Virus Research 158 (2011) 235–245

Contents lists available at ScienceDirect

Virus Research

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

C-terminal truncation of the transmembrane protein of an attenuated lentiviral vaccine alters its *in vitro* but not *in vivo* replication and weakens its potential pathogenicity

Cheng-Gang Jiang[a,b], Xu Gao[a,d], Jian Ma[a], Yue-Zhi Lin[a], Xue-Feng Wang[a], Li-Ping Zhao[a], Yue-Ping Hua[c], Di Liu[b], Jian-Hua Zhou[a,*]

[a] State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin 150001, China
[b] Heilongjiang Academy of Agricultural Sciences, Livestock Research Center, Postdoctoral Workstation, Harbin 150086, China
[c] Northeast Forestry University, Postdoctoral Workstation, Harbin 150086, China
[d] Yanbian University, Veterinary Department of Agricultural College, Yanji 133002, China

ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 9 April 2011
Accepted 14 April 2011
Available online 22 April 2011

Keywords:
EIAV
gp45
Truncation
Replication
Pathogenicity

ABSTRACT

Preliminary studies revealed that the gene of the gp45 transmembrane protein (TM) of the attenuated equine infectious anemia virus (EIAV) vaccine strain $EIAV_{FDDV13}$ had a high frequency of a premature stop codon at position 261W, which generated a 154-residue truncation at the C-terminus. $EIAV_{FDDV\text{-}TM36}$, a recombinant virus with the TM truncated at the intracytoplasmic (CT) domain due to the presence of a stop codon, was constructed based on $EIAV_{FDDV}$3-8, which is a proviral derivative of the vaccine. $EIAV_{FDDV\text{-}TM36}$ had a significantly reduced replication capability compared to $EIAV_{FDDV}$3-8 in equine or donkey monocyte-derived macrophages and a decreased ability to induce apoptosis. However, both viruses raised a similar plasma viral load in inoculated horses and did not induce clinical symptoms of EIA. To further compare the *in vivo* behavior between $EIAV_{FDDV\text{-}TM36}$ and $EIAV_{FDDV}$3-8, inoculated horses were transiently immunosuppressed with dexamethasone. While three of the four horses inoculated with $EIAV_{FDDV}$3-8 demonstrated significant increases in viral loads after the drug treatment, none of the four horses inoculated with $EIAV_{FDDV\text{-}TM36}$ showed a statistically increased plasma viral load. Significantly increased neutralizing antibody levels were also observed in the group of horses inoculated with $EIAV_{FDDV}$3-8, but not $EIAV_{FDDV\text{-}TM36}$, after immunosuppression. Our results indicate that although the CT truncation of TM decreased viral replication in cultivated equine and donkey macrophages, the primary target cell of EIAV, and did not influence the plasma viral load of inoculated hosts, it weakened the potential pathogenicity of the vaccine. The host immunity is presumably responsible for the equal *in vivo* replication levels of viruses with either the CT-truncated or prototype TM.

1. Introduction

Induction of immune protection against lentiviruses, such as the human immunodeficiency virus (HIV)-1, is one of the central goals of viral immunology. Although they have potentially serious risks, such as integration into the host's chromosomes and reversion to virulence, attenuated live vaccines are currently the only type of lentiviral vaccine that can induce effective immune protection, especially against heterologous strains (Craigo et al., 2005; Wodarz, 2008). A full understanding of the nature of attenuated virulence, the conditions essential for attenuated lentiviral vaccines to induce immune protection, and the characteristics of that immune protection will provide important information for the development of safe and effective lentiviral vaccines. Monkeys infected with simian immunodeficiency virus (SIV) or SIV/HIV chimeric virus (SHIV) are currently the major animal model for the study of HIV-1 vaccines. To date, tested SIV/SHIV attenuated vaccines have been constructed by recombinant DNA technology based on viral infectious clones, and they can only induce immune protection against infection by the parental virus strain or highly homologous viruses (Genesca et al., 2008; Mansfield et al., 2008).

Equine infectious anemia virus (EIAV) is a macrophage-tropic lentivirus that can cause continuous infection and chronic disease in horses. In 1970s, Shen et al. developed the attenuated EIAV vaccine, $EIAV_{DLV121}$, by passing the parental pathogenic strain in donkey monocyte-derived macrophages (MDM) for 120 generations. This vaccine was successfully applied to control the pandemic of equine infectious anemia (EIA) in China (reference to "Equine

* Corresponding author.
E-mail address: jianhua_uc@126.com (J.-H. Zhou).

0168-1702/$ – see front matter.
doi:10.1016/j.virusres.2011.04.007

infectious anemia and its controlling in China", a document issued by the Chinese Ministry of Agriculture in 1997, which is written in Chinese and is available by request from the corresponding author). As the first successfully developed lentiviral vaccine, the attenuated EIAV vaccine has served as a meaningful model for studying protective immune responses and assessing the main factors that affect vaccine-induced immune protection. According to previous published studies by us and others, the lentiviral replication capability and cytotoxicity to target cells are important factors for inducing an effective immune response in the host (Ma et al., 2009; Wodarz, 2008).

A 4.2-kb mRNA is produced after transcription and single splicing of the EIAV *env* gene. The synthesized glycoprotein then is cleaved by cellular proteases to generate a surface unit (SU, gp90) and a transmembrane protein (TM or gp45). The EIAV TM contains an ecto-domain, a membrane-spanning domain (MSD) and a unique long C-terminal intracytoplasmic domain (ICD). Unlike other lentiviruses, the ICD of EIAV TM is further cleaved presumably by the viral protease to yield a cytoplasmic tail (CT) of 20 kDa (p20), which consists of about 175 amino acid residues (Rice et al., 1990). Lentiviral transmembrane proteins are involved in virus assembly, viral envelope expression on the cell surface, fusion between the virus and the cell and induction of cytotoxicity, and they may also associate with calcium channels (Costin, 2007; Micoli et al., 2006). Most of the immunodominant epitopes of EIAV TM are on the N-terminus, particularly from the extracellular functional region to the transmembrane anchor region. In contrast, the C-terminus of TM has a weak and unstable interaction with immunized horse serum (Chong et al., 1991).

The sequence of TM is relatively well conserved compared to that of SU and is an important viral structure for the binding of EIAV to cellular receptors and for the production of neutralizing antibodies (Howe et al., 2002). We previously found that the *gp45* gene of the EIAV vaccine strain $EIAV_{FDDV13}$, which is a donkey dermal fibroblast-adapted vaccine strain of $EIAV_{DLV121}$, had high frequency of translation termination mutations at 261W. The premature stop codon at this site causes a truncation of 154 amino acid residues at the CT domain of TM. This mutation of $EIAV_{FDDV13}$ *gp45* was frequently detectable in circulating virions and proviral genomic DNA up to 40 days post-infection in horses (Ma et al., 2010). To determine the effect of this truncation on the biological characteristics of the EIAV vaccine strain, we constructed an infectious molecular clone strain with a premature stop codon at 261W of $EIAV_{FDDV}$3-8, which is a proviral derivative of the vaccine strain $EIAV_{FDDV13}$, as the backbone. We then assessed the effect of this truncation on the replication characteristics of EIAV in cultivated MDM from horses and donkeys and fetal donkey dermal (FDD) cell-derived fibroblasts. Finally, we examined changes in physical symptoms and viral loads in horses inoculated with virus containing either the CT-truncated or the prototype TM.

2. Materials and methods

2.1. Viral strains, plasmids and cells

The infectious molecular clone strain $EIAV_{FDDV}$3-8 was previously derived from an FDD cell-adapted EIAV vaccine strain, $EIAV_{FDDV13}$ (He et al., 2003). FDD cells were prepared from EIAV-negative fetal donkeys and cultured in MEM culture medium plus 5% lactalbumin hydrolysate, 10% fetal bovine serum, 100 IU penicillin, and 100 μg/mL streptomycin. Equine and donkey MDM were prepared as follows. Briefly, 200 mL of anticoagulant horse or donkey blood was placed in a flask for natural sedimentation for 1 h. The upper layer of white blood cells was collected and centrifuged at 1000 rpm for 10 min. Cells were then washed with PBS twice and diluted to 2×10^7 cells/mL with RMPI 1640 culture medium containing 50% calf serum and cultured in flasks. After incubation at 37 °C for 24 h, non-adherent cells were removed by washing with PBS. The remaining adherent cells were predominantly macrophages.

2.2. Introduction of a premature stop codon in the intracytoplasmic domain of gp45

The 1054 bp *gp45* gene fragment was first amplified from a plasmid containing the proviral genome of the EIAV molecular clone strain $EIAV_{FDDV}$3-8 by PCR with primers P1 (5′-CCGCCAGTCGCTACCTGAGGAAAAG-3′) and P2 (5′-GCCAAGCTAGGCCTATAATTAACCC-3′), which contained an *Nru* I and an *Xho* I restriction enzyme site at each end, respectively. The PCR product was recovered using agarose gel electrophoresis and inserted into the pMD-18T vector. The resulting plasmid was named pMD-1054. The entire sequence of pMD-1054 was PCR-amplified with mutation primers M1 (5′-AGTTCTCCAGGAACAACTGAAATGGCGAATC-3′) and M2 (5′-TCAGTTGTTCCTGGAGAACTTCCGCATGTTGG-3′) to obtain the mutated plasmid pM-MD-1054. The *gp45* fragment in this plasmid contained a premature stop codon at nt 783, which encoded the transmembrane unit of the envelope protein truncated at 261W. After enzymatic digestion with *Nru* I and *Xho* I, the mutated sequence was used to replace the corresponding fragment of prototype *gp45* in $EIAV_{FDDV}$3-8 to obtain the mutated recombinant plasmid $pEIAV_{FDDV\text{-}TM36}$.

2.3. Transfection of viral plasmids and detection of mutated virus

The plasmid $pEIAV_{FDDV\text{-}TM36}$ was prepared using the Endo-Free Plasmid Mini Kit (Omega). FDD cells were transfected with the plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Recombinant viruses were harvested 10–12 days after transfection. After three freeze–thaw cycles, viruses in the cell lysates were collected and used for the next passage in FDD cells. The Reverse Transcriptase Assay Colorimetric Kit (Roche) was used according to the manufacturer's instructions to detect reverse transcriptase (RT) activity of viruses in the culture medium. To further confirm the rescue of recombinant virus $EIAV_{FDDV\text{-}TM36}$, FDD cells were plated at 2×10^5 cells/mL in 6-well plates and inoculated with the virus. After 10 days in culture, EIAV in infected cells was detected by an indirect immunofluorescent assay (IFA). Briefly, cells were fixed with 3.7% formaldehyde, incubated with a 1:100 diluted EIAV-positive horse serum for 1 h, and washed three times with PBS plus 0.5% Triton X100 (PBST). Cells were then incubated with a 1:5000 diluted, FITC-labeled rabbit anti-horse IgG (Sigma). After three washes with PBST, EIAV-containing cells that interacted with EIAV-positive horse serum were detected by fluorescence microscopy.

The correct introduction of the premature stop codon in the *gp45* of $EIAV_{FDDV\text{-}TM36}$ was determined by sequence analysis. The viruses obtained after transfection were used to re-inoculate FDD cells. After incubation at 37 °C for 10 days, viruses were collected and viral genomic RNA was extracted using a QiaAmp Viral RNA Mini Kit (Qiagen) and stored at −80 °C. The EIAV *gp45* fragment was amplified by RT-PCR using the forward primer (5′-ATGTCTTATATCGCTTTGACAGAA-3) and the reverse primer (5′-TGTTACATGAGATGTAGCTGGATTT-3′). RT-PCR products were recovered from agarose gels and ligated with the cloning vector pMD-18T. The resulting plasmids were then transformed into *Escherichia coli* JM109 competent cells. Twenty clones from each transformation were selected and sent to Invitrogen Inc. for sequencing.