Viral haemorrhagic fever and vascular alterations

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Zusammenfassung

Summary
Pathogenesis of viral haemorrhagic fever (VHF) is closely associated with alterations of the vascular system. Among the virus families causing VHF, filoviruses (Marburg and Ebola) are the most fatal, and will be focused on here. After entering the body, Ebola primarily targets monocytes/macrophages and dendritic cells. Infected dendritic cells are largely impaired in their activation potency, likely contributing to the immune suppression that occurs during filovirus infection. Monocytes/macrophages, however, immediately activate after viral contact and release reasonable amounts of cytokines that target the vascular system, particularly the endothelial cells.

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The term viral haemorrhagic fever (VHF) classifies a number of virus-induced acute diseases that are typically associated with fever and, in severe cases, haemorrhage and shock. Currently, members of four virus families are known to cause VHF in humans:

- arenaviruses,
- filoviruses,
- bunyaviruses, and
- flaviviruses.

The severity of VHF ranges from relatively mild illnesses to severe life-threatening cases characterized by virus-induced shock syndrome and multiorgan disease that is to some extent comparable to gram-negative bacteria-induced septic shock syndrome. Lethality likely results from overall impairment of cardiovascular regulation (e. g. blood pressure regulation, coagulation/anti-coagulation balance and control of fluid distribution between intravascular and interstitial spaces) and the inability to a sufficient immune response due to the destruction of immune competent cells.

VHFs are zoonotic diseases, which are characterized by pathogens capable to spread from animals or insects to humans or might spread from humans to humans, who are not their natural reservoir. Survival of zoonotic pathogens in a particular area depends on the presence of host organisms that serve as the natural reservoir. Primary human infection by VHF agents mostly occurs through close contact with an infected host (e. g. Ebola haemorrhagic fever occurs by close contact to gorillas or chimpanzees) (21). This might explain why Ebola haemorrhagic fever (EHF) and Marburg haemorrhagic fever (MHF) outbreaks in humans are irregular.

Among VHF families, filoviruses are the prototypic VHF agent causing the most severe human disease with case fatality rates up to 89% (21, 27), although asymptomatic infections have been reported (32). While filovirus outbreaks occurred sporadically until 1995 (mostly in central African countries) annual outbreaks have become regularly in the past decade (21, 27). Both increasing outbreak frequency and importation of Ebola and Marburg viruses from epidemic into non-epidemic areas have heightened efforts to understand filovirus epidemics and pathophysiology. Field studies and experimentations suggest that fruit bats might serve as natural reservoir for filovirus, but further studies are required for verification (37, 43, 65, 67).

Since the most dramatic symptoms are seen in response to filovirus disease, we will review vascular alterations caused by filovirus HF in humans and non-human primates.

Filoviruses and symptoms

Filoviruses are large enveloped filament-like viruses with a diameter around 80 nm and a length between 800–1400 nm. They
Aleksandrowicz et al. have an on-segmented single-stranded negative RNA (34, 35) and are thus grouped with paramyxov-, rhabdo-, and borna-viruses to the order of Mononegavirales. Filoviruses consist of two genera (20),
- Zaire Ebolavirus (EBOV) with four species:
  - Zaire ebolavirus,
  - Sudan ebolavirus,
  - Côte d’Ivoire ebolavirus,
  - Reston ebolavirus,
- Marburgvirus (MARV) with a single species.

With the exception of Reston ebolavirus, all these species cause severe haemorrhagic fever in humans. The molecular organization of filoviruses and an electron micrograph are shown in Figure 1.

After an incubation period of about 4–10 days in average, the first immediate symptoms include headache, anorexia, fever, chills and myalgia, partially resembling the flu. Sore throat, nausea, vomiting, cough, arthralgia, diarrhea, and pharyngeal conjunctival infections might accompany accelerated illness. Patients might become dehydrated, apathic, and develop maculopapular rash and erythema. In severe cases, haemorrhages frequently occur. Bleeding from petechia, puncture sites, mucous membranes and the gastrointestinal tract is common. Early symptom onset and development of a severe shock syndrome might predict fatal cases (14, 50).

Bleeding tendency might be explained in part by elevated partial thromboplastin time, the release of fibrin split products (D-dimers) due to consumption of coagulation factors, and decreased platelet count (23, 45). However, blood loss is moderate and does not contribute to hypovolaemic shock that likely arises from the disbalance of fluid distribution between the vasculature and the interstitium (21, 56), and from the release of nitric oxide from infected monocytes/macrophages and endothelium (51).

Ebola virus infection in human and non-human primates is further accompanied by lymphopenia and the release of immature and atypical leukocytes pointing to altered immune responses during infection (23).

Viral entry into host cells
Filovirus entry into host cells is mediated by trimers of their envelope glycoprotein (GP). The molecular mechanism of viral entry is largely unknown, although receptor-mediated endocytosis is believed to take place. Several molecules have been proposed to be filovirus receptors:
- asialoglycoprotein receptor ASGP-R (8),
- β1 integrins (66),
- dendritic cell-specific ICAM-3 grabbing non-integrin (DC-SIGN) and its homologue DC-SIGNR (2),
- folate receptor-a (FR-a) (12), and the
- tyro-3 tyrosine kinase family (61).

All these factors increase infectivity, or even restore susceptibility to infection in non-permissive cells (T-lymphocytes), but none of them has been shown to be absolutely required for filovirus’ entry. It is suggested that some of these molecules might serve as binding factors to enhance uptake (40). Nevertheless, the filovirus receptor remains elusive.

Filoviruses are thought to use raft/caveola-mediated endocytosis to enter cells. Studies revealed that infection depends on cholesterol and ganglioside M1 (GM1), and that virions colocalize with caveolin-1 (6, 17). Otherwise, it has been shown that host cell infection is strongly affected by both clathrin- and raft-mediated endocytosis inhibitors (49). In addition, other endocytotic routes such as macropinocytosis might also play a role in the uptake of these huge filovirus particles. Despite controversy over the entry pathway, it is clear that filoviruses require transport to a compartment with acidic pH.

Under these conditions, at least Ebola-GP is cleaved by cathepsins B and/or L, and triggers viral fusion with the endosomal membrane and the subsequent release of its RNA into cytoplasm (13, 58).

Pathogenesis
The current model of filovirus pathogenesis is illustrated in Figure 2. Infection in humans often occurs by close physical contact that allows the virus to enter the body via
skin lesions and mucous membranes. This is followed by infection of dendritic cells and monocytes/macrophages. It was proposed that cells infected during the primary wave travel via lymphatic and blood vessels to lymph nodes, liver and spleen where they easily can spread (54). Evidence of this infectious route was experimentally demonstrated in Cynomolgus monkeys by intramuscular injection of EBOV. A local productive infection, particularly in monocytes/macrophages and dendritic cells, was followed by increased infection of local lymph nodes and spreading of the virus to the liver and spleen (26). Thus, it appears that cells important in inflammation and immune responses, such as monocytes/macrophages and dendritic cells, are the primary targets in Ebola infections. Similar to septic shock, it is proposed that an uncontrolled release of cytokines (cytokine storm) from infected monocytes/macrophages contributes to the development of virus-induced shock.

Experimentally infected monocytes/macrophages become activated early after infection and release a reasonable amount of cytokines (19, 63). Interestingly, fatal and non-fatal infections in humans are accompanied by differences in cytokine profile. While high levels of interleukin (IL)-10, neopterin and...
IL-1 receptor antagonist are evident in fatal cases, IL-1β and IL-6 are found in non-fatal cases (3, 5, 36, 71). In contrast, EBOV- or MARV-infected dendritic cells are unable to release sufficient amounts of costimulatory cytokines, CD80 and CD86, which are important in T-cell proliferation (11, 39). In addition, considerable T-lymphocyte bystander apoptosis is seen in both humans and non-human primates at certain locations (4, 24, 44, 51). Furthermore, peripheral mononuclear blood cells transiently express a pro-apoptotic mediator, tumour necrosis factor related ligand (TRAIL), indicating a possible mechanism for the observed apoptosis (26, 30). These results clearly indicate early alterations to the innate and adaptive immune system upon infection.

**Endothelial cells and vascular dysfunction in filoviral VHF**

During Marburg and Ebola infection, the vascular system mainly alters due to host responses rather than direct effects of virus replication. The development of a filovirus-induced shock syndrome largely resembles septic shock, a condition defined as dysbalance of oxygen supply and demand. This results in organ hypoperfusion that leads to cellular energy loss and, in turn, organ and cellular dysfunction. Our understanding of the pathophysiological background of filovirus-induced shock is fragmentary.

The current hypothesis is based on cell culture and animal experimentation, as well as on the few human case reports. The development of virus-induced shock in humans and non-human primates appears to be due to loss of endothelial integrity, activation and fibrin deposition (disseminated intravascular coagulation, DIC) (21, 23, 31, 55). These parameters are assumed to significantly promote the fatality of filovirus haemorrhagic fever.

**Endothelial integrity and paracellular barrier function**

The vascular endothelium forms a unique selective barrier between the blood and tissue, controlling exchange of solutes and water. This balance of solutes and fluid homeostasis is regulated in two ways: Transport of:

- macromolecules, such as albumin, through the endothelium via a transcellular pathway (41),
- water and small solute exchange preferentially via a paracellular route through endothelial junctions, particularly in capillaries and post-capillary venules.

Under normal conditions, intercellular junctions are impermeable to macromolecules, except in the liver sinus endothelium, lymph nodes and spleen. In inflamed tissue, endothelial barrier function is decreased due to reduced intercellular adhesion that, in severe cases, can result in gap formation that allows extravasation of macromolecules and water (7, 70). However, a less dramatic loss of intercellular adhesion might lead to invisible intercellular gaps but still allow extravasation of small solutes and water. In these cases, cell adhesion molecules at cell junctions only moderately reorganize (53, 73).

In contrast to epithelial cells, endothelial junctions are complex in nature and do not create somatotropic separated adherens, tight and gap junctions (62). While expression of tight junction strands is very heterogeneous within vascular beds, adherens junctions are common structures throughout all endothelial cells, making them crucial for paracellular barrier function regulation. Particularly in post-capillary venules, adherens-type junctions are the predominant structures targeted to increase paracellular permeability and leuko-
cyte extravasation during inflammation and EBOV infection (10, 19, 38, 52, 69, 73). Although the molecular mechanisms of paraendothelial barrier function regulation are only partially understood, it is becoming clear that calcium-dependent vascular endothelial (VE-) cadherin and junction associated actin filaments are critical players.

Increased paraendothelial permeability is associated with changes in both VE-cadherin organization, loss of junction actin filaments, and stress fibre formation (7, 57, 74). VE-cadherin is a type I transmembrane protein that interacts with intracellular β- and γ-catenin via its carboxyterminal cytoplasmic domain (15, 70), and its connection to cortical actin filaments via a catenin may play a role in the dynamic reorganization of cell junctions (16, 75). The expression of both VE-cadherin- and junction-associated actin filaments in endothelial cells differ in vivo and in cell culture. However, application of fluid flow causes a reorganization of both VE-cadherin and actin filaments resembling the appearance of these complexes in vivo (59) (Fig. 3). Taken together, VE-cadherin-mediated cell adhesion is a critical component of junction regulation.

Supernatants derived from either MARV-infected monocytes/macrophages or recombinant TNF-α and interferon (INF)-γ applied to endothelial cell cultures cause breakdown of endothelial barrier function and reorganization of the VE-cadherin/catenin complex (52) (Fig. 3B). The mechanism of cell junction opening is incompletely understood, but tyrosine-phosphorylation/dephosphorylation of cell-junction proteins appears to be important. It was recently shown that mediators released from MARV-infected monocytes/macrophages, as well as recombinant TNF-α/H₂O₂ and IFN-γ, cause both opening of interendothelial junctions (Fig. 4B) and tyrosine phosphorylation of PECAM-1 (platelet endothelial cell adhesion molecule 1) (9). In contrast, no tyrosine phosphorylation of the VE-cadherin/catenin complex was observed after stimulation of endothelial cells with either supernatants of MARV-infected monocytes/macrophages or recombinant TNF-α or INF-γ (9). In agreement with these results, tyrosine phosphorylation of the VE-cadherin/catenin complex was shown to upregulate and maintain endothelial barrier function under fluid flow (59) and upon treatment with ATP or vascular endothelial growth factor (60).

In addition to the effects of cytokines and proinflammatory mediators on endothelial barrier function and activation, the production and release of viral proteins from infected host cells may also exert an impact on pathogenesis. Particularly, Ebola-infected cells release high amounts of a soluble glycoprotein (sGP) and delta peptide, which both are furin cleavage products of the precursor GP, pre-sGP (22). Pre-sGP is the primary expression product of the glycoprotein gene and RNA-editing is needed to express the full-length transmembrane glycoprotein GP which was shown to be cytotoxic. Thus, RNA-editing might control EBOV cytoxicity and eventually also impact on pathogenicity (1, 72, 64). In addition, high serum concentrations of sGP are proposed to also contribute to pathogenesis, particularly in relation to endothelial cells.

It may be assumed that soluble viral proteins cause cell destruction or have pathologic effects on endothelial cells. Surprisingly, it was found that sGP, also independent of C-mannosylation (18), accelerates recovery of endothelial barrier function after disruption by TNF-α application (18, 73) (Fig. 4A). This intriguing protective function of sGP could be interpreted as a viral anti-inflammatory weapon and is currently the only verified effect on host cells. In contrast, full-length GP of EBOV causes a breakdown of endothelial barrier function that is significantly increased in the presence of TNF-α (73). Thus, a decrease in endothelial barrier function can be simply provoked by GP or virus-like particle (VLP) binding to endothelial cells. A comparable effect was seen in monocytes/macrophages when inactivated EBOV caused cytokine release (63).
Endothelial cell activation

Cytokines are released from both EBOV and MARV-infected monocytes/macrophages, and were detected in reasonable amounts in Ebola-infected patients. These cytokines modulate, as outlined, immune responses and decrease endothelial barrier function. A further important pathophysiological function of cytokines (e.g. TNF-α) includes activation of endothelial cells, which, in turn, express cell adhesion molecules such as E-selectin, ICAM-1 or VCAM-1 (73) that mediate the multi-step process of leukocyte transmigration through endothelial cells (69). Likewise, application of recombinant cytokines, particularly TNF-α, live virus, inactivated virus or VLPs may induce these events (73). Activation and opening of endothelial cell junctions typically occur simultaneously, and these processes cannot be separated per se from each other. In addition, tissue factor (TF), an integral membrane protein of the cytokine receptor family, is expressed in endothelial cells and in cells of the innate immune system during inflammation caused by septic- and filovirus induced diseases (28, 42). While cell adhesion molecules are required for leukocyte transmigration in inflamed tissue, TF facilitates pro-coagulation activity and modulates intercellular adhesion and barrier function of endothelial cells in concert with the protease activated receptors (PAR-1 and PAR-2) (46).

An important role for the TF signalling pathway in Ebola infection was recently discovered. It was shown that blocking TF activity diminishes EBOV-induced lethality in non-human primates (25). TF initiates coagulation by binding the coagulation factor protease VIIa and its substrate factor X, which becomes activated to Xa (extrinsic tenase complex). When this complex binds to the physiological TF pathway inhibitor, blood coagulation is suppressed in endothelial cells. Release of factor Xa from the complex (33, 48, 68) causes
- thrombin and fibrin formation,
- platelet activation, and
- inflammation.

This process is strongly counteracted by the natural anticoagulant protein C pathway that may significantly regulate the development of septic- and VHF-induced shock (46, 47). Activated protein C (APC) is generated via thrombin while binding to its endothelial cell surface receptor thrombomodulin in connection with the endothelial protein C receptor. In conjunction with the cofactors protein S and phospholipids, APC subsequently degrades factors Va and VIIIa. In this way, APC not only provides physiologic anticoagulant activity but also exhibits both anti-inflammatory and anti-apoptotic functions (33, 48, 68).

Administration of APC reduces lethality in EBOV-infected non-human primates and is an established treatment of septic shock (29, 42, 46). In addition to APC, TF pathway inhibitors, e.g. the nematode anticoagulant peptide c2 (NAPc2) derived from the hookworm, are also capable to significantly reduce lethality in experimental EBOV-infected macaques (25, 29). Under NAPc2 application, a reduced level of D-dimers was observed, and TF-specific blood coagulation parameters were clearly reduced (25). Determining if and at what stages NAPc2 or APC can change endothelial activation and barrier function is a challenge for the future.

Conclusion

Pathogenesis of viral haemorrhagic fever, particularly of filovirus infections, is closely associated with
- alterations of the vascular system and
- insufficient immune response.

In the vascular system, the breakdown of endothelial barrier function, hypotension, blood coagulation and activation of endothelial cells seem to play a role in the severity of filovirus infections. Analyzing the underlying molecular mechanisms and signaling events, particularly in endothelial cells, the vascular and immune systems will help to understand pathogenesis of EBOV and MARV haemorrhagic fever.

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