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Research paper

Dirofilaria immitis exhibits sex- and stage-specific differences in excretory/secretory miRNA and protein profiles

Lucienne Tritten[a,1], Damian Clarke[a], Scott Timmins[b], Tom McTier[b], Timothy G. Geary[a,*]

[a] Institute of Parasitology and Centre for Host-Parasite Interactions, McGill University, 21,111 Lakeshore Road, Sainte-Anne-de-Bellevue, QC, H9X 3V9, Canada
[b] Zoetis Inc., 333 Portage Street, Kalamazoo, MI 49008, USA

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ABSTRACT

The canine heartworm *Dirofilaria immitis* releases excretory/secretory molecules into its host and in culture. We report analyses of the types, amounts and stage-dependence of microRNAs and proteins found in *D. immitis* culture media recovered after incubating 800,000 microfilariae for 6 days, 500 L_3 and 500 L_4 for 7 days, as well as 40 adult females and 40 adult males for 48 h, all separately. In addition, the presence of exosome-like particles was established by nanoparticle tracking analysis. Our results are in concordance with the *D. immitis* molecules previously detected in dog blood and in culture medium, but add additional insight into the sex- and stage-specificity of these processes. Of 131 miRNA candidates analyzed, none of the most abundant sequences was exclusively associated with one stage. Several isoforms of the nematode miR-100 family, miR-279, miR-71, were highly represented and overlapped substantially with the profile of heartworm miRNAs described from infected dog blood. lin-4 was primarily associated with males. We also report 4, 27 and 72 proteins in media from microfilariae, females and males, respectively. The only protein in common to all samples was actin, and only 9/88 proteins with a gene ontology description had not been reported in other studies of filarial secretomes. Exosomal proteins were well represented, dominated by cytoskeletal proteins, metabolic enzymes, zeta polypeptide, and chaperones.

1. Introduction

Molecules secreted by helminths have raised great interest for their immunomodulatory properties (McSorley et al., 2013). There is considerable evidence that the filarial nematode *Dirofilaria immitis*, also known as the canine heartworm, releases proteins (Geary et al., 2012; Sassi et al., 2014) and microRNAs (miRNAs) (Tritten et al., 2014a) in culture and in the host. miRNAs are short, non-protein coding RNAs with substantial regulatory roles in gene expression. Translation of targeted genes is usually impaired or the targeted mRNA is degraded by binding of cognate miRNAs (Bartel, 2004).

Helminth-derived miRNAs have been detected in host biofluids and represent potentially interesting biomarkers of infection (Hoy et al., 2014; Tritten et al., 2014a; Tritten and Geary, 2016). They are primarily found encapsulated in vesicles, and hence protected from enzymatic or pH degradation (Gallo et al., 2012). In general, these vesicles transport chemical messages in the form of RNA, proteins, lipids, etc. that serve roles in intercellular communication. In host-parasite interactions, they are involved in the modulation of the host immune system (Barteneva et al., 2013; Buck et al., 2014).

Although some options are available, sufficiently powerful and cost-effective tools to precisely measure filarial adult heartworm burdens are lacking (Genchi et al., 2007). Available antigenic tests are limited by the inability to detect male worms or to accurately reflect the number of adult females. Improved diagnostic methods are needed to identify optimal strategies for prophylaxis and treatment of these infections based on parasite burden.

Although some information is available about proteins and miRNAs released by *D. immitis*, little is known about stage-or sex-dependent variations in the menu of secreted molecules. We therefore investigated the set of miRNAs and proteins secreted by microfilariae, L3 and L4 larvae, and adult males and females of this species. In addition, we investigated the secretion of exosome-like vesicles in culture by adult *D. immitis*. Stage- and/or sex-specific biomarkers could provide the basis for novel diagnostics that could

* Corresponding author.
E-mail address: timothy.g.geary@mcgill.ca (T.G. Geary).
[1] Present address: Institute of Parasitology, University of Zurich, Winterthurerstrasse 266a, CH-8057, Zurich, Switzerland.

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quantitatively inform on adult worm burdens or the presence of larvae or pre-reproductive adults.

2. Materials and methods

2.1. Animals and D. immitis in vitro culture

Dogs were kept in Zoetis facilities. Maintenance and experimental protocols complied with all applicable animal welfare and Zoetis animal care and use protocols; all procedures were reviewed by appropriate welfare authorities and were conducted according to state and national/international regulations. For infections, *D. immitis* L_3 were harvested from *Aedes aegypti* mosquitoes (Liverpool strain), 15–17 days after membrane feeding on heparinized blood containing 2,500–5,000 mf/ml. Forty L_3 were injected per dog subcutaneously in the inguinal area. For recovery of adult worms, dogs were euthanized with a lethal dose (0.22 ml/kg) of Fatal-Plus® (Vortech Pharmaceuticals, LTD, Dearborn, MI), following intravenous heparin administration (1000 USP/ml). Adults were retrieved from the heart and lungs (precava, right atrium, right ventricle and pulmonary arteries) after clamping the anterior and posterior venae cavae. The pleural and peritoneal cavities were examined as well.

To obtain secretory miRNA and protein profiles, 40 adult male and 40 adult female worms were cultured separately by sex at 10 worms per 100 ml in serum-free RPMI-1640 media supplemented with 200 mM L-glutamine, 200 U/ml penicillin, 200 μg/ml streptomycin, 1% w/v D-glucose and 1% w/v sodium bicarbonate, pH = 7.2, for 24 to 48 h at 37 °C and 5% CO_2.

mf were retrieved from dog blood. Red blood cells were lysed for 5 min at 37 °C, using a water-based buffer containing 0.2% saponin and 0.85% NaCl. mf were subsequently filtered using a vacuum system and placed into Petri dishes containing culture media (RPMI-1640, 100 U/ml penicillin, 100 μg/ml streptomycin). mf were extensively washed 3 times in 50 ml media and pelleted by spins of 1000 × *g* for 5 min, before incubation started. 8,000 mf per ml were cultured for 6 days in 100 ml of the same culture media.

L_3 were obtained from dissected mosquitoes. 500 L_3 and 500 L_4 were cultured in the same culture media for 7 days. L_4 were created by molting the L_3 *in vitro* for 6 days in NCTC-135/IMDM medium (Abraham et al., 1987) without serum.

After removal of the worms manually (for adults) or by centrifugation at 1000 × *g* for 5 min (for larvae), media were filtered using polyethylsulphone or cellulose acetate filters (pore size: 0.22 μm), frozen at −20 °C, and shipped to McGill on dry ice. After thawing, pooled male media, pooled female media and mf media were concentrated separately by filtration through 3 kDa membranes (Ultracel 3 kDa membranes, and/or Amicon Ultra 15, 3 kDa, Millipore), to 1.5 ml and 5 ml, respectively. An RNA/protein purification kit was employed to immediately isolate both total RNA and protein (Norgen Biotek, Thorold, ON, Canada), following the manufacturer's instructions.

2.2. Nanoparticle tracking analysis

For exosome visualization, 5 male and 5 female worms were co-cultured in 100 ml culture media, which was processed as above. Concentrated media were subjected to exosome precipitation with the polymer ExoQuickTC (System Biosciences, Mountain View, CA, USA), resuspended in 100 μl sterile-filtered PBS and sent to System Biosciences for nanoparticle tracking analysis. NanoSight measurements were performed in triplicate and visualized on a LM10 NanoSight apparatus (Malvern Instruments, Montreal QC, Canada).

2.3. miRNA sequencing

Total RNA was shipped on dry ice to LC Sciences (Houston, TX, USA) for miRNA sequencing and analysis as previously described (Tritten et al., 2014a,b). RNA quality was assessed by LC Sciences and Illumina deep-sequencing of small RNAs was performed, following a proprietary pipeline using 398 ng RNA from mf, 110 ng from L_3, 105 ng from L_4, 938 ng from female, and 989 ng from male culture media, respectively. Low-quality sequences and other RNA populations (Rfam, Repbase; (Gardner et al., 2011; Jurka et al., 2005) were removed and the remaining sequences mapped to the *D. immitis* genome (http://nematodes.org/downloads/959nematodegenomes/blast/db2/Dirofilaria_immitis_2.2.fna.gz) (Godel et al., 2012) and to nematode miRNAs/pre-miRNAs in miRBase v.21.0 (Griffiths-Jones et al., 2008). Sequences were subsequently mapped to the dog genome (*Canis lupus familiaris* v3.1). Candidate sequences were named based on homology and assigned to groups reflecting both mapping properties and secondary structure. Only mature sequences of 18–25 nucleotides were retained. Normalization of sequence counts in each sample was performed by dividing the counts by a library size parameter of the corresponding sample (Eq. (1)). The library size parameter (s_j) is the median value of the ratio between the counts in a specific sample and in a pseudo-reference sample. A count number in the pseudo-reference sample is the count geometric mean across all samples.

$$sj = \underset{i}{median}\left(\frac{Cij}{\left(\prod_{k=1}^{m} Cik\right)^{1/m}}\right)$$

Eq. (1). Normalization of sequence counts. c_{ij} = the count number of sequence *i* of sample *j*; *m* = the total number of samples involved

The raw data reads were deposited in GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE84866.

2.4. Proteomic analysis

Protein concentration was assessed using the Pierce™ BCA assay (Thermo-Fisher Scientific, Waltham, MA). Samples were shipped on dry ice to the proteomics platform of the Quebec Genomics Center, University of Laval. 1 μg protein was analyzed separately from *D. immitis* mf, female and male culture media by LC–MS/MS. Proteins in solution were subjected to tryptic digestion overnight and analyzed as described (Bondu et al., 2014). Briefly, peptides were separated by reverse-phase nanoscale capillary liquid chromatography (Agilent 12,000 nano pump) and analyzed by electrospray tandem mass spectrometry (5600 mass spectrometer, AB Sciex, Farmingham, MA, USA). Separation occurred on PicoFrit columns (New Objective, Woburn, MA, USA) packed with Jupiter (Phenomonex) 5u, 300A C18 and eluted with a linear gradient of 2–30% solvent (acetonitrile, 0.1% formic acid). Spectra were acquired by Analyst software v.1.6. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version 2.4.1) and X! Tandem (The GPM, thegpm.org; version CYCLONE (2010.12.01.1)). Mascot was set up to search the TAX_Canidae_9608_20141103 database (unknown version, 99076 entries) assuming the digestion enzyme trypsin. X! Tandem was set up to search a subset of the TAX_Onchocercidae_6296_20141027 database also assuming trypsin. Scaffold (version Scaffold_4.4.1.1, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at > 28.0% probability to achieve a False Discovery Rate (FDR) < 1.0% by the Scaffold Local FDR algorithm. Protein identifications were accepted if they could be established at >99.0% probability to achieve an FDR < 1.0% and