

## 論文の内容の要旨

論文題目 Analyzing the Role of Sphingolipids in the Replication of Hepatitis C Virus  
(C型肝炎ウイルスの複製におけるスフィンゴ脂質の役割の解析)

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Hepatitis c virus (HCV) is considered as one of the most important pathogens that cause chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). HCV is an enveloped, plus-stranded RNA virus, classified into multiple genotypes and subtypes. HCV belongs to the genus Hepacivirus in the Flaviviridae family. HCV replicates mainly in hepatocytes. It enters into the target cells by clathrin-mediated endocytosis in the low pH-dependent endosomal compartment. After uncoating, the viral RNA is released into the cytoplasm and translated into a polyprotein that is cleaved by both host and viral proteases into ten structural (E1, E2 and Core) and non-structural (NS) proteins (P7, NS2, NS3, NS4A, NS4B, NS5A and NS5B). The uncoated plus-stranded RNA genome serves as a template for the synthesis of a minus-strand which in turn serves as templates for the production of several strands of plus-strand RNA that are subsequently used for polyprotein translation, the synthesis of new intermediates of replication or packaging into new viral particles. Plus-stranded RNA viruses depend on intracellular membranes in all aspects of their life cycles. Plus-stranded RNA viruses, including HCV, share a hallmark of remodelling the intracellular membranes creating membrane replication factories or vesicles where viral RNA replication resides. Electron microscopic studies defined the membrane replication factories as a complex structure of remodelled membranes with negative and positive curvatures that bear little resemblance to the parental organelles from which they are derived. Almost every intracellular membrane in eukaryotic cells, including endoplasmic reticulum (ER), Golgi, the outer membrane of the mitochondria and peroxisomal membranes, acts as a parent membrane for the biogenesis of membrane replication factories induced by plus-stranded RNA viruses. Morphologically, there are two distinct types of these vesicles; the invaginated vesicle/spherule type and the double membrane vesicles (DMVs) type. Despite the very distant evolutionary relationship, HCV, picornaviruses, and coronaviruses belong to the DMVs type, whereas the more closely HCV-related flaviviruses such as Dengue virus (DENV) and West Nile virus (WNV) induce invaginated vesicles within the ER. Membrane replication factories perform different functions in the viral replication process: (i) increasing local concentration of factors required for viral replication, especially in the early steps of viral infection, (ii) spatially coordinating different steps of the viral replication cycle (RNA translation, replication and assembly), and (iii) acting as a barrier between viral RNA replication and the cytosol which contains

innate immune sensors, RNases, and proteases to protect viral proteins and RNA. HCV NS proteins are seen to be associated with a membranous web (MW) which includes DMVs, HCV RNA and ER membranes. The MW in HCV-proteins-expressing cells appears to be induced by HCV NS4B possibly in combination with NS5A. The biogenesis of membrane replication factories is a complicated process of concerted actions of HCV NS proteins and many host factors. There are many host factors reported to be involved in the biogenesis of membrane replication factories induced in HCV infection such as Cyclophilin A (CypA) and Proline-serine-threonine phosphatase interacting protein (PSTPIP2). HCV is strongly dependent on the lipid kinase PI4KIII $\alpha$  and its product, phosphatidylinositol-4-phosphate (PI4P). PI4P recruits specific viral and/or host proteins with PI4P-binding sites to the membrane remodelling site. Oxysterol binding protein (OSBP) and Four-phosphate adaptor protein 2 (FAPP2) are two PI4P effectors that required to transfer cholesterol and glycosphingolipid, respectively to sites of viral replication. Despite the high advances in the understanding of the molecular characterization of HCV replication and the increasing list of host factors required for HCV replication, the understanding of the structure of these vesicles and concerted action between HCV proteins and host factors to remodel the ER membranes are not clear. Our group and others previously reported that HCV replication occurs in lipid raft-associated membranes enriched in cholesterol and sphingomyelin (SM). While several reports indicated the requirement of cholesterol which is the partner of SM in the lipid rafts structure to the stability of DMVs, the mechanism of the involvement of SM in HCV replication was not evident. SM belongs to sphingolipids with a general structure of fatty acid and sphingoid base linked together by an amide bond. SM biosynthesis starts in the ER by two different pathways, *de novo* and salvage pathways giving rise to ceramide which is transported from ER to Golgi by the action of ceramide transfer protein (CERT). Ceramide is then further modified into SM by SM synthase (SGMS). SM plays several roles in the HCV infection, including replication and represents a component of very low density lipoproteins (VLDL) whose assembly component and the pathway is required for HCV morphogenesis and secretion. In this study, we aimed to analyze the importance of SM in the biogenesis of the membrane replication factories.

We had shown, for the first time, lines of evidence that SM is essential for the biogenesis of membrane replication factories, the site of viral replication. We first analyzed the importance of SM in the replication of HCV through inhibition of SM biosynthesis pathways using either small molecule inhibitors or by knockout (KO) of CERT; one of the essential player proteins in the SM biosynthesis process. We used 3 different small molecule inhibitors for SM biosynthesis pathways including Myriocin; a well-known inhibitor of the *de novo* pathway, Fumonisin B1; a potent inhibitor of both the *de novo* and the salvage pathways and D609; the sole commercial and direct inhibitor of SGMS to analyze the importance of SM to HCV replication. Myriocin, fumonisin B1 and D609 decreased luciferase activity in

subgenomic replicon cells, either genotype 1a or 2a, in a dose-dependent manner that was confirmed by measurement of HCV-RNA copies with no significant effect on cell viability. We confirmed such suppression in HCV replication using HCV infection system as well. Inhibitors of SM biosynthesis suppressed HCV replication in concentration-dependent and genotype-independent manners. For confirming the importance of SM to the replication of HCV and the biogenesis of membrane replication factories, we established CERT-Knockout (CERT-KO) cells using CRISPR-Cas9 technology. CERT-KO cells showed complete abrogation of CERT expression and a significant reduction of SM level. HCV RNA replication was dramatically reduced in CERT-KO cells compared to control cells. Such reduction in HCV replication was rescued by either exogenous SM supply, which showed co-localization with HCV replicase proteins and dsRNA or by ectopic expression of the functional CERT protein. We then analyzed the importance of SM in the biogenesis of membrane replication factories that required for the replication of HCV. We used two different approaches; the first was by inhibition of SM biosynthesis pathways either by D609 or in CERT-KO cells. The second approach was by degrading SM content of the isolated membrane replication factories and analyzing such effect on the morphology and function of the isolated vesicles. Either replicon cells treated with D609 or CERT-KO cells transfected with HCV replicon RNA showed lower numbers of induced membrane replication vesicles. This result was confirmed in a replication-independent system that showed that driving HCV protein expression without replication induced a lower number of DMVs in CERT-KO cells compared to control. This result confirmed that SM is required for the biogenesis of the membrane which is required for HCV replication and an evident that the lower number of DMVs was not the result of the overall reduction in HCV replication but resulted from the SM biosynthesis inhibition. After isolation of the membrane replication factories or vesicles in the DRM fraction using detergent treatment and membrane floatation assay, we analyzed the effect of degradation of SM content on the morphology and function of the isolated vesicles. We showed that degradation of SM content of such vesicles caused deformation of these vesicles and shrinkage in their size too. Reduced DMVs diameters were also reported by depletion of cholesterol from purified DMVs. These data suggest that both SM and cholesterol, the main components of lipid rafts, are essential structural components of HCV-remodeled membranes, in consistent with the previous reports showing that membrane replication factories consist of lipid raft-associated membranes enriched in cholesterol and SM. The inhibition of RNA replication activity by either SM degradation or  $\beta$ -CD treatment, the compound that extracts cholesterol from the membrane, of the isolated vesicles supports the same conclusion. Degradation of SM content of the isolated membranes affected the protection function of these vesicles to the replication complex and the genomic RNA against protease and RNase treatment supporting the argument that membrane replication factories protect viral proteins and RNA from host

immune defences reviewed. We showed that SM degradation increased the sensitivity of HCV RNA and proteins to RNase and protease treatment, respectively. These results suggested that the presence of SM in the structure of membrane replication vesicles may protect HCV RNA against cytoplasmic RNA sensors, therefore, keeping the replication of HCV. The requirement of SM is not exceptional for HCV viral replication; our data showed the evidence that poliovirus, another plus-stranded RNA virus, requires SM for its replication. It is noteworthy too that both HCV and poliovirus share the same morphological type, DMVs. We also showed that DENV that induces invaginated vesicle type did not require SM, but it had an inhibitory effect on DENV replication. Further analysis is required to prove the importance of SM in the biogenesis of DMVs induced by other plus-strand RNA viruses. In conclusion, these findings indicate that SM is required for HCV replication through being involved in membrane replication vesicles biogenesis.