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Tick Longicin Implicated in the Arthropod Transmission of *Toxoplasma gondii*

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Abstract

Antimicrobial peptides are major components of host innate immunity, a well-conserved evolutionarily ancient defensive mechanism. Infectious disease-bearing vector ticks are thought to have evolved to produce specific defense peptides implicated in controlling the infection and transmission of various pathogens. Longicin, a defensin peptide identified from the hard tick, *Haemaphysalis longicornis*, is known to have a significant deadly effect against both Gram-negative and Gram-positive bacteria and other microorganisms. In this study, female *H. longicornis* ticks were experimentally injected with *Toxoplasma gondii* tachyzoite parasites, and the transcription profiles of *longicin* in ticks demonstrating the amplification of *T. gondii B-1* gene fragments were examined to determine whether and how ticks may respond immunologically in controlling *T. gondii* infections. As a result, 10 days after parasite injection, ticks indicated the upregulation of the *longicin* gene, consistently with the presence of *T. gondii*. The effects of recombinant longicin on the morphology of *T. gondii* tachyzoites were also examined *in vitro*. Tachyzoite parasites incubated with recombinant longicin induced pathological changes in cell morphology followed by a marked reduction in the number of parasites. These findings suggested that recombinant longicin could impair parasite membranes, leading to the destruction of *Toxoplasma* parasites.

Keywords: *Haemaphysalis longicornis*; Longicin; Longicin P4 peptide; Parasiticidal activity; *Toxoplasma gondii*; Vector

Introduction

Ticks surpass all other ectoparasitic arthropods in the number of diseases they transmit to animals and humans [1]. The hard tick *Haemaphysalis* is also the primary vector of the pathogens causing babesiosis in humans and domestic animals in Japan [2]. Antimicrobial peptides are a major component of the innate immune system in host defense, which is a well-conserved and evolutionarily ancient mechanism [3]. Vector ticks were recently shown to possess specific gene products that mediate innate protective responses against transmitted pathogens. Previously, we identified longicin, a defensin peptide from the midgut of *H. longicornis* ticks, which shows a significant killing effect on tick-borne protozoan *Babesia* parasites *in vivo* and *in vitro* [4,5]. Longicin consists of 74 amino acids, including a signal peptide of 22 residues, and is produced mainly in the midgut epithelial cells. Longicin and its 16-22 residue fragments (P1-P4) were chemically synthesized on the basis of their deduced amino acid sequences [4]. The synthetic peptides P1 (residues 23-37), P2 (33-45), and P3 (42-57) did not exert any bactericidal effects, but P4 (53-73) consistently exhibited remarkable dose-dependent killing activities against *Babesia* parasites and four bacteria species.

Toxoplasmosis is a widespread zoonotic disease caused by a parasitic protozoan, *Toxoplasma gondii*. It may pose a serious public health problem as a congenital infection causing cerebral and ocular damage in newborns and as an acquired infection in immunocompromised individuals, such as AIDS patients [6]. So far, oral transmission by consumption of raw meat, food, and, rarely, water contaminated with cat feces containing infective *T. gondii* oocysts is regarded as the only route of primary infection. However, this route hardly explains the common occurrence of *T. gondii* in a variety of hosts, such as herbivorous mammals, wild rodents, and birds, which are unlikely to have contracted the primary infection orally with meat or cat feces [7]. Thus, some other transmission routes of *T. gondii* have been considered, and previous studies have raised the possibility of transmission by blood-sucking arthropods, in particular, ticks [8-11]. Sroka et al. [11] recently detected *T. gondii* DNA in *Ixodes ricinus* ticks collected from the woodlands of northwestern Poland, suggesting that this tick species may be involved in the spread of toxoplasmosis under natural conditions. However, it is unclear whether and how ticks may respond immunologically in controlling *T. gondii* infections.

In the current study, we examined the amplification of *T. gondii B-1* gene fragments and the endogenous transcription profiles of the *longicin* gene in female *H. longicornis* ticks experimentally injected with *T. gondii* tachyzoite parasites; next, we explored whether or not the morphological changes might be induced in *T. gondii* tachyzoites incubated with recombinant longicin *in vitro*.

Materials and Methods

Ticks

Haemaphysalis longicornis (Okayama strain) was maintained by feeding on Japanese white rabbits (SPF), as described previously

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[12], in our laboratory. Rabbit care was approved by the Kagoshima University Animal Care and Use Committee.

Parasite and cells

Tachyzoites of the *T. gondii* RH strain was maintained by continuous passage through adherent monkey kidney fibroblasts (Vero cells) cultured in Eagle's Minimum Essential Medium (EMEM; Sigma Chemicals Co., St. Louis, MO, USA) containing 8% heat-inactivated fetal bovine serum (FBS). To purify tachyzoites, parasites and host cell debris were washed in saline solution, and the final pellet was resuspended in phosphate-buffered saline (PBS) before passage through a 27-gauge needle and 5.0 µm Nucleopore filters (Millipore Co., Billerica, MA, USA). The purified suspension was separated by centrifugation at 450 g for 10 min, and the supernatant was removed. The parasites suspended in PBS were counted using a hemocytometer and washed three times with PBS.

T. gondii injection

A suspension containing fifty *T. gondii* tachyzoites was injected (0.5 µl) into unfed adult female ticks, through the fourth coxae into the hemocoel, and the injected ticks were left for 10 days at 25°C in an incubator. DNA was extracted from PBS-injected ticks or *T. gondii* tachyzoite-injected whole ticks using an extraction buffer [100 mM Tris-HCl (pH8.0) containing 0.5% SDS, 100 mM NaCl, and 10 mM EDTA]. After that, the lysis solution was incubated with proteinase K (100 µg/ml), and phenol/chloroform extraction was carried out. Detection of *T. gondii* DNA based on the amplification of 35-fold-repetitive gene B1 fragments in two nested PCR reactions was performed using gene B1 fragment-specific primers (S1-F and S1-R of the first reaction and S2-F and S2-R of the second nested-PCR reaction, listed in Table 1) designed from *T. gondii B1 gene* fragments (GenBank accession no AF179871) with the method described by Grigg and Boothroyd [13]. Twenty microliters of the PCR amplification mixture consisted of 0.4 µl each primer (100 pmol/ml), 1 µl template DNA, 10 µl AmpliTaq GOLD PCR Master Mix (Applied Biosystems, Foster City, CA, USA), and 8.2 µl nuclease-free water. Thirty cycles were performed in a 2-stage reaction. Each of the cycles was carried out at 94°C for 30 s, 60°C for 30 s, and 72°C for 90 s. The reaction products of first-stage amplification (1 µl) were used for the second-stage PCR. Additionally, at each stage, initial denaturation (5 min at 94°C) and final elongation (5 min at 72°C) were performed. The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Determination of longicin gene expression in ticks after *T. gondii* injection

All ticks were surface-sterilized with gentle agitation in 70% ethanol for 10 min and then rinsed with PBS prior to processing. Ticks were homogenized using a Pellet Pestle Mortar (Kimble Kontes, NJ, USA) and stored at -80°C until use. To determine the gene expression profile of longicin, total RNA was extracted from the ticks after 10 days of *T. gondii* tachyzoite injection using the TRI reagent (Sigma, St. Louis, MO, USA). Single-strand cDNA was generated by reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland) according to the manufacturer's instructions. The reverse transcription reaction was carried out at 50°C for 30 min, and PCR was then conducted using longicin-specific primers (Longicin-F and Longicin-R, listed in Table 1) designed from the *H. longicornis longicin* gene (GenBank accession no. AB105544). The PCR conditions were 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min for 35 cycles. Control amplification was carried out using actin-specific primers (Actin-F and Actin-R, listed in Table 1) designed from the *H. longicornis β-actin* gene (GenBank accession no. AY254898). The PCR products were electrophoresed on a 1.5% agarose gel and stained with ethidium bromide.

Name	Sequence (5'-3')
S1-F	TGTTCTGTCCTATCGCAACG
S1- R	ACGGATGCAGTTCCTTTCTG
S2 -F	TCTTCCCAGACGTGGATTTC
S2-R	CTCGACAATACGCTGCTTGA
Longicin-F	CAAGATGACGAGAGT
Longicin-R	CTACTTGCGGTAGCAC
Longicin *Bam* HI-F	AC*GGATCC*CCAAGATGACGAGAGT
Longicin *Bam* HI-R	AC*GGATCC*CTACTTGCGGTAGCAC
Actin-F	CCAACAGGGAGAAGATGACG
Actin-R	ACAGGTCCTTACGGATGTCC

Restriction enzyme sites are shown in italics

Table 1: Gene-specific primers used for nested-PCR and RT-PCR.

Expression and purification of recombinant longicin in *Escherichia coli*

The coding of longicin cDNA was amplified by PCR using a plasmid as the template and a gene-specific forward primer (Longicin *Bam* HI-F, listed in Table 1) and a reverse primer (Longicin *Bam* HI-R, listed in Table 1) containing a recognition site for *Bam* HI. The PCR product and the pRSET-B vector (Invitrogen, Carlsbad, CA, USA) were digested by *Bam* HI, purified using a gel purification kit (GEANCLEAN II kit, MP Biomedicals, OH, USA), ligated, and transformed into DH5 α competent cells. The sequence and correct ORF of the sub cloned products were verified by DNA sequencing using T7 sequencing primers. For overexpression, the target clone was transformed into BL21. The overnight culture of the plasmid containing BL21 competent cells was inoculated into 500 ml of a fresh LB medium and incubated in a shaker until the OD_{600} reached 0.4-0.6. Then, isopropyl 1-thio- β -D-galactopyranoside (IPTG) was added to a final concentration of 1 mM, and the culture was incubated for another 4 h. Cells were collected by centrifugation at 1,500 g for 30 min and resuspended in PBS. After the cells were broken by sonication, the supernatant was collected by centrifugation at 20,000 g for 30 min at 4°C.

Purification was performed on the BioLogic DuoFlow Base system (BIO-RAD, Tokyo, Japan) using a HisTrap FF column (GE Healthcare UK, Ltd., Buckinghamshire, UK) according to the method of Tanaka et al. [14]. The purified recombinant longicin solution was concentrated by VIVASPIN 500 (Statorius Stedim, Gottingen, Germany) and stored at -30°C until use.

The analysis of proteins by electrophoresis onto 15% polyacrylamide gels was performed according to the Laemmli method [15].

Protein determination

The total protein concentration was determined by the bicinchoninic acid procedure (MicroBCA, Pierce, Rockford, USA) with bovine serum albumin as the standard.

Parasiticidal assay

Tachyzoite parasites (5×10^6/ml) were washed three times by centrifugation at 450 g for 10 min, resuspended in PBS, and then incubated with 50 µM longicin for 60 min at 37°C in a final volume of 1.0 ml of PBS. Recombinant lysozyme was also used as a control [14]. After centrifugation at 16,000 g for 5 min to remove the reaction buffer,

parasites were resuspended in PBS. Staining was immediately assessed by light microscopy to examine the morphological state of parasites, after which staining with Giemsa solution was conducted.

Results

Injection of *T. gondii* into *H. longicornis*

Five ticks were collected as a sample of Days 2, 4, 7, and 10 after *T. gondii* injection. PCR analysis revealed that *T. gondii B-1* gene fragments amplified in all five female ticks immediately after tachyzoite infection were not observed in ticks 2 and 4 days after injections (Figure 1, lanes 1,2). However, the weak but positive fragments of the *B-1* gene were detected in ticks 7 and 10 days after injections (Figure 1, lanes 3,4).

Transcription profiles of longicin gene in ticks after *T. gondii* injection

RT-PCR was used to examine the gene transcription profiles of *longicin* and *actin* in ticks on Days 2, 4, 7, and 10 after a single injection with *T. gondii* or PBS (Day 10; tick results shown in Figure 2A). The relative expression (%) of *longicin* mRNA against *actin* mRNA indicated that the endogenous *longicin* transcription was upregulated in ticks injected with *T. gondii* but not in those injected with PBS at 10 days after injection (Figure 2B). However, the gene transcription of longicin was not observed in ticks on Days 2, 4, and 7 after injection with *T. gondii* or PBS.

Anti-*Toxoplasma* activities of recombinant longicin

A recombinant fusion protein carrying a tag of six histidine residues was produced in *E. coli*. The cDNA fragment encoding longicin was amplified by PCR. The PCR product was inserted into a *Bam* HI site of the pRSET-B vector. Finally, the His_6-tagged protein was purified by affinity chromatography on a HisTrap FF column containing chelating Sepharose with nickel ions (Figure 3). We examined the putative anti-*Toxoplasma* activity of recombinant longicin. The pathological morphology and proliferation of *T. gondii* were examined after *in vitro* incubation with recombinant longicin for 60 min. Figure 4C shows that, after exposure to recombinant longicin, the cytoplasm and nuclei of most of the parasites were barely stained with Giemsa solution, and the parasites showed marked reduction in number. In contrast, more than 90% of the parasites incubated with PBS or recombinant lysozyme for 60 min were normally stained with Giemsa solution, and normal proliferation of the parasites was confirmed (Figure 4A and 4B).

Discussion

The detection of the *T. gondii B1* gene amplified in *H. longicornis* female ticks 10 days after injection with tachyzoites in this study suggests that ticks can harbor *T. gondii* parasites, suggesting that tick transmission can result in an epidemic spread of toxoplasmosis. In a few previous studies, it has been reported that *Toxoplasma* parasites were isolated from various tick species, which suggests the possibility of transmission of *T. gondii* infection by ticks [8]. Derylo et al. [9] microscopically observed the presence of *T. gondii* parasites in the tissues of nymphs and females of *I. ricinus* ticks and reported that *T. gondii* was experimentally transmitted through blood feeding by nymphs of *I. ricinus* to mice infected with *T. gondii*. In the current study, the gene transcription of *longicin* in *H. longicornis* female ticks was upregulated by experimental *T. gondii* injections, raising the possibility that endogenous longicin was induced as a defense factor for parasite infection control. Activation of insect immune genes is now

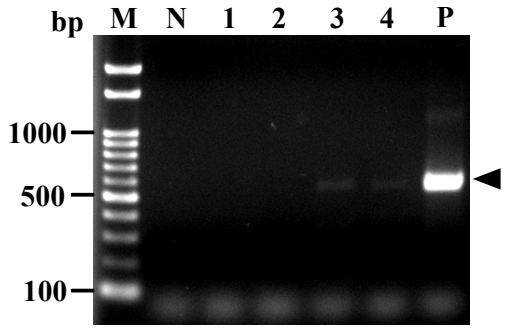

Figure 1: Detection of *T. gondii B-1* gene in adult females injected with *T. gondii* tachyzoites or a control PBS into hemocoel. 531-bp fragments of *T. gondii B-1*gene amplified by nested-PCR. The arrowhead indicates the position of the expected band. Lane M, molecular marker; lane N, negative control (ticks 10 days after PBS injection); lane 1, ticks 2 days after *T. gondii* injection; lane 2, ticks 4 days after *T. gondii* injection; lane 3, ticks 7 days after *T. gondii* injection; lane 4, ticks 10 days after *T. gondii* injection; lane P, positive control (*T. gondii* DNA sample).

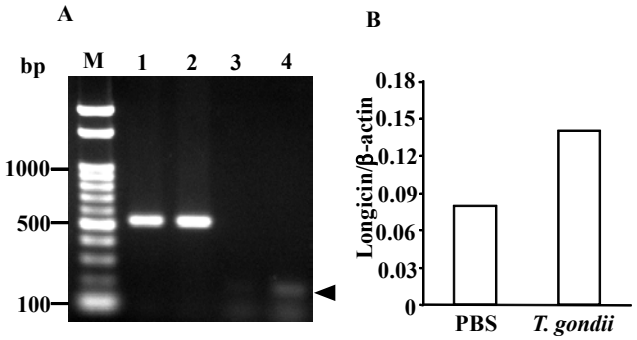

Figure 2: Upregulation of *longicin* gene transcripts in female ticks 10 days after injections with *T. gondii* tachyzoites. (A) Gene expression of *longicin* amplified by RT-PCR in ticks 10 days after *T. gondii* or PBS injection. Bands in lanes 1 and 2 indicate 540-bp fragments of the constitutively expressed *β-actin* of *H. longicornis*. A band in lane 4 (arrowhead) indicates a 159–bp fragment of *longicin*. Lane M, molecular markers; lanes 1 and 3, ticks injected with PBS; lanes 2 and 4, ticks injected with *T. gondii*. (B) The data are expressed as the ratio of the density of *longicin* to the density of *β-actin* products from the same template. This data is shown to represent data in 3 different experiments. Similar results were observed in 3 different experiments.

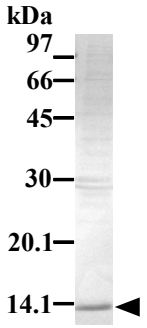

Figure 3: SDS-PAGE of recombinant longicin expressed in *E. coli*. Recombinant His-longicin purified using a nickel Sepharose column was electrophoresed on a 15% SDS-polyacrylamide gel and then stained with CBB.

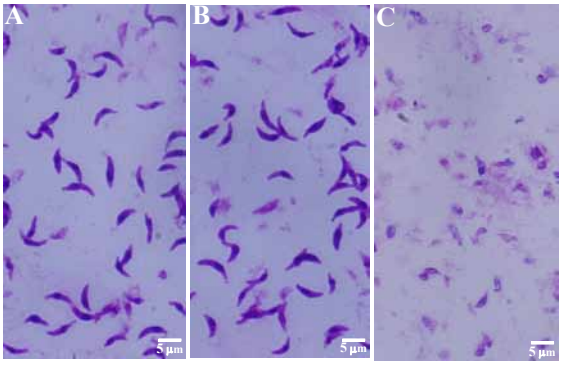

Figure 4: Giemsa staining of *Toxoplasma gondii*. Tachyzoite parasites (5.0×10^6) were exposed for 60 min at 37°C to PBS alone (A), 50 μM recombinant lysozyme (B), or 50 μM recombinant longicin (C) in 1 ml reaction volumes.

considered as a homologous response to vertebrate innate immunity since this process is mediated by phylogenetically conserved Toll/IMD pathways [16]. A regulatory pathway of tick immunity triggered by *T. gondii* appears to be involved in the gene expression of the tick defensin called longicin, although the detailed pathway is not yet clear.

Considerable evidence has indicated that the antimicrobial activities of defensins protect the host against a wide variety of bacteria, fungi, virus, and parasites as a part of the innate immune responses of humans, animals, arthropods, and plants [17]. Tick defensins (often having several isoforms) were so far identified in nearly twenty hard and soft tick species [18]. Defensins are known to exhibit a broad spectrum of antimicrobial activities against bacteria, including multidrug-resistant strains from humans and animals [4,5,17]. For anti-parasite activities, previous studies have demonstrated that human defensins can inhibit spore germination of different microsporidia species and contribute to the defense of the intestine against infection by luminal microsporida spores [19]. Moreover, it was reported that human defensins also display a parasiticidal role against *T. gondii* [20], *Trypanosoma cruzi* [21], and *Cryptosporidium parvum* [22].

The molecular mechanisms of defensin-mediated antimicrobial activities are still unclear. However, it is clear that defensins primarily target the negatively charged phospholipids of a cell membrane [23]. There is also evidence that defensins may have enhanced activity as oligomers [24]. Most defensins are amphipathic molecules that have clusters of positively charged amino acid side chains and amino acid hydrophobic side chains. The electrostatic attraction and transmembrane bioelectric field pull the peptide molecules towards and into the cell membrane [25]. As the peptide molecules accumulate in a carpet-like manner, the membrane is strained, and the peptides transition into another formation to lower the strain. However, this results in the formation of membrane pores [25].

Longicin and Longicin P4 peptide inhibited the growth and killed *Babesia* parasites [4,5] and *T. gondii* parasites by membrane disruption of the parasites [26]. Tachyzoites parasites incubated with longicin or P4 peptide had been induced the pathological changes of cell morphology followed by the marked reduction in number. Longicin P4 was located at the surface of *T. gondii* tachyzoites, as demonstrated by fluoresce microscopic analysis. An Electron microscopic analysis and a fluorescence propidium iodide exclusion assay of tachyzoites exposed to longicin P4 revealed pore formation in the cellular membrane, membrane disorganization, and hollowing as well as cytoplasmic vacuolization. These findings suggested that longicin P4 conceivably impaired parasite membranes, leading to the destruction of *Toxoplasma* parasites. Longicin has a well-defined β-sheet at the amino C terminus.

Longicin P4 is composed of 21 amino acids with the sequence, SIGRRGGYCAGIIKQTCTCYR [4,5]. The activity of shorter peptide fragments, SIGRRGGYCAG (AMP1) and IIKQTCTCYRK (AMP2) from longicin P4 against bovine Babesia parasites, *B. bovis* and *B. bigemina*, was studied by Galay et al. [27]. From the two peptide fragments, only AMP1 consistently demonstrated a significant reduction in parasitemia of *B. bigemina*. Gao et al. [28] reported that all defensins contain a common highly exposed motif, located in the m-loop, which is associated with their antiparasitic activity. For longicin P4, the corresponding m-loop sequence is GRRGG. Among the two peptide fragments, only AMP1 contains this sequence and is most probably responsible for its anti-babesial activity, suggesting that longicin P4 could possess antiparasitic activity. In contrast to insect defensins, in which the recognition site is located in the α-helical region, the active site of longicin is localized in the terminal β-sheet [4,5]. In a previous study, we characterized the antimicrobial motif of synthesized longicin P4 peptide by analyzing some structural features using carious bioinformatics tolls and/or circular dichroism (CD) spectroscopy [29]. From the chemicophysical characteristics, longicin P4 is suggested to be a cationic peptide with hydrophobic and amphipathic character. The predicted secondary structure indicated the existence of a β-sheet, which was also observed in the modeled tertiary structure. CD spectroscopy results also showed the existence of a β-sheet and transition to a helical conformation in the presence of membrane-mimicking conditions. These structural observations of longicin P4 suggested that the antimicrobial activity could be due to the β-sheet as well as the α-helix.

Based on the present results and previously reported studies, we postulate that the efficient killing activities of longicin show the potential of these peptides as a novel toxoplasmacidal drug. Further studies will be necessary to elucidate the kinetics of interaction between longicin and the membrane of the parasites to clarify the mechanisms of the parasiticidal effect of longicin.

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