Veterinary Parasitology 221 (2016) 84–92

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Research paper

A preliminary proteomic characterisation of extracellular vesicles released by the ovine parasitic nematode, *Teladorsagia circumcincta*

Thomas Tzelos[a,*], Jacqueline B. Matthews[a], Amy H. Buck[b], Fabio Simbari[b], David Frew[a], Neil F. Inglis[a], Kevin McLean[a], Alasdair J. Nisbet[a], C. Bruce A. Whitelaw[c], David P. Knox[a], Tom N. McNeilly[a]

[a] *Moredun Research Institute, Pentlands Science Park, Bush Loan, EH26 0PZ, Edinburgh, UK*
[b] *Institute of Immunology and Infection Research, School of Biological Sciences, University of Edinburgh, King's Buildings, EH9 3JL, Edinburgh, UK*
[c] *The Roslin Institute, University of Edinburgh, Easter Bush, Midlothian,EH25 9RG, Edinburgh, UK*

ARTICLE INFO

Article history:
Received 14 December 2015
Received in revised form 14 March 2016
Accepted 15 March 2016

Keywords:
Nematode
Teladorsagia circumcincta
Proteomics
Extracellular vesicles

ABSTRACT

Teladorsagia circumcincta is a major cause of ovine parasitic gastroenteritis in temperate climatic regions. The development of high levels of anthelmintic resistance in this nematode species challenges its future control. Recent research indicates that many parasite species release extracellular vesicles into their environment, many of which have been classified as endocytic in origin, termed exosomes. These vesicles are considered to play important roles in the intercellular communication between parasites and their hosts, and thus represent potentially useful targets for novel control strategies. Here, we demonstrate that exosome-like extracellular vesicles can be isolated from excretory-secretory (ES) products released by *T. circumcincta* fourth stage larvae (*Tci*-L4ES). Furthermore, we perform a comparative proteomic analysis of vesicle-enriched and vesicle-free *Tci*-L4ES. Approximately 73% of the proteins identified in the vesicle-enriched fraction were unique to this fraction, whilst the remaining 27% were present in both vesicle-enriched and vesicle-free fraction. These unique proteins included structural proteins, nuclear proteins, metabolic proteins, proteolytic enzymes and activation-associated secreted proteins. Finally, we demonstrate that molecules present within the vesicles-enriched material are targets of the IgA and IgG response in *T. circumcincta* infected sheep, and could potentially represent useful targets for future vaccine intervention studies.

1. Introduction

Teladorsagia circumcincta is the principal cause of ovine parasitic gastroenteritis (teladorsagiosis) in temperate climatic regions and has been reported as the most predominant nematode species in ovine flocks in the UK (Bartley et al., 2003; Burgess et al., 2012). In the northern hemisphere, teladorsagiosis is usually seen from July to October, coincident with an exponential increase in infective third stage larvae (L3) on the pasture on which the animals graze. The main clinical manifestations are reduced weight gain/condition loss and dehydration due to diarrhoea (Scott, 2007). Faecal contamination of the fleece in the perineal area may also attract blowflies, which can lead to myiasis. Teladorsagiosis has a significant economic impact on the industry: studies have estimated that losses in excess of £84 million *per annum* in the UK are associated with ovine parasitic gastroenteritis due to reduced productivity and the cost of the treatment alone (Nieuwhof and Bishop, 2005), with *T. circumcincta* being the major contributor. Moreover, the cost of sub-clinical infection, which is likely to be significant, is not included in the above figures (Nieuwhof and Bishop, 2005).

Control of *T. circumcincta* is largely based on the administration of broad spectrum anthelmintics (Kohler, 2001); however, resistance to these drugs appears to be widespread including reports of multiple drug resistant *T. circumcincta* isolates (Sargison, 2011; Wrigley et al., 2006). The introduction of two new classes of anthelmintic, monepantel in 2008 (Kaminsky et al., 2008) and derquantel in 2010 (Little et al., 2010), have helped to fill the gaps created in the control management of parasitic helminths. However, recent studies have shown that *T. circumcincta* and *Trichostrongylus colubriformis* have developed resistance to monepantel (Scott et al., 2013) and although derquantel is still effective

* Corresponding author.
E-mail address: Thomas.Tzelos@moredun.ac.uk (T. Tzelos).

http://dx.doi.org/10.1016/j.vetpar.2016.03.008
0304-4017/

in sheep, experience from other anthelmintic drug classes would suggest that development of resistance to derquantel is likely.

Vaccination represents an alternative control strategy against *T. circumcincta* as sheep develop protective immunity against the parasite after experimental multiple infections (Seaton et al., 1989; Smith et al., 1983). Recent work has identified several vaccine candidates (Nisbet et al., 2010a, 2009, 2010b, 2011; Redmond et al., 2006) and a "cocktail" of eight recombinant proteins targeting the fourth larval stage of *T. circumcincta*, which is the stage most intimately associated with the host, has been shown to stimulate significant levels of protection against experimental *T. circumcincta* challenge (Nisbet et al., 2013). However, the levels of protection achieved using this recombinant protein vaccine were variable, with mean reductions in egg output and adult worm burdens ranging from 58 to 70% and 56 to 75%, respectively, over two replicated experimental trials (Nisbet et al., 2013). These data indicate that further optimisation of the vaccine may be required.

Extracellular vesicles (EVs) are membrane vesicles which originate from either endosomal membranes (exosomes) or plasma membranes (microvesicles) (Raposo and Stoorvogel, 2013). These vesicles exhibit differences size ranges, with exosomes ranging between 30 and 100 nm (Thery et al., 2006; Urbanelli et al., 2013) and microvesicles ranging between 100 and 1000 nm in size (Muralidharan-Chari et al., 2010). EVs can be secreted by multiple mammalian cell types and the nature and function of these vesicles, and exosomes in particular, has been intensively studied over the last decade (Bobrie et al., 2011; Couzin, 2005; Mathivanan et al., 2010; Montaner et al., 2014; Simons and Raposo, 2009; Thery, 2011). Growth in this field is largely due to the discovery that exosomes play a key role in intercellular signalling and cell–cell communication; for example, B-lymphocytes (Raposo et al., 1996) and dendritic cells (Zitvogel et al., 1998) secrete exosomes which contain molecules that affect host immune responses (Andreola et al., 2002; Thery et al., 2002).

Research regarding EVs has now expanded to other organisms including parasitic helminths (Montaner et al., 2014). Studies showed that the trematodes *Fasciola hepatica* and *Echinostoma caproni* actively release exosomes (Chaiyadet et al., 2015; Marcilla et al., 2012), as do the nematode species, *Caenorhabditis elegans* (Liegeois et al., 2006), *Heligmosomoides polygyrus* (Buck et al., 2014) and *Trichuris suis* (Hansen et al., 2015). Marcilla et al. (2012) showed that rat intestinal cells actively take up exosomes secreted by *F. hepatica* and *E. caproni*. A recent study demonstrated that small RNAs contained in exosomes secreted by *H. polygyrus* can regulate genes of the host (mouse) innate immune system (Buck et al., 2014). These observations support the hypothesis that exosomes play roles in host-parasite communication. Furthermore, based on these observations, it has been suggested that exosomes could contain candidates for vaccines and/or targets for pharmaceutical intervention (Marcilla et al., 2012). To our knowledge there have been no reports on whether ruminant parasitic nematodes release EVs.

The work here examined whether EVs could be found in the excretory/secretory (ES) products of *T. circumcincta*. We focused on ES material from fourth stage larvae (L4) as previous studies have indicated that local humoral and cellular responses to this larval stage are critical in the acquisition of protective immunity in lambs (Smith et al., 1986, 1985; Stear et al., 1995; Strain et al., 2002). Furthermore, a detailed proteomics analysis was conducted to characterize the contents of these vesicles. Finally, an important consideration was to examine whether the vesicle proteins are targets of the host immune response, which would provide evidence of their release *in vivo*. Thus, immunoblots were performed to examine the immunogenicity of the contents of EVs released by *T. circumcincta*.

2. Materials and methods

2.1. Parasite material

EVs were prepared from the ES products of *T. circumcincta* L4 (*Tci*-L4ES). The L4 were harvested following methods described previously (Knox and Jones, 1990; Redmond et al., 2006; Smith et al., 2009). Briefly, 5 helminth-free lambs were infected, each with approximately 150,000 *T. circumcincta* L3 (strain MTci2; an anthelmintic-susceptible laboratory isolate from Moredun Research Institute). Seven days later, the lambs were euthanized to retrieve the L4. Each abomasum was removed and processed individually. The L4 were retrieved from the mucosa and from the abomasal contents as described previously (Knox and Jones, 1990). The L4 were pooled, washed with Phosphate Buffered Saline (PBS) and cultured in nematode culture medium [RPMI 1640 Gibco®, Life technologies™ (500 ml) supplemented with sterile L- glutamine (10 ml of 100 mM), D-glucose solution (5 g in 50 ml), penicillin/streptomycin (5 ml of 10,000 μg/ml), amphotericin B (62.5 mg), gentamycin sulphate (12.5 mg) and Hepes solution (10 ml; 1 M Sigma-Aldrich)] as described in previously published methods (Redmond et al., 2006).

The protocol for the collection of the ES material after 24, 48 and 72 h of culture was based on previously published studies (Smith et al., 2009). The pooled ES products from these time-points were filtered using a 0.22 μm syringe filter (millex® GP). The filtered ES products were divided into two equal aliquots (one to be processed for EVs purification and the other for total *Tci*-L4ES preparation) and frozen at −80 °C until further processing.

2.2. Extracellular vesicle purification

EVs purification was carried out following the protocol described in Buck et al. (2014). Briefly, *Tci*-L4ES samples were thawed at room temperature and ultracentrifuged at 100,000 × *g* for 2 h using a SW40 swing out rotor (cooled overnight at 4 °C). The supernatant (termed EV-free *Tci*-L4ES) was removed and stored at −80 °C prior to processing. Pelleted material was washed twice with 12.5 ml PBS. After each wash, the samples were ultracentrifuged at 100,000 × *g* for 2 h and the supernatant was removed. Finally, pelleted material was re-suspended in 100 μl PBS (termed EV-enriched *Tci*-L4ES) and protein concentration measured with Qubit® 2.0 Fluorometer (Life technologies™). An aliquot of the pelleted material was immediately processed for microscopy as described below, and the remaining material stored at −80 °C prior to proteomic analysis.

2.3. Transmission electron microscopy (TEM)

An aliquot (8 μl) of the pelleted material from 2.2 was fixed with an equal volume of paraformaldehyde 4% (Fisher scientific). The sample was prepared by the Electron Microscopy (EM) unit of the University of Edinburgh (School of Biological Sciences, King's Buildings). The technicians of the EM unit prepared the sample for the TEM as described in Buck et al., 2014.

2.4. Concentration of total and EV-free Tci-L4ES

Total *Tci*-L4ES and EV-free *Tci*-L4ES were thawed at room temperature and Amicon Ultra-15 centrifugal filter units (MWCO 10 kDa; Sigma Aldrich) were used to concentrate the material according to the manufacturer's instructions to a final volume of ~1 ml. Finally, the filter units were washed twice with pre-chilled PBS to buffer exchange the material into PBS. The concentration of the protein in these samples was measured using the Pierce® BCA