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Review Article

Therapeutic Applications of MicroRNAs in Polio Vaccines. Current Status and Strategies

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Abstract

Vaccines that are live and attenuated still offer the most economical and safest defense against viral diseases. Many significant viruses lack an effective live vaccination because live vaccine strains are created empirically and the basis for attenuation is typically poorly established. Here, we outline a basic plan for the logical creation of live vaccines that are both safe and efficacious, using the machinery of microRNA-based gene silencing to regulate viral replication. We show that short miRNA homology sequences may be inserted into a viral genome to limit tissue tropism, which prevents pathogenicity and results in an attenuated viral strain using poliovirus as a model. The central nervous system is unable to replicate poliovirus strains designed to be targets of neuronal-specific miRNAs, which resulted in a marked reduction in the neurovirulence of infected animals. Significantly, these viruses were still able to multiply in organs other than neurons. These modified miRNA-regulated viruses thereby strongly induced protective immunity in mice without causing illness.

Keywords: mRNA; Vaccination; Neurovirulence; Poliomyelitis; Immunity

Abbreviations

mRNA: microRNA

Introduction

One of the biggest advancements in public health in the past century is the use of vaccines to prevent viral infections. More than 99.9% fewer people die each year in the US from diseases caused by RNA viruses, such as poliomyelitis, measles, mumps, and rubella (CDC, 1999). Numerous methods for developing viral vaccines have been devised, such as the generation of viral proteins, inactivated viruses, live attenuated viruses, and gene delivery vehicles that express viral antigens [1]. Because live attenuated virus vaccines activate all immune system components and elicit a broad humoral and cellular response in addition to a balanced systemic and local immune response, they are the most effective form of vaccination [2]. Along with production and distribution challenges, the possibility of attenuated viruses returning to the pathogenic phenotype is a significant worry. A major obstacle in the creation of live vaccines is the absence of well-thought-out methods for reducing virus pathogenicity. Vaccination against a virus should ideally multiply robustly in a small number of tissues to trigger a sophisticated and long-lasting immune response while preventing the virus from spreading to other tissues where it could cause illness. For example, poliomyelitis is caused by motor neuron infection in the brainstem and spinal cord, while poliovirus replication in the gut is not fatal [3]. Vaccine development may be facilitated by the logical creation of viral strains that are specifically prevented from replicating in a particular tissue. The traditional technique for virus attenuation and vaccine strain generation involves changing a virus's cell tropism by passage it via cell culture. Attenuation of the pathogenic phenotype technique is frequently the consequence of the pathogenic virus adapting to the specific cell line or lines, which lowers virus fitness in particular organs and cell types within the vaccinee [3]. Several virus vaccines have been generated thanks to this method, however, the empirical nature of such passaging may change the virus in unanticipated ways, and over time, many more vaccines have been developed.

Recent research has detailed a novel method of controlling the mistake rate of the viral polymerase to restrict the pathogenic potential of poliovirus [4]. In an animal model of infection, the poliovirus population's genetic diversity was limited and its virulence was reduced by raising polymerase fidelity [5,6]. Later, attenuated poliovirus vaccines with increased stability were created using this method [7]. Nonetheless, a crucial component of creating attenuated virus vaccinations continues to be the problem of restricting viral reproduction to particular tissues. In this review, we present a logical approach to modulate viral replication through the utilization of the regulatory potential of the RNA interference (RNAi) gene-silencing apparatus this family of non-coding genes regulates gene expression post-transcriptionally. Mature miRNAs are produced by two processing events: first, the RNase-III enzyme Drosha processes the nascent miRNA transcripts into pre-miRNA precursors (~70 nucleotides in length) in the nucleus; second, the pre-miRNA is cleaved by another RNase-III enzyme, Dicer, in the cytoplasm to produce the ~21–25-nucleotide mature miRNAs [8-16].

To direct cleavage, miRNAs can also function as siRNAs of a well-matched series [17]. Within the framework of the RNA-induced silencing complex (RISC), the endonuclease Argonaute2 (Ago2) mediates this mRNA-cleavage stage of RNAi [18,19]. Many facets of cellular physiology and development are regulated by miRNAs. Because of this, the patterns of miRNA expression are tissue-specific and developmentally regulated, meaning that a distinct set of miRNAs characterizes each tissue [20,21]. One such miRNA that regulates the developmental transition from the L4 stage into the adult is encoded by let-7a, which has been found in worms [22-24]. Through a variety of methods, the neuron-specific miRNA miR-124 encourages the differentiation of mammalian neurons [25-27].

The rationale for creating stable attenuated viruses

Polioviruses were engineered with two complementary miRNA target sequence insertions to see whether the miRNA gene-silencing mechanism could be utilized to logically design viruses with tissue tropism identified by the miRNA expression profiles. The two insertions were made between the structural and nonstructural genes in the coding area as well as into the "variable" section at the 3' end of the 5' untranslated region (UTR). Large insertions have been demonstrated to be tolerated at these sites without negatively impacting viral replication [28]. The viruses containing these insertions were created as PV-L7 and PV-124, respectively, and the miRNA target sequences used for this investigation are complementary to let-7a or miR-124a. Additionally, the creation of control viruses with altered miRNA target sequences impedes these miRNAs' ability to recognize and cleave the viruses known as PV-L7M and PV-124M, respectively.

Attenuation of viral replication is mediated by the miRNA machinery

Viral replication in HeLa cells transfected with miRNA inhibitors (anti-miRs) has been assessed to directly investigate the role of miRNA let-7a in PV-L7 viral proliferation. Single-stranded nucleic acids called anti-miRs are chemically altered to selectively bind to and inhibit endogenous miRNA molecules. Transfection with a let-7a specific anti-miR partially restored PV-L7 replication, but PV-L7 growth persisted to be suppressed in HeLa cells transfected with a control anti-miR). In cells transfected with let-7a anti-miR, the final virus titers (24-hour time point) for wild-type poliovirus were 100 times higher than those levels found for PV-L7. Anti-miR transfected HeLa cells most likely exhibit partial inhibition of viral replication due to newly processed mature miRNA or residual cellular let-7a. Transfection efficiency is also typically not ideal even in those untransfected cells, virus growth is still suppressed. However, these findings suggest that let-7a availability is a prerequisite for the suppression of PV-L7 replication.

The pathogenicity of poliovirus is significantly reduced by endogenous miRNAs regulating viral replication

Because the miRNA apparatus effectively inhibited PV-L7 proliferation in tissue culture cells, scientists were able to evaluate the effect of miRNAs on poliovirus pathogenesis using an animal infection model. 50% fatal dosage (LD50) for the wild type, PV-L7, PV-L7M, PV-124, and PV-124M viruses in transgenic mice expressing the human poliovirus receptor (cPVR), the infectious particle count (PFU) of the wild-type poliovirus is 2.2 × 106 was first determined. In contrast, the viruses harboring PV-L7 and PV-124 LD50s were more than the maximum administered dose of 1 × 108 PFU. The control viruses PV-L7M and PV-124M displayed LD50 values that were similar to the wild type, as would be expected. Upon intramuscular injection of 108 PFU of the wild-type virus into cPVR mice, the central nervous was rapidly invaded.

Poliovirus tissue tropism is regulated by miRNAs

It has been postulated that in areas where let-7a and miR-124a miRNAs are produced, they will selectively regulate the replication of viruses containing complementary sequences. Moreover, studies have anticipated that the observed variations in the virulence of the modified viruses would be explained by this tissue selectivity. To investigate these theories, investigators have looked at viral replication in a variety of tissues taken six days following intravenous injections of either wild-type or modified viruses in mice. In the brain and spinal cord of the infected animals, both control viruses, PV-L7M and PV-124M, accumulated to wild-type levels. However, the PV-L7 and PV-124 viruses either did not proliferate in these tissues or did so very sparingly. PV-L7 was not found in the brain or spinal cord of the mice that received injections. Three of the five infected mice had brains with detectable low levels of PV-124 (102 PFU), and one of these mice had a spinal cord titer that was similarly low six days after injection. Nevertheless, no neuropathogenic side effects linked to poliovirus infection were evident in these animals. Notably, all viruses showed wild-type levels of replication in the spleen, except PV-L7, which was discovered at a lower level.

Promising candidates for vaccination are polioviruses limited by miRNA

The ability of the modified viruses PV-L7 and PV-124 to elicit an immune response in mice given intraperitoneal injections, one important indicator of the effectiveness of the poliovirus vaccine is neutralizing antibodies. Four weeks following injection, sera from inoculated mice have shown that the PV-124 virus had produced substantial levels of neutralizing antibodies specific to the poliovirus. The reaction induced by mice inoculated with UV-inactivated wild-type poliovirus was comparable to that of mice immunized with PV-L7, while the titers obtained from mock-immunized mice (PBS) were below the assay's detection threshold. Compared to G64S-immunized mice, PV-124 induced a three-fold greater neutralizing antibody response. It has been demonstrated that the high-fidelity replication variant, G64S, was a more potent immunogen than the existing Sabin type 1 vaccine strain [7]. These findings significantly encourage the further application of this technology for the development of virus vaccines, as they showed that PV-124's immunogenic potential is on par with or superior to that of vaccinations now in use.

The vaccinated mice appeared to be immune to the deadly challenge of a wild-type pathogenic virus, based on the high neutralizing antibody titers found in the animals. To investigate this possibility, twenty mice from each batch were challenged with a lethal dose (10LD50) of wild-type poliovirus intramuscularly four weeks following intraperitoneal vaccines. When challenged with a lethal dose of wild-type poliovirus, 18 out of 20 mice inoculated with the modified poliovirus PV-124 were fully protected. By contrast, 50% of the animals that received the PV-L7 vaccination showed protection. Comparing UV-inactivated wild-type poliovirus vaccination-affected mice to control mice treated with PBS, the former showed only a limited protective effect. According to these findings, neutralizing antibody responses elicited by PV-124 and PV-L7 provided protective immunity against a highly specific viral infection test. The percentage of immunized mice who survived a lethal dose of the pathogenic virus was positively linked with the generation of neutralizing antibodies in those mice. Similar tests have been performed with IFNAR mice to investigate the protection provided by PV-L7 vaccinations in more detail. Mice inoculated with 107 PFU PV-L7 were challenged with either 10LD50 or 104LD50 via intramuscular injection one month following intraperitoneal injections. All of the inoculated mice were protected against a 10LD50 injection of the wild-type poliovirus, and 70% of the mice survived the extremely deadly 104LD50 dose. On the other hand, mice given PBS plus UV-inactivated virus did not receive any protection at all. These findings support the viability of using these viruses as vaccine candidates and the feasibility of this method for logically developing live attenuated viral vaccinations.

Methods adopted in viral Cloning of Polio Virus

To simplify the cloning of the miRNA target sequences into the plasmid, the wild-type poliovirus type 1 Mahoney cDNA plasmid prib (+) XpAlong was designed to have unique restriction sites in the 5'-UTR (BssHII and SacI) and 2A site (EcoRI and XhoI) [29]. After being annealed for two minutes at 90 °C in 40 mM Tris pH 7.5, 20 mM MgCl2, and 50 mM NaCl, complementary oligos (Elim Biopharmaceuticals, Hayward, CA) were allowed to gradually cool to room temperature. EcoRI and XhoI were introduced on restriction site-flanked segments into the poliovirus genome's cDNA at position 2A to generate the viruses. A second BssHII and SacI restriction site-flanked fragment was later inserted into the cDNA in the 5'-UTR using positive clones. To preserve the right amino acid, the codon sequence for the control viruses (PV-L7M and PV-124M) was changed by the poliovirus amino acid usage. Using the modified plasmids above, 20 µg of electroporated in vitro transcribed RNA was put into 800 µl NTERA-2 cells (ATCC HTB-106, 5×106 cells/ml) in a 4 mm cuvette with the following pulse: 300 V, 24 °C, and 500 µF. This allowed the preparation of virus stocks. The progeny were passaged twice in NTERA-2 cells at 37 °C and a high multiplicity of infection (MOI=10); the latter passed virus was utilized in the experiments that are reported.

Viral RNA from infected cells using RT-PCR

The experiments presented here from previous studies have been somewhat altered from data that has already been published [7]. A 6-well plate was seeded with 1 × 106 HeLa cells in each well, and the next day, the viruses PV-L7, PV-L7M, or wild type were added at a multiplicity of infection of 10 (MOI=10). With a small adjustment, the infections were carried out similarly to the one-step growth curves previously mentioned. To permit receptor engagement while blocking cellular internalization, virus absorption was done at 4°C. Following two cold PBS washes, the cells were put in to warm DMEM/High glucose medium supplemented with 10% fetal bovine serum (Sigma). Total RNA was extracted from adherent cells using Trizol (Invitrogen, CA) following the manufacturer's rec-

ommended methodology at different points after infection. 200ng of total RNA were amplified into a 269nt fragment covering the 5' target insertion site using a ThermoscriptTM RT-PCR kit (Invitrogen, CA). For the PCR procedure, the forward and reverse primer sequences are, respectively, GGCTGCTTATGGTGACAATCACAG and GTGGTGTAATTAATGGTAGAACCACC. After 25 cycles of the PCR process, the fragments were separated on a 2% agarose gel.

Pathogenesis of polio Infection in Mice

6-to 8- weeks-old cPVR and IFNAR transgenic mice that expressed the poliovirus receptor in these studies have been used to carry out these studies. During sedation, the following injections were given to them: two millilitres (250 µl) per quadriceps, one mL for every muscle, and one mL per vein (100 µl, tail vein). Every day, the mice were checked for signs of paralysis, and when complete paralysis appeared, they were put to death. Twenty mice were intramuscularly injected with successive virus dilutions to calculate the LD50 values using the Reed and Muench method. Entire organs were removed from five mice in each group that had received an intravenous injection to examine viral tissue tropism. Serum from five mice (from a set of twenty cPVR animals) was used for neutralization experiments one month after the mice were intraperitoneal immunized with PBS, 1×107 PFU UV-inactivated wild type, PV-L7, or PV-124 virus. Serial dilutions of sera (conducted in octuplicate) were used to treat a wild-type poliovirus at 100 times its 50% tissue culture infectious dose (TCID50) for two hours. Through a week of incubation on 1×104 HeLa cells (DMEM, 2% final FBS content) in 96-well plates, the serial dilution that neutralized 100TCID50 was found. The dilution of the neutralizing antibody was calculated using the Reed and Muench method. The graph represents the neutralizing antibody titer, which is the reciprocal of this dilution.

Insertion of MicroRNA sequence

MicroRNAs are short RNA molecules found in eukaryotic cells that regulate gene expression. They typically bind to complementary sequences in messenger RNA (mRNA) molecules, leading to the degradation of the mRNA or inhibition of its translation. To attenuate a virus, researchers can engineer its genome to include sequences that are complementary to specific miRNAs that are abundant in the target tissues of the virus e.g., neuronal cells for neurotropic viruses. When the virus infects these tissues, the miRNAs present in the cells will bind to the complementary sequences in the viral genome. This binding can result in the degradation of the viral RNA or prevent its translation, thereby reducing viral replication and pathogenicity in these tissues. For vaccine production, the attenuated virus can be grown in cultured cells that do not express the specific miRNA. This allows the virus to replicate efficiently in the production environment, while still being attenuated in the target host tissues due to the presence of the miRNA. Despite the attenuation, the virus retains its wild-type immunogenicity, meaning it can still elicit a strong immune response in the host. This makes it an effective vaccine candidate [49]. Developing new vaccines using novel approaches, such as the attenuation of viruses through miRNA manipulation holds promise but also presents several challenges. Initial experiments using these novel approaches have shown potential for developing nonpathogenic polioviruses with wild-type antigenicity. This suggests that these methods could lead to effective vaccines. Despite promising preliminary results, more research is required to determine if inactivated poliovirus vaccines (IPVs) made from these modified strains are feasible to produce and effective in practice. Combining different genome modification techniques might enhance the efficacy and safety of the resulting strains. Regulatory issues should be addressed early in the development process to ensure a smooth path to licensure. This involves demonstrating comparable efficacy to existing vaccines. Clinical trials directly measuring protection against infection are not feasible. Instead, licensure decisions rely on surrogate markers, like the ability to induce neutralizing antibodies. Differences in the immunochemical structures of formalin-treated Sabin viruses and conventional IPV complicate dosage formulation and efficacy prediction against various strains of the same serotype. A new vaccine that is not immunochemically different from the Salk IPV would likely face fewer regulatory hurdles, as it would simplify the evaluation process [50].

Discussion

Here, we discuss a method for logically designing live attenuated viral vaccines. Previous strategies significantly restrict virus replication in tissues where the virus causes sickness by utilizing the nucleic acid-based RNAi response and the tissue-specific expression patterns of miRNAs. The miRNA/RNAi machinery targets the miRNA complementary sequences that are inserted into the virus genome, thereby preventing virus replication in a tissue-specific manner. Crucially, these modified viruses showed reduced neurovirulence but maintained their ability to replicate, allowing for

the establishment of a successful immune response. High levels of neutralizing antibody production demonstrated these test strains' immunogenicity. Such innovative methods of rationally engineering live vaccines have a unique and intriguing advantage they can be applied to a broad range of viruses due to their adaptability. Every type of cell has a unique miRNA repertoire, and cellular miRNAs have been linked to basic biological activities [20]. With the help of these cellular miRNAs, it may be possible to precisely regulate the tissue-specific replication of several viruses with varying harmful profiles. For instance, transgene expression from lentiviral vectors has been successfully regulated based on tissue, lineage, and differentiation status using miRNA expression profiles [30]. The fact that viral replication is still robust in tissues in the absence of the targeted miRNA is another benefit of this strategy for vaccine manufacturing. This enables the activation of several immune system components to elicit a more comprehensive and durable protective response. Significantly, this suggests that this approach might also work with negative-strand RNA viruses and DNA viruses [31]. We believe that this approach could open the door for the safe and effective production of vaccines to combat a variety of viral illnesses, both established and emergent, that have proven resistant to previous attempts at vaccine development. The potential for attenuated vaccinations to revert to a virulent phenotype is one worry of viral escape from RNAi repression [32-36]. According to these publications, RNAi escape mutants were produced by whole deletions or single mismatches within the targeted region. Multiple complementary miRNA target sequences were inserted into the viral genome to stop it from escaping. Let-7a RNA escape mutants were not detected in the studies we conducted to look into PV-L7 viral replication. However, partial deletions of both miR-124a target regions were found in the virus that was recovered from the spinal cord of mice infected with the PV-124 virus. The cellular miRNA/viral target hybrid is disrupted by these alterations, but the mice did not exhibit any signs of the disease. PV-124 translation was probably suppressed as a result of the residual partial complementarity to miR-124a. Compared to animals immunized with PV-L7, mice immunized with PV-124 exhibit a greater neutralizing antibody response and total protection against lethal challenge. On the other hand, a decline in safety is correlated with the increased efficacy of PV-124. These findings clearly show that while PV-L7 is a safer vaccination strain than PV-124, the latter is just as effective as the Sabin vaccine that is now in use. Additionally, viruses might be modified to include a variety of miRNA target sequences to improve cell selectivity, reduce the possibility of cytopathic side effects, and restrict virus escape from replication-silencing activities. By adding polymerase fidelity into viruses that carry several miRNA target sequences, a logical combinational strategy might be employed to further mitigate potential issues related to the viral escape. Because of this, our strategy might be useful in conjunction with a recently published vaccine design technique that makes use of the finding that pathogenicity is reduced when replication fidelity is increased. When combined, these tactics could successfully boost genetic stability without sacrificing replication robustness and give these virus vaccines more protection, enhancing existing vaccinations. For many viruses, however, obtaining high-fidelity versions could not be simple. As a result, a novel miRNA-based strategy offers a more adaptable, all-encompassing substitute for logically developing attenuated live vaccines, which would enable the quick manufacture of vaccines in the event of an emerging viral threat.

Conflict of Interest

The authors have no conflict of Interest to declare. All authors have contributed equally.

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