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Dose-Dependent Inhibition of Melanoma Differentiation-Associated Gene 5-Mediated Activation of Type I Interferon Responses by Methyltransferase of Hepatitis E Virus

Jinjong Myoung* and Kang Sang Min

Korea Zoonosis Research Institute and Genetic Engineering Research Institute, Chonbuk National University, Jeonju 54896, Republic of Korea

Hepatitis E virus (HEV) accounts for 20 million infections in humans worldwide. In most cases, the infections are self-limiting while HEV genotype 1 infection cases may lead to lethal infections in pregnant women (~ 20% fatality). The lack of small animal models has hampered detailed analysis of virus-host interactions and HEV-induced pathology. Here, by employing a recently developed culture-adapted HEV, we demonstrated that methyltransferase, a non-structural protein, strongly inhibits melanoma differentiation-associated gene 5 (MDA5)-mediated activation of type I interferon responses. Compared to uninfected controls, HEV-infected cells display significantly lower levels of IFN-β promoter activation when assessed by luciferase assay and RT-PCR. HEV genome-wide screening showed that HEV-encoded methyltransferase (MeT) strongly inhibits MDA5-mediated transcriptional activation of IFN-β and NF-κB in a dose-responsive manner whether or not it is expressed in the presence/absence of a tag fused to it. Taken together, current studies clearly demonstrated that HEV MeT is a novel antagonist of MDA5-mediated induction of IFN-β signaling.

Keywords: Hepatitis E virus, interferon beta, methyltransferase

Introduction

Hepatitis E virus (HEV) is a (+)-sense, single-stranded RNA virus with two distinct structural features: enveloped in the bloodstream but non-enveloped in bile and feces [1].

When HEV was first identified, it was wrongly classified as a member of Calicivirus, however, it is now re-classified in the genus Orthohepevirus in the Hepeviridae family based on comparative phylogenetic analysis [2]. There are 8 HEV genotypes (GTs) known so far: among these, 4 are known to infect humans: GT1, GT2, GT3 and GT4. GT1 and GT2 are anthropotropic while GT3 and GT4 are enzootic [3–5]. Annual estimation of new HEV infections is over 20 million cases via the fecal-oral route. Most HEV infections are acute, asymptomatic and self-limiting while roughly 3.4 million acute HEV-related hepatitis and jaundice cases are reported worldwide [6, 7]. In addition, acute liver failure caused by HEV infection is responsible for over 55,000 deaths annually [8–11]. Mortality rates are especially high in pregnant and immunocompromised patients, suggesting that host immune responses play an important role in controlling HEV infections [6]. Interestingly, different HEV GTs are associated with distinct pathologies upon infection: HEV GT1 and 2 are far more associated with pancreatitis and high mortality in pregnant women while HEV GT3 and 4 are related with neurological diseases [12, 13].

Upon viral infections, type I interferons (IFN) are expressed and play an important role to counteract invading pathogens in both infected and neighboring cells. A number of pattern recognition receptors (PRRs) are known to recognize viruses, initiating a cascade of signal transduction that leads to type I IFN expression. Included are melanoma differentiation associated gene 5 (MDA5), retinoic acid-inducible gene I (RIG-I), and toll-like receptors (TLRs) [4, 14–16]. Both MDA5 and RIG-I sense and bind to double-stranded RNAs (dsRNAs) in the cytoplasm [17, 18] with subtle differences in the structural and chemical

features they recognize. RIG-I seems to require 5′-triphosphate RNA with blunt end for efficient recognition of its RNA ligand. In addition, relatively short dsRNAs are recognized by RIG-I while MDA5 recognizes long dsRNAs without the structural/chemical restrictions that RIG-I has [19–21]. Ligation of those PRRs by invading pathogens' RNA genomes results in induction of their conformational changes that facilitate the assembly of large oligomeric signalosomes that subsequently recruit and activate downstream signaling adaptors [22]. Activated RIG-I and MDA5 in turn induce mitochondrial antiviral signaling protein (MAVS) polymerization on the outer membrane of mitochondria [23–25], which leads to phosphorylation and activation of cytoplasmic kinase, Tank-binding kinase 1 (TBK1) and IκB kinase epsilon (IKKε) in the cytoplasm. TBK1/IKKε-mediated phosphorylation of IRF3 induces its self-dimerization and nuclear translocation, culminating in transcription of IFN-β.

Previously, we reported that HEV methyltransferase (MeT) inhibits RIG-I-dependent activation of IFN-β signaling [4, 26]. In fact, both RIG-I and MDA5 sense cytoplasmic double-stranded RNAs (dsRNA) with perceptive differences: RIG-I-mediated sensing requires 5′-triphosphate of relatively short dsRNAs while MDA5 recognizes long dsRNA with or without 5′-triphosphate [20, 21, 27–31]. Therefore, it is possible that MeT may also inhibit MDA5-mediated activation of IFN-β signaling. In this report, we demonstrated that HEV infection inhibits activation of IFN-β signaling in stably-infected A549 cells and HEV MeT was responsible for the inhibition by genome-wide screening of HEV proteins. Interestingly, MeT-mediated inhibition of IFN-β signaling was dose-dependent and an untagged form of MeT demonstrated similar dose-dependent inhibitory activity on MDA5-mediated activation of IFN-β and NF-κB. Taken together, these results clearly show that HEV-encoded MeT regulates MDA5-mediated innate recognition of HEV infection. Thus, it seems that HEV MeT represents itself as an attractive target of HEV-specific therapeutics for the future as it antagonizes both RIG-I- and MDA5-mediated innate immune sensing of HEV infection.

Materials and Methods

Cell Culture and Reagents

Human embryonic kidney 293T (HEK 293T) cells were purchased from ATCC and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS, Gibco, USA) and 1% penicillin/streptomycin (P/S, Thermo Fisher

Scientific, USA). A549/D3 and A549/HEV cells were generously provided by Dr. Reimar Johne and maintained in DMEM supplemented with 5% calf bovine serum and 1% P/S [4, 32, 33]. The cells were allowed to grow in a humidified incubator at 37ºC with 5% CO_2 [34, 35]. For plasmid transfection, polyethyleneimmine (PEI, Sigma-Aldrich) solution was prepared [15, 36]. When indicated, cells were stimulated with high molecular weight polyinosinic-polycytidylic acid (poly I:C) (Invivogen, USA). Anti-FLAG antibody (M2) was procured from Sigma-Aldrich and all other antibodies were purchased from Cell Signaling (USA): anti-mouse antibody conjugated with horseradish peroxidase (HRP), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [37].

DNA Constructs and Transfection

HEV genes were cloned into p3XFLAG-CMV10 as previously described. When indicated, HEV gene constructs were co-transfected with FLAG-tagged MDA5 in conjunction with IFN-β-luc and/or NF-κB-luc. Plasmid DNA and PEI complex was allowed to form at a 1:2 ratio at RT for at least 30 min [38, 39]. DNA and transfection reagent complex was added to cell monolayers dropwise. Cells were left incubated for 24 h before being subjected to harvest for luciferase reporter assay.

Luciferase Reporter Assay

To investigate whether HEV-encoded genes are capable of inhibiting MDA-induced induction of IFN-β expression, each HEV gene was co-transfected with MDA, β-gal control plasmid, IFN-β-luc or NF-κB. Cells were cultured for 24 h after transfection and lysed for luciferase assay. Luciferase activities were determined by the Glomax (Promega, USA) according to the manufacturer's instructions using luciferase assay system (Promega). Luminescence of experimental groups was normalized by the β-galactosidase activity of each well.

Statistical Analysis

Statistical significance was determined by the two-tailed Student's t test. P value of less than 0.05 was considered statistically significant. Data shown are one representative set from two independent experiments.

Results and Discussion

HEV Infection Antagonizes IFN-β Transcriptional Activation Induced by Sendai Virus

The A549/D3 cell line is a subclone of A549 cells and was first developed by the Johne group for its heightened susceptibility to HEV infection. Furthermore, HEV infection in A549/D3 cells led to the stable maintenance of HEV in A549/D3, and then termed A549/HEV. Using the A549/HEV cells, we tested if HEV infection antagonizes type I IFN signaling. The two cell lines were transfected with IFN-β-luciferase (IFN-β-luc) reporter and beta-galactosidase (β-gal)
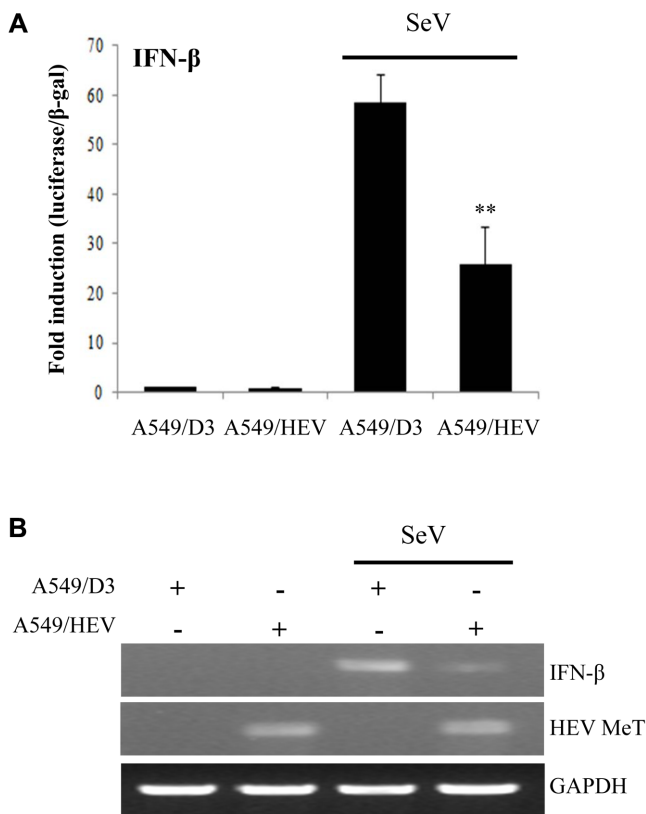
Fig. 1. Sendai virus-induced IFN-β promoter activation is decreased in HEV infected cells.

A549/D3 and A549/HEV cells were seeded in a 6-well plate and transfected with both IFN-β-luc construct and β-gal-expressing plasmid. After 24 h, cells were infected with 100 HAU/ml of SeV (**A** and **B**) and cultured for 12 h before harvest for IFN-β reporter assay (**A**) or qualitative RT-PCR (**B**). β-Galactosidase activity-normalized luciferase fold induction is plotted and one representative data set of two independent experiments is shown. **$p < 0.01$ by Student's t-test.

Fig. 2. HEV MeT, PCP, and ORF2 inhibit IFN-β and NF-κB signaling activation.

HEK293T cells were co-transfected with β-gal, MDA5 and viral protein expression plasmids together with IFN-β (**A**) or NF-κB (**B**) reporter construct. After 24 h transfection, cells were transfected with 5 μg/ml of polyI:C for 8 h before harvest for luciferase assay. β-Galactosidase activity-normalized luciferase fold induction is plotted and one representative data of two independent experiments is shown. *$p < 0.05$ and **$p < 0.01$ by Student's t-test, respectively.

plasmid, and infected with 100 HAU/ml of SeV, which is a powerful activator of type 1 IFN signaling. Interestingly, A549/HEV cells significantly downregulated (ca. 60%) IFN-β reporter activity upon SeV infection compared to A549/D3 (Fig. 1A). In addition, levels of IFN-β mRNA were similarly impaired (Fig. 1B). These results strongly suggest for the presence of HEV-encoded antagonistic mechanism(s) against type I IFN signaling.

HEV MeT, PCP, and ORF2 Proteins Inhibit MDA5-Mediated IFN-β and NF-κB Transcriptional Activation

Poly I:C is known to be recognized by two cytoplasmic sensors: MDA5 and RIG-I. Recognition of poly I:C by MDA5, in turn, activates downstream signaling molecules involved in the type 1 IFN pathway. When cells were
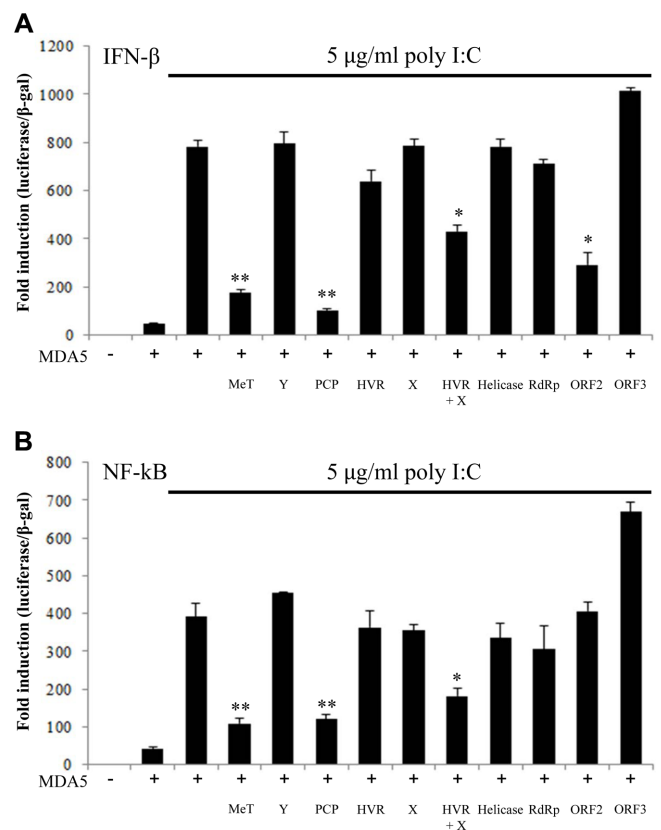
transfected with poly I:C alone, the fold increase was albeit low (~10-fold, unpublished data and [26, 36]). However, when co-transfected with MDA5-encoding plasmid, the fold increase of IFN-β luciferase activities reached roughly 800-fold (Fig. 2A), suggesting a synergistic action by polyI:C and MDA5. The same was true of the NF-κB luciferase assay (Fig. 2B); a 400-fold increase when MDA5 and polyI:C were co-transfected into HEK293 cells. To investigate which viral protein(s) inhibit RIG-I-mediated activation of IFN-β signaling, HEK293T cells were transfected with each viral gene and MDA5 together with IFN-β-luc (Fig. 2A) or NF-κB-luc (Fig. 2B), and subsequently transfected with poly I:C. As expected, PCP and ORF2 inhibited type I IFN responses [40−42] as they are known antagonists of type 1 IFN responses encoded by HEV. Interestingly, HEV
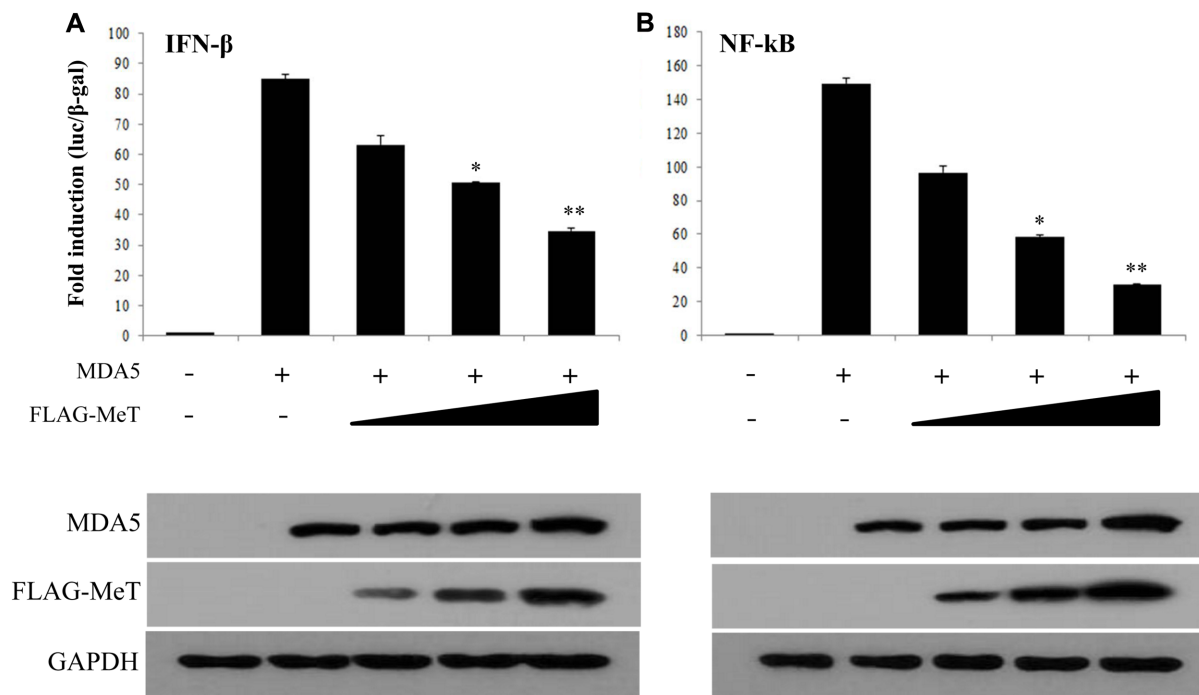
Fig. 3. HEV MeT inhibits MDA5-mediated IFN-β and NF-κB induction in a dose-dependent manner.

HEK293T cells were transfected for 24 h with IFN-β (**A**) or NF-κB (**B**) reporter plasmid together with constructs encoding β-gal, MDA5 and MeT (0.5, 1, and 2 μg) of FLAG-tagged MeT expression plasmid. Cells were harvested and subjected to luciferase assay. β-Galactosidase activity-normalized luciferase fold induction is plotted. A representative data set of three independent experiments is shown. Expression of MDA5 and MeT were assessed by western blotting (**A** and **B**, bottom panels). *$p < 0.05$ and **$p < 0.01$ by Student's *t*-test, respectively.

MeT strongly inhibited both IFN-β production (Fig. 2A) and NF-κB activation (Fig. 2B). Previously, we showed that MeT inhibits RIG-I-mediated IFN-β signaling at most by 50% [26]. Here, the data show that MeT inhibits MDA5-mediated IFN-β and NF-κB signaling by more than 75%. Interestingly, either hypervariable region (HVR) or X domain did not inhibit MDA5-mediated activation of NF-κB (Fig. 2B). However, when those domains were co-transfected, roughly 50% inhibition was evident (Fig. 2B), suggesting that HVR and X may function cooperatively. Further studies are warranted to elucidate the nature and magnitude of their interactions and cooperative action. Studies are currently under way to fathom the molecular mechanisms of how HVR and X proteins, when expressed together, inhibit MDA5-mediated NF-kB activation.

MeT Inhibits MDA5-Mediated Induction of IFN-β Production and NF-κB Activation in a Dose-Responsive Manner

To investigate the dose-kinetic relationship of MeT and inhibition of MDA5-mediated IFN-β signaling, increasing amount of MeT-expressing constructs were transfected together with MDA5-encoding and IFN-β-luc (Fig. 3A) or NF-κB-luc (Fig. 3B) plasmids into HEK293 cells. Interestingly, overexpression of MeT inhibited IFN-β production and NF-κB in a dose-responsive manner, demonstrating that MeT is a true antagonist of MDA5-mediated IFN-β signaling pathways. Of note, increasing levels of MeT did not perturb MDA5 protein expression (Fig. 3, bottom panels), suggesting that MeT-mediated antagonism of IFN-β signaling pathways is not mediated through inhibition of MDA5 protein expression or promotion of its degradation. Delineation of molecular mechanism(s) of MeT-mediated inhibition of MDA5-induced activation of IFN-β signaling cascades are currently under way.

A Native Form of MeT Inhibits IFN-β and NF-κB Induction in a Dose-Responsive Manner

It is well known that a native structure of a protein can be altered or mispresented depending on the presence and position of a tag fused to it. As 3XFLAG-tagged MeT was expressed and analyzed for its function in Figs. 2 and 3, it is of paramount importance to test whether a native form of
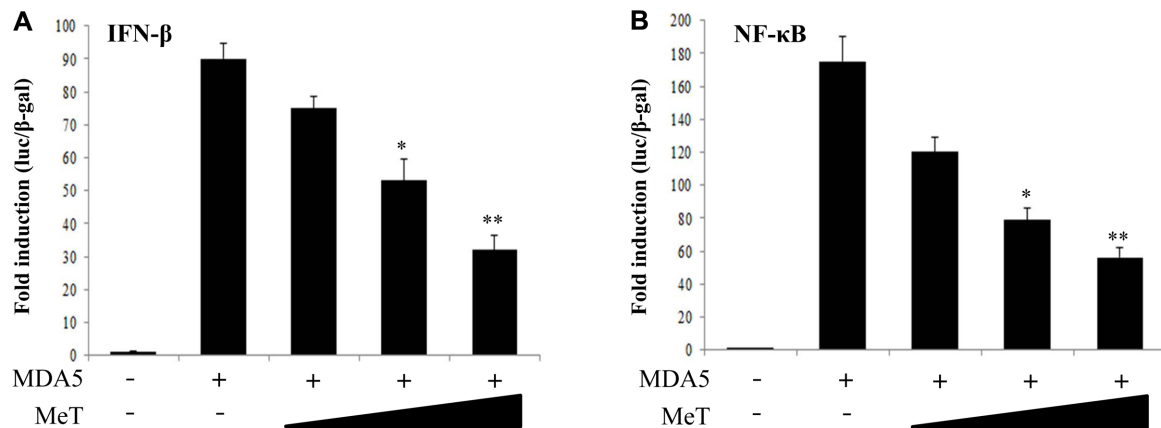
Fig. 4. A native form of HEV MeT inhibits MDA5-mediated IFN-β and NF-κB induction in a dose-responsive manner. HEK293T cells were co-transfected with β-gal, MDA5 as well as various amounts (0.5, 1, and 2 μg) of untagged MeT expression plasmids together with IFN-β (**A**) or NF-κB (**B**) reporter gene. At 24 h post-transfection, luciferase activity was assessed and normalized with β-galactosidase activity in a given sample. A representative data set of three independent experiments is shown. $*p < 0.05$ and $**p < 0.01$ by Student's t-test, respectively.

MeT has the same inhibitory activities on MDA5-mediated IFN-β signaling pathways. To this end, an untagged MeT was expressed together with MDA5 and its effects on IFN-β induction (Fig. 4A) and NF-κB activation (Fig. 4B) were analyzed. As demonstrated, untagged MeT displayed as strong inhibitory activities on IFN-β signaling pathways as 3xFLAG-tagged MeT, confirming that MeT is a true antagonist of MDA5-mediated activation of IFN-β signaling pathways.

Taken together, these results strongly suggest that MeT is a novel antagonist of IFN-β signaling induced by MDA5. First, SeV-mediated activation of IFN-β signaling was significantly suppressed (Fig. 1). Second, genome-wide screening of HEV-encoded proteins for their inhibitory activities against IFN-β revealed that MeT was responsible (Fig. 2). Third, FLAG-tagged, as well as untagged, MeT inhibited MDA5- and poly I:C-induced IFN-β and NF-κB activation over 75% (Figs. 3 and 4). Therefore, it seems clear that MeT is a novel HEV-encoded antagonist against type I interferon responses. Detailed investigation of its mechanisms will provide a way for the development of rationale-directed, virus-specific therapeutics.

Acknowledgment

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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