CD106	Immunoglobulin	VLA-4

Lymphocytes and monocytes follow the early transmigration of neutrophils and begin to arrive at a lesion about 4 h after an inflammatory stimulus. This change is reflected by changes in endothelial expression of adhesion molecules [53]. E-selectin begins to decline after 4-6 h, while ICAM-1 and VCAM-1 levels increase. Both ICAM-1 and VCAM-1 are induced by IL-1 and TNF and reaches a plateau after 24 h. Besides IL-1 and TNF, EC are known to produce several factors that are capable of inducing inflammation and recruiting leukocytes such as; IL-6, IL-8, GRO-α, MCP-1, G-CSF, M-CSF, and GM-CSF [54].

The endothelium as a target of tissue injury

In several vasculitis processes the endothelium itself is a target for injury and results in damage such as EC necrosis, denudation, or EC dysfunction that can lead to severe consequences [51]. Infectious agents can trigger vasculitis and most of them are immune-complex mediated (i.e., hepatitis B virus, hepatitis C virus) [55]. Complement-mediated lyses as well as neutrophil-mediated EC damage are the main mechanisms of EC injury in these processes. However, the role of EC as target of injury seems to be more prominent in small-vessel vasculitis [51].

Damage by neutrophils

ANCA was first described in relationship with glomerulonephritis by Davies *et al* [56] in 1982 and Hall *et al* in 1984 [57]. However, Van der Woude firmly associated them with WG in 1985 [58] suggesting that EC may suffer indirect damage owing to the actions of ANCA. There are two major types of ANCA [c-ANCA recognizing proteinase 3 (PR3) and p-ANCA recognizing myeloperoxidase (MPO)] and both c- and p-ANCA can activate primed neutrophils to mediate lysis of cultured EC [59-62]. However, stimulation of ANCA alone is not sufficient to cause EC injury [61, 63, 64].

The proposed ANCA-mediated mechanism in vasculitis is that TNF-primed neutrophils translocate PR3 and MPO to the surface and become further activated upon binding by ANCA, leading to a respiratory burst with release of reactive oxygen species, proteolytic enzymes, increased production of nitric oxide and cytokines/chemokines such as IL-1 and IL-8 that perpetuate the inflammatory response. Following ANCA activation, neutrophils undergo apoptosis and uptake of opsonised apoptotic neutrophils by macrophages evokes more production of TNF. The cytokine release cause further endothelial cell activation and upregulation of adhesion molecules affecting transmigration of neutrophils and the recruitment of other inflammatory cells [61, 63, 65].
There are also demonstrations that PR3 and MPO (highly cationic) can bind non-covalently to the EC surface and in this sitiuation they can be recognized and bound by ANCA. Neutrophils may then bind to ANCA via their Fc portion, activating the neutrophils and causing direct injury to the endothelium [66].
ANCA is a useful tool in diagnosis and disease monitoring, and the titres are supposed to correlate with disease activity. c-ANCA positivity is shown in more than 95% of WG patients and 75% of the MPA are p-ANCA positive. However, there are doubts about the true pathogenicity of ANCA in small vessel vasculitis [64, 67].

Injury associated with anti endothelial cell antibodies (AECA)

AECA is a heterogeneous group of antibodies detected in a variety of disorders connected with endothelial damage [68-71]. The presence of AECA in WG, Kawasaki disease, Behcet´s disease, Takayasu´s arteritis, MPA, SLE and RA are the most documented. In WG and MPA the AECA prevalence ranges between 55-80% [72]. In addition, the AECA titres have been proposed to change in line with the severity and correlate with the disease activity [18, 29, 49, 73-76].
The AECA bind to different endothelial structures, mainly via the $F(ab)_2$ portion of the IgG immunoglobulin with low avidity [70, 73, 75, 77-79]. The low avidity binding is probably the reason for *in vivo* absence of antibody deposits in the affected vessel wall as detected by immunohistochemistry technique [75]. AECA exhibit limited cross-reactivity with other cell types, mainly fibroblasts [73].

Bound AECA have the potential to mediate EC injury and lysis through either complement or cytotoxic effector cells ($CD8^+$, NK cells) or by more fine changes in EC function. The AECA have been found to fix complement in sera from SLE patients, but a defined cytotoxic effect has only been demonstrated in patients with haemolytic uremic syndrome (HUS) and thrombotic thrombocytopenic purpura [70, 73, 81].
Antibody dependent cellular cytotoxicity (ADCC) has been demonstrated in some patients with SLE and systemic sclerosis [82]. ADCC is a process by which specific antibody binds to the target cell and engages a natural killer cell (NK cell) via its Fc receptor causing subsequent lysis of the target EC cell. The importance of ADCC in vasculitides is not clear [34, 66]. However, AECA from WG and SLE patients have shown to:

i) increase expression of adhesion molecules such as CD62E, VCAM-1, and ICAM-1,
ii) leukocyte accumulation and adherence,
iii) enhanced secretion of proinflammatory cytokines and chemokines (IL-1, IL-6, IL-8, and MCP).

The cytokine secretion seems to support the up-regulation of adhesion molecules via an autocrine loop [74, 75, 83]. All of these effects might potentially cooperate in inducing the vessel wall inflammation seen in vasculitis *in vivo*. The absorption of anti-PR3 activity does not seem to affect the EC modulating effects of isolated IgG AECA from WG patients. However, IgG from ANCA positive sera (but AECA-negative) failed to induce adhesion molecule expression and leukocyte adherence. There are also WG patients positive for AECA, but negative for ANCA, representing patients with a higher risk for clinical relapse [74, 75]. One experimental model has provided further evidence that AECA can be pathogenic. A panel of mice were immunized with AECA-reactive IgG. The mice shortly developed histological signs of both renal and pulmonary vasculitis [74, 75, 84].

The antigen targets of AECA are not well defined. Antigen characterization using Western blotting and immunoprecipitation of AECA-postive sera with cell membrane preparations or whole EC extracts have given indecisive results (ranging between 25-200 kDa) [78, 79, 85] probably due to the technical difficulties and the wide range of hetergenous antigens [49, 75]. However, absorption studies have shown that AECAs antigen specificity does not seem to be; nuclear, ABH blood group or MHC complex antigens [69, 73]. The diagnostic influence of AECA and the understanding of its role in disease will improve when the autoantigens are defined.

Damage by the coagulant / thrombotic system

AECA may have further effects on the EC by promoting a procoagulant state, in concert with IL-1 and TNF, with increased production of tissue factor (TF) [61] and von Wilebrand factor (vWF) [86]. TF is the initiator of the extrinsic coagulation pathway and vWF mediates the process by which platelets adhere to exposed collagen and release their granular contents [61]. Furthermore, the thrombosis products themselves can then trigger EC activation, leukocyte recruitment and inflammation, for example: thrombin and fibrin activate EC to secrete IL-8. Fibrin is also an important chemoattractant for macrophages into the Bowman´s space [87].

Platelets may also contribute to vascular damage via a variety of surface receptors, including MHC class I molecules, IgG receptors, low affinity IgE receptors, vWF and fibrinogen receptors [66]. Normally, platelets do not adhere to the vascular endothelium or to each other, but when the vessel wall is damaged they accumulate and adhere to

macromolecules, such as collagen, that is exposed in the subendothelial tissue. Upon adhesion to the sub-endothelium, the platelets become activated, change shape, release their granular contents (which accelerate the formation of the platelet plug and play a role in tissue repair), and aggregate to form a first plug. The surface of activated platelets provides a procoagulant surface that triggers the sequential activation of clotting factors to produce the thrombin burst that result in fibrin polymerization [65, 66].

Damage by other immunocompetent cells

Other immunocompetent cells also have the potential to damage the endothelium. In autoimmune vasculitis it is possible that EC present autoantigen to either HLA class II or I restricted T cells, since EC have the capacity to present antigens to T cells *in vitro* [88]. Activated T cells, predominatly $CD4^+$, are found in biopsy specimens of the respiratory tract and kidneys and in PBMCs of WG patients [61]. There is no data yet relating to the potential cytotoxicity of monocytes, NK cells or eosinophils to the endothelium in vasculitic disorders [66].

CIRCULATING ENDOTHELIAL CELLS

Origin of circulating endothelial cells

Until recently, it was believed that vascular formation in adults resulted exclusively from proliferation, migration and remodelling of pre-existing vessels, a process referred to as angiogenesis. Angiogenesis is important for general development, reproduction and wound healing, but also for tumour growth and metastasis. In contrast, vasculogenesis is defined as the formation of new blood vessels from endothelial progenitor cells (EPCs) during embryogenesis. The first evidence for postnatal neovascularization by human peripheral blood-derived $CD34^+$ precursors (EPCs) was shown in an immunodeficient mice model of hindlimb ischaemia by Asahara and colleagues, who described the incorporation of progenitors into sites of active angiogenesis [89]. Angiogenesis and vasculogenesis is believed to occur simultaneously [90, 91]. A putative candidate called the "hemangioblast" is thought to be a common stem cell precursor for both EC and hematopoietic stem cells [43, 92, 93].
Circulating EPCs are presumed to be bone marrow derived and to be important in repair following vascular damage, while circulating mature EC are cells that randomly detach from the vessel wall and enter the circulation as a result of vascular injury [90, 94, 95]. There is no proof of biological function assigned to the circulating mature EC.

Vascular injury: detachment of endothelial cells

In normal conditions, 99% of endothelial cells are quiescent and the physiological turnover of the endothelium is reflected by low basal levels of circulating EC (CECs; EPC including mature EC). In healthy individuals, hardly any CECs are detectable in the peripheral blood. The potential mechanisms of EC detachment from the vessel are multiple [96, 97]. Mechanisms suggested to be associated with increased detachment of EC are summerized in Table 5.

Table 5. Possible mechanisms of endothelial cell detachment in vascular injury.

Activation of apoptosis
Cytokine and protease-mediated injury
Defective endothelial cell adhesion: intercellular, or to the extracellular matrix
Imbalance in pro-angiogenic and anti-angiogenic factors
Mechanical injury
Drugs, e.g. cyclosporine

Characterization of circulating endothelial cells

Conformity about the phenotypic differentiation of EPC and circulating mature EC is still lacking as these cells share several markers. The most widely used EPCs surface marker are Flk-1/KDR and CD133, although Flk-1/KDR is also expressed on mature EC [98-102]. Flk-1/KDR is a receptor for vascular endothelial cell growth factor (VEGF). This receptor seems to be important for embryonic endothelial cell differentiation and vasculogenesis. FLk-1 cells can give rise to both endothelial cells and vascular smooth muscle *in vitro* and *in vivo* [98, 103]. CD133 is downregulated upon differentiation and is therefore a marker for EPCs. CD133 positive cells have the capacity to differentiate into

EC *in vitro* [98, 99, 104]. A very small subset of cells (0.01 - 0.002%) of mononuclear cells in the peripheral blood stain positive for these two markers simultaneously [99, 100]. CECs are mostly detected with antibodies against CD146. The marker CD146 is however not specific for mature EC and detects both EPCs and mature EC in the circulation [94, 105-109]. Thus, currently there are no specific markers that help differentiate EPCs from mature EC. Therefore phenotypic markers that may help distinguish between these two EC types are needed.

Isolation of circulating endothelial cells

From a clinical point of view, markers of ongoing endothelial injury and damage are of great interest for the diagnosis, monitoring of the disease activity, as well as to make a decision about treatment.
The methodology for detecting CECs evolved from smears of peripheral blood [110] to immunomagnetic isolation [110, 111]. Since CECs are present in extremely low levels as compared with other cell populations in peripheral blood, the CD146-immunomagnetic isolation has become the golden standard for their detection and enumeration [105]. However, EPCs may also be CD146 positive, which make the method unreliable and needs futher evaluation [94]. The most common alternative to the CD146-immunobead method is flow cytometry, where whole blood is generally labelled with monoclonal antibodies. Flow cytometry is regarded to be more sensitive, often reporting up to 1000-fold higher numbers of CECs as compared with the immunobead method [94]. The variability in the numbers of CECs is not only a reflection of different methods used, but also the diversity of each disease studied. In Table 6 some studies of CECs enumeration in different diseases are summarized.

Table 6. Circulating endothelial cells (CECs) in vascular disorders.

Disease	Method used	Cases (CECs/ml)	Normals (CECs/ml)
SLE	Flow cytometry	89	10
Systemic sclerosis	Flow cytometry	243-375	77
ANCA vasculitis	Immunobead	136	5
Kawasaki disease	Immunobead	15	6
Throbotic thrombocytopenia	Immunobead	6-220	<3
Rickettsial infection	Immunobead	5-160	<3
Renal transplantation	Immunobead	24-72	6

Circulating endothelial cells in vascular disorders

Elevated levels of CECs are found in a variety of conditions characterized by vascular injury or vessel formation (Table 6) and the number of CEC have been shown to correlate with the degree of endothelial injury or neovascularization.
In ANCA associated vasculitis, SLE, Behcet´s disease, acute coronary syndromes, sickle cell patients, and in patients following allogenic stem cell transplantation - CEC levels were higher during the acute phase of the clinical syndrome and were found to predict both the severity and outcome in the vascular disease [106, 107, 111-113]. In remission, patients demonstrate only moderately elevated CECs numbers and with immuno-suppressive treatment the CECs numbers decline markedly. With ANCA-associated vasculitis, it has also been demonstrated that these CECs were necrotic together with pro-coagulant features [114, 115].

Endothelial cells (EC) are able to amplify the inflammatory response by adhesion molecule expression, cytokine production and angiogenesis (angiogenesis is not further discussed) [34]. EC act as the gatekeepers for tissues, regulating the quality and amount of leukocytes entering organs during both basal immune surveillance and inflammation. In order to do this EC respond to cytokines/chemokines and other proinflammatory agents that alter or induce the expression of molecules on the endothelium, leading to the ordered capture and later transendothelial migration of leukocytes from the blood [66].

Two such "stress"- induced molecules on the endothelium are the vascular adhesion protein 1 (VAP-1) and MHC class I – related chain A molecule (MICA). In this thesis, VAP-1 and MICA were shown to be highly expressed in kidney sections from active WG patients. Therefore, these two molecules will be further highlighted.
Another important feature of inflammation in vascular disease is the chemokines that direct the migration of leukocytes to the site of injury [116, 117]. A brief description of some chemokines detected in our studies will follow in Table 7.

MHC class I – related chain A molecule (MICA)

Major histocompatibility complex (MHC) class I chain related molecules (MIC) show homology (30%) with classical human leukocyte antigen (HLA) molecules, but they do not bind peptide nor combine with the β2 microglobulin, are not expressed on normal circulating lymphocytes, and are not upregulated by IFN-γ [118, 119]. The MIC genes are located within the MHC class I region of chromosome 6. Seven MIC loci (MICA-MICG) have been detected, of which only MICA and MICB encode expressed transcripts. There are more then 50 recognized human MICA alleles. MICB is less polymorphic, although 17 alleles have been described so far. The MICA locus encodes a membrane bound polypeptide of 383-389 amino acids with a relative molecular weight of 62 kDa, and the MICB locus encodes a protein of 383 amino acids bearing 83% homology with MICA [118]. The biological function of MICA and MICB still remains unknown, but in response to stress (e.g., virus, bacteria, heat, cold, oxygen deprivation), MIC proteins are expressed in endothelial cells, monocytes, epithelial cells, and fibroblasts, but not in $CD4^+$, $CD8^+$ or $CD19^+$ cells [118, 119]. MIC acts as a ligand for NK cells, γδ T-cells and $CD8^+$ T cells which express NKG2D receptor that co-localizes with DAP10, a transmembrane signaling adapter protein [118, 119]. When MICA is recognized by NKG2D receptors, this engagement is supposed to activate the cytolytic responses of the NKG2D bearing cells against the MICA expressing cells resulting in lysis of the cells [120]. In humans, the NKG2D receptor has a dual role: it functions as a stimulatory receptor in NK cells and as a co-stimulatory receptor in T-cells, however the mechanism underlying this functional duplicity is not fully elucidated. [121].

MIC molecules have been shown to be involved in areas like CMV infection, tumor biology and transplantation. For example, in transplantation, one study showed a significant correlation between pre- or post-transplant MICA specific antibodies and graft-loss. A total of 43,7 % of the patients with acute rejection had antibodies that reacted with kidney EC, wheras 90% of the non-rejectors did not [122].

MICA has also been involved in some autoimmune diseases. In RA, soluble MICA was found in large amounts in synoviocytes maybe causing autoreactive T cell stimulation [118]. In addition, as compared with healthy individuals, patients with WG, RA, and

multiple sclerosis have been shown to have increased frequencies of $CD8^+/CD28^{null}$ T-cells expressing killer cell inhibitory receptor (KIR) and/or NKG2D. One proposed idea is that the acquisition of stimulatory NK receptors by senescent T cells contributes to the breakdown of tissue tolerance in autoimmune diseases. The expression of stimulatory NK receptors could provide a mechanism through which the TCR activation can be amplified and through which the threshold for T cell activation by self-antigens can be lowered. Since NKG2D is constitutively expressed, acquisition of its stress induced MIC ligands might be the triggering incident that leads to breakdown of tolerance [121]. Whether breakdown of tolerance occurs in a similar manner in WG is not known.

Vascular adhesion protein 1 (VAP-1)

VAP-1 is a glycoprotein consisting of two identical subunits of 90 kDa upregulated upon inflammation from intracellular granules distinct from Weibel-Palade bodies. It is expressed in endothelial cells, pericytes, smooth muscle cells, fat cells, dendritic cells in germinal centers, but is absent in all leukocytes [123, 124]. VAP-1 functions as an adhesion molecule in the process of leukocyte trafficking to sites of inflammation. Only $CD8^+$ T cells and NK cells bind to VAP-1, while monocytes, B cells or T helper cells do not. The ligand couterpart for VAP-1 is not characterized [125-129].
Besides its adhesive property, it also possesses semicarbazide-sensitive amine oxidase (SSAO) enzyme activity. SSAO belongs to the copper-containing monoamine oxidases that catalyze the oxidative deamination of primary amines to the products: aldehyde, ammonium, and hydrogen peroxide [124, 130]. Both hydrogen peroxide and aldehydes are potent biological substances. At high concentrations they are cytotoxic and may contribute to different vascular lesions. Hydrogen peroxide may also have a signalling role in proliferation and gene expression of P-selectin in vascular EC and smooth muscle cells, further improving the leukocyte rolling [131]. VAP-1 SSAO activity is also required and critical for leukocyte transmigration, but how the adhesive and enzymatic properties of VAP-1 collaborate in this process remains unknown. Usage of the new SSAO inhibitor (BTT-2027) is the first evidence that blocking of the SSAO activity effectively ameliorates the development of an inflammatory reaction *in vitro* and the possibility of successfully combating inflammatory reactions via these small molecule compounds may open new ways for developing useful anti-adhesive therapeutics [130].

Chemokines

Table 7. Brief description of the chemokines used in this study [117, 132].

Chemokine	Attractant for	Expressed in
MCP-1	Monocytes, macrophages, T-cells	EC, fibroblasts, smooth muscle cells, epithelial cells, mononuclear leukocytes
IL-8	Neutrophils	EC, monocytes, macrophages, T-cells, fibroblasts, keratinocytes
GRO-α	Neutrophils	HUVEC, monocytes, fibroblasts, epithelial cells, melanocytes
GCP-2	Neutrophils	EC, fibroblasts
ENA-78	Neutrophils	EC, fibroblasts, platelets, mast cells
MIP-1α	Mainly CD8+ T-cells, but also B-cells, NK-cells, dendritic cells, eosinophils, macrophages	EC, T cells, B cells, monocytes

AIMS OF THE PRESENT STUDY

The exact mechanisms by which endothelial cells of the upper and lower respiratory tract and kidneys are damaged in Wegener´s granulomatosis are not known.
Therefore, the general aim of this thesis was to elucidate some of the mechanisms by which endothelial cells are damaged in Wegener´s granulomatosis.

The specific aims were:

Paper I.
To study the interaction and frequencies of AECA in WG sera with clinically relevant endothelial cells isolated from the small blood vessels of the nose, kidney and lung.

Paper II.
To isolate and characterize human nasal endothelial cells from the inferior turbinate.

Paper III.
To elucidate the role of circulating endothelial cells in the pathogenesis/progression of Wegener´s granulomatosis.

Paper IV.
To investigate the functional role of IgG AECA in the pathogenesis of WG.

METHODOLOGICAL CONSIDERATIONS

Most of the methods have been described in detail in Papers I-IV. The following section list some of the methods used together with some general considerations.
Informed consent was obtained from all patients prior to blood or tissue sampling.
All studies were approved by the ethics committee at Karolinska University Hospital in Huddinge (Dnr 407/01 and 263/03).

Patients and samples

In paper I, serum samples from WG, "limited Wegener´s granulomatosis" (LWG), microscopic polyangiitis (MPA) and renal limited vasculitis (RLV) were included.
All patients were defined according to the "Chapel Hill Consensus Conference on the nomenclature of systemic vasculitides". For comparison, patients with SLE, RA and healthy normal individuals were also included. All SLE and RA patients were under treatment at time of blood sampling. After blood sampling, serum was separated by centrifugation and stored in -20 °C until use.
In paper II, human nasal specimens (1-2 cm) were obtained from patients undergoing surgery due to some structural deformity, such as septal deviation, for the isolation of nasal EC. These patients were healthy and with no other pathological conditions apart from their septal predicament, that might have an effect on the actual outcome of the nasal EC isolation. After surgery, the biopsy was immediately placed in standard cell culture medium (RPMI 1640) until isolation was performed. Isolation of nasal EC was usually performed within 3-4 hours after surgery.
In paper III, blood samples were obtained from untreated active PR3-ANCA positive WG patients, treated WG patients in remission and normal healthy controls. All blood samples were analysed on the same day, in most cases within 1-2 hours.
In paper IV, sera from WG patients known to have high frequencies of AECA reactive against kidney EC (based on our previous results in paper I) were pooled and used for further isolation of IgG fractions.

Detection of AECA with flow cytometry

The detection of AECA in patient sera against endothelial surface antigens can be performed using various methods. The most common are flow cytometry assay and enzyme-linked immunosorbent assay (ELISA). For the flow cytometric assay, trypsinized and single-cell suspended cells were incubated with patient serum, followed by a secondary fluorescent-labelled anti-human antibody that was detected by the flow cytometer.
The advantage of the FACS technology is that it is rapid, quantitative and phenotypically different subsets of cells can be studied simultaneously. In addition, several measurements such as; debris, cell size, granularity, cell death, cell proliferation, cell cycle analysis, cytokine expression etc, can be applied by using the flow cytometry. When studying a known homogenous population of cells, flow cytometry may not be necessary. In such a case the ELISA method is equally quick, easy, less expensive and allows processing of larger number of samples per day than with the flow cytometer. However, flow cytometry is also used for uniform cell populations [80].
The advantage of cell based ELISA is that the cells are in their natural adherent monolayer appearance. The drawback is that the immobilization/fixation of the cells might alter the protein confirmation of the native protein making the true antigen undetectable, and might

give false positive or negative reactions [133, 134]. Second, sera have been found to react strongly with the gelatin coating used in ELISA plates to facilitate EC adhesion [80]. Whether the antibodies in the sera bind to the gelatin coating in a non-specific manner or reacting specifically against gelatin epitopes is not clear. In addition, the colored end product is proportional to the amount of protein in the whole sample, which does not permit single cell analysis.

Isolation of nasal endothelial cells

In one of our studies we used five different types of human microvascular EC. These were isolated from; nose, kidney, lung, liver and human umbilical cord (HUVEC).
Lung (HLMEC) EC and HUVEC were commercially purchased and were cultivated in their respectively recommended media. Kidney (HKMEC) and liver (HLSEC) EC were isolated according to methods described earlier in detail [135, 136].
However, for the isolation of the nasal endothelial cells (HNMEC) there did not exist any established protocol. Briefly, the nasal biopsy specimens were first cut/minced into small pieces and enzymatically digested with collagenase type IV solution. We found that collagenase IV and a digestion time of 5-7 minutes were enough to break down the native collagen that holds the nasal tissue together.
The HNMEC were isolated using anti-CD144 antibody coupled microbeads. This method is simple, quick and gave a general yield of approximately $2.5x10^5$ $CD144^+$ cells per 1.5 cm of nasal tissue specimens. Culture of $CD144^+$ cells demonstrated the presence of two heterogeneous EC populations with different morphology; one cobblestone and one fusiform. From these cultures we manually picked the cobblestone and the fusiform populations and placed these in new individual culture plates. This step needed precision, a firm hand and good knowledge of cell morphology. Both cell types were cultured under same conditions and maintained their characteristics during 11-13 passages. The vascular type was however more frequent in the $CD144^+$ cultures, yielding in general 70% vascular and 30% sinusoidal HNMEC.

Gene microarray

In one of the studies (paper II) we performed a microarray analysis. Total RNA from two different populations (vascular and sinusoidal) of unstimulated HNMEC was prepared. Labelled cDNA were hybridized to a Human Genome U133 Plus 2.0 Gene chip which represents around 38 500 genes, where some genes were represented by more than one probe set. The actual microarray procedure was performed by Karolinska Institute Core Facility using well-established standard protocols from Affymetrics, Inc. (Santa Clara, CA, USA). This method generates large amounts of genetic information, which can be obtained in a short period of time. However, it is tedious to handle all data properly for a correct analysis. The sample data is given as fold change e.g., increased or decreased in comparison between the two samples, and not absolute values of the relative mRNA abundance for each representative gene. The use of a third sample for comparison would give more accurate results. Unfortunately, the reproducibility is limited by the high cost of the assay. Another disadvantage is that data collected from different microarray platforms cannot be accurately compared due to the absence of a “common language” to exchange data between different groups.

Boyden chamber assay

The Boyden chamber is used to study the chemotaxis of leukocytes or other migratory cells. A solution containing a chemokine/chemotactic factor is placed in the bottom chamber and the cell suspension is placed in the upper chamber. A filter separates the lower and upper chamber. Cells are then allowed to migrate through the pores, across the thickness of the filter, and toward the source of chemoattractant in the lower chamber [137].
Another way of studying cell migration is by using a transwell system [138]. In this system the filters are inserted at the bottom of a dish ranging from 24-96 well sizes, which can be inserted in a suitable cell culture plate. The transwell system is much easier to handle since the size of the loading chambers are larger and operates with larger volumes of fluid. Small volumes of fluids often make bubbles when pipetting, which can disturb the assay especially in the Boyden assay where the volume in the upper chamber is only 56 µl. However, for small quantities of cells and fluid the Boyden assay is prefered to the transwell system. Cell-cell/cell-substrate/cell-matrix interactions and feeder-layers can also be studied with the transwell system since cell layer can be cultured on top of the filters. The filter can later be fixed and subjected to both transmission and scanning electron microscopy (EM). Neither cell culturing nor EM is possible with the Boyden chamber filters. However, both the Boyden and transwell system can be used to study chemotaxis, chemokinesis and haptotaxis.

Calcium mobilization

To evaluate if isolated IgG from WG patients had a capacity to mobilize calcium flux in EC, a dual-excitation fluorescent microscopy was used together with the calcium-sensitive ratiometric dye Fura 2-AM. Stimulus, WG IgG or normal IgG was added to the cells in a final concentration of 0.5 mg/ml. Total record time was 200 seconds. Images were acquired and the ratio between 340 nm/380 nm ratio images were calculated 'off-line' following background subtraction with commercially available software (Miracal, Life Science Resources Ltd). A measurement of Ca^{2+} levels with fluorescent probes is one of the most sensitive techniques known. The method is based on the principle that these compounds display shifts in their excitation or emission upon calcium binding [139-141]. Since there are no known reports about IgG AECAs role as a stimulating/activating agent on endothelial cells in vasculitides, this study was conducted in order to see if IgG AECA from WG patients had a capacity to mobilize a calcium flux in kidney EC. With this method, a first sign of cell activation can be determined, however, downstream events, such as signalling pathways, cannot be determined.

Purification of human anti-PR3 depleted IgG antibodies

Sera from WG patients known to have AECA reactive against HKMECs (based on our previous results in paper I) were pooled and total IgG fractions were isolated using goat anti-human IgG (Fc-chain specific) agarose beads according to standard procedure. Bound IgG was eluted by 0.1 M Glycine-HCl (pH 2.5) in fractions and neutralized with 1 M Tris-HCl (pH 7.5). Protein concentration was measured spectrophotometrically at 280 nm, IgG fractions were pooled, dialyzed, lyophilized, and resuspended in distilled water.

Since a role for neutrophils and c-ANCA has been implicated in the pathogenesis of WG, we depleted the WG IgG fractions from c-ANCA antibodies (directed against PR3). The isolated WG IgG fraction was then applied to a prepared PR3-column and the flow-

through was collected, dialyzed and concentrated as described above. The efficiency of anti-PR3 depletion was evaluated by measuring the ANCA activity by anti-PR3 ELISA (PR3-ELISA from Wieslab AB, Lund, Sweden) according to manufacturer's protocol. IgG concentration was determined by standard Mancini method. The PR3-depleted WG IgG was used for all subsequent analysis.

SDS-PAGE and Western blot

SDS-PAGE and Western blotting was performed to identify the autoantigen(s) recognized by anti-PR3 depleted WG IgG. HKMEC were labelled with biotin-7-NHS (D-biotinoyl-e aminocaproic acid-N-hydroxysuccinimide ester) solution and immunoprecipitated according to standard protocol. The biotin-labelled proteins were purified by immunoprecipitation (with 10 mg/ml of WG IgG) and protein G agarose beads. Immunoprecipitated proteins were then electrophoretically separated on an 8% resolving gel. After electrophoresis, the proteins were blotted onto a PVDF membrane and subsequently detected using a streptavidin-horseradish peroxidase conjugate and luminal/iodophenol as substrate. The chemiluminescence signal was visualized by exposure on X-ray film.
This method is sensitive and involves many steps that might give unexpected results. For example, it is difficult to assay if the immunoprecipitation with the primary antibody has worked successfully, and when loading the protein onto the gel the exact amount of protein is not known. Another drawback is that proteins of same size cannot be separated. For example, if we have many copies of two different proteins that were both of the same amino acids long, they would travel together through the gel in a mixed band. As a result, it is not possible to separate these two proteins from each other with SDS-PAGE method. A 2-dimentional gel (2D-gel) approach where the proteins are separated according to size and isoelectric point would give a better molecular identification of the proteins.

Organ specific anti-endothelial cell antibodies

AECA have been described in several vasculitis disorders both primary and secondary vasculitides, with a common effect resulting in endothelial damage [30, 70, 74, 75]. Although, the presence of AECA in ANCA-positive vasculitis and SLE are the most documented [142]. In most of these studies regarding AECAs pathogenic role, HUVEC cells have been traditionally used as a target cell line [39, 143, 144]. This may be misleading, due to the fact that the endothelium displays phenotypic and functional heterogeneity depending on the anatomical site. In WG it is the small to medium sized vessels that are damaged, whereby the use of a large vein EC becomes questionable. The anatomic localization of the affected vessel is also closely related to the clinical manifestations seen in vasculitides.

Therefore, in paper I, we isolated EC from the relevant target organs affected in WG namely, nose, lung and kidney, for the detection of AECA in sera from WG and MPA patients. Sera from RA, SLE and normal healthy controls were also included in the study. We found by flow cytometry, that significantly higher numbers of WG patients reacted specifically against unstimulated kidney (71%) and nose (61%) EC, as compared to unrelated target cells like HUVEC (7%) and liver (0%) EC ($p<0.0001$). These findings point out the importance of using the correct target EC when studying different vasculitis disorders, since EC have specialized functions depending on their tissue location. The same pattern of reactivity was also seen in WG patients in remission. However, the titres of the AECA were much lower.
Another pattern was shown with RA and SLE sera. AECA from RA patients reacted weakly (0-10%) in general (except with lung EC 60%) against all of the EC used, while in SLE, AECA reacted strongly (between 55-100%) against all EC types without any organ specificity. RA is an autoimmune disease that causes chronic inflammation mainly of the joints [145] and SLE is a chronic disease with many manifestations in glomeruli, skin, kidney, lungs, synovium and other places [146]. The different disease patterns in these two disorders correlate with the low percentage of AECA in RA sera and high percentage of AECA in SLE sera, respectively.

AECAs reactivity was also investigated against cytokine stimulated (TNF-α and IFN-γ) EC. Interestingly, significantly fewer sera reacted against stimulated kidney (29%) and nose (11%) EC ($p<0.001$). A similar pattern of decreased AECA reactivity upon cytokine stimulation has also been described in haemolytic uremic syndrome (HUS) [81]. Speculations on this decreased reactivity may be that TNF-α and IFN-γ induce a down regulation of the AECA target antigen, or induce an antigen alteration, alternatively induce endocytosis of the antigen making the antigen undetectable for AECA. Maybe this might explain the fact that WG patients show high levels of AECA in circulation and absence of *in vivo* antibody deposits in affected vessels [147].
Nor do most types of vasculitides show any *in vivo* complement depositions, indicating that AECA most probably are not lytic. Antibody dependent cellular cytotoxicity (ADCC) has only been established in a few studies concerning AECA role in vasculitis [147] and is not regarded as a mechanism involved in EC damage in vasculitides.

We found that WG sera were not able to lyse unstimulated nose or kidney EC in a complement-dependent cytotoxic assay. However, when using cytokine stimulated nose EC, WG sera lysed ~ 60% of the nose EC as compared to 10-15% in the unstimulated scenario. Perhaps the cytokine treatment makes the cells further susceptible to unknown

reactive components present in the sera. The same experiment was carried out after heat-inactivation (1 hour, 56 °C) of the WG sera, and the percentage of lyzed cells remained the same, indicating that the complement factors (e.g., are heat-labile) may not play a role in the lysis of cells. Furthermore, this increased lysis of nose EC was only observed with WG and MPA sera and not with RA or SLE sera, again reflecting the disease specificity in vasculitis. However, addition of sera from all patient groups to cytokine stimulated lung EC resulted in increased lysis. In addition, without adding rabbit-complement to the reaction, nose and lung EC were the two cell types most sensitive to cytokine treatment as detected with propodium iodide staining of dead cells. Another interesting finding was that addition of WG but not MPA sera, to cytokine stimulated nose EC resulted in aggregation or clumping of the cells. The biological significance of this observation is currently not known.
In summary, these results give additional confirmation that EC from different locations are in fact heterogeneous not only in their phenotype, but also in their response to different stimulus. This indicates the importance of using clinically relevant target EC for a better understanding about the processes underlying the disease mechanism in WG as well as in other vasculitides.

Nasal endothelial cells

We report a novel finding about the presence of two heterogeneous populations, vascular and sinusoidal, of human nasal microvascular EC isolated from the inferior turbinate. During the isolation of nasal EC in paper I, we observed the presence of two morphologically different EC in the cell cultures. Therefore, in paper II, we pursued this observation and studied in detail the two different EC types.
Both populations were examined by light and electron microscopy, flow cytometry and immunocytochemistry. Analysis demonstrated that the vascular population exhibit classic vascular endothelial markers (CD31, CD62E) with cobblestone-like morphology.
The sinusoidal population had fusiform morphology and did not express CD31 and CD62E. However, the sinusoidal population expressed another surface marker, L-SIGN, which is found in sinusoidal EC of the liver and lymph nodes [135]. The sinusoidal population also showed other features, such as fenestration, lack of tight junctions and was discontinuous. These EC characteristics resemble those of the liver EC where the arrangements of the liver sinusoids facilitate the exchange that takes place between the blood and hepatocytes [45, 46, 148]. In comparison, the microvasculature of the nose contains a network of capillaries with fenestrations allowing water to escape into the lumen airway, permitting evaporation and enabling conditioning of the inspired air [149, 150]. Perhaps the lack of CD31 and CD62E expression in the nose is a reflection of their different functions that may have impact on their leukocyte interactions at this anatomic site. Currently, there are no studies performed on how immune cells interact with nasal sinusoidal EC and the consequences of these interactions are not known.

In the liver, it has been suggested that the lack of important adhesion molecules might indicate an increased risk of recurrent infections [45, 46] and the loss of fenestrations may result in diseases like cirrhosis, fibrosis, atherosclerosis, hyperlipoproteinemia and cancer [48]. Our group has recently shown that autoantibodies against liver sinusoidal EC can transform these cells to a vascular type with no fenestration and tight junctions [135]. This transformation may play an important role in hepatocellular failure and portal hypertension in patients with autoimmune hepatitis and primary biliary cirrhosis. Whether capillarization of the nasal sinusoidal EC occurs in various nasal disorders is not known and will be interesting to investigate.

The existence of vascular and sinusoidal EC was further confirmed by *in vivo* staining of nasal biopsy sections. Here, sinusoidal EC (L-SIGN positive) were found mainly in the surrounding area of epithelial cells, whereas vascular EC were found in vessel areas. This typical pattern is also observed in the liver [45, 46, 48].
For additional insight in the EC heterogeneity, the vascular and sinusoidal EC were analysed by gene array. From the vast data generated, we selected genes considered to be involved in human EC biology. In general, the profile between the two populations was comparable, with a few exceptions. The sinusoidal EC showed higher expression of five genes: MMP-1, MCP-1, CD106, collagen type 1 and osteoprotegerin.
Further investigation is needed in order to explain if these differences have any physiological or clinical implications. However, it is important to note that this represents cells from one individual at one single time point, and it is likely that the gene expression will vary between individuals.
In conclusion, these two heterogeneous EC populations provide a unique *in vitro* system to study the biology of nasal vascular and sinusoidal EC in normal conditions as well as inflammatory processes in various nasal disorders such as: asthma, non-allergic inflammation, allergic rhinitis, vasculitides such as Wegener´s granulomatosis, nasal polyposis and other nasal diseases.

Circulating endothelial cells

Circulating endothelial cells (CECs) consist of at least two cell types: endothelial progenitor (EPCs) cells and mature inflammatory EC (IECs). EPCs are either bone marrow or peripheral blood derived and IECs are probably detached from the vessel wall and enter the circulation due to vascular injury. Several studies concerning vasculitis (as well as in other disorders) indicate the use of CECs as a disease marker and /or as a monitor marker for relapses [94, 107, 109, 110, 112, 114, 115, 151-158]. Most of these studies isolate CECs by using CD146 antibody coupled magnetic beads [94, 105, 109, 114, 158]. CD146 is expressed on both progenitor and mature EC [94, 114], but also in our experience on activated T cells (unpublished results), making the results hard to interpret. On the other hand, EPCs have been identified using monoclonal antibodies against several surface markers including; CD133, VEGFR-2, Tie-2, and CD34 [99, 101, 104, 159-166]. No general agreement of a consensus for EPCs and IECs markers exists today.

In paper III, we reported that WG patients with active disease displayed significantly higher number of circulating (IECs), as compared to WG patients in remission and healthy controls. A novel finding is that these circulating IECs could be distinguished from EPCs by the expression of vascular adhesion protein 1 (VAP-1) and MHC class-I related chain A (MICA). IECs expressed both VAP-1 and MICA, while the EPCs did not. Isolated IECs (with VAP-1 coupled beads) showed expression of MICA, several known endothelial cell surface markers, but were negative for stem cell, leukocyte, monocytes, B cell and NK cell staining, indicating their true endothelial origin.

EPCs were isolated by a two-step colony-forming assay resulting in colonies of EPCs consisting of multiple thin, flat cells originating from a central cluster of rounded cells [167]. Colonies were counted manually and stained positive for markers like CD133 and VEGFR-2, and e-NOS. EPC colonies were negative for VAP-1, MICA and i-NOS staining. By flow cytometry, single and double staining also confirmed the fact that VAP-1 and MICA might be used as markers to distinguish EPCs from IECs.

EPCs have been shown to contribute to the homing and regeneration of the vasculature in several injury-model studies [89, 90, 102] while no function has so far been ascribed to IECs after their detachment from the site of injury.

However, we showed in this study that IECs secreted various neutrophil activating chemokines (IL-8, ENA-78, MIP-1α and GRO-α) and expressed iNOS and supernatants from IECs induced increased neutrophil migration. In addition, supernatants from IECs had a significant negative effect on the proliferation, migration and eNOS expression of EPCs. WG patients with active disease also showed decreased amount of EPC colonies as compared to those in remission. Together, these results indicate that IECs may contribute to the vascular damage by impairing the functional capacity for repair by EPCs (see figure 2, page 50), and IECs might be helpful as a disease marker in the diagnosis of WG.

In vivo staining of kidney sections from WG patients with active disease showed expression of endothelial VAP-1 and MICA. Perhaps the circulating IECs observed in WG might be cells detached from the site of injury, due to the vasculitis in the kidneys. We also observed that increased levels of IECs were associated with increased organ involvement. Whether this is an outcome of the endothelial damage in different organs is at present not known.

Functional binding of IgG AECA

In paper IV, the general aim was to elucidate the functional role of IgG AECA (anti-PR3 depleted) from WG patients. Kidney involvement is seen in the majority of WG patients (85%) [13, 168]and kidney EC was therefore the choice of target cell used for studying the functional role of AECA. We had earlier observed an intense expression of MICA and VAP-1 in kidney biopsy sections taken from patients with active WG. Therefore one of the aims was to investigate if IgG AECA could induce expression of VAP-1 or MICA on kidney EC. We also attempted to identify the putative autoantigens recognized by IgG AECA.
We found that stimulation of kidney EC with WG IgG elicited a rapid Ca^{2+} flux (a primary signal of cell activation) within seconds, induced high levels of neutrophil/monocytes attracting chemokines MCP-1 and GCP-2, and up-regulated surface expression of MICA, but not VAP-1. MICA is known to be induced by external stress and is the ligand for NKG2D receptors found on mainly natural killer cells, $CD8^+$ T-cells, and γδ T cells [118]. The ligand engagement of NKG2D activates NK cells and potently costimulates effector T cells [121, 169, 170]. However, the biological significance of MICA remains unknown [119]. $CD8^+$ and γδ T-cells were the most frequent cell types when staining WG kidney sections for infiltrating cells.

Based on the above observations, we propose the following mechanism for endothelial dysfunction in WG in figure 2, page 50.

Western blot analysis of immunoprecipitated HKMEC proteins with IgG AECA revealed three bands of: 190-200 kDa, 70-73 kDa and 50-53 kDa. Further molecular identification and characterization of the immunoprecipitated proteins will be of importance for studying the nature of the autoantigens in WG.

CONCLUSIONS

In our studies, we demonstrate the following:

- WG patients have high frequency of non-lytic AECA against clinically relevant EC namely: nose, kidney, and lung endothelial cells, but not against unrelated liver EC and HUVEC, indicating organ specificity for the AECA.

- Nasal microvascular endothelium consists of two heterogeneous populations of cells - vascular and sinusoidal. These cells will provide a unique *in vitro* system to study the pathogenesis of nasal vascular diseases.

- VAP-1 and MICA are novel markers of endothelial inflammation that may help to distinguish between EPCs and IECs.

- IECs might be a new disease marker in WG.

- IECs may contribute to the vascular damage by impairing the functional capacity for repair by EPCs.

- In kidney EC, stimulation with WG IgG AECA elicited a rapid Ca^{2+} flux, induced high levels of chemokines and up regulated surface expression of MICA.

- Western blot of immunoprecipitated kidney EC with WG IgG AECA revealed three bands: 190-200 kDa, 70-73 kDa and 50-53 kDa.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Vårt immunförsvar är utvecklat för att skydda kroppen mot främmande ämnen, t ex bakterier, svamp och virus, som hotar att angripa vår kropp. Immunförsvaret gör detta genom att känna skillnad på vad som är kroppen själv och vad som är främmande (t ex bakterier som kommit in i kroppen), och därefter angripa det som är främmande medan den egna kroppen lämnas ifred. Tyvärr händer det att immunsystemet angriper en eller flera av kroppens egna vävnader, vilket kallas för autoimmunitet. Några välkända exempel på autoimmuna sjukdomar är multipel scleros (MS), systemisk lupus erythematos (SLE), reumatisk artrit (RA) och typ I diabetes. Orsaken till varför dessa sjukdomar uppkommer är oklar, men troligt är att det är en kombination av arv och miljö, d v s att en person med en viss genetisk uppsättning under vissa omständigheter utvecklar sjukdomen.

Wegener´s granulomatos (WG) är en systemisk vaskulit sjukdom (vaskulit = inflammation i blodkärl) som uppkallats efter den tyske läkaren Friedrich Wegener, född 1907-1990. Den årliga incidensen uppskattas till 10-15/miljon/år med mycket lätt manlig övervikt. Sjukdomen kan förekomma hos barn och upptill hög ålder. Det finns ingen känd orsak till varför man får WG, men sjukdomen är inte smittsam och det finns inga bevis på att den är ärftlig.
Hur den kliniska bilden yttrar sig beror dels på storleken av det kärl som är drabbat dels vilket eller vilka organ de drabbade kärlen försörjer. I WG är det de små till medelstora artärerna som blir drabbade. Alla organ kan i princip drabbas, men det vanligaste är luftvägarna och njurarna.
Vanliga och ospecifika symtom är nästäppa, rinnsnuva, näsblödningar, trötthet, feber, hosta mm. Dessa symtom misstolkas ofta som en besvärande förkylning/luftvägsinfektion som inte ger vika för antibiotika behandling. Det tidigaste tecknet på njurpåverkan vid WG är att urinen innehåller röda blodkroppar eller proteiner. Om detta går obehandlat kan njurfunktionen förloras inom några dagar. Mer än 95% av patienterna med WG har sk. ANCA-antikroppar mot cytoplasmatiska (i cellen) komponenter i neutrofiler (en typ av vit blodkropp). Vanligast är antikroppar riktade mot enzymet proteinas-3 (PR3-ANCA) som har hög specificitet för WG. Hos en del, men inte alla patienter med WG, följer halten av dessa antikroppar sjukdomens aktivitet.
Förloppet för många patienter är ofta dramatiskt, och individer kan på några veckor utveckla livshotande sjukdomstillstånd. Om inte behandling med steroider, cytostatika, plasmafores eller dialys tillgrips, leder WG till döden. Tyvärr vet man idag fortfarande inte hur dessa sjukdomar uppkommer och specifika behandlingsmetoder saknas. Det finns alltså ett stort behov av metoder för att upptäcka och förbättra behandlingen av dessa sjukdomar.

Frånsett ANCA antikroppar, har > 60 % av WG patienterna även förekomst av anti-endotelcells antikroppar (AECA) riktade mot ett/flera okända protein (er) på endotelets yta. Endotelet täcker insidan av kroppens alla kärl och bildar således en barriär mellan blodet och underliggande vävnad. Tidigare betraktades endotelet som passivt och statiskt, men det har visat sig att endotelet har en mängd viktiga funktioner varav en är reglering av kärltonus. Endotelet är också involverat i den inflammatoriska processen där adhesions-molekyler och andra substanser påverkar de vita blodkropparnas interaktion med kärlväggen. Det finns flera typer av endotel i kroppen som är förknippat med olika organ funktioner. Den vanligaste typen är kontinuerligt endotel som återfinns hos kapillärerna i skelettmuskel, hud, hjärtmuskel och bindväv. Fenestrerat (med öppningar) endotel återfinns i magtarmkanalens och bukspottkörtelns kapillärer samt i njurens glomerulus. Även i levern och mjälten uppvisar endotelet mycket stora diskontinuerliga fenestreringar. Även samma organ kan innehålla olika typer av endotel. Ett exempel är njuren där

kapillärerna i glomeruli är diskontinuerliga och fenestrerade i peritubulära kapillärer och kontinuerliga i andra delar.

Målet med denna avhandling var att studera interaktionen mellan AECA och endotelceller, i hopp om att försöka förstå någon/några av de mekanismer som bidrar till att endotel-cellerna blir skadade i de blodkärl som försörjer just luftvägarna och njurarna.
I artikel I har vi studerat förekomsten av AECA i sera från WG patienter. I de flesta studier angående AECA har man använt endotelceller från navelsträng (HUVEC). I denna studie har vi istället valt att använda endotelceller isolerade från sjukdomens målorgan, såsom näsa, lunga och njure. Vi fann att ett större antal WG patienter hade AECA riktade emot näs- (61%), lung- (25%) och njurendotel (71%), i jämförelse med HUVEC (7%), vilket ifrågasätter användandet av HUVECs i AECA studier.

I artikel II utvecklades ett nytt protokoll för isolering av humana endotel celler från nässlemhinna (inferior turbinate), som visade att det fanns två typer av endotel celler; en vaskulär med ”kullerstens” morfologi och en andra sinusoidal med fenestreringar och stjärnformig morfologi. Dessa två typer av endotelceller kan även hittas i levern och njurarna, och kan komma att bli viktiga i studier rörande endotelcellers biologi och deras interaktion med immunceller vid sjukdomar i näsregionen.

Frånsett, ANCA och AECA antikroppar kan perifert blod även innehålla s.k. cirkulerande endotelceller. Det finns två typer; den enda har sitt ursprung i benmärgen (EPC; endothelial progenitor cells) och den andra består av mogna endotelceller (IEC; inflammatory endothelial cells) som har avlägsnas från blodkärlets insida pga skada. Artikel III visar att WG patienter med aktiv sjukdom har högre nivåer av IECs jämfört med WG patienter i remission och friska individer. Dessa IECs kan särskiljas från EPCs med två nya markörer, VAP-1 (vascular adhesion protein 1) och MICA (MHC class-I related chain A), som EPCs inte uttrycker. Supernatanter från IECs cellkulturer visade sig också ha en negativ påverkan på EPCs prolifiering och migrationskapacitet. En möjlighet är att IECs bidrar till WG progress genom att störa EPCs funktionella kapacitet att reparera skadade kärl.

I artikel IV isolerade vi IgG (immunoglobulin av klass G) fraktioner från WG patienter med höga nivåer av AECA. Immunoprecipitering av njurendotel med WG IgG gav tre band; 190-200 kDa, 70-73 kDa and 50-53 kDa. Vid stimulering av njurendotel med dessa WG IgG påvisades en snabb kalcium mobilisering (ett första tecken på cell aktivering), uppreglering av MICA på cellytan och höga nivåer av neutrofil/monocyt attraherande kemokiner (utsöndrat protein). MICA är en molekyl som induceras av ”stress” (virus, bakterier, svamp, syrebrist, värme, kyla etc). Dess ligand, NKG2D receptorn, kan hittas på immunceller såsom T-celler och NK celler.

Sammanfattningsvis, baserat på ovanstående resultat föreslår vi att mekanismen (se figur 2, sidan 50) för endotel skada kan medieras av IgG AECAs inbindning till endotelet och att det därefter sker en pro-inflammatorisk loop genom rekrytering av immunceller via det uppreglerade MICA uttrycket, tillsammans med lokal produktion av kemokiner som ytterligare attraherar fler immunceller. Troligtvis aktiverar även inbindningen av IgG AECA till endotelet ett ökat uttryck av adhesionsmolekyler som vidare underlättar immuncellernas infiltration genom endotelet. WG IgG antas vara medierande i sjukdomsprocessen genom att bidra till en pro-inflammatorisk profil istället för att ha en direkt cytotoxisk (celldödande) verkan på endotelet. Parallellt med detta, kan cirkulerande IECs eventuellt bidra till sjukdomens progression genom att hindra EPCs kapacitet att reparera endotelskadan.

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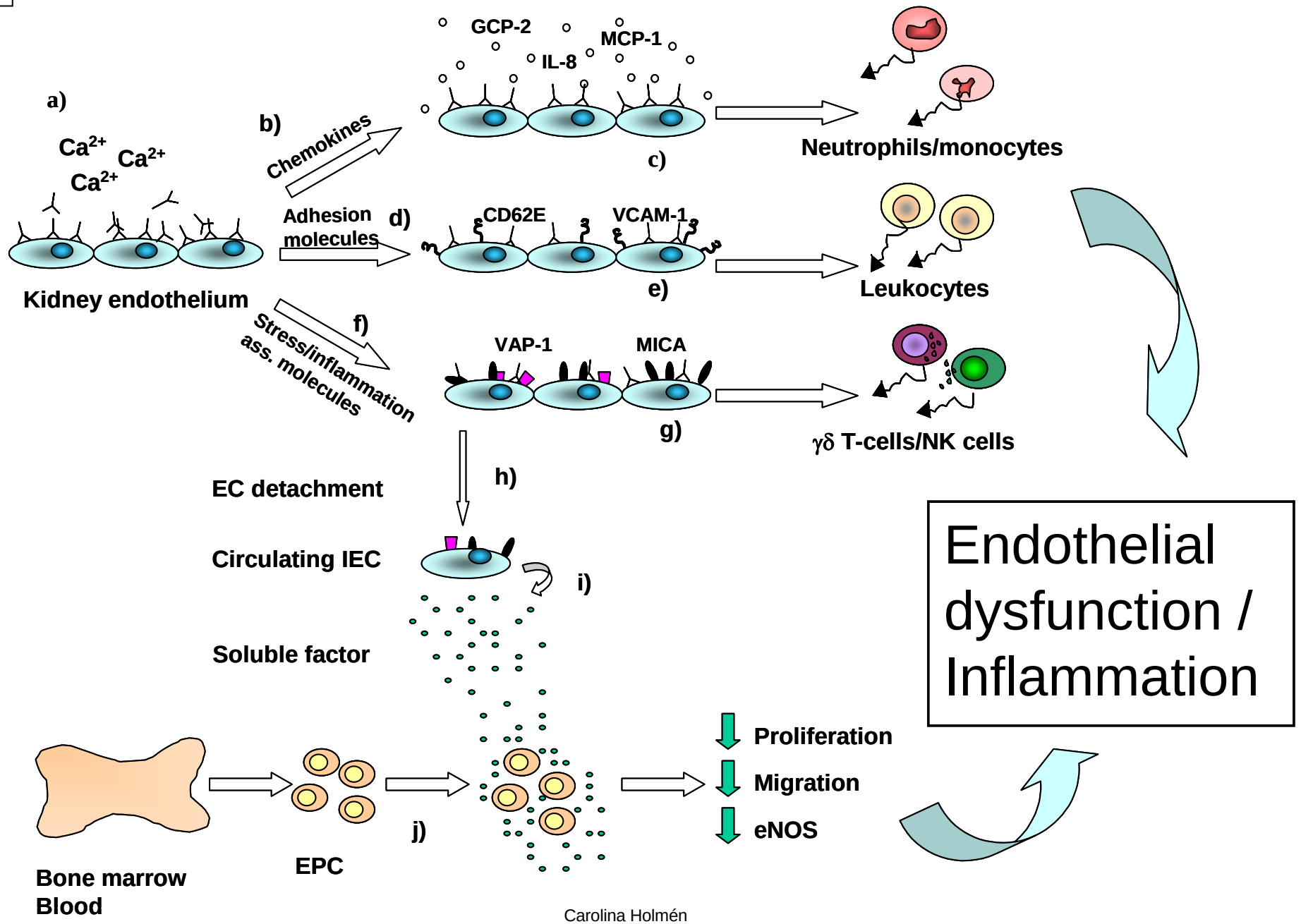
Figure 2.


a)
Ca2+
Ca2+
Ca2+
Kidney endothelium
b)
Chemokines
GCP-2
MCP-1
IL-8
c)
Neutrophils/monocytes
Adhesion molecules
d)
CD62E
VCAM-1
e)
Leukocytes
f)
Stress/inflammation ass. molecules
VAP-1
MICA
g)
γδ T-cells/NK cells
EC detachment
h)
Circulating IEC
i)
Soluble factor
Proliferation
Migration
eNOS
Endothelial dysfunction / Inflammation
Bone marrow
Blood
EPC
j)

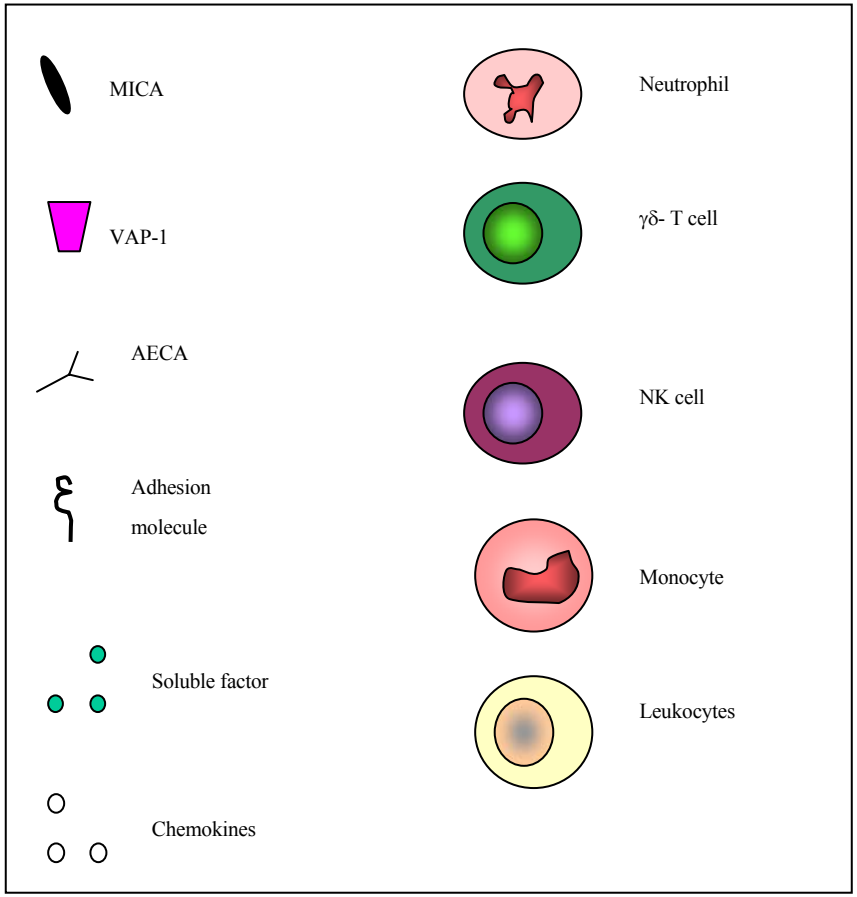


Figure 2. Upon IgG AECA binding;
A rapid calcium flux (**a**) generates an initial signalling process, stimulating the endothelium to secrete chemokines (**b**), which attracts monocytes and neutrophils (**c**) to the site of inflammation as well as release of IL-1, IL-6, IL-8 that may enhance the inflammation. The endothelium can also upregulate adhesion molecules (**d**) necessary for the rolling and transmigration of various leukocytes into the inflamed tissue. As the monocytes enter the tissue (**e**) they become macrophages, which efficiently mediate activation of T cells, which in return secrete more cytokines recruiting and activating more macrophages. This self-perpetuating response may lead to granuloma formation characterized by extensive tissue damage. IgG AECA binding also stimulates the endothelium to express MICA (**f**), which can be recognized by (**g**) NK cells and γδ T cells via their NKG2D receptor. Via the receptor-ligand engagement, T and NK cells are stimulated to release various substances, which might further enhance the established pro-inflammatory loop. The inflamed endothelium also expresses the inflammation-associated molecule vascular-adhesion protein-1 (VAP-1). Endothelial cells from the inflamed organ may detach (**h**) and enter the circulation. The circulating inflammatory endothelial cells (IECs) release soluble factors (**i**) that have a negative effect on endothelial progenitor cells (EPCs) (**j**) derived from the bone marrow or peripheral blood. IECs may decrease the proliferative or migratory capacity of EPCs, as well as down regulate eNOS expression. Thus, IECs may contribute to EPC dysfunction by interfering with their functional capacity for vessel wall repair.