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Rabies Glycoprotein: A Benefit to the Virus, us or both?

Susan Cherian[1*], Rajendra Singh[1], Anjaneya[1], Singh KP[2]

[1]Division of Pathology, Indian Veterinary Research Institute, Izatnagar, U.P-243122, India.

[2]Division of Pathology, CADRAD, Indian Veterinary Research Institute, Izatnagar, U.P-243122, India.

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***For Correspondence**

Susan Cherian, Division of Pathology, Indian Veterinary Research Institute, Izatnagar, U.P, India 243122.

E-mail: cpasusan18@gmail.com

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ABSTRACT

Rabies virus glycoprotein play a critical role in the pathogenesis of the disease by enabling binding of the virus to the potential receptors at site of inoculation, entry into the neurons, fusion of virus with endosomal membrane, transcription and translation of viral genome, replication , its retrograde transport to higher neurons and production of neutralizing antibodies. The ability of glycoprotein to activate the immune system and subsequent production of neutralizing antibodies have been exploited for the production of recombinant vaccines which may turn out in future to be an effective choice than the available alternatives for immunization of both human beings and animals. Oral bait vaccines for wildlife currently under use is based on this technology which proclaims the practical utility and field application of this technology. Monoclonal antibodies against glycoprotein are being utilized for epitope marking in various rabies virus isolates and it can also be a superior choice for post exposure prophylaxis in human beings. Retrograde transport property warranted by glycoprotein is utilized in brain research to unveil the complex neuronal connections to explore the normal functions of brain which may also improve our understanding of brain disorders and the possible treatment alternatives. Also, glycoprotein based peptides and pseudotyped viral vectors can be a better choice for drug or gene delivery to brain in various nervous disorders which have the advantage of overcoming the BBB in non-invasive way. Thus rabies glycoprotein has both detrimental as well as beneficial effects.

INTRODUCTION

Rabies is a fatal encephalomyelitic viral zoonotic disease of mammals. Rabies virus (RV) belongs to genus Lyssavirus under Rhabdoviridae family. Out of the seven genotypes, Classical RV include laboratory fixed strains as well as majority field strains which are prevalent in large parts of the world while remaining six members form a group of viruses named Rabies related viruses (RRV) which have a restricted geographical distribution [1,2].

RV is bullet shaped with a length of about 180nm and a diameter of 75 nm [3]. The genome is non-segmented with negative sense single stranded RNA. The genome encodes five structural proteins 3' N-P-M-G-L 5'; N (nucleoprotein), P (phosphoprotein), M (matrix protein), G (glycoprotein) and L (RNA dependent RNA polymerase) [4,5]. The leader and trailer untranslated regions at the 3' and 5' ends of the genome flank the structural genes and between the protein encoding regions there are non-coding intergenic sequences; N-P, P-M, M-G, G-L [6].

Among the five structural proteins, G is present on the envelope is the only protein exposed out. Glycoproteins are proteins which are covalently bonded with sugar units [7]. Rabies virus glycoprotein (RVG) is a trimeric type I trans-membrane protein which are single pass type with extracellular N-terminus and cytoplasmic C-terminus for cell membrane [8-10]. The precursor RVG is 524 amino acid (AA) in length (522 aa in Mokola virus), consisting of a signal peptide of 19 aa in the N terminal [11-13]. The mature

protein has N terminal ectodomain (439 aa), a transmembrane segment (22 aa) and cytoplasmic tail /endodomain/ ENDO (44 aa) [11,14]. Various components are involved in RV pathogenesis, but here we attempt to glance through these events in relation to RVG.

ROLE OF RVG IN PATHOGENESIS

RVG binding and interaction with cell receptors enabling viral entry

RV through saliva reaches the neuromuscular junctions (NMJ) and sensory terminations upon animal bite. RVG exist in native (N) state on the viral surface at pH above 7 which enable receptor binding [15]. Phospholipids, gangliosides and proteins have been proposed as of viral receptors on cell surface [16,17]. Among the protein receptors, nicotinic acetylcholine receptor (nAchR) is present at NMJ on myotubes and in neurons of CNS, while it is localized on post synaptic membrane of motor neurons. RVG segment having aa sequence similarity with snake venom neurotoxin and binds to the residues 173-204 of α-1 subunit of nAchR [18-20]. This binding may concentrate the virus at NMJ which enable uptake by nerve terminals by binding of RVG to microdomains formed by concentration of another protein receptor neural cell adhesion molecule (NCAM) through gangliosides. Pre-synaptic membranes of neurons of CNS and cell bodies as well as non-myelinated axons of peripherl nervous system have these receptors. Low affinity growth factor/p75NTR is the third protein receptor expressed at synapses of neurons [21]. However mutations at aa positions 318 and 352 in RVG could terminate the interaction with receptor [22]. After binding with the receptors, RV gets endocytosed and the enveloped virus inside the vesicles are transported along the axons in retrograde direction whereas binding with p75 NTR enable transcytosis.

Fusion of RVG with endosomal membranes

On reaching the cytoplasm, RVG attains activated (A) hydrophobic state, immediately after acidification with in the endosomes. pH threshold for fusion is 6.3 0.1 [15]. Protonation at pH below 6.7 induce conformational changes exposing the RVG fusion peptide (aa 103-179) which interact with endosomal membrane [15,23]. More than one RVG trimer is required for fusion and about 13-19 trimers are involved in formation of fusion site [15,24]. After the formation of fusion site, there occurs the transition of RVG towards fusion inactive (I) state releasing energy to overcome fusion activation energy barrier. A stalk connecting the outer leaflets of the fusing viral and endosomal membrane is formed with a ring of RVG which later transform to a hemifusion diaphragm by radial expansion of stalk. This stage is reversible and a second protonation of atleast three residues per trimer at pH below 6.3 break ring of RVG and induces complete fusion [24,25]. Upon fusion of the membranes, viral ribonucleoprotein is released into the cytoplasm.

Transcription and translation

G proteins are translated on endoplasmic reticulum (ER) bound ribosomes and then co-translationally translocated into ER in an unfolded form.

RNA dependent RNA polymerase of the virus sequentially transcribe the mRNA from the five structural genes by pausing at each intergenic region and re-initiating the synthesis at next gene which may lead to relatively high level of 3' terminal N mRNA while progressively less P,M,G and L transcripts [26,27]. Contrary to this, Palusa et al. [28] reported higher G mRNA even reaching to level of N mRNA in human T293 cells infected with Challenge virus standard -11 (CVS-11). This increased level of G mRNA may be attributed to synthetic rate and increased stability of the transcript rendered by the interaction between 3'UTR of G mRNA and host cellular protein poly(rc)- binding protein 2 (PCBP2), a ribosome binding protein [28,29]. Similarly, N and G mRNA level were similar and higher in mice infected with fixed virus [30].

Morimoto et al. [31] reported that there is slight difference in the levels of G mRNA by RV variants and it's level was more in primary neuron culture infected with less pathogenic RVs. G transcript levels were similar in brains of mice infected with CVS-24 and street virus, silver-haired bat rabies virus [32]. But in contrast to above findings G mRNA in mice inoculated with street viruses or rRV expressing the G from the street RV were found lower than that N mRNA [30].

N-glycosylation and folding of RVG

N-linked glycosylation of the ectodomain of RVG is essential for the proper folding, assembly, intracellular transport, cell surface expression, secretion and antigenicity of the virus. Inside the host cell after translation core oligosaccharide units are added to the specific consensus sequence when the polypeptide chains enter the lumen of ER [33]. RVG ectodomain have three potential N-linked glycosylation sequences (sequons) Asn-X- Ser/Thr at Asn37, Asn247 and Asn319 [11]. Asn247 and Asn319 are effectively core glycosylated; Asn247 is glycosylated in some strains but not in all while Asn319 is always glycosylated [34]. The hydoxy aa in the sequon influence N linked glycosylation [35] and it occurs when X is any aa except proline [36]. Out of the three sequons, only one or two are glycosylated depending on strain of RV. Most street viruses have one (Asn319) or two sequons (Asn37 and Asn 319) glyosylated while fixed viruses may have additional sequons at positions 158, 204, 247. Sequon 319 is conserved in G protein of RV [37]. Additional N-glycosylation may lead to reduced pathogenicity [38]. TMD and ENDO do not affect the efficiency of core N-glycosylation at any sequon in ECTO while C-terminal deletions of ECTO decrease the glycosylation efficiency at Asn319 [39]. Other factors contributing to effective core glycosylation might be the aa sequence surrounding the sequon, speed of translocation.

Folding of RVG occurs in ER after glycosylation and only correctly folded G is transported out of ER. Glycosylation of sequon 319 is essential for folding of G in street viruses [38]. The ECTO, TMD and ENDO form independent folding domains. Unfolded RVG also associate with chaperons. t1/2 for folding is about 20 min. RVG acquires its trimeric organization in ER [8,40]. After folding, nascent RVG is transported through Golgi apparatus in fusion inactive (I) conformation to avoid non-specific fusion during its transit in acidic compartment [41] and gets incorporated to budding virions upon interaction of ENDO with M protein or M-RNp complex. The intracellular transport of RVG to the plasma membrane requires N-linked glycosylation [42].

Replication of RV

It reported that RVG play a role in viral replication. Chimeric recombinant virus where G or M gene or both of attenuated strain when replaced by corresponding gene of pathogenic virus strain result in decreased rate of viral replication. The level of mRNA and genomic RNA was detected to be lower by quantitative RT- PCR. Similarly the rate of viral replication deduced from the viral RNA transcripts was found to be lower in mice infected with street viruses or rRV expressing the G from the street RVs by qRT-PCR [30].

Retrograde axonal transport and transsynaptic spread

After multiplication in the cytoplasm, virus is transported to the neuronal dendrite and its further propogation is achieved by retrograde trans-neuronal transfer to pre-synaptic terminals of higher order neurons [43,44]. This process also depends on the presynaptic receptors in the nervous system. G gene deficient recombinant RV complemented phenotypically with RVG has been used to demonstrate its requirement in transsynaptic spread both in vitro in cell culture and in vivo in murine models. RVG pseudotyped lentiviral vectors prove that RVG confers retrograde axonal transport to the retrovirus core [45-51]. Recombinant Vesicular stomatitis virus (rVSV) with RVG spread rapidly from neuron to neuron in retrograde manner in vivo [52]. N-terminal segment of the RV-G extracellular domain of 439 amino acids appears to be involved in the retrograde gene transfer.

RVG expression in neurons

RV adopts stealth to avoid stimulating the neutralizing host immune response by regulating the RVG expression at the site of infection and in neural tissues during its transit towards central nervous system. There is an inverse correlation between pathogenicity and RVG expression level. RVG expression was found to be about four folds higher in less pathogenic RV variants in primary neuron culture by immunofluoresence analysis [31].The expression of the RVG was detected to be stronger in brain of CVS- 24 than SHBRV-infected mice using TUNEL assay [32]. G gene may determine the level of G expression and it was detected low in street viruses by western-blot analysis and immuno-histochemistry [30]. This was in agreement to findings of Yan et al., [32], Sarmento et al. [53], Wang et al. [54] and Kuang et al. [55]. However, Wirblich and Schnell [56] concluded that RVG expression level does play a role in pathogenicity, but it is not a critical dominant factor and will not attenuate a pathogenic RV. This was observed in a study where the effect of differences in glycoprotein aa sequences on virulence were overcome by using recombinant variant of pathogenic CVS-N2c strain with synthetic codon optimized or de-optimized N2c which have same aa sequence but differ in glycoprotein expression.

Pathogenic virus regulates RVG expression by degradation. Morimoto et al. [31] reported that in less pathogenic variants, RVG degraded slowly implicating the role of post translational events in determining the stability and therefore the differential expression of RVG.

Antigenic sites on RVG

The ECTO which is the most variable region of the RVG contains the major antigenic sites which induce production of neutralizing antibodies. Both conformational and linear antigenic sites have been identified and mapped based on binding of neutralizing Abs and subsequent sequencing of mutants that escape neutralization. There are four major antigenic sites (I, II, III, IV) and a minor site (site a). Antigenic sites II and III are the two major conformational epitopes. Antigenic site II is discontinous extending from aa 34-42 (IIa) and from aa 198-200 (IIb) [57], while the continous antigenic site III extends from aa 333-338 [58]. Minor site a include aa 342 and 343 which is separated from site III by three aa which include a proline. Antigenic site I have both conformational and linear epitopes spanning from aa 226-231 [59]. Another major site IV includes aa 263-264. Along the antigenic domain in ECTO there are about 14 highly conserved cysteine residues. There are several isolated epitopes in addition to those mentioned above. It was identified that a linear epitope at N-terminal of ECTO aligned between aa 14 and 19 of mature glycoprotein.

RV variants with Arg-Gln mutation at position 333 of antigenic site III, were unable to kill adult immunocompetant mice [58,60] which may be due to influence of RVG sequence in the spread of virus in neurons, related to the differential usage of receptors [21,61] and differential expression of RVG in infected cells. Antigenic site III of RVG binds specifically to nAchR blocking further binding of acetyl choline.

APPLICATIONS OF RVG

Vaccine development- RVG is responsible for inducing and reacting with neutralizing antibodies.

There is a direct positive correlation between G expression and immune protection [58,62]. Next generation rabies vaccines aim to provide protection from rabies without raising any safety issues. These include viral recombinant vaccines, DNA vaccines, subunit vaccines and replication deficient live virus vectors.

Recombinant rabies glycoprotein expressed in viral vectors; vaccinia virus, canary pox virus, canine adeno virus and parainfluenza virus were found to be effective as vaccine candidates in mouse models [63-66]. Purevax (Merial) a rabies recombinant vaccine where canarypox virus vector express G of Evelyn-Rotkitniki-Abelseth (ERA) has been licensed and recommended for use in cats in the USA and Canada [64].Tackling rabies in wildlife is a great challenge and oral administration is the only route appropriate for vaccination of wild animals. Recombinant vaccines expressing RVG which can be given along with oral baits are relied upon towards this effort. Vaccinia-recombinant glycoprotein (V-RG) is an oral bait vaccine with recombinant, live vaccinia virus vector containing the glycoprotein gene from the ERA strain. WHO has recommended V-RG bait vaccine for immunization of wildlife [67]. RABORAL V-RG (Merial) is licensed in the USA for ORV in coyotes and raccoons [68] and is used under experimental license in gray fox. ONRAB (Artemis Technologies Inc., Guelph, Ontario, Canada) a live adenovirus recombinant oral vaccine, consisting of a human adenovirus type 5 vector containing the ERA G gene is currently under field trial in skunks and raccoons in Canada [69].

DNA vaccines where bacterial plasmid DNA vectors encoding rabies glycoprotein when given intramuscular [70], intradermal [71] and non-invasive intranasal route [72] were effective in providing protection in dogs. Tesoro-cruz et al. [73] described higher survivability in cats upon intradermal delivery of DNA vaccine against Mexican rabies isolate.

In protein subunit vaccine, glycoprotein is expressed in insect cells infected with recombinant baculovirus vectors [74] or in transgenic plants namely tobacco and maize [75,76] enabling large scale production which induce protection in mice.

Replication-deficient RV-based recombinant vaccine vector in which phosphoprotein gene is deleted which express two copies of the RVG gene (SPBN-ΔP-RVG) produce immune response against both glycoprotein and ribonucleoprotein as well as survival in mice [77].

Monoclonal antibody (MAb)

Most of the rabies specific antibodies are directed to epitopes on the RVG [6]. MAb against RVG is mostly utilized for typing of RV strains in epidemiological investigations [78], identification of the various lyssavirus serotypes and to study functional epitopes [59,79,80].

A combination of monoclonal antibodies targeting the RVG can offer a better alternative for human rabies immunoglobulin (HRIG) in future as a therapeutic. Three mouse monoclonal antibody (MoMAb) combinations of five MoMAb; E559, 1112-1, 62-7-13, M727-5-1 and M777-16-3, were equally effective as HRIG in vivo in hamster [81]. CL184, a combination of two human MAbs; CR 57 (binding site I) and CR4098 (binding site III) produced by MAbstract technology and PER.C6 cell line which is in clinical trial now could effectively neutralize all street RV in vitro yielding no escape mutants, have global coverage of RV isolates and does not interfere with rabies vaccine [82-85]. There also other potential candidates of MAbs targeting the glycoprotein. Another human MAb, RAB1 against site III derived from transgenic mouse (Medarex, Inc, Princeton, NJ, USA) could neutralize most of RABV isolates [86].

Capture ELISA based on MAb against RVG could detect immune-complexes in CSF enabling ante-mortem diagnosis in human [87]. Thus MAb also enable diagnosis of rabies cases especially if it is imported.

Brain research

Research that target normal brain functioning concentrate mainly on the attempts to decipher the complex neural circuits which may in turn also contribute to the treatment as well as prevention of brain disorders. CVS-11 strain has been used for studying the neural circuits exploiting its ability for retrograde trans-neuronal spread [43,44]. Glycoprotein gene-deleted SAD-B19 RV (SADΔG) has enabled the tracing and functional investigation of monosynaptic connections of defined neurons [88]. Modifiying SADΔG RV by pseudotyping also enables better tracing of neuronal connections [89-91]. Pseudotyping of viral vectors is achieved by deletion of the disease promoting genes and replacement of native glycoprotein with that from another virus helps in achieving tropism to desired target cell. Neuronal circuit for hand dexterity in primates was tracked using Highly efficient retrograde gene transfer (HiRet) vector made by pseudotyping a Human Immunodeficiency virus HIV-1 vector with a fusion envelope glycoprotein (FuG-B) in which the ENDO of RVG was substituted by the corresponding part of vesicular stomatitis virus glycoprotein [92,93]. Neuron-specific retrograde gene transfer (NeuRet) vector constructed by pseudotyping the (HIV-1) vector with fusion glycoprotein C type (FuG-C), in which a short C-terminal segment of the ECTO and TM/ ENDO of RV-G were replaced with the corresponding regions of VSV-G was employed for neural tract targeting in primate brain [94,95]. Beier et al. [52] proposed the possibility of using VSV vectors pseudotyped with RVG to study the connectivity of neuronal circuitry. Endocytic trafficking of RVG pseudotyped lentiviral vectors enable studies on axonal retrograde transport in primary motor neurons [96].

Drug or gene delivery

In disease conditions associated with central nervous system or brain, the effective delivery of therapeutic agents in a non- interventional way by peripheral route (by i/v) still remains a Herculean task due to the impediment offered by blood-brain barrier (BBB). So the research in this direction aims for the development of delivery systems that can effectively transport the drugs or therapeutic molecules to the target site in brain across the BBB. The mechanism by which RVG transports the virus across the nervous system was simulated. A chimeric peptide with monomer arginine residues at carboxy end of a short 29 aa peptide derived from RVG (RVG-9R) delivered SiRNA targeting Japanese encephalitis virus to the neurons in brain result in efficient silencing [97]. Pulford et al., [98] demonstrated effectiveness of Liposome- SiRNA- RVG-9R peptide complex in protecting SiRNA from serum degradation. RVG peptide linked nano carrier could successfully transport miRNA into brain enabling targeted gene therapy of miRNA associated neurological diseases [99]. A 39-amino acid peptide derived from the RVG (RDP) was exploited as an efficient protein carrier for brain-targeting delivery in mice [100]. Kim et al. [101] reported the potential of RVG and chitosan conjugated pluronic based nano-carrier for diagnosis and therapy of brain diseases.

Pseudotyped viral vectors when tagged with a gene of interest enable reliable experimental manipulation of gene expression yielding valuable insights into the mechanisms underlying a disease condition or a means for testing putative therapeutic approaches by effecting the ectopic expression of molecules such as growth factors or antioxidants [102]. Lentivirus pseudotyped with RVG enabled strong transduction offering possibilities for designing therapeutic strategies for different neurological diseases [45,47,49-51]. NeuRet and HiReT vectors also offer a promising technology for gene therapy of neurological diseases through enhanced retrograde gene transfer [103].

CONCLUSION

RVG plays essential role in the pathogenesis of the disease by binding to the specific receptors and enable viral entry in cells followed by fusion with endosomal membrane, transcription, translation and replication, retrograde axonal transport and trans-synaptic spread with regulated expression to remain stealth to the immune system. Thus RVG remains critical to the virus for its survival and existence. The ability of RVG to induce and react with neutralizing antibodies are being utilized in development of vaccine and production of monoclonal antibodies. The viral recombinant vaccines, DNA vaccines, subunit vaccines and replication deficient live virus vectors offer safe and effective protection stategies against rabies. Monoclonal antibody both murine and human raised against RVG could be utilized for its diagnostic value in typing RV strains and have therapeutic potential as a cost effective and better alternative to HRIG. RVG thus could be exploited as a potential tool for protection and therapy of the very disease it cause. G gene pseudotyped RV and lentiviral vectors are being employed in deciphering neural circuits and studying the retrograde axonal transport, providing us new insights to the normal functioning of brain. RVG derived peptides could deliver SiRNA and protein in brain overcoming the BBB offering possibilities of the development of delivery systems that can effectively transport the drugs or therapeutic molecules. RVG pseudotyped lentiviral vectors are promising tools for gene delivery studies in brain. RVG derived peptides and pseudotyped lentiviral vectors could be the future drug/ gene delivery systems in various diseases affecting brain. Thus RVG is beneficial to virus for its survival and the same RVG could be exploited for prevention, diagnosis and treatment of rabies and can be utilized for studying the neural circuits and in developing drug delivery system in brain thus benefiting mankind.

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