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Immunohistopathology of liver in autopsy cases of dengue shock syndrome

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Dengue hemorrhagic fever (DHF) is a severe febrile disease, characterized by abnormalities in hemostasis and increased vascular permeability, which in some cases results in hypovolemic shock and in dengue shock syndrome. The clinical features of DHF include plasma leakage, bleeding tendency and liver involvement such as enlargement of the liver. During the peak period of outbreak from July through October, of 2005 and 2006, a total of 13 fatal cases of dengue hemorrhagic fever (DHF)/ dengue shock syndrome (DSS) were collected from Intensive Care Unit, Yangon Children Hospital to study their immunohistopathology of DHF. The liver seems to be a target for dengue virus, so postmortem examinations were performed to investigate histopathological changes and whether the virus and viral antigens were present in target cells of the liver. We detected Kupffer cell hyperplasia in 100%, hemorrhage in 92%, mixed midzonal and centrilobular necrosis in 85%, and hemophagocytosis in 77% by using hematoxylin and eosin stain of routine histopathological standard method. Dengue virus antigens of the structural (envelop) and non-structural (NS1) proteins were detected in 54% and 77% respectively in both hepatocytes and Kupffer cells, using immunohistochemistry. There was no recruitment of polymorphonuclear cells; however, minimal degree of portal infiltration by lymphocytes was detected in the liver lesions of patients who died from DHF. Thus, our findings conclude that the hepatocytes and Kupffer cells may be target cells supporting virus localization and replication to provide more information for understanding the pathogenesis of liver involvement in DHF.

INTRODUCTION

Only a few histopathologic studies of liver has been published that describe the cellular and organ damage done by dengue viruses and the accompanying host responses that characterize dengue hemorrhagic fever/ dengue shock syndrome (DHF/DSS) [1-5]. No general histopathologic studies on fatal dengue infections have been published within the past twenty-five years. Conventional studies on dengue pathology have been supplemented by efforts to isolate dengue virus or localize dengue viral antigens in peripheral blood leukocytes, biopsies or organs at autopsy using fluorescent antibody or in situ hybridization [6-11]. French workers have demonstrated dengue 1 antigen in hepatocytes from a patient dying during a dengue infection; these cells also showed apoptosis [12]. Recently, studies of autopsy tissues have been complemented by efforts to grow dengue viruses in human tissue explants, primary cells and human cell lines. Several studies have shown the inability of dengue 1 virus to replicate in mature Kupffer cells *in vitro*, while hepatocytes and cell lines do become infected but

then promptly die as the result of apoptosis [13-16]. These studies raise questions concerning the sites of infection during human dengue infection. An under-standing of the mechanisms that produce symptoms of disease and the fatal vascular permeability, hepatic damage and metabolic acidosis rests ultimately with gaining definitive understanding of dengue virus-host cell interactions during infections. To achieve this goal, a comprehensive study of liver obtained at autopsy is essential. The liver seems to be a target for dengue virus, so autopsy is essential for immunohistopathological studies of DHF/DSS.

MATERIALS AND METHODS

Thirteen autopsies of DHF/DSS cases were collected following a designated protocol, at Yangon Children's Hospital (YCH) during 2005 and 2006. Ethical clearance had been taken from Ethical Review Committee, DMR (LM). After taking written inform consent from parents/guardian, autopsies were conducted as soon as possible after death in order to minimize autolysis and post-mortem artifacts using a proforma protocol. Subjects were placed in the mortuary cold room of YCH, the internal temperature of which is kept at 4-8°C constant. Age, sex, date of onset of fever, date of hospitalization and date of death were recorded for each study subject. During autopsy, gross observations of liver were also recorded. The liver tissue specimens were not allowed to dry out during the preparation phase and were placed into fixatives 10% neutral buffered formalin and Optimal Cutting Temperature (OCT) compound.

Staining methods

The following methods were applied to serial section: hematoxylin and eosin (H&E) and immunohistochemical staining using EnVision™ kit (Dako). This kit is a peroxidase-conjucated polymer backbone, which, in addition, also carries secondary antibody molecules directed against mouse immunoglobulins. Endogenous biotin will not affect staining results. The primary monoclonal antibody against NS1 and envelop protein and positive control of dengue virus injected suckling mouse brain were produced and provided by Medical Molecular Biology Unit, Siriraj Hospital. They were applied for 30 minutes at room temperature for primary antibody and 30 minutes for ready to use EnVision™ reagent. Counter stain was done by Mayer's hematoxylin.

RESULTS

The ages of the patients varied from 3 years to 9 years. Seven cases were aged 3 to 3 and half years, 2 cases were 4 to 5 years, 2 cases were 7 years and 2 cases were 9 years old. Five cases were males and 8 cases were females. All the patients went through two clinical stages, febrile and shock, before succumbing to the disease. Hospital stay varied from 1 to 8 days. The children who died within 24 hours were 2 cases, within 48 hours were 4 cases, 3 days and 4 days were 3 cases each and 8 days were 1 patient. All cases have hepatomegaly on clinical examination.

Gross autopsy findings

Most of the subjects appeared well developed and well nourished. Hemorrhage appeared as petechial rashes or purpura, especially around needle punctures sites. The rashes were particularly striking over the lower limbs; frank hemorrhage appeared in patches in subcutaneous tissue. Hemorrhage presented in the mucosa of the nose, gums and gastrointestinal tracts, as well as in liver. The livers were enlarged and subcapsular hemorrhages were noted in most fatal cases. The cut surface of the liver revealed yellowish colour suggesting fatty metamorphosis.

Microscopic findings

Microscopic examination of the liver was made in 13 patients. Necrosis of the liver cells was present in 11 cases, some of which showed only a few necrotic liver cells in a single focus, while in others a major part of

the hepatic lobules was involved. Where necrotic foci were small they occurred focally or in the paracentral part of the lobules, usually on one side of the central veins separated by a few rows of intact liver cells. The liver cells in the necrotic foci were swollen, their cytoplasm was acidophilic and vacuolated. The nuclei showed pyknosis, kavorrhexis and karyolysis, and occasionally had disappeared. The foci of necrotic liver cells were usually surrounded by histiocytic mononuclear cells or altered Kupffer cells. Polymorphonuclear leucocytes were rarely present. Centrilobular necrosis, hemorrhage, Kupffer cell hyperplasia and hemophagocytosis in necrotic areas were graded as in Table 1.

Table 1. Grading of histological changes in liver of DHF/DSS

Centrilobular necrosis	
Grade I	Necrosis area extend 1/3 from central vein to portal triad
Grade II	Necrosis area extend 2/3 from central vein to portal triad
Grade III	Necrosis area extend from central vein to portal triad
Hemorrhage	
Grade I	Hemorrhagic area extend 1/3 from central vein to portal triad
Grade II	Hemorrhagic area extend 2/3 from central vein to portal triad
Grade III	Hemorrhagic area extend from central vein to portal triad
Kupffer cell hyperplasia	
Grade I	With effort
Grade II	Occasional
Grade III	Easy to find
Hemophagocytosis in necrotic areas	
Grade I	With effort
Grade II	Occasional
Grade III	Easy to find

Histological changes in liver were observed by two pathologists at the same time who are investigators and this grading classification is based on their working definition.

In two cases, the necrotic foci were small and were seen in only a few lobules. Five cases showed moderate-sized foci, while in four cases the necrosis was fairly extensive (Table 2). In one case, no necrosis of the liver cells or haemorrhage was observed. In all cases the Kupffer cells showed hyperplasia and acidophilia of the cytoplasm. Councilman bodies were noted in one case of liver. Fatty metamorphosis of varying degree was noted; where it was not extensive the central and paracentral zones were mostly involved. Typical features of hepatitis were not seen in all fatal cases.

Table 2. Grading of histological changes in liver

	GI (%)	GII (%)	GIII (%)	No changes	Total number
Liver					
Centrilobular necrosis	2 (15%)	5 (38%)	4 (31%)	2 (15%)	13
Hemorrhage	3 (23%)	5 (38%)	4 (31%)	1 (8%)	13
Kupffer cell hyperplasia	0 (0%)	6 (46%)	7 (54%)	0 (0%)	13
Hemophago-cytosis	7 (54%)	2 (15%)	1 (8%)	3 (23%)	13

G= Grade

Immunohistochemical results

A total of 13 liver tissues, 11 cases (85%) were NS1 positive and 9 cases (69%) were envelop protein positive.

DISCUSSION

Despite the mixed midzonal and centrilobular necrosis of the liver, minimal degree of portal infiltration by lymphocytes was noted in all cases of DHF autopsy. But Kupffer cell hyperplasia was observed in Grade II and III of these cases. In this series of 13 autopsy cases, only one case showed Councilman bodies, frequently described in the literature for common pathologic change in the liver of dengue infection at autopsy. The causes of midzonal necrosis and centrilobular necrosis of the liver should be different. Regarding the vascular supply of the hepatic lobules, the centrilobular necrosis seems to be secondary to hypoxia. The midzonal necrosis seems to be immunologically induced, but it is difficult to explain why zone 1 and 3 are spared. The observation of the dilatation of subcapsular lymphatic spaces (Fig. 1) without any evidence of inflammation needs correlation with the existence of right pleural effusion and ascites from the clinical and gross autopsy profile in each case.

In the histopathological analysis of this fatal case of DENV-3 infection, in Rio de Janeiro,

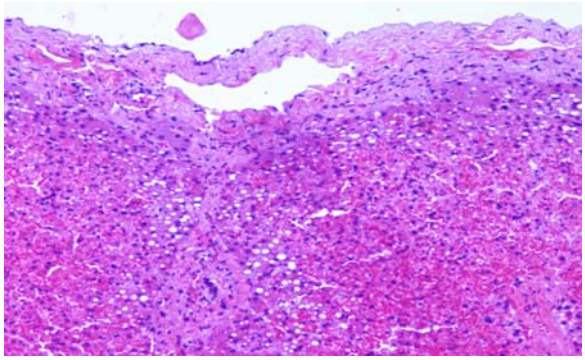

Fig.1. Histological changes of capsular lymphatic dilatation in liver (H & E)

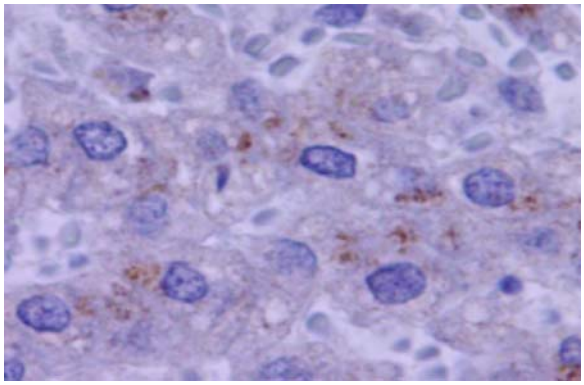

Fig. 2A. Dengue viral antigens (NS1) detected in hepatocytes and Kupffer cells (Magnification x 40)

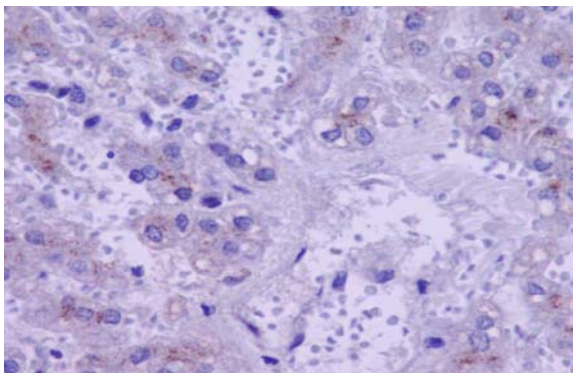

Fig. 2 B. Dengue viral antigens (E protein) detected in cytoplasm of hepatocytes and Kupffer cells (Magnification x 10)

Brazil found severe damage to the vascular system, hepatic injury, and mononuclear cell infiltration in many target organs. These data corroborate the conception that the liver is an important site of dengue virus replication. Intense injury to many organs was demonstrated, such as focal vacuolization of hepatocytes, and increasing concentrations of inflammatory cells inside sinusoid capillaries. This suggested the participation of cytokines and inflammatory mediators, causing alteration of vascular permeability [17]. In our patient, viral antigen was found in hepatocytes and Kupffer cells. Similarly, dengue virus has previously been identified within liver tissue, and dengue virus antigen was identified within Kupffer cells of infected individuals [18]. That expression of the NS1 and envelope protein antigens of dengue in the hepatocytes and Kupffer cells in the paraffin-embedded tissue is not quite distinct as that observed in the paraffin-embedded tissue of suckling mouse brain that was inoculated by heavy dosage of dengue viruses used as a positive control. Elevation of serum aminotransferase activity is almost universal in individuals with DHF [19]. The histopathology showed marked macrovesicular fatty changes, midzonal hepatocellular necrosis and focal lymphocytic and polymorphonuclear leucocyte infiltration in a case of dengue hemorrhagic fever with fulminant hepatic failure [20]. In our patient, hepatocellular injury was also observed, and focal microvesicular steatosis was found around the centrallobular vein. The hepatic alteration consisted of diffuse necrosis of sinusoid capillaries, suggesting release of cytokines and activation of the complement system, as well as endothelial dysfunction. In conclusion, the patient in our study, infected with dengue virus died with considerable hepatic injury.

The observation that the immunohistochemical assay was as sensitive in detecting the number of infected cells may have several explanations. First, the fatal form of the disease is clearly associated with massive viral proliferation in the liver with a concomitant large amount of viral protein being synthesized, resulting in ample amounts of target. Second, the multistep method employed allows a greater sensitivity in general for immunohistochemical detection of a variety of proteins. The immunohistochemical assay showed that the viral protein localized to the cytoplasm in a punctate pattern (Fig 2A & 2B). In this study, NS1 protein antigen was 85% positive in all liver tissue of patients,

envelop protein antigen as positive in 6% of these cases. Thus, the NS1 protein was found predominantly associated with the liver, where hepatocytes appeared to represent major target cells.

Dengue fever is a common disease that, in most cases, is self-limited. Presumably, this represents an active and successful immunologic response to the virus. In the rare and often fatal forms of dengue fever, such as hemorrhagic dengue fever, the virus is able to apparently escape immunologic surveillance and rapidly and massively proliferate at certain sites, such as the liver as documented in this study. The marked hepatic dysfunction associated with the viral infection certainly could explain, in part, the hemorrhagic tendencies characteristic of the disease. A better understanding of pathogenesis and viral proliferation in dengue infection need to study changes in mutiorgans by using IHC using viral markers and other markers as macrophages, dentritic cells, endothelial cells, B and T lymphocytes, compliments, cytokines and molecular techniques such as in situ hybriddization and RT-PCR.

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