ISSN 0974-3618 (Print)
0974-360X (Online)
www.rjptonline.org

RESEARCH ARTICLE

Study on Genetically Engineered Vesicular Stomatitis Virus for the Application of Treating Malignant Diseases using Gene Therapy

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ABSTRACT:
Vesicular Stomatitis Virus (VSV) is a non-pathogenic negative stranded RNA enveloped virus which is genetically engineered to generate recombinant VSV producing suicide gene product, thymidine kinase (TK) or cytokine interleukin 4(IL-4). To target cancer cells or to stimulate immunity against diseases such as AIDS or influenza, the VSV is engineered which is ascetically harmless. The recombinant virus induces apoptosis in the cancerous cells while the normal cells are protected by the interferons and has the effective oncolytic activity, both in vitro and in vivo. When injected in vitro, high level of biologically active TK and IL-4 is and no defects in replication are observed. VSV leukemolytic properties is well established in treating leukemia cell lines by killing it efficiently, keeping the normal clonogenic bone marrow progenitor cells remarkably safe from VSV infection. Recombinant VSV is employed as an emerging anti-cancer approach. To improve oncoselectivity, safety, oncotoxicity and stimulus of tumor-specific immunity, VSV-based recombinant viruses have been engineered through reverse genetics. A complete decrease in the number of tumors as well as the high anti-tumor activity in the malignant disease was observed. The recombinant VSV is a methodology to various other malignant diseases, those anchorages a range of genetic defects and neoplastic diseases.

KEYWORDS: Vesicular Stomatitis Virus (VSV), Interferons, Oncolytic virotherapy, Acute Myelogenous Leukemia (AML), Rhabdoviridae.

INTRODUCTION:
Gene therapy is a therapeutic method to various incurable diseases by presenting genetic material into the cell to recompense for abnormal genes. Genetically engineered vector is used as a carrier to insert gene instead of directly injecting gene. Certain virus, such as retroviruses integrate their DNA into the chromosomes of cell. Vectors can be injected or given intravenously into specific tissue for treatment.

There are different types of disorders such as autoimmune, malignant etc. In autoimmune disorder, the immune system that is intended to protect the body against foreign substance, such as infectious agents and tumor cells, while not retorting the self- molecules, attacks the latter due to immunological tolerance to autoreactive immune cells.

Received on 12.02.2019 Modified on 14.04.2019
Accepted on 30.05.2019
Research J. Pharm. and Tech. 2019; 12(11):5371-5378.
DOI: 10.5958/0974-360X.2019.00932.6

It is linked with the genetic, infectious, environmental factors. It includes insulin dependent diabetes mellitus, hydroiditis, multiple sclerosis, scleroderma etc. Potentially malignant disorders are recognized by WHO as the risk of malignancies being present in the lesion or during the time of initial diagnosis. They are classified into two sub-types, (a) precancerous lesions, having superior risk of transmuting to malignancies and (b) precancerous condition, a disease related with the greater risk of cancer development in that specific tissue.

NEED FOR GENE THERAPY:
Gene therapy is transferring the genetic material needed for curing a particular disease in a patient, which is achieved by transforming virus into the shuttle vector and deliver the gene of interest into the host cell.[1] The mortality rate of the various malignant cancers, autoimmune disorders have marginally improved and this calls for several alternative biological strategies[2,3] such as "somatic gene therapy"[4-7]. This therapeutic efficacy depends on several other factors (i) prime of target cell type, (ii) appropriate tissue- specific delivery system, (iii) high transfection rate and (iv) a suitable promoter or enhancer for the optimal gene expression.

Several techniques have been employed for transferring gene across the cell membrane[8,9] such as physical method, chemical method and recombinant viral vector based on the manipulation[10,11].

RNA virus based vectors are derived form simple retrovirus such as murine leukemia virus. DNA-derived minigenomes or small defective RNAs from rhabdoviruses and paramyxoviruses have predefined system of replication and transcription, where RNAs are accrued into nucleocapsid[12] inside the cells expressing the viral N protein and polymerase proteins. Although been very beneficial, these system do not allow genetic manipulation of the full length genome of infectious viruses[13]. Anti-viral therapies have been used widely. Ribavirin is a nucleoside analogue showing antiviral activity and highly effective when used in combination with the interferon-α.[14] Artificial Viral Envelope (AVE) are the lipid vesicles that mimic retrovirus envelope to target the moieties such as Viral Binding Protein and increase the efficiency of the viruses delivery.[15]

VESICULAR STOMATITIS VIRUS (VSV):

Vesicular Stomatitis Indiana Virus, commonly called as the VSV is an arbovirus of family Rhabdoviridae, a membrane enveloped viruses that replicates in the cytoplasm and infects the vertebrate, invertebrate and plants. Rhabdoviruses is widely distributed, have single negative strand RNA genome. It is enveloped of negative sense RNA genome consisting of 11161 nucleotides that encodes five viral structural gene, (i) nucleocapsid protein, N for the encapsidation of genomic RNA during replication, (ii) phosphoprotein, P, (iii) RNA dependent RNA polymerase, L, (iv) matrix protein, M, (v) attachment glycoprotein, G[16-18]. VSV can have at least 4.5kb of foreign RNA and expressed at high levels from two additional mRNA. There are two types of serotypes: Indiana and New Jersey. These two serotypes were differentiated by specific neutralizing anti-serum directed against single glycoprotein species (G) forming spikes developed from the viral envelope[19].

The order of the protein is as 3'-N-P-M-G-L-5'[20]. Gene expression is precised by the highly conserved order of genes relative to the single transcriptional promoter[21]. RNA dependent RNA polymerase, started at the promoter site near 3' end, highly sequential[22-25]. Gene close to 3'end transcribed at much higher rate than those distant ones[26]. Rearranging three genes: P, M, and G in possible orders from the cDNA of VSV. The recovered viable viruses were examined with the level of gene expression, its growth latent in culture and its virulence towards the mice. Some rearranged genomes showed better growth, while some showed decrease in replication, although all were mortal to mice in comparison to the wild type virus. Translocation of N gene for the nucleocapsid protein of VSV from its proximal position to 2nd, 3rd and 4th position in gene order caused reduction of the protein synthesis. Even altering the glycoprotein could change the immune response in animals as well as their expression. Lack of the recombination showed immobilization of the ancestral gene. Altered genome results in phenotypic change of the viruses and a novel approach for the synthesis of expression vector and recombinant vaccine [27].

When livestock is naturally infected with VSV, it grounds diseases involving vesiculation and ulceration of tongue and oral epithelia and appearance of lesions on feet and teats[28-30]. Gene expression of the VSV is controlled systematically via manipulating its gene arrangement. VSV infection persuades strong cellular and antibody response to its own as well as the recombinant protein. These viruses can infect murine neuroepithelial cells and thus represent a model system for the examination of acute neurotropic virus infections [31].

In human cells, the VSV cannot reproduce since it is highly sensitive to antiviral action of interferon (ITN)[32, 33]. Being a member in the family cytokines, interferon is produced in response to the infection (providing innate immunity). IFN inducible double stranded RNA dependent protein kinase (PKR) is the essential component of anti-viral host defense. Cells in absence of this system are highly susceptible to VSV infection. VSV infection induces the activation of PKR, which potently inhibits the protein synthesis by phosphorylating eIF2α.

The IFN-inducible gene product(s) responsible for the inhibition of VSV replication has not been identified. In most cases the major or exclusive site of action of IFN against VSV is at the level of viral RNA and protein synthesis, although defects in viral entry and morphogenesis have been demonstrated in some cell lines[34].

When infected with recombinant VSV, human cancer cell lines undergo cytolysis, thereby proving as an attractive and effective therapy against the malignant disease. There are various advantages of using VSV, such as non-pathogenic nature, genetic malleability of the virus, genetically manipulative nature, recombinant can be feasibly created, no recognized transforming abilities, gene not attenuated which affects replication and oncolytic tumor activity[35]. VSV can replicate in variety of tumorigenic cells and not in selective tumor suppressor gene such as p53.

RECOMBINANT VSV:

The infectivity of the virus is done by generating VSV from DNA. The difficulty is that neither fully length genomic DNA nor the anti- genomic RNA's are infectious, but only the genomic RNA destined to 1250 subunits of nucleoplasid (N) protein. VSV was recovered from a full length cDNA clone of viral genome while the bacteriophage T7 RNA polymerase from recombinant vaccinia virus was used to synthesize the positive sense transcript of VSV,[36] that is the approach of expressing the full-length positive strand[37]. This renders the VSV to be completely available to genetic manipulation[38]. For the negative sense strand, the VSV sub genomic replicons were recovered from the cDNA clones[39].

On adding an unselective gene, it remains stable in the virus. Owing to the significantly slow mutation rate in the virus, foreign gene can be easily expressed even during extensive passaging. Single passage yields virus to carry out injection at industrial level[40]. But errors in the polymerase during the replication posed as a great hurdle in utilizing VSV to express a foreign gene[41]. Mutation frequencies in VSV was observed as 1 to 10^3 to 10^4 for a specific nucleotide in the genome[42]. Owing to its various advantages, the genetic engineering of VSV provides an effort to develop new generation of custom made VSV vectors having the ability of high anti-tumor activity. It involves the designing of the VSV vector carrying TK (Thymidine Kinase) and IL-4(Cytokine interleukin) gene to create recombinant VSV carrying immune modulatory gene. Live attenuated vaccines, however, have not been discovered for VSV[43].

On phosphorylating non-toxic prodrug Ganciclovir (GCR) by the TK protein, they merge into the cellular DNA during replication resulting in chain termination and eventually cell death. The tumor killing is drastically augmented due to cell death transferred from transduced cell to the adjacent cell. This phenomenon is called the bystander effect. This effect not only act within the tumor, but also the between distant tumor. Natural Killer, encounter not only the target cells but also the non-target bystander cells. The presence of the bystander cells augments the killing efficiency and NK cell migration.

The IL-4 induces the development of the effector cells, the eosinophil and Antigen Presenting Cells (APC) which regulates the T-helper cell and stimulates humoral response. APC is a cell that provides antigen joined with major histocompatibility complex (MHC) on the antigen surface. Engineered IL-4 cells decrease the number of malignancies, melanoma, glioma and colon carcinoma. Glioma is a type of tumor that occurs in the brain and spinal cord, instigates in the glial cells that edge the nerve cells. There are three types of glial cells: astrocytomas, ependymomas and oligodendrogliomas. When injected with the VSV, the mice has its T- cell mediated immune response against the latter showing the cytotoxic activity, Viruses induces the formation of detectable cytotoxic T- cells which is specific in two ways, (i) towards virus and (ii) towards the host self-marker[44-46].

MECHANISM OF INTERFERON:

Interferon belongs to the family of associated pleiotropic cytokines with persuasive antiviral activities[47]. They are separated as two main types: Type I (α and β) and Type II (α and β). It is a factor that bound to the cell surface receptor, inducing both anti-viral response and growth inhibitory (apoptotic signals in the cells), thus killing the tumor cells. In order to limit the development of the tumor, INF pathway is hindered by the cancer specific mutations of the gene product. Reduction in the viral production to less than 1000 viral particle per ml was observed on pre-treating the normal cell cultures with the interferon.

In melanoma culture, normal primary cells were defensive and remained unaffected from the infectious cell in presence of the interferon. Interferon is an effective circulating factor in defending the normal cells from the killing of VSV, but unable to do the same for wide ranges of tumor types. VSV is a selective oncolytic agent. The interferon mechanism was subjugated to limit the tumors growth, but expected enlarged results were not obtained due to cancer specific mutation in the interferon pathway[48-53].

VSV IN TREATMENT OF VARIOUS MALIGNANT DISEASES:

B16 Melanoma Treatment:

Melanoma is a lethal type of skin cancer, starting in melanocytes and characterized by a change in the existing mole. Pathogenesis of metastasis is done by invading the tissues, blood vessels having its origin from the primary cancer[54]. They are released into the circulation, initially the tumor is arrested but some recirculate and get trapped in other organs[55]. Mere presence of the tumor doesn't initiate and process the metastasis in the body since most of them rapidly die with 0.1% survived for the secondary growth[56-58].

When immunized syngeneic lymphocyte mixed with B16 melanoma cells was injected into the mice containing tumor cells, drastic decrease in pulmonary metastasis was observed[59]. *In vitro* injection of low doses of lymphocytes resulted in the clumps of cells. High lymphocytes to the tumor cell lines ratio results high clumping, thus inhibition of metastasis. Injection of tumor cells from successive lines, that is, from F1 generation till F11 showed that (i) pulmonary tumor

colonies increased with successive tumor lines, (ii) tumor cell lines from F11 generation was more metastatic yielding more pulmonary tumor module than the tumor cells from F1 cell lines.

B16 melanoma tumor cells cause pulmonary tumor colonies in their host. Two lines were shaped, low metastatic and high metastatic B16 tumor cell lines. They are distinguished on the basis of their difference in the electrophoretic mobility surface, glycoprotein, etc. High metastatic activity results larger nodules. The metastatic invades the tissue, blood vessels forming primary tumor, it circulates and enters the parenchyma forming the secondary tumor. Owing to the property of reduced adhesiveness, the malignant tumor invades efficiently. Malignant is due to the high proteolytic and fibrinolytic activity as compared to the normal cells. The cell emboli arrested in capillary bed occurs on the basis of the factors like, (i) tumor cell surface charge, (ii) level of surface enzymes, (iii) interface between the tumor cells and host immune cells, (iv) host blood clotting mechanism. This resolved that the immune response may have dual role in its relationship to the development and spread of the cancer. The tumor cells growth is mainly due to weak immune response and on activation can inhibit the former's growth[60-62].

VSV treating AIDS:

More than 20 years ago, AIDS became epidemic, a matter of concern. HIV cause AIDS which takes 8-10 years to develop. It weakens and destroys the defense system of the body eventually the patient dies·[63] Anti-Retroviral Therapy (ART) is an effective method of controlling the AIDS to which high adherence is an important determining factor for the treatment.[64] Apart from the current therapies including the Highly Active Antiviral Therapies (HAART) and Salvage Treatment, latest include the use of Raltigravir, an antiretroviral drug, with additional potency and durability than the previous drugs.[65]A safe and effective vaccine easily produced on a large scale is an immediate action. Live virus vaccination results in high level synthesis of viral proteins, an effective AIDS vaccine, but testing is delayed due to the risk of disease associated with the live attenuated HIV immunization. VSV has been efficient in the murine models, that is, recombinant VSV expressing influenza virus hemagglutin (VSV-HA) protects the against the influenza virus. In immunodeficiency virus infection control, Cellular immunity plays an vital role [66-68].

VSV is a high- level expression vector. This is done by introduction of foreign glycoprotein after the mixed infection with several enveloped virus including influenza virus. HA gene is incorporated between the genes encoding G and L protein. NA gene is injected between gene encoding M and L protein (figure 4.1) Influenza virus A has RNA genomes encoding hemagglutinin (HA) that recognizes receptor containing sialic acid at the cell surface and neuraminidase (NA) to remove the sialic acid from the cells. This would inhibit the self- aggregation or virions. This could be used as a model for live vaccine development[69-71].

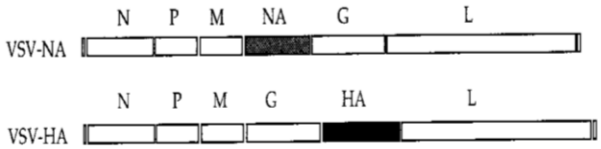

Figure 4.1: The gene order of VSV- Neuraminidase (VSV-NA) and VSV- Hemagglutinin (VSV-HA)[69]

As an alternative to AIDS treatment, VSV/HIV recombinant expressing the envelope glycoprotein of an HIV can be provided. Studies proved that the vaccination of VSV recombinants expressing Env and Gag protein in the models is highly protective from the AIDS. If the boosting system using a vector with heterologous VSV G protein were used, it could be a powerful AIDS vaccine. The route of vaccination is a combination of either oral and intramuscular or intranasal and intramuscular routes.

The infection of the immune deficiency virus depends on cellular immunity. Necrotic cell death produces components that can function as the initiation of cellular immune response[72]. Strong cellular immune response is the basis of the AIDS vaccine research[73] as they DNA vaccine has been used to enhance the immunity as they are liable for early control of viral replication before the neutralizing antibodies are formed[74]. Presence of the envelope glycoprotein (Gnv) of HIV is a poor target for the antibodies. Measures has been adopted to reduce the replication of challenge virus, preservation of CD+4 T cells and AIDS prevention. Immune response is immunized by the four injection of DNA expression plasmid encoding viral protein and interleukin 2. Another method is DNA priming, tailed by recombinant pox-virus booster which boosts up higher frequencies of T- cells[75].

VSV in Cancer treatment:

Cancer is an abnormality in DNA known as mutation. This results in continuous growth of cells converting the normal cells to tumor cells.[76] Conventional therapy for cancer is bone marrow although the culture of the latter didn't show produced VSV particles. After the *in vitro* culture, infected bone marrow culture is similar to MOCK infected medium culture in forming the normal spectrum of hematopoietic cell types. Another method is nanotechnology treating cancer. It is preferred over chemotherapy as they endow with poor supply and

incapability to deliver the drugs at the required tumor site efficiently cause hair loss, digestive problem, lack of energy, mouth ulcer.[77,78] It overcomes the challenge such as poor bioavailability, *in vivo* stability, sustained and targeted delivery to site of action.[79] Nanoparticles carry the potential for embattled and time-release drugs by functionalizing the surface of nanocarrier with ligands like antibody or peptides to bind to tumor specific antigen. Even though the carriers are associated with the drug‘s longevity in the blood being increased, patient protection. They have disadvantages like (a) trigger immune response and allergic response and (b) generate free radical and reactive oxygen species.[80]

Acute Myelogenous Leukemia (AML) cell lines, OCI/AML3, OCI/AML4 and OCI/AML5 are vulnerable to VSV infection. Selective oncolytic property of VSV was observed in the co-culture of the leukemic OCI/AMI3 cell line mixed with the normal bone marrow, infected with VSV both in the presence and absence of the growth factor. In the presence of the growth factors, both were grown, whereas in the absence only the leukemic cell lines growth was obtained. After a certain period, these growth factor independent leukemic cells were removed and normal bone marrow precursors were revived. This showed the selective destruction of the leukemia cells in the presence of the potential of VSV cells.

Mice model with the human tumor xenografts was treated with VSV for the study of the potential anti-cancer therapeutics[81]. This method is restricted due to the immunological impairment of the mice. This can cause the death of the mice due to the VSV infection[82-84]. VSV induced mortality was protected via interferon. Intratumoral injection of live VSV led to the partial followed by the complete suppression of the tumor in the mice, but some were killed due to severe tumor burden.

VSV shows “leukemolytic property” that is, the VSV remove bone marrow culture *ex-vivo* having considerable significances in autologous bone marrow transplantation. Another study showed the mixing of the spleen cells from the immunized mice with the target tumor cells and injected with the thymecotized recipients. When in smaller amounts, it increases tumor growth, while in larger amount, it controls and inhibits the tumor growth[62]. Recent demonstration showed improvised transfer of herpes simplex virus in treating the ovarian cancer using virus producing cells rather than the virus alone[30].

OTHER APPLICATIONS:

Retroviral viruses derived from the Moloney murine leukemia viruses (MoMLV) are substantial for the constant transfer of gene into the mammalian cells, which has been inadequate due to its limited host cell range and it’s incapability to produce high titer virus[2]. These has been used to study gene expression and gene regulation for human gene therapy.

To overcome these problems, the G-glycoprotein of VSV substitutes the retroviral envelope glycoprotein, whose infectivity is demolished by revelation to anti VSV antibodies, to facilitate the gene therapy model studies. Several studies were considered for the construction of the pseudotypes of the retroviral vectors by the substitution. The genome of one virus is encapsulated by the envelope of the second virus resulting in the formation of retroviral pseudotypes[85]. The host range of the pseudotype virus is by the virus of the enveloped protein[86,87]. VSV has an enormously broad cell range and can be concerted by ultracentrifugation without the loss of the infectivity. It has been reported that the retroviral vector pseudotype containing the G glycoprotein of VSV (VSV-G) was capable of injecting the hamster cell[88].

The interface between the viral envelope protein and the specific cell surface receptor proteins is essential for retroviral infection. The phospholipid content of the cell membrane interacts with the VSV-G to mediate viral entry.

CONCLUSION:

For the last few decades, many reports regarding the correlation of the virus infections with the tumor regression[89,90]. Molecular events related to the generation and evolution of Malignancies have been studied extensively, thus the tailoring and selection of viruses for their ability to replicated preferentially in tumor cells[91].

The need of the gene therapy is dominant and the role of the recombinant virus is essential. Limited success has been gained with an efforts to develop subunit- or DNA-mediated vaccines for VSV[92]. Suicide gene therapy is an approach to the malignant treatment and an early indication of anti- tumor response in several patients.

FUTURE PERSPECTIVE:

DNA/RNA viruses appeared as useful eukaryotic vectors, integrating large amount of foreign genes in the viral genome without the loss of infectivity and processing of expression protein. This is a technique to provide protection to wide range of diseases. Up to present, it was expected at providing gene therapy and various other therapies in medical research. Such as the cancer therapies, where recombinant VSV encoding suicide cassettes or immune response to eradicate cancer cells They are the tools as substitute viruses to study the entry mechanism, identification of novel cellular

receptors, screening antiviral libraries, etc. Further studies and application of VSV vectors will provide with benefits for biological sciences and medical research.

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