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Short communication

Surface associated antigens of *Dirofilaria immitis* adult worms activate the host fibrinolytic system

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ABSTRACT

Cardiopulmonary dirofilariosis (*Dirofilaria immitis*) is characterized by apparent contradictory events, like the long-term survival of adult worms in the circulatory system of the infected hosts and the development of life-threatening events like thromboembolisms and others. Thus parasite mechanisms, like the activation of fibrinolytic system, are key to the survival of both the worms and the host. The aim of this study was to investigate the interaction between *D. immitis* adult worms surface-associated antigens (DiSAA) and the fibrinolytic system of the host. We demonstrate that DiSAA extract is able to bind plasminogen and generate plasmin, with the latter occurring in a tissue plasminogen activator (t-PA) dependent manner. Additionally, 11 plasminogen-binding proteins from DiSAA extract were identified by proteomics and mass spectrometry (MS) (actin-5C, actin-1, enolase, fructose-bisphosphate aldolase, GAPDH, MSP domain protein, MSP 2, beta-galactosidase-binding-lectin, galectin, immunoglobulin I-set domain-containing protein and cyclophilin Ovcyp-2). Because in a previous work we have shown the positive interaction between the excretory/secretory antigens of *D. immitis* (DiES) and the host fibrinolytic system and many of the molecules identified here are shared by both antigens, we hypothesize that DiSAA cooperate in host fibrinolytic system activation promoting the fibrin clot lysis.

1. Introduction

Dirofilaria immitis is the causative agent of canine and feline cardiopulmonary dirofilariosis and human pulmonary dirofilariosis. It is a vector-borne transmitted disease with a cosmopolitan distribution (Genchi et al., 2001). *D. immitis* adult worms can survive for years (7 or more) in the pulmonary arteries and right ventricle of dogs (Quiroz-Romero, 1984), causing a chronic vascular disease mainly associated with inflammatory reactions. Moreover, the simultaneous death of groups of adult worms can trigger an acute pathology characterized by the exacerbation of the inflammatory reactions and the occurrence of serious thromboembolisms (Venco, 2007) that poses an immediate risk for the life of the affected hosts.

The fibrinolytic system activity is based on the conversion of plasminogen into plasmin, the enzyme responsible of fibrin clots lysis (Cesarman-Maus and Hajjar, 2005). This process is regulated by the t-PA, mainly synthesized by vascular endothelial cells. It has been recently demonstrated that the metabolic products excreted by *D. immitis* "in vitro" stimulate the host fibrinolytic system. Furthermore, this stimulation causes an over-expression of t-PA in vascular endothelial cells (González-Miguel et al., 2012), suggesting

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the existence of regulatory mechanisms of the thromboembolisms by this parasite in its intravascular habitat. On the other hand, parasite-surface molecules have been widely linked to key roles related to the host/parasite relationships (Fetterer and Rhoads, 1993) and some of them have been related to the plasminogen-binding activity in protozoa (Almeida et al., 2004; Vanegas et al., 2007; Mundodi et al., 2008) and helminth parasites (Jolodar et al., 2003; Erttmann et al., 2005; Ramajo-Hernández et al., 2007). In this work we demonstrate that different surface-associated molecules of *D. immitis* adult worms bind plasminogen and generate plasmin, activating the host fibrinolytic system.

2. Materials and methods

2.1. Collection of surface associated antigens from D. immitis adult worms (DiSAA)

DiSAA extract was obtained following the methodology described by Wedrychowicz et al. (1994) with minor modifications. In brief, live worms (7) obtained from a naturally infected dog were washed and then incubated in saline solution containing 0.25% CTAB with a cocktail of protease inhibitors (Maizels et al., 1991) at 37 °C for 4 h. The worms were separated from detergent and extracted proteins were precipitated with sodium acetate 0.002 M with nine volumes of 96% ethanol, at −20 °C for 48 h followed by centrifugation (10,000 × *g*, 10 min). The resulting pellets were re-suspended in PBS pH 7.2 and stored at −80 °C until use. Previously, protein concentration of DiSAA was measured by DC protein assay commercial kit (Bio-Rad).

2.2. Plasminogen binding assay

To determine whether the plasminogen would bind surface components of *D. immitis* an ELISA test was carried out as described previously (González-Miguel et al., 2012). In brief, multiwell microplates (Costar) were coated with 1 μg/well of DiSAA extract, blocked and then incubated with increasing amounts (from 0 μg to 3 μg) of human plasminogen (acris antibodies). After incubation with the corresponding antibodies and with a chromogen, the optical density was measured at 492 nm in an easy reader (Bio-Rad). In parallel, competition assays were performed by including 50 mM of the lysine analogue ε-aminocaproic acid (εACA) during plasminogen incubation.

2.3. Plasminogen activation assay

Plasminogen activation assay was performed in a test volume of 100 μl by measuring the amidolytic activity of generated plasmin (González-Miguel et al., 2012). In each well 2 μg of human plasminogen (acris antibodies) were incubated in PBS with 3 μg of the chromogenic substrate S-2251 (Sigma) in the presence of 1 μg of DiSAA extract. Activation of plasminogen was initiated by addition of 15 ng of t-PA (Sigma). In parallel, plasmin generation was also measured in the absence of t-PA. Plates were incubated at 37 °C for 2 h and the hydrolysis of the chromogenic substrate was monitored by measuring absorbance at 405 nm every 30 min. Each sample was analyzed in triplicate.

2.4. Two-dimensional electrophoresis (2-DE) of DiSAA extract and immunoblot assay

The 2-DE of DiSAA extract was performed as described before by us (González-Miguel et al., 2012). Briefly, DiSAA extract aliquots were supplemented with ampholytes, incubated and centrifugated, and then applied to 7 cm IPG strips (Bio-Rad) with linear pH ranges of 3–10, 5–8 and 7–10, using a Protean IEF Cell (Bio-Rad) for isoelectric focusing (IEF). After IEF, strips were reduced and alkilated, and second dimension was done in 12% acrylamide gels. Gels were then silver stained with the PlusOne silver staining kit, protein (GE Healthcare).

To determine which proteins of DiSAA extract bind plasminogen an immunoblot was performed (González-Miguel et al., 2012). The 2-D gels were transferred to nitrocellulose membranes which were blocked and then incubated overnight at 4 °C with 10 μg/ml of human plasminogen. After incubating the membranes with the corresponding antibodies, proteins were revealed with 4-chloro naphthol.

The 2-D gels and membranes were scanned and analyzed with the quantity one software v.4.6.5 (Bio-Rad). Matching of 2-D gels with the homologous Western blot to identify plasminogen-binding proteins was analyzed using the PDQuest Software v.8.0.1 (Bio-Rad).

2.5. MS and protein identification

In gel digestion of proteins and MS analysis were done as described before by us (González-Miguel et al., 2012). The spots containing plasminogen-binding proteins were excised manually from the gels and sent to the unit of Proteomics of the Centro de Investigación Principe Felipe (Valencia, Spain) for MS analysis. For peptide mass fingerprinting and the acquisition of LIFT TOF/TOF spectra, an aliquot of the digestion of each spot was deposited onto a 600 μm AnchorChip MALDI probe (Bruker-Daltonics). Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) in positive-ion reflector mode. The measured tryptic peptide masses were transferred through the MS BioTools software (Bruker-Daltonics) as inputs to search the National Centre for Biotechnology Information non-redundant database (NCBInr) using Mascot software (Matrix Science). When necessary, MS/MS data from the LIFT TOF/TOF spectra were combined with MS peptide mass fingerprint data for database searches.

2.6. Statistical analysis

The results from the plasminogen binding assay and plasminogen activation assay were analyzed with the Student's *t*-test. The results were expressed as the mean ± SEM of at least 3 independent experiments. In all experiments, a significant difference was defined as a p-value < 0.05 for a confidence level of 95%.

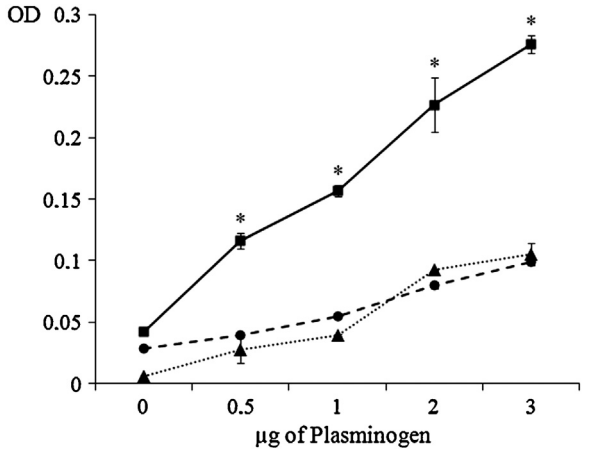

Fig. 1. Plasminogen binding to 1 μg of DiSAA extract of *D. immitis* measured over a range of plasminogen amounts using a microtiter plate method. (■) Incubation with increasing amounts of plasminogen, 0–3 μg. (●) Competition assay with 50 mM εACA included during plasminogen incubation. (▲) Negative control consisted of wells coated only with BSA. Each point is the mean of three replicates ±SD. The asterisk (*) designates significant ($p < 0.05$) differences.

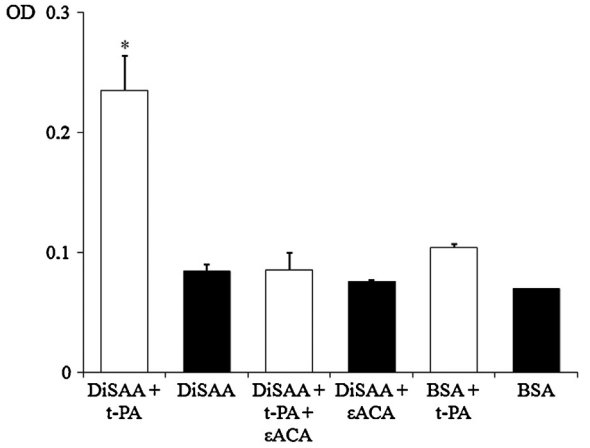

Fig. 2. Plasminogen activation and plasmin generation by DiSAA extract of *D. immitis*. (□) 15 ng of t-PA was added to mixtures containing 2 μg of human plasminogen, 3 μg of S-2251 (Sigma) and 1 μg of DiSAA extract (or BSA as negative control) in the presence or absence of 50 mM of εACA in a test volume of 100 μl. (■) No t-PA was added to reaction mixtures. Each point is the mean of three replicates ±SD. The asterisk (*) designates significant ($p < 0.05$) differences.

3. Results

3.1. Proteins of DiSAA extract bind plasminogen

The binding capacity of plasminogen to DiSAA extract was measured by ELISA. The test showed that DiSAA binds plasminogen obtaining optical densities statistically higher ($p < 0.05$) than those of the control wells (coated only with BSA) (Fig. 1). This binding is also directly proportional to the amount of plasminogen. The competition assay showed that the inclusion of 50 mM εACA inhibits the plasminogen-binding (Fig. 1), demonstrating that this union is dependent on lysine residues.

3.2. The plasminogen-binding activity of DiSAA extract generates plasmin

The ability to activate plasminogen by DiSAA extract and to generate plasmin was assessed by measuring the amidolytic activity of plasmin generated in the presence of the antigenic extract and plasminogen. This effect was measured in the presence or absence of t-PA, to observe the ability of the DiSAA extract proteins of activating plasminogen on their own. Negative controls replacing DiSAA by BSA or t-PA were also used. As shown in Fig. 2, the generation of plasmin by t-PA is enhanced by DiSAA reaching optical density values significative higher ($p < 0.05$) than the negative controls. Furthermore this effect is inhibited by 50 mM εACA, indicating the involvement of lysine residues in the process. However, DiSAA extract is unable to generate plasmin without t-PA resulting in optical density values identical to the negative control.

3.3. Two-dimensional analysis of DiSAA extract

To obtain an overall view of all the proteins of the DiSAA, this extract were first electrofocused using 3–10 linear immobilized pH gradient strips. Silver nitrate staining of these 2-D gels revealed about 315 spots in the *D. immitis* surface proteome, many sparsely settled, with isoelectric points (pIs) between 5 and 9.7, and a broad range of molecular weights (MWs) (10–145 kDa). Only 4 spots were observed with pI‹5 (not shown). In order to improve spot resolution and detection, once the spot MW and pI ranges were determined, the DiSAA extract were electrofocused in 5–8 and 7–10 IPG strips. With these new conditions, silver staining revealed a total of 347 spots, most of them (318) located between pIs 5 and 8. The remaining 29 spots had pIs between 8 and 9.8 (Fig. 3A and B).

3.4. Identification of plasminogen-binding proteins

To identify plasminogen-binding proteins, a ligand blotting with plasminogen of 2D gels of 5–8 and 7–10 pH was performed. As shown in Fig. 3C and D, 61 plasminogen-binding spots were revealed (17.58% for total spots revealed in the surface proteome). Most of them ($n = 42$) were resolved in a narrow range of MWs and pIs (between 40 and 100 kDa, and 5.4 and 8, respectively). In the control blots, in which plasminogen incubation was omitted, the anti-plasminogen antibody did not reveal any spots (not shown).

The matching of spots revealed by ligand-blotting with their homologous in the silver-stained 2-D gels allowed us to select a total of 53 plasminogen-binding spots of *D. immitis*, which were manually excised from 2-D gels and submitted to analysis by MS. Table 1 shows the identity of these proteins and their MWs and pIs (theoretical and experimental), the number of access to similar information available in the NCBI database, the sequence coverage and the Mascot score. Sixteen of 53 spots were identified (30.18%) and corresponded to 11 different proteins. Between 1 and 4 isoforms of each protein were identified. Most proteins were identified by their similarity to homologous

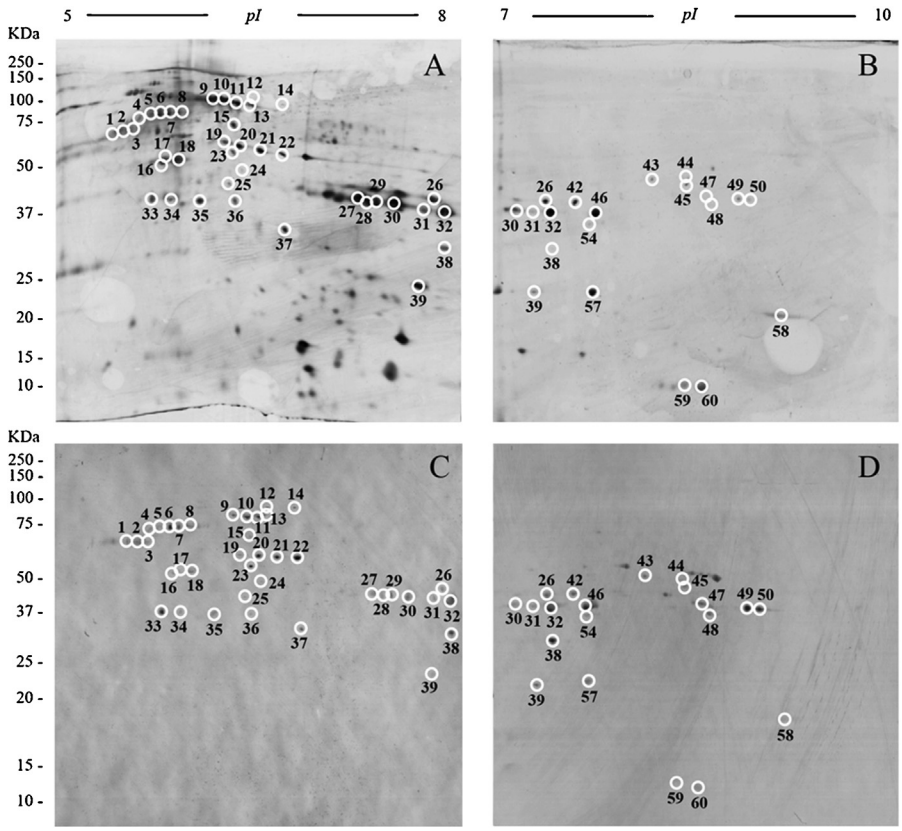

Fig. 3. Representative 2-DE of 60 μg of the DiSAA extract from adult *D. immitis* worms. The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained (A and B). Plasminogen-binding spots revealed on ligand blots from gels A and B (C and D). Reference molecular masses are indicated on the left. The plasminogen-binding spots analyzed by MS are circled and numbered.

Table 1
Plasminogen-binding protein spots of DiSSA extract identified by MALDI–TOF MS. GAPDH: Glyceraldehyde 3-phosphate dehydrogenase; MSP: major sperm protein; exp: experimental; theo: theoretical.

Spot number	Accesion code	Protein definition	Species	MW (kDa) theor/exp	pI theor/exp	Queries matched	Mascot score
17	EFV54220	actin-5C	*T. spiralis*	41.8/54.1	5.3/5.7	4	154
18	P02578	actin-1	*A. castellanii*	41.7/52.8	5.4/5.8	6	190
20	XP_001896281	Enolase	*B. malayi*	47.5/59.6	6.0/6.3	6	248
22	Q7YZX3	Enolase	*O. volvulus*	47.1/55.5	6.0/6.6	8	64
26	AAB52600	Fructose-bisphosphate aldolase	*O. volvulus*	39.2/40.5	7.7/7.7	8	247
42	AAB52600	Fructose-bisphosphate aldolase	*O. volvulus*	39.2/39.0	7.7/7.9	2	59
28	P48812	GAPDH	*B. malayi*	36.1/40.0	7.7/7.2	13	128
30	P48812	GAPDH	*B. malayi*	36.1/39.8	7.7/7.4	17	259
32	P48812	GAPDH	*B. malayi*	36.1/37.4	7.7/7.8	11	84
46	P48812	GAPDH	*B. malayi*	36.1/36.0	7.7/8.0	9	71
35	XP_001900868	MSP domain protein with Glu-rich domain	*B. malayi*	18.1/40.1	5.5/6.0	2	64
60	P13263	Major sperm protein 2	*O. volvulus*	14.3/15.7	7.8/8.8	18	265
37	AAA20541	Beta-galactosidase-binding-lectin	*O. volvulus*	32.0/33.6	6.0/6.6	20	143
38	XP_001900812	Galectin	*B. malayi*	31.8/30.3	6.4/7.8	3	68
39	XP_003139445	Immunoglobulin I-set domain-containing protein	*L. loa*	22.5/24.2	6.6/7.6	8	307
58	AAC47233	Cyclophilin Ovcyp-2	*O. volvulus*	18.5/20.9	8.3/9.4	1	71

proteins from other species of filarial worms. Thus of the 16 spots identified 14 corresponded to other filarial proteins (*Brugia malayi*, *Onchocerca volvulus* and *Loa loa*). The 2 remaining spots corresponded to a protein from the nematode *Trichinella spiralis* (actin-5C) and to a protein from the free-living protist parasite *Acanthamoeba castellanii* (actin-1).

4. Discussion

Cardiopulmonary dirofilariosis is caused by the long-term presence of *D. immitis* adult worms in the pulmonary arteries and right ventricle of their definitive host. In this location, worms are exposed to the effector mechanisms of the host immune system. Moreover, the death of the adult worms triggers some pathological processes that immediately threat the host's life, the thromboembolisms being one of the most serious. However, *D. immitis* can survive through different mechanisms of both immune evasion and modulation of its vascular environment (Simón et al., 2012). In the live worms there are two antigenic compartments participating in the parasite/host relationships, the excreted metabolic products (DiES antigens) and the surface-associated antigens, many of which are also excreted (Smith, 1991), taking part in the excretory/secretory antigens. In this study we demonstrate that the DiSAA bind plasminogen and stimulate plasmin generation. Surface-associated antigens have also been related to the plasminogen recruitment in both bacterial (Bergmann and Hammerschmidt, 2007) and parasitic infections (Jolodar et al., 2003; Ghosh and Jacobs-Lorena, 2011). It has also been postulated that plasmin produced by plasminogen activation plays a key role in the degradation of extracellular matrices, migration through the tissues (Bergmann and Hammerschmidt, 2007) and evasion of the immune response (Barthel et al., 2012). For the conversion of plasminogen into plasmin t-PA is necessary, being mainly synthesized and secreted by the vascular endothelium (Cesarman-Maus and Hajjar, 2005). Given that it has been previously shown that the DiES causes an over-expression of t-PA in cultured vascular endothelial cells (González-Miguel et al., 2012), it is possible that a combined action of both compartments (DiES and DiSAA) could exist. Thus *D. immitis* worms are not only capable of activating the fibrinolytic system in order to avoid clot formation in the systemic level by action of the DiES, but also in their immediate environment by the action of the DiSAA. This combined action would be of great importance as a parasite survival mechanism.

The combination of proteomic, immunomic and MS techniques allowed us to identify a total of 16 spots of the DiSAA extract that corresponded to 11 plasminogen-binding proteins. These were identified using the available information of filarial proteins in databases. Among them, various enzymes from the group of actins (Dudani et al., 2005; Ramajo-Hernández et al., 2007), enolase (Jolodar et al., 2003; Marcilla et al., 2007; Mundodi et al., 2008), fructose-bisphosphate aldolase (Ramajo-Hernández et al., 2007; de la Paz Santangelo et al., 2011) and GAPDH (Erttmann et al., 2005; Ramajo-Hernández et al., 2007) have been extensively studied due to their interaction with the fibrinolytic system in different types of pathogens. Furthermore, 3 isoforms of actin, 2 of GAPDH and the galectin were identified as plasminogen-binding proteins on the DiES antigens (González-Miguel et al., 2012). The other proteins identified in our study (MSP domain protein, major sperm protein 2, beta-galactosidase-binding-lectin, immunoglobulin I-set domain-containing protein and cyclophilin Ovcyp-2) have been identified as plasminogen binding proteins for the first time. These proteins are involved in different biological processes such as structural activity (MSP domain protein and MSP 2), carbohydrate binding (beta-galactosidase-binding-lectin) or protein folding (cyclophilin Ovcyp-2). Further studies are needed to know the real effect of the identified plasminogen-binding proteins on the survival mechanisms of *D. immitis*.

In summary, we have demonstrated the "in vitro" interaction between the surface-associated antigens of *D. immitis* and the host fibrinolytic system, supplementing the already demonstrated fibrinolytic activity of the excretory/secretory antigens of this species.

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