FINAL REPORT

การศึกษากระบวนการออโตฟาจีซึ่งเป็นสื่อกลางของปฏิสัมพันธ์ระหว่าง
ไวรัสไข้เลือดออกและเซลล์เจ้าบ้าน

Autophagy as a mediator of the dengue virus: host cell interaction

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12-2012

BRG5280006

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The work described in this report was supported by

The Thailand Research Fund

ACKNOWLEDGEMENTS

The work described in this report was supported by research grant number BRG528006 from the Thailand Research Fund whose support I gratefully appreciate and acknowledge. I am also extremely grateful to Nitwara Wikan, Phitchayapak Wintachai, Mingkwan Panyasrivanit, Atefeh Khakpoor, and Pathama Klomporn who, although being students in my laboratory, I am pleased to call colleagues, and without whom none of the work detailed in this report could have been undertaken. I also express my gratitude to my collaborators including Drs Thananya Thongtan, Chutima Thepparit, Rojjanaporn Pulmanausahakul, Sittiruk Roytrakul, Lukkana Suksanpaisan and Professor David Murphy all who gave freely of their time and expertise. I also wish to express my deep gratitude to Khun Anchalee Nirachanon for undertaking the financial record keeping, as well as for tirelessly helping with all other administrative details associated with running this grant.

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Project code: BRG5280006

Project title: Autophagy as a mediator of the dengue virus: host cell interaction
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Project period: **30/06/2009 to 31/12/2012**

บทคัดย่อ

มีการประมาณการกันว่าประชากรโลกราวสามพันล้านคนอาศัยอยู่ในบริเวณที่มีความเสี่ยงที่จะเกิดการติดเชื้อด้วยไวรัสเดงกี่และในแต่ละปีมีการติดเชื้อเกิดขึ้นกว่าหนึ่งร้อยล้านครั้ง ไวรัสเดงกี่จึงยังคงเป็นโรคที่เกิดจากแมลงพาหะที่พบมากที่สุด แม้ว่าจะมีงานวิจัยจำนวนมากที่ศึกษาปฏิสัมพันธ์ระหว่างไวรัสเดงกี่กับเซลล์เป้าหมายแต่กระบวนการที่ไวรัสเดงกี่มีปฏิสัมพันธ์กับเซลล์เป้าหมายก็ยังคงไม่ชัดเจนและยังมีความไม่แน่นอนที่ว่าเซลล์ใดของมนุษย์ที่เป็นเซลล์เป้าหมายที่แท้จริงของไวรัสเดงกี่ อย่างไรก็ตามมีการศึกษาพบว่าเซลล์มนุษย์สองชนิดคือ เซลล์โมโนซัยท์และเซลล์ตับสามารถติดเชื้อไวรัสเดงกี่ได้ นอกจากนี้เซลล์โมโนซัยท์ยังมีบทบาทสำคัญในการติดเชื้อไวรัสเดงกี่ครั้งที่สองอีกด้วย มีการวิจัยจำนวนมากได้แสดงให้เห็นว่าเมื่อมีการติดเชื้อไวรัสเดงกี่ในครั้งแรกจะมีการกระตุ้นให้เกิดภูมิคุ้มกันต่อเชื้อไวรัสเดงกี่นั้น แต่ภูมิคุ้มกันที่เกิดขึ้นจะจำกัดอยู่ที่ไวรัสเดงกีชนิดแรกเท่านั้นไม่สามารถป้องกันการติดเชื้อครั้งที่สอง ที่เกิดจากไวรัสเดงกี่ต่างชนิดได้ ที่สำคัญนอกจากนั้นคือ แอนติบอดี้ที่เกิดขึ้นจากการกระตุ้นของการติดเชื้อครั้งแรกยังสามารถช่วยเพิ่มระดับการติดเชื้อของไวรัสเดงกี่ที่เป็นการติดเชื้อครั้งถัดมาและเชื้อไวรัสเป็นเชื้อคนละชนิดกับเชื้อครั้งแรก ผ่านทางกระบวนการติดเชื้อที่เรียกว่า antibody dependent enhancement และเชื่อว่ากระบวนการนี้เกี่ยวข้องกับความรุนแรงของโรค ในระยะแรกของงานวิจัยเราได้แสดงให้เห็นว่ากระบวนการออโตฟาจี ซึ่งเป็นกระบวนการย่อยสลายสารหรือโมเลกุลภายในเซลล์ ได้ถูกกระตุ้นในเซลล์ตับที่ถูกทำให้ติดเชื้อไวรัสเดงกี่ นอกจากนี้เรายังแสดงให้เห็นว่าปฏิสัมพันธ์ของกระบวนการออโตฟาจีในเซลล์ตับกับชนิดของเชื้อไวรัสเดงกี่เป็นแบบจำเพาะ และยังแสดงให้เห็นว่าปฏิสัมพันธ์ของกระบวนการนี้กับไวรัสเดงกี่มีส่วนช่วยส่งเสริมการติดเชื้อไวรัสเดงกี่ในเซลล์ตับ นำไปสู่การเสนอแบบจำลองใหม่ที่เชื่อมโยง กระบวนการออโตฟาจี กระบวนการเข้าสู่เซลล์ และการจำลองตัวเองของไวรัสเดงกี่ในเซลล์ตับ ในการงานวิจัยนี้ได้นำผลการทดลองที่ได้มาทำการศึกษาและวิจัยเพิ่มเติมถึงปฏิสัมพันธ์ของกระบวนการออโตฟาจีและไวรัสเดงกี่ที่ติดเชื้อในเซลล์โมโนซัยท์ จากผลการวิจัย แสดงให้เห็นว่าปฏิสัมพันธ์ระหว่างกระบวนการออโตฟาจีและไวรัสเดงกี่มีความจำเพาะกับเซลล์ที่มีการติดเชื้อ และการกระตุ้นกระบวนการออโตฟาจีในเซลล์โมโนซัยท์จะทำให้เกิดการลดปริมาณการจำลองตัวเองของไวรัสซึ่งได้ผลตรงข้ามกับผลวิจัยที่ได้จากเซลล์ตับ นอกจากนี้ปฏิสัมพันธ์ของไวรัสเดงกี่กับกระบวนการต่างๆในเซลล์โมโนซัยท์ยังได้รับการศึกษาเพิ่มเติมในด้านความเกี่ยวโยงกับการเกิด ER stress และความเกี่ยวโยงกับกระบวนการตายของเซลล์ (apoptosis) ซึ่งทั้งสองกระบวนการต่างก็แสดงให้เห็นว่ามีความสัมพันธ์กันกับการเกิดกระบวนการออโตฟาจี ความรู้ที่ได้จากการวิจัยนี้ได้ช่วยเพิ่มความเข้าใจของเราต่อปฏิสัมพันธ์ของไวรัสเดงกี่กับเซลล์โมโนซัยท์

คำสำคัญ: ออโตฟาจี, การตายของเซลล์, ER stress ,เซลล์โมโนซัยท์

ABSTRACT

It has been estimated that some 3 billion people live in areas at the risk of infection with the dengue virus, and that up to 100 million infections occur each year, making dengue the most common arthropod-borne viral disease. Despite significant effort worldwide, much of how the dengue virus interacts with a host cell remains unclear, and indeed there is even considerable uncertainty in humans as to what exactly are the host cells. However, both monocytic cells and liver cells have been shown to be genuine targets for dengue virus infection and monocytic cells are of particular importance in secondary dengue infections. Much work has shown that a primary dengue infection raises a protective immune response, but that this only has limited or no protective effect against a second dengue infection with a heterotypic virus. More importantly antibodies raised against the first dengue infection can mediate the internalization of a heterotypic virus in a subsequent infection through the process termed antibody dependent enhancement of infection, and it is believed this process underlies the association between secondary dengue infections and severity of the disease. In an earlier series of papers we showed that autophagy, the cellular lysosomal degradation pathway is activated in cases of dengue virus infection of liver cells. We not only showed that there were serotype specific differences in the interaction, but showed that the interaction between autophagy and the dengue virus enhanced virus infection. We further proposed a novel model that linked virus entry and replication. In this study those results were expanded to investigate the interaction between the dengue virus and autophagy in monocytic cells. Surprisingly we found that the interaction between dengue and the host cell autophagy pathway was cell type specific, and that induction of autophagy decreased virus output as opposed to the increase seen in liver cells. The interaction between dengue virus and monocytic cells was further examined in a series of studies that investigated the links between ER stress and apoptosis, both pathways that show significant cross talk with the autophagy pathway. Combined, these studies have led to a significant increase in our understanding of the dengue virus/monocytic cell interaction.

Key words: autophagy, apoptosis, ER stress, monocytes

Executive Summary

Introduction

Approximately 2.5 billion people live in areas at risk of infection with the dengue viruses, and up to 100 million infections are believed to occur annually [Guzman and Kouri, 2002]. While the majority of these infections are believed to be asymptomatic, infection may result in a febrile disease termed dengue fever (DF) or it may result in hemorrhagic manifestations which are classified as either dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS) dependent upon severity [Halstead, 1989]. The causative agent of DF, DHF and DSS are the dengue viruses. These viruses are classified in the family Flaviviridae, genus Flavivirus, and species Dengue virus. There are four antigenically distinct viruses, termed dengue serotypes 1, 2, 3 and 4 (DENV 1 to DENV 4). The dengue viruses are enveloped positive-sense single-stranded RNA viruses of approximately 11 kb and encode three structural proteins (core, pre-membrane and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) in one open reading frame [Chang, 1997]. The dengue viruses are transmitted primarily by Aedes aegypti mosquitoes, although other Aedes species such as Aedes albopictus may also serve as transmission vectors.

Infection with any one of the four dengue viruses results in the induction of lifelong immunity to that dengue virus. However, at best only a very short lived transient protection is provided against the other three dengue viruses, and so multiple rounds of infection with multiple dengue viruses can occur [Guzman et al., 2000; Sangkawibha et al., 1984]. In a second infection with a heterotypic virus, antibodies from the first infection can recognize and bind to the heterotypic virus, but broadly speaking not neutralize the virus. However, the complex of heterotypic virus and antibody from the proceeding virus can interact with the Fc receptor of Fc bearing cells (such as monocytes), promoting infection of these Fc receptor bearing cells. This process is termed antibody dependent enhancement of infection, and is believed to be one of the primary mechanisms behind the association between increased disease severity and second infections [Halstead and O'Rourke, 1977; Halstead et al., 1980]. This may result both from the cytokine responses of the infected Fc receptor bearing cells, as well as a significantly increased target cell population. Monocytic cells are therefore a critical cell in the pathology of dengue infections.

In previous studies [Khakpoor et al., 2009; Panyasrivanit et al., 2009a] we showed that liver cells, another target of the dengue virus [Smith and Khakpoor, 2009] induce autophagy upon infection. Autophagy is a lysosomal degradative mechanism for degrading cytoplasmic materials in eukaryotic cells [Eskelinen and Saftig, 2009; Levine and Klionsky, 2004; Meijer and Codogno, 2006]. Autophagy can be activated by stress conditions such as amino acid starvation or virus infection. In cases of virus infection, the interaction between the autophagy mechanism and the invading virus is thought to follow one of two routes: defense or subversion. In the case of autophagy as a cellular defense mechanism autophagy is induced to clear the cell of the invading virus. However, some viruses have adapted to evade the autophagy mechanism, by down regulating the pathway (as occurs with Herpes simplex virus). More recently it has become clear that some viruses have evolved to subvert the autophagy process by using the autophagic membranes as sites for viral replication (as occurs with poliovirus) and that biochemical treatments to down regulate autophagy result in a

reduction of virus production. In our earlier studies we showed that the interaction between the dengue virus and autophagy in liver cells was one characterized by subversion [Khakpoor et al., 2009; Panyasrivanit et al., 2009a], and that inhibiting autophagy reduced virus output. We more over showed that there were serotype specific differences in the nature of the interaction of dengue virus and autophagy [Khakpoor et al., 2009; Panyasrivanit et al., 2009a]. As a result of our studies, we proposed that virus entry and replication were linked through the structures termed amphisomes, formed by the fusion of entry endosomes and autophagosomes [Panyasrivanit et al., 2009b].

This current study aimed to understand the interaction of the dengue virus and autophagy in monocytic cells infected under conditions of antibody dependent enhancement of infection to determine whether autophagy mediated the interaction of the dengue virus with these critical host cells.

Results and Discussion

To understand the role of cell type in mediating the dengue virus - autophagy interaction we followed our earlier studies on this interaction in liver cells [Khakpoor et al., 2009; Panyasrivanit et al., 2009a] with studies in monocytic cells. Liver cells are capable of infection in both primary and secondary dengue infections, while monocytic cells are involved predominantly as a dengue virus target in secondary infections when non-neutralizing antibodies from a primary infection facilitate entry into monocytic cells which bear Fc receptors. The model cell line utilized was U937 cells, which are frequently used as a model system to investigate the mechanism of antibody dependent enhancement (ADE) of dengue infection.

We initially sought to optimize the conditions of infection of U937 cells with dengue virus [Klomporn et al., 2011]. This was undertaken by first confirming that U937 cells did not support direct infection, and then determining the correct dilution of antibody to allow for ADE infection. Optimal conditions were determined for all four dengue viruses (DENVs).

To undertake the dengue/ autophagy interaction analysis we initially confirmed that autophagy was able to be modulated in this cell type by inducing autophagy through starvation and through the use of rapamycin, two well characterized inducers of autophagy in other cell types. The induction of autophagy was monitored through the increased association of LC3 and LAMP1 as observed by confocal microscopy and statistical analysis using Pearsons correlation coefficient. Results showed that both starvation and rapamycin treatment induced autophagy in U937 cells [Panyasrivanit et al., 2011].

Using the protocol established [Klomporn et al., 2011], U937 cells were infected with dengue virus serotype 2 (DENV 2) under conditions that resulted in at least 80% infection of U937 cells, and cells again examined for the induction of autophagy as assessed by colocalization of LAMP1 and LC3. Results showed that dengue virus infection significantly induced autophagy [Panyasrivanit et al., 2011].

To determine the effects of modulation of autophagy on dengue virus output, we first confirmed that two chemicals (rapamycin, and autophagy inhibitor and L-asparagine, an inhibitor of lysosomal fusion had the appropriate cellular consequence by looking by western blot for increase in the autophagic membrane associated form of LC3 (LC3-II). Results were consistent with the expected results, and both rapamycin and L-asparagine modulated autophagy in U937 cells as they have been show to do in other cell types such as liver cells [Khakpoor et al., 2009; Panyasrivanit et al., 2009a]. We further established that these chemicals were not cytotoxic to the cells.

When U937 cells were infected in the presence of rapamycin, results showed a surprising reduction of both extracellular and intracellular virus levels. This was surprising as liver cells showed a significant increase in virus production in the presence of this compound [Khakpoor et al., 2009; Panyasrivanit et al., 2009a]. In the presence of L-asparagine which inhibits lysosomal fusion of the autophagosome with the lysosome, only a marginal decrease in virus output was observed, suggesting that the process of lysosomal fusion only plays a marginal role in DENV production [Panyasrivanit et al., 2011].

Initial attempts to down regulate autophagy were unsuccessful, primarily because U937 cells were shown to be extremely susceptible to the cytoxocity of 3-methyladenosine (3-MA) a compound commonly used to down regulate autophagy. Experiments with other potential biochemical inhibitors of autophagy also failed to reduce autophagy without significant cytotoxic effects.

Down regulation of autophagy without significant cytotoxicity was finally achieved through the use of lentiviral vectors expressing dominant negative mutations of Vps34 (Vps34dn), a known inhibitor of autophagy. Experiments showed that this construct was able to down regulate autophagy, and cells transfected with this construct when subjected to DENV infection showed a slight, but significant increase in intracellular and extracellular virus production [Panyasrivanit et al., 2011].

The results from all arms of the study were therefore consistent, in that up regulating autophagy decreased virus production, while down regulating autophagy increased virus output [Panyasrivanit et al., 2011]. However, the results of modulation of autophagy while statistically significant, were modest, suggesting that autophagy is not a significant part of the dengue virus replication strategy in monocytic cells [Panyasrivanit et al., 2011]. Most importantly, these results were in contrast to our previous studies in liver cells [Khakpoor et al., 2009; Panyasrivanit et al., 2009a], and showed for the first time that the virus/autophagy interaction is cell type specific. It also suggests that attempts to use modulation of autophagy as a possible therapeutic regime against dengue infection is not a simple option and moreover that different results might occur in primary and secondary infections. We further showed that the induction of ER stress led to the activation of autophagy, and proposed a model whereby ER stress led to a reduction in the DENV replication capacity through the sequestration of ER membranes to autophagic membranes [Panyasrivanit et al., 2011].

To investigate further the interaction between dengue virus and monocytic cells, we also conducted a comprehensive analysis of the induction of ER stress and apoptosis, studying all four serotypes of the dengue virus based on the optimization of infection study described earlier [Klomporn et al., 2011]. ER stress is believed to be induced in viral infections by the influx of viral proteins into the ER, causing deficits in the cells ability to correctly complete protein folding. This results in the dissociation of the chaperone protein GRP78 from it cognate proteins (IRE-1, ATF6 and PERK) leading to down regulation of translation, increased folding capacity as well as, if the stress is unrelieved the induction of apoptosis.

Our results [Klomporn et al., 2011] showed that, contrary to a previous publication by other authors [Umareddy et al., 2007], all four dengue serotypes induced ER stress equally, and that several ER stress pathways were activated, including the unfolded protein response, and the NOXA/PUMA pathway. Interestingly, while ER stress lead to the induction of intrinsic apoptosis pathways, extrinsic pathways, most likely mediate through the action of TNFα, were also activated. Thus, multiple ER stress pathways were activated in response to dengue virus infection as well as multiple apoptosis pathways [Klomporn et al., 2011]. Interestingly, autophagy can also lead to the induction of apoptosis, and thus it is possible that as many as three independent cell death pathways are activated in response to dengue virus infection. The multiplicity of pathways probably reflect the importance of these cells to the body's defense mechanisms and serves to remove these infected cells from circulation as quickly as possible.

Details of research

All our previous studies undertaken on DENV virus infection in mammalian cells has been undertaken on directly infected cells, representative of primary infections [Chingsuwanrote et al., 2004; Ekkapongpisit et al., 2007; Jindadamrongwech and Smith, 2004; Jindadamrongwech et al., 2004; Khakpoor et al., 2009; Panyasrivanit et al., 2009a; Sithisarn et al., 2003; Suksanpaisan et al., 2007; Suksanpaisan and Smith, 2003; Suksanpaisan et al., 2009; Thongtan et al., 2004; Upanan et al., 2008]. It was therefore necessary to establish basic methodologies relating to infection of monocytic cells before commencing more detailed experiments. We initially established that monocytic cells were not directly infectable with dengue virus serotype 2 (DENV 2) by incubating the model monocytic cells (U937 cells) with different multiplicities of infection (m.o.i.) DENV 2 ranging between 1 and 20 and assaying for virus titer over 5 days post infection. The results showed no de novo virus production, showing that these cells are not able to be directly infected with DENV 2.

U937 cells were subsequently incubated with DENV-2 and either no antibody or serial dilutions of monoclonal antibody HB114, which we established to be non-neutralizing for infection (unpublished data). Virus titer over 5 days was quantitated as described previously and a significant enhancement of infection was seen when infection was undertaken in the presence of a 1:200 dilution of monoclonal antibody HB114. We subsequently investigated the percentage of infected cells under different infection conditions, and determined by flow cytometry that an optimized level of infection (>80% of cells) occurred when infection was undertaken with m.o.i. 50 in the presence of a 1:200 dilution of monoclonal antibody HB114 for DENV 2.

Using this optimized level as a basis, all four DENVs were individually optimized to obtain 80% infected cells, and individual optimized infection protocols were: DENV-1: m.o.i. 20; DENV-2: m.o.i. 50; DENV-3: m.o.i. 20; DENV-4: m.o.i. 10 all in the presence of a 1:200 final dilution of monoclonal antibody HB114. Using the optimized infection protocol, 2 main lines of investigation were conducted - firstly the autophagy/DENV interaction and secondly the pathways by which cell death was induced in response to DENV infection.

The autophagy dengue virus interaction in monocytic (U937) cells

To undertake an analysis of the interaction between DENV and autophagy in U937 cells, it was initially required to establish that the autophagy pathway was present in these cells, and that it was capable of being induced. This was achieved by subjecting these cells to two common autophagy induction techniques namely nutrient starvation and treatment with the chemical rapamycin. U937 cells were therefore either deprived of nutrients or incubated with rapamycin on glass slides for 1 hour, after which time the cells were fixed, and examined under a confocal fluorescent microscope for the co-localization of autophagy markers LC3 and LAMP1 [Klionsky and et al., 2012]. As expected, in response to these treatments, there was a significant increase of co-localization of these two markers and statistical analysis of the degree of co-localization in treated as compared to untreated cells by Pearson correlation coefficient analysis showed that the increase in co-localization was statistically significant. This result showed that the autophagy pathway was both present in U937

cells, and was inducible, showing that this cell line was a suitable experimental model system.

The induction of autophagy in these cells in response to DENV infection was subsequently investigated using both confocal analysis of co-localization of LC3 and LAMP1 as above (as compared to mock infected cells), and in addition a Western blot analysis of LC3, specifically one able to distinguish the autophagic membrane associated form of LC3 - LC3-II from the unprocessed form, LC3-I. Both analyses supported the induction of autophagy in response to DENV infection This result was in agreement with our earlier studies undertaken in liver cells [Khakpoor et al., 2009; Panyasrivanit et al., 2009a].

To determine the nature of the interaction between autophagy and DENV, the autophagy pathway was modulated in two ways. Firstly, cells were dual infected and treated with rapamycin to induce higher levels of autophagy, and secondly, cells were treated with L-asparagine during the infection process. L-asparagine has been shown to inhibit autophagic flux by inhibiting the step of fusion between autophagosomes and lysosomes, and both rapamycin and L-asparegine were shown to be non-cytotoxic to the cells. In both cases, both extracellular (secreted) and intracellular (internal) virus titers were determined by standard plaque assay.

When DENV infection occurred in the presence of rapamycin, a slight, but statistically significant reduction of virus titer (both extra- and intra-cellular) was observed. This result was in contrast to the results seen in liver cells, and suggests that the autophagy/DENV interaction was not subversive as has been seen previously. Indeed, the results would be consistent with an anti-viral role for autophagy. Interestingly, in the presence of L-asparagine (and disruption of autophagic flux) only a slight, and non statistically significant reduction in virus titer was observed, suggesting that autophagosome-lysosome fusion plays little role in the DENV-autophagy interaction, again in contrast to our earlier studies in liver cells.

To prove that autophagy serves to clear at least some of the virus in infected cells, it was necessary to down regulate autophagy in these cells. Experimentally, this proved to be very difficult. 3-Methyladenine (3-MA), a commonly used inhibitor of autophagy proved to be extremely cytotoxic to U937 cells, even at low levels. A number of other partially characterized inhibitors of autophagy were also examined, but these also either proved to be extremely cytotoxic, or had no effect upon autophagy in U937 cells.

Previous studies have shown that over-expression of a dominant negative mutant of Vps34 (Vps34dn), an autophagy regulatory molecule inhibits autophagy. We therefore constructed a lentiviral expression vector expressing Vps34dn which was transduced into HEK293T/17 and U937 cells, and the inhibition effect of over-expression of Vps34dn on autophagy was investigated by Western blot analysis of LC3. The results showed a reduction of LC3-II form in HEK293T/17 cells expressing Vps34dn on day 3 post transduction as compared to mock. Lower LC3-II expression was also observed in U937 cells expressing Vps34dn on days 3 to 5 post transduction as compared to mock infected cells. The expression of Vps34dn was not cytotoxic to cells, as shown by a flow cytometric analysis of transfected as compared to cells transfected with a lentivirus control expressing GFP.

Subsequently, U937 cells transfected with lentivirus constructs expressing either Vps34dn or GFP were infected with DENV-2 under conditions of ADE infection as described previously. Consistent with previous results a small but statistically significant increase in virus output (as assessed by standard plaque assay) was seen for both extracellular and intracellular virus titers. This result is consistent with autophagy playing a small, but defensive role in dengue virus infection of U937 cells. The effect of enhancement of infection seen in cells with down regulated autophagy was confirmed by the increased levels of viral proteins (E and NS1) seen in western blot analysis.

ER, ER stress and autophagy

A number of studies have proposed that DENV replication occurs in association with the ER. We therefore examined the co-localization of dsRNA and a predominantly ER resident marker, calnexin using immunocytochemistry and visualization by confocal microscopy as described earlier. We observed a high degree of co-localization between dsRNA and calnexin implying that the ER is the major site of DENV-2 replication in U937 cells.

As shown in the next section we showed that DENV infection of U937 cells induces ER stress and activation of the unfolded protein response (UPR) pathway. As other studies have shown that the induction of the UPR can lead to the activation of autophagy it is possible that the increase in autophagy seen in response to DENV infection results from ER stress and UPR activation. To test this hypothesis cells were treated with the sarco/endoplasmic reticulum Ca2+ ATPase inhibitor thapsigargin, a commonly used ER stress inducer and cells examined for the induction of the UPR (as evidenced through the generation of the stress specific splicing of the XBP-1 transcript), and for the induction of autophagy by western blot analysis to look for increases in the autophagic membrane associated form of LC3, LC3-II. Both UPR activation and autophagy activation were clearly shown, suggesting mechanistic link between these two processes.

To further establish the nature of the link, U937 cells were treated with thapsigargin, and subsequently infected with DENV and the production of extracellular and intracellular viruses established by standard plaque assay on days 1 to 3 post infection. Consistent with the results observed for the induction of autophagy through the use of rapamycin, a reduction of both intracellular and extracellular virus levels was observed. In addition, we also confirmed that rapamycin did not induce the UPR itself by treating cells with rapamycin and looking for UPR activation which was not observed.

ER stress and apoptosis

As noted earlier, we initially optimized infection of U937 cells with all four dengue virus serotypes. Interestingly, virus production of different DENVs from cells that are equally infected is different, and can differ by nearly 2log in virus produced. This suggests that some DENVs may inherently produce higher virus yields than others. As noted above, DENV infection of U937 cells induces the ER stress responses and the induction of the unfolded protein response. We established this by initially

looking for the stress specific splicing of the XBP-1 transcript, which is undertaken by the ER resident IreI protein. Upon the induction of ER stress, IreI, which is normally sequestered by the Er resident chaperone protein GRP78 is released, and processes the XBP-1 mRNA transcript. This can be readily observed by RT-PCR. We showed that, under conditions of equal infection, an equal activation of ER stress was observed for each of the four DENVs. This was particularly important as the studies of others have suggested that the UPR induction is serotype specific [Umareddy et al., 2007]. However, using optimized and non-optimized infection protocols we were able to show that non-optimized infection protocols (generating equal infection percentages) apparently gave serotype specific results as a result of non-equal infection of cells. When infection was optimized and the same experiment undertaken on cells infected with DENVs of different serotypes - but of equal percent infected cells - the induction of the UPR response was serotype independent. Thus we were not only able to disprove the results of the other group, but also show where their error lay [Klomporn et al., 2011].

Interestingly, in addition to the induction of the ER UPR stress response pathway, we were also able to document the induction of another ER stress response pathway, namely that mediated by NOXA/PUMA, two genes shown to be transcriptionally regulated by p53, and up regulated under conditions of ER stress. Prolonged ER stress is known to result in the induction of apoptosis, and we showed that one critical gene that is intermediate between the ER stress and apoptosis pathways, CHOP, was up regulated in DENV infection. DENV infection did result in an increase of cell death for all four serotypes in U937 cells as assessed by flow cytometry after annexinV/propidium iodide staining.

Mechanism of DENV induced cell death in monocytic cells

To investigate the mechanism of cell death in DENV infected monocytic cells, U937 cells were infected separately with all four DENVs under conditions of ADE. Western analysis was used to determine the activation of caspases 7, 8 and 9 which are respectively an executioner caspase and the initiation caspases for extrinsic and intrinsic apoptosis respectively. Interestingly, we observed activation of both the intrinsic and extrinsic cell death pathways. As this could result from cross talk between the two pathways (as opposed to the independent activation of both), we used a caspase inhibitor to specifically inhibit caspase 9 to determine if caspase 8 was still activated. The results showed that even when caspase 9 was not activated, caspase 8 was shown to be activated, suggesting the activation of both intrinsic and extrinsic pathways independently. Extrinsic cell death pathways are activated through the binding of a specific ligand, such as TNF-alpha or TRAIL. We showed through RT-PCR that TNF-alpha expression was increased in response to DENV infection, suggesting that this is the molecule that mediates the extrinsic induction of cell death, while ER stress leading to activation of the UPR and subsequent expression of CHOP leads to the induction of the cells intrinsic cell death pathway.

Conclusions

This study has provided significant insights into the interaction of dengue virus with one of the most important cell types in dengue infections, namely monocytic cells. Critically, we have shown that autophagy is induced in monocytic cells in response to

dengue virus infection, but the interaction is distinct from that seen in liver cells as reported by ourselves and others. While autophagy promotes viral production in liver cells, it inhibits viral production in monocytic cells. This suggests that therapies targeted against autophagy as part of an antiviral strategy, may have different results in primary and secondary infections. The mechanism by which autophagy inhibits dengue virus in monocytic cells remains unclear, but we have proposed that activation of authophagy reduces virus output as a consequence of recruitment of ER membranes to autophagic vacuoles (Bernales et al., 2006; Bernales et al., 2007) thus reducing indirectly the number of available sites of replication for DENV. Significantly we have shown both that dengue virus infection induces ER stress, and that ER stress can directly induce autophagy and that ER stress can induce apoptosis. Given that there is additional, as yet un-fully characterized cross talk between autophagy and apoptosis, it suggests that cellular response to dengue virus infection may results from a triangle of cross talking pathways mediating ER stress, apoptosis and autophagy.

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Output: Papers

1. Thongtan T, Thepparit C, Smith DR*. The involvement of microglial cells in Japanese encephalitis infections. Clin Develop Immunol. vol. 2012, Article ID 890586, 7 pages, 2012. doi:10.1155/2012/890586 Impact factor = 1.838 (ที่มา : Journal Citation Reports, 2011).

**2. Klionsky DJ, and 1269 others including Smith DR. Guidelines for the use and interpretation of assays for monitoring autophagy. Autophagy 2012;8: 445 - 544. Impact factor = 7.435 (ที่มา : Journal Citation Reports, 2011).

3. Thongtan T, Wikan N, Wintachai P, Rattanarungsan C, Srisomsap C, Cheepsunthorn P, Smith DR*. Characterization of putative Japanese encephalitis virus receptor molecules on microglial cells. J Med Virol. 2012;84:615-23. Impact factor = 2.820 (ที่มา : Journal Citation Reports, 2011).

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5. Pulmanausahakul R, Roytrakul S, Auewarakul P, Smith DR*. Chikungunya in Southeast Asia: understanding the emergence and finding solutions. Int J Infect Dis. 2011;15:e671-6. Impact factor = 1.938 (ที่มา : Journal Citation Reports, 2011).

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(** Note - Grant acknowledgement was not allowed for this paper, only affiliation address. It is however an autophagy review)

Output: Books

1. From the cell cycle to cancer: The birth, death and transformation of cells. Duncan R. Smith. Documentplus, Pathum-thani, Thailand, 2010. ISBN: 978-974-11-1307-1

2. A Flavivirus compendium. Duncan R. Smith. Institute of Molecular Biosciences, Mahidol University, Salaya, Nakhon Pathom, Thailand, 2012. ISBN: 978-616-279-097-3

Output: Book chapter

1. Smith, D.R. Encephalitic flaviviruses. In: Flavivirus Encephalitis. Daniel Růžek (ed) pp3-24. InTech, Rijeka, Croatia, 2011. ISBN 978-953-307-669-0.

Output: Proceeding

1. Sujitra Keadsanti, Sittiruk Roytrakul, Duncan R. Smith. Proteomic Analysis of Dengue Virus Infected U937 Cells. The 37th Congress on Science and Technology of Thailand (STT 37). 10-12 October 2011. Bangkok, Thailand.

2. Sirikwan Libsittikul, Kanokporn Triwitayakorn, Duncan R. Smith, Yun-kiam Yap. Construction of plant expression vectors for Dengue virus serotype 2 envelope protein domain III (D2EDIII). 1st ASEAN PLUS THREE GRADUATE RESEARCH CONGRESS. 1-2 March 2012, Chiang Mai, Thailand.

OUTPUT: ABSTRACTS PRESENTED AT SCIENTIFIC MEETINGS

Invited lecture

The cellular consequences of arboviral infection. Faculty of Science, Mahidol University. 8 December 2010

Oral Presentations

1. Atefeh Khakpoor, Mingkwan Panyasrivanit, Nitwara Wikan and Duncan R. Smith. DENV Serotype Specific Interaction with Autophagic Vacuoles as Sites for its Replication/Translation The 4th Asian Dengue Research Network Meeting. 8-11 December 2009, Singapore.

2. Mingkwan Panyasrivanit, Atefeh Khakpoor, Nitwara Wikan and Duncan R. Smith. The interaction between Autophagy and Dengue virus. 15th Biological Science Graduate Congress, December, 15th-17th, 2010. Kuala Lumpur, Malaysia.

Poster presentations

1. Smith D.R., Klomporn P., Khakpoor, A., Wikan, N. and Panyasrivanit, P. Induction of Apoptosis in Dengue Virus Infected U937 Cells. The 4th Asian Dengue Research Network Meeting. 8-11 December 2009, Singapore.

2. Pathama Klomporn, Mingkwan Panyasrivanit and Duncan R. Smith. Serotype-Independent Unfolded Protein Response Induction in Dengue Infection. The 4th Asian Dengue Research Network Meeting. 8-11 December 2009, Singapore.

3. Sujitra Keadsanti, Thitiporn Surit, Sittiruk Roytrakul and Duncan R. Smith. Proteomic analysis of dengue infected cells. The 5th Annual symposium of Protein Society of Thailand. "Protein Research: From basic approaches to modern technologies". 23-25 June 2010, Bangkok, Thailand.

4. Woraphol Rattanachuen, Nitwara Wikan, Lukkana Suksanpaisan and Duncan R. Smith. Infection of mammalian-derived and insect-derived dengue virus in various cell lines. The 3rd Commission on Higher Education Congress. 9-11 September 2010, Pattaya, Thailand.

5. Duncan R. Smith, Mingkwan Panyasrivanit, Atefeh Khakpoor, Nitwara Wikan. Autophagy as a mediator of the dengue virus: host cell interaction. TRF Junior-Senior Meeting. 14-16 October, 2010. Hua Hin, Thailand.

6. Pathama Klomporn, Mingkwan Panyasrivanit, Nitwara Wikan, Duncan R. Smith The mechanism of induction of apoptosis in dengue virus infected monocytes. Joint International Tropical Medicine Meeting 2010 (JITMM2010) and International Malaria Colloquium (IMC2010). 1-3 December, 2010, Bangkok, Thailand.

7. Kunlakanya Jitobaom, Chanida Fongsaran, Pham Ngoc Phuong, DuncanR. Smith, Chutima Thepparit. Analysis of Dengue virus E protein interacting proteins. The 7th International Symposium of the Protein Society of Thailand. 29-31 August 2012, Bangkok, Thailand.

Students

The following students completed their studies during the course of this grant:

Doctor of Philosophy (Ph.D.)

1. Dr Mingkwan Panyasrivanit passed his final Doctoral Degree exam. Thesis title: The interaction between autophagy and dengue virus.

2. Dr Atefeh Khakpoor passed his final Doctoral Degree exam. Thesis title: Mechanism of dengue virus-induced apoptosis in liver cells.

3. Dr Atichat Kuadkitkan passed his final Doctoral Degree exam. Thesis title: Characterization of mosquito dengue virus receptor proteins

Master of Science (M.Sc.)

1. Miss Pathama Klomporn passed her final Masters Degree exam. Thesis title: Linking ER stress pathways and apoptosis in dengue-infected monocytic cells.

2. Miss Sujitra Keadsanti passed her final Masters Degree exam. Thesis title: Proteomic analysis of DEN-2 infected cells.

Student awards

1. Dr Mingkwan Panyasrivanit : Mahidol University publication award 2010
2. Miss Pathama Klomporn : Mahidol University Distinguished thesis award 2011
3. Miss Sirikwan Libsittikul : Excellent Poster Award. The ASEAN Plus Three Graduate Research Congress, Chiang Mai University, 2012