Dengue Viral Non-Structural Protein Induced Host Cubic Membrane: A Target for Understanding and Combating Viral Infections

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The virus used in this study DENV-2 (Strain number DENGU-2 P-23085) was maintained in National Institute of Virology (NIV), Bangalore. Viral stock was obtained by inoculating a monolayer of BHK-21 cells. Infection and Replication efficiency was confirmed by cytopathic effects (CPEs). Briefly, monolayer of BHK-21 was infected at multiplicity of infection after 5 days, the medium was harvested, cleared from cellular debris by low speed centrifugation, aliquoted, andstored at −70°C.

Cell Line-C636

Aedes albopictus C636 cell line was maintained at the Cell culture Unit, National Institute of Virology. Mitsuhashi and Maramorosch Insect media (Hi-media) with containing 1% L-glutamine, 10% fetal bovine serum, 100 units/ml penicillin, 100 µg/ml streptomycin (Sigma)in 5% Co_2 at 37°C. Vero E6, Baby Hamster Kidney-21 (BHK-21),Human lung Carcinoma Cell line(A549) and Hela cell lines are also maintained in Eagle's minimal essential medium (MEM-Hi-media), containing 10% FBS at 37°C, 5% CO_2. Six well plates were seeded with $1x10^6$ cells/ ml of C6/36 per well and maintained for 1- 3 days at room temperature (25ºC – 28ºC).

Infection Monitoring By CPE

The cells of cell line at log phase were harvested, counted and transferred to three 25-cm^2 tissue culture flask (3×10^5 cells) were infected. The cultures were inoculated by 0.25 ml aliquots cell supernatants from dengue virus-infected Huh-7 cells were inoculated with 10-fold serial dilutions. The infected cultures were maintained at 25°C and examined every day for cytopathic effect and occurrence of occlusion bodies (OBs) in the nuclei. Photos were taken in Phase contrast Microscopy using Nikon LX 900 Camera 16 megapixel.

Transmission Electron Microscopy Analysis

Preliminary studies, which have demonstrated the formation of induced membranes in the course of DENV viral infection under the conditions of interfering with cellular cholesterol homeostasis, suggested the existence of direct link between viral infections, lipid

metabolisms and induced membrane formation. Based on this core evidence, we investigated the formation of induced membrane in C636 cells infected with DENV-2 at different time points 12 hours, 24 hours, 48 hours and 72 hours and the structural analysis was done by Transmission electron microscopy (TEM) from NIMHANS.

2D-SDS PAGE and MS analysis Techniques for the Identification of Proteins

2D-LC/MS Techniques was done from National centre for biological science, Hebbal, Bangalore. 48 hours infected cells were arrested and pelleted for MS analysis. In this we used online and offline multidimensional liquid chromatography (LC) in proteomics using an 1100 LC system are included. Proteome profiling by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) has become an important complement to gel-based protein analysis. The typical LC workflow involves protein digestion by trypsin followed by peptide separation by 2d Gell chromatography coupled to MS/MS detection. However, for complex peptide mixtures such as whole cell lysates, additional separation steps should be performed before LC-MS/MS to reduce complexity. These steps can be performed on either the protein or the peptide level that is, before or after enzymatic digestion, respectively, or on both levels.

Thin Layer Chromatography

We used Thin-layer chromatography (TLC) which is a widely used, fast and relatively inexpensive method of separating complex mixtures. The "lipids" is used here in a broad sense and comprises fatty acids and their derivatives as well as substances related biosynthetically or functionally to the specific cell line. We focused on lipids that are most abundant in membrane systems, i.e. cholesterol and its derivates, glycerides, sphingo- and glycolipids as well as phospholipids.

Results Achieved

Infection Monitoring By CPE using Phase Contrast Microscopy: Cytopathic effect (CPE) is noticed as the monolayer cells deteriorate as a result of the viral infection. This destruction of the tissue cells in monolayer allows for easy monitoring and assessment of viral growth. At early stage of infection after 48 hrs, the cytopathic effect such as hypertrophy of nuclei, heavy clumping and adherence of the cells tothe substratum were observed. Numerous large clumps of cells were observed. Some of the cell aggregates that exhibited infected cells were

removed from the infected cultures and examined under microscope. The cells were loaded with OBs in the nuclei depending on the cell size in each cell line.

Transmission Electron Microscopy: TEM analysis showed that DENV infection induced the formation of vesicles, lipid droplet, Autophagy and convoluted membranes. Intriguingly, we also observed a more intense convoluted membrane formation and Autophagy after 48hrs post infection as compared to 24 hrs. Electron micrograph where taken by using Carl Zeiss SMT - Nanotechnology Systems Transmission Electron Microscopy.

Two-Dimensional Gel Separation And Identification Of Protein Variation: The Cell proteins were fractionated by 2DE in a non-linear gradient pH 3–10. Approximately 150 protein spots, distributed in a molecular mass range between ~17 and ~110 kDa and a pI range between ~3.5 and ~9.5, were detected in the Coomassie blue G-stained gels . Forty different protein spot entries were found in infected cell band among the 2 protein were identified as stress related protein.

TLC Plate Showing Lipid Variation in Infected Cell Sample: After running Thin layer chromatography we confirmed the presence of lipid variation in infected sample which showed in TLC plate.

Our Preliminary TEM analysis, DENV-2 infection on C636 cell line have been found to be inducing the formation of convoluted membranes, vesicles and lipid droplet formation. This biogenesis, massive proliferation and membrane rearrangement of intra cellular membranes are link to be viral replication and assembly in the host cell. This investigation will help us to elucidate how DENV infection tend to form convoluted membrane formation and affecting the cholesterol level, this study will give some clue for further understanding of the molecular mechanism of DENV pathogenesis. Altering the cholesterol level in DENV-infected cells, might play a role in facilitating formation of induced membrane for viral replication (McMahon et al,. 2005). In the first step, modulating the cholesterol level for facilitating the viral entry (Welsh et al,.2009). Secondly, by increase in the cholesterol level in the host cell, DENV -2 could alter the fluidity of the ER, enabling formation of vesicles, convoluted membrane formation, lipid droplet formation for viral replication. These vesicles are the site for viral particles assembly and replication. A study by Westaway et al., (1997)

revealed that, convoluted membrane is the source for WNV replication. However the functions of DENV- induced convoluted membranes remains to be elucidated. However, finding the key viral factors like proteins and lipids which are responsible for modifying these factors or preventing the formation of induced membrane by blocking the molecular mechanism might thus disturb the viral replication and assembly could offer new avenues to treat the viral infections.

Dengue is an arthropod-borne RNA virus of the *Flaviviridae* family, which comes 4 serotypes that cause dengue fever or dengue haemorrhagic fever in humans. An approximately 2.5 billion people in over 120 dengue-endemic countries worldwide are at risk of dengue infection, but many believe that, this number is not quite accurate. According to WHO, dengue virus (DENV) infects more than 50 million people, with approximately 22,000 fatal cases [world health organisation].

The number of countries reporting dengue virus (DENV) cases has increased dramatically in the past decades as a reflection of the expanding habitat of the vector *Aedes* spp, a result of the poorly planned urbanization of many cities in developing countries, an increased number of susceptible human hosts, and the rapid spread of DENV serotypes through global human travel networks. Dengue virus can cause an array of diseases from asymptomatic infection to Mild dengue fever (DF) or severe Dengue haemorrhagic fever (DHF) and Dengue shock syndrome (DSS).

Currently, there is no approved vaccine or antiviral agents against clinical dengue, necessitating prompt strategies to design effective antiviral strategies against this infection. DENV is carried and spread to humans by the primary mosquito vector *Aedes aegypti* and to a lesser extent *Aedes albopictus*. This investigation will help us to elucidate how DENV infection tend to form convoluted membrane formation and affecting the cholesterol level, this study will give some clue for further understanding of the molecular mechanism of DENV pathogenesis. Altering the cholesterol level in DENV-infected cells, might play a role in facilitating formation of induced membrane for viral replication.

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