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Complement-mediated injury and protection of endothelium: Lessons from atypical haemolytic uraemic syndrome

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Abstract

The complement system provides a vital defence against invading pathogens. As an intrinsic system it is always 'on', in a state of constant, low level activation. This activation is principally mediated through the deposition of C3b on to pathogenic surfaces and host tissues. C3b is generated by spontaneous 'tick over' and formal activation of the alternative pathway, and by activation of the classical and lectin pathways. If the deposited C3b is not appropriately regulated, there is progression to terminal pathway complement activation via the C5 convertases, generating the potent anaphylotoxin C5a and the membrane attack complex C5b-9. Unsurprisingly, these highly active components have the potential to cause injury to bystander host tissue, including the vascular endothelium. As such, complement activation on endothelium is normally tightly controlled by a large number of fluid-phase and membrane bound inhibitors, in an attempt to ensure that propagation of complement activation is appropriately restricted to invading pathogens and altered 'self', e.g. apoptotic and necrotic cells.

The kidney is increasingly recognised as a site at particular risk from complement-mediated endothelial injury. Both genetic and acquired defects which impact on complement regulation predispose to this susceptibility. The thrombotic microangiopathy, haemolytic uraemic syndrome (HUS), will be used to illustrate the mechanisms by which the endothelial cell injury occurs. Finally, the underlying rationale for current and future potential therapeutic interventions in HUS and also the opportunities for enhancing endothelial defence to prevent relapsing disease through increased complement cytoprotective strategies will be summarised.

Keywords

Complement; Endothelium; Microvascular injury; Haemolytic uraemic syndrome; Eculizumab; Cytoprotection

The endothelium

Endothelial cells line the interior surface of all blood vessels in the body, occupying a strategic location between the blood vessel walls and the blood stream. The endothelium is able to detect and respond to mechanical stimuli, such as pressure and shear stress, and to hormonal stimuli such as vasoactive mediators. Endothelial cells have important roles in

health and disease. Endothelial cell functions include the release of agents which regulate vasomotor function, trigger inflammatory processes and affect haemostasis in addition to contributions to angiogenesis and vascular permeability (Endemann and Schiffrin 2004). Normal vascular endothelium expresses an anti-inflammatory phenotype and does not support complement activation (Hindmarsh and Marks 1998).

The morphological and biological responses of endothelial cells vary depending on the vascular system from which they originate, a concept termed *endothelial cell heterogeneity* (Langenkamp and Molema 2009). Variation in endothelial structure and function is seen at all levels of the vascular tree. As yet, little is known about the differences in complement regulation in individual vascular beds.

The kidney glomerular endothelium is a highly specialised vascular bed; the endothelial cell layer is attached to the glomerular basement membrane which is covered at the urinary side by visceral epithelial cells, the podocytes. A characteristic feature of glomerular endothelial cells is the presence of numerous nondiaphragmed fenestrae, which in humans are about 100 nm in diameter, and whose maintenance is vascular endothelial growth factor (VEGF)-165 dependent (Ballermann 2005). The kidney is increasingly recognised as a site at particular risk of complement-mediated endothelial injury. Using atypical haemolytic uraemic syndrome (aHUS) as the example, the mechanisms of complement-mediated injury relevant to endothelium will be considered in greater detail in this review, as they have relevance for our understanding of the choice and mechanism of action of potential therapies.

Haemolytic uraemic syndrome (HUS)

HUS is a type of thrombotic microangiopathy and is characterised by the formation of fibrin-platelet clots in arterial microcirculations. There is an apparent predilection for the renal glomerular capillaries and arterioles, resulting in kidney failure, although the simultaneous involvement of other microvascular beds in the heart, brain and pancreas to a greater or lesser extent, is recognised and reported in all subtypes of HUS. Endothelial cell injury (swelling, detachment, and endotheliosis) is a pathological feature common to all subtypes of haemolytic uraemic syndrome. We are increasingly able to sub-classify HUS as the specific aetiological triggers are dissected.

Triggers with a direct ability to cause endothelial injury include verotoxin (diarrhoeal)-associated HUS (Zoja et al. 2010), anti-endothelial cell antibody (AECA) associated-HUS, *Streptococcus pneumoniae*-associated HUS (Copelovitch and Kaplan 2008), anti-VEGF-associated HUS (Eremina et al. 2008) and atypical HUS (aHUS), in which a significant proportion of patients have been found to carry a genetically inherited predisposing factor in one or more complement components with activity in the alternative pathway (AP) (Kavanagh and Goodship 2010). To date, mutations in the AP regulators Factor H (FH), membrane cofactor protein (CD46, MCP), Factor I (FI), the terminal pathway regulator clusterin, and the AP activators C3 and Factor B have been reported. Risk-associated haplotypes in the FH and FH-related genes and the CD46 promoter, autoantibodies to FH, and complex genetic deletions/rearrangements involving FH and the FH-related genes, forming hybrid genes confirm the importance of these regulatory AP proteins. Furthermore,

recent reports of a HUS-associated polymorphism in the predominantly classical pathway regulator, C4 binding protein (C4bp) (Blom et al. 2008) and mutations and polymorphisms in the anti-coagulant protein thrombomodulin (Delvaeye et al. 2009) have uncovered previously unsuspected AP regulatory roles and highlighted the multi-faceted protective strategies against complement-mediated injury employed by host cells, including endothelium.

Whilst the presence of endothelial cell injury is a key component of all subtypes of aHUS, the unifying mechanisms linking all the recognised aetiological triggers and complement proteins mutations is poorly understood and an active focus for many researchers in the field. The diverse nature of the triggers implies that there may be more than one mechanism. Whilst the alternative complement pathway activation and regulation is fundamentally involved, it is likely that both the classical pathway and the mannose-binding lectin/ mannose-binding lectin associated serine protease (MBL/MASP) pathway of complement activation also contribute in specific settings, for example, the vascular injury and renal thrombotic microangiopathies seen in association with the connective tissue disorders, systemic lupus erythematosis (SLE), antiphospholipid syndrome (APLS), anti-endothelial cell antibodies (AECA), humeral rejection of renal allografts, viral and bacterial infections. This is an under-researched area and highlights the possibility that new therapeutic options specifically regulating the complement pathway(s) will also have a role to play in the management of these conditions.

The complement cascade

A principal biological function of the complement system is the rapid recognition and elimination of pathogens. Key mechanisms include opsonisation by C3b, activation of the inflammatory cascade by the generation of the anaphylotoxins C3a and C5a and lysis by the membrane attack complex (MAC; C5b-9). The complement cascade is activated by the classical pathway (CP) (Kojouharova et al. 2010), the alternative pathway (Harboe and Mollnes, 2008 and Wallis et al., 2010) or the MBL/MASP pathway (Wallis et al. 2010). The CP is initiated when IgM or IgG antigen/antibody complexes, C-reactive protein (CRP), necrotic or apoptotic cells bind to and activate C1q. This is followed by C4 and C2 dependent cleavage of C3 (by C3 convertase C4b2a) and ultimately the cleavage of C5 by formation of a C5 convertase (C4b2a3b) (Kojouharova et al., 2010 and Wallis et al., 2010). The AP is activated by the presence of bacterial lipopolysaccharide (LPS), proteins and lipids, by properdin (Spitzer et al. 2007) and by the spontaneous generation of C3b following hydrolysis of the internal C3 thioester bond, a process known as 'tick-over'. In this pathway, C3 binds to Factor B and is cleaved by Factor D, to form the alternative C3 convertase (C3(H₂O)Bb). Properdin acts here as an amplifier and positive regulator, stabilising this complex, and enabling further C3b to bind, forming the alternative C5 convertase (C3bBb3b) (Harboe and Mollnes, 2008 and Spitzer et al., 2007). The MBL/MASP pathway is initiated by the binding of MBLs or ficolins (which are structurally homologous to C1q) to mannose and glucosamine residues on bacterial cell walls. This triggers activation of MBL-associated proteases (MASP1-3), resulting in the cleavage of C4 and C2 and subsequent activation of C3, as for the classical pathway (Wallis et al. 2010). All pathways therefore utilise C3 and cleave C5 to form C5a and C5b.

Endothelial protection against complement-mediated injury

The complement system is tightly controlled by natural fluid phase and membrane bound inhibitors, to restrict propagation to invading pathogens or altered self cells.

Membrane bound complement regulators expressed on endothelium

The membrane-bound complement regulatory proteins decay accelerating factor (DAF, CD55), membrane cofactor protein (MCP, CD46) and CD59 are constitutively expressed on unstimulated macrovascular human umbilical vein endothelial cells (HUVEC), conditionally immortalised human (dermal) microvascular endothelial cells (HMEC-1) (Mason et al. 1999), conditionally immortalised glomerular endothelial cells ciGEC (Roumenina et al. 2009) and primary GEC (Richards et al. unpublished data). Complement receptor 1 (CR1, CD35), which has both decay accelerating activity and cofactor activity for Factor I, is not expressed on the surface of quiescent conditionally immortalised GEC (Roumenina et al. 2009). The membrane-bound regulators provide protection of the endothelium against the risk of complement-mediated injury caused by the constant low level activity ('tickover') of the AP which constantly deposits C3b onto host as well as pathogenic surfaces (Brooimans et al., 1992a, Brooimans et al., 1992b, Liszewski et al., 2008 and Lublin and Atkinson, 1989).

The mechanisms by which each of these factors control complement are distinct, reinforcing the multi-level control systems in place to protect host cells from bystander injury. CD55 prevents the formation, and accelerates the decay of C3 and C5 convertases (Lublin and Atkinson 1989) thereby reducing the deposition of C3b and C5b-9 and the generation of the anaphylotoxins C3a and C5a. CD46 binds to C3b and C4b and acts as a cofactor for their breakdown by Factor I to iC3b and C4c and C4d respectively (Liszewski et al. 1991). CD46 mutations are reported in aHUS and confer reduced resistance to host cell injury through this impaired complement regulation (Richards et al. 2007). CD59 is an 18-20 kDa membrane glycoprotein anchored to the membrane by glycosyl-phosphatidylinositol (GPI). CD59 inhibits the membrane attack complex (MAC, C5b-9) by incorporating into the forming complex at the C5b-8 stage and blocking the uptake and insertion of C9 molecules (Davies et al., 1989 and Okada et al., 1989).

Soluble complement regulators protecting endothelium

The soluble complement regulators of relevance to aHUS due to their identified roles in endothelial cell protection are FH and FI. FH is a 155 kDa glycoprotein which prevents the spontaneous and non-specific activation of the AP in plasma and on cell surfaces. It has decay accelerating activity for the AP convertases and is a cofactor for the FI-mediated cleavage of C3b. It is a mediator of 'self' vs. 'non-self' recognition, mediated through the binding of the carboxy-terminus of FH to polyanionic markers, primarily sialic acid and sulphated polysaccharides expressed on endothelial cell surfaces (Morgan et al. 2011). Mutations, hybrid genes and autoantibodies to FH are the commonest genetic cause of aHUS (>30%). Mutations in the C terminus of FH permit complement activation on the surface of human endothelial cells (Ferreira et al., 2009 and Manuelian et al., 2003). FI is a serine protease which, in the presence of one of its cofactors (FH, CD46 or CD35), cleaves

C3b to iC3b and C4 to C4c and C4d. These breakdown products cannot participate in further propagation of the complement cascades. FI mutations also predispose to the development of aHUS (Fremeaux-Bacchi et al., 2004 and Kavanagh et al., 2008).

Less commonly considered but potentially important regulators with respect to the pathogenesis of aHUS are carboxypeptidase N (CPN), clusterin and vitronectin. Carboxypeptidase N is an important biological enzyme with many functions. These include the degradation of the anaphylotoxins C3a and C5a to C3adesArg and C5adesArg respectively by removal of the carboxy-terminal arginine (Arg) residue. CPN can also be associated with endothelial cell membranes (as carboxypeptidase M, CPM) (Skidgel and Erdos 2007). Clusterin (apolipoprotein J) is a lipoprotein expressed in most human tissues which acts as a multifunctional cytoprotective molecule that inhibits complement-mediated cytotoxicity by binding to C5b-6 and preventing formation of MAC (Jenne and Tschopp 1992). A mutation in clusterin (Q433P), which showed limited binding to C5b-7 by Biacore, has been associated with recurrent aHUS, in a pedigree who also carry a CD46 mutation (Stahl et al. 2009). Vitronectin (protein S) is a multifunctional human glycoprotein which assists in the regulation of MAC formation by interacting with the C5b-7 complex preventing its insertion into cell membranes. It also limits on-going membrane-associated pore formation by inhibiting C9 polymerization (Milis et al. 1993). The existence of membrane bound (CD59) and two soluble regulators (vitronectin and clusterin) of C5b-9 formation is striking and suggests a very important need for tight regulation of MAC on host tissues.

The specific contributions of the different *soluble* and *membrane* bound complement regulatory molecules to the protection of specific endothelial beds during inflammation remains to be determined (Liszewski et al., 1996 and Liszewski et al., 2008). Evidence to date suggests that the relative hierarchy in terms of the physiological importance of complement regulation for the membrane regulators is CD59 > CD55 > CD46 (Brooimans et al., 1992a and Brooimans et al., 1992b) whilst other studies suggest that CD55 is particularly important in providing additional EC protection during subacute and chronic inflammation (Ali et al., 2009, Kinderlerer et al., 2008, Kinderlerer et al., 2009, Mason et al., 2002a, Mason et al., 2002b, Mason et al., 2001 and Mason et al., 2004).

Clearance of complement split products and MAC

Processes such as antibody-mediated rejection are characterised by the deposition of complement C3 and C4 split products on endothelial cells. C3 split products are critical mechanistically because once C3b is covalently bound to tissues it can initiate an amplification loop through Factor B. On host cells, this process is controlled by regulatory proteins although the covalently bound split products, iC3b and C3d, still act as ligands for complement receptors on leucocytes, leading to neutrophil, monocyte and macrophage infiltration. Haun et al. demonstrated that over half the deposited C3b/iC3b and a third of the C3d deposited following exposure of cultured HUVEC to IgM to β_2 -microglobulin was cleared from the cell surface during a 3-7 h incubation period with human serum. Neither hypoxia-reperfusion nor IL-1 β increased amounts of C3 split product deposition and had little effect on clearance rates (Haun et al. 2005). The mechanism was not identified but may involve shedding (with/without antibody) or exocytosis/endocytosis (as described for MAC

on neutrophils) (Morgan et al. 1987). These processes represent potentially important additional mechanisms for limiting host endothelial cell injury by complement.

Endothelial cell activation and injury in HUS

Specific insights into the pivotal role of endothelial activation and injury as a trigger and a determinant of outcome in HUS will aid in understanding pathogenic mechanisms.

Unravelling the links between genetic predisposition, inflammatory triggers, complement and the coagulation cascade will potentially identify therapeutic pathways with wider relevance for other conditions in which thrombotic vascular injury is a key feature, for example, ischaemia-reperfusion injury (IRI), preeclampsia and atherosclerosis.

Production of complement components by endothelium during inflammation

During inflammation, protective mechanisms are activated which enhance complement regulation. For example, the stimulation of HUVEC with interferon gamma ($\text{IFN}\gamma$) leads to an increase in endothelial production of the soluble regulators FH and FI (Brooimans et al., 1989 and Ripoche et al., 1988). However, endothelium also has the capacity to produce complement activatory molecules such as properdin (Bongrazio et al. 2003), C3 (Dauchel et al., 1990 and Sheerin et al., 1997) and Factor B (Dauchel et al. 1990) at sites of local inflammation or injury. This local production of complement components may be a factor mediating specific endothelial bed susceptibility to inflammation (Sheerin et al. 1997).

Exposure of endothelial cells to proinflammatory cytokines also results in marked phenotypic changes including the upregulation of adhesion molecules, secretion of soluble mediators, and changes in vascular tone and permeability (Cines et al., 1998 and Molema, 2010). The concept of differential vascular bed response to cytokines is gaining increased support (Molema 2010). Lipopolysaccharide is an important determinant of pathogenesis in verotoxin-induced HUS (Keepers et al. 2006).

The balance between anti and pro-inflammatory cytokines and the production, and regulation of individual complement component production at times of injury will contribute to the overall phenotype and severity of injury. This is illustrated by the identification of a polymorphic risk variant in the promoter region of CD46 which was shown to decrease the transcriptional activity of the promoter and increased the likelihood of developing aHUS in susceptible pedigrees (Esparza-Gordillo et al. 2006). It was hypothesised to lead to reduced levels of CD46 expression at times of need, conferring reduced cell surface complement regulatory capacity.

Complement dysregulation

Vascular endothelium is continuously exposed to autologous complement-mediated challenge generated by the local or systemic activation of the CP, AP and MBL/MASP pathways of complement. This is enhanced during inflammation, significantly increasing the risk of injury to bystander host tissue. Complement activation products such as C1q, C3a,

C5a and C5b-9 can directly act on endothelial cells and adversely influence their function (Morgan 1989) (Fig. 1).

For example, there are many possible mechanisms by which C5b-9 can mediate endothelial injury in thrombotic microangiopathies. Whilst it is well known that C5b-9 can induce cell lysis, the numerous roles of sublytic amounts of C5b-9 inserted in the membrane of nucleated cells (which are generally more resistant to cell lysis than non-nucleated cells), is perhaps less appreciated. Sublytic MAC can induce secretion of multimers of endothelial von Willebrand factor (Hattori et al. 1989), stimulate endothelial prothrombinase and tissue factor activity (Saadi et al. 1995) and activate platelets and fibrin deposition generating a pro-thrombotic endothelial cell surface (Sims and Wiedmer 1995). Furthermore, complement induces endothelial morphological changes, e.g. cell retraction, exposing pre-existing inducers of thrombosis in the underlying matrix to plasma clotting factors and platelets (Saadi and Platt 1995). The ability of C5b-9 to upregulate expression of leucocyte adhesion molecules on the endothelial cell might also contribute to platelet localisation and adhesion (Kilgore et al. 1995) as well as increased leucocyte adhesion and subsequent cytokine and growth factor production. Complement injury may also cause release heparan sulphate proteoglycans from endothelium (Platt et al. 1990).

Glycocalyx injury

In normal blood vessels, heparan sulphate proteoglycans and other glycocalyx components, e.g. sialic acid, on the surface of endothelial cells function to maintain an anticoagulant environment by localised activation of anti-thrombin III (Marcum et al., 1986 and Matzner et al., 1985), a potent inhibitor of thrombin generation. Heparan sulphate also renders endothelial cell surfaces relatively impermeable to transit of blood cells and plasma proteins (Gallagher et al., 1986 and Matzner et al., 1985). Glycocalyx injury is an under-appreciated cause of endothelial cell injury and is a prominent pathogenic mechanism in conditions such as *S. pneumoniae*-related haemolytic uraemic syndrome whereby a secreted bacterial pathogenic factor, Neuraminidase, is responsible for de-sialylating the glycocalyx. This is believed to contribute to aHUS onset due to exposure of the large T-antigen, which reacts with natural IgM antibodies in serum, activating complement through the classical pathway (Copelovitch and Kaplan 2008). The relevance of this mechanism for glycocalyx injury has also been studied in xenotransplantation. Platt et al. (1990) exposed cultured porcine endothelium to human serum as a source of natural antibodies and observed release of >50% of endothelial cell proteoglycans within 1 h. Proteoglycan release depended on activation of the classical pathway (inhibited in the presence of C2-deficient serum) and preceded irreversible cell injury. Ischaemia-reperfusion injury also results in shedding of the glycocalyx. In the fenestrated endothelium of the kidney, this may increase exposure of the underlying glomerular basement membrane which, due to lack of intrinsic regulators, supports both complement activation and is pro-coagulant (Hindmarsh and Marks, 1998 and Platt et al., 1990).

Anti-endothelial cell antibodies

Mechanisms implicated in complement deposition on the surface of endothelial cells include activation of the CP by immune complexes and anti-endothelial cell antibodies (AECA). The binding of specific subtypes of antibodies to endothelium may result in complement activation. AP activation and amplification plays a major but under-appreciated role in mediating the effects of 'classically activated' Classical and Lectin pathways. Harboe and Mollnes recently demonstrated that amplification by the AP contributed to 80-90% of total C5 activation when the initial activation was highly specific for the classical pathway (Harboe et al. 2004). Thus, the interpretation of experimental models of human disease where direct activation of the AP has been assumed may need revision to identify CP and LP triggers and place a greater emphasis on alternative pathway amplification rather than activation.

An example of the devastating impact of antibody-mediated injury is seen in hyperacute allograft rejection which results in overwhelming endothelial cell death, intravascular coagulation and extravasation of blood elements (Robson et al., 1995 and Saadi and Platt, 1995). AECA have also been associated with aHUS and levels appear to correlate with outcome but their presence has not been systematically evaluated. Their removal during plasma exchange is likely to correlate with short-term improvement in renal function with prognosis determined by ongoing presence or successful suppression. However, there is a lack of correlation with the level of antiphospholipid antibodies (which recognise β_2 glycoproteins on endothelial cells), and an individual's susceptibility to the development of renal thrombotic microangiopathy. The *in vitro* observation that monoclonal antiphospholipid antibodies do not bind to quiescent cultured endothelial cells, nor cause endothelial activation, suggests that an additional endothelial cell activatory insult is required to manifest disease (Chen et al. 2004). These observations support the use of cytoprotective enhancement strategies in patients at risk of complement-mediated endothelial cell injury.

Mechanical/shear stress

Unidirectional laminar shear stress (LSS) contributes to vascular endothelial homeostasis by contributing to resistance to apoptosis and maintenance of an anti-proliferative, anti-oxidant, anti-thrombotic, anti-adhesive endothelial cell barrier (Traub and Berk 1998). In thrombotic microangiopathies like aHUS, the endothelial cell activation and intravascular platelet-fibrin thrombus formation causes a loss of LSS and the development of turbulent flow. This has implications for complement-mediated endothelial cell injury. Kinderlerer et al. demonstrated a significant increase in cell surface expression of CD59 following exposure of macrovascular human umbilical vein endothelial cells and the dermal microvascular endothelial cell line (HMEC-1) to 24-48 h of LSS. The increased expression was functionally significant, reducing C9 deposition and complement-mediated lysis of flow-conditioned endothelial cells by 50%. In contrast to LSS, disturbed flow failed to induce endothelial cell CD59 protein expression (Kinderlerer et al. 2008). Urbich et al. (2000) had previously demonstrated an increase in clusterin protein expression after 18 h of LSS.

Thus, loss of LSS will predispose to increased complement-mediated endothelial cell injury by reduced expression of both a soluble and membrane-bound inhibitor of MAC. This is very likely to be functionally important as CD59 is considered to be the most potent endothelial regulator of MAC (Brooimans et al., 1992a and Brooimans et al., 1992b). Endothelial injury may be further exacerbated by the effects of turbulent flow, as in addition to this reduced protection against complement, it may increase complement activation by enhancing alternative pathway activation. The synthesis of endothelial properdin (a stabiliser of the AP C3 and C5 convertases), is markedly upregulated following exposure of endothelial cells to turbulent flow *in vitro* (Bongrazio et al. 2003). Management of the associated hypertension, which exacerbates endothelial cell injury from disturbed flow in patients with aHUS, is thus an important therapeutic adjunct.

Anti-VEGF therapies

The vascular endothelial growth factors (VEGF) represent a family of multifunctional glycoproteins centrally involved in vasculogenesis, angiogenesis, regulation of vascular permeability and cytoprotection (Ferrara et al. 2003). The recent observations that anti-VEGF inhibitors used in oncological and ophthalmic practice are, in rare cases, associated with the development of marked hypertension, proteinuria and a renal thrombotic microangiopathy, has suggested a new pathway for complement mediated injury. The particular renal endothelial susceptibility to loss of VEGF was demonstrated by *in vivo* studies utilising a doxycycline-dependent Cre murine model where the targeted deletion of murine VEGF-164 (human VEGF-165 equivalent isoform) from the glomerular epithelial podocytes of mature mice led to a glomerular thrombotic microangiopathy with endothelial cell injury analogous to that seen in aHUS (Eremina et al. 2008). In human glomerular endothelium, VEGF-165 is critical for maintaining a fenestrated endothelial phenotype although how the loss of fenestrations predisposes to thrombotic microangiopathy is not yet understood. Likely mechanisms include an increased susceptibility to endothelial cell injury caused by the vascular haemodynamic changes, hypertension and hyperfiltration resulting from reduced fenestration density and to changes in complement activation and regulation, as described above for turbulent flow. Furthermore, both VEGF-165 and basic fibroblast growth factor (bFGF), but not placental growth factor (PlGF), have been shown to upregulate CD55 on the surface of macro and microvascular endothelium (HUVEC, HAEC and HMEC-1) *in vitro*. CD55 expression was upregulated three-fold on HUVEC following treatment with VEGF-165 and provided enhanced protection from complement-mediated injury. The cytoprotection was mediated through the VEGFR2 receptor and was inhibited by cyclosporin A in a dose-dependent manner (Mason et al. 2004). Unsurprisingly, VEGF supplementation with VEGF-121 has been shown to facilitate vascular repair during nephritis and thrombotic microangiopathy (Ostendorf et al. 1999), and more recently to have beneficial effects on blood pressure and renal function in rat models of pregnancy-induced hypertension (Gilbert et al. 2010) and preeclampsia (Li et al. 2007).

Therapeutic relevance

Complement inhibition in aHUS

Current treatment protocols for aHUS are based on plasma exchange/infusion. The rationale for this treatment, which has significantly improved morbidity and mortality in individual cases, but has not been formally evaluated in a clinical trial, is logical. It may beneficially supplement levels of deficient complement components contained in plasma, e.g. Factor H or Factor I, secondly, it removes mutated proteins which may exert a dominant negative effect on complement regulation and thirdly it removes pathogenic autoantibodies, for example anti-FH or AECA (Waters and Licht 2010). However, it is not uniformly effective, is time-consuming and there is significant procedure-related morbidity, for example access-related infections. Future potential developments in this field include the development of a plasma-derived FH concentrate (Fakhouri et al. 2010) or recombinantly produced Factor H (Schmidt et al. 2011) for those in whom there is a therapeutic rationale for this approach (reduced Factor H levels, efficacy of plasma infusion, evidence of ongoing AP turnover).

Secondly, a specific engineered complement inhibitor, Eculizumab, has been used successfully in an increasing number of cases of aHUS, including post-transplant recurrence and is now under investigation in the setting of clinical trials in adults and children with plasma-sensitive and plasma-resistant aHUS (Waters and Licht 2010). Eculizumab is a humanised murine monoclonal antibody against C5, which prevents C5 cleavage and hence limits both C5a and 5b production by any of the three complement pathways. As outlined above, at the most fundamental level of understanding, the pathogenesis of all subtypes of aHUS is likely to reflect an increased production of the terminal complement components C5a, C5b and subsequently C5b-9, due to enhanced alternative pathway activation caused by defective regulation or excess activation. This supposition is supported by several different strands of *in vitro* and *in vivo* evidence. The levels of circulating soluble C5adesArg and soluble C5b-9 (sC5b-9) are elevated in the serum of patients with active aHUS. Secondly, an elegant *in vivo* study has demonstrated that the development of murine aHUS can be completely prevented by crossing the homozygous transgenic aHUS mouse (which carries a deletion of the C-terminal CCPs16-20 of Factor H on a homozygous Factor H deficiency background and develops a spontaneous phenotype resembling human aHUS) with a C5 knockout mouse (de Jorge et al. 2011). Finally CD59 (the inhibitor of C5b-9) has been shown to protect glomerular endothelial cells from immune mediated thrombotic microangiopathy in rats. The model was generated by selective renal artery perfusion with either control F(ab)₂ fragments or F(ab')₂ fragments of anti-CD59 monoclonal antibody prior to perfusion with either a goat anti-rat glomerular endothelial cell antibody or control goat IgG. Neutralisation of CD59 in rats who received control goat IgG did not result in any significant functional or histological changes. Neutralisation of CD59 in rats who received the goat anti-rat GEC antibody resulted in more C5b-9 formation in the glomerulus. This was accompanied by increased platelet and fibrin deposition, more severe endothelial cell injury and reduced renal function - a histological and phenotypic replication of human HUS (Nangaku et al. 1998).

Glycocalyx modulation

An improved understanding of glycocalyx biology may have specific relevance as a potential therapeutic option for HUS. As described above, production of neuraminidase by viruses and bacteria is a potent virulence factor predisposing to development of HUS through endothelial desialylation (Copelovitch and Kaplan 2008). Secondly, mutations in the carboxy terminus of FH, which contains a heparin/sialic acid binding site (Blackmore et al. 1998) are strongly associated with development of aHUS (Richards et al. 2001). Thirdly, the subtilase cytotoxin (SubAB) (a virulence factors produced by verotoxin-producing *Escherichia coli*) is highly toxic to eukaryotic cells, and when injected intraperitoneally into mice, causes pathology resembling that associated with human HUS. SubAB exhibits a strong preference for glycans terminating in alpha2-3-linked N-glycolylneuraminic acid (Neu5Gc), a sialic acid that humans are unable to synthesize, due to a genetic lack of the necessary enzyme. However, humans who eat Neu5Gc-rich food, such as red meat and dairy, incorporate Neu5Gc on the surfaces of endothelial and intestinal cells, facilitating toxin binding and subsequent endothelial cell injury (Lofling et al. 2009).

It is therefore of great interest that several recent publications have described a role for modulation of the glycocalyx as a potential treatment in other renal diseases. The landmark paper in this field described how oral dietary supplementation with the sialic acid precursor N-acetyl-d-mannosamine (ManNAc) given to pregnant mice, successfully improved survival in a murine model with an otherwise fatal knock-in mutation in the *GNE* gene (M712T). This gene encodes the key enzyme of sialic acid biosynthesis: uridine diphospho-N-acetyl glucosamine 2-epimerase/N-acetyl mannosamine kinase. This mutation is the commonest cause of an adult-onset, spontaneous inclusion body myositis in humans, who have no renal phenotype. The knock-in mice unexpectedly developed a severe, rapidly fatal renal phenotype, with haematuria, proteinuria and structural glomerular defects, apparently caused by a remarkable reduction in sialylation of podocalyxin on podocytes and subsequent loss of filtration barrier integrity (Galeano et al. 2007). Additional support for such an approach was provided by a further recent publication which reported success using ManNAc supplementation in improving outcome in a podocyte-specific transgenic rodent model overexpressing angiopoietin 4 (a secreted glycoprotein), which normally develops nephrotic syndrome (Clement et al. 2011). In human kidneys, the podocytes and also basement membrane and glomerular endothelial cells are highly sialylated. However, the phenotypic differences for the M712T mutation reported above would imply that there are likely to be important differences in sialylation levels in mouse and human kidneys, with respect to wider generalisation of results.

Finally, the results of a trial to investigate the role of oral sulodexide (a mix of the glycocalyx components heparan and dermatan sulphate) in dense deposit disease; a complement-mediated glomerulopathy characterised by defective systemic regulation of AP due to FH deficiency or C3 nephritic factors which stabilise C3 convertases, are awaited (Smith et al. 2007). In support of this approach, sulodexide was recently reported to increase both the sublingual and the retinal glycocalyx dimensions in participants with Type 2 diabetes with a trend to reduction in albuminuria after 2 months of therapy (Broekhuizen et al. 2010).

Endothelial cytoprotection strategies utilising complement

Endothelial cells are an increasingly important target for therapeutic manipulation. The concept of enhancing endothelial protection against injury by mechanisms which upregulate complement is not new but represents a potentially cost-effective area of intervention, not just in aHUS but many other conditions, e.g. organ transplantation, IRI, preeclampsia and atherosclerosis. Important candidate endothelial cytoprotective genes are CD55, eNOS, thrombomodulin, and haem oxygenase 1, which exert anti-inflammatory, anti-thrombotic, and anti-oxidant effects. An increasing number of commonly used drugs can be shown to have additional direct effects on endothelium, inducing higher levels of functionally relevant CD55 expression. Such agents include the statins (Mason et al., 2002a and Mason et al., 2002b) and inducers of haem oxygenase 1 (HO-1), e.g. haemin (or haem arginate) (Kinderlerer et al. 2009). Statins do not have a role in the current management of thrombotic microangiopathies but are worthy of consideration given their existing safety data, cost-effectiveness and therapeutic rationale based on their ability to enhance complement-mediated endothelial cell protection. Of note, the recent description of an association between functionally significant complement polymorphisms and mutations in CD46 and Factor I and a significantly associated risk (odds ratio of 8.2) of preeclampsia in patients with SLE/APLS (Salmon et al. 2011) suggests that the results from the ongoing trial of statin therapy (Statins to Ameliorate Preeclampsia; StAmP) (Ahmed 2011) in this condition will be of great interest with respect to this hypothesis.

Conclusions

Consideration of the interactions between complement and the endothelium is critical for understanding the pathophysiology of conditions with prominent endothelial cell injury. Unanswered questions in aHUS relate to reasons for the renal endothelial susceptibility; the determinants of penetrance in those with a genetic predisposition and the balance between soluble and membrane bound complement regulation at endothelial and basement membrane surfaces. Many aspects of endothelial cell biology are relevant when considering approaches to treatment. For example, our developing pathophysiological understanding of the causes of HUS over the last decade has informed our understanding of complement-mediated defence of host tissues, including endothelial cells. The requirement for full functionality of all regulators in context of an endothelial cell insult highlights the non-redundancy and specific importance of each individual regulator. Knowledge gained from situations in which renal thrombotic microangiopathy occurs as a side-effect of a new therapeutic approach, e.g. VEGF inhibition has identified new pathways of endothelial cell injury and refocused attention on historical *in vivo* studies of thrombotic microangiopathies where VEGF supplementation was beneficial. Advances made in understanding of completely unrelated conditions, e.g. inclusion body myositis may also suggest novel therapeutic pathways by virtue of understanding their pathobiology. Thus, by considering rarer conditions with prominent endothelial cell dysfunction, we may gain new insights into pathophysiology and treatment options for more common endothelial injury conditions and thus determine how best to apply them.

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Abbreviations

AP	alternative pathway
CPN	carboxypeptidase N
CP	classical pathway
CR1	CD35, complement receptor 1
ciGEC	conditionally immortalised glomerular endothelial cells
CRP	C-reactive protein
DAF	CD55, decay accelerating factor
HUS	haemolytic uraemic syndrome
HMEC-1	human (dermal) microvascular endothelial cells
IRI	ischaemia-reperfusion injury
LPS	lipopolysaccharide
MBL/MASP	mannose-binding lectin/mannose-binding lectin associated serine protease pathway
MAC	C5b-9, membrane attack complex
MCP	CD46, membrane cofactor protein
TMA	thrombotic microangiopathy
VEGF	vascular endothelial growth factor

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Fig. 1. Activation of the complement cascade by the classical, lectin or alternative pathway leads to the generation of biologically active complement components.

C3a and C5a are potent anaphylotoxins which recruit leucocytes and platelets to the endothelial cell surface following upregulation of adhesion molecules, e.g. ICAM-1 and P-selectin. C5a also promotes endothelial cell retraction, exposing underlying basement membrane, and upregulates the expression of procoagulant tissue factor. C5b participates in the formation of the membrane attack complex C5b-9. Downstream effects of sublytic MAC on endothelium additionally include secretion of multimers of endothelial von Willebrand factor and release of heparan sulphate proteoglycans from endothelial glycocalyx. These changes favour a switch from an anti-inflammatory, anti-coagulant endothelial phenotype to a highly active, pro-coagulant phenotype. In atypical HUS, the downstream effects of excessive complement activation in the vicinity of the glomerular endothelium leads to thrombotic microangiopathy within capillaries and arterioles, causing renal failure.