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Studies of the pathophysiology and epidemiology of vasculitis

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**LUND
UNIVERSITY**
Faculty of Medicine

DOCTORAL DISSERTATION

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<p>Abstract</p> <p>Vasculitis is a group of diseases characterized by inflammation in and around vessel walls that may cause secondary tissue damage to affected organs. Microvesicles, as well as the kinin and complement systems, have been implicated in the pathogenesis of vasculitis. In this thesis, the role of microvesicles, bearing kinin-receptors or complement, in the pathogenesis of vasculitis was investigated and the incidence of pediatric primary systemic vasculitis was studied in a defined population.</p> <p>Plasma from patients with vasculitis were shown to have high levels of leukocyte- and endothelial-derived microvesicles and these microvesicles carried kinin B1-receptors on their surface. In addition, endothelial-derived microvesicles exhibited deposits of complement factors C3 and C9 on their surface. Plasma from vasculitis patients and microvesicles derived from B1-receptor transfected cells were chemotactic for neutrophils. The chemotactic property of microvesicles, dependent on the presence of the B1-receptor, was regulated by the presence of C1 inhibitor. Unexpectedly, the presence of complement deposits on endothelial-derived microvesicles was regulated by kinin receptor antagonists. Microvesicles could transfer functional B1-receptors to recipient cells of a different cell type, in vitro. Renal biopsies from vasculitis patients demonstrated that this phenomenon could potentially occur in vivo in the kidney, thus potentiating the inflammatory response.</p> <p>The epidemiological study of primary systemic vasculitis in Skåne identified 556 pediatric patients treated between 2004-2014. The annual incidence rate per million children (CI 95%) was estimated to be 200 (range 183-217) for all cases, further divided into incidences for IgA-vasculitis, Kawasaki disease, granulomatosis with polyangiitis, microscopic polyangiitis, polyarteritis nodosa, eosinophilic granulomatosis with polyangiitis and Takayasu's arteritis. No deaths occurred during the follow up period.</p> <p>In conclusion, this thesis demonstrates that both the kinin and complement systems are activated on microvesicles in patients with vasculitis and suggests that inhibition of the kinin and complement systems by B1- or B2-receptor antagonists or C1-inhibitor should be explored as potential therapeutic targets in vasculitis. Primary systemic vasculitis was shown to be rather common in childhood and affected adolescents more severely.</p>		
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*To my family,
without you I am nothing*

*A dream you dream alone is only a dream.
A dream we dream together is reality.
John Lennon*

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List of papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

- I. Mossberg M, Ståhl A, Kahn R, Kristoffersson AC, Tati R, Heijl C, Segelmark M, Leeb-Lundberg FM, Karpman D. C1-inhibitor decreases release of vasculitis-like chemotactic endothelial microvesicles. *J Am Soc Neph* 2017 in press.
- II. Mossberg M, Ståhl A, Tati R, Kristoffersson AC, Kahn R, Segelmark M, Leeb-Lundberg LMF, Karpman D. The kinin system modulates complement activation on endothelial cell microvesicles in vasculitis. Manuscript.
- III. Kahn R, Mossberg M, Ståhl A, Johansson K, Lapatko Lindman I, Heijl C, Segelmark M, Mörgelin M, Leeb-Lundberg LMF, Karpman D. Microvesicles transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis. *Kidney Intl* 2017; 91: 96-105.
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Abbreviations

AAV	ANCA-associated vasculitis
ANCA	Anti-neutrophil-cytoplasmic antibodies
ACR	American College of Rheumatology
BVAS	Birmingham vasculitis activity score
CRP	C-reactive protein
EVs	Extracellular vesicles
EMVs	Endothelial microvesicles
EGPA	Eosinophilic granulomatosis with polyangiitis
EMA	European medical agency
ESR	Erythrocyte sedimentation rate
EULAR	The European League against Rheumatism
GPA	Granulomatosis with polyangiitis
HK	High-molecular-weight kininogen
IgAV	IgA Vasculitis (a.k.a. Henoch-Schönlein purpura)
KD	Kawasaki disease
MPA	Microscopic polyangiitis
MVs	Microvesicles
PAN	Polyarteritis nodosa
PRES	Paediatric Rheumatology European Society
PRINTO	Paediatric Rheumatology International Trial Organisation
PSV	Primary systemic vasculitis
TAK	Takayasu's arteritis
TNF	Tumor necrosis factor- α

Abstract

Vasculitis is a group of diseases characterized by inflammation in and around vessel walls that may cause secondary tissue damage to affected organs. Microvesicles, as well as the kinin and complement systems, have been implicated in the pathogenesis of vasculitis. In this thesis, the role of microvesicles, bearing kinin-receptors or complement, in the pathogenesis of vasculitis was investigated and the incidence of pediatric primary systemic vasculitis was studied in a defined population.

Plasma from patients with vasculitis were shown to have high levels of leukocyte and endothelial-derived microvesicles and these microvesicles carried kinin B1-receptors on their surface. In addition, endothelial-derived microvesicles exhibited deposits of complement factors C3 and C9 on their surface. Plasma from vasculitis patients and microvesicles derived from B1-receptor transfected cells were chemotactic for neutrophils. The chemotactic property of microvesicles, dependent on the presence of the B1-receptor, was regulated by the presence of C1 inhibitor. Unexpectedly, the presence of complement deposits on endothelial microvesicles was regulated by kinin receptor antagonists. Microvesicles could transfer functional B1-receptors to recipient cells of a different cell type, *in vitro*. Renal biopsies from vasculitis patients demonstrated that this phenomenon could potentially occur *in vivo* in the kidney, thus potentiating the inflammatory response.

The epidemiological study of primary systemic vasculitis in Skåne identified 556 pediatric patients treated between 2004-2014. The annual incidence rate per million children (CI 95%) was estimated to be 200 (range 183-217) for all cases, further divided into incidences for IgA-vasculitis, Kawasaki disease, granulomatosis with polyangiitis, microscopic polyangiitis, polyarteritis nodosa, eosinophilic granulomatosis with polyangiitis and Takayasu's arteritis. No deaths occurred during the follow up period.

In conclusion, this thesis demonstrates that both the kinin and complement systems are activated on microvesicles in patients with vasculitis and suggests that inhibition of the kinin and complement systems by B1- or B2-receptor antagonists or C1-inhibitor should be explored as potential therapeutic targets in vasculitis. Primary systemic vasculitis was shown to be rather common in childhood and affected adolescents more severely.

Introduction

Vasculitis is a group of diseases characterized by inflammation in and around blood vessels affecting various organs such as the kidneys, skin, joints and lungs, among others. Vasculitis can affect both adults and children and the disease spectrum can be mild and transient to life-threatening. The etiology of vasculitis is unknown.

The pathogenesis of vasculitis depends on the specific disease entity. In general, inflammatory cells are present in the affected organs, and in some vasculitides there are circulating immune complexes that deposit in the tissues. Activation of the kinin- and the complement systems contributes to the inflammatory response and is believed to play an important role in the pathogenesis of vasculitis.

Microvesicles are small membrane-bound structures shed from cells under resting conditions but more so during stress and senescence. They are highly active particularly in intercellular communication. Microvesicles are elevated in vasculitides and levels correspond to disease activity (1, 2).

Activation of the kinin system results in liberation of highly potent vasoactive kinins, binding to either the B1- or B2-receptors, causing the classical signs of inflammation. The B2-receptor is constitutively expressed while the B1-receptor is upregulated during inflammatory conditions (3). C1-inhibitor is the major inhibitor of the kinin system (4). The kinin system has been shown to be activated in vasculitis (5).

The complement system is part of the innate immune system and its byproducts play an important role in inflammation. The complement system can thereby play a major role in inflammatory diseases. Activation via the classical pathway is important in systemic lupus erythematosus (SLE) while the alternative pathway is activated in vasculitis (6).

The aim of this thesis was to study the levels and effect of leukocyte and endothelial cell-derived microvesicles in vasculitis and the contribution of the kinin B1-receptor and complement on microvesicles to the inflammatory process, and attempt to block these effects. An additional aim was to describe the epidemiology of pediatric primary systemic vasculitis in a well-defined area.

Vasculitis

Vasculitis is a collective term for conditions characterized by inflammation in and around vessel walls that may cause secondary tissue damage to various organs such as the kidney, skin, joints and lungs, among others. Vasculitides can vary from mild, transient, conditions to life-threatening diseases. Vasculitis affects children as well as adults and has a slightly different disease spectrum within different age-groups. The etiology of vasculitis is multifactorial in which genetic predisposition, environmental factors, autoimmune processes as well as infectious triggers may play important roles (7-9). Vasculitis can be localized to one organ such as the skin or be systemic affecting many organ systems. Primary vasculitis has no association to other diseases processes whereas secondary vasculitides are associated with malignancies, infections, drug reactions or other autoimmune diseases. This thesis focused on primary systemic vasculitides.

Classification

Vasculitis is a heterogeneous group of diseases that can be classified, according to the predominantly engaged vessel, into small-, medium- and large-sized vessel vasculitis according to the Chapel Hill Consensus Conference on the Nomenclature of Vasculitides (10). Small vessels are capillaries, venules and arterioles. Medium-sized vessels are small arteries and veins with their proximal branches. Large vessels include the aorta and its proximal branches as well as the pulmonary arteries and corresponding veins (Figure 1).

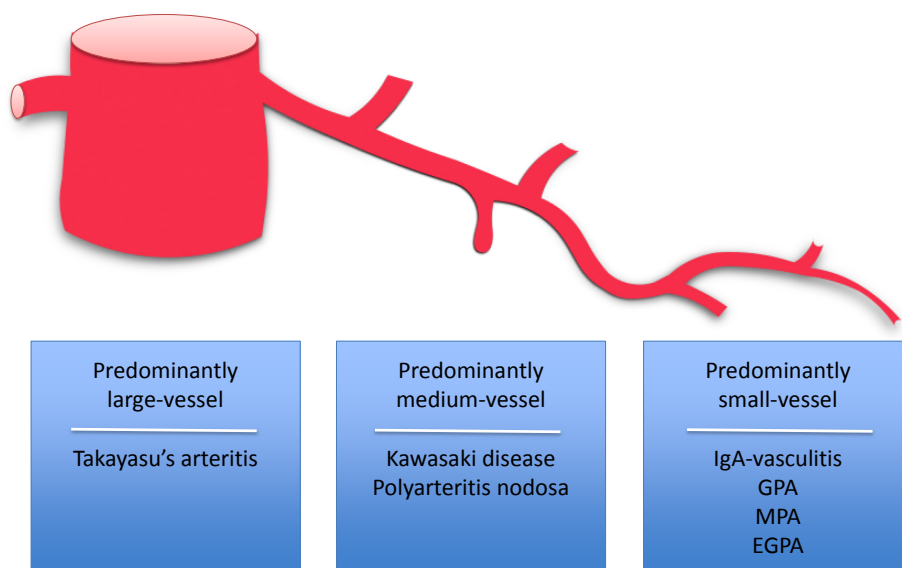


Figure1. Classification of vasculitis according to the predominantly engaged vessels.

GPA: Granulomatosis with polyangiitis, MPA: Microscopic polyangiitis, EGPA: Eosinophilic granulomatosis with polyangiitis.

Primary systemic vasculitis

In the following section the various primary systemic vasculitides included in this thesis will be described.

IgA vasculitis

IgA vasculitis (IgAV), previously known as Henoch-Schönlein purpura, is the most common vasculitis in children with an annual incidence of 101-204 per million children (11, 12). The majority of children affected are under 10 years of age. IgAV is less common in adults with an annual incidence between 4-50 per million (13, 14). IgAV has a seasonal variation coinciding with the peaks of infectious diseases (15, 16).

Clinical manifestations

IgAV is a self-limiting vasculitis which may have a relapsing course. Children usually present after a history of an upper respiratory tract infection (17) with palpable purpura (predominantly on lower limbs), arthritis and/or arthralgia,

gastrointestinal pain or bleeding (18) as well as scrotal pain and swelling (19). Some patients develop severe forms of glomerulonephritis, which is more common in adults than in children (20). Table 1 presents a pediatric classification.

Table 1. Classification criteria of pediatric IgA vasculitis

IgA vasculitis classification criteria ¹	
Purpura or petechial (mandatory) with lower limb predominance plus one of the four following	
•	Abdominal pain
•	Arthritis or arthralgia
•	Renal involvement
•	Histopathology showing predominant IgA deposition

¹, Classification according to EULAR/PRINTO/PRES (21). EULAR: European League Against Rheumatism, PRINTO: Paediatric Rheumatology International Trials Organisation, PRES: Paediatric Rheumatology European Society.

Laboratory parameters

There are no specific laboratory findings in IgAV, although it is important to rule out thrombocytopenia or coagulopathies. In cases with renal involvement hematuria and/or proteinuria may be present as well as elevated creatinine.

Pathology

The histopathology of cutaneous lesions in IgAV exhibits profound inflammation consisting of neutrophils and monocytes and deposits of IgA seen around the small vessels. These skin lesions are referred to as leukocytoclastic vasculitis. In the kidneys there is mesangial proliferation consisting of cells and matrix expansion. Immune deposits contain IgA, as determined by immunofluorescent staining. Deposits of complement proteins consist of C3, properdin and the membrane attack complex (MAC) C5b-9 suggesting activation of the alternative and terminal complement pathways (22). In some cases mannose-binding lectin, ficolin-2, mannan-binding lectin serum protease 1 of the lectin pathway and C4d are deposited (23). As the disease progresses crescent formation, segmental sclerosis, necrosis, glomerulosclerosis and interstitial fibrosis may develop.

Pathogenesis

IgA play an important role in the immunopathogenesis of IgAV and is deposited as IgA complexes in the skin and renal mesangium of patients with IgAV (24). IgA is secreted as two isotypes (IgA₁ and IgA₂) that can be monomeric or polymeric. IgAV is associated with IgA₁ in which the O-linked sugars in the hinge-region exhibit abnormal glycosylation resulting in galactose-deficient polymeric IgA₁. Blood samples from patients with IgA nephritis have elevated levels of underglycosylated polymeric IgA₁ compared to controls (25). Underglycosylated polymeric IgA₁ alone or in complex with complement aggregates in target organs causing inflammation by release of inflammatory

mediators (26). Underglycosylated polymeric IgA₁ has a proliferative and inflammatory effect on mesangial cells (26).

The kinin system is activated both locally and systemically in patients with IgAV as kinins were detected in kidney and skin biopsies as well as in the blood samples (5).

Complement activation occurs via both the alternative and lectin pathways, when there is deposition of IgA₁ and circulating immune complexes in the glomerulus resulting in influx of inflammatory cells, fibrin deposition, cytokine production and subsequent epithelial proliferation of the cells in the Bowmans capsule (26). GWAS gives support for the alternative pathway of complement being part in the pathogenesis in IgAV (27), however deposition of complement components from the lectin pathway is associated with severe glomerular inflammation in patients with IgAV (23). There is no correlation between the presence of nephritis and serum levels of C3 and C4 in patients with IgAV (28).

Current treatment

Most cases of IgAV do not require treatment as the condition is often mild and self-limited (29). Symptomatic treatment with non-steroidal anti-inflammatory drugs can be used in patients with isolated arthritis. Corticosteroids have been used for severe skin lesions or gastrointestinal involvement (30). Patients with severe renal disease are often treated with corticosteroids, immunosuppressive drugs such as mycophenolate mofetil or cyclophosphamide and antihypertensive medication even though corticosteroids do not prevent renal disease (30, 31). Treatment with B-cell depletion by rituximab has been tried with promising results (32).

Prognosis

In children with IgAV the prognosis is very good and most children will have an uneventful recovery. However, the overall risk in pediatric IgAV of developing end stage renal failure is 2-5 percent (33). Adults have a worse prognosis in which 10-30 percent progress to end stage renal failure (20).

Anti-neutrophil cytoplasmic antibody associated vasculitis

Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is a small vessel vasculitis comprising Granulomatosis with polyangiitis (GPA), Microscopic polyangiitis (MPA) and Eosinophilic granulomatosis with polyangiitis (EGPA). These conditions are rare in childhood but more common in adulthood. A study from southern Sweden reported the adult incidence rate of AAV to be 20.9 per million inhabitants (34), which is slightly higher than the incidence in other European countries. There is limited information on the

incidence of AAV in childhood. However a French study showed a childhood incidence of 0.5 per million children per year (35).

Clinical manifestations

AAV usually presents with weeks of flu-like symptoms such as fever, headache, malaise, weight loss and pain in muscles and joints. In addition to these general symptoms there can be symptoms from the ear (chronic/recurrent otitis media), nose and/or throat (ulcerations) that do not respond to antibiotics as well as inflammatory symptoms from the eyes, respiratory tract (tracheal/bronchial stenosis, hemoptysis/alveolar hemorrhage), skin (petechiae, ulcerations) and renal manifestations (hematuria, hypertension). Table 2 presents a pediatric classification.

Table 2. Classification of pediatric ANCA- associated vasculitis

ANCA-associated vasculitis	
Granulomatosis with polyangiitis¹	<p>At least three of the following:</p> <ul style="list-style-type: none"> • Histopathology (granulomatous inflammation) • Upper airway involvement (inflammation) • Laryngo-tracheo-bronchial stenosis • Pulmonary involvement (X-ray showing nodules, cavities or fixed² infiltrates) • ANCA positivity • Renal involvement (abnormal urinalysis/ impaired renal function)
Microscopic polyangiitis³	<ul style="list-style-type: none"> • Predominantly affects small vessels • Necrotizing arteritis • Few or no immune deposits in histopathology • Necrotizing glomerulonephritis • Pulmonary capillaritis • Granulomatous inflammation is absent
Eosinophilic granulomatosis with polyangiitis⁴	<p>At least three of the following:</p> <ul style="list-style-type: none"> • Asthma • Eosinophilia >10% • Neuropathy, mono or poly • Pulmonary infiltrates, non fixed² • Paranasal sinus abnormality • Extravascular eosinophilia

¹, Granulomatosis with polyangiitis classified according to EULAR/PRINTO/PRES (21). ², Fixed infiltrates must be present on chest x-ray for more than one month, while non fixed change locations (36). ³, Microscopic polyangiitis classified according to the Chapel Hill Consensus Conference definitions of vasculitides (10). ⁴, Eosinophilic granulomatosis with polyangiitis classified according to the American College of Rheumatology (37). EULAR: European League Against Rheumatism, PRINTO: Paediatric Rheumatology International Trials Organisation, PRES: Paediatric Rheumatology European Society.

GPA and EGPA often affect the airways and patients with EGPA may have a medical history of asthma (38, 39). MPA, on the other hand, more commonly affects the kidneys at presentation (40) and has a worse renal prognosis compared to the other AAVs (41).

Laboratory parameters

ANCAs are predominantly IgG antibodies directed against myeloperoxidase (MPO) or proteinase 3 (PR3), and found in 91 percent of all AAV patients (41). GPA is mostly associated with PR3-ANCA (42, 43) whereas EGPA and MPA are mostly associated with MPO-ANCA (44-46). ANCA levels are higher in active GPA than in remission allowing them to be used as disease markers (42). In EGPA eosinophilia in the peripheral blood count is seen. Patients with AAV often have hematuria and/or proteinuria and urinary casts suggesting glomerular affection.

Pathology

AAV causes glomerulonephritis in the kidneys characterized by necrotizing inflammation without any visible deposits of immunoglobulins or complement thus resulting in a pauci-immune glomerulonephritis. Fibrinoid necrosis, crescent formation and proliferative changes are typically seen. In GPA and EGPA granulomas are present and in the latter, tissue eosinophilia can also be seen. In the granulomas T-cells and neutrophils are abundant. T-cells are considered important in the formation of the granulomas in AAV (47).

Pathogenesis

PR3 is stored in azurophilic granules, the secretory vesicles and specific granules of neutrophils and monocytes. Upon neutrophil degranulation PR3 is released in active form and due to its potency leads to a multitude of inflammatory reactions, such as release of tumor necrosis factor into circulation (48) and activating endothelial cells to release IL-8 (49). PR3 degrades extracellular matrix proteins thereby promoting neutrophil infiltration via basement membranes (50). PR3 specifically activates the kinin system by releasing a 13-amino acid long peptide from high molecular weight kininogen (51) and may thus be involved in the pathogenesis of vasculitis (51). The role of the kinin system in vasculitis will be discussed below.

MPO is also stored in azurophilic granules of neutrophils and released upon degranulation. MPO partakes in producing highly cytotoxic substances (hypochlorous acid and tyrosyl radicals) by catalyzing hydrogen peroxide and halides and oxidizing tyrosine (52, 53) aimed at killing pathogens. Alpha-1 antitrypsin is the main inhibitor of PR3 (54). Patients with alpha-1 antitrypsin deficiency have a 10 fold increase of incidence and prevalence of GPA compared to the general population (55). Ceruloplasmin is an inhibitor of MPO in vivo (56).

ANCAs have been suggested to be important in the pathogenesis of AAV even though immunoglobulins are not visualized in the renal biopsies of patients. When pro-inflammatory cytokines, such as Tumor necrosis factor- α (TNF- α), prime neutrophils and monocytes, PR3 or MPO are detected on the cell surface allowing

ANCA to bind (57). PR3-ANCA and MPO-ANCA have pro-inflammatory effects on neutrophils triggering them to degranulate (57), release cytokines (58, 59) and activate complement (6). PR3-ANCA can activate the endothelium directly causing expression of tissue factor and IL-1 (60) as well as inducing release of IL-8 (61).

Mouse models have been used to study the pathogenesis of AAV. There are very few PR3- ANCA mouse models of AAV that resemble the human AAV, however an MPO-ANCA mouse model has been developed. MPO knock-out mice developed anti-MPO after being immunized with mouse MPO. The anti-MPO IgG was in turn injected into recombinase- activating gene-2 deficient mice (lacking functional B and T cells) and wild-type mice, both mice types developed pauci-immune necrotizing and crescentic glomerulonephritis even though the wild-type had milder symptoms (62). Lipopolysaccharide exacerbated the symptoms. This effect was mediated by TNF- α as anti-TNF administration decreased symptoms (63). Wild-type B6 mice were antibody-treated to deplete neutrophils and then injected with anti-MPO IgG. These mice were protected against glomerulonephritis thus demonstrating that neutrophils are essential for the process (64).

Direct evidence of the pathogenicity of ANCAs was demonstrated in a neonate that developed transient glomerulonephritis and pulmonary hemorrhage (65) after transplacental transfer of MPO-ANCA. However, in other neonates transplacental MPO-ANCA transfer did not induce disease (66). In patients with MPO-ANCA associated vasculitis, the presence of circulating ANCA does not always predict serious disease, but most patients that develop serious flares are ANCA-positive (67, 68).

Mouse models have also shown that activation of the alternative pathway of complement is important for the development of vasculitis (6). The role of the complement system in vasculitis will be elaborated on below.

Current treatment

Rapid induction of remission is vital in acute AAV. Remission may be induced by cyclophosphamide or B-cell depletion by infusion of rituximab (30). High dose corticosteroids are given during the acute phase and tapered over several months to years. Azathioprine or mycophenolate mofetil can be used as maintenance therapies. In limited disease methotrexate can be used as induction therapy. Prophylaxis with co-trimoxazole is given to avoid opportunistic infections during the induction phase.

Prognosis

Seventy percent of all patients with AAV develop ANCA-associated nephritis (34) and 30-40 percent of all AAV patients will develop a need for renal replacement therapy. MPA carries a worse renal outcome than GPA (41). The five-year survival of AAV in a French study of pediatric AAV was 94 percent (35), while in adults in Sweden it was 70 percent (41).

Kawasaki Disease

Kawasaki disease (KD) is a self-limiting disease of unknown origin predominantly affecting medium sized arteries (69). KD is the second most common vasculitis in childhood, 80 percent of affected children are below the age of five (70). There is a geographical as well as an ethnical variation in the epidemiology of KD. KD has an annual incidence in Japan of 2648 per million children (71), while in UK the incidence is 81 per million children (11). KD has a postulated seasonal variation coinciding with the peaks of infectious diseases, suggesting a possible infectious cause (69, 72).

Clinical manifestations

Patients with KD present with high fever lasting for five days or more, conjunctivitis, unilateral cervical lymphadenopathy, polymorphic rash, cracked or hyperemic lips and/ or mucous membranes, and reddening of soles and palms, edema and subsequent desquamation (Table 3) (73). Not all symptoms need to be present. During the course of KD patients may develop coronary artery aneurysm, ischemic heart disease, myocardial infarction as well as sudden death (74). KD is the most common cause of pediatric acquired heart disease in Japan, Europe and the US (11, 70, 71). Table 3 presents diagnostic criteria of KD.

Table 3. Diagnostic criteria of Kawasaki disease

Kawasaki Disease diagnostic criteria¹

Fever at least five days plus four of the following principal clinical features:

- Extremity changes (erythema of palms and soles, edema of hands and feet and subsequent desquamation)
- Polymorphous rash
- Bilateral conjunctival injection without exudate
- Changes in the lips and oral cavity (red cracked lips, strawberry tongue, mucositis)
- Cervical lymphadenopathy

Kawasaki disease may be diagnosed with fewer than four features if coronary artery abnormalities are present

¹, Classification according to the American Heart Association (75).

Laboratory parameters

There is no specific laboratory test confirming KD. C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and liver function tests may be elevated.

Hypoalbuminaemia is a common feature. Thrombocytosis is often noted after two to three weeks of disease.

Pathology

The medium-sized muscular arteries are affected in KD. Especially the coronary arteries are affected causing dilatations, aneurysms or giant aneurysms detected by echocardiography.

The vasculitic lesions consist of neutrophil infiltration at the luminal side of the vessel causing necrotizing destruction of the intima and media. Lesions may also originate from the adventitia progressing towards the lumen with infiltration of cytotoxic lymphocytes, plasma cells, IgA and fibroblasts causing inflammation by secretion of inflammatory mediators causing fusiform arterial dilatations (76).

Pathogenesis

The pathogenesis is unknown but an infectious trigger has been suggested causing an immunological reaction (69). A super-antigen causing nonspecific activation of T-cells resulting in pronounced inflammation through cytokine release has been proposed (77) but these pathogenetic proposals are, at present, speculative. Endothelial and platelet-derived microvesicles were shown to be elevated in plasma from pediatric vasculitis patients. In this study, 15 out of the 29 children included had KD (1).

Current treatment

Patients with suspected KD are treated with intravenous immunoglobulins and acetylsalicylic acid (30). Immunoglobulin treatment usually resolves the fever promptly and reduces other symptoms. If the patient is not afebrile within 24 hours, an additional dose of immunoglobulins is given. High risk cases are, in addition, treated with intravenous corticosteroids.

Prognosis

Coronary artery aneurysm is a serious complication of KD that may cause significant morbidity but also mortality. It develops in 15-25 percent of untreated patients with KD (78). Treatment with immunoglobulins has shown to reduce the risk of coronary aneurysm significantly (71).

Polyarteritis nodosa

Polyarteritis nodosa (PAN) is associated with aneurysmal nodules targeting mainly medium-sized muscular arteries predominantly affecting the skin, kidneys, muscles and gastrointestinal tract. The annual incidence rate of polyarteritis

nodosa (PAN) in adults is 0.9 per million inhabitants in southern Sweden (34), which is comparable to North Germany (79) while in Norfolk in the UK the incidence is as high as 8 per million (80). In the adult population PAN occurs more commonly in males compared to the pediatric population in which there is an equal gender distribution (81). There is limited information of the incidence of PAN in childhood, the studies performed have used different diagnostic criteria resulting in difficulties to compare the data (82).

Clinical manifestations

Patients usually present with fever, weight loss, arthralgia and myalgia. Dermatological symptoms are common (livedo reticularis and necrotic lesions). Affection of the renal arteries may result in kidney infarction and contribute to hypertension. Peripheral neuropathy is common (83, 84). The vasculitic lesions may affect the gastrointestinal tract causing bleeding, ileus as well as perforation (85). Table 4 presents a pediatric classification.

Table 4. Classification criteria of pediatric Polyarteritis nodosa

Polyarteritis nodosa classification criteria¹

Histopathology or angiographic abnormalities (mandatory) and one of the following:

- Skin involvement (livedo reticularis, subcutaneous nodules)
- Myalgia/ muscle tenderness
- Hypertension
- Peripheral neuropathy
- Renal involvement (abnormal urinalysis/ impaired renal function)

¹, Classification according to the EULAR/PRINTO/PRES (21). EULAR: European League Against Rheumatism, PRINTO: Paediatric Rheumatology International Trials Organisation, PRES: Paediatric Rheumatology European Society.

Laboratory parameters

Patients with PAN often have anemia, leukocytosis (neutrophilia) thrombocytosis, elevated CRP and ESR during the active phase of disease. When the kidneys are affected patients have elevated creatinine and may develop hematuria (86).

Pathology

Inflammatory infiltrates with fibrinoid necrosis are visible in the vessel wall of the affected organs such as the skin and kidneys (87), with segmental narrowing of the vessel giving rise to aneurysms seen by angiography. The vessels affected in the kidneys are the lobar and arcuate arteries which may be partially or totally occluded causing ischemia and infarction (82).

Pathogenesis

Genetic predisposition is believed to be part of the pathogenesis of PAN suggested because PAN has been associated with familiar Mediterranean fever as well as

PAN occurring in siblings (82). The pathology in PAN is due to immunological processes involving cytokines, neutrophils, macrophages and lymphocytes (88).

Current treatment

PAN is treated with corticosteroids alone or in combination with chemotherapeutic drugs, such as cyclophosphamide, to induce remission (30, 89). Biological drugs such as infliximab (anti- TNF) and rituximab have also been successfully used (30, 90, 91). Acetylsalicylic acid is given to prevent platelet aggregation (92). In life-threatening situations plasma exchange can be carried out (93).

Prognosis

The prognosis of childhood-onset PAN is better than in adult-onset disease. The reported mortality rate in childhood PAN is 1-4 percent (85, 94). A French study in adults reported a mortality rate of 25 percent (95).

Takayasu's arteritis

Takayasu's arteritis (TAK) is a granulomatous large vessel vasculitis affecting the aorta and its proximal branches (96). TAK has an incidence of 0.7 per million inhabitants in Sweden, which is comparable to other studies in Europe (97). The incidence of TAK in East Asians is said to be 100-fold higher (81). TAK more often affects female patients under 40 years (98). The disease is rare in childhood but may affect infants (96). The incidence of TAK in childhood is to my knowledge not defined.

Clinical manifestations

Patients usually present with unspecific symptoms as fever, weight loss, headache or dizziness. Other symptoms include chest pain, claudication, absence of peripheral pulses, diplopia or decreased vision and transient ischemic attacks or strokes. Vascular hypoperfusion gives rise to symptoms depending on the affected organ. Table 5 presents a pediatric classification.

Table 5. Classification criteria of pediatric Takayasu's arteritis

Takayasu' arteritis classification criteria¹
<p>Angiographic abnormalities of the aorta or its major branches and pulmonary arteries showing aneurysm/dilatation (mandatory criterion) plus one of the following:</p> <ul style="list-style-type: none"> • Pulse deficit or claudication • Four limb blood pressure discrepancy • Bruits • Hypertension • Acute phase reactant elevation

¹, Classification according to EULAR/PRINTO/PRES (21). EULAR: European League Against Rheumatism, PRINTO: Paediatric Rheumatology International Trials Organisation, PRES: Paediatric Rheumatology European Society.

Laboratory parameters

Patients with TAK have no specific laboratory or serological features but elevated inflammatory parameters such as CRP and ESR are seen (99, 100).

Pathology

The histopathology of the inflammatory lesions show infiltrating leukocytes, neovascularization as well as granulomatous inflammation but in children the granulomatous inflammation is absent (100, 101).

Pathogenesis

The perivascular granulomatous inflammation consists of activated leukocytes releasing proinflammatory cytokines leading to more recruitment of inflammatory cells to the vessel wall. Lesions start in the adventitia progressing to the media resulting in stenosis, occlusion and aneurysm of the affected vessels (99).

Current treatment

Patients with TAK are treated with corticosteroids and/or other immune suppressive medications such as methotrexate, azathioprine and cyclophosphamide as well as biologic agents such as anti-TNF and anti-IL-6 to induce remission (30). Surgical vascular interventions may be necessary to prevent ischemia causing damage to end-organs (81).

Prognosis

The five-year mortality rate in children is 35 percent (102), which is much higher than the mortality rate in adults.

Disease activity assessment

Assessment of disease activity is used to follow diseases over time. It assists the physician in making therapeutic decisions and assessing the response to treatment. Disease scores enable the comparison of disease activity in varying populations when carrying out epidemiological studies. The Birmingham Vasculitis Activity Score (BVAS) includes 56 items (103, 104) divided into nine organ systems including general, cutaneous, mucous membranes/eyes, ear, nose and throat, chest, cardiovascular, abdominal, renal and nervous system. Each item is given a score with a maximum score for each organ system (104). The assessment is carried out when the patient has new/worsened symptoms or persistent symptoms, giving higher points for new symptoms. The BVAS has been validated for standard assessment of systemic vasculitis in both clinical practice and in clinical trials in

adults (104). The BVAS is not adequate for assessing childhood systemic vasculitis because the clinical manifestations and comorbidities in children vary from those in adults. For the purpose of assessment of childhood vasculitis the Pediatric Vasculitis Activity Score (PVAS) was developed (105). In PVAS there are 64 items divided into nine organ systems, as for the BVAS. As with BVAS, each manifestation gives a score with a maximal score for each organ system.

Epidemiology

Epidemiological studies are important to help researchers understand the pattern of diseases in relation to varying study populations with regard to age, gender, geographic distribution as well as variation over time. Furthermore, epidemiological studies can define the disease burden to society and the health care system enabling redistribution of resources accordingly.

Vasculitis has a global distribution in adults and children. A specific geographical distribution is more commonly seen in certain vasculitides. Examples are KD and TAK that are more common in Asia than the rest of the world. Age distribution is exemplified by IgAV and KD that are more common in childhood than adulthood while AAV is more common in adults than in children. Takayasu's arteritis affects mainly women before the fourth decade of life while AAV is more common in the sixth or seventh decade of life. Seasonal variation is seen in IgAV and KD suggesting an infectious trigger.

In Skåne, an area in southern Sweden, population-based validated epidemiological studies have been performed on all adult systemic vasculitides and the epidemiological data has contributed significantly to the present knowledge of adult vasculitides (34, 41, 97, 106-109).

In the pediatric population, there are few studies comparing the entire spectrum of diseases within a confined geographical area, i.e. in some of the diseases limited information on incidence rates as well as other epidemiological parameters are available.

In epidemiological studies classification criteria are used in order to compare the same disease in different studies. Several different systems have been developed for the purpose of diagnosis and classification as presented in Table 6. Specific classification criteria have been developed and validated for pediatric vasculitides including IgAV, GPA, PAN and Takayasu's arteritis by the European League Against Rheumatism/ Paediatric Rheumatology International Trials Organization /Paediatric Rheumatology European Society (EULAR/PRINTO/PRES) (21).

Table 6. The different vasculitis grouping systems and diseases covered

	Chapel Hill Consensus Conference	American College of Rheumatology	European Medicines Agency	EULAR/ PRINTO / PRES	American Heart Association
Definitions	+	-	-	-	-
Diagnostic criteria	-	-	+	-	+
Classification criteria	-	+	+	+	-
IgAV	+	-	-	+	-
GPA	+	+	+	+	-
MPA	+	-	+	-	-
EGPA	+	+	+	-	-
KD	-	-	-	-	+
PAN	+	+	+	+	-
TAK	+	+	-	+	-

The different vasculitis grouping systems have different properties which can be to define, diagnose or classify the vasculitis described in the table. Definition is how the disease is defined, diagnostic criterias are used when diagnosing a patient while classification criteria are used to categorize into a specific vasculitides. Chapel Hill Consensus Conference (10); American College of Rheumatology (110); European Medicines Agency (36); EULAR/ PRINTO/ PRES (21); American Heart Association (73). EULAR: European League Against Rheumatism, PRINTO: Paediatric Rheumatology International Trials Organisation, PRES: Paediatric Rheumatology European Society, IgAV: IgA vasculitis, GPA: Granulomatosis with polyangiitis, MPA: Microscopic polyangiitis, EGPA: Eosinophilic granulomatosis with polyangiitis, KD: Kawasaki disease, PAN: Polyarteritis nodosa, TAK: Takayasu arteritis, +: applicable, -: not applicable.

Microvesicles

The pathophysiological mechanisms in vasculitis differ depending on the disease involved. Microvesicles, the kinin system and the complement system are involved in the pathogenesis of vasculitides and were the focus of this thesis.

Definition

Extracellular vesicles (EVs) are membrane-bound vesicles released by cells in physiological as well as in pathological conditions. They are believed to play an important role in intercellular communication as well as in cellular waste disposal. EVs can be divided into three subgroups, exosomes, microvesicles and apoptotic bodies. Exosomes are the smallest (30-100 nm), produced by inward budding of the endosomal membrane and released from multivesicular bodies. Microvesicles are vesicular structures 0.1-1.0 μm in diameter shed by cells during resting conditions but more so during stress, senescence or apoptosis. The largest extracellular vesicles (1-5 μm) are apoptotic bodies that are formed during the late stages of apoptosis (111).

Microvesicles carry membrane-derived receptors, proteins, lipids and genetic material. Their contents may reflect their cellular origin (112, 113). Circulating microvesicles are mainly of platelet, erythrocytes, leukocyte and endothelial origin (114-117).

Microvesicle release

Microvesicles are released from cells during physiological conditions especially during cell growth (118). Microvesicle shedding is increased when the cells are activated due to cell injury, proinflammatory stimulants, hypoxia, oxidative stress or shear stress (119, 120).

Microvesicles are formed by outward protrusion or budding of the plasma membrane of the parent cell. This process is initiated by an increase of cytosolic

calcium that activates proteases such as calpains (121). This leads to remodeling of the cytoskeleton, by cleaving the actin protein network, enabling blebbing to occur. The plasma membrane is composed of a lipid bilayer in which phosphatidylserine is located in the inner leaflet of the resting cell. The enzymes flippase, floppase and scramblase control phospholipid asymmetry (122). When the cell is activated, the increased cytosolic calcium activates floppase (allowing lipid movement to the outer membrane) and scramblase (enabling bi-directional lipid movement), while flippase (allowing lipid movement to the inner membrane) is inactivated resulting in the negatively charged phosphatidylserine to be flipped to the outer leaflet of the phospholipid bilayer (123). However, this process does not always occur as some microvesicles do not expose phosphatidylserine on their outer leaflet (123) (Figure 2).

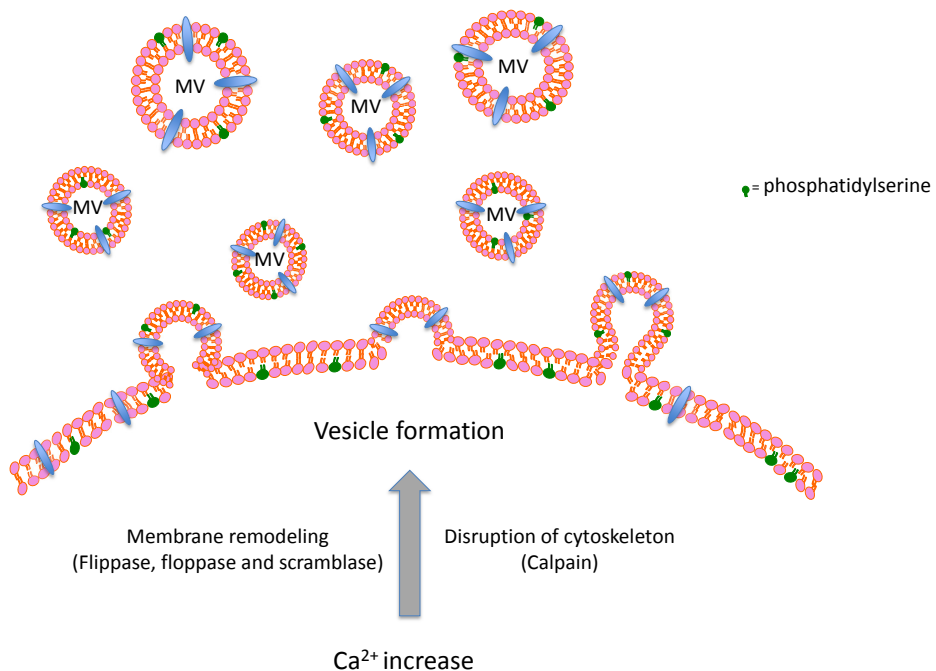


Figure 2. Schematic representation of microvesicle release.

The release of microvesicle is initiated by increased cytosolic calcium leading to secondary cytoskeletal disruption and membrane remodeling followed by shedding of the microvesicle. MV: microvesicle.

Microvesicles may contain cytokines, chemokines, enzymes, growth factors, signaling proteins, lipids, receptors as well as genetic material (micro-RNA and mRNA), depending on the parent cell, the microenvironment as well as the triggers preceding their release (123-125).

Microvesicles may express a slightly different repertoire of surface receptors or cytoplasmic components compared to the parent cell due to a selective process during shedding (126). In the same manner, microvesicles released from activated cells do not express exactly the same surface receptors as microvesicles shed during resting conditions (127). For example, endothelial-derived microvesicles from activated endothelial cells have higher levels of CD62E than endothelial-derived microvesicles from resting cells (2). Platelets-derived microvesicles may also exhibit a higher concentration of surface markers compared to the parent cell (126).

Microvesicle uptake

Microvesicles released into the circulation have a half-life of a couple of minutes to a few hours (128) in which they may be taken up by neighboring or distant cells. There are various mechanisms for the cellular uptake of microvesicles depending on the cargo of the vesicle, the intended intercellular communication as well as the microenvironment of the cell.

The most common mechanism is endocytosis whereby the microvesicle is engulfed into the recipient cell (128). There are several mechanisms of endocytosis such as clathrin-dependent or independent, caveolin-mediated, macropinocytosis, phagocytosis and lipid raft-mediated (129). Endocytosis and phagocytosis can also be a means to get rid of unwanted substances on or within the vesicles. Another mechanism for microvesicle uptake is fusion, whereby the microvesicles fuse with the membranes of the recipient cell and the content of the vesicle is released into the cell. Platelets expressing P-selectin fuse with tissue-factor-rich monocyte-derived microvesicles increasing the procoagulability of the platelet (126). During fusion, the two plasma membranes should be of the same fluidity (pH dependent), which in turn requires an acidic microenvironment (130) (Figure 3).

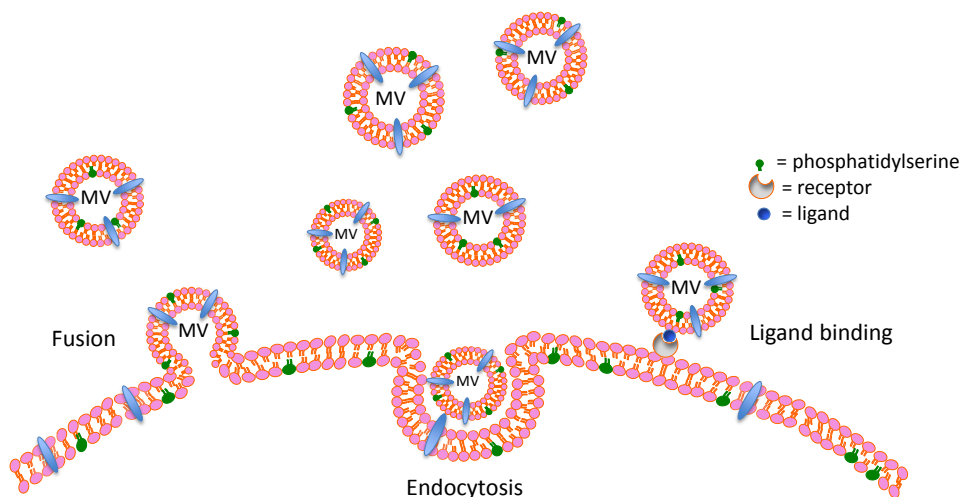


Figure 3. Schematic representation of microvesicle uptake.

Microvesicles can be taken up or induce signals in target cells by fusion, endocytosis or ligand binding. MV: microvesicle.

Detection

Microvesicles are mostly detected in blood samples but also in cerebrospinal fluid (131), urine (132), synovial fluid (133), bronchoalveolar lavage fluid (134), breast milk (135), bile (136), saliva (137) and uterine fluid (138). The techniques for microvesicle detection used in this thesis are described below and other methods are listed in Table 7.

Flow cytometry

The flow cytometer detects microvesicles as small as 300 nm in diameter (depending on the sensitivity of the instrument). The principle of detection is based on microvesicles passing through a laser beam. The size of the vesicle as well as the granularity are determined by the pattern of the light scatter from the laser beam. Modern flow cytometers have many lasers and fluorescence detectors, which allow for labeling with multiple antibodies in the same sample (139). Annexin V is used to detect phosphatidylserine. Many microvesicles, but not all,

have phosphatidylserine on the outer membrane making it possible to use annexin V for their detection.

Although flow cytometry is widely used to detect microvesicles, it has some limitations. Flow cytometry does not detect the smallest microvesicles as individual events. Multiple small microvesicles may be detected collectively as a single event, a phenomenon termed swarm detection (140). In addition, small microvesicles may have a limited number of antibody binding sites, hampering multiple staining (141). Thus, both the number of small microvesicles and their surface expression may be underestimated.

Transmission electron microscopy

The transmission electron microscope (TEM) visualizes small structures ($> 2\text{-}5\text{nm}$) due to the high resolution of the technique. Immune electron microscopy entails adding a conjugated antibody to detect a specific antigen in the sample (142). Negative staining is performed when the surrounding media is stained leaving the vesicles unstained and the contrast clearly visualizes the vesicles.

Both microvesicles and exosomes may be detected by TEM. However, quantification and multiple labeling of EVs is complicated and uncertain.

Nanoparticle tracking analysis

Nanoparticle tracking analysis (NTA) analyzes microvesicles in the liquid phase by a laser beam that determines the size and concentration by filming the light scattering when the particles move under Brownian motion (143). The technique detects microvesicles with a size of $0.05\text{-}1\text{ }\mu\text{m}$. NTA can be used in fluorescent mode thus detecting labeled vesicles (143).

NTA with fluorescent mode provides both quantitative and qualitative information of the EVs in suspension. However, as of today flow cytometry still provides better qualitative information by the use of multiple labeling of microvesicles in this technique.

Table 7. Methods for the detection of microvesicles

Method	Size limitations	Quantification	Phenotyping	Reference
Flow cytometry	> 300nm	+	+	(144, 145)
Nanotracking analysis	50 nm-1 μ m	+	+	(143)
Transmission electron microscopy	> 2-5 nm	-	+	(142, 146)
Atomic force microscopy	> 0.5-1 nm	-	-	(147)
Resistive pulse sensing	> 40 nm	+	-	(148)
Dynamic light scattering	> 300 nm	+	-	(149)

Several methods are used to detect microvesicles. Flow cytometry, nanotracking analysis and transmission electron microscopy are described in the text. Atomic force microscopy is a high-resolution type of scanning probe microscopy. Resistive pulse sensing is impedance flow cytometry. Dynamic light scattering generates size distribution using the Brownian motion of particles. This table categorize methods used for detection of Evs based on size limitations, possibility of quantification and phenotyping (i.e. possibility of cell surface characterization). +: applicable, -: not applicable.

Microvesicles in physiological and pathological processes

Microvesicles are released under physiological as well as pathological processes. In the following section the role of microvesicles in these processes will be discussed.

Intercellular communication

Microvesicles participate in intercellular communication by transfer of proteins, lipids, receptors, mRNA as well as microRNA from the parent cell to recipient cells in which they may induce phenotypic changes.

Release of microvesicles and transfer of proteins and lipids

The release of microvesicles may be essential for the cell in order to rid the cell of unwanted substances. For example, inhibition of the release of endothelial microvesicles containing caspase-3 resulted in cell detachment and apoptosis (150). Microvesicles transport proteins such as cytokines, chemokines and growth factors to neighboring or distant cells, resulting in modulation of the target cell. On

the other hand, upon release vesicles may shelter proteins that would otherwise be phagocytosed in or neutralized in free form in plasma. Vesicles thus protect their content from the host response (114). This mode of transport can also be utilized by bacterial and viral components to evade the host response (114, 151). Bioactive lipids, such as sphingosine 1-phosphate and arachidonic acid, are also transported within microvesicles (152). Lipids in platelet microvesicles can increased adhesion between endothelial cells and monocytes (153) thus microvesicles not only effect recipient cells but also other cells in their microenvironment.

Transfer of mRNA and microRNA

Microvesicles can transfer mRNA and microRNA horizontally to target cells that thereby is translated, changing the recipient cell's phenotype. Murine microvesicles derived from embryonic stem cells transferred microRNA caused epigenetic changes in adult hematopoietic stem/progenitor cells (154). Horizontal transfer of mRNA from endothelial progenitor cells carrying green fluorescence protein (GFP) could via microvesicles transfer mRNA activating an angiogenic program (155). Horizontal transfer of genetic material and the changes seen in the target cells were even demonstrated between cells of different species (156).

Transfer of receptors

Microvesicles are capable of transferring functionally active receptors to recipient cells that lacked the receptor. This is exemplified by the CCR5 and CXCR4 co-receptors that are important for the HIV-1 virus to enter cells. Transferred CCR5 enabled HIV-1 to be internalized in cells previously not susceptible to the virus (157). In a similar manner, megakaryocytic and platelet-derived microvesicles were able to transfer the CXCR4 co-receptor to cells lacking the CXCR4 receptor that are not primary targets of the HIV-1 infection (158) suggesting that this may be a mean of disseminating HIV infection. Hematopoietic cells can receive specific adhesion molecules from platelet-derived microvesicles that modulate their biological functions (159, 160). As seen in hematopoietic stem cell transplantations, bone marrow cells covered with platelet microvesicles engrafted faster than cells without microvesicles in lethally irradiated mice, proposing a specific role for the transfer of receptors in stem cell transplantation. Furthermore, microvesicles released from aggressive glioma cells transfer the oncogenic epidermal growth factor receptor (EGFR) vIII to tumor cells causing a propagation of oncogenic activity (161).

Ligand binding

Surface-exposed ligands or receptors on microvesicles can bind to target cells (Figure 3). Microvesicles from platelets expressing P-selectin were shown to bind to P-selectin glycoprotein ligand-1 on the surface of leukocytes causing leukocyte

accumulation and aggregation (162). In addition, microvesicles bearing Sonic hedgehog (Shh) bind to the Shh receptor, this binding promotes differentiation of megakaryocytes, production of endothelial nitric oxide as well as in vitro angiogenesis and in vivo neovascularization. The effects seen are reversed when silencing the Shh receptor (163).

Metastasis

In cancer biology microvesicles have been extensively studied because their capacity for intercellular communication may cause malignant cells to potentiate their survival and spread (123). Microvesicles are released by cancer cells and more aggressive forms of malignancies produce more microvesicles than less aggressive forms. Microvesicles can be taken up by neighboring cancer cells potentiating their survival and growth, and, when taken up by non-cancerous cells, potentiate the spread of tumor cells (164). For example, platelet microvesicles incubated with breast or lung cancer cells facilitate adhesion and invasion of the tumor cells (165, 166).

Vessel integrity and thrombosis

The endothelium lining the inner wall of the vessels has an important barrier function but also partakes in maintaining vascular tone and regulating inflammation, angiogenesis and coagulation. Endothelial- and platelet- derived microvesicles protect the endothelial cell lining by promoting cell survival (155, 167, 168). Microvesicles are believed to play a role in the pathogenesis of many diseases characterized by decreased vascular function such as atherosclerosis (169), acute coronary syndromes (170), hypertension (171), pre-eclampsia (172), sepsis and vasculitis (1). Endothelial microvesicles may modulate vascular injury, inflammation and thrombosis especially in pathological conditions by affecting nearby or distant cells by their surface molecules or soluble mediators. They might also cause decreased nitric oxide formation in endothelial cells (173), causing alterations in vascular tone. Microvesicles of neutrophil origin have been shown to activate the endothelium (174) and cause endothelial cell dysfunction.

Microvesicles play an important role in coagulation, platelet aggregation and thrombosis. Under physiological conditions microvesicles may have antithrombotic effects (175, 176) that becomes prothrombotic during pathological conditions. Microvesicles exposing phosphatidylserine on the outer leaflet of the phospholipid membrane are negatively charged, which in turn generates a prothrombotic surface capable of binding coagulation factors (prothrombin, factors Va and Xa) (177, 178). However, microvesicles derived from monocytes (126), and possibly even platelets (179) or endothelial cells (180) have been shown to be

prothrombotic by a tissue factor-dependent mechanism. This in turn will generate thrombin via the extrinsic pathway of coagulation (180).

Immune response

Microvesicles play an important role in the normal physiology of immune response by having both pro- and anti-inflammatory properties.

Microvesicles from leukocytes may activate the endothelium to produce cytokines such as IL-6, IL-8 as well as upregulate adhesion molecules for leukocytes on endothelial cells (174). Furthermore, leukocyte-derived microvesicles can activate platelets that in turn bind to the endothelium causing release of proinflammatory cytokines and upregulation of monocyte adhesion molecules (153). Platelet-derived microvesicles may also increase immunoglobulin production by B-cell cells (181).

Complement activation results in complement deposits on cells and consequently opsonization leading to elimination by phagocytes. Deposition of the membrane attack complex leads to cell lysis. Microvesicle release by cells with complement deposits on their surface may be a cytoprotective mechanism to avoid complement-mediated cell death (182). Microvesicles can also bear C1q in vivo (183) and thus activate the classical pathway of complement. The complement system is discussed in more detail below.

Tissue regeneration and angiogenesis

Microvesicles may cause endothelial regeneration by direct interaction with endothelial cells or by affecting endothelial progenitor cells to support repair (155). Extracellular vesicles from injured tissues are transmitted to stem cells affecting in the process of tissue repair (184). On the other hand stem cell microvesicles can harbour a variety of lipids, proteins, and nucleic acids, that may mediate phenotypic and functional changes in progenitor cells, increasing their regenerative potential (185, 186).

Microvesicles may carry proangiogenic factors and thus participate in the process of angiogenesis. Endothelial microvesicles promote angiogenesis at lower concentrations (187) and platelet microvesicles may affect endothelial cells by inducing survival, proliferation and migration as seen in vitro. When injecting platelet microvesicles in the myocardium post-ischemic neovascularization was demonstrated (188). In the case of tumor cells that are dependent on neovascularization to reach the high demand of oxygen and nutrients for the survival of the malignant cell, microvesicles have been shown to attract and

activate fibroblast and endothelial cells as well as increase the expression of proangiogenic factors (166)

Microvesicles in inflammatory disease

Microvesicles play an important role in inflammatory diseases through a variety of mechanisms involving blood cells, endothelial cells as well as the complement and the kinin system. Endothelial microvesicles may be markers of ongoing disease in vasculitis (1), preeclampsia (189), acute coronary syndrome (170), hypertension (171), and diabetes (190).

Rheumatoid arthritis is an autoimmune disorder causing chronic joint inflammation. Microvesicles are elevated in plasma from patients with rheumatoid arthritis compared to controls, and intense treatment with anti-inflammatory agents does not reduce the number of circulating microvesicles (191). Microvesicles mainly derived from leukocytes are elevated in synovial fluid. These microvesicles bear tissue factor and may cause hypercoagulability and fibrin deposits in inflamed joints (192).

In Systemic Lupus Erythematosus platelet-derived microvesicles are significantly increased and correlate to thrombin generation, suggesting a role in the thromboembolic state (193). No correlation was seen to other aspects of disease activity or antiphospholipid antibodies (193).

Antiphospholipid syndrome is an autoimmune disease causing hypercoagulability due to antiphospholipid antibodies. Patients with antiphospholipid syndrome have elevated endothelial and platelet-derived microvesicles compared to controls (193, 194).

Multiple sclerosis is a demyelinating disease in the central nervous system in which microvesicles play a role in the pathogenesis. Endothelial microvesicles carry metalloproteases leading to disruption of the blood brain barrier (195). Furthermore, microvesicles from brain endothelium, platelets and leukocytes cause release of chemokines from endothelial cells as well as increase of adhesion molecules participating in the inflammation seen in multiple sclerosis (196). Microvesicle levels have been suggested to predict relapses in multiple sclerosis (197, 198).

Systemic sclerosis is a systemic connective tissue disorder. Microvesicles derived from platelets, leukocytes and endothelial cells are elevated in systemic sclerosis (199). The total number of microvesicles were inversely correlated to the modified Rodnan skin thickness score (199).

Microvesicles in vasculitis

Microvesicles from endothelial cells, platelets and leukocytes are increased during the acute phase of vasculitis, returning to normal levels during remission (2, 117, 200). Endothelial microvesicle levels in pediatric vasculitis correlated to the BVAS, C-reactive protein and erythrocyte sedimentation rate (1) whereas endothelial microvesicles in adults AAV correlated to the BVAS (2) and thus could be used as a biomarker for vasculitis activity (1, 2).

ANCAs circulating in patients with AAV activate neutrophils causing them to release microvesicles (201). In patients with vasculitis neutrophil microvesicles are increased (200) and may activate endothelial cells causing them to release cytokines (174, 201, 202). Neutrophil microvesicles may expose PR3 and MPO on their surface enabling ANCA to bind. Microvesicles which bear tissue factor are pro-thrombotic, as previously described, and may contribute to the thromboembolic complications seen in vasculitis (126, 179).

The kinin system

Overview

The kinin system, also known as the contact system, causes classical signs of inflammation when activated. The kinin system can be activated in the circulation by plasma kallikrein that cleaves high- molecular weight kininogen (HK) releasing bradykinin and in tissues by tissue kallikrein that cleaves low molecular weight kininogen releasing kallidin (4). The two kinins are further processed to des-arg⁹-bradykinin or des-arg¹⁰-kallidin, respectively. An alternative pathway of activation may be particularly relevant in vasculitis as it is induced when PR3 cleaves HK releasing PR3-kinin, a vasoactive kinin (51).

Activation of the kinin system in plasma

HK is formed mainly in the liver and circulates in complex with prekallikrein (203). The HK-prekallikrein complex binds to a receptor complex on the cell surface. Activation of the kinin system is initiated when prolylcarboxypeptidase cleaves prekallikrein to kallikrein. Cleavage of prekallikrein may also be achieved by factor XIIa, resulting in kallikrein that in turn cleaves HK into a heavy chain, light chain and the vasoactive nonapeptide bradykinin (4, 204). Plasma kallikrein exerts a positive feedback loop by activating more factor XII to factor XIIa. Carboxypeptidases further process bradykinin to des-arg⁹-bradykinin (205, 206). Bradykinin and des-arg⁹-bradykinin exert their effect via two kinin receptors, the B2-receptor and B1-receptor, respectively. Angiotensin-converting enzyme (ACE) degrades the effector kinins to inactive metabolites (207) (Figure 4). An alternative pathway of activation of the kinin system was described by our group, in which PR3 from neutrophils cleaves HK releasing the 13 amino-acid long peptide PR3-kinin that in turn can bind to the B1-receptor (51). It is reasonable to assume that the PR3 pathway is activated during vasculitis, as there is a massive infiltration of PR3-positive neutrophils during vasculitis (51).

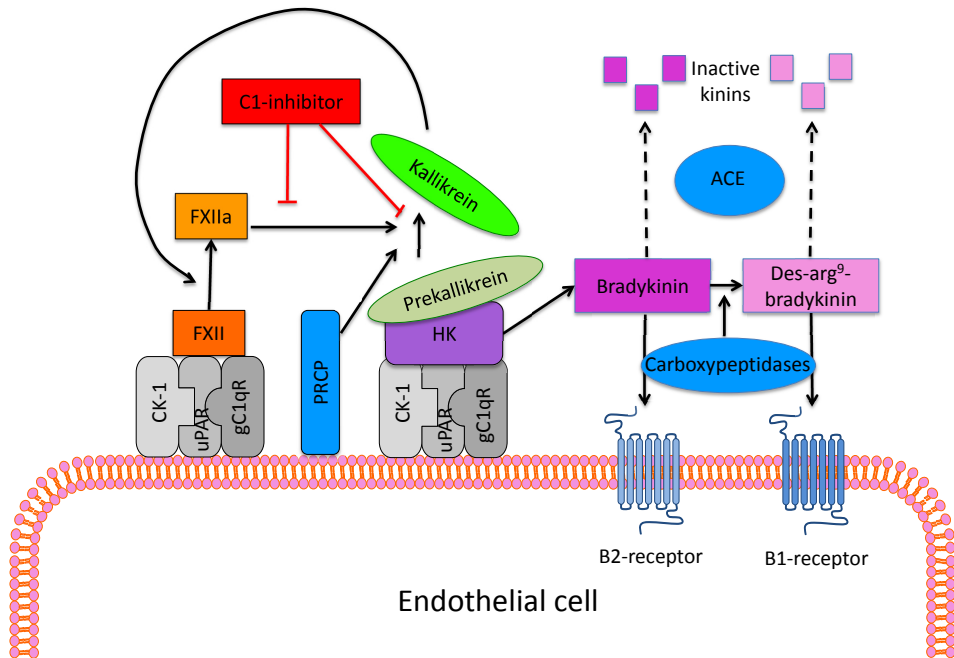


Figure 4. Schematic presentation of the kinin system.

The kinin system is activated when high molecular weight kininogen and prekallikrein circulating in complex bind to their cellular receptor. Prolylcarboxypeptidase or factor XIIa cleave prekallikrein to kallikrein. Plasma kallikrein exerts a positive feedback loop by activating more factor XII to factor XIIa. The kinin system activation is inhibited by C1-inhibitor. HK: High molecular weight kininogen, CK-1: cytokeratin-1, uPAR: urokinase plasminogen activator receptor, gC1qR: receptor for the globular heads of C1q (p33 as it is 33kDa), PRCP: prolylcarboxypeptidase, ACE: angiotensin-converting enzyme.

The components of the plasma kinin system

High molecular weight kininogen

HK is a plasma protein produced in the liver as well as by neutrophils, endothelial cells and platelets (4). HK consists of 6 domains (D1-6) divided into a heavy chain (D1-3), a light chain (D5-6) and the D4 domain (21 peptides) containing the nonapeptide bradykinin.

Plasma kallikrein and factor XII

Plasma prekallikrein is a serine protease produced in the liver and released to the circulation. It is cleaved and activated by factor XIIa or prolylcarboxypeptidase into plasma kallikrein, which in turn cleaves HK releasing bradykinin. Plasma kallikrein is also a chemoattractant for neutrophils (208) inducing them to release elastase (209). Plasma kallikrein can activate factor XII via a positive feedback loop and at the same time initiate the intrinsic pathway of coagulation when

activated factor XII activates factor XI, ultimately resulting in thrombin formation (210).

Kinins

Kinins are potent vasoactive pro-inflammatory peptides that promote inflammation, control local blood pressure and activate pain receptors (Figure 5).

Bradykinin

Bradykinin binding to its receptor induces a signal that modulates several endothelial cell functions such as increased production of nitric oxide causing increased vascular permeability and vasodilatation (3), formation of superoxide (211), enhanced expression of prostacyclins leading to vascular relaxation and platelet inhibition (212, 213) as well as increasing profibrinolytic properties by release of tissue plasminogen activator (214). In fibroblasts bradykinin induces the release of IL-6 and IL-8 (3) and in neural cells it induces pain (215).

Des-arg⁹-bradykinin

Des-arg⁹-bradykinin binding to the B1-receptors exerts similar effects to those induced by bradykinin. It stimulates endothelial cells to release nitric oxide (216) as well as prostacyclin (217, 218).

PR3-kinin

PR3-kinin binds to the B1-receptor but not the B2- receptor in vitro (51). In vivo PR3-kinin lowers blood pressure in mice in a manner similar to the B2-receptor agonist bradykinin. In B1-receptor overexpressing rats, PR3-kinin was more potent than bradykinin suggesting that PR3-kinin is degraded to bradykinin in plasma thus affecting both the B1-receptor and the B2-receptor (51).

Kallidin

Kallidin is a decapeptide released by cleavage of low molecular weight kininogen by tissue kallikrein (Figure 5). Kallidin is a B2-receptor agonist.

Des-arg¹⁰-kallidin

Des-arg¹⁰-kallidin is formed after carboxypeptidase processing of kallidin. Des-arg¹⁰-kallidin binds to B1-receptors and it has 1000 times higher affinity than des-arg⁹-bradykinin (3).

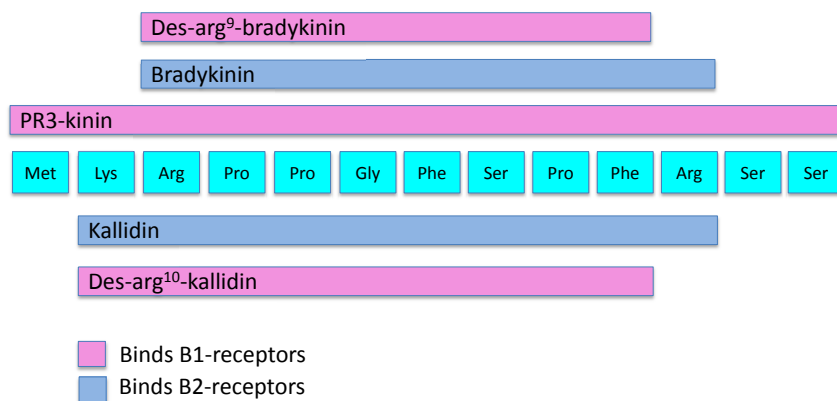


Figure 5. Kinins and their binding to the B1- and B2-receptor.

Bradykinin receptors

The kinin receptors, B1- and B2-receptors, are found on endothelial cells, smooth muscle cells, leukocytes, fibroblasts, epithelial cells as well as neural cells (3). The receptors are G protein coupled receptors and have a homology of 36 percent (219).

B1-receptor

The B1-receptor is generally not present on the surface of a resting cell in large amounts but sequestered intracellularly in the endoplasmic reticulum from which it recirculates when not bound by an agonist. It is internalized from the cell membrane to the cytoplasm and the endocytosomal reticulum and then back to the cell membrane (3). When injury occurs or cells are exposed to inflammatory mediators such as lipopolysaccharides, interleukins or cytokines the receptor expression is increased on the cell surface (220). Des-arg⁹-bradykinin, des-arg¹⁰-kallidin and PR3-kinin are all B1-receptor agonists. The binding of an agonist to the receptor upregulates its expression (221, 222), and more receptors are externalized to the cell membrane, due to delayed endocytosis (3). When the B1-receptor is activated it exerts a prolonged and sustained signal because the receptor is not desensitized and internalized.

When an agonist binds to the B1-receptor it stimulates phospholipase C to increase phosphatidylinositol hydrolysis and enhance cytosolic calcium that initiates a cascade of events resulting in arachidonic acid release, eicosanoid production

(217) as well as endothelial nitric oxide synthase (eNOS) activation resulting in nitric oxide production (3).

Inhibition of B1-receptors reduced neutrophil chemotaxis in a murine model pre-treated with IL-1 β . In addition, des-arg9-bradykinin induced migration of neutrophils in air pouches in mice pre-treated with IL-1 β , an effect decreased by administration of a B1-receptor antagonist (223). Migration of neutrophils in inflammatory tissue was substantially decreased in B1-receptor knock-out mice thus demonstrating that B1-receptors are important for the inflammatory tissue damage induced by neutrophil influx (224). Activating the B1-receptor on endothelial cells releases proinflammatory cytokines such as TNF- α and IL-1 that will activate leukocytes (223, 225).

There are many B1-receptor antagonists described, in this thesis the peptide R715 is used (3).

B2-receptor

The B2-receptor is constitutively expressed on cell membranes as described above. Bradykinin and kallidin are B2-receptor agonists. The B2-receptor is desensitized and internalized and recycled upon ligand binding (3). This recycling of the receptor causes a transient signal. When an agonist binds to the B2-receptor there is a transient increase of cytosolic calcium (3) that initiates a cascade of events similar to those described above for the B1-receptor. Activation of the B2-receptor on primary sensory neurons leads to the acute pain response seen in acute inflammation (215). In chronic inflammation the B2-receptor is postulated to be downregulated (226), however it may cause upregulation of the B1-receptor (227) during the chronic inflammatory state. Cytokines such as TNF- α and interleukin-1 β upregulate the B2-receptor on fibroblasts (228), and bradykinin in turn causes fibroblasts to release IL-6 and IL-8, as previously mentioned.

Several commercially available B2-receptor antagonists have been described (3) In this thesis HOE-140 was used. It is used in clinical practice and marketed as Icantibant (Shire, Zug Switzerland) for the treatment of hereditary angioedema.

Inhibitors of the kinin system

The most important inhibitor of the kinin system is C1-inhibitor, discussed below. Alpha-2 macroglobulin is a major inhibitor of kallikrein and plasmin (229). Another inhibitor is alpha 1-antitrypsin which inhibits the PR3 pathway of activation. The PR3 pathway circumvents all other inhibitors of the kinin system.

C1-inhibitor

C1-inhibitor is a serine protease inhibitor, part of the serpin superfamily, in the plasma. C1-inhibitor is the most important physiological inhibitor of the kinin system and exerts inhibition on both plasma kallikrein and fXIIa (4, 204). C1-inhibitor also inhibits the classical and lectin pathways of complement, as described below. PR3 released from neutrophils and monocytes during inflammation may degrade C1-inhibitor, causing activation of the kinin system by reducing the inhibitory mechanisms (230).

C1-inhibitor deficiency leads to hereditary angioedema in which there is recurrent uncontrolled activation of the kinin system and release of vasoactive kinins causing swelling, due to vasodilation and leakage of fluids into the extracellular space, which can be potentially life-threatening (231).

Kinin system activation in vasculitis

The kinin system is activated in children (5) and adults (51) with vasculitis. Skin and kidney biopsies from patients with vasculitis have shown deposition of kinins in inflammatory areas (5). The B1-receptor was demonstrated in biopsies from patients with glomerulonephritis including those with vasculitis (232). Bradykinin levels were elevated in the circulation in vasculitis patients (51). This indicates that the kinin system is activated both locally and systemically (5).

Neutrophils present at inflammatory sites in vasculitis could potentially activate the kinin system by release of PR3 during the inflammatory response.

In a mouse model in which glomerulonephritis was induced by nephrotoxic serum, mice were protected by treatment with a B1-receptor antagonist resulting in reduced renal pathology and improved kidney function (233). This suggests that B1 receptor antagonists may protect from renal inflammation.

Complement system

Overview

The complement system plays an important role in host innate immunity. Its main physiological functions are to

- Defend against invading organisms
- Clear immune complexes and unwanted cells
- Enhance the adaptive immune response

The complement system consists of more than 35 proteins circulating in plasma and when activated a cascade of interactions leads to the formation of the C3 and C5 convertases with simultaneous release of by-products which are antimicrobial, anaphylatoxins, opsonins and chemoattractants. Ultimately, the membrane attack complex (MAC) is formed. Due to its pro-inflammatory and cytotoxic properties the complement system need to be tightly regulated.

The complement system can be initiated by three pathways, the classical, the lectin and the alternative pathway, and although they diverge in their activating surfaces or molecules, all will be amplified by the so-called amplification loop within the alternative pathway followed by activation of the terminal pathway.

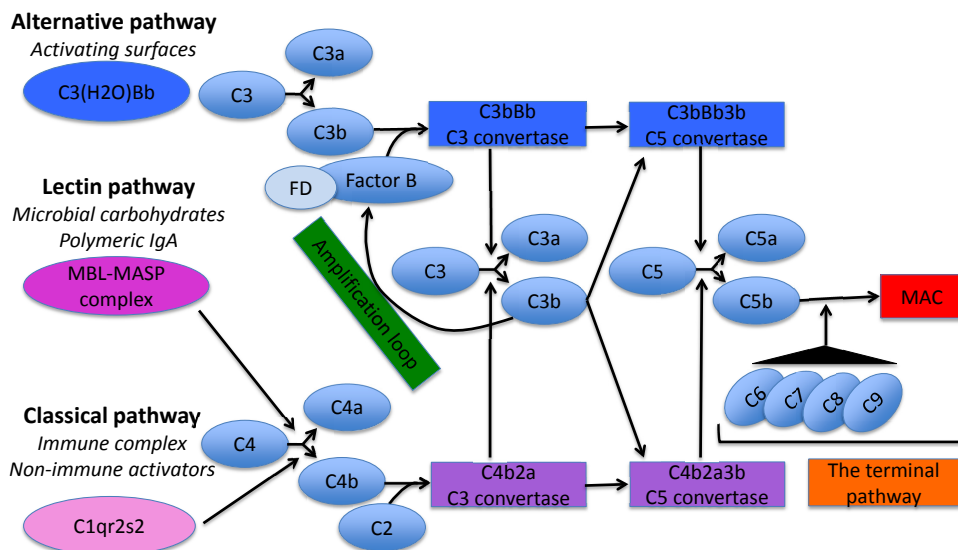


Figure 6. An overview of the complement system.

The complement system can be activated by three pathways, the classical, the lectin and the alternative pathway. The three pathways converge into the common pathway that leads to the formation of the membrane attack complex. FD: factor D, MAC: membrane attack complex.

The classical pathway

The classical pathway is activated by immune complexes or nonimmune molecules, such as CRP. C1q is mainly released from dendritic cells and macrophages whereas most other complement proteins are produced in the liver (234, 235). The globular head of C1q binds to the Fc region of an antibody (IgG or IgM) in complex with its antigen. The binding of C1q to an immune complex initiates the process leading to the formation of C1qr₂s₂ in the presence of Ca²⁺. C1qr₂s₂ enzymatically cleaves both C4 and C2 in the presence of Mg²⁺, resulting in the classical pathway C3 convertase (C4b2a) that in turn can cleave C3. When an additional C3b binds to C4b2a the C5 convertase (C4b2a3b) is formed capable of cleaving C5 into C5a and C5b (236).

The lectin pathway

The lectin pathway is activated when carbohydrates on bacterial surfaces or polymeric IgA bind mannose binding lectin (MBL)/collectins (237) or ficolins (238) in the presence of MBL-associated serine proteases (MASPs). MASPs cleave C2 and C4 thus forming the C3-convertase (C4b2a) (239). Following this

step, the pathway converges with the classical pathway of complement activation and is amplified by the alternative pathway. MASP-3 may, however, also activate the alternative pathway directly (240). Furthermore, MASP-2 can cleave prothrombin to thrombin thus promoting thrombin generation (241) providing a link with the prothrombotic system.

The alternative pathway

C3 is spontaneously hydrolyzed to C3(H₂O) in the presence of Mg²⁺. C3(H₂O) interacts with factor B cleaved by factor D resulting in a preliminary C3 convertase C3(H₂O)Bb that can cleave C3 into C3a and C3b. The newly formed C3b subsequently binds factor B to form C3bBb in the presence of factor D. This is the C3 convertase capable of cleaving more C3 into C3a and C3b thus triggering the amplification loop (242). C3bBb has a short half-life but is stabilized by properdin (243, 244). When an additional C3b binds to the C3bBb the C5 convertase (C3bBb3b) is formed cleaving C5 into C5a and C5b.

The terminal pathway

The C5b formed, in the common pathway, is still bound to the C5 convertase. C5b will bind to C6 and C7 after which the C5 convertase is released and the hydrophobic complex is inserted into the lipid bilayer (245). The addition of C8 to the hydrophobic complex on the bilayer makes the cell slightly permeable. The addition of multimers of C9 to the C5b-8 complex produce a pore spanning through the cell membrane. The C5b-9 complex is called the membrane attack complex (MAC) causing the cell to lyse (246).

Function of the complement system

One of the main functions of the complement system is to lyse target cells. This is achieved by insertion of the MAC, a pore like structure, into the cell membrane (247). Another important task of the complement system is to opsonize unwanted substances by release of the opsonins C4b, C3b and C5b (248) marking pathogens and apoptotic cells as well as other debris for removal by phagocytosis. At the same time the complement system has the ability to attract immune cells such as neutrophils and monocytes to sites of inflammation or infection, by C3a and C5a (248), which are strong chemoattractants enabling the immune cells to kill or phagocytose the unwanted cells. C5a is also able to prime neutrophils causing

them to display PR3 and MPO on the cell membrane (249). C3a and C5a also have antimicrobial properties and C4a, C3a and C5a are anaphylatoxins (250).

Complement regulation

The complement system requires tight regulation in order to prevent injury to the host. Several inhibitors control complement activation along the activation pathways. These complement inhibitors may be soluble or membrane-bound (Table 8). Properdin is the only positive regulator of the complement system as it stabilizes the C3 convertase of the alternative pathway.

Table 8. Regulators of the complement system

Complement regulator	Pathway	Major function	Localization	Reference
C1-inhibitor	CP,LP	Binds to activated C1, dissociating the complex , or binds to MASPs	FP	(251)
C4-binding protein	CP, LP	Promotes dissociation of convertases. Cofactor for factor I in cleavage of C4b	FP	(252)
Factor I	CP, AP	Degrades C3b and C4b in the presence of cofactors factor H, C4 binding protein, MCP and CR1	FP	(253)
CD46 (MCP)	CP, AP, T	Co-factor for factor I in cleaving C3b	MB	(254)
CD55 (DAF)	CP, AP, T	Promotes dissociation of convertases	MB	(255)
Complement receptor 1 (CR1, CD35)	CP, AP, T	Regulation of of C3 degradation and cofactor for factor I	FP & MB	(256)
Properdin	AP	Stabilizes C3bBb	FP	(257)
Factor H	AP	Promotes dissociation of the C3 convertase, cofactor for factor I in cleavage of C3b, host cell recognition	FP	(258)
Clusterin	T	Inhibits MAC formation	FP	(259)
CD59 (protectin)	T	Inhibits MAC formation	MB	(260)
Vitronectin	T	Inhibits MAC formation	FP	(261)

CP, classical pathway; LP, lectin pathway; AP, alternative pathway; FP, fluid phase; T, terminal pathway. MB, membrane-bound; MAC, membrane attack complex.

C1-inhibitor

The classical and lectin pathway is inhibited by C1-inhibitor. C1-inhibitor binds to activated C1s and C1r, dissociating the C1 macromolecule or binds to MASPs cleaving them to the inactivated form (251).

C1-inhibitor is also the major inhibitor of the kinin system, as described above. Hereditary deficiency of C1-inhibitor primarily results in symptoms related to overwhelming kinin system activation (262). Lack of symptoms from the complement symptom may be due to the multitude of other inhibitors capable of regulating activation of the classical pathway.

Complement activation in vasculitis

Complement activation partakes in the inflammatory process during vasculitis. Plasma levels of C3a, C5a, soluble C5b-9 and Bb are elevated in patients with active AAV compared to samples taken at remission or controls (263). Factor Bb levels correlated to disease activity (264). Elevated factor Bb indicates activation of the alternative pathway (264). Factor B, C3 as well as the membrane attack complex were detected in the glomeruli of patients with renal involvement (265, 266). In IgAV there is activation of the complement system leading to deposition of complement but also of IgA1 in the skin and the mesangium. Both the alternative and lectin pathway are activated locally, in glomeruli, as well as systemically (267).

MPO knock-out mice were used to produce anti-MPO IgG as previously described. Wild-type mice (B6) developed glomerulonephritis while C5-deficient did not when injected with anti-MPO IgG (6). Pretreatment of the wild-type mice with cobra venom factor (to deplete C3) inhibited the development of glomerulonephritis and decreased influx of neutrophils and monocytes to the tissue (6). Anti-MPO was injected in mice deficient in C4 or factor B showing that the mice deficient in factor B did not develop disease while the wild type as well as the C4-deficient mice were not protected (6). Pretreating the wild-type mice with monoclonal antibody against C5 prevented development of ANCA-mediated glomerulonephritis (268). Taken together, these results indicate that complement activation via the alternative pathway is important in AAV.

C5a is able to prime neutrophils causing them to display PR3 and MPO on their surface and making it possible for ANCA to activate neutrophils (269). Neutrophils activated by MPO- and PR3-ANCA cause complement activation (6, 270). This will result in the release of C5a resulting in the recruitment of more neutrophils by this potent chemoattractant. Furthermore, C5a stimulates tissue factor expression on endothelial cells (271) and on neutrophils (272) causing them to be prothrombotic. The accumulated activated neutrophils in the vessel wall release reactive oxygen species and proteolytic enzymes causing endothelial tissue damage.

The presence of complement on microvesicles in vasculitis will be addressed in this thesis.

The interactions between the kinin and the complement systems

The kinin and complement systems are involved in the inflammatory process in vasculitis. Both systems promote inflammation and interact with the coagulation or thrombotic systems. There are components within the kinin and complement system that can activate the coagulation cascade. For instance, kallikrein may activate factor XII leading to activation of the intrinsic pathway of coagulation and induction of tissue factor on cells making them procoagulant (126, 179) as described above.

Thrombin, plasmin and coagulation factors FXIa, Xa and IXa can cleave C3 and C5 to biologically active C3a and C5a in the absence of C3 and C5 convertases (273). C5a causes endothelial cells to express P-selectin, to release von Willebrand factor, IL-8, IL-1 β and upregulates adhesion molecules.

Both the kinin and the complement system have the same inhibitor, C1-inhibitor, as described above.

The present investigation

Aims

- To demonstrate the presence of B1-receptors on endothelial- and leukocyte-derived microvesicles in patients with vasculitis
- To investigate if the presence of the B1-receptor on microvesicles could cause neutrophil chemotaxis
- To explore if C1-inhibitor could affect the release of B1-receptor-positive endothelial-derived microvesicles from glomerular endothelial cells and if its presence regulates neutrophil chemotaxis
- To investigate if endothelial-derived microvesicles in plasma from patients with vasculitis bear complement components
- To study if blocking the kinin B1 and B2 receptors affects complement deposition on endothelial microvesicles
- To investigate if microvesicles bearing the B1-receptor can transfer functional receptors to a recipient cell
- To define the incidence of pediatric primary systemic vasculitis in a defined population in southern Sweden

Experimental conditions and results

The following is an overview of the applied methods, study design and results from the papers included in this thesis. For further and more in depth information please refer to Papers I-IV.

The study was conducted with the approval of the Regional Ethics review board of Lund and Linköping Universities. Samples from patients and controls were taken with the written consent from the patients, their parents and the controls.

Paper I:

C1-inhibitor decreases the release of vasculitis-like chemotactic endothelial microvesicles

Our group has previously shown that the kinin system is activated in patients with vasculitis. In this thesis we studied the role of the kinin system in vasculitis and examined the expression of kinin receptors on microvesicles.

Plasma from patients with vasculitis (n=12) and controls (n=15) was investigated for B1-receptor-positive endothelial-derived microvesicles analyzed by flow cytometry. B1-receptor-positive microvesicles from transfected human embryonic kidney (HEK) cells and vasculitis plasma were used as chemoattractants for neutrophils in a Boyden chamber. We utilized a perfusion system to study the release of microvesicles from glomerular endothelial cells. Plasma samples from patients and controls (n=6, each) were run through artificial capillaries coated with glomerular endothelial cells, mimicking a small vessel, and the collected samples were analyzed by flow cytometry. In addition, patient plasma with reduced levels of microvesicles as well as C1-inhibitor-depleted plasma were perfused through the flow system. In certain samples we added B1-receptor antagonist or C1-inhibitor before perfusion.

Plasma from vasculitis patients had more endothelial microvesicles than controls and these microvesicles expressed proportionally more B1-receptors than those from controls. Plasma from vasculitis patients induced neutrophil chemotaxis mediated by the B1 receptor on the membrane fraction, as it was reduced by a B1-receptor antagonist and also reduced by lowering the microvesicle load. A similar effect on neutrophil chemotaxis was demonstrated using microvesicles released from B1-receptor-transfected HEK cells. The effect on neutrophil migration was abrogated by adding the B1-receptor antagonist. Perfusion of vasculitis plasma over glomerular endothelial cells released significantly more microvesicles, and particularly B1-receptor-positive microvesicles, than plasma from controls. A

similar effect was demonstrated when C1-inhibitor depleted plasma was perfused over the cells releasing B1-receptor positive microvesicles, which were decreased when C1-inhibitor or B1-receptor antagonist were added before perfusion.

Paper II:

The kinin system modulates complement activation on endothelial cell microvesicles in vasculitis

In paper II we set out to study if complement C3 and C9 were deposited on endothelial microvesicles during active vasculitis, and to investigate if kinin-receptor inhibitors or C1-inhibitor could reduce the release of endothelial microvesicles coated with complement.

Flow cytometry was used to analyze plasma from patients with vasculitis (n=22) and controls (n=21) for endothelial-derived microvesicles bearing complement. We used the same perfusion system as in paper I to flow plasma from patients with vasculitis and controls (n=6, each) over glomerular endothelial cells and used flow cytometry to analyze the release of microvesicles in general, as well as those coated with C3 or C9. We compared the effect of plasma taken during the acute phase to plasma taken at remission. Complement-positive endothelial-derived microvesicle release was studied in the presence of B1-receptor or B2-receptor antagonists (alone or in combination) or C1-inhibitor added to vasculitis plasma or to C1-inhibitor-depleted plasma.

Vasculitis plasma exhibited significantly higher levels of C3- and C9-positive endothelial-derived microvesicles than controls. Plasma from patients with vasculitis released significant more glomerular endothelial-derived microvesicles, positive for complement, than control plasma, an effect diminished by reducing the amount of microvesicles in the plasma by centrifugation. Plasma obtained during active disease phase released more endothelial-derived microvesicles, as well as more microvesicles bearing complement, than samples obtained at remission. B1- and B2-receptor antagonist or C1-inhibitor reduced the number of released endothelial-derived microvesicles as well as the microvesicles bearing complement. C1-inhibitor depleted plasma perfused over the glomerular endothelial cells mimicked the effect of vasculitis plasma, inducing the release of endothelial-derived microvesicles coated with complement C3 and C9. This effect was reduced when samples were run in the presence of C1-inhibitor or B2-receptor antagonist, alone or in combination with the B1-receptor antagonist.

Paper III:

Microvesicles transfer of kinin B1-receptors is a novel inflammatory mechanism in vasculitis.

In paper III we studied if kinin B1-receptors are present on leukocyte microvesicles and if functional B1-receptors could be transferred between cells by microvesicles.

Plasma from patients with vasculitis and controls (n=9, each) were investigated for leukocyte-derived microvesicles bearing B1- and B2-receptor, demonstrated by flow cytometry. Kidney biopsies from two patients with vasculitis were investigated for localization of microvesicles using transmission electron microscope. B1-receptor positive microvesicles released from B1-receptor transfected HEK cells and stimulated leukocytes were incubated with wild-type (B1-receptor-negative) HEK cells as well as glomerular endothelial cells. A calcium influx assay was performed to elucidate if the transferred receptor was functional.

The B1-receptor as well as the B2-receptor were present on microvesicles from leukocytes, mainly of neutrophil and monocyte origin, in vasculitis plasma. The paper continued to focus on the importance of the B1-receptor. In kidney biopsies neutrophil microvesicles bearing B1-receptor were shown to dock onto the glomerular endothelial cells demonstrating that the proximity could allow the transfer of B1-receptors from neutrophil-derived microvesicles to glomerular endothelial cells. Furthermore, microvesicles from leukocytes and HEK cells transfected with the B1-receptor were shown to transfer the B1-receptor to recipient cells of a different cell type (wild-type HEK cells and endothelial cells). The functionality of the transferred receptor was confirmed by measuring calcium influx, dependent on the B1-receptor.

Paper IV:

Epidemiology of primary systemic vasculitis in children – a population-based study from southern Sweden.

In paper IV the epidemiology and incidence of pediatric vasculitis was studied focusing on primary systemic vasculitis (PSV).

The Skåne Healthcare Registry, a database registering all healthcare contacts in Skåne, was screened for cases of PSV below 18 years of age between 2004-2014 using diagnosis codes from the International Classification of Diseases, tenth version (ICD-10): M300-M319 and D690-D699. Medical charts of the individual cases were reviewed to ascertain diagnosis and cases were divided into IgA

Vasculitis (Henoch-Schönlein Purpura), Kawasaki disease, granulomatosis with polyangiitis, microscopic polyangiitis, eosinophilic granulomatosis with polyangiitis, polyarteritis nodosa and Takayasu's arteritis.

In total, 556 cases of PSV were found in Skåne resulting in an annual incidence rate of 200 per million children (range 183-217, CI 95%). The most common childhood vasculitis in Skåne was IgAV with an annual incidence rate of 175.5 (160-191, CI 95%), the second most common was KD with an incidence rate of 20.1 (14.9-25.4, CI 95%), both exhibiting seasonal variations corresponding to infectious diseases. For GPA and MPA the incidence rate was 1.4 (0-2.8, CI 95%), for PAN 0.7 (0-1.7, CI 95%) and for EGPA and TAK 0.4 (0-1.1, CI 95%).

Discussion

This thesis demonstrates a novel role of microvesicles in the pathogenesis of vasculitis. We found that activation of the kinin system on microvesicles plays an important role in the propagation of inflammation, by inducing neutrophil chemotaxis and horizontal transfer of kinin receptors. Furthermore, we show that complement components are present on microvesicles, possibly as a cytoprotective effect aimed at preserving endothelial cell integrity after complement deposition. Importantly, the presence of C1 inhibitor and kinin receptor antagonists could modulate the activation or deposition of kinins and complement on endothelial microvesicles.

The results indicate that high levels of microvesicles in vasculitis patient plasma may induce the release of endothelial microvesicles as reducing plasma microvesicles decreased the release of endothelial microvesicles. Moreover, microvesicles play an important role in the pathogenesis of vasculitis by bearing kinin receptors and thereby sustaining inflammation by spreading kinin signaling between cells and inducing neutrophil influx.

Previous studies of complement activation in vasculitis have suggested that complement is triggered by ANCA-stimulated neutrophils (6, 270). In the present study there were no cells in the plasma, although circulating neutrophil-derived microvesicles (demonstrated in paper III) could possibly have a similar effect to the whole cells and thereby induce complement deposition on the endothelial microvesicles. Furthermore, we could not specifically demonstrate an effect of ANCA as endothelial microvesicle release was not altered in samples from which IgG was depleted.

An unexpected finding was that complement deposition on endothelial microvesicles could be decreased by kinin receptor antagonists. There is no direct

explanation for the observed complement inhibition incurred by blocking kinin receptors. Kinin receptors activate multiple intracellular signaling pathways leading to inflammatory responses (274) and we assume that the effect of kinin receptor antagonism on complement activation is secondary to a decreased inflammatory signaling on the endothelial cell surface. Regardless of the mechanism the finding may have important therapeutic potential as kinin receptor blockade is relatively inexpensive and currently the only complement inhibitor commercially available is also one of the world's most expensive drugs, eculizumab (275).

Kinin receptors have been studied with regard to their chemotactic potential. The presence of B1-receptors on cells rendered them chemotactic to neutrophils (223, 224, 276). This property has not been studied on microvesicles although other vesicle-associated proteins have been shown to induce chemotaxis (160, 277). The presence of the B1 receptor on microvesicles may be an important mechanism for inducing inflammation in vasculitis, as we could show the specific effect of the receptor for inducing chemotaxis. Furthermore, the transfer of functional B1-receptor between cells by microvesicles will escalate the inflammatory response. Another potentially important therapeutic implication of the findings was the regulatory role of C1 inhibitor. Although C1 inhibitor is currently only marketed for the treatment of hereditary angioedema our results suggest it should be investigated for the treatment of acute vascular inflammation in vasculitis.

In light of our results, it is tempting to suggest inhibition of microvesicle release as a therapeutic remedy. However, it is important to keep in mind that microvesicles play an important role in physiological interactions between cells and inhibiting their release, at least for an extensive period of time, could potentially be harmful. Cells expressing kinin receptors or bearing complement deposits on their surfaces may release microvesicles as a means to maintain cellular integrity. All the same, the results suggest that inhibiting kinin receptors or treatment with C1 inhibitor may have a beneficial effect that should be pursued in future studies. In addition, we aim to elucidate the inhibitory mechanism by which blocked kinin receptors inhibit activation of the complement system.

The studies herein focused on microvesicles that are vesicular structures with a diameter of 0.1-1.0 μm while exosomes have a diameter of 30-100 nm. Microvesicles and exosomes are released by different mechanisms, however current methodology does not allow to discern one vesicle from the other with complete certainty, and some overlap in detection may occur. Within plasma or cell supernatants exosomes may have also contributed to the registered effects. The term extracellular vesicles which includes exosomes and microvesicles is often used due to the difficulty of separating the vesicles and their effects.

One major limitation in the field of microvesicle research is that the methods available for vesicle analysis vary in their capacity to quantify and phenotype the vesicles particularly when they are not within pure systems, as in bodily fluids. Flow cytometry is often used but the technique is both instrument-dependent (certain instruments have a higher capacity for detection of submicron particles) as well operator-dependent resulting in difficulties in assessing results from different studies. Clearly more robust techniques that give reproducible results regarding detection and phenotyping of extracellular vesicles are needed.

Another limitation in our study was the availability of plasma from vasculitis patients. Due to a limited amount of available plasma, the perfusion system experiments, requiring quite a lot of patient plasma, were conducted using somewhat fewer patients than we initially set out to.

From papers I-III we concluded that microvesicles bearing kinin and complement may partake in the pathogenesis of vasculitis by attracting leukocytes as well as transferring kinin receptors to cells lacking them and thereby cause an increased inflammatory response. We suggest that inhibition of the kinin and complement systems by kinin receptor antagonists or C1-inhibitor should be explored as potential therapeutic targets in vasculitis.

In **paper IV** we investigated the epidemiology of the full spectra of pediatric PSV within a defined geographic area, which has previously been lacking. We observed that vasculitis is more common in children (200 cases/million children) than in adults (73 cases/million inhabitants). The most common vasculitis in childhood was, as expected, IgAV affecting 88 percent of the children in our study, followed by KD affecting 10 percent. Many of the other childhood vasculitides, affecting 2 percent, are very rare and this paper will shed some light on their annual incidence. The Swedish system with social security numbers, in which every resident has a unique number, makes it possible to identify each unique case in epidemiological studies, and remove duplicate cases. The Skåne healthcare registry documents every contact with the public healthcare system, which is the main healthcare provider in the region of Skåne. In our study all cases were reviewed and the diagnosis validated, except for IgA vasculitis in which only 20 percent of cases were reviewed fully. This makes our study more reliable in comparison to other studies in which the investigators relied on questionnaires, filled out by physicians retrospectively for the number of cases seen during a certain time period, or prospectively reported monthly. Both these designs have limitations due to low returns of questionnaires and difficulties in retrospectively finding cases. Other studies have been carried out in tertiary hospitals that would miss milder cases of IgAV, for example (60 percent in our study only had outpatient visits) giving a skewed incidence. In KD 16 percent of all cases developed coronary artery aneurysm. This is a rather high incidence, considering that

coronary artery aneurysm was previously reported to be present in 15-25 percent of untreated cases (75). This surprising finding, could be due to the fact that pediatric cardiologists followed up all cases, and hence no cases were lost to follow-up. The cause of this high incidence of coronary artery aneurysms will be the focus of future studies. Although being a population-based, validated study, our study had some limitations. One being strictly following classification criteria thereby resulting in exclusion of cases in which IgAV suspected but not confirmed, as purpura alone without additional symptoms were not considered to be IgAV and biopsies are often not carried out in children with milder cases of IgAV. In summary, pediatric primary systemic vasculitis in Skåne has a good prognosis with no deaths occurring during the follow-up period, but some serious complications were seen such as coronary artery aneurysm in KD and end stage renal failure in MPA.

Conclusions

- Patients with vasculitis have elevated circulating endothelial and leukocyte microvesicles bearing the kinin B1 receptor.
- Endothelial microvesicles were C3 and C9-positive in vasculitis plasma.
- B1-receptors on microvesicles induce neutrophil chemotaxis, an affect regulated by the presence of C1 inhibitor and decreased by a B1 receptor antagonist.
- The release of complement-positive endothelial microvesicles was decreased by kinin receptor antagonists and C1 inhibitor.
- Functional B1-receptor can be transferred from one cell to another via microvesicles and thus perpetuate inflammation.
- The incidence of the full spectrum of primary systemic vasculitis in children was determined in a validated population-based study.
- The incidence of vasculitis is higher in children than adults.

Populärvetenskaplig sammanfattning

Vaskulit är en grupp sjukdomar som orsakar inflammation i och runt kärl, och kan medföra skada av de olika organen som drabbas i kroppen. Vaskuliter brukar delas in i grupper beroende på storleken av de drabbade kärlen. Olika vaskuliter har olika benägenheter för att drabba organ såsom njurar, lungor, hjärtats kärl samt huden. Vaskuliternas svårighetsgrad kan variera mellan lätta, snabbt övergående, besvär till svåra kroniska besvär som kräver avancerad sjukhusvård.

Mikrovesikler är små runda membranblåsor som är 0.1-1.0 μm i diameter och avges av de olika blodkropparna men även av kärlväggens celler. Dessa avges helt naturligt hos alla celler men i en högre grad då cellerna är stressade eller på annat sätt aktiverade. Dessa vesikler kan bära olika ämnen på ytan men även inuti som kan påverka andra celler både nära och på avstånd.

Kininsystemet är ett av de immunologiska försvarssystem som är aktiverat vid vaskulit. Det orsakar inflammation genom att avge ämnen som heter kininer. Kininer påverkar vävnader genom att binda till speciella kininreceptorer på cellytan och orsaka de klassiska symptomen vi ser vid vaskulit såsom rodnad, svullnad, värmeökning och smärta.

Komplementsystemet är en del av vårt immunförsvar och består av ett 30-tal proteiner vars huvudsyfte är att försvara oss mot främmande ämnen som har kommit in i vår kropp. Detta görs genom att olika delar av dessa komplementproteinerna sätter sig på cellytan av den oönskade cellen och orsakar att den förstörs genom att göra ett hål i den eller göra den mer aptitlig så att immunförsvarets celler äter upp den. Vissa komplementproteiner är också kapabla på att kalla på just immuncellerna.

Både kininsystemet och komplementsystemet är kraftfulla system som måste kontrolleras noggrant för att inte orsaka onödig vävnadsskada hos individen, detta görs genom att dessa system kan skilja på vad som är egen vävnad samt vad som inte är det. När något går fel i detta system orsakas vävnadsskada som leder till en sjukdomsgrupp som kallas inflammatoriska sjukdomar där vaskulitsjukdomarna ingår.

I artikel ett visade vi att mikrovesikler från endotelceller hade kininreceptorer på sin yta. Vidare visade vi att dessa mikrovesikler med kinin receptorer på sin yta

kunde attrahera en speciell sort vita blodkroppar som är involverad i skadorna som man ser vid vaskulit.

I artikel två visade vi att komplementsystemet också är aktiverat på mikrovesiklerna från kärl. Vi visade också att genom att blockera kinin systemet minskade effekten av komplementsystemet.

I artikel tre visade vi att kininreceptorerna också finns på mikrovesikler från de vita blodkropparna. Dessutom har vi visat att man kan flytta en receptor med hjälp av mikrovesikler från en cell till en annan cell och att receptorn i den nya celler börjar fungera.

I artikel fyra studerar vi utbredningen av vaskulitsjukdomar hos barn i Skåne mellan åren 2004 till 2014. Vi såg att majoriteten av barn under tio år drabbades av de ofta övergående vaskuliterna IgA vaskulit och Kawasaki sjukdom medan barnen över tio år oftare drabbades av de mer kroniska och allvarliga sorters vaskuliter.

Vi har i denna avhandling visat att mikrovesikler bär delar av både kininsystemet och komplementsystemet på sin yta. Kininreceptorerna på en mikrovesikel har förmågan att dra till sig vita blodkroppar och de kan även föra över kininreceptorn till en annan cell och där få den att fungera. Ämnen som hämmar både kininsystemet och komplementsystemet tror vi skulle kunna vara ämnen som skulle kunna användas i behandlingen av vaskulit. Vi har även fått en uppfattning om hur vanliga de olika sorters vaskulitsjukdomarna är bland barn i Skåne.

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