EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR DURING NURSE CELL FORMATION IN *Trichinella spiralis* AND *Trichinella pseudospiralis* INFECTIONS

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Abstract- The two most common *Trichinella* species causing human disease are *T. spiralis* and *T. pseudospiralis* belonging to encapsulated and non-encapsulated clades, respectively. Upon invasion, the larva of *T. spiralis* not only occupies and develops inside the muscle cell but also transforms it into a nurse cell surrounded by the vascular network called circulatory rete. It has been postulated that vascular endothelial growth factor (VEGF) is involved in the rete formation leading to the development and maintenance of nurse cell. This study showed that the expression of VEGF in the nurse cell of *T. spiralis* and *T. pseudospiralis* was detected by *in situ* hybridization. Basal expression of VEGF was found in uninfected muscle by real-time PCR technique and Western blot analysis. Nevertheless, both methods were not able to demonstrate the increase of VEGF expression above the basal expression in muscles infected with *T. spiralis* or *T. pseudospiralis*. The unexpected results are probably due to the relatively small number of infected nurse cells which could not produce enough positive signals to show significant increase in VEGF expression. It is likely that VEGF plays a role in the formation of circulatory rete and nurse cell in *T. spiralis* infection. However, there has been no experiment to directly validate this hypothesis. This area of study should be further investigated.

Keywords- *Trichinella*, VEGF, Nurse cell, Real-time PCR

Introduction

Parasitic nematodes of the genus *Trichinella* are the etiological agents of the disease called trichinellosis which has a wide host range including birds, reptiles, several mammals as well as human. *Trichinella* behaves as an intracellular parasite of the striated muscle cell. There are two clades in this genus encompassing encapsulated and non-encapsulated species differentiated by the presence or absence collagen capsule around the nurse cell. *T. spiralis*, the most common encapsulated species, infects the muscle cell and induces remarkable changes that transform the infected muscle cell into a nurse cell that supports the growth and development of the parasite. This process is termed nurse cell formation which involves the loss of muscle proteins, enlargement and division of nurse cell nuclei, mitochondrial damage and formation of collagen [1]. Another obvious feature of the nurse cell in *T. spiralis* infection is the presence of a vascular network called circulatory rete surrounding it [2]. This feature probably corresponds to many observations indicating that larval and nurse cell energy metabolisms are anaerobic [3]. The anaerobic condition could be seen mimicking tissue hypoxia that stimulates angiogenesis through angiogenic factors such as vascular endothelial growth factor (VEGF) in response to hypoxic events [4]. In *T. spiralis* infection, it was shown that VEGF was expressed in the nurse cell from day 7 up to 8 months and angiogene-sis around the nurse cell began at approximately day 12 and ceased by day 26 [1,5]. Additionally, the *in vitro* production of VEGF was directly stimulated by *T. spiralis* antigens but not by those of *T. pseudospiralis* [6]. A recent study showed that VEGF protein expression increased in *T. spiralis*-infected muscles at an early phase of infection beginning 7 days after infection and diminished after 3 weeks [7]. The non-encapsulated *T. pseudospiralis* is also able to form the nurse cell but the collagen capsule, unlike in *T. spiralis*, is poorly developed [8, 9]. Nevertheless, there has not been any study to demonstrate the presence of angiogenesis around the nurse cell of *T. pseudospiralis*. VEGF has been hypothesized to be involved in the development of the nurse cell-parasite complex in *Trichinella* infection. However, there has been so far no quantitative study of VEGF transcripts during the development of the nurse cell of both *T. spiralis* and *T. pseudospiralis*. In the present study, we attempt to determine VEGF expression in both mRNA and protein levels and localize VEGF mRNA transcript during nurse cell formation in *T. spiralis* and *T. pseudospiralis* infections.

Materials and Methods

Parasite Maintenance and Isolation

The laboratory strain of *T. spiralis* used in this study was isolated from a human in Mae Hong Son, Thailand, in 1986 [10]. The labora-

tory strain of *T. pseudospiralis* was kindly provided by Professor Yuzo Takahashi, Gifu University, Japan. These parasites were maintained by consecutive passages through ICR mice. *T. spiralis* and *T. pseudospiralis* infectious first stage larvae (L1) were recovered from muscles of infected mice by digestion with 1% and 0.8% pepsin in acidified water, respectively [11]. Digestion was carried out at 37°C for 2 hrs.or longer by slowly stirring in a hot air incubator (500 ml digestive fluid/mouse). Undigested materials were removed by pouring the digestion through gauze. Larvae were isolated by passing the gauze-filtered digestion through a 200-mesh sieve (Fisher Scientific). The retained larvae were collected from the sieve by washing and suspended in normal saline containing 2% (w/v) gelatin. Mice were orally inoculated with approximately 250 L1 by gavaging. Three of infected mice were sacrificed at each time point on days 5, 10, 15, 20, 25, 30, 45 and 60 of infection. Thigh muscle samples were used for analysis of VEGF transcript levels and protein expression whereas gastrocnemius muscle samples were used for RNA *in situ* hybridization. The protocol for animal handling was approved by the animal research ethics committee, Faculty of Medicine, Chiang Mai University.

Isolation of Total RNA and Reverse Transcription

Total RNA was isolated from mouse thigh muscles (uninfected or infected with *T. spiralis* or *T. pseudospiralis*) or *T. spiralis* L1 larvae using PureLink™ RNA Mini Kit (Ambion). Genomic DNA was removed from the RNA samples by RNase-free DNase I (Fermentas). Reverse transcription was performed on 0.2-2 mg of total RNA samples using RevertAid™ H Minus Reverse Transcriptase (Fermentas) according to the manufacturer's recommendations.

Preparation of digoxigenin-labeled RNA Probes

Mouse VEGF DNA was amplified by PCR on the reverse transcription product using a pair of specific primers appended with T7 and T3 promoter sequences at their 5' end [forward: 5'-(T7) GTAC-CTCCACCATGCCAAGT-3'; reverse: 5'-(T3) AATGCTTTCTCCGC TCTGAA-3']. The primers used for synthesizing *T. spiralis* 18S rDNA were 5'-(T7) GATCGCAGCAAGATGAACAA-3' and 5'-(T3) AGACAAATCGCTCCACCAAC-3'. The primers were designed based on the sequences from mouse VEGF gene (GenBank accession number: M95200) and *T. spiralis* 18S ribosomal RNA gene (GenBank accession numbers: U60231 and AY497012), respectively [12].

The sense and antisense RNA probes were generated from VEGF DNA or *T. spiralis*18S rDNA PCR product by *in vitro* transcription using digoxigenin (DIG)-11-UTP (Roche).

Detection of VEGF mRNA in Muscle Tissue Sections by *In-Situ* Hybridization (ISH)

Mouse gastrocnemius muscles infected with *T. spiralis* or *T. pseudospiralis* at 30 days post-infection were snap-frozen in 2-methylbutane chilled with liquid nitrogen and sectioned at 5 mm thickness using a cryostat. The sections were mounted on the positively charged slides and proceeded to detect the expression of VEGF in the muscle cells by modified ISH method [5,13]. Briefly, the sections were fixed in freshly prepared 4% paraformaldehyde in PBS for 10 min and followed by washing with DEPC-treated PBS for 5 min twice. The sections were further prehybridized with hybridization cocktail/50% formamide (Amresco) overnight at RT in a chamber humidified with 5X SSC. Hybridization was performed by incubating the sections with hybridization cocktail/50% formamide containing 200 ng/ml DIG-labeled sense or antisense VEGF RNA

probe in a chamber humidified with 5X SSC/50% formamide overnight at 65°C. The hybridization mixture was heated to 80°C for 5 min, and quickly chilled on ice before applying on the slides. Washing step was done after hybridization by placing the slides in 0.2X SSC at 65°C for 30 min twice and at RT for 5 min. Prior to immunological detection, the slides were washed with 100 mM Tris-HCl pH 7.5, 150 mM NaCl (buffer 1) for 10 min and subsequently blocked by covering the sections for 30 min with blocking solution (buffer 1 containing 0.1% Triton X-100 and 2% fetal bovine serum). To detect bound RNA probes, the sections were incubated with 1:5,000 anti-DIG antibody conjugated with alkaline phosphatase overnight at 4°C. The sections were then washed with buffer 1 for 10 min three times and further equilibrated for 10 min with 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂ (buffer 2). The color reaction was developed by nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indoryl phosphate (BCIP) ready to use (Amresco) in the dark until the desired intensity was reached. Following terminating the color reaction by immersing the slides in 10 mM Tris-HCl pH 8.0, 1 mM EDTA (buffer 3) for 5 min, the sections were mounted with a water-based mounting medium (Kaiser's glycerol gelatin, Merck). The positive reaction was observed as purple color on the tissue under a microscope. Antisense probe of 18S rRNA of the parasite was used as a positive control.

Measurement of VEGF Gene Expression Levels During Nurse Cell Formation by Real-time PCR

Each reverse transcription product was subjected to real-time PCR in triplicate in 25-ml reaction containing 1X FastStart Universal SYBR Green Master with ROX (Roche) and 0.3 mM each of forward and reverse primers. The reactions were accomplished using Applied Biosystems 7500 Real-time PCR System instrument according to the thermal cycling profile of 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The primers used in real-time PCR for VEGF were 5'-AATTGAGACCCTGGTGGACA-3' and 5'-CTGCATGGTGATGTTGCTCT-3', and those for GAPDH were 5'-CTCATGACCACAGTCCATGC-3' and 5'-TTCAGCTCTGG GATGACCTT-3'. Following the real-time PCR, the relative expression ratios of VEGF at various time points during the nurse cell formation were calculated based on Ct values of the target (VEGF) and reference (glyceraldehyde-3-phosphate dehydrogenase, GAPDH) genes and their amplification efficiencies using the equation of Pfaffl [14] as shown below.

$$Ratio = \frac{(E_{VEGF})^{\Delta Ct_{VEGF}\,(uninfected\,-\,infected)}}{(E_{GAPDH})^{\Delta Ct_{GAPDH}\,(uninfected\,-\,infected)}},$$

Where E_{VEGF} and E_{GAPDH} are the real-time PCR efficiencies of VEGF and GAPDH transcripts, respectively, ΔCt_{VEGF} (uninfected - infected) is the difference between the mean of Ct ($n = 3$) of VEGF gene of uninfected mouse muscles and that of infected mouse muscles, and ΔCt_{GAPDH} (uninfected - infected) is the difference between the mean of Ct ($n = 3$) of GAPDH gene of uninfected mouse muscles and that of infected mouse muscles. An experiment was performed to determine the real-time PCR efficiencies and it was found that E_{VEGF} and E_{GAPDH} were 1.88 and 1.81, respectively. This ratio indicates the relative quantification of the target gene in relation to the reference gene.

Measurement of VEGF Protein Levels by Western Blot Analysis

Approximately 50 mg of mouse thigh muscle infected with *T. spiralis* or *T. pseudospiralis* was homogenized in 500 ml protein extraction buffer containing 5 mM Tris-HCl pH 7.4, 1 mM EDTA (pH 8.0), 10%

SDS and 1X mammalian protease inhibitor cocktail (Amresco). To remove insoluble material, the lysate was centrifuged at 10,000 x g for 10 min. The supernatant was determined protein concentration by the bicinchoninic acid (BCA) protein assay system (Pierce).

Fifty mg of protein extracts were electrophoresed on 12.5% SDS-PAGE and transferred to PVDF membranes. After blocking with PBS containing 5% nonfat dry milk and 0.05% Tween-20 overnight, the membranes were probed at 4°C overnight with 1:5,000 rabbit anti-VEGF polyclonal Ab (Abcam) and 1:20,000 mouse anti-GAPDH monoclonal Ab (Abcam) to detect their respective protein antigens. The membranes were washed with PBS containing 0.05% Tween-20 for 10 min three times. The membranes were further incubated at RT for 1 Hr. with species-appropriate horseradish peroxidase (HRP) conjugated secondary antibody. Following the washing step as before, bound antibody was visualized with 3,3'-diaminobenzidine (DAB, Amresco). The intensities of bands were determined using GeneTools program version 4.02 (SynGene). The relative expression of VEGF protein was normalized by dividing the intensities of VEGF bands by those of GAPDH bands.

Results

VEGF mRNA Expression Levels During Nurse Cell Formation

The real-time PCR experiment showed that mean relative expressions of VEGF gene during nurse cell formation in *T. spiralis* infection were 0.99, 1.33, 1.40, 1.08, 1.35, 0.60, 0.84, 1.01 and 1.43 for uninfected muscles, muscles at 5, 10, 15, 20, 25, 30, 45 and 60 days post-infection (DPI), respectively [Fig-1a]. There were no significant differences of VEGF expression among various time points during nurse cell formation of *T. spiralis* (Kruskal-Wallis, $p = 0.095$).

The results obtained for VEGF expression during nurse cell formation in *T. pseudospiralis* infection are shown in [Fig-1b]. The mean relative expressions of VEGF gene during nurse cell formation in *T. pseudospiralis* infection were 1.00, 1.13, 1.94, 1.11, 1.09, 1.27, 1.03, 1.33 and 1.25 for uninfected muscles, muscles at 5, 10, 15, 20, 25, 30, 45 and 60 DPI, respectively. Like *T. spiralis*, there were no significant differences of VEGF expression among various time points during nurse cell formation of *T. pseudospiralis* (Kruskal-Wallis, $p = 0.122$).

Localization of VEGF Transcripts by *In-Situ* Hybridization

In order to determine the localization of VEGF mRNA in *T. spiralis*- or *T. pseudospiralis*-infected muscles, RNA *in situ* hybridization was performed. With the VEGF antisense probe, the purplish stain was observed in *T. spiralis*-infected muscle cell but not in the larva indicating that VEGF mRNA was localized to the cytoplasm of the nurse cell [Fig-2a]. However, the VEGF antisense probe hybridized not only to the cytoplasm of the nurse cell but also to the larva in *T. pseudospiralis*-infected muscle [Fig-2c]. As expected, the VEGF sense probe did not hybridize to *T. spiralis*-infected muscle cell [Fig-2b]. Nevertheless, the sense probe showed weak background hybridization to *T. pseudospiralis*-infected muscle cell [Fig-2d].

Determination of VEGF Protein Expression During Nurse Cell Formation by Western Blot Analysis

Western blot analysis was performed to measure VEGF protein expression during nurse cell formation in *T. spiralis* and *T. pseudospiralis* infection. Relative expression of VEGF protein was determined by the intensities of VEGF bands normalized by those of GAPDH bands. The 43-kDa VEGF protein and the 37-kDa GAPDH protein in muscle protein extracts from individual mice were recog-

nized by anti-VEGF polyclonal antibody and anti-GAPDH monoclonal antibody, respectively [Fig-3]. The results showed that there were no significant differences of the relative VEGF protein levels among uninfected muscles, muscles at 5, 10, 15, 20, 25, 30, 45 and 60 DPI during the nurse cell formation of both *T. spiralis* and *T. pseudospiralis* (Mann-Whitney U test, all *p* values > 0.05).
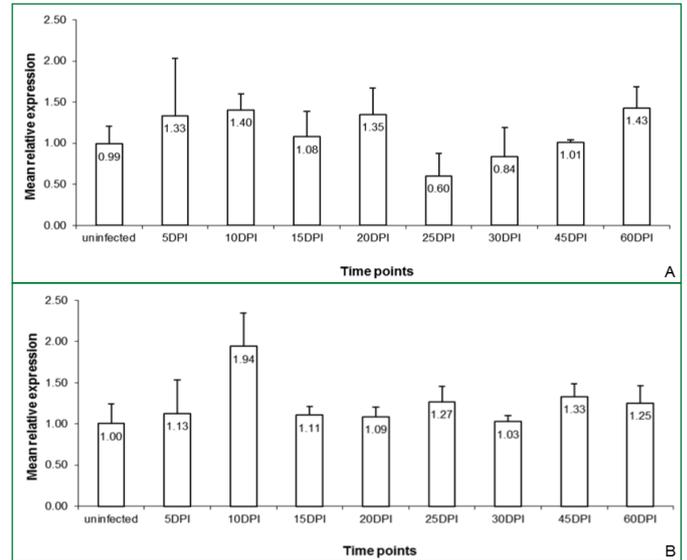

Fig. 1- Mean relative expression of VEGF at various time points during the nurse cell formation of *T. spiralis* (A) and *T. pseudospiralis* (B). Ct values were determined for VEGF gene in triplicate and normalized to Ct for GAPDH by the method of Pfaffl. Values shown are means of three independent samples. The error bars indicate the standard deviation (SD). Both *T. spiralis* and *T. pseudospiralis* infections showed no significant differences during nurse cell formation by non-parametric Kruskal-Wallis test ($p = 0.095$ and 0.112, respectively).
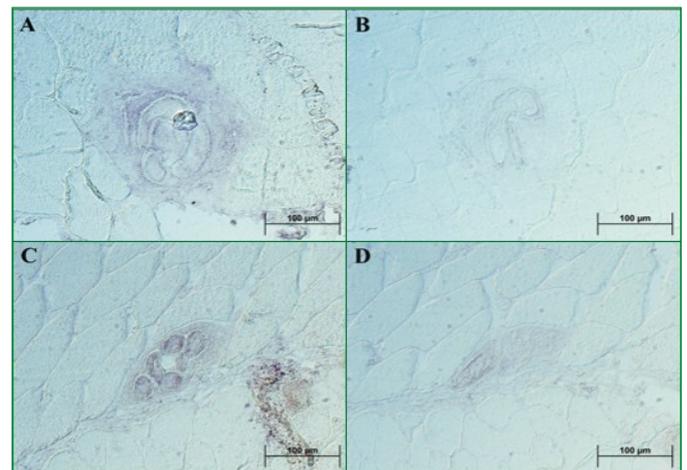

Fig. 2- Detection of mouse VEGF mRNA by *in situ* hybridization technique. *T. spiralis*-infected muscle (A and B) or *T. pseudospiralis*-infected muscle (C and D) 30 days post-infection were hybridized with mouse VEGF antisense probe (A and C) or mouse VEGF sense probe (B and D). The presence of mouse VEGF mRNA is indicated by the purplish stain. Mouse VEGF mRNA was detected in the cytoplasm of the muscle cell infected with *T. spiralis* (A) or *T. pseudospiralis* (C) by the antisense probe. The purplish stain was also observed in *T. pseudospiralis* larva. Hybridizations with the sense probe were shown in B and D as negative controls.
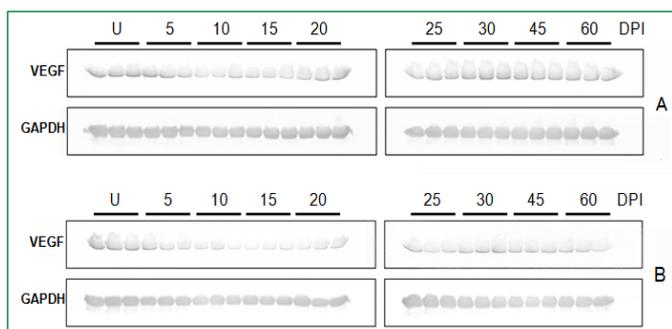
Fig. 3- Western blot analysis of VEGF protein expression in *T. spiralis* (A) and *T. pseudospiralis* (B) infected muscles. The expressions were analyzed for uninfected muscles (U), muscles at 5, 10, 15, 20, 25, 30, 45 and 60 days post-infection. Muscle extract of three different mice per each time point was recognized with anti-VEGF and anti-GAPDH antibodies.

Discussion

Angiogenesis is a mechanism of adaptive changes to tissue starving and hypoxia due to the limited supply of nutrients and oxygen [4,15]. VEGF is one of the most potent regulators of angiogenesis. VEGF binds to its receptors on the surface of endothelial cells in which this incidence induces a variety of signal transductions leading to the formation of new blood vessels [16]. VEGF-induced angiogenesis plays an important role in many conditions such as tumor growth [17,18], psoriasis [19], diabetic retinopathy and retina disorders [20,21] as well as wound repair [22]. *Trichinella* parasites are very unique in that, upon invasion of the host muscle, their larvae induce not only the transformation of muscle cells into nurse cells but also angiogenesis resulting in the vascular network surrounding the nurse cells [2]. These observations have been studied extensively only in *T. spiralis* infection. The supposed function of the vascular network is to promote the growth of the parasite by supplying nutrients and transporting waste products [1,23]. It has been suggested that VEGF might be involved in the vascular formation surrounding the nurse cell since VEGF mRNA was detected in the cytoplasm of the nurse cell from day 7 up to 8 months of *T. spiralis* synchronous infection by RNA *in situ* hybridization technique [5]. The highest level of VEGF mRNA was detected on 15 DPI in either nurse cell or surrounding inflammatory cells by estimating the reaction intensity. In our study, we confirmed that VEGF mRNA was present in the cytoplasm of the nurse cell of both *T. spiralis* and *T. pseudospiralis* infections but not in the uninfected muscle cells using RNA *in situ* hybridization technique. Additionally, the more sensitive real-time PCR technique to measure VEGF mRNA expression was performed in this study. The relative quantification by real-time PCR showed that there were no significant differences in the expression of VEGF mRNA during nurse cell formation in either *T. spiralis* or *T. pseudospiralis* infection. Furthermore, we found that uninfected muscles had a basal expression of VEGF which could be detected by real-time PCR and Western blot analysis. To confirm the above results, another experiment was repeated and the data showed similar patterns of VEGF expression as the previous experiment with insignificant fluctuations of VEGF mRNA levels among various time points during nurse cell formation. The real-time PCR results showing basal VEGF expression in uninfected muscles do not seem to correlate with the *in situ* hybridization results that did not give any positive signals. This may be explained by the fact that the real-time PCR technique is much more sensitive than the *in situ* hybridization. Despite its high sensitivity, the real-time PCR technique could not show significant increase in VEGF expression in infected muscles above the basal level whereas VEGF mRNA could be observed in the cytoplasm of nurse cells by *in situ* hybridization. Presumably, the expression of VEGF in the nurse cells, which constitute a tiny fraction of infected muscle, was not enough to show significant overall elevation by real-time PCR. However, the *in situ* hybridization, being able to show the localization of positive signals, may better reflect VEGF mRNA expression in the nurse cells indicating that VEGF transcripts were increased in the infected nurse cells. Regarding the expression of VEGF protein in *T. spiralis*-infected muscles during nurse cell formation, a recent study showed that there was an increase of VEGF protein above the basal level in uninfected muscles beginning at day 7 of infection and remained highly expressed until day 42 of infection [7]. In contrast, our study using Western blot analysis showed that VEGF protein expression was not significantly different among various time points (uninfected muscles, muscles at 5, 10, 15, 20, 25, 30, 45 and 60 DPI) during nurse cell formation in *T. spiralis* and *T. pseudospiralis* infections. Since there was basal expression of VEGF in uninfected muscles in our study, it is possible that VEGF protein expressed in the nurse cells, like its mRNA, was not enough to show significant increase above the basal expression.

The report on different gene expression patterns of the muscles infected with encapsulated parasite, *T. spiralis* and non-encapsulated parasite, *T. pseudospiralis* at 23 days after oral infection using cDNA microarray technique demonstrated that there was an up-regulation of VEGF only in *T. pseudospiralis*- but not in *T. spiralis*-infected muscles (3.09 and 0.98 times of uninfected muscles, respectively) [24]. The microarray data on the expression of VEGF in *T. spiralis* infection corresponded to our real-time PCR data which showed no difference of VEGF expression between uninfected and infected muscles since both techniques measured the overall expression of VEGF mRNA in the whole muscle. However, our study could not demonstrate the up-regulation of VEGF in *T. pseudospiralis*-infected muscles as in the microarray study.

An *in vitro* study using crude larval extracts showed that *T. spiralis* but not *T. pseudospiralis* antigens could induce the expression of VEGF mRNA and protein in rat alveolar macrophages [6]. The results indicated that *T. spiralis* antigens were able to stimulate VEGF expression via unknown mechanism. These data support the findings that VEGF mRNA and protein were expressed in infected muscle cells [5,7]. These may lead to future studies that could elucidate specific protein(s) responsible for stimulation of VEGF production and as well as its mechanisms.

Up until now there have been only a few publications on VEGF expression in *Trichinella* infection which still show some conflicting data among those studies and our present study. We therefore still do not completely understand the relationship between VEGF expression and nurse cell formation in *Trichinella* infection. Although VEGF seemed to increase during nurse cell formation and was likely to induce angiogenesis, it has not been shown that VEGF is directly responsible for the formation of circulatory rete around the nurse cell. Therefore, elucidating the angiogenesis process and VEGF function during nurse cell-parasite complex formation of *Trichinella* spp. are needed. The experiment that could down regulate the VEGF gene expression (e.g., RNA interference) or interfere with the function of VEGF (e.g., VEGF inhibitors such as chemical inhibitors and anti-VEGF antibody) would directly prove that VEGF

is indeed involved in the formation of circulatory rete and ultimately the nurse cell-parasite complex. Many recent studies have used anti-VEGF mAbs, which were produced by phage display technology [25], to inhibit functions of VEGF in many diseases such as intestinal adenoma, pituitary adenoma and psoriasis using mouse model [26-28]. An experiment that administers one of the anti-VEGF mAbs to *Trichinella*-infected mice could be tried to see its effects on the larval development and nurse cell formation. Another point of interest is that we have shown for the first time the expression of VEGF mRNA in *T. pseudospiralis*-infected nurse cell. However, there have not been any studies on the angiogenesis surrounding the nurse cell of any non-encapsulated *Trichinella* species. It is therefore a good opportunity to study the vascular network surrounding the nurse cell of *T. pseudospiralis* just as has been previously done in *T. spiralis* [2,29].

Conflict of Interests : The authors declare no conflict of interests.

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