Virology 536 (2019) 119–124

Contents lists available at ScienceDirect

Virology

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

Integrin α3 is involved in non-enveloped hepatitis E virus infection

Tomoyuki Shiota[a], Tian-Cheng Li[a], Yorihiro Nishimura[a], Sayaka Yoshizaki[a], Ryuichi Sugiyama[a], Masayuki Shimojima[b], Masayuki Saijo[b], Hiroyuki Shimizu[a], Ryosuke Suzuki[a], Takaji Wakita[a], Masamichi Muramatsu[a], Koji Ishii[c,*]

[a] Department of Virology II, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo, 208-0011, Japan
[b] Department of Virology I, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo, 208-0011, Japan
[c] Department of Quality Assurance and Radiological Protection, National Institute of Infectious Diseases, Gakuen 4-7-1, Musashi-murayama, Tokyo, 208-0011, Japan

ARTICLE INFO

Keywords:
Hepatitis E virus (HEV)
Non-enveloped HEV
Receptor
Integrin α3

ABSTRACT

Hepatitis E virus (HEV) causes acute and fulminant hepatitis worldwide. Although enveloped (e) and non-enveloped (ne) forms of HEV have been discovered, host factors involved in infection, including receptors, remain to be elucidated. Here, we identified integrin α3 (encoded by *ITGA3*), a protein that binds and responds to the extracellular matrix, as an essential host factor for HEV infection. Integrin α3 expression was lower in four HEV-non-permissive cell subclones than in an HEV-permissive subclone. *ITGA3* knockout cells lost HEV permissibility, suggesting that integrin α3 is critical for HEV infection. Stable expression of integrin α3 in an HEV-non-permissive subclone provided permissibility only to infection by neHEV; expression of integrin α3 lacking the ectodomain did not. Direct interaction between neHEV and the integrin α3 ectodomain was confirmed by co-precipitation using a soluble integrin α3-Fc. These results strongly suggest that integrin α3 is a key molecule for cellular attachment and entry of neHEV.

1. Introduction

Hepatitis E virus (HEV) infection is a serious public health problem, causing waterborne outbreaks in developing countries and serving as an emerging zoonotic disease in developed countries (Kupferschmidt, 2016; Teshale et al., 2010). There are estimated to be 20 million cases annually, including more than 3 million patients who develop acute hepatitis every year; the fatality rate is at least 1%, but can be up to 30% in pregnant women (Boccia et al., 2006), resulting in 50,000 hepatitis E-related deaths annually (Lozano et al., 2012; Rein et al., 2012). HEV causes acute and fulminant hepatitis in immunocompetent hosts and chronic infection in immunocompromised hosts (Hoofnagle et al., 2012; Yamada et al., 2009), while extrahepatic HEV infection recently has been reported in neurological and obstetrical diseases (Dalton et al., 2016; Stevens et al., 2017).

HEV belongs to the genus Hepevirus in the family *Hepeviridae* (Smith et al., 2014). The HEV RNA genome is approximately 7.2 kb in size, and includes 3 open reading frames that encode a non-structural polyprotein, a major structural protein (capsid), and a minor structural protein (Debing et al., 2016). HEV had previously been considered a non-enveloped (ne) virus. However, an enveloped (e) version of HEV was discovered in 2010 (Takahashi et al., 2010). Therefore HEV in serum and culture supernatant is now considered to be a mixture of eHEV and neHEV (Nagashima et al., 2017). Although neHEV-like particles produced in insect cells interact with heparan sulfate proteoglycans (Kalia et al., 2009), eHEV and neHEV have different characteristics and entry mechanisms (Yin et al., 2016).

We previously showed that cells of the human hepatocarcinoma cell line PLC/PRF/5 are a mixture of HEV-permissive and -non-permissive cells (Shiota et al., 2015). Given that the HEV-non-permissive subclones have HEV replication competence after transfection with the HEV RNA genome, we speculated that these cells do not express HEV-specific entry receptor(s) on the cell surface (Shiota et al., 2015). Based on these insights, we performed microarray analysis to compare gene expression profiles between HEV-permissive and -non-permissive cells. As we report here, we identified integrin α3 (encoded by *ITGA3*) as a candidate HEV receptor. Integrins are transmembrane proteins that localize to the cell surface and are involved in diverse functions such as cell migration, inflammation, and thrombus formation (Takada et al., 2007). The integrin family is composed of 18 kinds of α subunit and 8 kinds of β subunit that together can form 24 different heterodimers. However, integrin α3 associates only with integrin β1 subunits, yielding Very Late Antigen 3 (VLA3), a protein that binds to fibronectin, laminin, and collagen (Sanchez-Madrid et al., 1986; Takada et al., 1991). In the

* Corresponding author.
E-mail address: kishii@nih.go.jp (K. Ishii).

https://doi.org/10.1016/j.virol.2019.07.025
Received 12 June 2019; Received in revised form 29 July 2019; Accepted 29 July 2019
Available online 30 July 2019

present study, we characterized the function of integrin α3 in HEV infection at the cellular and molecular levels.

2. Materials and methods

2.1. Cells, viruses, infectious clones, and antibodies

PLC/PRF/5 cells (cell line number JCRB0406; lot number 01272003) were obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). PLC/PRF/5 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/mL penicillin G, and 100 mg/mL streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. HEV-permissive (4-21) and non-permissive (3-12, 3-13, 4-20, and 4-29) subclones were described previously (Shiota et al., 2015). HEV genotype 3 and infectious clone G3-HEV83-2-27 (AB740232.1) were used as described previously (Shiota et al., 2013). neHEV and eHEV were generated from the supernatant of HEV-infected cells as reported previously (Nagashima et al., 2014). Anti-HEV polyclonal antibody was described previously (Li et al., 1997); anti-HEV monoclonal antibody (clone: MAb58, mouse IgG2aκ) was raised against G3 HEV virus-like particles that had been produced in insect cells. Anti-integrin α3 monoclonal antibody ASC-1 was purchased from BioLegend (CA, USA). Purified mouse IgG1κ (MOPC-21) and IgG2aκ (G155-178) isotype controls were purchased from BD Pharmingen (CA, USA).

2.2. Microarray analysis comparing gene expression between HEV-permissive and -non-permissive subclones

Total RNA was prepared from HEV-permissive subclone 4-21 and from non-permissive subclones 3-12, 3-13, 4-20, and 4-29 using RNeasy mini kits (Qiagen, Hilden, Germany). The microarray analysis was performed by Pharmafrontier (Kyoto, Japan) using a Whole Human V2 Genome Microarray kit (4 × 44K) (Agilent Technologies, CA, USA). To compare up- or downregulated-genes with Venn diagrams from 34,127 genes, ratios (non-log scaled fold-change) and Z-scores (Quackenbush, 2002) were calculated. The arithmetic mean of the intensities for gene expression in 4-21 cells was used as a control. The criteria for the regulated genes were as follows: ratio ≥2 and Z-score ≥2.0, upregulated genes; ratio ≤0.5 and Z-score ≤-2.0, downregulated genes. Heat maps were generated using MeV software (Saeed et al., 2003). The microarray data analysis was supported by Cell Innovator (Fukuoka, Japan).

2.3. Flow cytometric analysis

Cells were washed with phosphate-buffered saline (PBS) and re-suspended in flow cytometry buffer (PBS supplemented with 2% FBS). Cells then were incubated with 5 μg/mL anti-integrin α3 monoclonal antibody for 30 min on ice; the isotype control (mouse IgG1κ) was used as a negative control. After washing with flow cytometry buffer, cells were incubated in a 1:1000 dilution of Alexa Fluor 488 (Invitrogen) -conjugated anti-mouse antibody for 30 min on ice. Cells then were washed and re-suspended in flow cytometry buffer. Fluorescence signals were acquired with a FACSCalibur flow cytometer (Becton Dickinson, CA, USA) running Cellquest ver. 6.0 software. Layout and mean fluorescence parameters were analyzed by FlowJo ver. 9.7.7 software (Tree Star, OR, USA).

2.4. ITGA3 knockout in HEV-permissive subclone

A single-guided RNA (sgRNA) targeting *Homo sapiens ITGA3* was designed based on the *ITGA3* cDNA sequence (NM_005501.2); the sgRNA was designed and synthesized by the Takara Bio Dragon Genomics Center (Mie, Japan). The sgRNA sequence (5′-CATGGCTAC ACCAACCGGACTGG-3′) was inserted into an sgRNA expression plasmid (pRGEN_Human-ITGA3_U6_SG_1). PLC/PRF/5 subclone 4-21 cells were electroporated with pRGEN_Human-ITGA3_U6_SG_1 and pRGEN-CAS9_EF1a. After cloning the cells, we extracted the genomic DNA and amplified the target region by PCR for direct sequencing. We selected clone #22 as the *ITGA3* knockout strain; this clone harbors a homozygous deletion of a guanine corresponding to nucleotide position 724 of the *ITGA3* cDNA sequence (NM_005501.2).

2.5. Construction of integrin α3 and deletion mutant expression plasmids, and establishment of stable expression cell lines

The *ITGA3* cDNA (NM_005501.2) was synthesized and cloned into pUC57 by Genscript (Tokyo, Japan); the resulting plasmid was used as a template for PCR. The *ITGA3* cDNA was amplified using the primer pair M13 Forward (-20) (5′-GTAAAACGACGGCCAG-3′) and M13 Reverse (5′-CAGGAAACAGCTATGAC-3′). The amplified DNA fragment was cloned into the pEF6-V5-His TOPO plasmid (Life Technologies, CA, USA) to generate pEF6-ITGA3. We generated pEF6-ITGA3Δ, a deletion mutant lacking the codons for amino acids (aa) 75-813 of the integrin α3 ectodomain (aa 1-991 in NP_005492.1), by using pEF6-ITGA3 and restriction enzymes. The resulting plasmids were used to express integrin α3 and the deletion mutant stably in HEV-non-permissive 3-12 cells. Stable transfectants were selected with 7.5 μg/mL blasticidin S HCl (Invitrogen, CA, USA). The resulting 3-12ITGA3Δ cells and 3-12emp cells, a stable cell line transfected with the empty vector, were used without cloning.

2.6. Viral infection assay

We investigated the optimal copy number of the HEV genome for infection by using a 10-fold dilution series to generate inocula ranging from 10^7 to 10^2 genomes. The inoculation of 10^4 genome copies yielded inconsistent infection, with only some wells exhibiting HEV replication. Therefore, we used 10^5 genome copies of HEV for the infection assay to obtain the replicable results. Confluent cells (0.5 mL per well in a 24-well plate) were inoculated with the supernatant of HEV-infected cells (using a volume corresponding to 1.5×10^6 genome copies/well), eHEV (2.5×10^5 genome copies/well), or neHEV (2.5×10^5 genome copies/well). After inoculation, cells were incubated at 25 °C for 1 h (Tanaka et al., 2007). After incubation, viral supernatants were removed completely and replaced with maintenance medium consisting of a 1:1 (v/v) mixture of DMEM and medium 199 (Invitrogen, CA, USA) containing 2% FBS, 30 mM $MgCl_2$, 100 U/mL of penicillin G, and 100 μg/mL streptomycin. Maintenance medium was collected and replaced every 3 or 4 days. To detect HEV replication, the viral genome and antigen were quantified by real-time reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay, respectively, as previously described (Shiota et al., 2013).

2.7. Generation of the soluble integrin α3 protein

The majority of the integrin α3 ectodomain (aa 1-871) -encoding sequence was amplified using the primer pair ITGA3FcFw (5′-TATCG GTCCGATAAATATGGGCCCCGGCCCCAGCC-3′) and ITGA3-ect-wo-dbr R (5′-GAACGGACCGCTGTGGGGATGATGGCCTG-3′); the resulting amplicon was cloned into the SanDI site of pEF6-S-IgG2a-His to generate pEF6-ITGA3-Fc-His, which encoded the integrin α3 ectodomain fused to the immunoglobulin Fc fragment of mouse IgG2a followed by a 6xHis tag. To generate the expression plasmid encoding a deletion mutant protein (pEF6-ITGA3Δ-His), the nucleotides encoding aa 75-819 of integrin α3 on pEF6-ITGA3-Fc-His were removed by mutagenesis using the PrimeSTAR mutagenesis kit (Takara Bio, Shiga, Japan) with primer pair pEF6-ITGA3sectPaDel-Fc-F-Mod (5′-GCGGATGATTATCAACCCTC TCAACC-3′) and pEF6-ITGA3sectPaDel-Fc-R-Mod (5′-TTGATAATCATC CGCTCACAGTCAT-3′).

As unrelated negative control Fc-fused proteins, the two receptors

for enterovirus 71, P-selectin glycoprotein ligand-1 (PSGL-1) (Nishimura et al., 2009) and scavenger receptor class B member 2 (SCARB2) (Yamayoshi et al., 2009), were produced as soluble Fc-fused proteins using the expression plasmids pEF6-PSGL-1-Fc-His and pEF6-SCARB2-Fc-His, respectively. Constructs were transfected into 293T cells using Lipofectamine 2000 (Invitrogen, CA, USA). At 3 days post-transfection, the supernatants were collected and briefly centrifuged to remove debris. The secreted recombinant proteins were purified from the supernatants using Dynabeads His-tag isolation (Invitrogen, CA, USA). The various Fc-fused molecules of the expected molecular sizes was confirmed by western blotting using an anti-Fc antibody (Supplementary Fig. 1). The expression level of integrin α3-Fc was much lower than that of the other Fc-fused proteins. Therefore the relative expression levels of the various fusion proteins was calculated based on the western blotting and this information was used to normalize the amount of the individual Fc proteins in the co-immunoprecipitation assay.

2.8. Co-immunoprecipitation assay

neHEV was prepared as reported previously (Nagashima et al., 2017), with minor modifications. Namely, the cell culture medium containing HEV was centrifuged at 11,000 × *g* for 30 min at 4 °C to remove cell debris. Then the supernatant was recovered and filtered through a 0.22-μm membrane filter for use in the co-immunoprecipitation assay. The production of integrin α3-Fc was much lower than that of the other Fc fusion proteins. The relative expression levels of the various Fc fusion proteins were estimated based on signal strength in a western blot probed with anti-Fc antibody (Supplementary Fig. 1), and these data were used to normalize the amounts of the fusion proteins in the co-precipitation reactions. neHEV (1.0×10^7 genome copies per reaction) and the Fc-fused proteins were combined, and the mixture was incubated at 4 °C for 2 h with gentle agitation. Dynabeads Protein G (Invitrogen, CA, USA) were added to the mixture. After incubation at 4 °C for 30 min with gentle agitation, beads were washed three times with immunoprecipitation buffer (20 mM Tris-Cl, 135 mM NaCl, 1% Triton X-100, and 10% glycerol, pH 7.4). Beads then were washed and resuspended in ultra-pure distilled water (Gibco, CA, USA) and boiled for 5 min in order to denature protein structures. The eluted viral genome was quantified by real-time RT-PCR, as previously described (Shiota et al., 2013).

3. Results

In order to identify the essential host factor(s) for HEV infection or replication, we first used microarray analysis to compare the mRNA expression levels of whole human genome transcripts in the HEV-permissive and HEV-non-permissive subclones (Fig. 1A). The gene expression patterns were not homogeneous among the four clones. To identify a candidate for the gene encoding a host factor essential for HEV infection, we focused on genes that encoded transmembrane proteins, specifically screening for those with consistently lower expression levels in the four HEV-non-permissive subclones compared to that in the HEV-permissive subclone; we paid special attention to genes previously reported to encode functional receptors for other viruses. As a result, we identified integrin α3, a protein that is encoded by *ITGA3* gene and is a functional receptor for Kaposi's sarcoma-associated herpesvirus (Akula et al., 2002). *ITGA3*, but no other members of the integrin gene family, showed lower expression in all four of the HEV-non-permissive cells (Fig. 1B). Additionally, integrin α3 protein expression on the cell surface was lower in HEV-non-permissive subclones than in the HEV-permissive subclone (Fig. 1C).

We analyzed the involvement of integrin α3 in HEV infection. Cells of the HEV-non-permissive subclone 3-12 were transfected with pEF6-ITGA3 and transfectants were selected using 7.5 μg/mL blasticidin. The resulting cells (designated 3-12ITGA3) then were used for the experiments without cloning. 3-12ITGA3 cells showed strong expression of integrin α3 (Fig. 2A). Additionally, 3-12ITGA3 cells secreted particles containing the HEV RNA genome as assessed at 1.5 months post-infection, while cells of the parental 3-12 subclone did not (Fig. 2B).

We confirmed the importance of integrin α3 in HEV infection by generating *ITGA3*-knockout PLC/PRF/5 cells. Specifically, starting from the HEV-permissive subclone (4-21 cells), we established *ITGA3* homozygous knockout cells (designated line #22) that lacked protein expression of integrin α3 (Fig. 3A). As expected, #22 cells lacked HEV permissibility, resembling the 3-12 cells (Fig. 3B). Taken together, these results indicated that high-level integrin α3 expression is critical for HEV infection.

Next, we examined the distinct involvement of integrin α3 in infection by eHEV and neHEV. Starting from the primary 3-12ITGA3 transfectant, we established a 3-12ITGA3 clone that exhibited stable high-level exogenous expression of integrin α3 (Fig. 4A). We also established another 3-12-derived line, which we designated 3-12ITGA3Δ, that expressed integrin α3 lacking the majority of its extracellular domain (Fig. 4A). Interestingly, 4-21 was permissive only for neHEV (Fig. 4B), suggesting a specific role of the ectodomain in permitting infection by neHEV present in the supernatant (Figs. 2B–3B). In contrast to the parental 3-12 subclone, 3-12ITGA3 cells, but not 3-12ITGA3Δ cells, exhibited permissibility for infection by neHEV (Fig. 4B).

To investigate the direct interaction between integrin α3 and neHEV, we generated a soluble form of integrin α3 by fusing *ITGA3* sequences to those encoding an Fc fragment. We then used the resulting integrin α3-Fc protein in a co-precipitation assay (Fig. 4C). Integrin α3-Fc co-precipitated with neHEV, demonstrating a specificity for HEV similar to that seen with the MAb58 anti-HEV antibody. neHEV was not co-precipitated with the negative control fusion proteins (either the protein encoded by a deletion mutant lacking the ectodomain, or the integrin-unrelated proteins PSGL-1 and SCARB2) or an isotype control (Fig. 4D). These results indicated that the ectodomain of integrin α3 specifically interacts with neHEV.

4. Discussion

We identified integrin α3 as a host factor essential for infection by neHEV. Based on microarray analysis, we focused on integrin α3, a protein that was previously reported to serve as a functional receptor for Kaposi's sarcoma-associated herpesvirus (Akula et al., 2002). We confirmed the direct interaction between integrin α3 and neHEV (Fig. 4D). The strength of the direct interaction was similar to that of anti-HEV antibody for HEV. This insight implied that integrin α3 is a functional receptor for neHEV.

Integrin α3 was expressed not only on HEV-permissive cells but also on HEV-non-permissive cells, although the level of expression in the latter was lower than that in the former. Notably, 3-12, a subclone that is non-permissive, acquired HEV permissibility when endowed with exogenous expression of integrin α3, suggesting that comparatively strong expression of integrin α3 is needed to provide permissibility for HEV infection. The expression level of integrin α3 in 4-21 (Fig. 1C) and 3-12ITGA3 (Figs. 2A–4A) was comparatively stronger, exhibiting almost 10-fold higher fluorescence intensity than that observed by flow cytometric analysis in the non-permissive subclones (Fig. 1C). Thus, permissibility for HEV infection appears to be conferred by strong expression of integrin α3. Interestingly, integrin α3 promoted infection by neHEV, but not that by eHEV (Fig. 4B). eHEV presumably uses an as-yet unknown strategy to infect cells and escape from neutralizing antibodies targeting the HEV capsid (Takahashi et al., 2010; Yin et al., 2016). The antibodies targeting the capsid would neutralize the infection only for neHEV (Nagashima et al., 2017), suggesting that the capsid protein of neHEV, not that of the enveloped one, interacts with integrin α3 to facilitate entry into the cell. eHEV presumably uses an unidentified receptor(s) other than integrin α3 to infect hepatocytes.

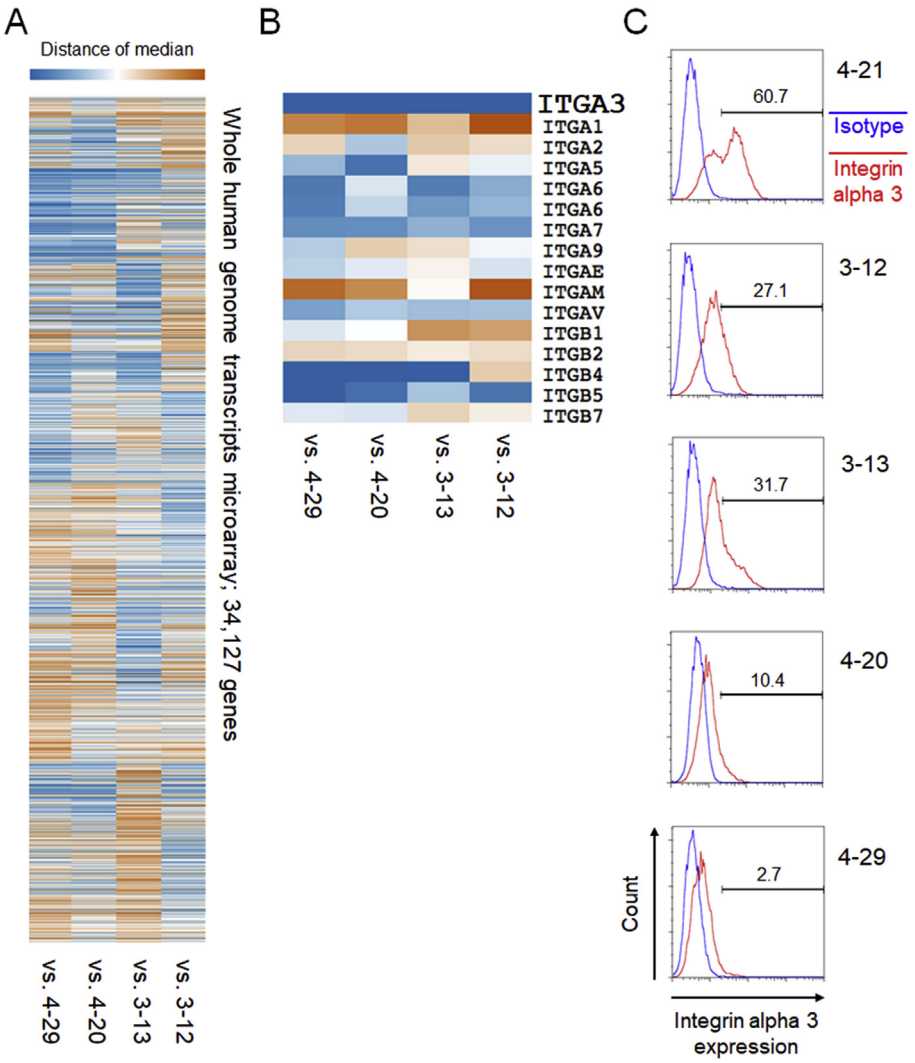

Fig. 1. Global and integrin-encoding gene expression and integrin α3 protein expression in HEV-permissive and non-permissive subclones of the PLC/PRF/5 cell line. (A) Global gene expression. A hierarchical clustering image, which shows human genome transcripts from 34,127 genes, revealed differences between the HEV-permissive subclone (4-21) and the non-permissive subclones (3-12, 3-13, 4-20, 4-29). Blue and red indicate lower and higher expression (respectively) of genes when compared to 4-21. (B) Expression of genes encoding members of the integrin family. mRNA expression of the genes encoding integrin α (*ITGA*) and integrin β (*ITGB*) was compared between permissive (4-21) and non-permissive subclones, as assessed by global gene expression analyses using microarrays. *ITGA6* is listed twice because the same gene is detected via two separate oligonucleotides within the microarray. (C) Cell surface expression of integrin α3 by flow cytometry. Blue and red lines indicate isotype control and anti-integrin α3 antibody staining, respectively. Values in the panel indicate percentage of strongly integrin α3-positive cells.

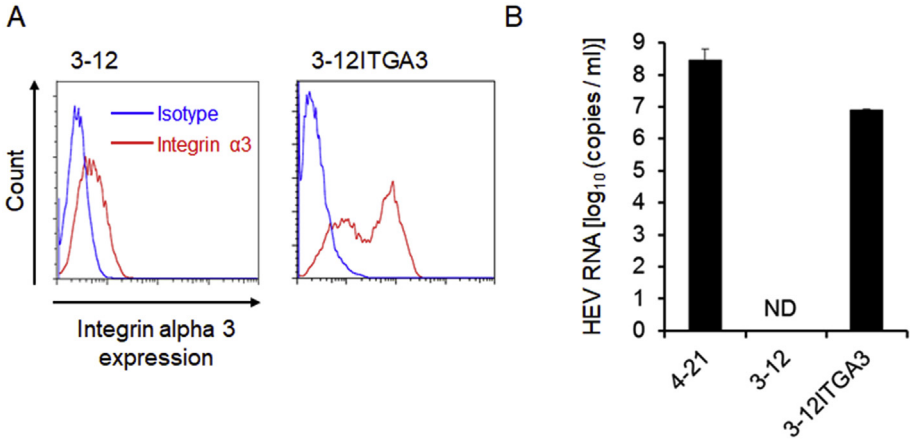

Fig. 2. Enhanced integrin α3 expression confers permissibility for infection by HEV. (A) Integrin α3 expression on non-permissive 3-12 cells and on crude 3-12ITGA3 cells (3-12 cells stably expressing exogenous integrin α3; these transfectants were tested without cloning). Blue and red lines indicate isotype control and anti-integrin α3 antibody staining, respectively. (B) Rescue of HEV replication in non-permissive cells by exogenous expression of integrin α3. 3-12ITGA3 cells secreted the viral genome at 1.5 months post-inoculation with HEV supernatant. Experiments were performed in duplicate; standard deviations are shown as error bars. ND means not detected (under the detection limit).

Although we examined a series of commercially available monoclonal and polyclonal anti-integrin α3 antibodies, none of them inhibited infection of 4-21 cells by neHEV (data not shown). This lack of inhibition might be due to the low antigenicity of the HEV-interacting region of human integrin α3. Establishment of an anti-integrin α3 antibody capable of inhibiting the interaction between neHEV and integrin α3 would enable confirmation of their specific interaction and the involvement of integrin α3 in the cellular entry of neHEV.

Members of the integrin family are extracellular matrix receptors with diverse functions. Integrin α3β1 acts as a receptor for laminin, the major component of the basement membrane in epithelial cells, and mediates the cell migration of neuronal and tumor cells (Campbell and Humphries, 2011). Integrin α3 is broadly expressed in the human body (de Melker et al., 1997), including tissues known to correspond to *in vivo* sites of HEV replication. Integrin α3-dependent infection by neHEV might not play a major role in the hepatic infection and pathology,

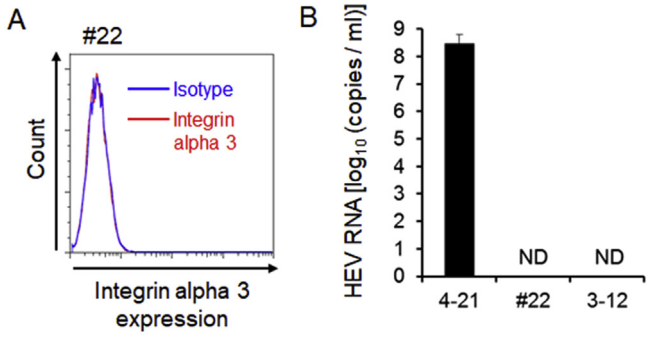

Fig. 3. *ITGA3* knockout in 4-21 abolishes permissibility for HEV infection. (A) Integrin α3 expression analyzed by flow cytometry. Both copies of the *ITGA3* gene in HEV-permissive clone 4-21 were mutated, yielding homozygous knockout clone #22. Blue and red lines indicate isotype control and anti-integrin α3 antibody staining, respectively. (B) *ITGA3* homozygous knockout effect on HEV infection. #22 showed non-permissibility to HEV at 1.5 months post-inoculation with HEV supernatant (same time point as that used in Fig. 2B). 3-12 is an HEV-non-permissive cell line. Experiments were performed in duplicate in parallel with the experiments in Fig. 2B. Therefore, the same values for HEV RNA copy numbers are plotted in Figs. 2B–3B. Standard deviations are shown as error bars. ND means not detected (under the detection limit).

given the low expression of integrin α3 in normal hepatocytes (de Melker et al., 1997; Volpes et al., 1991, 1993). Thus, eHEV might play the dominant role in hepatic pathological change. Studies in animal models have shown that HEV replicates not only in liver, but also in the small intestine, colon, lymph nodes, kidney, spleen, stomach, placenta, and central nervous system (Debing et al., 2016; Pischke et al., 2016; Wang et al., 2019); therefore, integrin α3 might be involved primarily in non-hepatic infection. Indeed, it has been reported that orally ingested HEV may infect integrin α3-expressing cells in the small intestine (Beaulieu, 1992; Choy et al., 1990). Additionally, HEV may cross into blood vessels via activated lymphocytes expressing integrin α3 (Shimizu and Shaw, 1991). Considered together with the results of the present study, these observations suggest that integrin α3 could be involved in hepatic and extrahepatic hepatitis E pathology.

We have shown that integrin α3 is an essential host factor for infection by neHEV. Knowledge regarding integrin α3 and its role in HEV interaction is expected to yield new strategies for specific treatment and prevention of HEV infection. Further investigation of the mechanism of eHEV infection and identification of the responsible receptor will be needed to provide a comprehensive understanding of HEV pathogenesis. Nonetheless, identification of integrin α3 as an essential host factor for infection by neHEV is expected to catalyze our understanding of the HEV life cycle, leading to fundamental solutions for HEV treatment.

Acknowledgments

We thank I. Shiota for helpful discussions and critical reading of the manuscript. This research was supported in part by the Research Resident Program and grants-in-aid from AMED to T.S. and K.I. (Grant Number JP18fk0108018). This work also was supported in part by grants-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to K.I. (Grant Number JP16K08825). This work additionally was supported by grants to T.S. from the Sasakawa Scientific Research Foundation from the Japan Science Society (Grant Number 24-428) and from JSPS KAKENHI (Grant Numbers JP24790452 and JP15K19118).

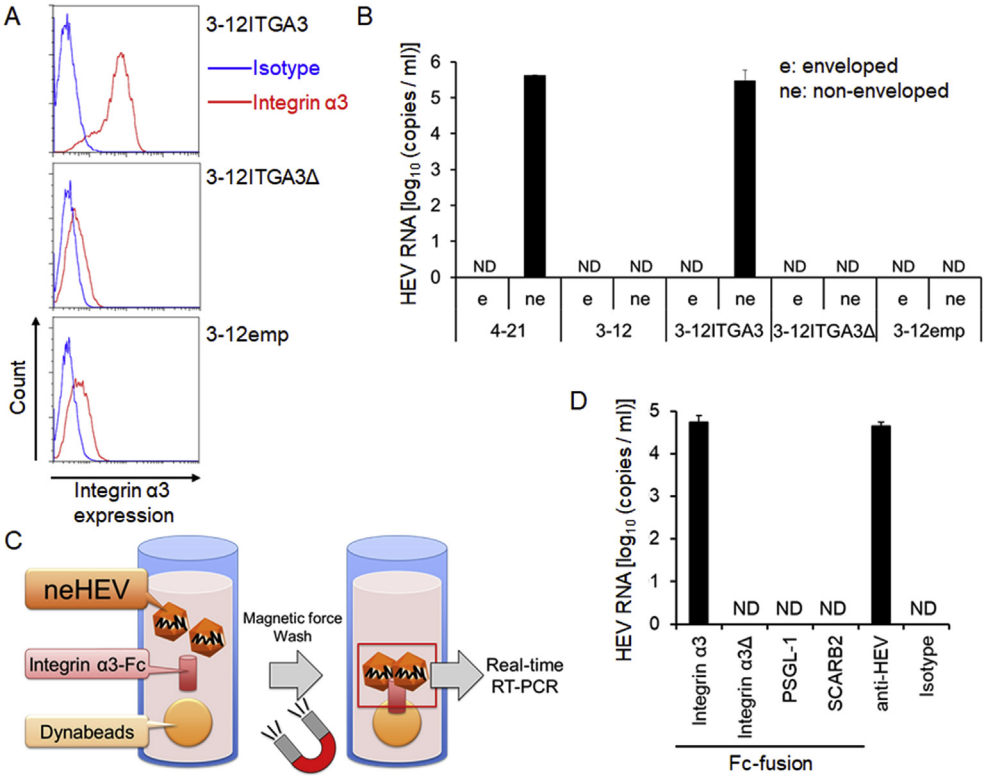

Fig. 4. The extracellular domain of integrin α3 is involved in non-enveloped HEV-specific permissibility and interaction. (A) Flow cytometric analysis of integrin α3 expression on cells established from HEV-non-permissive cell line 3-12. Cloned 3-12ITGA3, 3-12ITGA3Δ, and 3-12emp are stable cell lines transfected with expression vectors harboring *ITGA3*, harboring a mutated *ITGA3* lacking sequences encoding the ectodomain, or lacking an insert (i.e., empty vector), respectively. 3-12ITGA3 strongly expressed integrin α3. Blue and red lines indicate isotype control and anti-integrin α3 antibody staining, respectively. (B) Replication of enveloped (e) and non-enveloped (ne) HEV. The stable cell lines were infected with eHEV or neHEV. 4-21 had permissibility only for infection by neHEV. Exogenous expression of full-length integrin α3 in 3-12 (yielding clone 3-12ITGA3) endowed the subclone with neHEV-specific permissibility. Exogenous expression of integrin α3 with a substantial deletion of the extracellular domain in 3-12 (yielding clone 3-12ITGA3Δ) did not confer neHEV permissibility. HEV RNA copies at approximately 3 weeks post-infection are shown. ND means not detected (under the detection limit). (C) Schematic representation of the integrin α3-Fc binding assay. neHEV bound to integrin α3-Fc was precipitated by Dynabeads Protein G and magnetic force. After washing, the precipitated neHEV particles were quantified by real-time RT-PCR. (D) Detection of neHEV co-precipitated with integrin α3-Fc. The secreted Fc-fusion forms of integrin α3, integrin α3Δ, PSGL-1, and SCARB2 were used for co-precipitation assays. The amounts of fusion proteins were normalized based on relative expression levels determined by western blotting using an anti-Fc antibody. An anti-HEV monoclonal antibody (MAb58) and an IgG2aκ isotype control were used as positive and negative controls, respectively. Experiments were performed in duplicate (B) and triplicate (D); standard deviations are shown as error bars.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2019.07.025.

References

Akula, S.M., Pramod, N.P., Wang, F.Z., Chandran, B., 2002. Integrin alpha3beta1 (CD 49c/29) is a cellular receptor for Kaposi's sarcoma-associated herpesvirus (KSHV/HHV-8) entry into the target cells. Cell 108, 407–419.

Beaulieu, J.F., 1992. Differential expression of the VLA family of integrins along the crypt-villus axis in the human small intestine. J. Cell Sci. 102 (Pt 3), 427–436.

Boccia, D., Guthmann, J.P., Klovstad, H., Hamid, N., Tatay, M., Ciglenecki, I., Nizou, J.Y., Nicand, E., Guerin, P.J., 2006. High mortality associated with an outbreak of hepatitis E among displaced persons in Darfur, Sudan. Clin. Infect. Dis. 42, 1679–1684.

Campbell, I.D., Humphries, M.J., 2011. Integrin structure, activation, and interactions. Cold Spring Harb. Perspect. Biol. 3.

Choy, M.Y., Richman, P.I., Horton, M.A., MacDonald, T.T., 1990. Expression of the VLA family of integrins in human intestine. J. Pathol. 160, 35–40.

Dalton, H.R., Kamar, N., van Eijk, J.J., McLean, B.N., Cintas, P., Bendall, R.P., Jacobs, B.C., 2016. Hepatitis E virus and neurological injury. Nat. Rev. Neurol. 12, 77–85.

de Melker, A.A., Sterk, L.M., Delwel, G.O., Fles, D.L., Daams, H., Weening, J.J., Sonnenberg, A., 1997. The A and B variants of the alpha 3 integrin subunit: tissue distribution and functional characterization. Lab. Investig. 76, 547–563.

Debing, Y., Moradpour, D., Neyts, J., Gouttenoire, J., 2016. Update on hepatitis E virology: implications for clinical practice. J. Hepatol. 65, 200–212.

Hoofnagle, J.H., Nelson, K.E., Purcell, R.H., 2012. Hepatitis E. N Engl J Med 367, 1237–1244.

Kalia, M., Chandra, V., Rahman, S.A., Sehgal, D., Jameel, S., 2009. Heparan sulfate proteoglycans are required for cellular binding of the hepatitis E virus ORF2 capsid protein and for viral infection. J. Virol. 83, 12714–12724.

Kupferschmidt, K., 2016. Europe's new hepatitis problem. Science 353, 862–863.

Li, T.C., Yamakawa, Y., Suzuki, K., Tatsumi, M., Razak, M.A., Uchida, T., Takeda, N., Miyamura, T., 1997. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J. Virol. 71, 7207–7213.

Lozano, R., Naghavi, M., Foreman, K., Lim, S., Shibuya, K., Aboyans, V., Abraham, J., Adair, T., Aggarwal, R., Ahn, S.Y., Alvarado, M., Anderson, H.R., Anderson, L.M., Andrews, K.G., Atkinson, C., Baddour, L.M., Barker-Collo, S., Bartels, D.H., Bell, M.L., Benjamin, E.J., Bennett, D., Bhalla, K., Bikbov, B., Bin Abdulhak, A., Birbeck, G., Blyth, F., Bolliger, I., Boufous, S., Bucello, C., Burch, M., Burney, P., Carapetis, J., Chen, H., Chou, D., Chugh, S.S., Coffeng, L.E., Colan, S.D., Colquhoun, S., Colson, K.E., Condon, J., Connor, M.D., Cooper, L.T., Corriere, M., Cortinovis, M., de Vaccaro, K.C., Couser, W., Cowie, B.C., Criqui, M.H., Cross, M., Dabhadkar, K.C., Dahodwala, N., De Leo, D., Degenhardt, L., Delossantos, A., Denenberg, J., Des Jarlais, D.C., Dharmaratne, S.D., Dorsey, E.R., Driscoll, T., Duber, H., Ebel, B., Erwin, P.J., Espindola, P., Ezzati, M., Feigin, V., Flaxman, A.D., Forouzanfar, M.H., Fowkes, F.G., Franklin, R., Fransen, M., Freeman, M.K., Gabriel, S.E., Gakidou, E., Gaspari, F., Gillum, R.F., Gonzalez-Medina, D., Halasa, Y.A., Haring, D., Harrison, J.E., Havmoeller, R., Hay, R.J., Hoen, B., Hotez, P.J., Hoy, D., Jacobsen, K.H., James, S.L., Jasrasaria, R., Jayaraman, S., Johns, N., Karthikeyan, G., Kassebaum, N., Keren, A., Khoo, J.P., Knowlton, L.M., Kobusingye, O., Koranteng, A., Krishnamurthi, R., Lipnick, M., Lipshultz, S.E., Ohno, S.L., Mabweijano, J., MacIntyre, M.F., Mallinger, L., March, L., Marks, G.B., Marks, R., Matsumori, A., Matzopoulos, R., Mayosi, B.M., McAnulty, J.H., McDermott, M.M., McGrath, J., Mensah, G.A., Merriman, T.R., Michaud, C., Miller, M., Miller, T.R., Mock, C., Mocumbi, A.O., Mokdad, A.A., Moran, A., Mulholland, K., Nair, M.N., Naldi, L., Narayan, K.M., Nasseri, K., Norman, P., O'Donnell, M., Omer, S.B., Ortblad, K., Osborne, R., Ozgediz, D., Pahari, B., Pandian, J.D., Rivero, A.P., Padilla, R.P., Perez-Ruiz, F., Perico, N., Phillips, D., Pierce, K., Pope 3rd, C.A., Porrini, E., Pourmalek, F., Raju, M., Ranganathan, D., Rehm, J.T., Rein, D.B., Remuzzi, G., Rivara, F.P., Roberts, T., De Leon, F.R., Rosenfeld, L.C., Rushton, L., Sacco, R.L., Salomon, J.A., Sampson, U., Sanman, E., Schwebel, D.C., Segui-Gomez, M., Shepard, D.S., Singh, D., Singleton, J., Sliwa, K., Smith, E., Steer, A., Taylor, J.A., Thomas, B., Tleyjeh, I.M., Towbin, J.A., Truelsen, T., Undurraga, E.A., Venketasubramanian, N., Vijayakumar, L., Vos, T., Wagner, G.R., Wang, M., Wang, W., Watt, K., Weinstock, M.A., Weintraub, R., Wilkinson, J.D., Woolf, A.D., Wulf, S., Yeh, P.H., Yip, P., Zabetian, A., Zheng, Z.J., Lopez, A.D., Murray, C.J., AlMazroa, M.A., Memish, Z.A., 2012. Global and regional mortality from 235 causes of death for 20 age groups in 1990 and 2010: a systematic analysis for the Global Burden of Disease Study 2010. Lancet 380, 2095–2128.

Nagashima, S., Takahashi, M., Jirintai, S., Tanggis, Kobayashi, T., Nishizawa, T., Okamoto, H., 2014. The membrane on the surface of hepatitis E virus particles is derived from the intracellular membrane and contains trans-Golgi network protein 2. Arch. Virol. 159, 979–991.

Nagashima, S., Takahashi, M., Kobayashi, T., Tanggis, Nishizawa, T., Nishiyama, T., Primadharsini, P.P., Okamoto, H., 2017. Characterization of the quasi-enveloped hepatitis E virus particles released by the cellular exosomal pathway. J. Virol. 91.

Nishimura, Y., Shimojima, M., Tano, Y., Miyamura, T., Wakita, T., Shimizu, H., 2009. Human P-selectin glycoprotein ligand-1 is a functional receptor for enterovirus 71. Nat. Med. 15, 794–797.

Pischke, S., Hartl, J., Pas, S.D., Lohse, A.W., Jacobs, B.C., Van der Eijk, A.A., 2017 May. Hepatitis E virus: Infection beyond the liver? J Hepatol 66 (5), 1082–1095. https://doi.org/10.1016/j.jhep.2016.11.016. Epub 2016 Nov 29. Review. PubMed PMID: 27913223.

Quackenbush, J., 2002. Microarray data normalization and transformation. Nat. Genet. 32 (Suppl. l), 496–501.

Rein, D.B., Stevens, G.A., Theaker, J., Wittenborn, J.S., Wiersma, S.T., 2012. The global burden of hepatitis E virus genotypes 1 and 2 in 2005. Hepatology 55, 988–997.

Saeed, A.I., Sharov, V., White, J., Li, J., Liang, W., Bhagabati, N., Braisted, J., Klapa, M., Currier, T., Thiagarajan, M., Sturn, A., Snuffin, M., Rezantsev, A., Popov, D., Ryltsov, A., Kostukovich, E., Borisovsky, I., Liu, Z., Vinsavich, A., Trush, V., Quackenbush, J., 2003. TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34, 374–378.

Sanchez-Madrid, F., De Landazuri, M.O., Morago, G., Cebrian, M., Acevedo, A., Bernabeu, C., 1986. VLA-3: a novel polypeptide association within the VLA molecular complex: cell distribution and biochemical characterization. Eur. J. Immunol. 16, 1343–1349.

Shimizu, Y., Shaw, S., 1991. Lymphocyte interactions with extracellular matrix. FASEB J. 5, 2292–2299.

Shiota, T., Li, T.C., Yoshizaki, S., Kato, T., Wakita, T., Ishii, K., 2013. The hepatitis E virus capsid C-terminal region is essential for the viral life cycle: implication for viral genome encapsidation and particle stabilization. J. Virol. 87, 6031–6036.

Shiota, T., Li, T.C., Yoshizaki, S., Kato, T., Wakita, T., Ishii, K., 2015. Establishment of hepatitis E virus infection-permissive and -non-permissive human hepatoma PLC/PRF/5 subclones. Microbiol. Immunol. 59, 89–94.

Smith, D.B., Simmonds, P., International Committee on Taxonomy of Viruses Hepeviridae Study, G., Jameel, S., Emerson, S.U., Harrison, T.J., Meng, X.J., Okamoto, H., Van der Poel, W.H., Purdy, M.A., 2014. Consensus proposals for classification of the family Hepeviridae. J. Gen. Virol. 95, 2223–2232.

Stevens, O., Claeys, K.G., Poesen, K., Saegeman, V., Van Damme, P., 2017. Diagnostic challenges and clinical characteristics of hepatitis E virus-associated guillain-barre syndrome. JAMA Neurol 74, 26–33.

Takada, Y., Murphy, E., Pil, P., Chen, C., Ginsberg, M.H., Hemler, M.E., 1991. Molecular cloning and expression of the cDNA for alpha 3 subunit of human alpha 3 beta 1 (VLA-3), an integrin receptor for fibronectin, laminin, and collagen. J. Cell Biol. 115, 257–266.

Takada, Y., Ye, X., Simon, S., 2007. The integrins. Genome Biol. 8, 215.

Takahashi, M., Tanaka, T., Takahashi, H., Hoshino, Y., Nagashima, S., Jirintai, Mizuo, H., Yazaki, Y., Takagi, T., Azuma, M., Kusano, E., Isoda, N., Sugano, K., Okamoto, H., 2010. Hepatitis E Virus (HEV) strains in serum samples can replicate efficiently in cultured cells despite the coexistence of HEV antibodies: characterization of HEV virions in blood circulation. J. Clin. Microbiol. 48, 1112–1125.

Tanaka, T., Takahashi, M., Kusano, E., Okamoto, H., 2007. Development and evaluation of an efficient cell-culture system for Hepatitis E virus. J. Gen. Virol. 88, 903–911.

Teshale, E.H., Hu, D.J., Holmberg, S.D., 2010. The two faces of hepatitis E virus. Clin. Infect. Dis. 51, 328–334.

Volpes, R., van den Oord, J.J., Desmet, V.J., 1991. Distribution of the VLA family of integrins in normal and pathological human liver tissue. Gastroenterology 101, 200–206.

Volpes, R., van den Oord, J.J., Desmet, V.J., 1993. Integrins as differential cell lineage markers of primary liver tumors. Am. J. Pathol. 142, 1483–1492.

Wang, L., Teng, J.L.L., Lau, S.K.P., Sridhar, S., Fu, H., Gong, W., Li, M., Xu, Q., He, Y., Zhuang, H., Woo, P.C.Y., Wang, L., 2019 Mar 21. Transmission of a Novel Genotype of Hepatitis E Virus from Bactrian Camels to Cynomolgus Macaques. J Virol (7), 93. https://doi.org/10.1128/JVI.02014-18. pii: e02014-18. Print 2019 Apr 1. PubMed PMID: 30700602; PubMed Central PMCID: PMC6430554.

Yamada, K., Takahashi, M., Hoshino, Y., Takahashi, H., Ichiyama, K., Nagashima, S., Tanaka, T., Okamoto, H., 2009. ORF3 protein of hepatitis E virus is essential for virion release from infected cells. J. Gen. Virol. 90, 1880–1891.

Yamayoshi, S., Yamashita, Y., Li, J., Hanagata, N., Minowa, T., Takemura, T., Koike, S., 2009. Scavenger receptor B2 is a cellular receptor for enterovirus 71. Nat. Med. 15, 798–801.

Yin, X., Ambardekar, C., Lu, Y., Feng, Z., 2016. Distinct entry mechanisms for nonenveloped and quasi-enveloped hepatitis E viruses. J. Virol. 90, 4232–4242.