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Application of recombinant adenoviruses expressing glycoprotein or nucleoprotein of rabies virus to Korean raccoon dogs

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Purpose: A new rabies vaccine for animals, including raccoon dogs, in Korea is needed to eradicate rabies infection. In this study, we constructed two recombinant adenoviruses expressing the glycoprotein or nucleoprotein of the rabies virus (RABV). We then investigated the safety and immunogenicity of these strains in raccoon dogs, depending on inoculation route.

Materials and Methods: Recombinant adenoviruses expressing the glycoprotein (Ad-0910G) or nucleoprotein (Ad-0910N) of rabies were constructed in 293A cells using an adenoviral system. One-year-old raccoon dogs underwent intramuscular (IM) inoculation or oral administration of the recombinant Ad-0910G and Ad-0910N. Clinical symptoms were observed and virus-neutralizing antibodies (VNA) against RABV were measured at 0, 2, 4, and 6 weeks after the immunization. Raccoons were considered positive if VNA titers were ≥ 0.1 IU/mL.

Results: Raccoon dogs inoculated with the combined Ad-0910G and Ad-0910N virus via the IM route did not exhibit any clinical sign of rabies during the observation period. All raccoon dogs (n = 7) immunized IM had high VNA titers, ranging from 0.17 to 41.6 IU/mL at 2 weeks after inoculation, but 70% (7/10) of raccoon dogs administered viruses via the oral route responded by 6 weeks after administration against RABV.

Conclusion: Raccoon dogs inoculated with Ad-0910G and Ad-0910N viruses showed no adverse effects. Immunization with the combined Ad-0910G and Ad-0910N strains may play an important role in inducing VNA against RABV in raccoon dogs.

Keywords: Adenovirus type 5, Rabies virus, Raccoon dogs

Introduction

Rabies is one of the most severe zoonotic diseases, caused by infection with the rabies virus (RABV) of the genus Lyssavirus (family *Rhabdoviridae*, order *Monogegavirales*). Until recently, wild animals, such as raccoon dogs (*Nyctereutes procynoide koresis*) and badgers (*Meles meles*), have been the rabies reservoir in South Korea and especially raccoon dogs have played a key role in transmitting rabies since 1993. Thus, raccoon dogs have become the main target animal for controlling rabies [1-3]. As the disease spread into southern regions of Gyeonggi province in 2013, the Korean Veterinary Authority strengthened preventative measures against raccoon dogs [4].

Oral rabies vaccination (ORV), one of the rabies elimination programs, is a socially acceptable disease control method for wildlife reservoirs and oral bait vaccine can be distributed over wide areas in a limited period of time [5-7]. ORV requires a safe and effective vaccine strain that is proven via several routes and a vaccine delivery package

suitable for the target species. In Gangwon and Gyeonggi provinces in South Korea, animal rabies cases in the raccoon dog population have been reduced through large-scale ORV using a vaccinia-rabies virus glycoprotein recombinant virus (V-RG) that expresses the glycoprotein of the Evelyn-Rokitnicki-Abelseth (ERA) strain. Similar results have been obtained from European countries with attenuated or modified strains of RABV (SADBern, SADB19, SAG1, and SAG2) [8,9]. Although recombinant and live-attenuated vaccines have proven successful for fox rabies control, further safe rabies vaccines are required because of residual pathogenicity in a variety of species, including humans [10,11]. An alternative ORV tactic involves the use of recombinant vaccines constructed from virus vectors that express the RABV glycoprotein and nucleoprotein. It has been reported that these recombinant vaccines provide improved safety and they have been found to be effective in animals [12]. In many countries, the V-RG has proven effective via the oral route in raccoons and red foxes, but efforts to immunize raccoon dogs and skunks with V-RG were less successful [13].

Recombinant vaccines using adenoviruses as vectors have also been considered and ONRAB, prepared in Canada, is one of the recombinant oral rabies vaccines that use a human adenovirus vector to express the RABV glycoprotein [14,15]. The adenovirus vector system has an advantage in that the recombinant vector can create a replication-incompetent adenovirus that can be used to deliver and transiently express RABV gene in dividing or non-dividing mammalian cells.

In this study, we investigated the safety and immunogenicity of recombinant adenoviruses expressing the glycoprotein and nucleoprotein of RABV constructed using the ViraPower Adenoviral System (Invitrogen, Carlsbad, CA, USA) in Korean raccoon dogs after oral administration or intramuscular injection.

Materials and Methods

Cells and viruses

The 293A cells were maintained in Dulbecco's modified Eagle medium (DMEM) with 2 mM L-glutamine, 0.1 mM non-essential amino acids, 10% heat inactivated fetal bovine serum (FBS), and antibiotics (100 IU/mL penicillin, 10 μg/mL streptomycin, and 0.25 μg/mL amphotericin B). The 293A cells were used for propagating recombinant adenoviruses expressing the glycoprotein or nucleoprotein of RABV in DMEM supplemented with 5% FBS and grown at 37°C in a 5% CO_2 incubator. The CVS-11 strain was propagated in BHK-21 cells and used for the virus neutralizing antibody (VNA) test.

Construction of the recombinant adenoviruses (Ad-0910G and Ad-0910N)

Recombinant human adenoviruses expressing the complete G and N proteins of KRVB0910 strain were constructed using the ViraPower Adenoviral System (Thermo Fisher Scientific Inc., Waltham, MA, USA) according to the manufacturer's protocol. Briefly, the amplified reverse transcription polymerase chain reaction products were cloned into the entry vector of the adenoviral expression system to construct the recombinant entry plasmids, pENTR-G, and pENTR N. Then, each adenoviral expression clone was generated by performing the attL×attR recombination reaction mediated by Gateway LR Clonase enzyme mix (Life Technologies, Carlsbad, CA, USA) between the entry plasmid and the replication-deficient, E1 deleted, human adenovirus (Ad) type 5, pAd-DEST vector. Two generated adenoviral expression clones (pAd-G and pAd-N) were digested with *Pac*I, and then transfected into 293A cells. After cytopathic effect (CPE) appearance, cell cultures were harvested and the two recombinant adenoviruses were propagated in 293A cells. Single recombinant virus was purified by isolation from individual viral plaques on 293A cells. After the third passage of constructed recombinant adenoviruses, the two viruses, named Ad-0910G and Ad-0910N, were deposited with the Korea Veterinary Culture Collection (accession Nos. KVCC-VR1500041 and KVCC-VR1500042). Titration of both Ad-0910G and Ad-0910N was conducted in 96-well plates with 10-fold serial dilutions. The viral titers with CPE were calculated according to the Reed and Muench method and expressed by 50% tissue culture infectious dose per mL ($TCID_{50}$/mL).

Identification of Ad-0910G and Ad-0910N by indirect fluorescent antibody (IFA) test

The recombinant Ad-0910G and Ad-0910N strains were propagated in 293A cells. After removing supernatants, the cells were fixed with 80% chilled acetone for 15 minutes. For staining, the cells were reacted with specific monoclonal antibodies against the glycoprotein or nucleoprotein (QIA, Anyang, Korea) of RABV for 45 minutes, and then stained with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG+IgM. After washing in phosphate-buffered saline (PBS), the specific fluorescence in the infected cells was visualized using a fluorescence microscope (Nikon, Tokyo, Japan).

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Safety and immunogenicity of recombinant adenoviruses in raccoon dogs

The experimental design was submitted to Laboratory Animal Ethics Committee (QIA, Korea) and was approved by the committee (QIA2013-668). The animal experiment was conducted in the wildlife rescue center of the College of Veterinary Medicine, Kangwon National University. One-year-old raccoon dogs, seronegative against RABV, were divided into three groups. Group 1, consisting of 10 Korean raccoon dogs, was inoculated with the recombinant adenoviruses (Ad-0910G and Ad-0910N) combined at a 3:1 ratio (1 mL, $10^{8.0}$ $TCID_{50}$/mL) via an intramuscular route. Second, inoculation with the same dose was performed 2 weeks after the first vaccination. In group 2, Korean raccoon dogs were administered 2 mL of recombinant mixture via the oral route. A second administration with the same dose was carried out 2 weeks after the first immunization. Four raccoon dogs in the control group received no treatment. In the experimental raccoon dogs, special attention was paid to any change in animal behavior. All raccoon dogs were monitored daily for adverse effects, such as anorexia, prostration, anxiety, agitation, aggression, and paralysis. Following inoculation or administration, at 0, 2, 4, and 6 weeks, blood was collected from all raccoon dogs to measure neutralizing antibodies against RABV.

Serological assay

The VNA titer against RABV was determined by a fluorescent antibody virus neutralization test [16]. Briefly, a positive reference serum from World Health Organization, adjusted to 0.5 IU/mL, was used as a positive control. Each serum sample and the positive and negative controls were distributed in four consecutive wells, and then serially diluted three-fold. The RABV (CVS-11 strain) containing around 100 $FAID_{50}$/50 μL was then added to each well. After incubation at 37°C for 60 minutes, 50 μL of BHK-21 cell suspension containing 4×10^5 cells/mL was added to each well and the microplates were incubated for 72 hours in a humidified incubator with 5% CO_2 at 37°C. The cells were fixed in cold acetone (–20°C) for 20 minutes. After three successive washings with PBS (pH 7.2), the plates were reacted with a specific monoclonal antibody against rabies for 45 minutes at 37°C, and then stained with FITC-conjugated goat-anti mouse IgG+IgM. After rinsing with PBS, the microplates were air-dried and examined at ×200 using a fluorescence microscope (Nikon). The titers of serum samples were expressed in international units per milliliter (IU/mL) by comparing results obtained with those of the positive standard. Raccoon dogs were considered positive if VNA titers were ≥0.1 IU/mL.

Results

Construction of recombinant RABV

The complete genes encoding glycoprotein and nucleoprotein were cloned into the entry vector, pENTR/D-TOPO. After performing the LR recombination reaction using the entry clone containing the rabies gene and pAd/CMV/V5-DEST vector, each recombinant clone containing the glycoprotein or nucleoprotein gene was transfected into 293A cells. Two recombinant adenoviruses, designated as Ad-0910G and Ad 0910N, were constructed and grown in 293A cells. CPE, characterized by rounding and detachment, was detected after a 5-day incubation period in 293A cells inoculated with the Ad-0910G or Ad-0910N strain. Each 293A cell inoculated with the Ad-0910G or Ad-0910N strain was fixed with cold acetone and reacted with specific monoclonal antibodies against the glycoprotein or nucleoprotein of RABV. As shown in Fig. 1, RABV-specific fluorescence appeared in the infected cells. The Ad-0910G and Ad-0910N strains propagated well in 293A cells and exhibited a titer of $>10^{8.0}$ $TCID_{50}$/mL.

Safety and immunogenicity of the Ad-0910G and Ad-0910N viruses in raccoon dogs

The raccoon dogs exhibited no clinical sign of rabies during the experiment whether the viruses were administered orally or inoculated via the intramuscular route. As shown in Table 1, all raccoon dogs in group 1 (n=10) administered the combined Ad-0910G and Ad-0910N viruses orally developed very low rabies VNA titers, ranging from 0.07 to 0.5 IU/mL (geometric mean, 0.16 IU/mL) at 2 weeks after oral administration and had VNA titer of geometric mean 0.18 IU/mL at 6 weeks post-administration. Seven of 10 raccoon dogs responded after the second dose. All raccoon dogs in group 2 (n=7) inoculated with 1 mL of Ad-0910G and Ad-0910N viruses showed high rabies VNA titers, ranging from 0.17 to 41.5 IU/mL (geometric mean, 24.6 IU/mL) at 2 weeks after inoculation and moderate rabies VNA titer, ranging from 0.5 to 13.7 IU/mL (geometric mean, 3.6 IU/mL) at 6 weeks post-inoculation. The four raccoon dogs in group 3 (n=4) remained seronegative against RABV throughout the experiment, confirming that no contact transmission had occurred between vaccinated and control raccoon dogs.

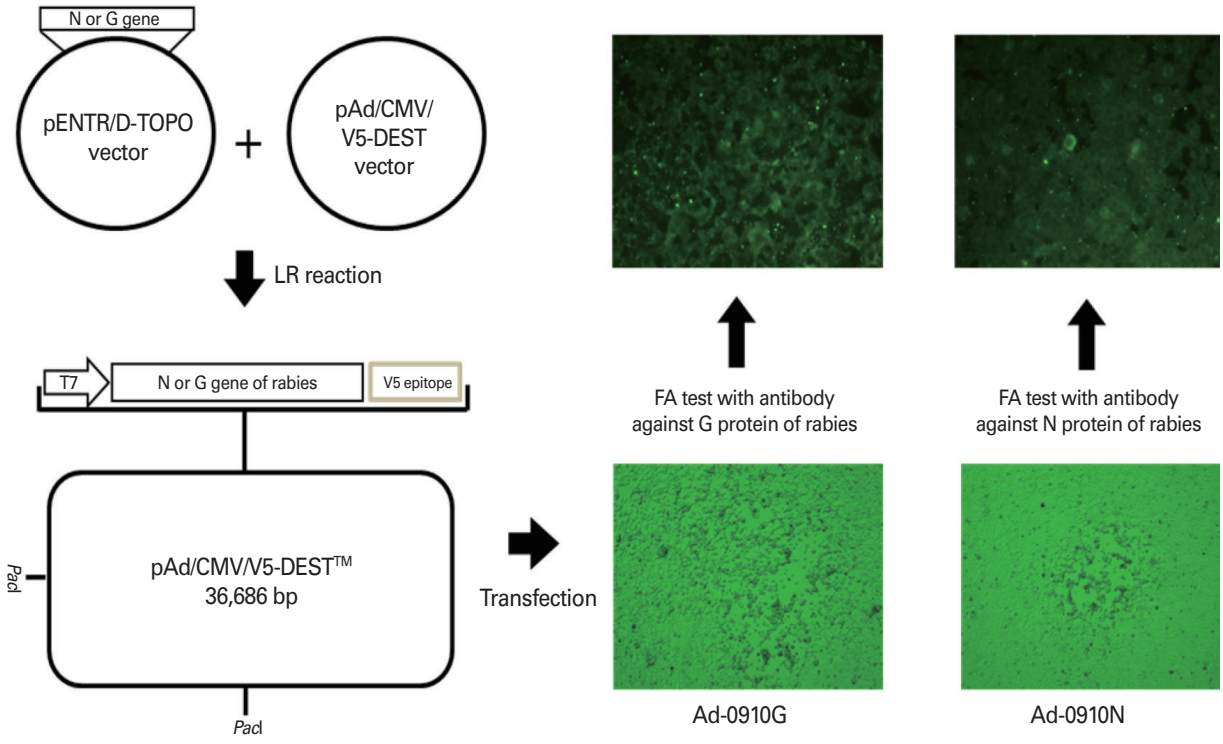

Fig. 1. The recombinant adenoviruses expressing glycoprotein or nucleoprotein of rabies virus were constructed by the ViraPower Adenoviral System. Each adenoviral expression clone was generated by performing an attL × attR (LR) recombination reaction between the entry plasmid and replication-deficient, E1-deleted, human adenovirus (Ad) type 5, pAd-DEST vector. Two generated-adenoviral expression clones (pAd-G and pAd-N) were digested with *Pac*I, and then transfected into 293A cells. The viral titer of Ad-0910G and Ad-0910N strain propagated in 293A cells reached $10^{8.0}$ $TCID_{50}$/mL. After fixing the Ad-0910G or Ad-0910N infected cells with cold acetone, fluorescent assay (FA) was conducted with monoclonal antibodies against rabies virus.

Discussion

In South Korea, approximately 500 people are bitten annually by various animal species, such as dogs, cat, cattle, raccoon dogs, wild animals, and bats. Of those bitten, the majority receive rabies post-exposure prophylaxis (PEP) [17]. Also, 106 Koreans who visited foreign countries between July 2006 and December 2012 received rabies PEP [18]. As most human rabies is caused by being bitten by rabid animals, a rabies vaccine for animals is considered a major preventative measure in countries with a high incidence of rabies. Rabies in humans and animals has occurred in most Asian countries, with the exceptions of Brunei, Hong Kong, Japan, Malaysia, and Singapore. Many people living in Asia are at high risk of exposure to rabid animals [19]. Thus, each country in Asia has set up control measures, including mass vaccination of pets and domestic animals, and has carried out elimination strategies against animal rabies. Despite these efforts, it seems there are limits to individual mass vaccinations of animals.

The glycoprotein of RABV is critical for the induction of VNA and is related to protection of animals against challenge with virulent RABV [20]. The nucleoprotein is the major component of the viral ribonucleoprotein complex, and can induce protective immunity [21]. In this study, glycoprotein and nucleoprotein genes of RABV isolated from rabid Korean cattle in 2009 were prepared. The amplified G or N genes of KRVB0910 strain were cloned into the entry vectors of an adenoviral expression system to construct the recombinant entry plasmids, pENTR-G, and pENTR-N. After transfection, two recombinant adenovirus strains, Ad-0910G and Ad-0910N, were rescued in 293A cells. The Ad-0910G or Ad-0910N viruses induced CPE in 293A cells at 5 days after inoculation and were confirmed by IFA test using specific monoclonal antibodies, indicating that the Ad-0910G and Ad-0910N viruses (patent No. 10-1479668) could propagate well in 293A cells.

The adenoviral expression system is designed to allow high-level, transient expression of recombinant fusion proteins in 293A cells. Advantages of adenoviral systems include that they allow the generation of high-titer recombinant adenovirus, delivering the fusion gene to actively dividing cells, and

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Table 1. The VNA titer of raccoon dogs administrated or inoculated with recombinant adenoviruses (Ad-0910G, Ad-0910N) via oral or IM route

No. of raccoon dogs	Dose	Inoculation route	Titer of VNA (wk)			
			0	2	4	6
1	$10^{8.0}$	Oral	0.07	0.07	0.07	0.07
2	$TCID_{50}$/mL		0.07	0.17	0.07	0.07
3	(2 mL)		0.07	0.17	0.17	0.07
4			0.07	0.1	0.17	0.17
5			0.07	0.50	0.10	0.10
6			0.07	0.17	1.51	0.17
7			0.07	0.07	0.17	0.5
8			0.07	0.07	0.17	0.17
9			0.07	0.07	0.07	0.17
10			0.07	0.17	0.17	0.29
Mean			0.07	0.16	0.27	0.18
1	$10^{8.0}$	IM	0.07	125.00	41.50	13.70
2	$TCID_{50}$/mL		0.07	41.50	13.70	4.50
3	(1 mL)		0.07	13.70	1.50	1.50
4			0.07	72.20	7.90	2.60
5			0.07	1.50	4.50	1.50
6			0.07	0.17	0.17	0.50
7			0.07	0.50	0.50	0.87
Mean			0.07	36.37	9.97	3.60
1	-	Control	0.07	0.07	0.07	0.07
2			0.07	0.07	0.07	0.07
3			0.07	0.07	0.07	0.07
4			0.07	0.07	0.07	0.07
Mean		-	0.07	0.07	0.07	0.07

VNA, virus-neutralizing antibodies; IM, intramuscular; $TCID_{50}$, 50% tissue culture infectious dose.

allowing production of a replication-incompetent virus that enhances the biosafety of the system [22,23]. The Ad-0910G and Ad-0910N strains propagated in 293A cells and exhibited a titer of $10^{8.0}$ $TCID_{50}$/mL, demonstrating that the adenoviral expression system facilitates propagation of high levels of the recombinant Ad-0910G and Ad-0910N strains.

To date, many recombinant adenoviruses expressing proteins of infectious agents have proven effective as vaccines [15,24]. It was reported that an E1-deleted recombinant adenovirus expressing the RABV glycoprotein induced an immune response against RABV in mice [25]. A recombinant adenovirus-vector vaccine, ONRAB, expressing the RABV glycoprotein originating from the ERA strain was developed and distributed in Canada for controlling rabies in raccoons [14].

It is critical to evaluate the safety and immunogenicity of any ORV candidate in the major target animals of RABV; raccoon dogs are responsible for transmitting RABV in Korea. In our study, we demonstrated that Korean raccoon dogs administered Ad-0910G and Ad-0910N viruses containing $10^{8.0}$ $TCID_{50}$/mL twice orally or intramuscular (IM) were safe for 6 weeks, displaying no clinical signs. All raccoon dogs inoculated with combined Ad-0910G and Ad-0910N viruses IM had high VNA titers, ranging from 0.5 to 13.7 IU/mL at 6 weeks post-inoculation. Thus, it was assumed that raccoon dogs inoculated with combined Ad-0910G and Ad-0910N viruses IM have protection against challenge with virulent RABV, which provides a basis for use of a live RABV strain. However, oral administration did not induce high VNA titers in raccoon dogs versus intramuscular inoculation. Brown et al. [14] reported that ONRAB bait vaccine containing a viral titer of 10^{10} $TCID_{50}$/mL has proven effective in several animal species.

Our study revealed that 70% (7/10) of raccoon dogs receiving Ad-0910G and Ad-0910N viruses responded to the administration of recombinant adenoviruses and had low levels of VNA within 6 weeks. From these results, it seems that the Ad-0910G and Ad-0910N virus titers were low compared with the ONRAB vaccine [26] and the administration of the combined adenoviruses was not ideal. Additionally, adenovirus preparations with low viscosity might be spilled from the oral cavity. Although our study did not show complete immune responses in raccoon dogs administered the vaccine via the oral route, those inoculated with Ad-0910G and Ad-0910N viruses IM showed better responses.

In conclusion, intramuscular immunization with the Ad-0910G and Ad-0910N viruses in raccoon dogs was safe and induced high neutralizing antibody titers. These results, together with safety and immunogenicity in raccoon dogs, make the combined Ad-0910G and Ad-0910N strains an alternative to the attenuated rabies vaccine used previously for animal rabies control. Preparation and application of the concentrated Ad-0910G strain, which exhibited a viral titer over 10^{10} $TCID_{50}$/mL, to raccoon dogs will result in high VNA titers. Additionally, further study concerning the effectiveness of ORV in accordance with the National Standard Assay for Veterinary Biologic Products in dogs and raccoon dogs is needed.

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