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Published in:
Hämostaseologie

DOI:
10.5482/HAMO-12-12-0025

2013

Complement activation in thrombotic microangiopathy

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Keywords
Complement

Summary
The endothelium lining the vascular lumen is continuously exposed to complement from the circulation. When erroneously activated on host cells, complement may generate a deleterious effect on the vascular wall leading to endothelial injury, exposure of the subendothelial matrix and platelet activation.
In this review the contribution of complement activation to formation and maintenance of the pathological lesion termed thrombotic microangiopathy (TMA) is discussed. TMA is defined by vessel wall thickening affecting mainly arterioles and capillaries, detachment of the endothelial cell from the basement membrane and intraluminal thrombosis resulting in occlusion of the vessel lumen. The TMA lesion occurs in haemolytic uraemic syndrome (HUS) and thrombotic thrombocytopenic purpura (TTP). HUS is further sub-classified as associated with Shiga toxin-producing *Escherichia coli* (STEC-HUS) or with complement dysregulation (atypical HUS) as well as other less common forms. The contribution of dysregulated complement activation to endothelial injury and platelet aggregation is reviewed as well as specific complement involvement in the development of HUS and TTP.

Schlüsselwörter
Komplement

Zusammenfassung
Das Endothel, die zum Lumen ausgerichtete Gefäßinnenwand, ist im ständigen Kontakt mit zirkulierenden Komplementfaktoren. Wenn Komplement irrtümlich auf Wirtszellen aktiviert wird, kann es eine zerstörerische Wirkung auf die Gefäßwand auslösen, die zu Endothelschädigung, Exposition der subendothelialen Matrix und Thrombozytenaktivierung führt.
In dieser Übersicht wird der Beitrag diskutiert, den die Komplementaktivierung zur Entstehung und Aufrechterhaltung der pathologischen Läsion, der so genannten thrombotischen Mikroangiopathie (TMA), leistet. Die TMA ist definiert durch eine vorwiegend arterioläre und kapilläre Gefäßwandverdickung, Ablösung der Endothelzellen von der Basalmembran und Thrombosierung des Lumens, die zum Gefäßverschluss führt. Die TMA-Läsion tritt bei urämisch-hämolytischen Syndrom (HUS) und thrombotisch-thrombozytischer Purpura (TTP) auf. HUS wird unterteilt in mit Shiga-Toxin-produzierende *Escherichia coli* assoziierte HUS (STEC-HUS) und mit Komplementregulationsstörung assoziierte HUS (atypische HUS) sowie andere seltene Formen. Wir betrachten den Anteil, den eine Fehlregulation der Komplementaktivierung bei der Endothelschädigung und der Thrombozytenaggregation hat, sowie die spezifische Beteiligung des Komplements bei der Entstehung von HUS und TTP.

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* Figure 1 and Table 1 are part of the PhD thesis of Dr. Ramesh Tati.

Komplementaktivierung bei thrombotischer Mikroangiopathie
Hämostaseologie 2013;33:-
DOI: 10.5482/HAMO-12-12-0025
received: December 22, 2012
accepted in revised form: January 23, 2013
prepublished online: February 15, 2013

The main functions of the complement system are disposal of foreign cells, such as bacterial pathogens, or altered host cells, such as apoptotic cells, by opsonization and cytolysis. Upon activation complement components are released with anaphylatoxic, anti-microbial and chemotactic properties. Complement activation is strictly controlled by numerous regulators to prevent undesirable activation on host cells. In spite of this massive regulation complement may become activated on host cells, with deleterious effects, in various infectious, genetic and autoimmune diseases. In these conditions complement activation either overwhelms the regulators, or the regulators are dysfunctional, thus allowing inappropriate activation to harm host cells. Two such conditions are reviewed here:

- haemolytic uraemic syndrome (HUS),
- thrombotic thrombocytopenic purpura (TTP).

Activation of the complement cascade in HUS was demonstrated in the 1970–80s (1). HUS is a syndrome associated with

- non-immune haemolytic anaemia,
- thrombocytopenia,
- acute renal failure,
- thrombotic microangiopathy (TMA) lesions in the renal glomeruli.

The quest to understand how complement activation was involved in this syndrome led to the finding that some patients had mutations in the main fluid phase complement regulator factor H (2, 3).

The form of disease primarily associated with complement dysregulation is known as atypical HUS (aHUS). Since the initial finding many mutations in complement regulators and factors have been described, as well as autoantibodies to factor H, and various mechanisms by which complement activation can cause and/or con-

tribute to the development of aHUS have been described.

There is evidence for complement activation even in the other main form of HUS, caused by Shiga toxin-producing *Escherichia coli* (STEC) infection. In this subtype of HUS complement activation appears to be secondary to the primary toxin-induced endothelial cell injury and platelet activation. Nonetheless, complement activation may potentially contribute to disease progression.

Likewise, complement is activated during TTP. TTP similarly manifests as:

- non-immune haemolytic anaemia,
- thrombocytopenia,
- renal manifestations,
- neurological manifestations,
- fever.

TTP is associated with deficiency or dysfunction of the von Willebrand factor (VWF)-cleaving protease ADAMTS13 due to mutations or auto-antibodies. The pathological lesion in HUS and TTP, termed TMA, is very similar. In this review the mechanisms leading to the development of HUS and TTP will be described with reference to the contribution of complement activation.

The complement system

The complement system plays an important role in the host innate immune response. Activation of complement under physiological conditions will result in removal of foreign or unwanted cells and cellular debris, such as bacteria or apoptotic cells, and disposal of immune complexes. The complement system is comprised of three activation pathways defined as the classical, lectin and alternative pathways (▶Fig. 1), varying with regard to the activating surface or molecule, and a common terminal pathway.

- The classical pathway can be activated by immune complexes, apoptotic cells, C-reactive protein as well as other non-immune activators (4).
- The lectin pathway is activated by the binding of ficolins and mannose-binding lectins (MBL) to the surface of pathogens and dying cells (5) and to polymeric IgA (6). The classical and lectin pathways converge at the formation of the C3 convertase C4b2a, capable of cleaving C3 into C3a and C3b. Binding of a C3b molecule to the C3 convertase forms the C5 convertase cleaving C5 into C5a and C5b.
- The alternative pathway is activated on the surface of foreign or altered host cells.

The alternative pathway undergoes constant low-grade activation in the circulation, so called tick-over by low-rate hydrolysis of C3 into $C3(H_2O)$. In the presence of Mg^{2+} $C3(H_2O)$ interacts with factor B, cleaved by factor D into Bb, thus forming $C3(H_2O)Bb$. This initial form of the C3 convertase cleaves C3 into C3a and C3b. Cleavage into C3b exposes cell-binding sites allowing covalent binding to cell surfaces (7). C3b binding to factor B will form the C3 convertase (C3bBb) and promote more cleavage of C3. The convertase is stabilized by binding to properdin. The

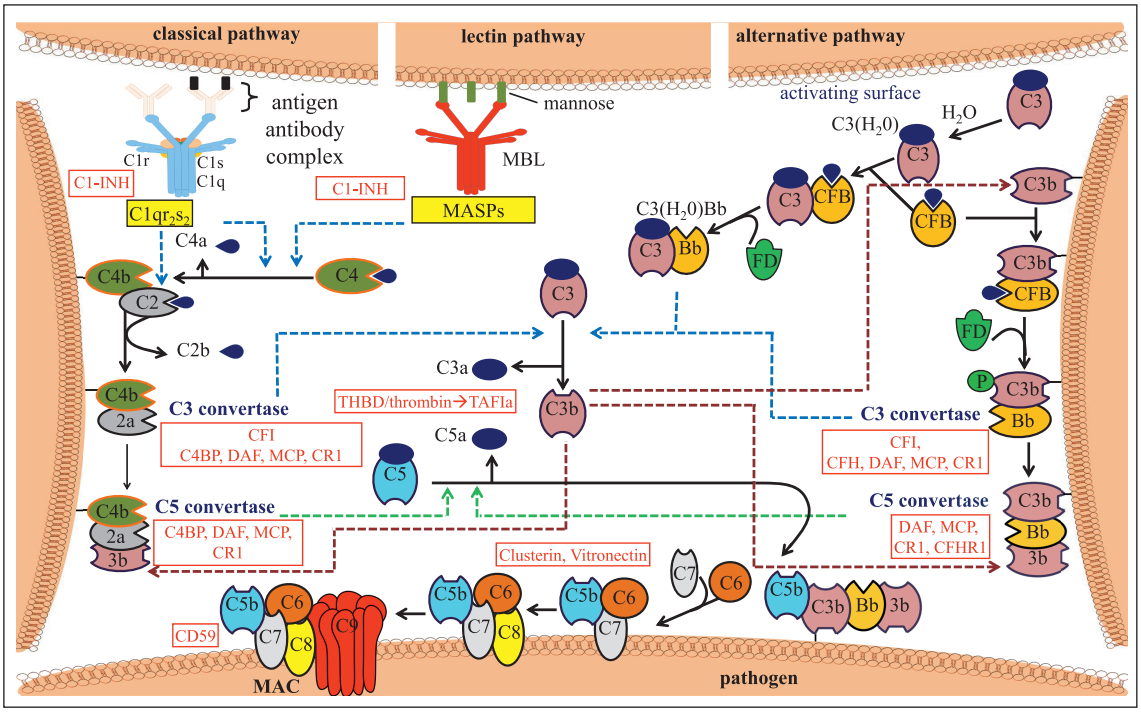

Fig. 1 The classical, lectin and alternative pathways of complement activation. Complement activation occurs on the surface of foreign cells and altered host cells. Regulators are marked in red rectangles. CINH: C1 inhibitor; MBL: mannose-binding lectin; MASP: MBL-associated serine protease; CFB: factor B; FD: factor D; P: properdin; THBD: thrombomodulin (in complex with thrombin); TAFIa: thrombin-activatable fibrinolysis inhibitor; CFI: factor I; CFH: factor H; C4BP: C4 binding protein; DAF: decay accelerating factor; MCP: membrane cofactor protein; CR1: complement receptor 1, MAC: membrane attack complex

Tab. 1 Inhibitors of the complement system

complement		fluid-phase or membrane-bound	mechanism of inhibition	reference
regulator	pathway			
factor H	alternative	fluid phase	cofactor for factor I in cleavage of C3b; accelerates decay of the C3 convertase and preferentially recognizes host cells	(95)
factor H-related protein 1	terminal	fluid phase	inhibits the C5 convertase	(96)
factor I	alternative and classical	fluid phase	cleaves C3b or C4b to the inactive forms in presence of cofactors factor H, C4 binding protein, MCP or CR1	(97)
CD46 (MCP)	alternative, classical and terminal	membrane-bound	cofactor for factor I in cleavage of C3b	(98), (99)
thrombomodulin	alternative, classical and terminal	membrane-bound	enhances factor I-mediated inactivation of C3b in the presence of factor H; generation of TAFIa[1], which inactivates C3a and C5a	(100)
C1-inhibitor	classical and lectin	fluid phase	binds to C1r and C1s removing them from C1q, or binds to MASPs	(101)
C4 binding protein	classical, lectin and terminal	fluid phase	decay accelerating activity (C3 and C5 convertases) and cofactor for factor I	(102)
complement receptor 1 (CR1, CD35)	alternative, classical and terminal	membrane-bound	decay accelerating activity and cofactor for factor I	(103)
CD55 (DAF)	alternative, classical and terminal	membrane-bound	inhibits assembly and promotes decay of C3 and C5 convertases	(104)
clusterin	terminal	fluid phase	inhibits MAC formation	(59)
vitronectin	terminal	fluid phase	inhibits MAC formation	(105)
CD59	terminal	membrane-bound	inhibits MAC formation	(106)
carboxypeptidase N	alternative and terminal	fluid phase	cleavage and partial inactivation of C3a and C5a	(107)

MCP: membrane cofactor protein; DAF: decay accelerating factor; MAC: membrane attack complex; MASP: mannose-binding lectin-associated serine protease; TAFI: thrombin-activatable fibrinolysis inhibitor; [1]Thrombomodulin is a cofactor for thrombin-mediated activation of TAFI to TAFIa. TAFIa is a plasma procarboxypeptidase B that inactivates C3a and C5a by removal of an arginine.

reaction is amplified by further binding of factor B to C3b.

> Thus, after C3b is formed, activation of all pathways of complement can be enhanced through the amplification loop of the alternative pathway.

Binding of a molecule of C3b to the C3 convertase (C3bBb) will also form the C5 convertase thus cleaving C5. C5b binds to C6 and C7 and attaches to the cell wall (8). This is followed by binding of C8 and numerous C9 molecules to form the C5b-9 lytic complex, also known as the membrane attack complex (MAC). The C9 molecules surround a pore in the cell membrane thus causing cell lysis when sufficient C5b-9 structures are formed (▶Fig. 1).

Complement possesses other potent effects, in addition to cell lysis. Peptides generated during activation, i.e. C3a, C4a and C5a, possess antimicrobial and/or anaphylatoxic and chemotactic properties. Furthermore, C3b, iC3b and C4b binding to cells function as opsonins allowing these cells to undergo phagocytosis.

> It is thus evident that complement is a very powerful system that, upon full-blown activation, removes foreign and apoptotic cells by induction of
> - inflammation,
> - phagocytosis and
> - cell lysis.

Left unchecked this system could be damaging to healthy host cells, especially as the alternative pathway undergoes constant low-rate activation. To prevent this, multiple complement regulators function on the cell surface and in the fluid phase.

Regulation

Activation of the complement system is tightly regulated. The regulators inhibiting the various pathways are shown (▶Fig. 1) and listed (▶Tab. 1). The main fluid phase regulator functional in the alternative pathway is factor H possessing three regulatory mechanisms:

- cofactor to factor I in cleavage of C3 to iC3b,
- prevention of formation and accelerated decay of the convertase (C3bBb),
- host recognition, i.e. discrimination between host and foreign cell surfaces based on the presence of polyanions such as glycosaminoglycans and sialic acids on host cell surfaces (9).

Factor H will thereby preferentially bind to non-activating surfaces, such as host cells, and thus complement activation via the

alternative pathway will be prevented. Foreign cells, lacking polyanions on their surface, will be susceptible to the destructive effects of complement as they do not bind factor H.

Complement activation may play an important role in the evolvement of thrombotic lesions.

> Overwhelming or dysregulated complement activation may enhance and maintain
> - endothelial injury,
> - neutrophil chemotaxis and
> - platelet activation.

Complement activation in thrombotic microangiopathy (TMA) will thus promote thrombosis and sustain formation of the lesion.

Thrombotic microangiopathy

TMA defines a pathological lesion consisting of

- vessel wall thickening, primarily affecting arterioles and capillaries, with swelling or detachment of the endothelial cell from the basement membrane,
- accumulation of hyaline amorphous material in the subendothelial space,
- intraluminal thrombosis and partial or complete occlusion of the vessel lumen (10).

Endothelial cell injury in the microvasculature and platelet activation are central to the pathogenesis of TMA. Several clinical syndromes are associated with the TMA lesion, predominantly affecting the kidney. These include HUS and TTP.

- HUS is classified based on etiology into various subtypes (11) of which two major subtypes are
  - STEC-HUS, also called typical HUS (12, 13) and
  - aHUS associated with complement dysregulation secondary to mutations or autoantibodies as described (14).
- TTP is associated with deficiency or dysfunction of ADAMTS13, the von Willebrand factor (VWF)-cleaving protease, due to a genetic or acquired disorder (15, 16).

Complement activation in TMA

Complement activation may occur as the primary event leading to endothelial perturbation and platelet activation (in aHUS) or as a secondary event associated with thrombus formation after toxin-mediated injury (STEC-HUS) or due to ADAMTS13-deficiency (TTP).

In aHUS endothelial damage and platelet activation are primarily associated with dysregulated complement activation allowing the harmful effects of complement to occur on host cells (17–19). Complement activation during STEC-HUS is principally associated with endothelial cell injury induced by bacterial virulence factors Shiga toxin and lipopolysaccharide and enhanced by the host response (20, 21). Endothelial disruption may, in turn, lead to platelet activation, or platelets may be directly activated by the toxin (22).

In TTP ultra-large forms of VWF circulate due to deficiency or dysfunction of ADAMTS13. Complement activation can occur as a secondary phenomenon related to endothelial injury and thrombus formation (23). Thus, although the TMA lesion is similar in these three conditions (STEC-HUS, aHUS and TTP), the triggering event leading to lesion formation and complement activation differs.

Complement activation on endothelial cells and platelets and its role in TMA

Intact endothelium allows normal blood flow and homeostasis. Endothelial cells separate the vascular space from the extravascular tissue. Endothelial cells express heparan sulphate and release thrombomodulin and tissue factor inhibitor thus preventing clot formation (24, 25). Endothelial activation and/or injury promotes

- leukocyte rolling and migration,
- platelet aggregation,
- loss of thromboresistance.

Stimulation of endothelial cells with IL-1, mimicking inflammation, generates a procoagulant state characterized by increased synthesis of tissue factor and plasminogen activator inhibitor and decreased thrombomodulin activity (26).

During the inflammatory process the endothelium is targeted by the complement system. Tissue factor expression and release by endothelial cells is regulated by complement and mediated by IL-1α (27). Endothelial cells express and secrete complement factors and regulators as well as receptors (28, 29). Complement activation on the endothelium will lead to cell activation, with subsequent

- expression of adhesion molecules (30, 31),
- release of cytokines and chemokines (32–35),
- release of cell-derived microparticles (36),
- MAC formation and ultimately cytolysis.

C3a and C5a released during activation of the complement cascade bind to endothelial cells and induce cytokine release (37, 38). C5a and IL-8 (upregulated in the endothelium) are chemotactic for polymorphonuclear leukocytes. Vascular permeability is increased by activation of the kinin and complement systems on the endothelium (39) attributed in part to C3a and C5a (25) as well as the terminal complement complex, C5b-9, which also induces increased endothelial permeability (40) allowing leukocyte migration into the extravascular space (41). Thus, complement activation on the endothelium during the inflammatory process promotes cell injury and enhances vascular permeability as well as leukocyte recruitment to the vascular wall and underlying tissues.

Endothelial cell activation and damage has been documented in TMA as well as the interaction of endothelial cells with complement.

> Shiga toxin has a direct cytotoxic effect on endothelial cells (21).

The toxin was also shown to induce the expression of P selectin on microvascular endothelial cells thus binding C3, activating the alternative pathway and reducing thrombomodulin. This process promoted thrombus formation under perfusion con-

ditions in vitro, and in a mouse model in vivo (42).

Atypical HUS mutations affecting complement factors C3 and factor B (43, 44) as well as the main fluid-phase regulator factor H (17, 18) allows unrestricted complement activation to occur on endothelial cells due to hyperfunction of C3 or factor B or decreased regulatory capacity of factor H. Glomerular endothelial cells stimulated with pro-inflammatory mediators (TNFα and IFN α) and exposed to aHUS serum with hyperfunctional C3 expressed tissue factor and thus a procoagulant phenotype (43).

Serum from TTP patients with ADAMTS13 deficiency induced C3 deposition and MAC formation on microvascular endothelial cells and promoted neutrophil-mediated endothelial cytotoxicity. These effects were abrogated by complement inhibition (23). Taken together, there is in vitro and in vivo evidence for complement activation on endothelial cells in subtypes of TMA, which may initiate and sustain the endothelial cell injury.

Platelet activation during TMA may occur as a result of direct activation of platelets, or secondary to endothelial injury.

Platelets roll on the surface of activated endothelial cells and on VWF and collagen in the subendothelium exposed during perturbation of the endothelial lining (45). Platelets can bind C3 and C4, and express complement receptors P selectin and CR2, capable of binding C3b and C3d, respectively (46–50), as well as CR4 (51), CR3 (52) and the C1q receptor (53). Binding of complement components from the alternative, classical and terminal pathway can activate platelets (54–57). The converse has also been reported, the complement system can become sequentially activated on platelets (46). To prevent excessive complement activation platelets express and bind complement regulators (58–64).

Thrombocytopenia occurring during TMA is a subsequence of platelet consumption in the microangiopathic lesion.

In STEC-HUS bacterial virulence factors Shiga toxin and lipopolysaccharide circulate bound to platelets as well as to other blood cells (22, 65). Platelets are activated after binding Shiga toxin and/or lipopolysaccharide. Our group has demonstrated that activated plaletets form aggregates with leukocytes during STEC-HUS. Platelet- and monocyte-derived microparticles are thus released. Platelet-leukocyte aggregates as well as cell-derived microparticles were shown to be coated with complement C3 and C9 (66) suggesting complement activation on these blood cells. In aHUS platelet activation may occur as a result of complement-mediated endothelial injury (17) or due to complement activation on platelets as a result of dysfunctional inhibition (19) or hyperfunctional C3 (67). Atypical HUS-associated factor H mutations were shown to allow complement activation to occur on platelets thus leading to their activation (19). In TTP VWF-platelet strings are formed under perfusion in vitro due to lack of ADAMTS13 (68). Platelet and endothelial microparticles are released into the circulation (69, 70). Although complement activation has not been demonstrated on the VWF-platelet strings, or the microparticles, it is plausible to assume that complement activation occurs in the TTP setting of platelet activation on the endothelial surface. Thus in the various sub-forms of TMA complement activation can occur on the endothelium and platelet thrombi, and may thereby augment the process.

Complement activation in the renal TMA lesion

The TMA lesion affects renal glomerular capillaries. The glomerular vasculature and basement membrane are particularly vulnerable to complement activation. This may be due to the fact that the glomerular basement membrane is devoid of inherent complement regulators and thus dependent on soluble regulators (71, 72). Furthermore, glomerular endothelial cells are exposed to shear stress, which may have, together with nitric oxide, a role in regulating the permeability of the glomerular capillary wall (73). Shear stress affects the endothelium and platelets and unfolds VWF exposing the scissile cleavage site. ADAMTS13 will normally dock onto VWF and cleave it into smaller multimers (74).

The glomerular endothelium may be particularly susceptible to injury in the TMA setting.

For example, renal glomerular endothelial cells are more susceptible to the cytotoxic effect of Shiga toxin due to increased basal expression of the Gb3 toxin receptor (75). Likewise, lack of functional ADAMTS13 at sites exposed to higher shear stress may promote thrombus formation when the endothelial layer is perturbed. Thus, the glomerular vasculature may be more prone to develop the TMA lesion and complement deposition on thrombi and injured endothelial cells.

Complement activation in STEC-HUS

There is clear evidence of systemic complement activation during STEC-HUS. Patients may exhibit low levels of serum C3 (1, 76) and elevated levels of complement products C3a, factor Bb and soluble C5b-9 (66, 77), as well as complement C3 and C9 on platelet-leukocyte aggregates and cell-derived microparticles, as described. In vitro studies have shown that Shiga toxin 2 can activate the alternative pathway of complement in serum and bind to factor H at host surface recognition sites thus inhibiting its regulatory effect (78). Furthermore, in vitro experiments showed that Shiga toxin (both 1 and 2) could induce the formation of platelet-leukocyte aggregates and the release of blood cell-derived microparticles, both coated with C3 and C9, an effect enhanced in the presence of *E. coli* O157 lipopolysaccharide (66).

Further support for the role of complement in STEC-HUS came from a report describing the successful use of eculizumab, an anti-C5 antibody approved for the treatment of aHUS, in three children with STEC-HUS and neurological complications (79). Based on this report approximately 200 STEC-HUS patients were treated with eculizumab during the large *E. coli* O104:H4 outbreak in Germany in 2011. Reports from this outbreak did not, however, demonstrate a beneficial effect of this treatment (80–82). This may be due to

administration late in the course of disease. Thus, randomized clinical trials are required to determine if certain patients could benefit from this treatment and at which time point eculizumab should be administered.

Complement activation in aHUS

Atypical HUS (aHUS) has been associated with mutations in complement regulators factor H, factor I, membrane cofactor protein (MCP, CD46), clusterin, thrombomodulin, or complement factors C3 or factor B (14, 83, 84). Mutations are usually heterozygous and certain patients have mutations in more than one factor. Patients may have auto-antibodies to factor H, which have been associated with deletions or rearrangements in factor H and factor H-related proteins (CFHRs) resulting in hybrid genes. This form of HUS has been termed DEAP-HUS (DEficient for CFHR proteins and factor H Autoantibody Positive) (85). As described, the mutations have been shown to trigger complement activation thus leading to deposition on renal endothelial cells (43, 44) and patient platelets (19, 67, 84). Patients may have C3 deposition in glomeruli (►Fig. 2) (86) suggesting that complement activation occurs systemically in the circulation and locally in the renal vasculature.

Patients with aHUS have benefited from treatment with eculizumab, monoclonal antibody targeting human C5, during relapses and prophylactically (87). This treatment has also proved to be efficient in preventing and treating recurrences of aHUS after renal transplantation (88). The efficacy of eculizumab in treating aHUS proves unequivocally the role of complement dysregulation in inducing this condition.

Complement activation in TTP

In TTP the kidney is affected with typical TMA lesions. Endothelial and platelet involvement occurs due to the release of ultra-large VWF multimers capable of binding platelets and consequently forming VWF-platelet aggregates which cannot be cleaved due to dysfunctional/deficient ADAMTS13, thus leading to thrombosis (89). Complement does not have an initiating role in this process but there is considerable evidence that complement activation occurs. Patients with TTP have elevated circulating C3a and soluble C5b-9 during acute episodes (90) or low levels of C3 (91). Renal and myocardial tissue from TTP patients autopsied in the 70s showed complement deposition (92, 93). More recently complement C3d, C4d and C5b-9 were observed in the skin of a patient with acquired TTP (94). The patient described in this case report was successfully treated with eculizumab, also suggesting a role for complement activation in TTP in this case.

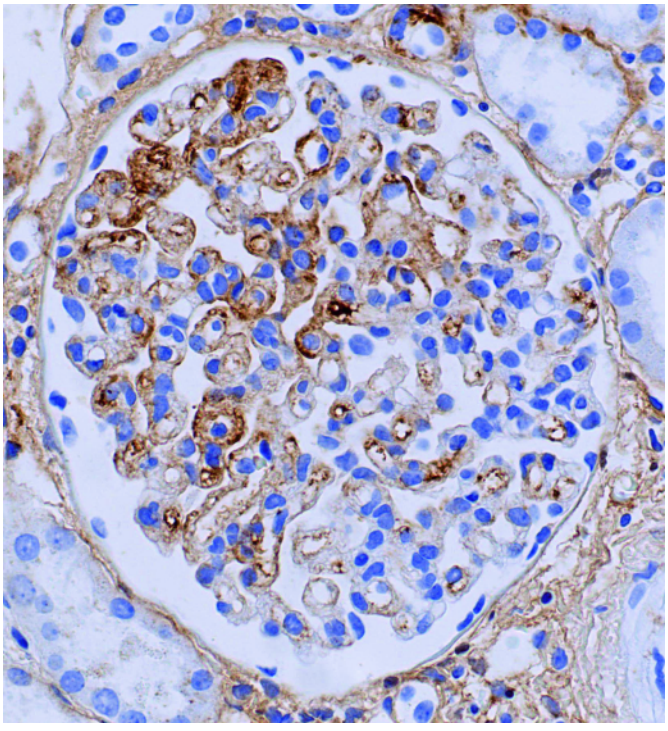

Fig. 2
Renal biopsy from a child with aHUS and mutations in C3 and MCP (86). C3 deposition (brown labeling) was demonstrated in capillaries and thrombi in glomeruli. The photomicrograph is available by courtesy of Dr. Sabine Leh, Department of Pathology, Haukeland University Hospital, Bergen, Norway

Conclusions

Although the TMA lesion observed in HUS and TTP is very similar, the primary cause of disease differs.

- In STEC-HUS the disease is initiated by gastrointestinal bacterial infection.
- In aHUS disease is initiated by complement dysregulation.
- In TTP the disease process is triggered by microvascular thrombosis induced by ADAMTS13 deficiency.

Thus, complement involvement is not a primary phenomenon in all cases. All the same, complement activation may contribute to sustaining the pathological lesion by enhancing endothelial damage, platelet activation and deposition of thrombi on the subendothelial matrix and basement membrane.

The beneficial effect of eculizumab in the treatment of aHUS suggests that targeting complement involvement in this subtype of HUS is essential for hindering disease development, whereas in the other forms of TMA alternative therapies should be designed in order to target the initial triggers of disease.

Financial support

Diana Karpman is supported by grants from The Swedish Research Council (K2013–64X-14008–13–5), The Torsten Söderberg Foundation, Crown Princess Lovisa's Society for Child Care, and The Konung Gustaf V:s 80-årsfond.

Conflict of interest

Diana Karpman was the national coordinator in Sweden of the multi-center inter-

national trial of eculizumab (Alexion Pharmaceuticals) in patients with atypical haemolytic uraemic syndrome (2010).

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