MECHANISMS OF DENGUE VIRUS INDUCED APOPTOSIS IN LIVER CELLS

PIMJAI CHINGSUWANROTE

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ABSTRACT

Apoptosis is an active process of cellular self-destruction, which is required for normal development, immune cell proliferation and tissue homeostasis in multicellular organisms. Inappropriate apoptosis can contribute to the cause and progression of many diseases.

Infection with any of four serotypes of dengue virus can cause dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Dengue-induced hepatocyte apoptosis has been implicated in pathogenesis of the severe forms of dengue virus infection, DHF and DSS. However, the underlying mechanism of apoptotic induction in response to dengue virus infection is still unclear. Infection of the HepG2 (human hepatoma) cell line with dengue virus serotype 1-4 (DEN1-4) was shown induction apoptotic response in a serotype specific manner by using DNA fragmentation. The ladder was observed clearly after infection with DEN-1, 2 and 4, while low signal of apoptosis was shown after infection with DEN-3.

The 14 members of cysteine proteases, caspase family, are the main regulators of cell death. From studying in a mouse system, caspase-12 serves as the key mediator of ER-stress induced apoptosis. Dengue virus utilizes the host cell ER as an integral part of their proliferation, and may induce an ER-stress response and also ER-stress mediated apoptosis. Infection with DEN-2 but not DEN-3 induced ER stress indicated by increasing levels of GRP78 but the activation of caspase-12 was not observed by infection with both serotypes. This may result from the use of HepG2 cells which is a cell line derived from a human source. It has been reported that the function of caspase-12 was lost in humans, and as such another caspase may responsible for regulation of ER stress mediated cell death in human. Alternatively, other ER specific cell death pathway may operate simultaneously.

Infection with DEN-2 was shown to decrease pro-caspase-7 levels suggesting the involvement of a caspase dependent pathway. Inhibition of caspase mediated apoptosis by treatment with a broad caspase inhibitor, z-VAD-fmk showed a decreasing both the number of cells dying and virus production in HepG2 cells but not in Hep3B cells. This result suggests that a caspase independent pathway may mainly be responsible for induction of cell death in Hep3B. The number of dead cells correlated with the amount of virions production, and thus possible, the virus may induce apoptosis to facilitate the release of virion progeny.

KEYWORDS: DENGUE VIRUS/ APOPTOSIS/ ER STRESS/ CASPASE

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กลไกการก่อให้เกิดการตายแบบ apoptosis ของไวรัสเดงกี่ในเซลล์ตับ
(MECHANISMS OF DENGUE VIRUS INDUCED APOPTOSIS IN LIVER CELLS)

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บทคัดย่อ

Apoptosis คือกระบวนการทำลายตัวเองของเซลล์ซึ่งจำเป็นต่อพัฒนาการ การเพิ่มจำนวนของเซลล์ในระบบภูมิคุ้มกัน และการรักษาสมดุลของเนื้อเยื่อในสิ่งมีชีวิต การสูญเสียการควบคุมของกระบวนการนี้เป็นสาเหตุของการเกิดพยาธิสภาพของโรคหลายชนิด

อาการของกลุ่มโรคไข้เลือดซึ่งเกิดจากการติดเชื้อไวรัสเดงกี่สามารถทวีความรุนแรงขึ้นได้โดยการเหนี่ยวนำให้เกิดกระบวนการ apoptosis ของเซลล์ตับ แต่กลไกการเหนี่ยวนำในระดับโมเลกุลนั้นยังไม่เป็นที่ทราบแน่ชัด จากการศึกษาด้วยเทคนิค DNA laddering ในเซลล์มะเร็งตับชนิด HepG2 พบว่าการติดเชื้อของไวรัสเดงกี่ที่ก่อให้เกิดกระบวนการ apoptosis ขึ้นอยู่กับความจำเพาะเจาะจงต่อซีโรไทป์ของไวรัสโดยการติดเชื้อของซีโรไทป์ 1, 2 และ 4 สามารถก่อให้เกิด apoptosis อย่างชัดเจน ในขณะที่การติดเชื้อของซีโรไทป์ 3 ไม่เห็นผลชัดเจนนัก

caspase-12 คือโปรตีนที่ควบคุมกระบวนการ apoptosis โดยการเหนี่ยวนำอย่างจำเพาะจาก ER stress ไวรัสเดงกี่ซึ่งมีการเพิ่มจำนวนโดยอาศัย ER จะส่งผลให้เกิดให้เกิด apoptosis โดยผ่านการเหนี่ยวนำจาก ER stress การติดเชื้อโดยซีโรไทป์ 2 สามารถเพิ่มปริมาณโปรตีน GRP78 ซึ่งแสดงถึง ER stressแต่ไม่พบการเพิ่มปริมาณของโปรตีนชนิดนี้จากการติดเชื้อของซีโรไทป์ 3 อย่างไรก็ตามการติดเชื้อของทั้งสองซีโรไทป์ไม่พบการกระตุ้นของ caspase-12 ซึ่งอาจเป็นผลมาจากการที่ caspase-12 สูญเสียหน้าที่ไปในเซลล์มนุษย์และมีกระบวนการอื่นทำหน้าที่แทน

การติดเชื้อโดยซีโรไทป์ 2 ทำให้ระดับของ procaspase-7 ลดลง และจากการศึกษาโดยการใช้ตัวยับยั้ง caspase พบว่า ระดับของผลิตผลของไวรัสและเซลล์ตายลดลงในเซลล์ HepG2 แต่ไม่พบปรากฏการณ์นี้ในเซลล์ Hep3B ซึ่งอาจเป็นเพราะกลไก apoptosis ในเซลล์ Hep3B ไม่จำเป็นต้องอาศัย caspase นอกจากนี้ ยังพบว่าปริมาณของเซลล์ที่ตายเกี่ยวเนื่องไปในทางเดียวกับปริมาณผลิตผลของไวรัส ซึ่งเป็นไปได้ว่าไวรัสอาจเหนี่ยวนำ apoptosis เพื่อสนับสนุนการปลดปล่อยอนุภาคไวรัส

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LIST OF ABBRIVIATIONS

Å	Angstrom
BSA	Bovine Serum Albumin
ºC	Degree Celsius
cm	Centemetre
DEN-1, DEN-2, DEN-3 and DEN-4	Dengue virus serotype 1 to 4
DF	Dengue fever
DHF	Dengue haemorrhagic fever
DMEM	Dulbecco's Modified Eagle's medium
DNA	Deoxyribonucleic acid
DSS	Dengue shock syndrome
DTT	1, 4- Dithiothreitol
EBSS	Earl's Balance Salt Solution
EDTA	Ethelynediamine trichloroacetic acid
FBS	Fetal bovine serum
ΔFBS	Heat inactivated fetal bovine serum m
FCS	Fetal Calf serum
g	gram
g/cm^3	Gram per cubic centemetre
HBSS	Hank's balance salt solution
kb	Kilobase
M	Molar
M199E	Medium 199 / EBSS
mg/L	microgram per litre

LIST OF ABBRIVIATIONS (Cont.)

mm	millimeter
mm^3	Cubic millimeter
mM	millimolar
moi	multiplicity o infection
PBS	Phosphate buffer saline
pfu/ml	Plaque forming unit per milliliter
pi	post infection
PMSF	Phenylmethylsulfonylfluoride
RIPA	Radioimmunoprecipitation
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	revolutions per minute
SDS	Sodium dodecyl sulphate
TBS-T	Tris base saline-Tween20
µg	microgram
µg/ml	microgram per milliliter
µl	microlitre
UV	Ultraviolet
V	Volt
v/v	Volume by volume
w/v	Weight by volume
YE-LAH	Yeast extract- Lactabumin

CHAPTER I
INTRODUCTION

1. General consideration and epidemiology

Dengue is a mosquito-borne viral disease that has become a major international public health concern for over the past 40 years. Infection of humans causes a variable spectrum of illness from, asymptomatic, undifferentiated febrile illness (viral syndrome) to dengue fever (DF) and the more severe forms, dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) which can be fatal. The first epidemics of dengue-like disease were reported in the medical literature in 1779 and 1780, when outbreaks occurred in Jakarta, Cairo, and Philadelphia, and the outbreaks re-emerged every 10-30 years until 1939-1945 [1;2]. During most of this time, dengue was a benign, nonfatal disease that was characterized by high grade fever and severe bone and back pain [3]. The epidemiology changed dramatically in Asia and the Pacific during and after World War II, mainly because of the transportation of the susceptible allied and Japanese soldiers into the endemic areas, which provided the condition for the co-circulating of multiple dengue virus serotypes, combined with the uncontrolled population densities of the mosquito vectors, *Aedes aegypti* resulting from the ecological changing by war materials [3;4]. The first epidemics of DHF occurred in the Manila, Philippines in 1953-1954 and Bangkok, Thailand in 1958. In the 1980s and 1990s, epidemic DHF/ DSS spread into Cambodia, China, India, Indonesia, the Lao People's democratic Republic, Malaysia, Maldives, Myanmar, Singapore, Sri Lanka, Viet Nam, and several Pacific Island groups [5].

Nowadays, more than 100 countries experience pandemic dengue (figure 1). Some 2,500 million people, or two fifth of the world population are now at risk for dengue virus transmission. It is currently estimated by the World Health Organization (WHO) that there may be 50-100 million cases of dengue infection and 200,000-500,000 cases occur as the severe, life-threatening form, DHF,

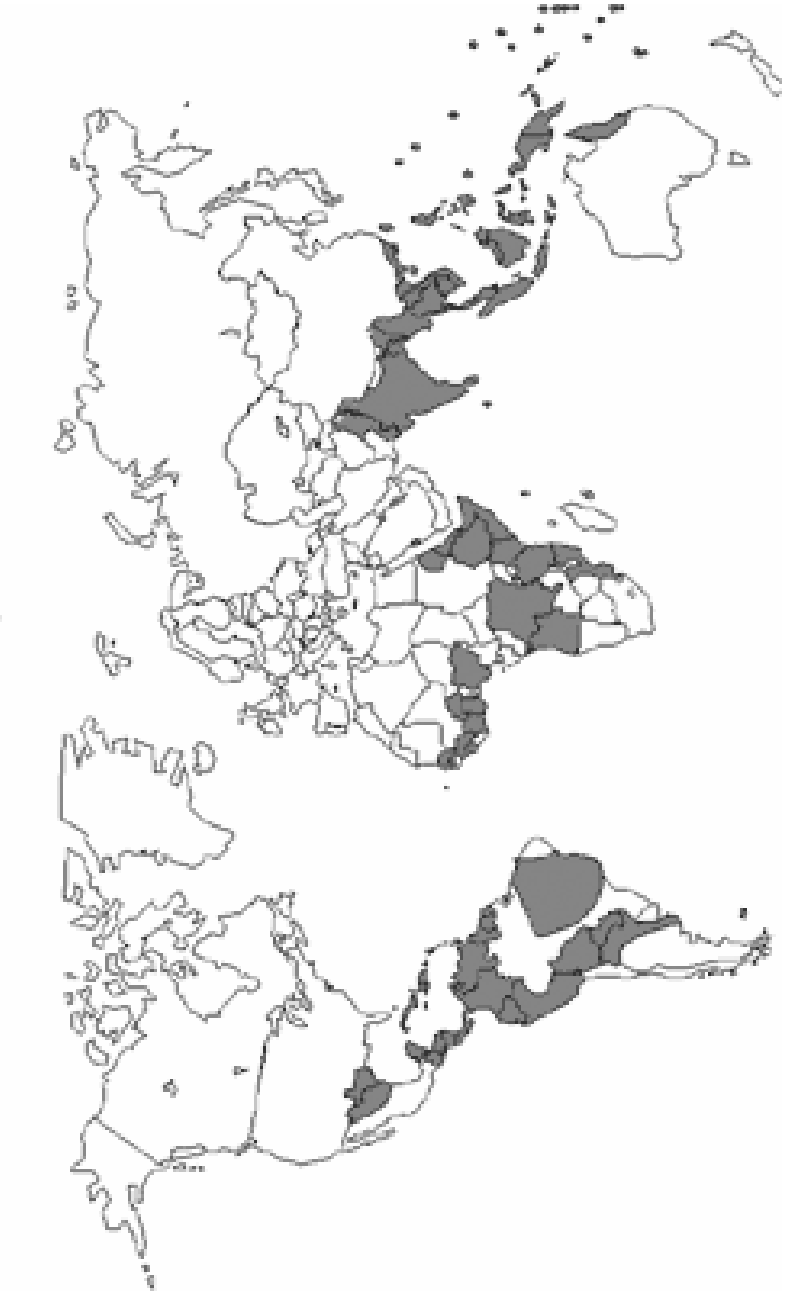

Figure 1: The General distribution of Dengue and/or Dengue Haemorragic fever in year 2001 [6]

The map shows the countries and areas reporting dengue cases in gray color.

resulting in around 24,000 deaths annually [6].

In Thailand, since the first large outbreak of DHF in 1958 in which 2158 cases and 300 deaths were reported, the reported number of cases has been gradually increasing. In the beginning, the epidemic of DHF occurred once every two years and the majority of cases were children aged between 2 to 6 years. The disease was mainly found in Bangkok and the surrounding area and spread from urban to rural area through out the country. Since 1965, the disease has been reported in all regions. In 1997 and 1998, the situation of DHF deserved serious attention when 101,689 and 126,348 cases were reported respectively. At the present, DHF is considered to be one of the top five most common communicable diseases in Thailand, which are, diarrheal disease, conjunctivitis, pneumonia, DHF and malaria [7].

2. Clinical manifestations and pathogenesis

2.1 Clinical manifestations

The clinical features of dengue may vary with the age of the patients and with repeated infection, which range from the clinically inapparent infection or lead to mild febrile disease, even death (figure 2). The incubation period is 4-7 days (range 3-14) after the mosquito bite. Infants and young children often have an undifferentiated febrile illness that is similar to other viral infections. In contrast, the more severe form is usually seen among older children, adolescents, and adult. Classic DF is characterized by acute onset of high fever (≥39ºC), frontal headache, retro-ocular pain, muscle and joint pain, incapacitating myalgias and arthralgias, nausea, and vomiting. The febrile period lasts for 5-6 days, and may leave the patient feeling tired for several more days. Sometimes the fever can return to almost normal in the middle of the period (biphasic or saddle-back temperature curve). More than 50% of patients report a rash that may be a flushing of the face, neck, and chest initially in the febrile period, or a centrifugal maculopapular rash and become confluent and sparing small islands of normal skin [8;9]. Haemorrhagic manifestations do occur in some cases such aspetechaie, epitaxis, gingival bleeding, gastrointestinal bleeding, microscopic Haematuria, and hyper menorrhoea [10;11].

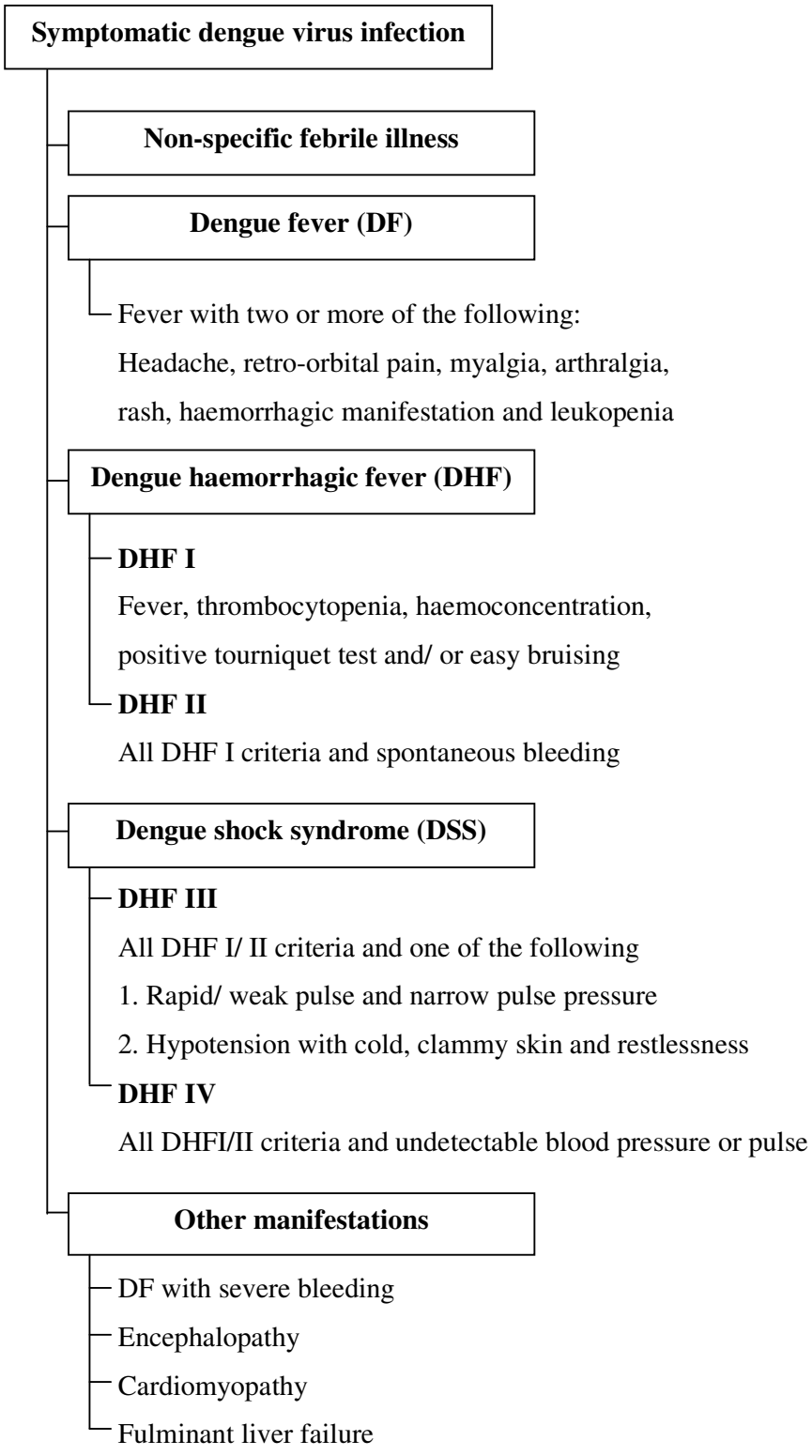

Figure 2: Classification of symptomatic dengue virus infection [3]

Children who experience secondary infection are more likely develop DHF than DF and more commonly seen in children less than 15 years in hyperendemic area. DHF is characterized by acute febrile illness with haemorrhagic manifestation, thrombocytopenia ($\leq 10^5$ cells/ mm^3), and evidence of plasma leakage due to increasing vascular permeability such as serous effusion and haemoconcentration (haematocrit $\geq$ 20%). Liver enlargement has been reported in up to 40% of patients and liver enzymes are usually mildly abnormal but jaundice is rare [12;13]. The condition of these patients may rapidly evolve into a critical stage of shock (DSS) and die within 12-24 hours, or quickly recover after appropriate volume replacement. The World Health Organization (WHO) has classified DHF into four grades according to clinical severity (figure 2).

It has been reported for the other unusual manifestation in the area that has high rate of infection. These are DF with severe of haemorrhage, fulminant liver failure, cardiomyopathy, and neurological phenomena such as altered consciousness, convulsion, and coma by encephalopathy which result from the leakage of plasma into serous spaces, haemorrhage, shock and metabolic disturbance [14-16].

2.2 Pathogenesis of DHF

The severe pathological changes in DHF/DSS occur from two main causes. One is an increased vascular permeability that gives rise to loss of plasma from the vascular compartment. Among possible mediators that cause increased vascular leakage, complement activation fragments C3a and C5a, both anaphylatoxins, and vasoactive cytokines, such interferon-γ and TNF-α have been implicated [17;18]. The second change is a disorder in haemostasis involving vascular changes, thrombocytopenia and thrombocytopathy. The simultaneous occurrence of several risk factors related to individual, epidemiological, and viral aspects determine whether a person in a given population will present with the clinical picture of DHF and DSS and whether an epidemic follows.

Epidemiological studies, conducted mainly in Thailand in the 1960-70s, suggested that severe manifestation occurred predominantly in children experiencing a second infection with a dengue virus serotype different from the one encountered in the previous infection. A mechanism that may involve in the development of DHF/DSS is called antibody dependent enhancement (ADE). In secondary infection

with a virus of different serotype, the presence of cross-reactive antibodies which do not neutralize the virus may enhance the efficiency with which the dengue virus infects susceptible cells. This in turn may result in the activation of cross-reactive CD4+ and CD8+ cytotoxic lymphocytes from the primary infection and the release of a number of cytokines and chemicals mediators [19;20]. These cytokines may induce capillary leakage through direct and indirect effects on the vascular endothelium. However, DHF and DSS in patients with primary infection have been reported also [21]. This suggests that other factors play a role of progression from DF to DHF/DSS [22].

There are the other individual risk factors which correlate with the development of severe form of disease such as age, sex, nutritional status, genetic predisposition, and viral virulence [23-25]. Viral risk factor is an alternative hypothesis for the pathogenesis of DHF/DSS which may explain the difference manifestation from absent clinical feature to life threatening. It is the capacity of virus to produce disease in a host such as ability to infect more cells and generate more progeny virus resulting in a high infectious viral load and gives a greater severity. It has been reported that the peak of viral titer of patients who develop DSS was 100- to 1000- fold of those with DF in dengue-infected in Thai children. The serotype and strain difference are also involved in severity. Several studies indicated that patients with secondary infection of serotype 2 have higher risk to DHF/DSS than other serotypes [26-28]. Non-virulent strain have previously been shown to replicate less well in ADE assay [29]. Furthermore, structural differences have also been found among various isolates of DF and DHF patients [30]. The virulent strains of dengue serotype 2 bound to monocyte and were internalized in the same way as the avirulent strains but that virulent strains were able to fuse with the phagosomal membrane at more basic pH [31].

Liver involvement is one of the factors which may be associated with the development of severe disease. It is suggested to be the major organ of dengue virus infection and replication. The impact of the dengue virus to the liver will be discussed in detail later. However, the mechanisms of pathogenesis of DHF/DSS remain elucidated.

3. Liver involvement

The pathogenesis of DHF/DSS is not well understood. Thus, whether the pathogenesis of severe dengue disease is due to mainly to viral virulence or to host susceptibility is unclear. However, the number and type of infected cells in target tissues and organs, the interaction between virus replication and cell function, and the nature of immune response to dengue virus infection govern the outcome of the disease.

Although the monocytic cells have long been considered to be the target cells of dengue virus infection [32], attention has been drawn to the involvement of the liver in DHF, since defective coagulation factors which can cause haemostatic abnormalities, has been report in severe patients and the liver is known to be the site of synthesis of most coagulation factors. Therefore, the impact of dengue virus to the liver may somewhat contribute to the severe manifestation. The reduced levels of coagulation factors are either the result of increased consumption or impaired synthesis. In DHF/DSS, the liver is always enlarged. Elevated serum transaminase levels found in dengue patients indicate the liver tissue is damage and the degree of AST level elevation correlated with that of haemorrhage [33;34]. In liver tissue obtained of fatal cases of dengue virus infection, there are histological changes with focal necrosis of hepatic cells, swelling and hyaline necrosis of the Kuffer cells, and the appearance of Councilman bodies (apoptotic cells). TUNEL tests are positive in necrotic areas, with positive cells forming clusters, suggesting that an apoptotic mechanism is involve in liver tissue injury. Furthermore, dengue virus antigen has been detected together with the apoptotic marker in the hepatocytes from necrotic areas of DHF patients by immunohistochemistry [35]. Several viruses have been shown to induce programmed cell death which often implicated to their cytopathogenic effects in host cells [36-42]. This evidence suggests that the liver is a real target organ of dengue virus infection and replication, and liver cell apoptosis in response to dengue virus infection may contribute to severe pathogenesis.

4. Dengue virus

4.1 Classification and particle structure

The dengue is classified as belonging to the genus *Flavivirus* of the family *Flaviviridae* which consists of over 60 arthropod borne viruses. Other viruses in this family, such as yellow fever virus (the family prototype), Japanese encephalitis virus and hepatitis C virus, are also important human pathogens. The four serotypes of dengue virus, known as dengue serotype 1, 2, 3 and 4 (DEN1, DEN2, DEN3 and DEN4) are antigenically different but closely related. The size of the mature dengue virus virion is approximately 50 nm diameter and consists of a positive single stranded RNA genome surrounded by an icosahedral or isometric nucleocapsid which is approximately 30 nm diameter. The nucleocapsid is covered by a lipid bilayer containing the envelope (E) protein and membrane (M) associated protein is about 10 nm in deep (figure 3). The virion has density of about 1.23 g/cm^3 as measure by equilibrium centrifugation in deuterium oxide-sucrose gradients and a sedimentation coefficient of around $210s_{20,w}$.

4.2 The viral RNA genome

The positive polarity single strand RNA genome is approximately 11 kb in length. The viral genome of the four serotype of the dengue virus consist of one open reading frame which contain 10,188, 10,173, 10,170, 10,158 nucleotides and 3,396, 3,391, 3,390 and 3,386 amino acids for DEN1, DEN2, DEN3 and DEN4 respectively. The genome is flanked by 5' and 3' nontranslated region (5' and 3' NTR) which proposed to regulate translation initiation at 5' NTR. The 5' NTR of type I 5'-cap is involved in secondary structure formation. The 3' NTR lack of a 3'-poly A tail is proposed to regulate viral replication [43]. The viral protein is translated into one long polyprotein precursor in order NH2-C-PrM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-COOH. The precursor protein is processed by proteolytic cleavage to generate individual proteins composed of 3 structural proteins which are capsid (C), membrane (prM/M) and envelope (E) proteins, together with 7 nonstructural proteins named NS1 to NS5 respectively. The maturation of the viral proteins is accomplished by co- and post- translational proteolytic cleavage with two different host proteases and one viral protease.

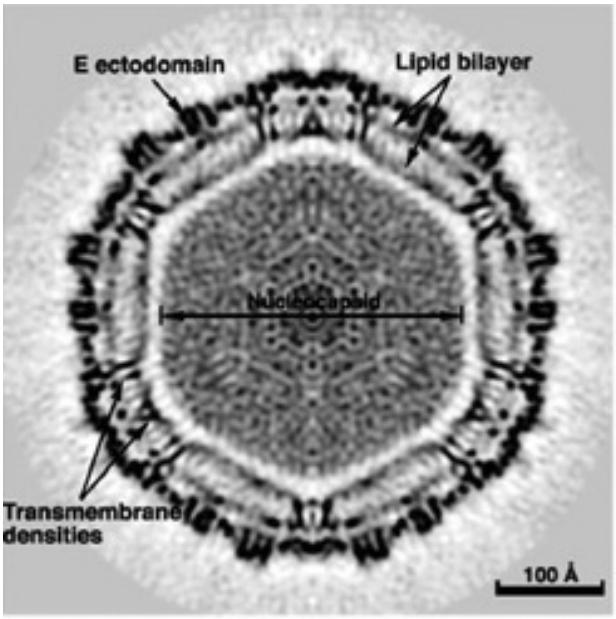

Figure 3: Structure of dengue virus virion [44]

A central cross section of dengue virus particle, viral RNA genome and capsid protein forms complex of nucleocapsid which surrounded by envelope protein and membrane protein. The scale bar presents 100 Å.

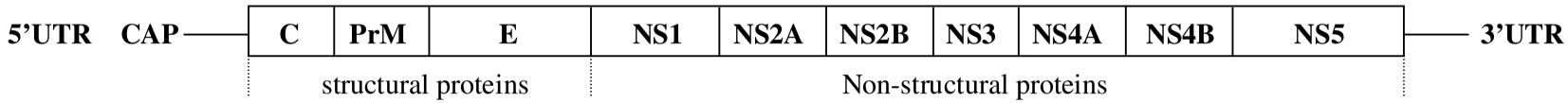

Figure 4: The dengue viral RNA genome

The figure shows gene order of the dengue viral RNA genome. The three structural (C, prM/M and E) and seven non-structural proteins (NS1to NS5) were presented. Each dengue serotype has the different ORF nucleotides and amino acid length.

DEN-1: 10,188 nucleotides; 3,396 amino acids

DEN-2: 10,173 nucleotides; 3,391 amino acids

DEN-3: 10,170 nucleotides; 3,390 amino acids

DEN-4: 10,158 nucleotides; 3,386 amino acids

4.3 Replication cycle

Similar to other viruses, the first step of dengue virus infection is attachment and penetration into susceptible host cells (figure 5). It has been reported that dengue virus can enter the target cells by two mechanisms, directed membrane fusion and receptor mediated endocytosis [45-47]. The approximated 60 kDal dengue E protein is the major viral antigen which mediates virus attachment to host cell receptors by acidic pH inducing an irreversible conformational change and mediated membrane fusion or by high affinity epitopes binding and triggering endocytosis [48-51]. In the case of receptor mediated endocytosis, two known mechanisms were proposed. The first one is immune infection enhancement or antibody dependent enhancement (ADE). The complexes of dengue viruses and non-neutralizing, but antivirion, immunoglobulin G (IgG) antibodies can be associated with Fc receptor-bearing cells and facilitate the uptake of viruses into host cell. The second one, dengue virus may interact with the host cell via a trypsin-sensitive virus receptor directly [45]. However, the identification of dengue virus receptor on target cells is still not definitive.

The uncoating step is occurs by a lowering of the pH inside the endocytic vesicle. The acidic condition of the endosome induce an irreversible conformational changed of virus E protein from dimer to trimer [52]. Then, the virion envelope may fuse to the host cell endosomal membrane and the nucleocapsid is released into the cytoplasm. This is followed by immediate translation of the polyprotein precursor from the uncoated viral genome. Early translation which occurs in association with rough endoplasmic reticulum (RER) facilitates localization of the viral proteins in their characteristic luminal, membrane or cytoplasmic context [53]. The host cell and viral proteases play a role in co-translational polyprotein processing to generate individual viral proteins.

The RNA replication appears to occur in the perinuclear region of the infected cells in association with smooth membrane. The viral replicase complex which formed by several NS proteins, binds specifically to the 3' untranslated region of the viral genome and subsequently copies the nascent positive-strand RNA into a negative-sense intermediate RNA strand. In this step, it generates partially RNase-sensitive heterogenous RNA called replicative intermediate RNA or RI-RNA.

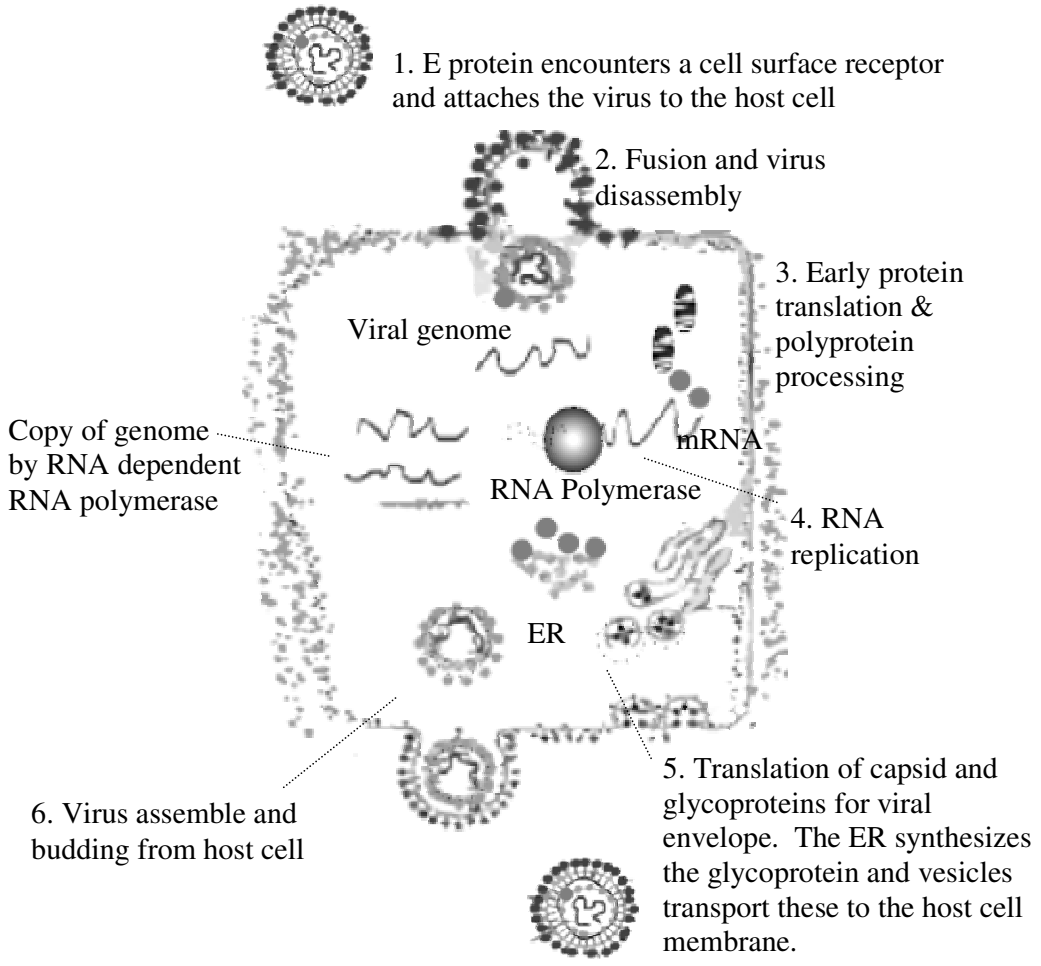

Figure 5: The life's cycle of Flaviviruses

Infection by a Flavivirus begins when its envelope E protein encounters a cell surface receptor and attaches the virus to the host cell. The host cell delivers the virus to a membrane-enveloped organelle called the endosome. The low pH of the endosome activates the E protein by promoting conformational changes of the protein. The activated E protein catalyzes merging of the viral membrane with the endosomal membrane, thereby allowing the viral RNA to escape into the cell. Then, early translation and polyprotein processing are occurred. RNA dependent RNA polymerase makes multiple copies of original positive strand RNA. Viral proteins and RNA are assembly and budding from host cell membrane. This picture was modified from reference [54].

This negative-strand serves as a template for positive-strand synthesis which generates another RNA form called, replicative-form RNA or RF-RNA. RF-RNA is RNase resistant, composed of a duplex genome length positive and negative stranded RNAs and can be used as a recycling template for the semi-conservative replication. Extensive membranous organelle, probably RER- and glogi-derived, appears to be a unique feature of flavivirus infected cells [55;56] , with these structure apparently compartmentalizing various aspects of flavivirus replication [57-60].

Nucleocapsid assembly from C protein and RNA and become envelope by budding through membrane which contained integral E and prM protein. The maturation of virion occurs in the exocytic vesicle by cleavage of the prM protein, resulting in a reorganization of the virion surface.

5. Apoptosis

5.1 Features of apoptosis

In 1972, Kerr et.al. coined the term apoptosis, after the Greek word meaning leaves falling from a tree, to describe an intrinsic cell suicide program involved in the normal turnover of hepatocytes [61]. Apoptosis or programmed cell death (PCD) is an essential mechanism required for development and tissue homeostasis of multicellular organisms. Disruption of this normal physiological cell death process can result in either excessive or insufficient apoptosis, which can lead to various disease states and pathology. Cell morphologic manifestations of apoptosis include condensation and fragmentation of the nucleus, shrinkage of the cytoplasm and the formation of apoptotic bodies that are small membrane-bound vesicles and are phagocytosed by neighboring cells before there is any leakage of cellular content. Because the plasma membrane of apoptotic cell remained intact, this mechanism does not induce an inflammatory response. In contrast to cell apoptosis, cells that die accidentally in response to an acute injury often do so by an uncontrolled process called cell necrosis. In necrosis, cells often undergo swelling and eventual rupture. The releasing of cytoplasmic contents, in addition to other events, triggers a pronounced inflammatory response [62;63] (figure 6).

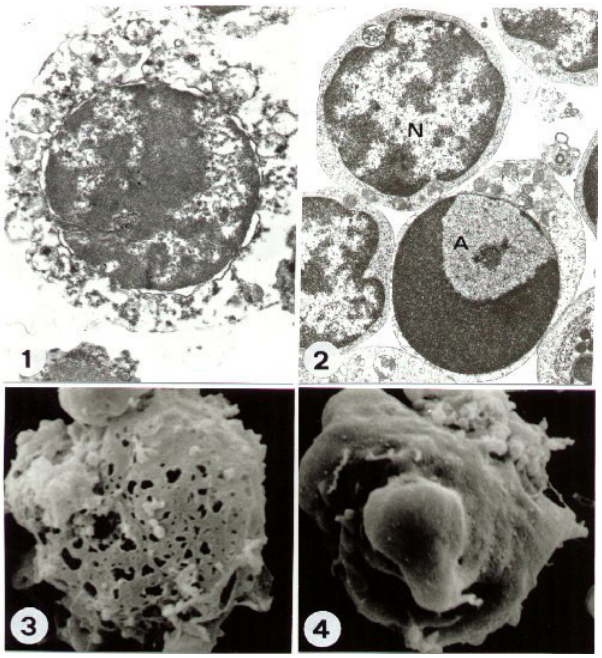

Figure 6: Morphological feature of cell apoptosis compare with cell necrosis [64].

The pictures show morphology of cell apoptosis (left) and cell necrosis (right). (1) The disruption of plasma membrane and organelles is observable including with a relative preservation of nuclear morphology appears in necrosis cell (original magnification: x 10,000). (2) TEM of an apoptotic (A) and a normal (N) cell. The characteristic chromatin rearrangement appears in (A), strongly different from its normal organization (N). The good preservation of membrane and organelles is also evident (original magnification: x 8,000). (3) SEM of a necrotic cell. Numerous lesions appear on the cell surface (original magnification: x 5,000). (4) SEM of an apoptotic cell. Surface blebbing is evident. (Original magnification: x 5,000).

When cells die via apoptosis, they undergo a number of morphological and biochemical changes that are stereotypical for this type of death. The affects cover all the alteration of cells from the plasma membrane to the nucleus. The lipids of the planar membrane are normally composed of a variety of both aminophospholipids (phosphatidylserine, PS and phosphatidylethanolamine) and choline phospholipids (phosphatidylcholine and sphingomyelin) [65]. In general, the cell maintains an asymmetry of orientation of phospholipids between inner and outer membrane by actively translocating PS from the outer to inner leaflet. During apoptosis, the equilibrium distribution of PS changes by an increased appearance in the outer leaflet. This exposed PS can be detected by the PS-binding protein, annexin-V, and serves as the function of recognition and phagocytosis by macrophages to remove the apoptotic cells [65;66]. Another prominent apoptotic change affecting the structure of the plasma membrane is the formation of blebs, or small, membrane-enclosed pieces of cytoplasm and condensed nuclear material. One of the final signs to appear during apoptosis is the inability of the plasma membrane to exclude dyes (e.g., trypan blue, ethidium bromide, and propidium iodide) which provides a convenient way to monitor cell death [67-69].

For the cytoplasmic changes cell shrinkage is another classical feature of apoptosis and can be observed from the very first report on the programmed cell death process [70;71]. This includes the degradation of cytoskeletal proteins and loss of cytoplasmic volume which results from membrane blebs. It has been shown that the loss of potassium gradient which is normally maintained by the plasma membrane may facilitate cell shrinkage [72].

In apoptosis, three mitochondria alterations are occur, osmotic swelling of the matrix, rupture of the outer mitochondrial membrane and the release of the pro-apoptotic proteins such as respiratory protein cytochrome *c*, apoptosis-inducing-factor (AIF), and in some cells, caspase-2, 3 and 9, from the inter-membrane space to the cytoplasm. These alterations result from the loss of maintenance of the mitochondrial membrane voltage ($\Delta\psi_m$) and pH gradient across the inner membrane and outer membrane. Although mitochondrial swelling is also found in necrotic cell death during apoptosis, the mitochondrial swelling is usually only a transient phenomenon.

In its normal state, the nucleus is a spherical structure with a relatively diffuse staining pattern. This staining pattern reflects different chromatin states with darker regions indicating inactive stretches of DNA bundled together with histones [73]. Using microscopy, several dramatic alterations are observed as a result of apoptosis. These changes include condensation of the nucleus such that it occupies less space and stains more intensely [61]. In addition, the nucleus often fragments into several sections [74]. These sections can also be released from the cell as part of apoptotic bodies [75]. Hence, a reduction in the total DNA content of the cell is a sign of an apoptotic cell death. On a biochemical level, these nuclear changes are associated with the activation of several different endonucleases. The action of the DNA degradative enzymes results in the formation of small fragments of DNA, often in multiples of 180 base-pairs reflecting basic nucleosome structure and resulting in a ladder pattern when analysed by agarose gel electrophoresis [68;75].

5.2 Mechanisms of caspases-dependent apoptosis

The mechanisms of apoptosis can be divided into three stages [76]. The first stage is the receiving of the apoptotic signal. The sensitivity of a cell to a variety of apoptotic stimuli depends upon the cell type. Cell receives apoptotic signal from both internal and external stimuli, includes perturbations of intracellular homeostasis, ligation of plasma membrane death receptors, removal of essential growth factors, or exposure to various chemical agent. Moreover, the exposure of cell to UV light or ionizing radiation, heat and changes to osmolarity can induce apoptosis [77-81]. In the second stage of apoptosis, the cell integrates the various signal and may, or may not commit to apoptosis. This process involves several signal transduction pathway such as the activation (or inactivation) of serine/threonine and tyrosine kinases and phosphatases, the synthesis of lipid second messengers including ceramides, altered gene expression, and the activation of specialized proteases known as caspases. Many cytoplasmic organelles are shown to integrate apoptotic stimuli such as mitochondria, nucleus, endoplasic reticulum, golgi apparatus, endosomes and lysomes [82]. The last stage is the activation of a common degradative/signaling pathway which triggers the acquisition of the characteristic morphological features [76].

Caspases are a family of cysteine aspatate proteases central in mediating cellular signals in apoptosis and inflammation [83]. They are among the most specific

proteases, with an absolute requirement for cleavage only after aspartic acid residues. Caspases are synthesized as inactive single polypeptide chain, procaspases, which contains three domains, an NH_2-terminal prodomain, a large subunit (p20) and a small subunit (p10) (figure 7). Caspases are usually activated by proteolysis that leads to conformational change of their catalytic parts [84]. The mature caspases are heterotetramers consist of two large and two small subunits aligned in a head-to-tail configuration and consist of an active site in each of the opposite ends. Activation occurs by one of two mechanisms. One of the activation mechanisms is initiation of autocleavage by interaction with other proteins. For example, caspase-8 is activated as a result of its interaction with Fas-associated death domain (FADD), and caspase-9 is activated through an interaction with cytochrome c, dATP (or ATP), and apoptotic protease activating factor-1 (Apaf-1). In the other mechanism, caspases are activated by cleavage of other caspases or other proteins. For example, both active caspase-8 and caspase-9 can cleave and activate caspase-3.

To date, at least 14 mammalian members of caspases have been identified, which are of human origin except for murine caspase-11 and caspase-12 that have no known human counterparts. Base on the sequence similarity among the domains of the large and small subunits, these caspases can be classified into three groups. Group I caspases are involved in inflammatory processes including caspases 1, 4 and 5 [85]. They are sometimes known as ICE-like caspases from another name of caspase-1, Interlukin-1 converting enzyme (ICE). Group II caspases contain caspases 6, 8, 9 and 10 [86]. These enzymes are known as initator caspases from their function which can activate the other caspases and initiate signaling cascade. The final category is group III caspases or effector caspases including with caspases 2, 3 and 7. These enzymes are activated by other caspases and go to cleave many cellular targets resulting in the acquisition of apoptotic morphology (figure 8). Activation of the effector caspases generally resulting in an irreversible commitment cell death [87;88]. These effector enzymes are the most specific, with a near absolute requirement for an aspartic acid in the first and fourth position prior to the cleavage site [86].

Caspases are responsible for cleaving numerous cellular targets, including structural elements, nuclear proteins and signaling proteins. Cytoskeletal proteins cleaved by caspases include lamin, α-fodrin, and actin [84]. It is possible that

cleavage of these proteins is associated with the dramatic morphological alterations seen in apoptosis [84]. Caspases have been shown to cleave and inactivate many nuclear elements including the U1 (70kDa) ribonuclear protein involved in RNA splicing and two enzymes involve in DNA repair, poly (ADP-ribose) polymerase (PARP) and DNA-dependent protein kinase (DNA-PK) (84;87;89-91). Caspases also activate two factors that lead to DNA fragmentation. These include a DNA fragmentation factor and inhibitor of caspase-activated DNase [84].

5.3 Mechanisms of ER stress-mediated apoptosis (role of caspase-12)

The endoplasmic reticulum (ER) is the cellular organelle which plays a critical role in a variety of process including the maintenance of intracellular Ca^{2+} homeostasis, synthesis and posttranslational modification and folding of membrane proteins. In almost all non-muscle cells, storage, release and uptake of Ca^{2+} are regulated by proteins of the ER. Accumulation of misfolded proteins in the ER lumen or interferance of Ca^{2+} homeostasis can induce ER stress and unfold protein response (UPR) which either improves local protein folding or results in cellular demise. The accumulation of unfolded proteins can be induced by agents such as tunicamycin, which blocks N-linked protein glycocylation, brefildin, which inhibits ER-Golgi transport, or dithiothreitol, which impairs the formation of disulphide bonds. Calcium ionophores such as thapsigargin which disrupt intracellular calcium homeostasis can also induce ER stress.

There is much evidence that shows the ER plays an important role in the apoptotic signaling process, which lead to activation of downstream caspases and other proteases. Caspase-12 is specifically localized on the cytoplasmic side (outer membrane) of the ER and is thought to play a role in ER stress-mediated cell death [92]. Caspase-12 is activated by ER stress stimuli such as tunicamycin, brefeldin A, and thapsigargin, but not by membrane- or mitochondrial-targeted apoptotic signals.

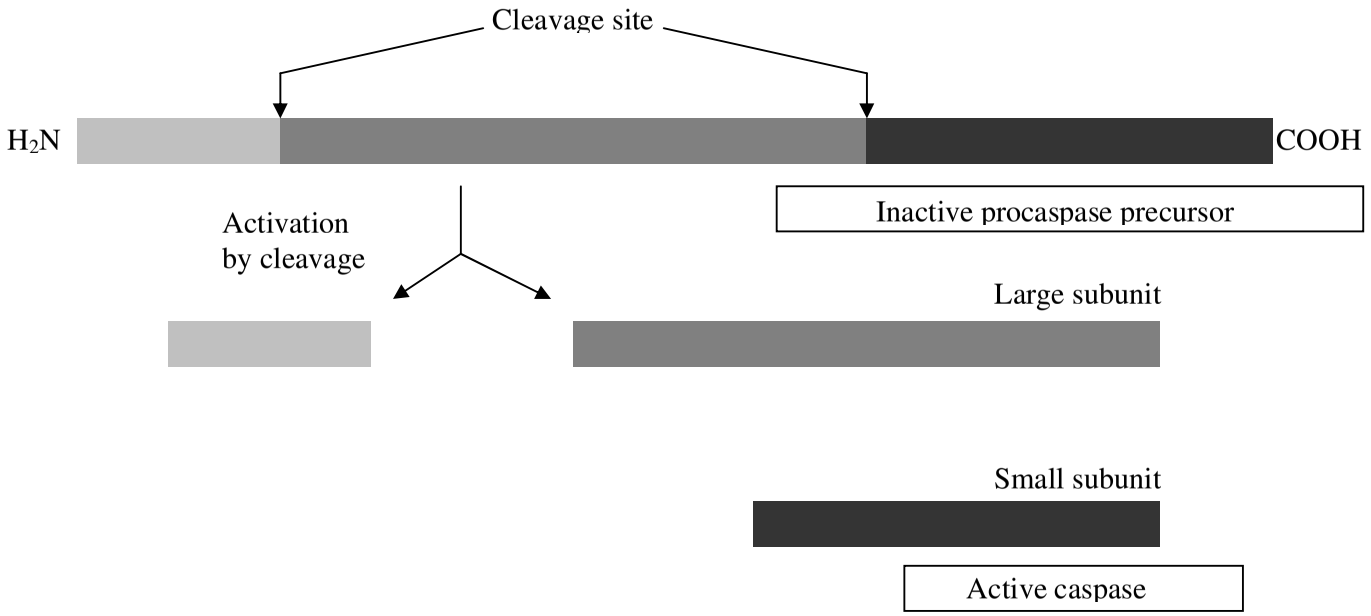

Figure 7: Activation of a caspase.

The protein is made as a large inactive precursor (a procaspase), which is activated by cleavage at two aspartic acids. The 'prodomain' is discarded, and the large and small subunits form the active enzyme. It is thought that there are two large and two small subunits in an activated caspase (not shown). The activating cleavages are usually catalyzed by caspase or procaspase molecules themselves.

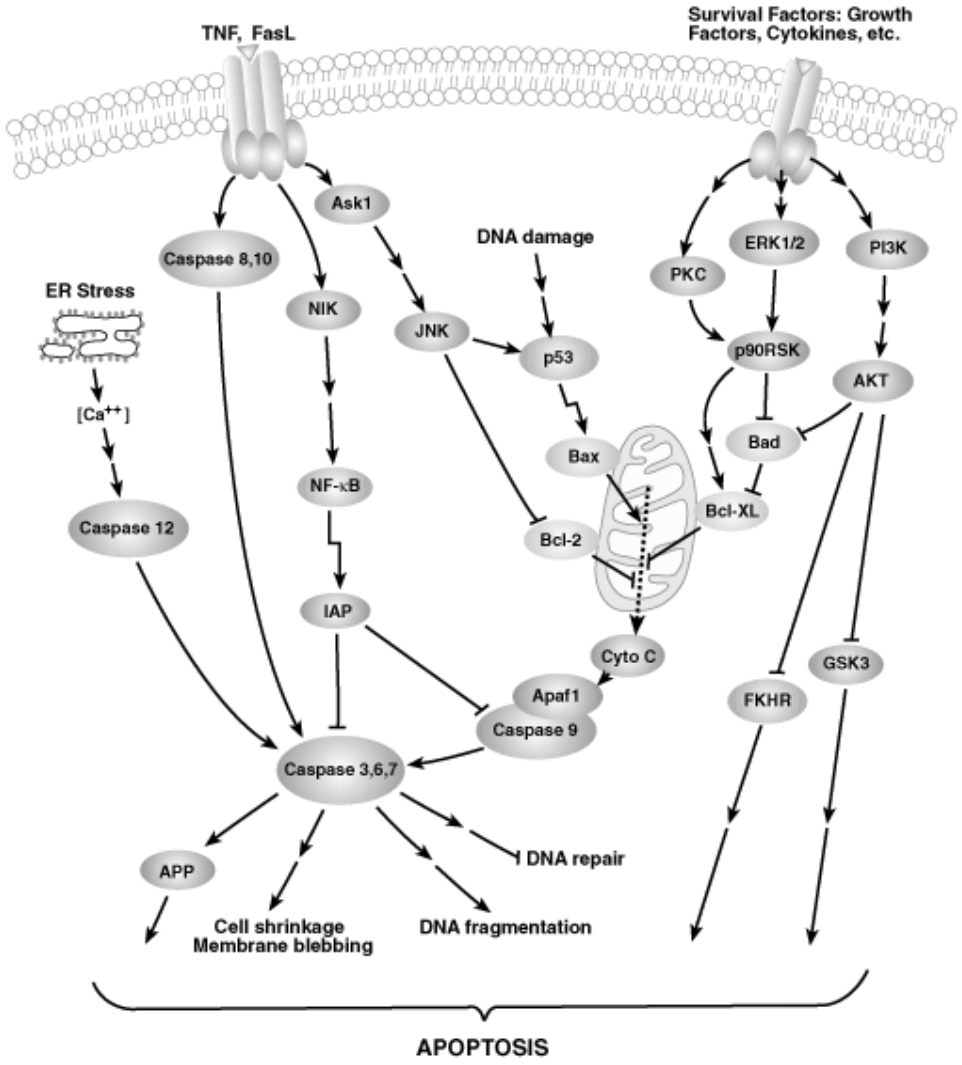

Figure 8: Overview regulation of apoptosis [93]

Caspases, a family of cysteine proteases, are central regulators of apoptosis. Initiator caspases (including 8, 9, 10 and12) are closely coupled to pro-apoptotic signals. Once activated, these caspases cleaved and activated downstream effector caspases (including 3, 6 and 7) which in turn cleave cytoskeletal and nuclear proteins and induce apoptosis. Cytochrome *c* release from mitochondria is coupled to the activation of caspase-9, a key initiator caspase. Pro-apoptotic stimuli include the FasL, TNF, DNA damage and ER stress. Fas and the TNFR activate caspase-8 and 10. DNA damage leads to the activation of caspase-9 and ER stress leads to the calcium-mediated activation of caspase-12.

Caspase-12, like most other members of the caspase family, requires cleavage of the prodomain to activate its proapoptotic form. So far, several possible molecular mechanisms for the processing of caspase-12 have been postulated. Nakagawa and Yuan (2000) reported that m-calpain, another cysteine protease, is responsible for cleaving procaspase-12 to generate the active caspase-12. Caspase-12 is initially processed at the N-terminal region by calpain, activated by ER stress. Caspase-12 is then activated and autoprocessed at D^{318}. Thus, calcium released from the ER may trigger a novel apoptotic pathway involving calcium-mediated calpain activation and crosstalk between the calpain and caspase families (figure 9).

The glucose regulated protein 78 (GRP78) is involved in polypeptide translocation across the ER membrane, and also acts as an apoptotic regulator by protecting the host cell against ER stress-induced cell death. Treatment of cells with ER stress inducers such as brefeldin A and thapsigargin causes GRP78 to redistribute from the ER lumens, so that subpopulations exist in the cytosol and as ER transmembrane protein [94;95] and induces the expression of the caspase-12 protein and also leads to the translocation of cytosolic caspase-7 to the ER surface. GRP78 forms a complex with caspase-7 and caspase-12 and prevents the release of caspase-12 from the ER, but upon excess ER stress, caspase-7 associates with caspase-12 and cleaves the prodomain at D^{94} to initiate the processing of caspase-12 at D^{341}, resulting in increased cell death [96].

The other molecule, which complexes with caspase-12 is tumor necrosis factor receptor-associated factor 2 (TRAF-2). Caspase-12 is shown to be released from TRAF2 complexes by ER stress and is then autoprocessed via homodimerization [97]. In that study c-Jun N-terminal inhibitory kinase (JIK) was identified as a binding partner of IRE1-α. JIK modulates the IRE1-α -TRAF2 complex formation and the resultant alteration to JNK signaling from IRE1-α. In response to ER stress, TRAF2 plays crucial roles, not only in the signaling of the c-Jun N-terminal kinase (JNK) pathway but also in the activation of caspase-12, to transduce signals from IRE1-α. Thus, TRAF-2 is a missing link in the ER stress-induced apoptosis signaling pathway, one which connects the stress sensor molecule IRE1-α and the activation of caspase-12.

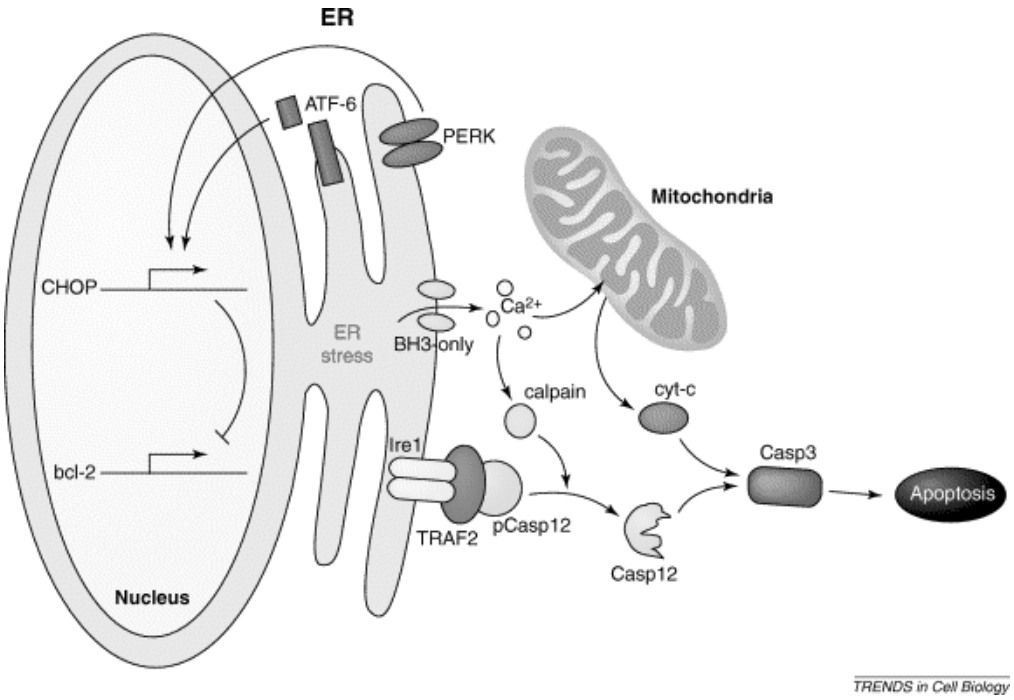

Figure 9: Regulation of calcium-mediated calpain activated caspase-12 [98]

Caspase-12 is specifically localized on the cytoplasmic side of the ER. It is activated by ER stress and the mobilization of the intracellular Ca^{2+} ion stores. Caspase-12 is proteolytically activated by m-calpain, a Ca^{2+} -responsive cytosolic cysteine protease. During the UPR, Ire1-α recruits TRAF-2, which in turn interacts with procaspase-12 and promotes its clustering and its activation by ER stress. Activation of caspase-12 leads to cleavage of caspase-3 independent of mitochondrial participation resulting in cell apoptosis.

6. Dengue virus and apoptosis

There have been few studies of the interactions between dengue virus and liver cells and the mechanisms underlining of dengue virus induced programmed cell death are remain unclear. According to the replication cycle of the RNA viruses, viruses that use the ER as an integral part of their life cycle may contend with the ER stress response and the downstream sequences of the ER stress signaling and also ER stress-mediated apoptosis. There are many studies shown that the infection of virus can initiated apoptosis signaling via ER stress response. Infection with the Bovine Viral Diarrhea Virus (BVDV), one of the viruses in family *Flaviviridae,* shown to activate ER stress response and trigger host cell apoptosis correlated with the expression of caspase-12 [99]. Respiratory syncytial virus (RSV) infection induced programmed cell death and caspase-12 activation [100]. Infection with Japanese encephalitis virus, another flaviviruses, initiates endoplasmic reticulum stress and an unfolded protein response [101].

It has been shown that the transcription factor, nuclear factor kappa B (NF-κB) was activated in associated with infection of human hepatoma cell line (HepG2 cells) by dengue virus and may involve in the mechanisms of virus induced apoptosis [102]. The activation of NF-κB occurs via the phosphorylation and degradation of an inhibitory protein, IκB-α, thereby releasing NF-κB from the cytoplasm and allowing its translocation into the nucleus. NF-κB binds to nuclear κB elements of target genes and activates transcription of genes mediating cell growth, differentiation, inflammation, oncogenesis, pro- and anti-apoptotic reactions, etc. In general, NF-κB activating stimuli seem to use oxidative stress as a common signal transduction pathway to elicit their response but NF-κB also shown to activated as the downstream consequence of ER stress response due to protein accumulation in the intracellular membrane [103]. It is possible that intracellular accumulation of the dengue viral protein between their replication cycle trigger apoptosis by inducing the ER stress-induced apoptotic pathway. Although, the mechanisms of dengue virus induced host cell apoptosis are remain elucidated.

CHAPTER II
OBJECTIVES

Dengue viruses (serotype 1 to 4) are mosquito borne flaviviruses which cause significant morbidity in most tropical and subtropical areas. A lot of studies indicated that the viral induction of liver apoptosis contributes at least in some parts of the pathogenesis of this disease. The molecular mechanisms underlying the dengue virus induced hepatocyte apoptosis is still unclear. The replication of flaviviruses which utilized the ER as the integral part of viral protein synthesis and genome replication has been showed to induced ER stress response and ER stress-mediated apoptosis via activation of caspase-12. These reasons lead to the aim of this project is to investigate the involvement of ER stress-mediated caspase-12 activation in response to dengue virus infection in hepatocytes. In addition, the effects of caspase dependent pathway to dengue virus induced apoptosis and virus proliferation was investigated by treatment with the broad caspase inhibitor, z-VAD-fmk.

CHAPTER III
MATERIALS AND METHODS

1. Source of materials

1.1 Chemicals

Acrylamide (electrophoresis grade)	Sigma
Ammonium persulfate	Sigma
Aprotinin	Sigma
Bis-acrylamide (N, N'-methylene bis acrylamide)	Sigma
Bovine Serum Albumin (BSA) Fraction V	Sigma, Missouri,USA
Crystal violet	Bio Basic Inc, Ontario, Canada
1, 4- Dithiothreitol (DTT)	Sigma
Ethelynediamine trichloroacetic acid (EDTA)	Bio Basic Inc, Ontario, Canda
Formaldehyde	Sigma
Fungizone	Brystal-Myers Squibb, Princeton, NJ,USA
Gentamycin	T.P. Drug Laboratories, Bangkok, Thailand
Lactalbumin hydrolysate	Sigma, Missouri, USA
Leupeptin	Sigma
Penicillin / Streptomycin	Hyclone, Utah, USA
PMSF	Sigma
Prostratin	Sigma
RNaseA	Sigma
Seakem LE agarose	Bio Whittaker Molecular Applications
SDS (Sodium dodecyl sulphate)	Sigma
Standard BSA	Sigma

1.2 Culture media reagents

Dulbecco's Modified Eagle's medium (DME / High Glucose powder)	GIBCO BRL, Maryland,USA
Fetal bovine serum (FBS)	GIBCO BRL, Maryland,USA
Fetal Calf serum (FCS)	GIBCO BRL, Maryland,USA
Medium 199 / EBSS (M199E)	Hyclone, Utah, USA
Trypsin (1:250)	GIBCO BRL, Maryland,USA

1.3 Cell lines

1.3.1 HepG2: Hepatoma cell line from *Homo sapiens* (human)

1.3.2 Hep3B: Hepatoma cell line from *Homo sapiens* (human)

1.3.3 Vero: Kidney cell line from *cercopithecus aethiops* (African green monkey)

1.4 Dengue virus

1.4.1 Dengue virus serotype-1 strain 16007

1.4.2 Dengue virus serotype-2 strain 16681

1.4.3 Dengue virus serotype-3 strain 16562

1.4.4 Dengue virus serotype-4 strain 1036

1.5 Antibodies

1.5.1 Primary antibodies

Monoclonal antibody mouse anti-actin	Chemicon
Polyclonal antibody goat anti-caspase-7	Santa Cruz
Polyclonal antibody rabbit anti-caspase-12	US Biological
Polyclonal antibody rabbit anti-human GRP78 H-129	Santa Cruz

1.5.2 Secondary antibodies

Horseradish peroxidase-conjugated anti-goat IgG	Pierce
Horseradish peroxidase-conjugated anti-mouse IgG	Sigma
Horseradish peroxidase conjugated anti-rabbit IgG	Pierce

1.6 Miscellaneous materials

Coomassie Plus Protein Assay Reagent Kit	PIRCE
1 kb plus DNA ladder	GIBCO BRL
Prestained SDS-PAGE Standards	BIO-RAD
PROTAN Nitrocellulose Transfer membrane	Schleicher&Schuell

ECL+ Western blotting detection reagents Amercham

Other general chemicals, solvents and materials used but not listed here were purchased from a variety off suppliers (Fluka, Sigma, Merk, Gibco BRL, Promega, Bio Basic Inc). All chemicals used were analytical grade.

2. Culture medium preparation

2.1 Culture medium for HepG2 cells

Dulbecco's Modified Eagle's medium (DMEM), 10% heat inactivated fetal bovine serum (ΔFBS), 100units/ml penicillin and 100µg/ml streptomycin

2.2 Culture medium for Hep3B cells

DMEM, 10% ΔFBS, 100units/ml penicillin and 100µg/ml streptomycin

2.3 Culture medium for Vero cells

Dulbecco's Modified Eagle's medium (DMEM), 5% heat inactivated fetal calf serum (ΔFCS), 100units/ml penicillin and 100µg/ml streptomycin

3. Recovering cells from liquid nitrogen storage

Frozen cells in cryogenic vials were removed from liquid nitrogen storage and immediately thawed in a water bath at 37ºC. Cell suspension was transferred to a centrifuge tube containing culture medium and resuspended before centrifugation at 200 x g for 5 minutes at room temperature. The supernatant was discarded and cell pellet was resuspended with culture medium supplement with FBS. The cells were seeded onto 100 mm culture plate and up into the CO_2 incubator.

4. Counting cells

The number of cells was determined by hemocytometer. The cells were trypsinized into single cells and resuspended to cell suspension in culture medium. A drop of cell suspension was delivered to a side of the hemocytometer and the number of cells was counted under microscope in ten squares of 0.1 mm^3 chamber formed by the 1x1 mm square and 0.1 mm high of the cover slipped of the hemocytometer. The number of cells was calculated further to an accurate cell number per milliliter of suspension (cells/ml).

5. Subculturing cells

The cells were subcultured into the next passage after they were grown until they reached confluence. The old culture medium was discarded and the cells were washed with 0.15M PBS at least one time. Then the solution of 0.25% Trypsin/1mM EDTA in 1xPBS was applied and rinse throughout the plate. For Vero and HepG2 cell, the cells was incubated at 37ºC, 5%CO_2 for 5 minutes and room temperature, 5%CO_2 for 5 minutes for Hep3B cells. The new culture medium was added and the culture was resuspended to complete the detached cells into single cells. The single cells suspension was add into the new culture plate in an appropriated amount and the new medium was added to provide the growing conditions. The cell were incubated at 37ºC, 5%CO_2 for Vero and Hep3B cell and 37ºC, 10%CO_2 for HepG2 cells.

6. Virus stocks preparation

Sub-confluent Vero cells were used for virus preparation. The cells were infected with dengue virus moi of 1 and incubated in DMEM supplemented with 5% heat inactivated FBS at 37ºC, 5%CO_2. The medium containing the virus was clarified by centrifugation at 1,200 rpm at room temperature for 5 minutes on the day which had the highest virus yield. The supernatant was supplemented with 20% (v/v) FBS and keep at -80ºC.

7. Virus infection

After the cells reached sub-confluence or confluence, the old medium was discarded and the cells were rinsed 1 time with 0.15M PBS. The cells were incubated with virus diluted in BA-I diluent buffer for 1.5 hours at 37ºC, 5%CO_2 for Vero and Hep3B cells and 37ºC, 10%CO_2 for HepG2 cells. Then the new culture medium was added and infected cells were incubated further in condition which appropriated to the cells.

8. Virus quantitation by plaque assay

Vero cells were seeded and grown in 6 wells culture plates at 37ºC, 5%CO_2 until reached their confluence. The virus samples were diluted with BA-I diluent buffer in ten-folded serial dilutions and used to infect confluent Vero cells at 37ºC, 5%

CO_2. The plates were incubated for 1.5 hours with agitation every 10 minutes. The overlay solution which made from 1.6%Seakem LE agarose and 2x nutrient was prepared during the infection period. After the virus absorption, the overlay solution was added into each well of the plates. The plated were leaved at room temperature until the overlay solution was solidified following with incubation at 37ºC, 5% CO_2 for 7 days to allowed the plaque formed. After that, the cells were fixed with 3.7% formaldehyde in PBS for at least 1 hour at room temperature. The plaques were visualized by staining with crystal violet in 10% ethanol. The number of plaque were counted and calculated in plaque forming unit per virus sample 1 ml (pfu/ml).

Titer (pfu/ml) = number of plaques x sDF x iDF

sDF = serial dilution factor of each well (e.g. 10^6)

iDF = infection dilution factor (Volume used for infection)

9. DNA fragmentation

The mock infected or virus infected cells were incubated at 4ºC for 15 minutes to stop cell activities and collected by mildly scrape in culture medium. The cells suspension was transferred into 10 ml conical tube following with centrifugation at 1,500 rpm for 5 minutes. Then the medium was discarded and the cell pellet was resuspended with 1 ml ice-cold HBSS buffer. The cells were fixed by added 10 ml of 70% ice-cold ethanol and stored at -20 ºC generally for 24-72 hours, some samples, however , were kept up for 4 weeks. The cells were then centrifuged at 800g for 5 minutes and the ethanol was throughout removed. The cells were resuspended in 40µl of phosphate-citrate (PC) buffer, consisting of 192 parts of 0.2M Na_2HPO_4 and 8 parts of 0.1M citric acid pH 7.8. Then the cells suspension was transferred into 1.5 ml centrifuge tube and incubated at least 30 minutes at room temperature. After centrifugation at 1,000g for 5 minutes, the supernatant was transferred to new tube and concentrated by vacuum until the solution decrease to approximately 10 µl. A 3 µl aliquot of 0.25% Nonidet NP-40 in distilled water was then added, followed by 3 µl of a solution of 1mg/ml RNaseA in distilled water. After 30 minutes incubation at 37ºC, 3 µl of a solution of 1 mg/ml proteinase K was added and the extract was incubated for an additional 30 minutes at 37ºC. After incubation, the extract was mixed with loading buffer and loaded into horizontal 0.8%agarose. The electrophoresis was

performed at 30V for 4 hours. After staining with ethidium bromide, the DNA in the gel was visualized under uv light [104].

10. Total protein extraction

Mock infected or dengue infected cells were incubated at 4ºC for 15 minutes for inactivating the activities of cells. The cells were mildly scrape in medium and transferred into the 10 ml conical tube. After centrifugation at 1,500 rpm for 5 minutes at 4ºC, the medium was discarded and the cell pellet was washed three times with ice-cold PBS. Then the RIPA extraction buffer was added followed by incubated on ice for 30 minutes with vortex several times. The DNA was sheared by passing the cell lysate through the 23g needle 3-4 times. The lysate was centrifuged at 14,000 rpm, 4ºC for 30 minutes. The cleared supernatant was taken into the new 1.5 ml centrifuge tube and the protein samples were stored at -80ºC until used.

11. Measuring protein concentration

Protein concentration was determined by using Coomassie reagent protein assay. Briefly, the 10 µl diluted protein extract was mixed with the 300 µl of coomassie reagent dye. The solution was mixed in a 96-well flat bottom Sero-Well microtiter plate and incubated at room temperature for 5 minutes before measuring an absorbance at 595 nm on the SpectraMAX 250 ELISA plate reader (Molecular device). Then the concentration of samples was calculated from the BSA standard curved.

12. SDS-Polyacrylamide Gel electrophoresis

The SDS-PAGE was performed using the Bio-Rad Mini-Protein II system. The samples were prepared by mixing the samples with the 5xloading buffer and boiled for 5 minutes before vortex several times and loading onto the gel. The gel system is composed of stacking and separating gel. Electrophoresis was performed by constant voltage 100V at room temperature with running buffer. After electrophoresis, the gel was analyzed further by Western blot analysis or visualized by 1 hour soaking in staining solution following with de-staining with de-staining solution overnight or until the background was cleared.

13. Western Blot analysis

13.1 Electrotransfer of protein from SDS-PAGE to nitrocellulose membrane

The protein samples separated by SDS-PAGE were transferred to the nitrocellulose membrane by wet blot transfer technique. The gel was soaked with ice cold transfer buffer for 10 minutes. The four pieces of filter paper and one pieces of nitrocellulose membrane which were cut into the same size as the gel, were immerged in the ice cold transfer buffer for 5 minutes. Wet-blot Electrophoretic Transfer cell (BIO-Rad) was used for transferring. A presoaked fiber pad was placed onto the cassette, follow with two pieces of filter paper and the SDS gel. The nitrocellulose membrane was then layered on the gel and covered with two pieces of filter paper and the second presoaked fiber pad. Finally, the entire cassette was put into the electrophoresis tank which filled with the pre-cooled transfer buffer. Electroblotting was carried out at constant voltage of 30V at 4ºC for overnight.

13.2 Immunodetection

After the electrotransfer process was completed, the apparatus was disassembled and the membrane was rinsed once with TBS-T. The membrane was stained with Ponceau S staining solution to check that the transfering process was worked. The membrane was washed with distilled water to remove the staining solution following with preblocked by immerging in blocking solution at 4ºC for overnight. Then the membrane was incubated with primary antibody diluted in blocking solution. The dilutions and conditions used for each primary antibody were showed in table 1.

Table1. The dilutions and conditions for primary antibodies

Primary antibody	dilution	condition
Anti-actin	1:1,000	4ºC, overnight
Anti-caspase-7	1:250	RT, 3 hours
Anti-caspase-12	1:1,000	4ºC, overnight
Anti-GRP 78	1:1,000	RT, 3 hours

After incubation, the membrane was washed three times with TBS-T and further incubated in new blocking solution containing 1:3,000 of secondary antibody at room temperature for 1 hour with shecking. Then the membrane was washed three times with TBS-T. The signal on membrane was developed by using ECL Plus western blotting analysis as described in the manufacturer's instruction before the chemiluminescence signal would be detected on X-ray film.

13.3 Strip blot for reprobing with other antibody

The membrane that already processed through chemiluminescence signal detection was washed three times with TBS-T with shacking before immerged in blocking solution overnight at 4ºC. Then the membrane was ready for detection with other species of antibody.

14. Treatment of caspase inhibitor (z-VAD-fmk)

The $2x10^5$ cells of HepG2 or Hep3B cells were seeded onto six well culture plates and incubated at culture condition for 24 hours to allow the cells to attach to the plate. The old culture medium was discarded and the cells were three times washed with PBS and one time washed with new culture medium. Then the cells were incubated with caspase inhibitor diluted in culture medium for 2 hours in culture condition with shacking every 10 minutes. After incubating, the inhibitor was discarded and the cells were then three times washed with PBS and one time washed with culture medium. The new culture medium was added and the cells were incubated in culture condition for 1 hour following by infection with dengue virus serotype 2 with moi of 1. After infection, the cells were culture for five days and the medium containing dengue virus was collected at day 1 to 5 post infection for tittering the virus by plaque assay. At day five post infection, the detached and attached cells were collect to count the number of cells. This experiment was done by individual duplicated experiment.

15. Counting attached and detached cells

The detached and attached cells were count by using haemocytometer. The culture plate was incubated at 4ºC for 15 minutes before the culture medium containing detached cells was transfer to the 10 ml conical tube which pre-incubated

on ice. The medium was mixed and 10 µl of cell suspension was transferred to the haemocytometer to count the detached cell. Then the attached cells were trypsinized to remove from the culture plate and the cells suspension was pipette up and down to split the cells into single cells before the 10µl of cell suspension was used to count the attached cells. The number of cells counted by haemocytometer was calculated back to the number of total cells in the culture plate. Both the detached and attached cells were count in duplicated.

16. Data statistical analysis

The intensity from the result of Western blot analysis of GRP78 and caspase-12 were read by OneDScan software (CSP inc., Billerica, MA). The data from the experiment of the effect of caspase inhibitor (z-VAD-fmk) to virus production were analyzed by using GraphPad prism software (GraphPad Software Inc., San Dieago, USA). ANOVA was used to analyze for more than two sets of the data at $p<0.0001$.

CHAPTER IV
RESULTS

1. Four serotypes of dengue virus induced apoptosis in HepG2 cells by DNA fragmentation

In cells undergoing apoptosis, a fraction of nuclear DNA is fragmented to the size equivalent of DNA in mono- or oligonucleosomes. When such DNA from apoptotic cell is analyzed by agarose gel electrophoresis, it generates a characteristic "ladder" pattern of discontinuous DNA fragments. To establish the profile of 4 serotypes of dengue virus induced apoptosis in HepG2 cells, HepG2 cells ($2x10^6$ cells) were plated onto 10 cm diameter plates and were incubated at 37^oC, 10%CO_2 for 24 hours before mock-infection or infection with DEN-1 to 4 moi of 0.1 and 10 before monitoring the DNA ladder at days 1, 3, 5 and 7 post-infection by using the low molecular weight DNA extraction as described in materials and methods.

In mock-infection, the result showed no signal from the apoptotic cells but only faint smear in day 7 post-infection which indicated that the HepG2 cells can be grew for further 7 days without apoptotic induction in this condition (figure14). In dengue virus infection, the result from infection with DEN-1 showed the DNA ladder only at day 7 post-infection in both moi 0.1 and 10 (figure 10) but these results were difficult to distinguish from mock infection at day 7 post infection which showed a faint smear (figure 14). In contrast, the DNA ladders were clearly observed by infection with DEN-2 in both moi of 0.1 and 10 at day 5 and 7 post-infection and low signal by infection with DEN-2 moi of 10 at day 3 post-infection (figure 11). Surprisingly, infection by DEN-3 showed low signal from apoptotic cells (figure 12). Finally, infection with DEN-4, the DNA ladders were clearly observed at day 7 post-infection by using moi 0.1 and at day 5 and 7 by using moi 10 (figure 13).

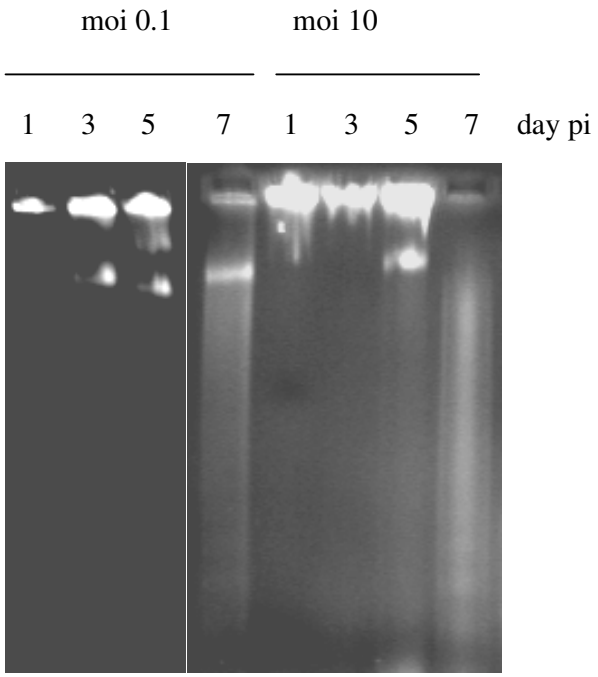

Figure 10: Infection with DEN-1 induced HepG2 cells apoptosis.

HepG2 cells were infected with DEN-1 moi 0.1 and 10. Low molecular weight DNA in cytoplasm of apoptotic cells was extracted at day 1, 3, 5 and 7 post-infection. DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis.

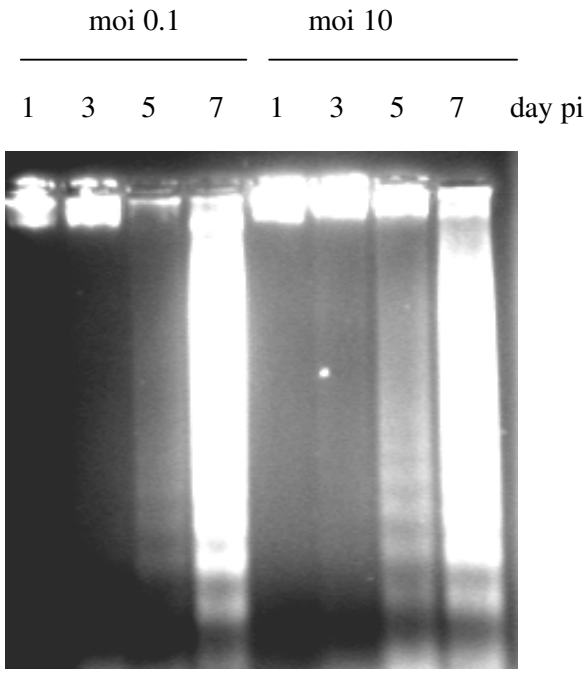

Figure 11: Infection with DEN- 2 induced HepG2 cells apoptosis.

HepG2 cells were infected with DEN-2 moi 0.1 and 10. Low molecular weight DNA in cytoplasm of apoptotic cells was extracted at day 1, 3, 5 and 7 post-infection. DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis.

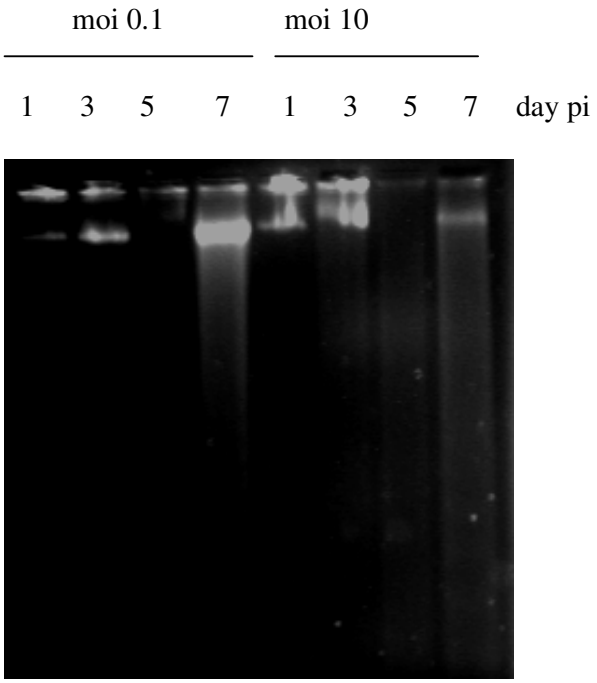

Figure 12: **Infection with DEN- 3 induced HepG2 cells apoptosis.**

HepG2 cells were infected with DEN-3 moi 0.1 and 10. Low molecular weight DNA in cytoplasm of apoptotic cells was extracted at day 1, 3, 5 and 7 post-infection. DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis.

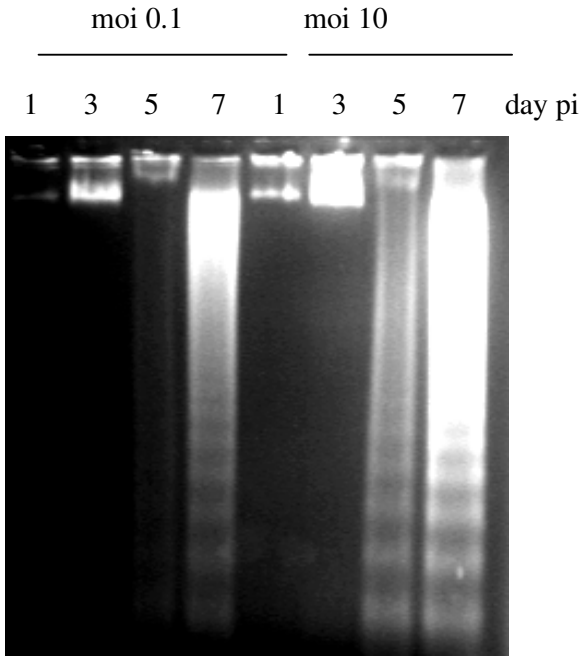

Figure 13: Infection with DEN-4 induced HepG2 cells apoptosis.

HepG2 cells were infected with DEN-4 m.o.i. 0.1 and 10. Low molecular weight DNA in cytoplasm of apoptotic cells was extracted at day 1, 3, 5 and 7 post-infection. DNA fragmentation was analyzed by 1.5% agarose gel electrophoresis.

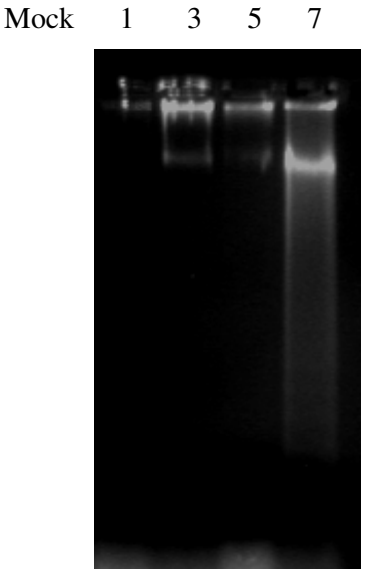

Figure 14: Analysis of DNA fragmentation in mock infection

Mock infection were prepare by incubation HepG2 cells with BA-1 medium and extracted low molecular weight DNA at day 1, 3, 5 and 7 post-infection and analyzed by 1.5% agarose gel electrophoresis.

2. The ER stress (GRP78 induction) in response to dengue virus infection in HepG2 cells.

To determine the mechanisms of dengue virus induced apoptosis, two serotypes of dengue viruses, DEN-2 and DEN-3 were selected to demonstrate after DEN-2 showed clearly apoptosis induction by DNA laddering but not for DEN-3. Dengue virus replicates in the cytoplasm of infected cells and utilizes the ER as the site of polyprotein processing and genomic replication. Based on the premise that the ER-tropic viruses induce ER stress, the effect of dengue virus infection on the expression of selected ER stress marker gene product was examined.

Glucose regulated protein 78 (GRP78) is a resident ER chaperone whose expression is up-regulated in response to ER stress. To determine whether GRP78 was induced by dengue virus infection, Western blot analysis was performed. The HepG2 cells were pre-grew for 24 hours to allow the cells attached to culture plates before mock or dengue virus infection. Total protein was extracted using RIPA buffer as describe in materials and method from mock infection at 16 hours and day 5 post-infection and from DEN-2 and DEN-3 infection at 16, 18 hours and day 1, 3 and 5 post-infection. The total proteins were analyzed by SDS-PAGE and Western blot using anti-GRP78 antibody. In mock infection, the result showed the similar intensity of GRP78 at both time points which indicated that no ER stress induction in this cell growth condition (figure 15 and 16). The results from DEN-2 infected cells showed the up-regulation of GRP78 proteins at day 3 post-infection which correlated with the induction of apoptosis measured by DNA fragmentation of DEN-2 infected cells (figure 15). In DEN-3 infected cells, the result showed the similar level of GRP78 proteins in every time points. The internal control, actin indicated the equal loading of total protein in each lane. The results from figure 16 and 18 showed the intensity read from GRP78 band against the internal control, actin from DEN-2 and DEN-3 infected cells respectively. The results also showed the induction of GRP78 in DEN-2 infected cells at day 3 post-infection but not presented in DEN-3 infected cells at any time points. These results indicated that infection with DEN-2 but not DEN-3 induced ER stress in HepG2 cells.

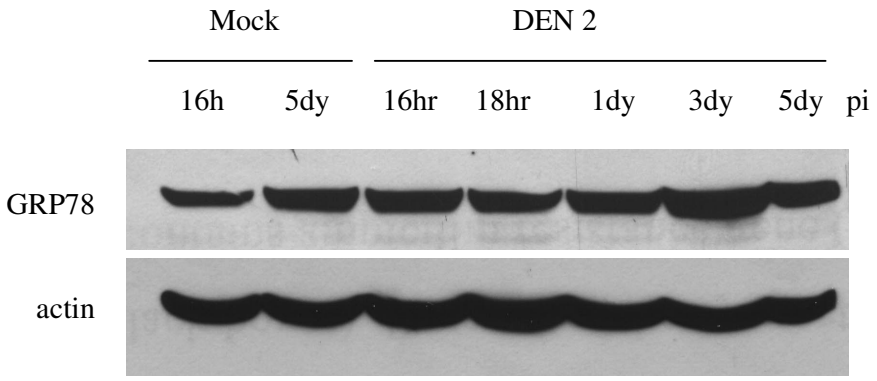

Figure 15: Infection with DEN- 2 induced the up- regulation of GRP78 in HepG2 cells.

The $2x10^6$ HepG2 cells were plated for 24 hours before infected with DEN-2 at moi of 10. The total protein was extracted at any time point with RIPA buffer and 80 μg of protein was loaded and separated by 15% SDS-PAGE. The up- regulation of GRP78 was analyzed by using GRP78 antibody at dilution 1: 1,000. Membrane was stripped and re- probed with anti- actin dilution 1:1,000

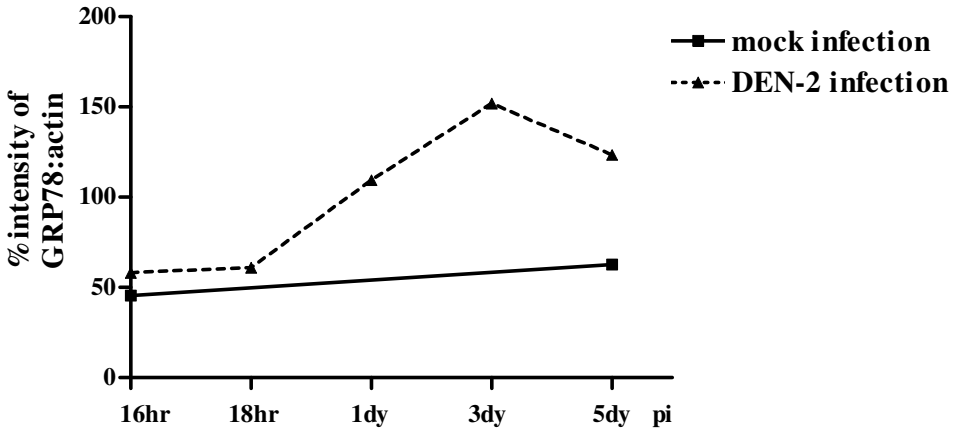

Figure 16: The intensity of GRP78 against actin by infection of HepG2 cells with DEN- 2.

The results from Western blot analysis of GRP78 and actin from figure15 was scanned and the intensity of each band was read by OneDScan program. The intensity read from actin was adjusted to 100% and the %intensity of GRP78 was calculated against the result read from each band of actin.

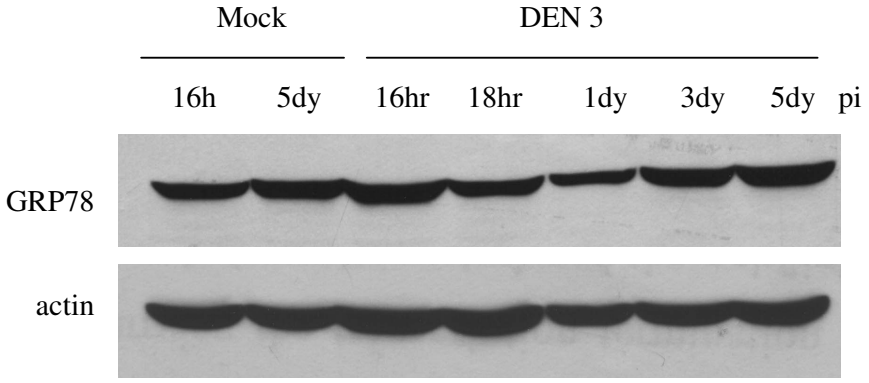

Figure 17: The Western blot analysis of GRP78 by infection HepG2 cells with DEN-3.

The $2x10^6$ HepG2 cells were seeded and infected with DEN-3 with moi of 10. The total protein was extracted at any time point with RIPA buffer and 80 μg of protein was loaded and separated by 15% SDS- PAGE. The level of GRP78 was analyzed by using GRP78 antibody at dilution 1: 1,000. Membrane was stripped and re- probed with anti- actin dilution 1:1,000.

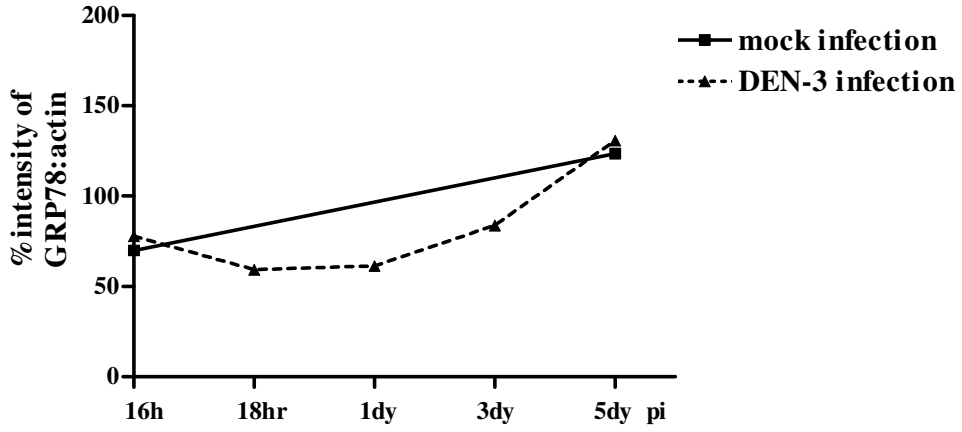

Figure 18: The intensity of GRP78 against actin by infection of HepG2 cells with DEN- 3.

The results from Western blot analysis of GRP78 and actin from figure 17 was scanned and the intensity of each band was read by OneeDScan program. The intensity read from actin was adjusted to 100% and the %intensity of GRP78 was calculated against the result read from each band of actin.

3. The effect of dengue virus infection to caspase-12 activation.

A hallmark of ER-stress induced apoptosis is the activation of caspase-12, an ER membrane associated cysteine protease that is activated by ER stress inducing agents (105;106). Since the infection by DEN-2 but not DEN-3 appears to induce ER stress, it was interesting to compare the results of caspase-12 activation from infection between serotype 2 and 3. The $2x10^6$ cells of HepG2 cells were seeded for 24 hours to allow the cells to attach to the culture plate and the cells were mock-infected or DEN-2 or DEN-3 infected. The total protein was extracted from mock-infected at 16 hours and 5 days post-infection. For DEN-2 and DEN-3 infection, the total lysates were extracted at 16, 18 hours and 1, 3 and 5 days post-infection. After that, the 100µg of protein lysates were separates by 15%SDS-PAGE and the activation of caspase-12 was measured by Western blot analysis using anti-caspase-12 antibody and the same membrane was wash and re-probed with anti-actin antibody. The result showed that the intensity of caspase-12 by infection with DEN-3 was similar to the intensity of mocked-infection at every time point and the activation of caspase-12 could not be observed (figure 21). The result of the intensity of actin showed the equal loading of the protein in each lane. The ratio of the intensity of caspase-12 against actin showed the similar result which indicated that there is no activation or up-regulation of capase-12 by infection of HepG2 cells with DEN-3 (figure 22).

It was surprising that infection with DEN-2 which showed the induction of ER stress showed the similar result to the infection with DEN-3. The signal of Western blot analysis from DEN-2 infection showed the similar intensity between mock-infection and DEN-2 infection at any time points and no active form of caspase-12 could be detected (figure 19). The same amount of protein loaded in each lane in the gel was indicated by the similar intensity of the internal control, actin. The result from calculation of the intensity among caspase-12 against actin showed that the ratio of the intensity from DEN-2 infection at every time point is closed to mocked infection (figure 20). These results indicated that infection of HepG2 cells with DEN-2 could not induced both the activation and up-regulation of caspase-12.

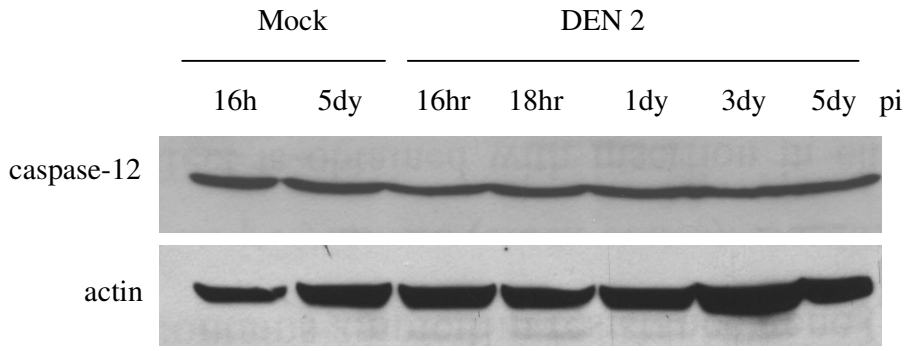

Figure 19: The Western blot analysis of caspase-12 by infection HepG2 cells with DEN- 2.

The $2x10^6$ HepG2 cells were plated and infected with DEN-2 at moi of 10. The total protein was extracted at any time point with RIPA buffer and 80μg protein was loaded and separated by 15% SDS- PAGE. The level of caspase-12 was analyzed by using caspase-12 polyclonal antibody at dilution 1: 1,000. Membrane was stripped and re- probed with anti- actin dilution 1:1,000

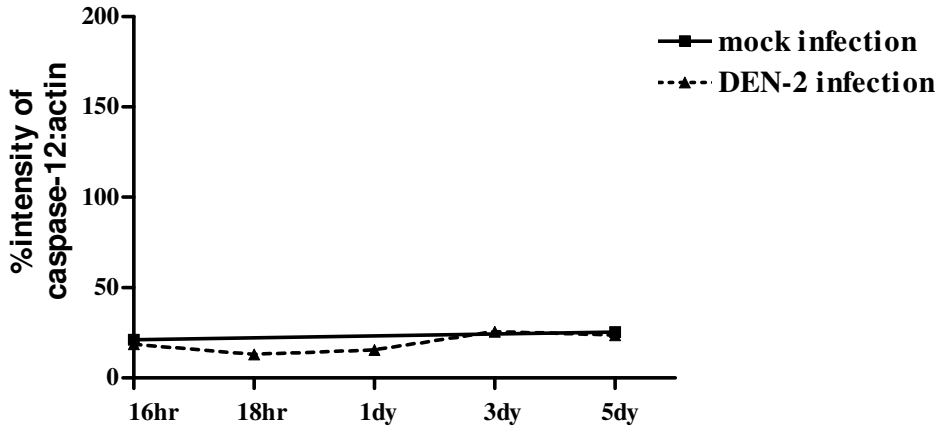

Figure 20: The intensity of caspase-12 against actin by infection of HepG2 cells with DEN- 2.

The results from Western blot analysis of caspase-12 and actin from figure19 was scanned and the intensity of each band was read by OneDScan program. The intensity read from actin was adjusted to 100% and the %intensity of caspase-12 was calculated against the result read from each band of actin.

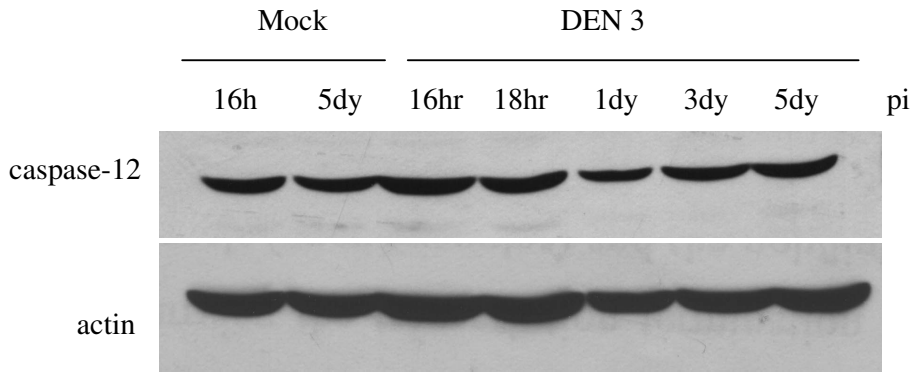

Figure 21: **The Western blot analysis of caspase-12 by infection HepG2 cells with DEN-3.**

The $2x10^6$ HepG2 cells were plated and infected with DEN-3 at moi of 10. The total protein was extracted at any time point with RIPA buffer and 80μg protein was loaded and separated by 15% SDS- PAGE. The level of caspase-12 was analyzed by using caspase-12 polyclonal antibody at dilution 1: 1,000. Membrane was stripped and re- probed with anti- actin dilution 1:1,000.

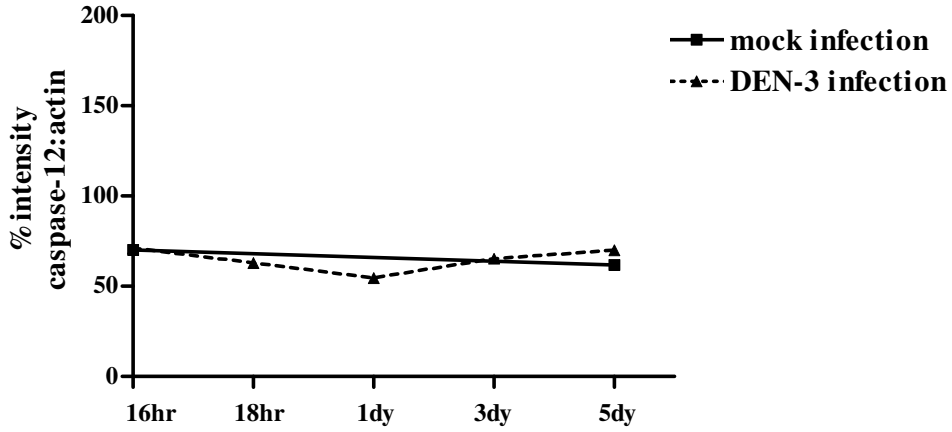

Figure 22: The intensity of caspase-12 against actin by infection of HepG2 cells with DEN-3.

The results from Western blot analysis of caspase-12 and actin from figure 21 was scanned and the intensity of each band was read by OneDScan program. The intensity read from actin was adjusted to 100% and the %intensity of caspase-12 was calculated against the result read from each band of actin.

4. The effect of dengue virus infection to caspase-7 activation.

One of the regulations of caspase-12 is cleavage by caspase-7. It has been shown that treatment with the ER stress inducer such as thapsigargin or brefeldin-A leads to the translocation of cytosolic caspase-7 to ER surface. Caspase-7 associated with procaspase-12 and cleaves the caspase-12 prodomain to generate active caspase-12 resulting in an increase cell death (96). The involvement of caspase-7 in response to dengue virus infection was investigated by the Western blotting. Cell extract from mock infected HepG2 cell at 16 hours and 5 days post infection showed the similar level of 35kDal pro-caspase-7 indicated that the condition for cell culture does not effect the expression or activation of caspase-7. From cell lysates prepared from DEN-2 infected HepG2 at 16, 18 hours and 1, 3 and 5 days post-infection, although the expected band of 10KDal active caspase-7 was not observed at any time points, the expression pattern of caspase-7 was showed the decreasing of the 35kDal pro-caspase-7 level. The equal loading of total protein in each lane was indicated by the similar level of actin (figure 23).

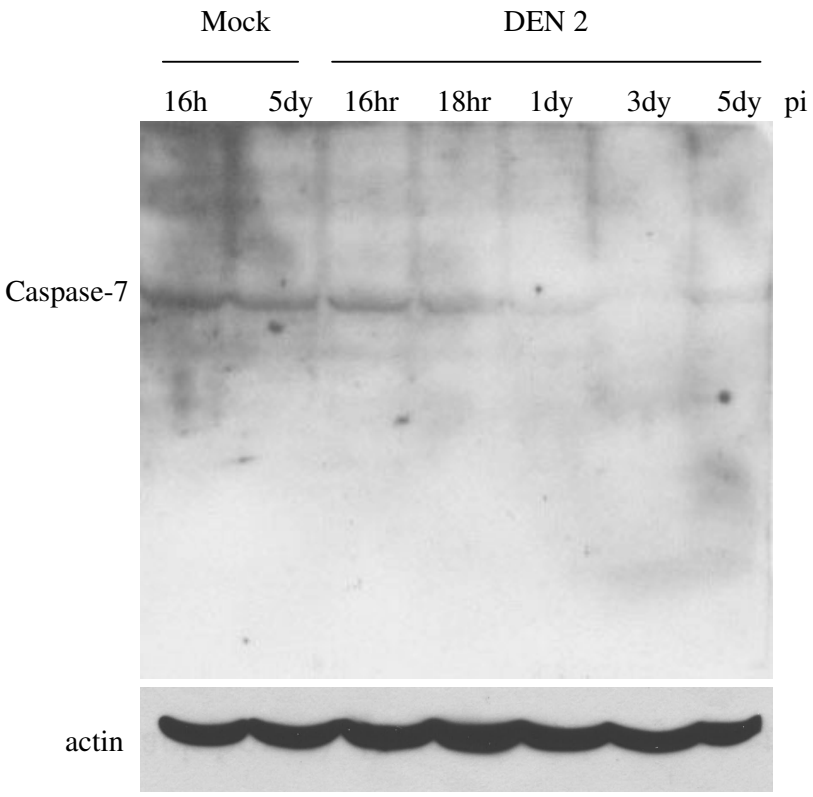

Figure 23: The Western blot analysis of caspase-7 by infection HepG2 cells with DEN-2.

The $2x10^6$ HepG2 cells were plated and infected with dengue virus serotype 2 at moi of 10. The total protein was extracted at any time point with RIPA buffer and 80µg protein was loaded and separated by 15% SDS- PAGE. The level of caspase-7 was analyzed by using caspase-7 polyclonal antibody at dilution 1: 250. Membrane was stripped and re- probed with anti- actin dilution 1:1,000

5. The effect of caspase inhibitor (z-VAD-fmk) to cell death and virus production.

To further investigate whether caspase activities are involved in dengue virus induced cell death, 50μM z-VAD-fmk, a cell permeable pancaspase inhibitor, was used to treat HepG2 cells before incubation with DEN-2. The cell culture medium was collected at day 1 to 5 post-infection for plaque assay to monitor the production of virus. At day 5 post-infection, the detached cell (dead cells) in medium were count by using haemocytometer, after that the attached cells were collected by trypsinization and separated by pipetting up and down. Then the attached cells were pooled together with the detached cells to count the number of total cells. The same experiment was done in parallel using Hep3B cells, another human hepatoma cell line which susceptible to dengue induced apoptosis (107), to compare the effect of inhibitor. For HepG2 cells, the cells were detached 1.23±0.43% in mocked-infection and 44.11±5.43% in dengue infected at day 5 post-infection (table 2). The detached cells of HepG2 cells were decreased to 26.17±2.93% after treatment with z-VAD-fmk which is approximately 40.67% decrease (figure 24). By contrast, the mock-infection of Hep3B cells were detached 6.77±2.64%. Dengue-infected Hep3B cells were detached 67.22±3.66% and the detached cells were decreased to 58.18±3.64% in z-VAD-fmk treated which is only 13.45% decrease (table 2, figure 25). In HepG2 cell, the virus production was significantly decreased after treatment with pan-caspase inhibitor (figure 26). On the other hand, pretreatment with caspase inhibitor in Hep3B cell did not significantly effect to the production of the virus (figure 27).

	%detached HepG2 cells	**%detached Hep3B cell**
Mock infected	1.23±0.43	6.77± 2.64
DEN2 infected	44.11±5.43	67.22± 3.66
Z-VAD-fmk/DEN2	26.17±2.93	58.18± 3.64

Table 2: Percentage of detached cells at day 5 post infection.

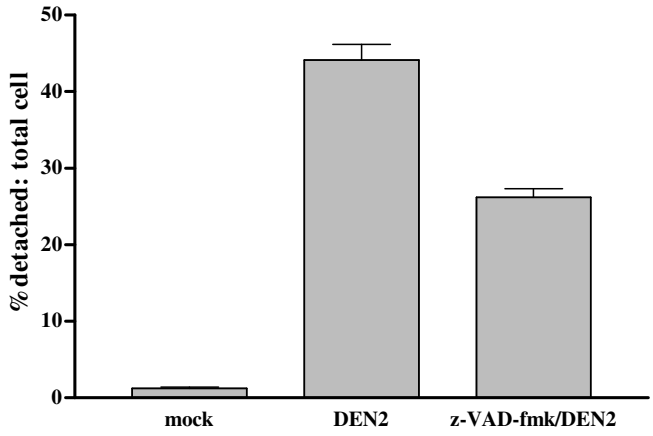

Figure 24: Comparison of the percentage detached cell between treatment and non-treatment with caspase inhibitor (z-VAD-fmk) in HepG2 cell.

HepG2 cells were non-pretreated or pretreated with 50μM of z-VAD-fmk before infection with DEN-2 at moi of 1. The cells were culture in the condition which appropriated to each cell type (see materials and method) for further 5 days. The number of detached cells and total cells were counted and calculated into %detached cells/total cells. Pretreatment of the inhibitor to HepG2 cells reduced the %detached cells/total cells for 40.67%. The error bars represent SEM.

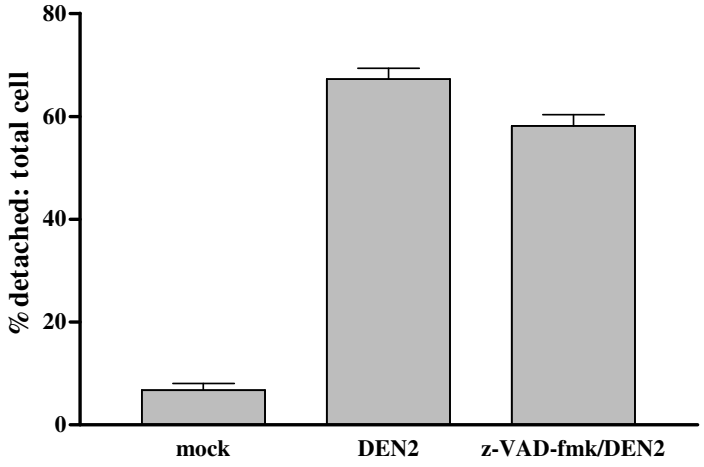

Figure 25: Comparison of the percentage detached cell between treatment and non-treatment with caspase inhibitor (z-VAD-fmk) in Hep3B cell.

Hep3B cells were non-pretreated or pretreated with 50μM of z-VAD-fmk before infection with DEN-2 at moi of 1. The cells were culture in the condition which appropriated to each cell type (see materials and method) for further 5 days. The number of detached cells and total cells were counted and calculated into %detached cells/total cells. Pretreatment of the inhibitor to Hep3B cells reduced the %detached cells/total cells only 13.45%. The error bars represent SEM.

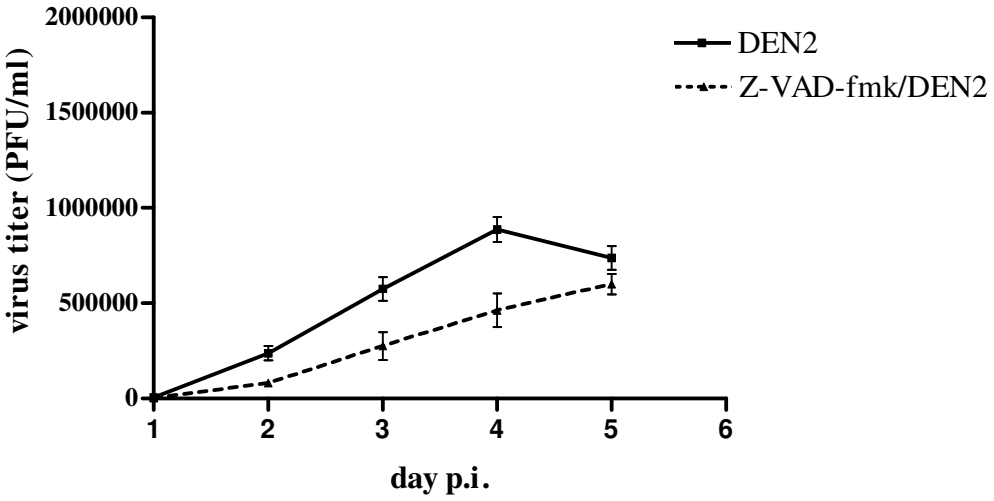

Figure 26: The effect of broad caspase inhibitor (z-VAD-fmk) to dengue virus production on HepG2 cells.

The figure showed the production of virus after treatment with 50μM of caspase inhibitor (z-VAD-fmk) and infected with DEN-2 at day 1 to 5 post infection in HepG2 cells (dotted line) compare with the positive control (solid line). Effect of the inhibitor to the virus production was showed significantly. The error bars represent SEM. ($P<0.0001$)

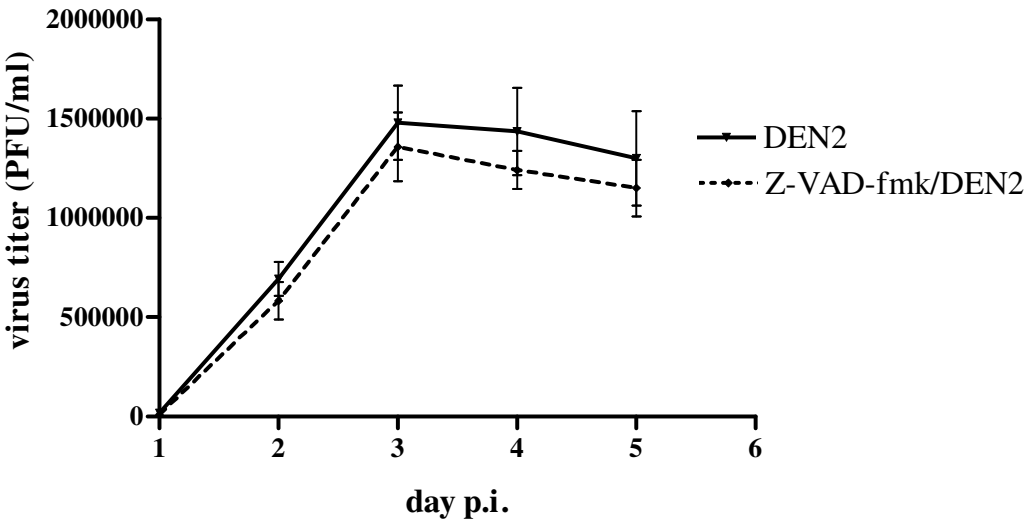

Figure 27: The effect of broad caspase inhibitor (z-VAD-fmk) to dengue virus production on Hep3B cells.

The figure showed the production of virus after treatment with 50µM of caspase inhibitor (z-VAD-fmk) and infected with dengue virus serotype 2 at day 1 to 5 post infection in Hep3B cells (dotted line) compare with the positive control (solid line). There was no significant difference of the production of virus. The error bars represent SEM. ($P<0.0001$)

CHAPTER V
DISCUSSION

Apoptotic cell death participates in the pathogenesis of many metabolic and infectious diseases [108]. Several viruses can induce programmed cell death which contributes directly to their cytopathogenic effects on the host cells [109]. The susceptible target cells for dengue virus infection are still not specified, although it has been shown that the dengue virus can infect a variety of cell types *in vitro*. At autopsy, dengue virus antigen has been found in several organs but predominantly in the liver, spleen, thymus, lymph node and lung cells [6]. Although the most recognized target cells for this virus in humans are the mononuclear phagocytes, several studies indicated that the involvement of the liver contributes to the severe pathogenesis of some dengue patients. Increasing level of transaminase and the defective of coagulation factor synthesis indicates the destruction of liver tissues [33;34;110]. There are many evidences of dengue virus induced hepatocytes apoptosis which may cause liver injury. Liver biopsy from fatal cases of dengue infection show the co-staining of dengue virus antigen with the DNA fragmentation, apoptotic hepatocytes by in situ detection [111;112]. To date, how the virus and host specific factors which have been implicated in the virulence of dengue disease are poorly understood. While the dengue virus induced liver cells apoptosis may participate in some parts of severe manifestation, the mechanisms underlying this effect are less well established.

The human hepatoma cell line, HepG2 was selected for studying the mechanisms of dengue virus induced apoptosis. Further studies demonstrated that infection of dengue virus serotype 1 in this cell line show morphological and biochemical changes consistent with the induction of cell death [102]. In this study, the effects of the viral specified factors in each serotypes for apoptotic induction was firstly investigated. From the results of four serotypes of dengue virus induced apoptosis by DNA fragmentation, only infection with DEN-1, 2 and 4 clearly showed

a DNA ladder pattern which is a hallmark of apoptotic cells. When compared with the negative control, infection of DEN-3 showed low signal of DNA laddering. It is interesting about the factors which make the DEN-3 different from other serotypes for apoptotic induction. To be notice, the production of DEN-3 was quite lower than the other serotypes [113]. Possible, in the replication of DEN-1, 2 and 4 which produce higher level of virus progeny and viral protein synthesis may cause the protein accumulation in the ER lumen or disruption of the ER-golgi transportation, resulting in the ER stress and induction of ER-stress mediated apoptosis.

To investigate whether the viral specific factor can cause the difference in ER stress response which may cause the different pattern of apoptosis, DEN-2 and 3 were selected to demonstrate. A high level of glucose regulated protein 78 (GRP78) expression is indicative of ER stress [114-116]. From the result of the induction of ER stress in response to dengue virus infection, the total protein extract from infection HepG2 cells with DEN-2 showed increasing of the GRP78 level whereas serotype 3 showed similar level of GRP78 when compare with mocked infection. It is interesting that the up-regulation of GRP78 level appeared at day 3 post-infection which consistent with the induction of apoptosis detection by DNA fragmentation when infection with DEN-2 at moi of 10. This result showed that the replication of dengue serotype 2 can induce ER stress and may support the initiation of apoptosis response via the signal transduction from ER.

In general, ER stress is induced by alteration in calcium stores or accumulation of unfolded or unassembled proteins in the ER lumen. Dengue virus and other flaviviruses such as HCV encode viral envelope glycoproteins that accumulate at high levels in the ER lumen [117-120]. The previous study in HCV showed that the HCV envelope proteins exist as high molecular- weight aggregates that are not recognized by conformation- specific antibodies, suggesting that they exist in nonnative conformations [121]. Overexpression of HCV glycoproteins activates *grp78* and *grp94* promoters in transient gene expression assays, consistent with the induction of ER stress response [122]. In addition, expression of HCV protein in HepG2 cells induces apoptosis [123]. Therefore, it is temping to speculate that dengue virus-induced ER stress is mediated through the signal that emanate from the ER lumen and are cause by the presence of unfolded or unassembled viral glycoproteins.

The main function of ER is viewed as a site of processing plant for protein folding and posttranslational modification of secreted and integral membrane proteins. Various conditions can disturb ER functions resulting in presenting of significant stress to cell. In response to ER stress, several signal transduction pathways can be activated. Recently, the ER was shown to be another subcellular compartment implicated in apoptotic execution. One of major local factor which initiates apoptosis by the signal transduction from ER stress is caspase-12, a caspase that has been described in mice and whose human homologue is still elusive. After replication of DEN-2 in HepG2 cells showed the induction of ER stress which correlated with the cellular apoptosis. The western blot analysis was performed to investigate whether infection of virus can induce caspase-12 activation. But from the results, the activation of caspase-12 cannot be observed.

From 14 members of identified mammalian caspase, only 11 of human caspases have been confirmed which are caspase-1 to caspase-10 and caspase-14 [124;125]. Caspase-11 exists in the mouse only while caspase-13 turn out to be the bovine homologue and dose not belong to the human caspase family [126;127]. So far, the function of human caspase-12 is not well characterized.

Recently, it has been reported that the function of caspase-12 is lacking in human. Alignment of the murine caspase-12 cDNA with the human genome sequence indicates that the human *caspase-12* gene is localized as a single locus within the caspase-1/ICE gene cluster including caspase-1, caspase-4 and caspase-5 on chromosome 11q22.3 (figure 23). RT-PCR and molecular cloning revealed that this gene is expressed in nine alternatively spliced transcripts, but a frame shift mutation and a premature stop codon, which are presented in all splice variants, preclude the expression of all full length protein (figure 29). Furthermore, loss-of-function mutation within the SHG box, a critical site in caspases, prohibits any proteins, if any are produced, from acting catalytically. Therefore, the absent of caspase-12 activation in response to dengue virus infection may causing from the lost of function of caspase-12 in HepG2 cells which is the cell line obtained from human source. The signal from approximated 35 kDa protein may be the truncated protein expressed in HepG2 cell.

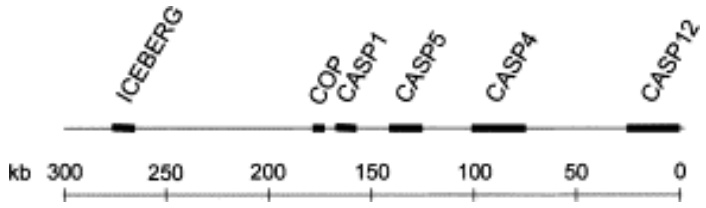

Figure 28: Organization of the human gene locus at 11q22.3 [128]

The gene of caspase-12 is located within the region of genes of the interleukin-1β converting enzyme (ICE) subfamily of caspases (caspase-1, 4 and 5) and its adapter molecules CARD-only protein (COP) [129] and ICEBERG [130]. Genes are indicated by black boxes.

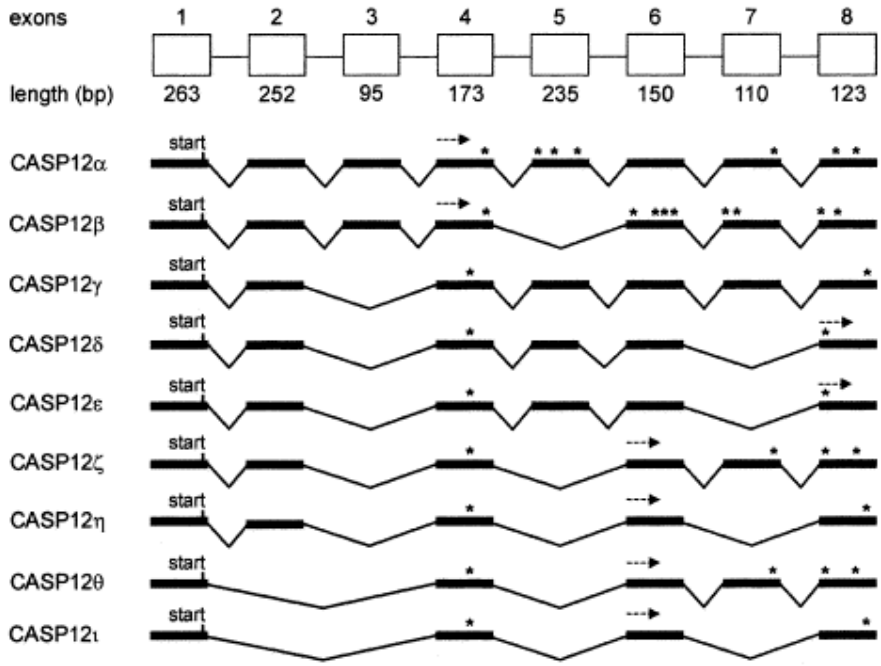

Figure 29: Exon structure of nine splice variants of human caspase-12 [128]

The open reading frame (ORF) of caspase-12 splice variants containing exon 3 (caspase-12 variant α and β, GenBank Acc Nos.AF486844 and AF486845) is shifted after this exon which introduced a frame shift (dotted arrow), causing several premature stop codon (asterisk). Moreover, all other variants, variants γ as well as splice variants δ to ι(GenBank Acc Nos. AF486847, AF464191, AF464192, AF464193, AF464194 and AF464195) contained early stop codons.

In place of caspase-12, other caspases may play roles in the ER stress-mediated cell death of human cells. Candidate caspases involved are caspase-4 and caspase-5. Procaspase-4 and procaspase-5 amino acid sequences are 59 and 54% identical to mouse procaspase-11, respectively. Procaspase-4 and procaspase-5 have an amino acid sequence identity of 77% (the next identity score being 55% between procaspase-1 and procaspase-4) and are also localized at the locus 11q22.3. On the basis of these results, human caspase-4 and caspase-5 are considered to be duplicated counterparts of mouse caspase-11 [131].

However, both caspase-4 and caspase-5 have a CARD domain at the N-terminal and also show the high similarity to mouse caspase-12 (figure 30). Procaspase-4 and procaspase-5 amino acid sequences are 48 and 45% identical to procaspase-12, respectively [132-134] (figure 30). Furthermore, the tetrapeptide around the cleavage site of caspase-12, VETD, is similar to the human caspase-4, VEKD. Very recently, it has been shown that human caspase-4 is located at the ER and activated by ER stress [135]. Thus, caspase-4 is involved in the ER stress-mediated cell death in human. At present, however, it is not clear whether caspase-4 and caspase-12 are associated with the same molecules located at the ER via their CARD domain and activated by the common molecular mechanism.

On the other hand, it is possible that other apoptotic mechanisms might also operate simultaneously (figure 31). Several possible pathways have been postulated for ER stress induced apoptosis. The other ER-associated proapoptotic molecules have been reported. Bap31, an ER transmembrane protein that binds to nascent membrane proteins in transit between ER and golgi apparatus, can form the complex with caspase-8 and the antiapoptotic regulator Bcl-2 or Bcl-x_L [136;137]. Cleavage of Bap31 by caspase-8 gives rise to a p20 subunit fragment which stimulates Ca^{2+}-dependent mitochondria fission, enhancing the releasing of cytochrome *c* in response to this initiator caspase [136]. The caspase-derived fragment of Bap31 was proposed to be a coordinator of cell death signals between the ER and mitochondria. It has been reported for the Tula Hantavirus, the envelope spherical negative stranded RNA virus of the family Bunyaviridae which infection initiates caspase-8 mediated apoptosis and several death programs that are intimately associated with ER stress and also changes in the expression pattern of Bap31 by down-regulation of the full-length and p20-

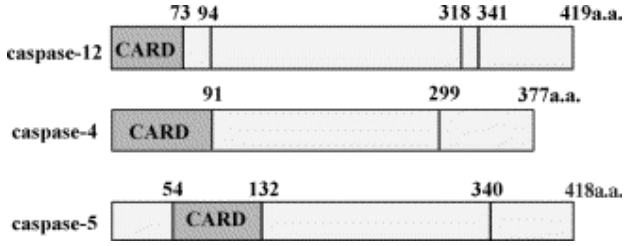

Figure 30: Comparison of molecular structure of mouse caspase-12, human caspase-4 and caspase-5 [138]

Amino acid sequences of mouse caspase-12 are 48 and 45% identical to those of human caspase-4 and caspase-5 respectively. Mouse caspase-12 has putative cleavage sites at D^{318} ($ATAD^{318}$) and D^{341} ($VETD^{341}$), while caspase-4 and caspase-5 have their cleavage site at D^{299} ($VEKD^{299}$) and D^{340} ($EEKD^{340}$), respectively. The tetrapeptide at cleavage site of caspase-4 at D^{299} is very similar to that of caspase-12 at D^{341}. Caspase-12, caspase-4 and caspase-5 have CARD domain at their N-terminal regions, 4-73, 2-91 and 54-132 respectively.

subunit of this protein [139]. Furthermore, human Bap31 can bind Bcl-2/Bcl-X_L and Ced-4 when this nematode protein is express in human cells [140].

The c-Jun amino-terminal kinase pathway (JNK; also known as stress-activated protein kinases/SAPK) is another regulation pathway which activated by ER stress though the IRE1-TRAF2-ASK1 complex formation [141] (figure 31). The antiapoptotic protein, Bcl-2 is phosphorylated and inactivated by the co-expression of ASK1 and JNK [142]. Furthermore, several reports showed that Bim, a BH3-only Bcl-2 family member, is induced by the JNK- c-Jun pathway [143;144]. Previous data suggested a significant pro-apoptotic role for the JNK/activator protein-1 (AP-1) signaling pathway in several forms of hepatocellular apoptosis [145]. Infection of dengue virus has been shown to activate JNK signaling pathway by increasing of both the JNK activity and level of phosphorylated AP-1 transcription factor subunit c-Jun in Huh-7 cells, another human hepatoma cell line [146]. It is interesting whether the activation of JNK pathway is the signal transduction initiated from ER stress.

Another family of apoptotic proteins which their function were initiated by ER stress is Bcl-2 family. Bcl-2 family proteins are divided into two groups which are anti-apoptotic and pro-apoptotic. Anti-apoptotic Bcl-2 family members include Bcl-2 and Bcl-X_L. Pro-apoptotic Bcl-2 family members are subdivided into a BH3-only class (including Bid and Bim) and multidomain class (including Bax and Bak). Bcl-2 proteins and their apparent roles in cell death have been evolutionary conserved and these proteins play key roles in regulation the integrity of the ER and mitochondrial membrane. A significant fraction of endogenous Bcl-2 family member proteins including Bcl-2, Bcl-X_L, Bax, Bak and Bik has been shown to be associated with the ER [137;147-149] where among other potential effects, these proteins function in the maintenance of Ca^{2+} homeostasis. The overexpression of Bax and Bak leads to Ca^{2+} efflux from the ER, Ca^{2+} influx into the mitochondria and increased cytochrome *c* release- induced ER Ca^{2+} efflux, leading to cell death that is inhibitable by Bcl-2 [150;151]. Infection with Japanese encephalitis virus, another virus in the family *Flaviviridae*, triggers the ER stress response in fibroblast BHK-21 cells and in neuronal N18 and NT-2 cells resulting in apoptotic cell death. Overexpression of Bcl-2 diminished JEV-induced apoptosis [101]. The recently study was shown that small membrane (M) protein of dengue virus was involved in the apoptosis induction.

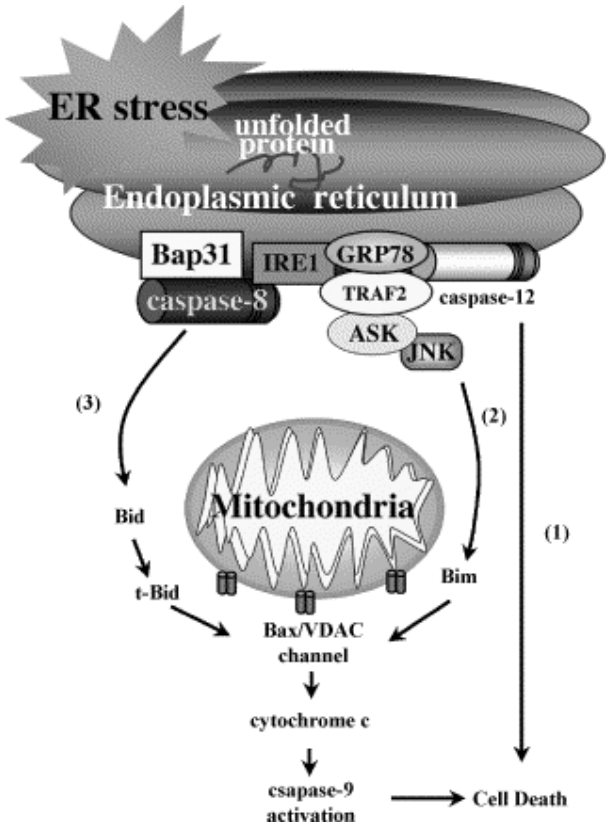

Figure 31: ER stress-mediated apoptotic pathways in various mouse cells [138]

Three major apoptotic pathways have been reported in the ER stress-mediated cell death: (1) caspase-12-dependent apoptotic pathway; (2) ASK/JNK pathway, which induces cytochrome *c* release from mitochondria and caspase-9 activation; (3) Bap31 and caspase-8 pathway, which also induces cytochrome *c* release from mitochondria and caspase-9 activation.

Evidence is provided that intracellular production of the M ectodomains (residues M-1 to M-40) of all four dengue serotypes triggered apoptosis in host cells such as mouse neuroblastoma Neuro 2a and human hepatoma HepG2 cells. The export of the M ectodomain from the Golgi apparatus to the plasma membrane appeared to be essential for the initiation of apoptosis. The study found that overproduction of Bcl-2 protected HepG2 cells against the death-promoting activity of the dengeu M ectodomain suggesting the involvement of this anti-apoptotic protein [152].

From the result of the effect of dengue virus infection to caspase-7 activation, no signal from 10kDal active caspase-7 has been observed but the decreasing of 35kDal pro-caspase-7 was shown. It is possible that the decreasing of pro-caspase-7 level is cause from its cleavage. From the reason that caspase-7 is the caspase which activation occurred at the late stage of apoptosis, the 10kDal active caspase-7 may degraded from the proteolysis process of apoptotic cell resulting in low amount of protein which cannot be detected by Western blotting. At least, this result was showed the involvement of caspase pathway in response to dengue infection.

The z-VAD-fmk is a highly specific cell-permeable irreversible inhibitor of caspases which variety used for study the effect of caspase dependent pathway to induction programmed cell death by several cell death signals. The study on Japanese encephalitis virus showed that treatment with this inhibitor can diminish virus induced apoptosis of BHK-21 cells. In addition, the death-promoting activity of dengue M ectodomains (ApoptoM) which showed to induce apoptosis in Hela cell, was partially inhibited with this general caspase inhibitor. This result from this study suggests that caspase-mediated pathway was involved in dengue ApoptoM induced apoptosis. From the result of the effect of caspase inhibitor to cell death, pretreatment of z-VAD-fmk was shown highly effect of inhibition of cells death in response to dengue virus infection on HepG2 cells. Interestingly, this inhibition was not efficient in p53-deficient Heb3B cells. It has been reported that the expression of caspase-8 is regulated by p53. The increase in caspase-8 gene transcription depends on the p53 status of the hepatoma cell line. Moreover, subsequent promoter deletion analysis in combination with luciferase reporter assays identified a p53-responsive element downstream of the transcriptional start site. These results implied that caspase-8 is a target gene of p53 [153]. Although the transcription regulation of other caspase by

p53 is still unclear, possible, the caspase-mediated pathway may defect in some part of their regulation in Hep3B cells. From these reasons, dengue virus may operate other caspase-independent pathway to induced apoptosis in Hep3B cells. Therefore, the broad caspase inhibitor give low effect to the inhibit dengue virus induced cell death in this cell line. Inhibition of caspases in HepG2 cells reduced dead cell approximately 40%, suggesting the noncaspase-mediated pathway also activated in parallel with the caspase dependent pathway in HepG2 cells but less influence than Hep3B cells.

For the effect of caspase inhibitor to virus production, the result showed the effect by reduce the virus production only in HepG2 cells but not in Hep3B cells. To be notice, the ability of host cells to produce viral progeny is correlated with the amount of dead cells. RNA viruses are though to induce apoptosis to facilitate release of virions. A growing number of viruses are believed to induce apoptosis actively at the late stage of infection. This process may represent a final and important step in the spread of the progeny to neighboring cells while also evading host immune inflammatory responses and protecting progeny virus from the host enzyme and antibodies. However, apoptosis needs to be delayed long enough for the virus to complete its replication cycle and to produce sufficient quantities of progeny. Very recently, it has been shown that dengue virus and Japanese encephalitis virus can activate phosphotidylinositol 3-kinase (PI3K) to block caspase dependent apoptosis at the early stage of infection. Further more, Bcl-2 apperars to be a crucial mediator downstream of PI3K/Akt signaling to inhibit apoptosis, since the overexpression of Bcl-2 reduced virus-induced apoptosis even the PI3K activation was repressed. The activation of the PI3K/Akt in flavivirus infected cells appears to be transient, probably due to the following mechanism. The study showed that the decrease of the Akt protein levels in flavivirus infected cells during the late stage of viral infection is likely mediated by the virus activated caspase, since the down regulation of Akt level could be blocked by a caspase inhibitor. To be note, even though the blocking of PI3K can increase virus induced cytopathic effect but the inhibition did not effect to the virus proliferation. This result indicating that a caspase independent translation mechanism might be adopted by these viruses when PI3K is blocked [154].

In the other hand, since the inhibition of caspase dependent pathway can reduce the virus production in HepG2 cells, it is interested that how the inhibition of

caspases activation effect the virus production. It has been reported that Langat Flavivirus NS3 protease bound to caspase-8 at both protease and helicase domain which enhance caspase-8 mediated apoptosis [155]. Interaction of caspases with viral proteins may play somewhat in the stage of replication especially at the step of polyprotein processing. Anyway, explanation of this mechanism is remained unclear.

Finally, rather than being single linear mechanisms, infection with dengue virus seem to induced host cells apoptosis in several pathways which can cross-interact. Moreover, the initial step of cell death response to infection of virus is depended on cell type. The exact mechanisms of dengue virus induced liver cell death remain largely explored.

CHAPTER VI
CONCLUSIONS

1. Induction of apoptosis in hepatocyte by dengue virus is mediated by serotype and cell type specific factors.
2. There is a correlation between the degree of cellular apoptosis and the amount of virus release, suggest that hepatocyte apoptosis is induced to enhance virus yield.
3. The evidences suggest that apoptosis is induced by multiple pathway both caspase dependent and caspase independent pathway, and that these pathways exhibit some degree of cell death.
4. The replication of dengue virus in HepG2 cells can induce ER stress.

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APPENDIX

APPENDIX
REAGENT PREPARATION

1. Solution for plaque assay

1.1 1xPBS

For 1 liter solution

Nacl	8 g
KCl	0.2 g
Na_2HPO_4	1.15 g
KH_2PO_4	0.2 g

Distilled water up to 1,000 ml sterile by autoclaving

1.2 BA-I medium

For 100 ml solution

10x M-199 E	10 ml
1M Tris-HCl (pH7.6)	5 ml
BSA fraction V	1 g
100x Penicillin / streptomycin	1 ml
7.5% $NaHCO_3$	1 ml

Tissue culture graded dH_2O up to 100 ml sterile by filtration

1.3 20x Earl's Balance Salt Solution (EBSS)

For 500 ml solution

$CaCl_2 \cdot H_2O$	2.65 g
KCl	4 g
$MgSO_4 \cdot 7\ H_2O$	2 g
NaCl	68 g
$NaH_2PO_4 \cdot H_2O$	1.25 g
Glucose	10 g

Tissue culture graded dH_2O up to 500 ml sterile by autoclaving

1.4 2x nutrient solution

For 100 ml solution

20x EBSS	9.8 ml
YE-LAH	6.6 ml
ΔFBS	6 ml
Gentamycin (80mg/ml)	0.5 ml
Fungizone (2.5mg/ml)	0.1 ml
7.5% $NaHCO_3$	6 ml
Tissue culture graded dH_2O to 100 ml	

1.5 YE-LAH

For 100 ml solution	1 g
Yeast extract	5 g
Lactabumin hydrolysate	
Tissue culture grade dH_2O to 100 ml sterile by autoclaving	

1.6 Seakem LE Agarose 1.6% autoclave

1.7 Crystal violet 1% (w/v) in 10% Ethanol

1.8 3.7% formaldehyde in PBS

2. RIPA buffer

For 50 ml solution

1 M Tris-HCl pH 7.5	2.5 ml
5M NaCl	1.5 ml
0.5 M EDTA	1 ml
10% SDS	0.5 ml
10% Triton X-100	5 ml
10% Sodium deoxycholic acid	5 ml
Distilled water up to 50 ml	

Protease inhibitor cocktail (1 mM PMSF, 5 µg/ml aprotinin, 5µg/ml leupeptin and 5 µg/ml prostratin) added fresh

3. Solution for SDS-PAGE and Western blotting

3.1 5x running buffer (Tris-glycine buffer)

For 1 liter solution

Tris base	15.1 g

Glycine	94 g
10% SDS	50 ml
Conc. HCl	4.3 ml

Adjust pH 8.3 and add distilled water up to 1,000 ml

3.2 1x transfer buffer (Wet blot)

For 1 liter solution

Tris base	1.93 g
Glycine	9 g

Distilled water up to 1,000 ml

3.3 1x TBS-T

For 1 liter solution

1M Tris-HCl	20 ml
5M NaCl	28 ml
Tween-20	1 ml

Distilled water up to 1,000 ml

3.4 Staining solution

For 1 liter solution

Methanol	400 ml
Acetic acid	150 ml
Coomazzie brilliant blue	1 g

Distilled water up to 1,000 ml

3.5 De-staining solution

For 1 liter solution

Methanol	100 ml
Acetic acid	75 ml

Distilled water up to 1,000 ml

3.6 Blocking buffer

5% skim milk in TBS-T

3.7 5x loading buffer

60 mM Tris-HCl pH 6.8

4% (w/v) SDS

25% (v/v) glycerol

0.1% bromophenol blue

100 mM DTT

4. HBSS buffer

$CaCl_2$	140 mg/L
$MgCl_2$-$6H_2O$	100 mg/L
$MgSO_4$-$7H_2O$	100 mg/L
KCl	400 mg/L
KH_2PO_4	60 mg/L
$NaHCO_3$	350 mg/L
NaCl	8,000 mg/L
Na_2HPO_4	48 mg/L
D-Glucose	1,000 mg/L

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Adaptation of the plaque assay methodology for dengue virus infected HepG2 cells

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Abstract

The HepG2 cell line is a useful tool for studying dengue virus–cell interactions but as it grows in clumps rather than monolayers, it does not readily adapt itself to the standard plaque assay technique. We therefore sought to develop an indirect plaque assay methodology. Initially HepG2 cells were infected with dengue virus serotype 2 and post-infection incubated for between 0 and 16 h before being treated with trypsin to separate the cells, followed by dilution and plating onto pre-grown monolayers of Vero cells in six well plates. After 7 days incubation and crystal violet staining, plaques were observed at all time points, although there was a relationship between number of plaques and post-infection incubation time, with the longest post-infection incubation time giving the highest number of plaques. To validate the assay with respect to virus input, the experiment was repeated at both the 0 and 16 h post-infection incubation times with different virus: cell levels. At both post-infection incubation times the response of input virus to plaque number was linear. This is a useful adaptation of the plaque assay methodology and one that may be applicable to other virus/cell line combinations.

Keywords: Flavivirus; Hepatocyte; Infection; Liver; Vero

Cell lines that are used commonly in plaque assays in dengue virus research include Vero (African Green monkey kidney) cells (Rao, 1976; Way et al., 1976; Matsumura et al., 1971), LLC-MK2 (Rhesus monkey kidney) cells (Morens et al., 1985; Kraiselburd et al., 1985) and BHK-21 (baby hamster kidney) cells (Malewicz and Jenkin, 1979). While these are useful cell lines for determining the levels of viruses, they are arguably less useful in studies investigating the mechanisms of entry of dengue viruses into cells given that they may not represent true target tissues, and as such their appropriateness may be somewhat limited. In dengue fever, the involvement of the liver in the pathogenesis of the disease is well documented (Kuo et al., 1992; Edelman et al., 1975; Bhamarapravati et al., 1967), and the dengue virus has been recovered from the liver of fatal cases of dengue fever (Rosen et al., 1999, 1989). As such, liver cell lines such as HepG2 which are derived from human hepatomas, probably more accurately represent a true target tissue. However, HepG2, while a useful cell line to study dengue virus: cell interactions has the disadvantage of growing in clumps rather than in a monolayers. As such, studies to detect the virus binding and internalization in this cell line are severely hampered due to an inability to maintain a concordance between an infected cell and a countable output, such as a plaque. We therefore sought to develop an indirect plaque assay by plating infected HepG2 cells onto monolayers of cells used commonly in the plaque assay technique, namely Vero cells.

We have established previously that it takes 18 h post-infection for mature infectious dengue serotype 2 viruses to be produced in HepG2 cells (Thepparit et al., Internalization and propagation of the dengue virus in human hepatoma (HepG2) cells, in press). Hence, if a dengue virus infected HepG2 cell was plated onto pre-grown monolayers of Vero cells, and the HepG2 cell was able to survive for 18 h a plaque should form. However, given that the conditions for growth under the overlay and under CO_2 levels required for maintenance of the Vero cells, it is possible that the HepG2 cells would not survive sufficiently long to produce mature viruses, or that this would be very in-

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efficient. To over come this problem, we attempted to incubate the dengue virus infected HepG2 cells under optimal conditions and then treat the cells with trypsin to separate the clumps and then dilute and plate. Under these conditions, the dengue virus infected HepG2 cells would have to survive under sub-optimal conditions for a much shorter period of time before producing mature viruses. Given that by 18 h the HepG2 cells would produce mature viruses (Thepparit et al., Internalization and propagation of the dengue virus in human hepatoma (HepG2) cells, in press), a cut off of 16 h post-infection incubation was selected to avoid plaques being produced in the Vero cells directly by progeny viruses in the cell supernatant. Under this length of post-infection incubation, the dengue virus infected HepG2 cells would only have to survive for two hours before producing viruses. We initially therefore selected a range of time points, ranging between no post-infection incubation (HepG2 would have to survive under overlay for 18 h) and 16 h post-infection incubation (HepG2 cells would have to survive for 2 h).

To determine the time course profile, HepG2 cells (2×10^6 cells/plate) were plated onto 5 cm diameter plates and propagated as described previously (Suksanpaisan and Smith, 2003). After overnight incubation, cells were incubated with dengue virus serotype 2 (strain 16681) in BA-1 buffer (1 × medium 199/Earle's balanced salts; 0.05 M Tris–HCl, pH 7.6, 1% BSA fraction V, 7.5% $NaHCO_3$, 100 units penicillin–streptomycin per ml) buffer at a virus to cell ratio of 1:1 for 90 min to allow adsorption and internalization (Huang et al., 2000; Suksanpaisan and Smith, 2003), subsequent to which, virus/cell mixture was treated with acid glycine (pH 3.0) to inactivate any uninternalized viruses (Hung et al., 1999). The infected cells were then post-infection incubated at 37 °C in 10% CO_2 in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin for 0, 4, 8, 12 and 16 h. After post-infection incubation cells were trypsinized with 0.25% Trypsin-EDTA (Gibco BRL, Gaithersburg, MD, USA) for 5 min at 37 °C and centrifuged at 1000 rpm for 10 min and resuspended in BA-1 buffer. The post-infection cell suspensions were serially diluted and 250 μl of each dilution was then plated onto pre-grown Vero monolayers in six well plates. A total of 4 ml of 1 × nutrient mixture (Earle's balanced salt solution supplemented with 0.5% (w/v) yeast extract, 2.5% lactabumin hydrolysate, 3% FBS) and 0.8% Seakem LE agarose (BMA, Walkersville, MD, USA) was added per well. Plates were incubated at 37 °C, 5% CO_2 for 7 days following which wells were treated with 3.7% formaldehyde for 1 h at room temperature and plaques developed by staining with 1% crystal violet in 10% ethanol. Experiment was undertaken independently in triplicate, with duplicate assay of plaque titer. Results (Fig. 1) show plaques were present at all time points of post-infection incubation examined. Fewest plaques are noted at the shorter post-infection incubation times, suggesting that not all of the infected HepG2 cells survive under the overlay for sufficient time to produce viruses. The response is however, relatively linear.

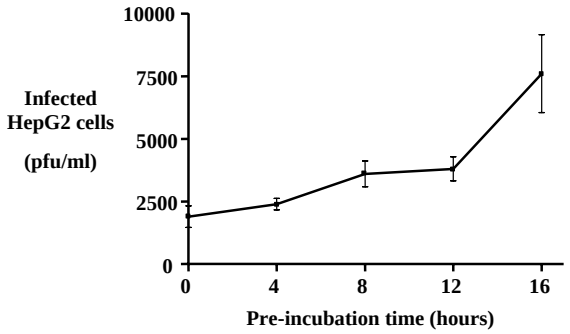

Fig. 1. Effect of pre-incubation on plaque number in an indirect plaque assay. HepG2 cells were infected with dengue virus serotype 2 and incubated post-infection for various times before being trypsinized and plated onto pre-grown Vero cell monolayers. Final plaque number is directly related to length of post-infection incubation time. Error bars represent S.E.M. of three independent experiments with duplicate assay of each point.

To investigate the linearity of the response with respect to input level of virus the experiment was essentially repeated, but this time only two time points (0 h post-infection incubation and 16 h post-infection incubation) were assayed, and the level of the input virus was varied for both times, and included virus: cell ratios of 0.1:1, 0.5:1, 1:1 and 5:1. Results (Fig. 2) show that with both 0 h post-infection incubation and with 16 h post-infection incubation a linear response between input virus and plaque number is obtained. The absolute number of plaques obtained at each virus input level is greatest for the 16 h post-infection incubation, suggesting that this methodology has the greatest sensitivity as more of the infected HepG2 cells survive to produce infectious viruses.

The use of trypsin to separate the HepG2 cells prior to plating on the Vero monolayers, will of course, have

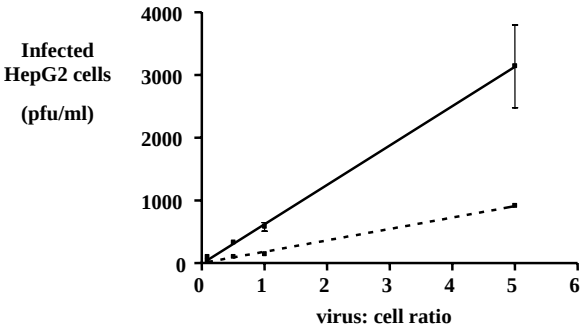

Fig. 2. Relationship between input virus and plaque number. HepG2 cells were infected for 90 min at different virus: cell ratios (0.1:1, 0.5:1, 1:1 and 5:1) and subsequently incubated prior to plating for either 0 h (dotted line) or 16 h (solid line) before being trypsinized and plated onto pre-grown monolayers of Vero cells. Both post-infection incubation times show a linear response, although pre-incubation of HepG2 cells for 16 h prior to plating shows a greater sensitivity. Error bars represent S.E.M. of three independent experiments with duplicate assay of each point.

significant effects upon the cells as a result of removal of most extracellular proteins. However, as stated earlier, this technique will have applications mainly in investigating the early mechanisms of binding and intenalization of the dengue virus. As such, the trypsin stage is post-internalization and will not affect this process. Other types of studies however may have to take this into account.

Overall the sensitivity of this methodology is relatively low, and calculations suggest that less that 1% of input viruses result in a plaque. However, provided that a constant post-infection incubation time is selected, the results, in respect of input virus, is linear and as such this method will have several applications in investigating the relationship between the dengue virus and liver cells, and may possibly be applicable to other virus/cell line combinations.

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