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Original article

Liposome mediated double-stranded RNA delivery to silence ribosomal protein P0 in the tick *Rhipicephalus haemaphysaloides*

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ABSTRACT

Control of ticks has been achieved primarily by the application of acaricides, which has drawbacks such as environmental contamination leading to the selection of pesticide-resistant ticks. The potential of dsRNA to suppress genes critical for tick survival due to its sequence specificity suggests that dsRNAs could be developed as tailor-made pesticides. In this study, the dsRNA of P0 gene from the tick, *Rhipicephalus haemaphysaloides*, was evaluated as a potential anti-tick agent. Effects of using different dsRNA delivery methods were tested by quantitative RT-PCR and tick bioassays to determine survival, feeding and reproduction. The results showed that P0 dsRNAs could be effectively delivered into ticks and silenced by incubating with liposomes. Incubation time was found to be the most important factor in dsRNA delivery and gene silencing compared with liposome types and dsRNA concentration. The effects of P0 dsRNA treatment on ticks were found to be significant on blood feeding, molting or reproduction. These data show that anti-tick agents based on dsRNAs could have potential use in tick control.

1. Introduction

Ticks are obligate hematophagous ectoparasites of wild and domestic animals as well as humans. They are considered to be second only to mosquitoes as global vectors of human diseases (de la Fuente et al., 2008; Jongejan and Uilenberg, 2004). Control of ticks has been achieved primarily by the application of acaricides, a method that has drawbacks such as environmental contamination and selection of pesticide-resistant ticks. These issues reinforce the need for alternative approaches to control tick infestations (de la Fuente and Kocan, 2006). RNA interference (RNAi), which is the sequence-specific degradation of mRNA mediated by homologous double-stranded (dsRNA), has become a valuable tool in gene knockdown in eukaryotes (Fire et al., 1998; Yu et al., 2013a). The potential of dsRNA to suppress genes critical for insect survival due to its sequence specificity suggests that dsRNAs could be developed as tailor-made pesticides (Huvenne and Smagghe, 2010; Zhang et al., 2013).

dsRNA-mediated gene silencing has been extensively used for the analysis of gene functions in ticks. Particularly, long dsRNAs have been routinely applied successfully in many tick species for targeted gene knockdown in various stages of tick lifecycle, with evidence of systemic RNAi spread into subsequent stages (de la Fuente et al., 2007; Karim and Adamson, 2012). The main goal of this method is to study tick physiology and to discover new targets to control. Notably, sterile ticks were created by knocking down a molecule named subolesin using RNAi. Consequently, the release of subolesin-silenced ticks was proposed as a sterile acarine technique (SAT) for autocidal control of tick populations (de la Fuente et al., 2006). The efficacy of SAT with dsRNA has been demonstrated to control tick infestations alone or in combination with subolesin vaccination, although some limitations have to be considered when large numbers of subolesin-knockdown ticks will be released into the environment (Merino et al., 2011).

The application of RNAi-triggering molecules as therapeutic or control agents has been progressing in mammals and insects, although in ticks progress in this subject has been slow. There are many limitations in using RNAi-based technologies for tick control; selection of the target gene and reliable double-strand RNA (dsRNA) delivery are the two major challenges. For example, micro-injection, electroporation, and artificial capillary feeding, which are methods to introduce dsRNA in ticks, can only be successfully used in laboratory experiments (Karim et al., 2010; Karim and Adamson, 2012; Ruiz et al., 2015). Soaking tick tissues or cells in dsRNA has been reported (de la Fuente et al., 2007). A recent report indicated the effectiveness of immersing whole ticks in dsRNA to induce gene silencing (Galay et al., 2016). Although naked

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dsRNA can be internalized at high concentration in sterile conditions in the laboratory, dsRNA in field settings could be subject to rapid environmental degradation (Dubelman et al., 2014; Fischer et al., 2017). To prevent RNAi trigger degradation, abiotic and biotic delivery systems including the use of liposomes, chitosan nanoparticles, *E. coli* expression systems, and *Pichia pastoris* expression systems have been explored in insects (Whyard et al., 2015; Van Ekert et al., 2014, Zhang et al., 2010; Bedoya-Perez et al., 2013). However, there are no such reports for tick dsRNA delivery systems.

A previous study suggested that gene silencing of the ribosomal protein, P0, was lethal to the tick, *Haemaphysalis longicornis* (Gong et al., 2008). Administering anti-P0 protein in *Rhipicephalus* spp. ticks resulted in high mortality and poor egg hatch rate (Rodríguez-Mallon et al., 2012). These results indicated that the P0 gene could be used as the key target for killing ticks and decreasing reproduction.

This study focused on investigating the best method to deliver P0 dsRNAs *via* the surface absorption using different liposomes under different conditions in order to develop a novel anti-tick biological agent.

2. Materials and methods

2.1. Tick collection and maintenance

A colony of *R. haemaphysaloides* ticks was initiated from one engorged female collected from a buffalo in Hubei, China (Zhou et al., 2006). After three generations of rearing under laboratory conditions, the tick colony was established in the Shanghai Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Shanghai, China. Ticks were reared in a dark incubator at 25 °C with 92% relative humidity and fed on a New Zealand white rabbit. Larvae, nymphs and adult ticks were collected for experimental use.

2.2. Cloning and sequence analysis of R. haemaphysaloides P0 gene

An expressed sequence tag (EST) of the *R. haemaphysaloides* P0 gene was found in the salivary gland cDNA library previously constructed in our laboratory (Yu et al., 2015). To clone the full-length cDNA of the *R. haemaphysaloides* P0 gene, 3′ and 5′ RACE was used according to the manufacturer's instructions (Invitrogen, Life Technologies Corporation, USA). Amplified PCR fragments were cloned into the pEGM-T vector, and the nucleotide sequences of the positive clones were sequenced. The obtained full-length cDNA sequence of ribosomal P0 was then analyzed.

2.3. Preparation of dsRNAs

The P0 cDNA in the pGEM-T vector was amplified using PCR primers 5′-GGATCCTAATACGACTCACTATAGGCTTCAACTTCCTTTCATACGG-3′ and 5′-GGATCCTAATACGACTCACTATAGGCACCCTCAACGAATGCTG-3′ containing the T7 promoter sequence at their 5′ ends. In addition, the cDNA fragment encoding bacterial luciferase (Yu et al., 2013b) was also amplified using the primers 5′-GGATCCTAATACGACTCACTATAGGGCTTCCATCTTCCAGGGATACG-3′ and 5′-GGATCCTAATACGACTCACTATAGGCGTCCACAAACACAACTCCTCC-3′ containing the T7 promoter sequence at their 5′-ends. Luciferace dsRNA was used as the negative control. The PCR products were gel-purified and RNA was synthesized by *in vitro* transcription using the T7 RiboMAXTM Express RNAi System (Promega, USA). Then, dsRNAs were quantified by spectrometry at 260 nm, and their integrity was checked on 1% agarose gels, and stored at −20 °C until further use.

2.4. Real-time PCR assay for gene silence

Total RNA was extracted from *R. haemaphysaloides* using the RNeasy Mini Kit (QIAGEN, Germany) following the manufacturer's instructions. cDNA was synthesized from the total RNA using the Takara RNA PCR Kit (AMV) Ver. 3.0 (Takara Bio Inc. Japan). SYBR green real-time PCR amplifications were performed using 0.1 μg of cDNA and oligonucleotide primers specific for P0 in a final volume of 20 μl. Transcript abundance was measured using a Light Cycler 1.5 (Roche Instrument Center AG, Roikreuz, Switzerland) according to the manufacturer's instructions. The tick elongation factor 1α gene was used as an internal control (Nijhof et al., 2007). The $2^{-\triangle\triangle CT}$ method was used to calculate the relative expression levels of P0. Each analysis was repeated three times. The primers used for real-time PCR are as follows: 5′-TTCTCGTATGGTCTGAAGATTTT-3′ and 5′-AACAGTTGGGTATCCGATGG -3′ for the P0 gene, and 5′-CGTCTACAAGATTGGTGGCATT-3′ and 5′-CTCAGTGGTCAGGTTGGCAG-3′ for the elongation factor-1α gene.

2.5. Testing P0 gene silencing efficiency

Three kinds of lipid agents, Lipofectamine 2000, DMRIE-C and Cellfectin (Invitrogen, Life Technologies Corporation, USA) were tested in this study. Based on the concentrations suggested in the manufacturer's instructions for DNA and RNA transfections, we used 1 μg P0 dsRNA/μl liposome solution (the proportion of water and liposome was 1:1) as the test dsRNA concentration and soaked for 24 h. For the control group, 1 μg P0 dsRNA/μl RNase free water was used. To calculate the rate of relative expression, ticks soaked in water without P0 dsRNA were used as the control. Larvae, nymphs and adult ticks were all tested. In soaking tests, ~100 larvae, 50 nymphs, or 20 adults were placed into 2 ml tubes, to which 1 ml dsRNA with liposome or control dsRNA was added. Ticks were soaked by setting the tube on a vertical rotating device at room temperature for 24 h. Then, ticks were rinsed in distilled water and dried on paper towels. Afterward they rested for 5 d before RNA was extracted for real-time PCR analysis. Relative expression of the P0 gene against the tick elongation factor-1 was calculated using the $2^{-\triangle\triangle CT}$ method, and then the rate of relative mRNA expression against the control (ticks soaked in water without P0 dsRNA as 100% relative expression) was calculated for final results. Each test was repeated three times.

2.6. Observation of Cy3-labeled P0 dsRNA in R. haemaphysaloides after soaking

To observe the delivery of dsRNA into the tick body, the P0 dsRNA was labeled using the CyTM3 Silencer siRNA labeling kit (Ambion Thermo Fisher Scientific Inc, USA) following the manufacturer's protocol. Lipofectamine 2000 was used in this study, and larvae and nymphs were tested. P0 Cy3-labeled dsRNA was soaked in Lipofectamine 2000 (1 μg/μl) for 24 h. Then, the ticks were washed three times in 1 × PBS in a dark room and observed through an inverted fluorescence microscope. P0 Cy3-labeled dsRNA in RNase free water (1 μg/μl) was used as the control.

2.7. Effect of soaking on different time periods

Lipofectamine 2000 was used with larval, nymphal and adult ticks. P0 dsRNA was soaked in Lipofectamine 2000 (1 μg/μl) for 3, 6, 12 and 24 h. Then, ticks were washed and rested for 5 d before RNA was extracted for real-time PCR analysis. Unsoaked ticks were used as the control. Each test was repeated three times.