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PREMATURE EVENTS IN CHIKUNGUNYA VIRUS INFECTION AND THEIR INHIBITING STRATEGIES—FROM VIRUS CELL BINDING TO MEMBRANE FUSION

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ABSTRACT

Chikungunya virus is a rapidly emerging mosquito-borne alphavirus causing millions of infections in the tropical and subtropical regions of the world. This infection often leads to an acute self-limited febrile illness with debilitating myalgia and arthralgia. A potential long-term complex infection results severe joint pain, which can last for months to several years. There is no clear-cut mechanistic explanations of the occurrences and spreading of this virus and also still there is no vaccines or specific therapeutics to prevent or treat this infection. Therefore this review describe the latest mechanistic aspects of critical steps involved in chikungunya virus cell entry including cell tropism, virus-receptor binding, internalization and membrane fusion, spreading mechanism and description of the molecules, drugs, other medicinal substances and means that have been used to interfere with the infection and related complications. So the aim of the study is to provide a newer feature on cell entry and to provide a viewpoint on potential new avenues in chikungunya virus research.

KEYWORDS: Chikungunya virus, alphavirus, cell tropism, receptor, endocytosis, clathrin, fusion, neutralizing antibodies, entry inhibitors.

INTRODUCTION

Chikungunya virus is an arbovirus belonging to the family Togaviridae and the genus Alphavirus, which can be further classified into encephalitic and arthritic viruses. This disease is transmitted by mosquitoes of the *Aedes (Ae.)* species eg Aedes aegypti and Aedes

albopictus. Upon infection, about 75%–95% patients develop fever, characterized by high temperature, myalgia, polyarthralgia, joint pain, rash, and intense asthenia.[1,2] A common long-term difficulty occurs in about 12%–49% patients is severe, debilitating joint paint that can persist for months to years after infection.[3] Rarely, encephalopathy, encephalitis, myocarditis, hepatitis, and circulatory failure is seen.[4,5] In past, Chikungunya virus caused minor outbreaks in limited area of Africa and Asia. But the situation considerably changed by the end of 2004 with the first major Chikungunya virus outbreak.[6] Till to date, Chikungunya virus is contagion in large parts of Africa, Asia, and the tropical and related regions of the Americas.[7] Within the last 2 years, this virus has spread to more than 40 countries including Bangladesh and other sub tropical regions involving over 1 million infections.[8] Four Chikungunya virus lineages titled West African (WA), the Asian, the Eastern/Central/Southern Africa (ECSA) and the Indian Ocean lineage (IOL) are found in the world and the latter one is emerged from the ECSA lineage in 2004.[9,10] Some IOL strains adapted to a new vector, Ae. albopictus, without significantly compromising viral fitness for the initial vector, Ae. aegypti, there by increasing the epidemic potential of the virus caused by adaptive mutations within the viral spike proteins E1 and E2 of Chikungunya virus.[11,12] Still there is neither vaccine nor any specific antiviral treatment to prevent or treat this viral infection. A potential antiviral strategy involves the inhibition of the cell entry process. Cell entry occurs based on a series of dynamic events between the viral glycoproteins E1 and E2 and the host cell, including virus-cell attachment, internalization, intracellular trafficking and membrane fusion. In this review we will describe the current knowledge of cell tropism, the cell entry pathway of Chikungunya virus and will discuss the molecules identified to interfere with these processes.

CHIKUNGUNYA VIRAL STRUCTURAL FEATURES

Chikungunya virus is a member of alphavirus of the antigenic Semliki Forest Complex including amongst others, related O'nyong-nyong virus (ONNV), Semliki Forest virus (SFV), and Ross River virus (RRV). Others include Sindbis virus (SINV) and Venezuelan or Eastern Equine Encephalitis Virus (VEEV and EEEV respectively).[13,14] Till today, most studies have been performed with SFV, SINV, RRV, and VEEV and it reveals that alpha viruses are enveloped spherical particles with a diameter of 65–70 nm.[15,16] Its genome consists of a single-stranded positive-sensed 11.8 kB RNA molecule packaged by the C protein to form the nucleocapsid. This nucleocapsid is surrounded by a host-cell derived lipid bilayer with two inserted transmembrane glycoproteins, E1 and E2. Host-cell derived lipid

bilayer strongly resembles the plasma membrane of the infected host cell. For mammalian-derived Chikungunya virus virions, the membrane consists of cholesterol and phospholipids in a ratio of approximately 1:1. The E1 protein is 439 amino acids long and contains one conserved N-linked glycosylation site at position 141.[17] E1 is anchored in the lipid bi-layer with a 30 residue transmembrane helix at the carboxy-terminal end. The cytosolic region is only five residues in length and does not interact with the nucleocapsid. Among DI, DII, and DIII domain of nucleocapsid, DIII is situated at the C-terminal of the protein and closest to the envelope, followed by the central DI and DII at the tip, which contains a hydrophobic fusion peptide. The E2 protein has a length of 423 amino acid and is N-glycosylated at 263 and 345 positions. Blast analysis revealed that like for E1, the glycosylation sites of E2 are conserved between all four Chikungunya virus lineages. At the C-terminus, transmembrane helix of 26 residues is located, followed by a cytoplasmic domain of 33 residues. The cytoplasmic domain contacts the nucleocapsid and studies with other alphaviruses have shown that this interaction is important for the correct assembly and budding of progeny viruses from the plasma membrane of infected cells. On a mature virion, 240 copies of E1 and E2 are arranged as 80 trimeric spikes; a single spike consisting of three E2/E1 heterodimers within which, E1 laterally contacts E2 along the central domain II and III. The E1 hydrophobic fusion peptide is buried in a groove between domain A and domain B of E2, thereby preventing pre-mature activation of the membrane fusion machinery of the virus.[18]

CHIKUNGUNYA TARGETS AND TROPISM

The Chikungunya virus target organs include joints, muscle, skin, liver, kidneys, eyes and the central nervous system (CNS). Above mentioned organs is frequently associated with a marked infiltration of mononuclear cells such as monocytes/macrophages. Tropism is an orientation of an organism to an external stimulus, such as light, especially by growth rather than by movement. Infection starts when a Chikungunya virus -infected *Ae.* mosquito is feeding on a human host.[19] During feeding, Chikungunya virus particles are thought to be released within the dermis and into the subcutaneous capillaries of the skin.[20] Within 2–4 days, the virus reaches the blood and disseminates to other parts of the body. Although Chikungunya virus pathogenesis is still poorly understood, recent studies shed light onto the organs and cells involved in Chikungunya virus replication.[35] Development of a macromolecular prodrug for the treatment of inflammatory arthritis. Mononuclear cell infiltration and viral replication in the skeletal muscle progenitor cells, and joints are associated with debilitating arthralgia, myalgia, and in some cases, arthritis. While the acute

phase symptoms usually resolve within two weeks, the musculoskeletal pain may linger for weeks to months or even years. Chronic disease has been linked to persistent virus replication in the target cells and/or the establishment of a self-sustained inflammatory mechanism that leads to the tissue damage.

Assembly And Distribution Of Viremia

During the 7–12 days-long acute viremic period, Chikungunya virus load can reach 10^9–10^{12} viral particles per milliliter of blood. The observation revealed that chikungunya virus reaches a high titer in a relatively short time period is suggestive for replication in leukocytes.[21] Indeed, other alphaviruses replicate in immune cells including dendritic cells (e.g., SFV, RRV, and VEEV) and monocytes (e.g., RRV and VEEV). In contrast to the above-mentioned alphaviruses, peripheral blood mononuclear cells (PBMCs) do not seem to contribute significantly to the production of Chikungunya virus progeny In fact, *in vitro* analysis revealed that most blood-derived cell types such as lymphocytes, dendritic cells, and natural killer cells are refractory to chikungunya virus infection. Conflicting reports were published on the permissiveness of monocytes to chikungunya virus infection. However, it is clear that even though monocytes might harbor Chikungunya virus antigens, viral production supported by the primary cultures of monocytes cannot explain the titers detected in blood of acute phase patients. These observations suggest that local Chikungunya virus replication in dermal fibroblasts, migrating monocytes/macrophages, and endothelial cells are pivotal places for virus production.

CELL ENTRY: ATTACHMENTS, INTERNALIZATION

Receptor-Mediated Endocytosis

Viruses enter cells either by fusion with membrane components, or by receptor attachment and internalization, followed by fusion with intracellular membranes of endocytic vesicles. Receptor-mediated endocytosis is the predominant mode of entry, most often mediated by the formation of clathrin-coated pits, and the subsequent transport to early endosomes, where the low-pH environment triggers fusion. Alternatively, some viruses utilize clathrin-independent pathways to gain entry into cells. The caveolar/raft pathway transports internalized virus to neutral-pH caveosomes, before redistribution to the ER. There are also a number of clathrin-independent, caveolin-independent pathways that viruses use for cellular entry that rely on small GTPases, although these are not well understood.[22]

Receptor Binding

The first step in Chikungunya virus infection involves attaching of the virus to a host cell receptor that facilitates by supposed receptor binding sites of both the domain A and domain B of the E2 protein. E2 domain B contains a class III PDZ binding motif and mediate protein-protein interactions.[23] To date, prohibitin (PHB), phosphatidylserine (PtdSer)-mediated virus entry-enhancing receptors (PVEERs), and glycosaminoglycans (GAGs) have been suggested as chikungunya virus receptor proteins in mammalian cells and ATPsynthase β subunit in mosquito cells.

Prohibitin

Prohibitins (PHBs) are evolutionary conserved multifunctional membrane proteins, which are present in multiple cellular compartments.[24] PHBs play a role in mitochondrial integrity, cell proliferation, cell survival and endocytosis in white adipose tissue. Importantly, PHBs are ubiquitously expressed at the cell surface of numerous mammalian cells. Wintachai and co-workers showed that anti-PHB antibodies and siRNAs towards PHB reduced Chikungunya virus infection of micro glial cells up to two-fold. Chikungunya virus was also found to bind to PHB in U937 cells, but despite this interaction the cells did not support a productive infection. U937 cells are a model cell line used in biomedical research. They were isolated from the histiocyticlymphoma of a 37-year-old male patient[2] and are used to study the behavior and differentiation of monocytes. U937 cells mature and differentiate in response to a number of soluble stimuli, adopting the morphology and characteristics of mature macrophages. On the other hand, flavaglines, plant compounds that directly interact with PHB did inhibit Chikungunya virus infection in HEK-293T cells for up to 50%. Thus, it is clear that PHB facilitates virus-cell binding. PHB likely acts to capture and concentrate Chikungunya virus particles at the cell surface. However, since the inhibiting compounds only moderately reduced infectivity and that U937 cells are refractory to Chikungunya virus despite PHB binding demonstrates that other factors are required to mediate (efficient) infection. The precise role of PHB in Chikungunya virus cell entry remains to be elucidated.

Phosphatidylserine (PtdSer)-Mediated Virus Entry-enhancing Receptors

Over the past three years, receptors and receptor complexes have recently been identified that enhance entry of a diverse range of enveloped viruses. This group of viral receptors shares the ability to bind to PtdSer present on the viral envelope and enhance the viral entry and termed as PtdSer-mediated virus entry enhancing receptors or PVEERs. This group includes

growth-arrest-specific 6 (Gas6) and the tyrosine kinase receptor, Axl, T-cell immunoglobulin and mucin domain 1 and 4 (TIM-1 and 4) proteins and MFG-E8/integrin αvβ3 or αvβ5 complexes etc. Among all the above T-cell immunoglobulin and mucin domain (TIM) family members are expressed on various immune cells and a range of mucosal epithelia, and are known to regulate immune cell activity. Recently, TIM-1 was described to enhance the entry and infection of chimeric virus particles displaying the glycoproteins of Chikungunya virus or other viruses in HEK293T cells.[25] TIM-1 clears the apopptic bodies and binds to phosphatidylserine (PtdSer) in the viral envelope and functions to concentrate the virus at the cell surface. These receptors act on the basis of their long stalk region and PtdSer binding motif. Indeed, other unrelated proteins with a long stalk region and PtdSer motif were also able to support viral cell entry, demonstrating that virus-cell binding is not TIM-1 specific. These results further indicate that Chikungunya virus uses TIM-1 as an attachment factor but not as a specific receptor. The broad expression of these receptors and their ability to interact with PtdSer on a wide array of enveloped viruses has huge potential implications for virus infection. Most importantly, PVEERs enhance virus binding to cells and facilitate internalization.

Glycosaminoglycans

Glycosaminoglycans (GAGs) are large complex carbohydrate molecules that are expressed at the cell surface of most mammalian cell types. GAGs include among others heparan sulfate, keratan sulfate, chondroitin sulfate, dermatan sulfateand hyaluronic acid (HA).[26] These molecules can bind a wide variety of proteins and mainly function in cellular adhesion, growth, differentiation, cell migration and signaling. Several alphaviruses are known to use GAGs for cell entry. Natural isolates of EEEV and low passage strains of VEEV were found to depend on GAGs for efficient infection of cells. For other alphaviruses, heparan sulfate binding was related to virus-cell culture adaptation and an attenuated disease phenotype in mice. For some chikungunya virus, GAG expression was found to increase the binding and infection efficiency of both a clinical and a vaccine strain in CHO cells. Yet, Chikungunya virus like other alphaviruses readily adapts to GAGs. GAG utilization is facilitated by mutations to positively charged amino acids at E2-82 and E2-79. For example, an arginine at E2-82 or a lysine at E2-79 leads to enhanced infectivity in mammalian cells and attenuated virulence in mice.

ATP Synthase β Subunit

ATP synthase β subunit (ATPSβ)was recently found to interact with Chikungunya virus in mosquito cells. Furthermore, ATPSβ-down-regulation significantly reduced viral entry and virus production. The ATPSβ gene is widely conserved and is for example expressed in human endothelial and hepatic cells. Although involved in F1/ATPase catalysis in the mitochondria, ATPS is also located at the surface of the plasma membrane. There, it can bind ligands as apolipoprotein A-I, apolipoprotein E and angiostatin.[27] Therefore, it is of interest to examine whether this protein is involved in Chikungunya virus entry in mammalian cells and whether ATPSβ also exerts its function via increasing attachment of virions to the cell surface or whether other mechanisms are involved. Over the past few years, evidences indicate that adenosine triphosphate (ATP) is an energy source for the binding, maturation, assembly, and budding process of many enveloped viruses including Chikungunya virus. A study suggests that the F1-ATP synthase beta subunit (ATPsyn β, BP53) of the shrimp Litopenaeus vannamei (L. vannamei) might serve as a potential receptor for white spot syndrome virus (WSSV)'s infection. A 50-kDa Chikungunya virus -binding protein was identified as the ATP synthase β subunit (ATPSβ). Co-immuno precipitation studies confirmed the interaction, and co-localization analysis showed cell-surface and intracellular co-localization between Chikungunya virus and ATPSβ. Both antibody inhibition and siRNA-mediated down regulation experiments targeted to ATPSβ showed a significant reduction in viral entry and virus production. These results suggest that ATPSβ is a chikungunya virus -binding protein capable of mediating the entry of chikungunya virus into insect cells. The F1 ATPase complex is the catalytic core of F-ATPases and is composed of 5 subunits (alpha, beta, gamma, delta, epsilon).The F1 complexes are rotary motors in which the central gamma subunit forms the rotor inside the cylinder made of the alpha(3)beta(3) subunits. In F-ATPases, there are three copies each of the alpha and beta subunits that form the catalytic core of the F1 complex, while the remaining F1 subunits (gamma, delta, epsilon) form part of the stalks. There is a substrate-binding site on each of the alpha and beta subunits, those on the beta subunits being catalytic, while those on the alpha subunits are regulatory. The alpha and beta subunits form a cylinder that is attached to the central stalk. The alpha/beta subunits undergo a sequence of conformational changes leading to the formation of ATP from ADP, which are induced by the rotation of the gamma subunit, itself is driven by the movement of protons through the F0 complex C subunit.

Other Chikungunya virus cell adhesion Receptors and Heat shock protein 60 (HsP60

Another potential chikungunya virus receptor is the aV integrin (ITGAV) and b1 integrin (ITGB1), consisting of two members of the integrin super family forming various transmembrane dimers, which function as cell adhesion receptors. The aV integrin (ITGAV) and b1 integrin (ITGB1) dimer was found in the brain proteasome of mice early in chikungunya virus infection. Additionally, Heat shock protein 60 (HsP60) is a chikungunya virus receptor candidate mainly known as a mitochondrial molecular chaperone which is involved in protein folding.[28] The protein has also been detected at the cell surface of murine monocytes/macrophage, B lymphocytes and T lymphocytes and human T lymphocytes. HsP60 was found to interact with chikungunya virus by a two dimensional Virus Overlay Protein Binding Assay (2D-VOPBA).

MOLECULAR MECHANISM OF CHIKUNGUNYA VIRUS FUSION

The fusion process includes: (1) destabilization of the E2/E1 heterodimer, (2) integration of the E1 protein in the target membrane, (3) E1 trimerization, and (4) fusion pore formation. Destabilization of the alphavirus E2/E1 heterodimer is triggered once the virus is exposed to the mildly acidic pH within the endosomes. Histidines having a pkA of ~6–7, within the envelope glycoproteins of Chikungunya virus, control the pH-dependent conformational changes during fusion proces.[29] Earlier work on SFV showed that protonation of this residue reduces the stability of the E2/E1 heterodimer by disabling the hydrogen bond with E1-S57. Loss of E2/E1 interactions, the B domain of E2 moves away and the E1 fusion loop is exposed. Thereafter, the E1 protein adopts an extended form and the hydrophobic fusion loop inserts into the target membrane. For SFV, this interaction is both low pH and cholesterol-dependent. The presence of sphingomyelin strongly stimulates cholesterol- mediated E1 binding, but is not strictly required. It is likely that these lipid interactions are similar for chikungunya virus, as cholesterol and sphingomyelin in the target membrane greatly enhance the fusion potential of chikungunya virus. One of the amino acids important for lipid- and pH-sensing of SFV, SINV and chikungunya virus is situated at the E1-226 position. This residue lies within the central DII domain in close proximity to the fusion loop. Chikungunya virus strains with a valine instead of an alanine at the 226 position are more dependent on cholesterol and require a lower pH for infection and fusion. Another residue that has been reported to play a role in SFV lipid recognition is E1-V178. This residue is conserved among most alphaviruses, and experimental mutation of this residue to alanine leads to decreased cholesterol dependence of chikungunya virus fusion.[30]

When the fusion peptide inserts into the target membrane, E2 is presumably still in association with the E1 molecules, as has been shown for SINV. As the pH further decreases, the E2 molecules completely dissociate, which enables E1 trimerization. A highly conserved histidine residue (E1-H3) is essential in regulating low-pH-induced trimerization. For SFV, the first step in E1 trimerization involves the formation of a core trimer between DI and DII, which is dependent on low pH and most likely the presence of cholesterol and sphingomyelin in the target membrane. Furthermore, sphingolipids have been proposed to play a role in stabilizing the E1 trimer.[31] After formation and stabilization of the core trimer, domain III re-folds back independently of pH towards the core trimer to form a hairpin-like homotrimer. This process brings the two opposing membranes together and forces merging of the outer membrane leaflets (hemifusion). Subsequently, a fusion pore is formed and expands, through which the nucleocapsid gains access to the cytosol. For SFV, it has been shown that several E1 homotrimers assemble in a ring-like structure on the target membrane with recent research indeed suggesting that for chikungunya virus fusion, several trimers need to act simultaneously to mediate membrane fusion.[32]

INTERFERENCE OF CHIKUNGUNYA VIRUS INFECTION PROCEDURES

Interference of Chikungunya virus infection procedures includes antiviral treatments and prevention of disease incidence. Antiviral treatment is symptomatic and includes antipyretics, analgesics and anti-inflammatory (eg, Paracetamol, naproxen, meloxicum) etc for treating fever, pain and inflammation. Ribavarin and INF-alpha 2b gives synergistic effects and ribavarin with doxycycline and favipiravir decrease replication and inflammations. Treatment with bindarit also makes symptoms mild and short duration in animal model. Mouse studies have revealed that antibody-based therapy might prevent persistent infection. To date, multiple inhibitors have been recognized distressing different stages of the viral life cycle. Specifically they interfere with: (1) attachment of the virus to the target cell, (2) endocytosis, and (3) membrane fusion. Epigallocatechin-3-gallate (EGCG) was found to inhibit chikungunya virus attachment and infection of HEK293T cells with a broad antiviral activity and most likely it acts as competitive antagonist of heparan sulfates and sialicacid.[33] Flavagline interferes virus –receptor binding through prior binding to the chikungunya virus attachment factor PHB. Monoclonal antibodies (MAbs) like MAbs CHK-9, m242, and IM-CKV063 have been described to target the E2 putative receptor-binding domain A to prevent cellular binding. Furthermore, antibody-binding to chikungunya virus particles will target the immune-complex to Fc receptor-expressing cells which internalize the particle via interaction

of the antibody to the Fc receptor. Chlorpromazine has been implicated to block the formation of clathrin-coated pits as an antivirals.[34] The anti-malarial drug chloroquine, which inhibits acidification of endosomes, was also found to hamper infection although some weakness.

CONCLUSION

Since the re-emergence of chikungunya virus, scientists understanding of the biology of the virus have greatly improved. Important progress has been made in defining the cells targeted and the different pathways of occurrence. Identifying the cells that serve as the main viral factories during the viremic period and produce most virus progeny will not only increase understanding of chikungunya virus pathogenesis but will also guide the development of antiviral treatments and prevention and it is very important. Persistent of chikungunya virus replication is associated with chronic arthralgia, the understanding of the underlying mechanism is poor. More *in vivo* studies in animal models will be required to gain more insight into the correlation of chikungunya virus tropism and viral persistence. Still, there are several molecules that act as an attachment factor rather than an entry receptor, facilitating and pivotal for chikungunya virus infection. On the other hand, confusion may arise that an entry receptor exists or not, considering the broad range of cells being infected and the fact that no receptor is required for membrane fusion. However, chikungunya virus can utilize a variety of attachment factors which may be sufficient to enter a cell.

Chikungunya virus enters cells via clathrin-mediated endocytosis and fuses from within early endosomes. Interestingly, novel adaptations in the emerging IOL strains like the A226V mutation in E1 and substitutions in the acid-sensitive region of the E2 protein alter the pH-dependent membrane fusion properties of the virus. Furthermore, increased infection of mosquito mid gut cells was observed, which likely led to the enhanced fitness of the virus in *Ae. albopictus*. It will be interesting to investigate if there is a direct correlation between the higher infection rate and the altered pH-dependent membrane fusion properties. For example, the site of membrane fusion may be important for successful initiation of infection. Clinical study revealed that chloroquine, gave disappointing results. Continual discovery of antiviral inhibitors is of utmost importance given the high burden of chikungunya virus infection. Antibody-based therapy is an attractive approach especially as it has been shown that it might prevent persistent infection in mice and this therapy always consist of a set of antibodies is effective in humans is expected soon as a current clinical trial is evaluating the effect of anti-

chikungunya virus serum antibodies in preventing severe disease in neonates.[35] This is because chikungunya virus contains multiple receptor binding domains and it is unlikely that one antibody abolishes virus-receptor interaction in all chikungunya virus permissive cells. So we hence postulate that antibodies targeting the membrane fusion machinery of the virus represent the most robust entry inhibitors. Therefore, focus should be laid on the further identification of neutralizing antibodies prying with membrane fusion and other premature procedures.

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