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Dengue viral infection: untangling the host-viral knot

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Abstract

Dengue virus (DENV) infections cause significant morbidity worldwide. The complex host-viral interactions affecting viral replication and the host immune response result in variable disease severity in affected individuals. This oration describes the effect of DENV on the host stress response that occurs due to the overwhelming proteins trafficking in a cell during infection. Unfolded protein response (UPR), by the cell attempt to reduce this protein burden of the cell and avoid cell death. However, DENV preferentially activates some of the arms of this host response to increase virus replication and increase inflammatory mediator production.

Studies performed during the last few years on identifying this UPR and effect on oxidative stress in relevant primary cells *in vitro* are described in an attempt to understand the DENV disease pathogenesis better. Furthermore, these basic science findings were expanded to identify novel markers that could predict severe disease in clinical samples. These studies which identified transcripts and proteins as disease markers to predict dengue haemorrhagic fever in patients during early dengue infection are summarized. In addition, use of a class of drug, iminosugars that affect these host responses including oxidative stress is described as a host-directed approach in treating DENV infection. Overall, this reports how host responses could be exploited by DENV for pathogenesis and how we can use these markers as diagnostics and design host-directed therapy.

Introduction

DENV causes a re-emerging arthropod borne viral infection transmitted by the Aedes mosquito. Approximately one third of the global population is at risk of infection from this flavivirus which poses a significant health burden.[1] DENV serotypes 1-4 cause symptoms ranging from asymptomatic illness to severe haemorrhagic fever and death. Severe dengue disease is characterized by increased vascular permeability leading to hypovolaemic shock and multi-organ failure. Pathogenesis of severe manifestations is still poorly understood and the variable presentation is presumed to be due to complex host-viral interactions modulating viral replication and the host immune response. Many mediators such as cytokines (tumour necrosis factor α [TNFα], interleukin-1β [IL-1β], IL-6, IL-10), mast cell products (vascular endothelial growth factor, tryptase, chymase, histamine), lipid mediators (platelet activating factor, phospholipase-A2) and angiopoietin 1/2 have been implicated in affecting the endothelial barrier (reviewed in (2)). In addition, immune factors, viral factors and host responses influence the outcome of DENV infection.[3] The ability to distinguish between individuals likely to develop severe disease from non-severe disease during early DENV infection would greatly help clinicians to triage patients. In addition, it would reduce the healthcare burden by preventing admissions of patients who otherwise can be managed in an outpatient setting.

DENV utilizes the host cellular machinery, in particular the endoplasmic reticulum (ER), for the production of viral proteins.[4] ER stress caused by DENV infection mounts a prosurvival cellular reaction known as the UPR. This cascade of events, important not just in viral but also bacterial and non-pathogenic adverse conditions, maintains cell survival and facilitates eradication of the virus. The UPR has complex interactions with the cellular autophagy

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machinery, apoptosis mediators, innate immunity and pro-inflammatory reactions. To overcome this hostile host cell environment, DENV selectively modifies these host processes to enhance viral replication and has the ability to evade host innate immunity. As such, host-viral interactions play an important role in deciding the fate of the infection in affected individuals. It is important to explore the role of the UPR and its multiple cellular effects during DENV infection for a better understanding of the pathophysiology following infection, and to explore potential early markers of subsequent disease severity.

This oration describes the identification of host responses in DENV infection to,

a) Understand disease pathogenesis

b) Explore diagnostic potential; identifying novel markers of disease severity

c) Developing host-directed treatment

1. Understanding DENV disease pathogenesis: host-DENV interactions

The exact mechanism of increased vascular permeability in DHF is poorly understood. However, the occurrence of such severe manifestations in a minority of affected individuals and the predilection to develop severe fluid leakage and liver involvement in patients with a metabolic syndrome phenotype raises the possibility of complex host-viral interactions playing a role. One such mechanism is ER stress that occurs due to overwhelming amounts of protein in the endoplasmic reticulum of a cell during infection. This leads to the host cell responding with a stress relieving response, unfolded protein response (UPR). Literature revealed that this stress relieving mechanism per se has interactions with many inflammatory pathways, cell death pathways and immune response. We started summarising the literature available on DENV induced unfolded protein response and found that data are sparse. In addition, available studies have used cultured cell lines not relevant to a human dengue infection. The review was published highlighting the gaps in literature.[5] Following this review, experiments were designed to understand the effect of DENV infection on this stress response.

1.1 Study 1: Identifying the host unfolded protein response in DENV-infected primary macrophages

Methods and results

I isolated monocytes from buffy coats using a ficoll gradient approach and differentiated them to macrophages using granulocyte-macrophage colony stimulating factor (GM-CSF). Cells were then infected with DENV-2 grown in laboratory and cells were subjected to qRT-PCR or western blot to identify the UPR markers in the two main arms (PERK-ATF4 and IRE1α - XBP1) of this host response. Ethical clearance was obtained from OXTREC (Oxford Tropical Research Ethics Committee).

Results showed that DENV activates these different arms of the stress response in a time dependent manner and mainly one arm was activated strongly (IRE1α - XBP1). The effect on the PERK arm was not significant suggesting that viral infection has a differential effect on this host response.

After identifying that one arm of this host response is activated following DENV infection, I tried to delineate the effect of this activation. Therefore, I used chemical inhibitors to block these arms and measured the viral production and secretion of inflammatory mediators after DENV infection.

Results revealed that IRE1α -XBP1 arm which was preferentially activated by the virus in fact was pro-viral. There was reduced virus production if this arm was blocked and PERK arm did not have this effect. Inflammatory mediator production was also reduced by blocking these arms. In addition, significant differences in the behavior of DENV was seen in primary monocytes when compared to DENV-infected cultured cell line, THP1 cells and this highlights the importance of selecting the most appropriate cells for *in vitro* experiments.

Discussion

Overall, above results showed for the first time in a primary monocyte-macrophage cell model that virus can preferentially activate the cellular response (UPR) that develops in an attempt to get rid of the protein stress in the cell. Since activation of this UPR leads to increased inflammation in immune cells, the interaction of these cells with the endothelium or possibly mediators produced downstream of this activation could play a role in disease pathogenesis. In addition, activation of UPR could lead to cell death and such pathways if activated in hepatocytes during a DENV infection, could also play a role in the liver cell necrosis that occurs in some patients leading to acute liver failure.

This observation also raises another question. Could a higher UPR activation in patients with DHF be

a reason for the higher inflammatory mediators implicated in severe disease and could patients with diabetes, obesity have a higher UPR activation which could be a reason for the severe disease manifestations? Previous studies have suggested a possible role of the UPR in development of diseases such as diabetes, fatty liver disease and obesity.

In conclusion, I found that activation of the UPR following DENV infection is cell type dependent, time-dependent and a cell culture model such as primary cells would be most appropriate for *in vitro* experiments to understand disease pathogenesis better. Results revealed that DENV activates pro-viral and pro-inflammatory arm of the UPR in infected cells.[6]

To understand the role of UPR, it is important to have knockout cell lines with inactivated UPR genes and this allows a better comparison of the outcome following DENV infection. Available knockouts at present are in human fibrosarcoma cells which is not a relevant cell for understanding dengue infection. Since a cell culture model that closely resembles a human infection is needed, production of stem cell knockouts using CRISPR technology was initiated. Initial work started was completed by the group and the differentiated cells are being used to understand the effect of this pathway better. These could be differentiated to mimic hepatocytes, neural tissue and cardiac cells.

2. Identifying novel host-based diagnostic markers of severe dengue disease

As the previous experiments highlighted the important role of UPR in dengue viral replication and inflammatory mediator production, one question was whether this holds true in a human infection where complex cell-cell interactions could occur. As a clinician, the question arises whether this response could be an upstream event that might be determining the production of downstream mediator/mediators leading to endothelial dysfunction and fluid leakage. It is plausible that monocytes infected during the initial infection could be triggering the cascade of events leading to the cytokine storm.

Also, it might be the link between metabolic syndrome phenotype and severe dengue disease. If the UPR in fact is different in patients developing DHF, it should be upregulated when virus persist, therefore should occur in early dengue infection. As such, UPR markers could also serve as a diagnostic marker of severe disease. There could also be many other markers related to host-based pathways triggered in monocytes leading to severe disease apart from the protein stress response. Next experiments were planned to identify such markers in patient monocytes.

2.1 Study 2: Identification of host markers (UPR based markers) of severe dengue disease

Methods

To understand whether UPR genes are differently regulated, I isolated peripheral blood monocytes (PBMCs) from dengue-PCR positive patients during the first 72 hours of illness. Cells were isolated and RNA extracted using the Taqman Cell-Ct kit and subjected to qRT- PCR. Serum was subjected to dengue viral PCR for the confirmation of infection. Healthy blood samples were used as controls. After optimization and identifying several genes, 4 transcripts (sXBP1, ATF4, BiP, CHOP) were selected based on initial experiments. PBMCs of 15 healthy controls, 10 dengue fever (DF) and 5 DHF patients were used for the experiment. All samples were collected during the first 72 hours of illness and a repeat sample was collected 24 hours after resolution of fever. This repeat sample, labelled as “defervescence” indicated recovery in DF patients and peak critical phase in DHF patients. Samples were collected in Infectious Diseases Hospital in 2018 and laboratory investigations were performed in the University of Sri Jayewardenepura. Ethical clearances were obtained from ERC, University of Sri Jayewardenepura and OXTREC.

Results and discussion

Results indeed revealed that patients who subsequently developed DHF had significantly higher UPR transcripts related to both arms (IRE1-XBP1 and PERK-ATF4) during acute infection. In addition, the transcripts related to apoptosis (cell death) were reduced significantly in DHF patients during early infection suggesting that virus would potentially inhibit cell death pathways to persist in the infected monocytes and propagate. All transcripts normalized during defervescence. Dengue viral load in serum was not different in either group.

There are no previous reports of a relationship of UPR and dengue disease severity published so far. We have for the first time identified the role of UPR in a relevant *in vitro* model and in patient clinical samples.

Although this was quite interesting as well as exciting, doing qRT-PCR for identifying patients likely to develop severe disease is expensive, time

consuming and require expertise. Therefore, while validation in higher number of samples is in progress, further evaluation to simplify the diagnostic procedure is needed. On the contrary, proteins are much easier to identify and it is possible to develop rapid bedside tests using proteins as a marker. Therefore, a further study was performed to identify possible host-directed proteins that would be different in DHF patients compared to controls.

2.2 Study 3: Identification of host proteins of disease severity in dengue-infected patient monocytes

Methods

PBMCs were isolated from patients with PCR-confirmed dengue infection during first 72 hours of illness and lysed in RIPA buffer to produce cell lysates. These lysates were then subjected to mass spectrometry using a 10-plex TMT labelling. Mass spectrometry is a technique used to detect proteins by their mass. Since I was looking for proteins different among DF versus DHF samples, a tandem mass tag (TMT) labelling was done using 2 healthy controls, 4 DF and 4 DHF patient samples. Finally, all proteins identified in the samples were compared across to see whether there are any proteins significantly different (mean fold change>1.5, $p<0.05$) in the DHF group. Also, the quantity of identified proteins in the samples were used in STRING pathway analysis to identify the pathways upregulated or downregulated in DHF compared to DF. SPSS 25.0 and GraphPad Prism software were used to draw heatmaps and assess statistical significance. Ethical clearances were obtained from ERC, University of Sri Jayewardenepura and OXTREC.

This is the first such proteomic study done in monocytes of patients available in literature and the first study to obtain such data in early disease to predict a severe disease outcome.

Results and discussion

There were 7352 human peptides corresponding to 1931 proteins identified in the PBMC lysates by mass spectrometry. Results revealed that there were 90 proteins significantly different ($p<0.05$ and fold change >1.5) in DHF patient cell lysates during the first 72 hours of illness compared to DF cell lysates. A few pathways identified by STRING database for these proteins included neutrophil aggregation, platelet aggregation and activation, organization of the cyto-skeleton, regulation of body fluids and coagulation. Further validation of selected proteins that can be secreted from the monocytes and detected in serum will be performed in a larger group of DF and DHF samples.

Future work in progress:

Both UPR transcripts and protein markers (detected in serum) are being validated in larger number of patient samples to identify potential marker/markers with high specificity and sensitivity that can be used to identify DHF patients during the first 72 hours of illness.

3. Host targeted therapy for DENV disease: exploiting DENV-host interactions

Host directed therapy for viruses is important as it avoids resistance and lead to broad spectrum antiviral activity. DENV is an enveloped virus and need the endoplasmic reticulum associated enzymes (α-glucosidases) for proper folding and assembly of the virions. This host response essential for enveloped viruses with glycoproteins to produce infective virions, provide a target for therapy.

Iminosugars are sugar mimetics that can inhibit the glucosidases needed for proper folding. Our group has already published that treating DENV-infected primary monocyte-derived macrophages *in vitro* result in reduced viral output.[7,8] DENV infection is characterized by vascular leakage after the viraemic phase. Most patients get admitted to medical settings after 3-4 days of illness. Therefore, a drug that could potentially reduce the hyper-inflammatory reaction of patients would be most beneficial rather than pure antivirals.

In this section, I have described my work on a host-directed drug which was seen to have wide array of anti-inflammatory activity in addition to antiviral effects. I further looked at the effect of a selected drug on the host stress response, UPR to see whether these drugs could exploit this pathway for a therapeutic benefit.

3.1 Study 4: Antiviral and anti-inflammatory effect of iminosugars

To further supplement the *in vitro* data already available in primary macrophages, I looked at several iminosugar derivatives (synthesized to reduce toxicity

and increase efficacy) in another important cell, primary dendritic cells in DENV infection. In addition to reduced virus output at different drug doses, these drugs also reduced secreted TNFα in high doses of treatment. All the doses tested were not toxic in cell viability assays.

Previously, the group has published that low dose treatment (inhibits – α-glucosidase II) is adequate to reduce viral replication.[9] However, my data showed that high doses inhibiting both α-glucosidases I and II were important for anti-inflammatory activity and high doses potentiate the antiviral effect.[10]

3.2 Study 5: The effect of iminosugars on the UPR and oxidative stress

Following the anti-inflammatory effect of one iminosugar seen in *in vitro*, we wanted to understand whether this effect was due to reduction in virus infection seen in cells following treatment or an independent anti-inflammatory effect. Further experiments using bacterial toxin, lipopolysaccharide and the fungus, *Candida albicans* revealed that this anti-inflammatory effect was seen across all these treatments suggesting a broader anti-inflammatory role for these host-directed drugs. A transcriptomic experiment looking at genes differentially expressed during drug treatment suggested that possibly this drug would be exploiting oxidative stress or the UPR in achieving this effect.

I looked to see whether treatment with this drug would reduce oxidative stress or have a beneficial effect on antioxidant capacity. Results revealed that treatment with iminosugars reduced reactive oxidative species.[11]

Following the favourable effect seen in oxidative stress with this drug during treatment, I also looked at the effect on the UPR, the stress response that has an important role to play in dengue infection as described before. My *in vitro* experiments were done in primary monocyte-derived macrophages and cells were treated with a long chain iminosugar. I used several techniques to identify the effect of the drug on the UPR as described before. Results showed that drug increased the PERK-ATF4 arm non-significantly. However, there was reduction in the activation of the pro viral IRE1α - XBP1 arm by the drug and this effect was also seen following treatment of other stress inducers such as tunicamycin, DTT, thapsagargin suggesting that this host-directed drug reduces the detrimental arm of the UPR during DENV infection and situations of protein stress. Although the exact mechanism and the effect of this beneficial response on the UPR is being explored, it is clear that some of the anti-inflammatory effects or antiviral effects could be driven by these results.

In conclusion, host-directed therapy using iminosugars result in broad antiviral effects and the use of high doses (inhibiting α-glucosidases I and II) have a broader anti-inflammatory effect across many pathogens. Also, these drugs reduce oxidative stress during DENV infection and reduce the pro viral arm of the UPR to create a favourable milieu for the host in infected immune cells.

Future clinical trials with identified clinical end points using these host-directed drugs would pave the way for future treatment of dengue infection.

Conclusions

Studies described in this report describe the important role of the host stress response, UPR in dengue-infected immune cells described *in vitro*. Results from studies in clinical patient samples confirm the important role of this host response in development of severe disease manifestations in a human infection. Several host proteins have been identified by proteomics which will be a platform to validate novel marker/s to identify severe disease during initial infection. Host-directed treatment using iminosugars provide an attractive approach to treat DENV infections which demonstrates broad anti-inflammatory effects, protein and oxidative stress reduction and antiviral activity.

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