Virus Research 175 (2013) 155–159

Contents lists available at SciVerse ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres

Short communication

Coagulation factors, fibrinogen and plasminogen activator inhibitor-1, are differentially regulated by yellow fever virus infection of hepatocytes

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ARTICLE INFO

Article history:
Received 24 January 2013
Received in revised form 17 April 2013
Accepted 22 April 2013
Available online 29 April 2013

Keywords:
Yellow fever virus
Asibi virus
17-D vaccine
Hepatocytes
Fibrinogen
Plasminogen activator inhibitor-1

ABSTRACT

Yellow fever virus (YFV) infection poses a great risk to un-vaccinated individuals living or traveling in the endemic regions of Africa and South America. It is estimated that approximately 30,000 people die each year of this disease. The liver is the main target of YFV, where as many as 80% of the hepatocytes may become involved in the infection. The overwhelming infection of the liver is associated with the observed hemorrhagic disease manifestations such as petechiae, ecchymoses, and hematemesis which are all thought to be linked with the observed coagulation abnormalities that include prolonged clotting times, reduction in clotting factors, fibrin-split products (D-dimers) and elevated prothrombin times. Many factors involved in the coagulation pathway are produced by hepatocytes, such as fibrinogen (FBG) and plasminogen activator inhibitor-1 (PAI-1). Both of these proteins have been indicated in another flavivirus related disease, dengue, as having roles related to the bleeding abnormalities observed and overall outcome of infection. In this study we wanted to determine if FBG and PAI-1 expression levels by human hepatocytes was disrupted or altered by infection with either wild-type Asibi or vaccine strain17-D YFVs. Our findings indicate that YFV infection does affect the transcriptional and translational expression of FBG and PAI-1 in human hepatocytes and that these results are further affected by IL-6 during early stages of infection. These results may lead to further understanding of the molecular mechanism associated with bleeding abnormalities observed during late stage YFV infection.

The acute phase response (APR) is comprised of many behavioral, biochemical, nutritional and physiological changes designed to counteract challenges induced by inflammatory stimuli. The APR is designed to counteract pathogens, limit cell destruction, activate coagulation pathways and activate repair processes. Hepatocytes are a significant source of acute phase proteins (APP) in response to inflammatory stimulation such as IL-6, IL-1, glucocorticoids, and growth factors. Many of the APPs produced by hepatocytes also have critical roles in maintaining hemostasis by regulating the coagulation pathway (Ceciliani et al., 2002; Gabay et al., 2001; Guillen et al., 1996; Johnson et al., 2004; Mazuski et al., 1997; Migita et al., 2004; Raynes and Bevan, 1993).

Fibrinogen (FBG) and plasminogen activator inhibitor-1 (PAI-1) are APPs that primarily function in the coagulation pathway. FBG is the main precursor of fibrin and mediates platelet aggregation and plasma viscosity (Iacoviello et al., 2001). Further, the functions of FBG γ chain include the recruitment of leukocytes, additional clotting and growth factors, and to induce production of IL-1β which, in turn, suppresses FBG production from hepatocytes in a negative feedback process (Albrecht et al., 2007; Iacoviello et al., 2001; van der Beek et al., 2002; Vasse et al., 1996; Verschuur et al., 2004). PAI-1 is the main inhibitor of fibrinolysis. PAI-1 primarily targets two major plasminogen activators: tissue type (tPA) and urokinase type (uPA). Dysfunction or inhibited production of FBG and over-production of PAI-1 has been associated with bleeding abnormalities that occur after severe inflammatory events such as sepsis (Kornelisse et al., 1996).

Yellow fever virus (YFV) is a positive-stranded RNA virus and a member of the family *Flaviviridae* that is reported to cause 200,000 infections annually in endemic regions of South America and Africa. Wild-type YFV infection causes an abrupt onset of non-specific symptoms (such as fever, malaise and nausea) following a 3–6 day incubation with concurrent viremia (Lloyd, 1931; Monath and Barrett, 2003). After a brief period of remission (~48 h), the patient may develop a hemorrhagic form of disease with multiple organ

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0168-1702/$ – see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.virusres.2013.04.013

dysfunction. Clinical symptoms may include, but are not limited to fever, vomiting, epigastric pain, jaundice, oliguria, cardiovascular instability, and hemorrhaging (Elton et al., 1955; Monath and Barrett, 2003). This period of disease can either result in the patient recovering rapidly with no liver scarring or will proceed to death. Only about 20–50% of patients in the intoxication stage progress to late-stage; accounting for an estimated 30–40,000 deaths each year (Monath et al., 1980; Nasidi et al., 1989).

Yellow fever virus strain 17-D is a live-attenuated virus, is used as the vaccine for prevention of yellow fever and has been administered globally (>500 million doses) with relatively few serious adverse events. The 17-D vaccine strain differs by approximately 35 amino acid residues from wild-type Asibi virus, with the majority of mutations found in the envelope protein (Barrett and Higgs, 2007; Hahn et al., 1987; Monath and Barrett, 2003; Rice et al., 1985, 1989). Wild-type YFV is highly hepatotropic and the presence of viral antigen in hepatocytes and Kupffer cells at post-mortem examination has been well documented (Klotz and Belt, 1930; McGavran and White, 1964). However, it is unknown whether 17-D virus infects the liver of healthy vaccinees that do not develop serious adverse events. It is possible that 17-D vaccination does result in liver-cell infection in vaccinees as low-level (<20–10^2 plaque forming units/mL) viremia develops in the serum (Monath et al., 2002; Wheelock and Sibley, 1965). We have previously shown that 17-D virus is capable of replicating in human hepatocytes (PH5CH8 cells) and primary human Kupffer cells, and further induces a major pro-inflammatory response from both cells types, which is regulated by few anti-inflammatory cytokines (Woodson et al., 2011; Woodson and Holbrook, 2011).

Coagulation factors such as FBG and PAI-1 have not been readily studied in the yellow fever literature as they are either not analyzed or not reported in case studies regarding wild-type virus infection or vaccine-associated serious adverse events. Clotting and prothrombin times are typically reported in case studies and indicate a deficiency or dysfunction in the coagulation system, but there has been little indication of where those dysfunctions are located within the pathway. Coagulation pathway components have been studied in a related flavivirus disease, dengue, which can also cause hemorrhagic disease. Dengue virus, the causative agent of dengue, is closely related to YFV and is also transmitted by infected mosquitoes. Several studies have examined expression levels of PAI-1 and/or FBG from patients suffering from classic dengue fever to severe dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). These studies have demonstrated that reduced FBG and increased PAI-1 serum levels are associated with severe dengue illness and poor outcome in children and some adults (Djamiatun et al., 2000; Huang et al., 2001; Jiang et al., 2007; Van Gorp et al., 2002). In one study, the "quality" of the fibrin clot formed from patient blood was examined *ex vivo*. It was found that the fibrin and resulting meshwork were thinner with an impediment in deposition of additional fibrin, which was suggested to be due to additional sialic acid residues (Marchi et al., 2009). Further, the clots were more prone to lysis.

FBG has been reported to have overall higher expression in YFV infection in both vaccinated and wild-type virus infected patients, even though there is significant patient variability (van der Beek et al., 2002; Verschuur et al., 2004). However, fibrin split products were also detected in these patients as an indication of hemorrhage. This finding suggests that YFV may inhibit or decrease release of PAI-1. To our knowledge, no information is currently available on gene expression and/or protein production of coagulation factors (FBG and PAI-1) from human hepatocytes after infection with either wild-type or vaccine strain YFV. Due to the bleeding abnormalities observed in yellow fever patients we wanted this study to focus on fibrinogen (FBG) and plasminogen activator inhibitor-1 (PAI-1) because of their roles in maintaining hemostasis. Our previous studies have shown that YFV infected hepatocytes induce high concentrations of IL-6 after YFV infection (Woodson and Holbrook, 2011) and since IL-6 is a major regulator of the APR and thus production of coagulation factors by liver cells, we also examined gene expression and protein production of FBG and PAI-1 after IL-6 stimulation of hepatocytes. In summary, our findings indicated that FBG and PAI-1 are negatively affected by YFV infection.

PAI-1 was measured in PH5CH8 hepatocyte culture media by ELISA (AssayMax human plasminogen activator inhibitor-1 ELISA kit available from AssayPro) from both un-stimulated and IL-6 pre-stimulated PH5CH8 hepatocytes [stimulation treatment and culture of cells is previously described in Woodson and Holbrook (2011)] after mock, Asibi or 17-D virus infection (Fig. 1). 17-D virus induced significantly higher concentrations of PAI-1 in hepatocytes during late stage infection when compared to either mock or Asibi virus infected hepatocytes ($p < 0.05$, measured by either Student's *t*-test or Mann–Whitney, see supplemental Table 1). The un-stimulated hepatocyte group (Fig. 1A) produced significantly ($p < 0.05$) more PAI-1 overall when compared to the IL-6 pre-stimulated hepatocytes group (Fig. 1B) regardless of the infecting virus.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.virusres.2013.04.013.

FBG was detected in a bead-based assay (Milliplex Map cardiovascular disease panel 2-fibrinogen, single plex available from Millipore) using a Bio-Rad Bio-plex 200 system described previously (Woodson and Holbrook, 2011). FBG was only detected from IL-6 pre-stimulated hepatocytes (Fig. 1B) and concentrations were very low (<0.4 ng/mL) from all samples (mock, Asibi virus and 17-D virus infected) with high variability between replicates. FBG production was highest overall from mock-infected samples, suggesting YFV infection causes a decreased capacity for these hepatocytes to produce FBG. However, when the infected samples were compared to each other, FBG concentrations were only significantly higher ($p < 0.05$) from Asibi virus infected hepatocytes at 72 hpi. No FBG was detected at 1 hpi, presumably due to the repeated washes for removal of the virus inoculum after the initial infection period. Control wells stimulated with IL-6 or un-stimulated for 48 h but not used in the experiment contained ~1.0 ng/mL or ~0.5 ng/mL of FBG respectively in the culture media (data not shown).

Gene expression for the two coagulation factors was also measured in 17-D and Asibi virus infected hepatocytes and compared to mock-infected hepatocytes (Fig. 2). PAI-1 and FBG were both found to be up-regulated from mock-infected controls after infection with either virus. Mock-infected controls were used to normalize data, therefore mock are assumed to be "1.0" and not represented in Fig. 2.

Hepatocytes infected with 17-D virus had significantly higher PAI-1 gene expression levels compared to Asibi virus infected hepatocytes in both un-stimulated and IL-6 pre-stimulated hepatocytes ($p < 0.05$, measured by Student's *t*-test or Mann–Whitney please see supplemental Table 2) (Fig. 2A and B, respectively). One exception was observed at 72 hpi with the un-stimulated Asibi virus infected hepatocytes being significantly higher. When the un-stimulated and IL-6 pre-stimulated groups were compared, PAI-1 gene expression was significantly ($p < 0.05$) up-regulated overall in the IL-6 pre-stimulated group, regardless of the infecting virus type.

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FBG gene expression was detected in both un-stimulated and IL-6 pre-stimulated YFV infected PH5CH8 hepatocytes (Fig. 2). FBG gene expression appeared to follow a similar trend as PAI-1 with 17-D virus infected hepatocytes inducing significantly ($p < 0.05$) higher gene expression when compared to Asibi virus infected