Excretory/secretory antigens from *Dirofilaria immitis* adult worms interact with the host fibrinolytic system involving the vascular endothelium

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ABSTRACT

Dirofilaria immitis is the causative agent of canine and feline heartworm disease. The parasite can survive for long periods of time (7 years or more) in the circulatory system of immunocompetent reservoirs, producing usually a chronic inflammatory vascular disease. In addition, the simultaneous death of groups of adult worms can trigger an acute disease characterized by the exacerbation of inflammatory reactions and the emergence of serious thromboembolic events. In the context of the *D. immitis*/host relationships, the aim of this study was to investigate the interaction between the excretory/secretory antigens from *D. immitis* adult worms (DiES) and the fibrinolytic system of the host. Using an enzyme-linked immunosorbent assay we showed that DiES extract is able to bind plasminogen and generate plasmin, although this fact requires the presence of the tissue plasminogen activator (t-PA). Moreover, we established that DiES extract enhances t-PA expression in cultured vascular endothelial cells. Additionally, 10 plasminogen-binding proteins from DiES extract were identified by mass spectrometry (HSP60, actin-1/3, actin, actin 4, transglutaminase, GAPDH, Ov87, LOAG_14743, galectin and P22U). The data suggest that DiES antigens interact with the environment of the parasite regulating the activation of the fibrinolytic system of the host with involvement of the vascular endothelium in the process.

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1. Introduction

Heartworm disease (HD) is a serious and potentially fatal disease caused by the filaroid nematode *Dirofilaria immitis* that affects dogs and cats all over the world [1]. The adult worms lodge in the pulmonary arteries and the right ventricle of infected hosts where they can live for years [2], causing a chronic inflammatory pathology. Initially the damages affect the arteries (endarteritis and perivascular inflammation), spreading later to the lung parenchyma and the right heart chambers [3]. In addition, when groups of worms die naturally or as a consequence of filaricide treatment, very serious alterations occur, with the exacerbation of inflammatory reactions and the formation of massive thromboembolisms [4] that put the life of the infected animals in immediate risk.

Since *D. immitis* can survive in the long term in the vascular system of immunocompetent hosts, it is reasonable to assume that adult worms interact with their intravascular environment, modulating the immune response and the associated pathology by means of the action of their metabolic products [excretory/secretory (ES) antigens], as it occurs in other parasitic infections [5,6]. A key point of vantage in blood parasites is the hemostasis, which is closely associated to fibrinolysis, inflammatory reactions and angiogenesis [7]. During the fibrinolysis, plasminogen binds to specific receptors together with activators of the process from which the most important is the t-PA that is mainly synthesized and secreted by endothelial cells. This binding determines the transformation of plasminogen into serin protease plasmin, which is the enzyme that lyses the fibrin [8], degrading it into soluble products including the D-dimer [9]. Moreover, the activation of the plasminogen–plasmin system plays a key role in the degradation of extracellular matrices [10] that has been related to cell invasion and intra-organic migration of different pathogens [11,12]. Plasminogen and t-PA bind to receptors present on cells in the fibrin clots, to annexin-A2 of vascular endothelial cells and integrin $\alpha M\beta 2$ of leukocytes

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[8]. Activation of plasminogen by binding to molecules secreted by bacteria with which it forms complexes has also been described [13]. Related to parasites, several molecules associated to the surface of protozoa and helminths [14–19] as well as molecules of the ES products [12,20], that bind plasminogen, have been identified. Additionally, it has been described that such interactions are mediated by carboxyl-terminal lysine residues of the plasminogen receptors [21].

We have previously observed some facts that suggest the interaction of *D. immitis* with its vascular environment: (i) DiES promote vasodilation, stimulating the expression of prostaglandin E2 by vascular endothelial cells, also limiting the transmigration of monocytes to the perivascular tissue [22]. (ii) In another study we found that serum levels of D-dimer are significantly higher in the 47% of dogs with HD analyzed than in the healthy dogs used as controls, indicating the presence of thromboembolisms and their degradation [23].

Although *D. immitis* is able to survive in the circulatory system of its hosts for years, there are no data on the interaction of adult worms with the fibrinolytic system of their hosts. The aim of this study was to demonstrate that the DiES antigens can bind plasminogen, generate plasmin, and stimulate the increase of t-PA synthesis by vascular endothelial cells. Additionally, we identified some plasminogen binding proteins of DiES extract using immuno-proteomic techniques and mass spectrometry (MS).

2. Materials and methods

2.1. Collection of ES extract of proteins from D. immitis adult worms

DiES were prepared as previously described [22] with minor modifications and stored at $-80\,^\circ$C. In brief, live worms (25) obtained from a naturally infected dog were washed in sterile phosphate-buffered saline solution (PBS) pH 7.2 and incubated for 24 h in 50 ml of Eagle's minimum essential medium (EMEM) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin at $37\,^\circ$C. A cocktail of protease inhibitors was added to the medium following the methodology described by Maizels et al. [24]. The medium was dialyzed against water for 24 h and filtered through an Amicon YC05 membrane (Millipore). The protein concentration of DiES was measured by DC protein assay commercial kit (Bio-Rad). DiES was tested for the presence of endotoxin contamination using a quantitative *Limulus* amebocyte lysate test (BioWhittaker). The endotoxin quantity was under the sensitivity level of cell stimulation (<0.4 U/mg protein).

2.2. Plasminogen binding assay

To determine whether plasminogen would bind components of the DiES extract, an enzyme-linked immunosorbent assay (ELISA) was performed. Multiwell microplates (Costar) were coated with 1 μg/well of DiES extract diluted in carbonate buffer, pH 9.6, overnight at $4\,^\circ$C. The wells were blocked with 1% BSA in PBS and incubated successively with increasing amounts (from 0 μg to 3 μg) of human plasminogen (Acris Antibodies), with a sheep anti-human plasminogen IgG (Acris Antibodies) at 1:2000 dilution and then with a peroxidase-conjugated donkey anti-sheep IgG (Sigma) at 1:4000 dilution. All incubations were performed for 1 h at $37\,^\circ$C and between each step washed three times with PBS wash buffer (PBS containing 0.05% Tween$_{20}$). Ortho-phenylene-diamine was used as a chromogen. Optical densities (OD) were 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. Some wells coated with BSA only were used as negative controls.

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 [15]. 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 DiES. 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 $37\,^\circ$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. Cell culture and stimulation of endothelial cells

Vascular endothelial cells HAAE-1 from ATCC (LGC Promochem) were grown and treated as previously described [22]. In brief, endothelial cells were grown in Ham's F12k medium (ATCC) supplemented with 2 mM L-glutamine, 10% fetal bovine serum (FBS) (ATCC), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mg/ml heparin (Sigma) and 0.03 mg/ml endothelial cell growth supplement (ECGS) (Sigma). Plates were precoated with 0.1% pig gelatine (Sigma). Cells were cultured at $37\,^\circ$C in a humidified atmosphere in the presence of 5% CO_2–95% air. Medium was changed every 3 days. Endothelial cells (10^6 cells/plate) were plated on 100 mm culture plates and grown for 4 days to obtain confluent cultures and treated with 1 μg/ml of DiES for 24 h. Non-stimulated cells were used as controls under the same conditions.

2.5. Two-dimensional electrophoresis (2-DE) of DiES extract

The 2-DE of DiES was performed as described before by us for the somatic antigen of adult worms of *D. immitis* [25]. Briefly, DiES extract was purified with the ReadyPrep 2-D Cleanup Kit (Bio-Rad) and resuspended in rehydration buffer 2-D (7 M urea, 2 M thiourea, 4% 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS)). The samples were divided into 125 μl aliquots (containing 60 μg of protein) and stored at $-20\,^\circ$C until use. When they were used DiES aliquots were supplemented with ampholytes and DTT, incubated and centrifuged to remove all particulate material, 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 alkylated, and second dimension separation was done in 12% acrylamide gels. Gels were then silver stained with the PlusOne Silver Staining Kit, Protein (GE Healthcare) or transferred to nitrocellulose membranes for their immunoblot analysis. The 2-D images were scanned with the GS-800 Densitometer (Bio-Rad) and analyzed with the Quantity One Software v.4.6.5 (Bio-Rad).

2.6. Immunoblot assays

To determine which proteins of DiES extract bind plasminogen, they were electrotransferred from 2D gels to nitrocellulose membranes at 20 V for 30 min using a Trans-Blot SD Semi-Dry Transfer cell (Bio-Rad). Blots were blocked with 2% BSA in PBS wash buffer, for 1 h at room temperature. DiES membranes were incubated overnight at $4\,^\circ$C with 10 μg/ml of human plasminogen. Then, the blots were incubated with a sheep anti-human plasminogen IgG (Acris Antibodies) at 1:1000 dilution and with a peroxidase-conjugated donkey anti-sheep IgG (Sigma) at 1:2000 dilution, each incubation for 90 min. All incubations were performed at $37\,^\circ$C with shaking and between each step washed three times with washing

buffer for 5 min per wash. Protein bands were revealed with 4-chloro naphthol. Negative controls were also used in which the plasminogen had been omitted. In addition, competition assays were performed by including 50 mM εACA during plasminogen incubation. Membranes were digitized with the scanner GS-800 Densitometer (Bio-Rad) using 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, the assignment of molecular weights (MW) and isoelectric points (pI) of each protein were analyzed using the PDQuest Software v.8.0.1 (Bio-Rad). All assays were performed in triplicate to assess the reproducibility of the spot pattern.

Western blot analysis for the t-PA expression was performed as previously described [22]. Treated and non-treated vascular endothelial cells were lysed in ice-cold lysis buffer. Protein samples (20 μg) were separated by SDS–PAGE under reducing conditions and blotted onto polyvinylidine difluoride membranes. Membranes were blocked before incubation with the primary antibody rabbit anti-t-PA (Santa Cruz Biotechnology) at 1:1000. After incubation with HRP-conjugated anti-rabbit secondary antibody at 1:20,000 dilution, bands were visualized by a luminol-based detection system with p-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products) was used to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using Scion Image Software.

2.7. MS and protein identification

In gel digestion of proteins and MS analysis were done as described before by us [25]. The spots containing plasminogen-binding proteins were excised manually from the gels and sent to the Unit of Proteomics of the Centro Nacional de Investigaciones Cardiovasculares (Madrid, 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) [26] in positive-ion reflector mode. The measured tryptic peptide masses were transferred through the MS BioTools program (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 (PMF) data for database searches.

2.8. Statistical analysis

The results from the plasminogen binding assay, plasminogen activation assay and Western blots for the t-PA expression 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 of <0.05 for a confidence level of 95%.

3. Results

3.1. Proteins of DiES extract bind plasminogen

The binding level of plasminogen to DiES extract was studied by ELISA. This test showed that the DiES extract binds plasminogen and that this binding is directly proportional to the amount of plasminogen (Fig. 1). The negative control consisting of wells coated only with BSA showed some non-specific binding activity, but always in a value significative lower than that shown by the DiES extract ($p < 0.05$). To determine whether or not lysine residues
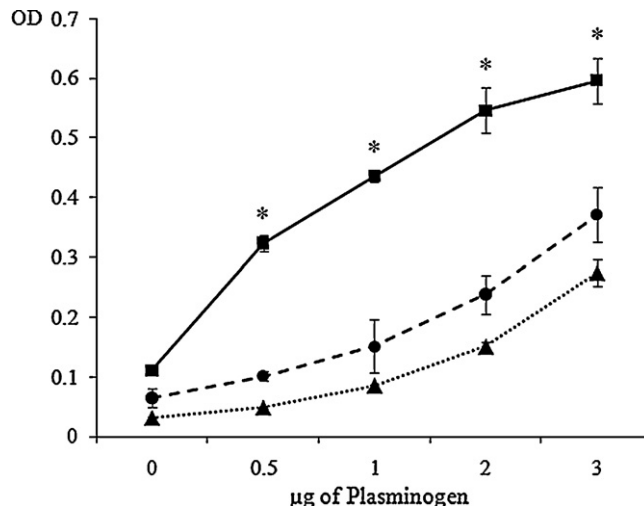

Fig. 1. Plasminogen binding to 1 μg of DiES extract of *D. immitis* measured over a range of plasminogen amounts per cavity using a microtiter plate method. (■) Incubation with increasing amounts of plasminogen, 0–3 μg. (●) Competition assay with 50 mM εACA included during plasminogen incubation. (▲) 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.

are involved in binding, a competition experiment including 50 mM εACA was carried out. In this case the binding between DiES extract and plasminogen was inhibited about 70%, resulting in slightly higher optical densities than the negative control (Fig. 1).

3.2. Plasminogen is activated by proteins of DiES extract and plasmin is generated

The ability to activate plasminogen by DiES 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 a physiological activator of the process, t-PA, to observe the ability of the DiES extract proteins of activating plasminogen on their own. Negative controls replacing DiES for BSA or t-PA were also used. As shown in Fig. 2, the generation of plasmin by t-PA
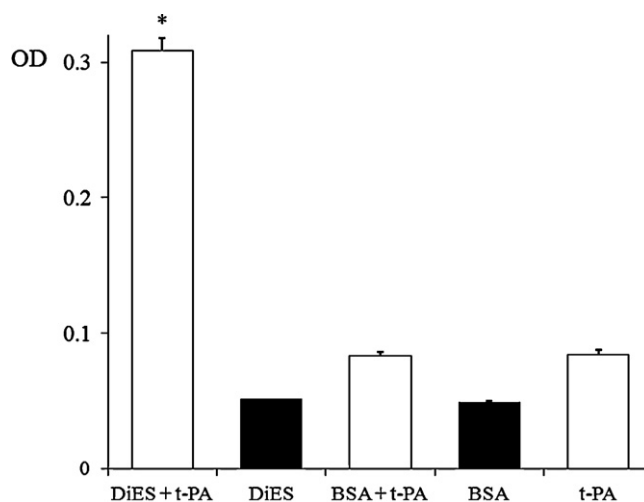
Fig. 2. Plasminogen activation and plasmin generation by DiES extract of *D. immitis*. (□) 15 ng of t-PA was added to mixtures containing 2 μg of human plasminogen and 3 μg of the chromogenic substrate S-2251 (Sigma) in the presence or absence of 1 μg of DiES (or BSA as negative control) in a test volume of 100 μl. (■) No t-PA were added to reaction mixtures. Each point is the mean of three replicates ± SD. The asterisk (*) designates significant ($p < 0.05$) differences.
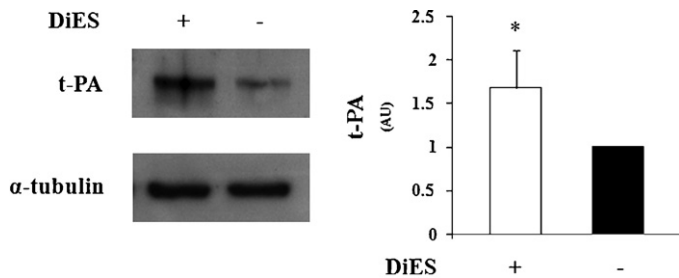
Fig. 3. Effect of DiES on the expression of t-PA in human vascular endothelial cells. Protein extracts from lysed DiES treated or untreated confluent cell cultures were analyzed by Western blot for t-PA. α-tubulin served as a protein control. Results were expressed as the mean ± SEM of at least 3 independent experiments. The asterisk (*) designates significant ($p < 0.05$) differences from control cells. (□) Stimulated endothelial cells. (■) Non-treated control cells. AU, arbitrary units.

is enhanced by DiES reaching optical density values significative higher ($p < 0.05$) than the negative controls in the presence of t-PA. However, DiES extract is unable to generate plasmin without t-PA resulting in optical density values identical to the negative control.

3.3. DiES extract stimulate t-PA expression in vascular endothelial cell cultures

D. immitis is an intravascular parasite for which we previously observed interactions of DiES antigens with the vascular endothelium. Additionally, the DiES extract activates the transformation of plasminogen to plasmin in a t-PA-dependent manner. Thus, the objective of this experiment was to determine whether or not DiES enhances the synthesis of t-PA in vascular endothelial cells (HAAE-1). Proteins from DiES-treated vascular endothelial cell extracts were separated by SDS–PAGE and analyzed by Western blotting using anti-t-PA antibody. As shown in Fig. 3, DiES induced a significative increase in t-PA ($p < 0.05$) protein expression after 24 h of stimulation.

3.4. Two-dimensional analysis of DiES extract

To obtain an overall view of all the proteins of the DiES, this extract were first electrofocused using 3–10 linear immobilized pH gradient strips. Silver nitrate staining of these 2-D gels revealed about 570 spots in the excretome of *D. immitis* with pIs between 5 and 9.8, and a broad range of MWs (10–150 kDa). Only 24 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 DiES extract were electrofocused in 5–8 and 7–10 IPG strips. With these new conditions, silver staining revealed a total of 636 spots, most of them (594) located between pH 5 and 8. The remaining 96 spots had pIs between 8 and 9.8 (Fig. 4A and B).

3.5. Identification of plasminogen-binding proteins

To identify plasminogen-binding proteins, ligand blotting of 2D gels of 5–8 and 7–10 pH with plasminogen was performed, after electrotransfering them to nitrocellulose membranes.

As shown in Fig. 4C and D, 81 plasminogen-binding spots were revealed. This represents a binding rate of 12.73% of total spots revealed in the excretome of *D. immitis*. Most of them ($n = 60$) were resolved in a narrow range of MWs and pIs (between 37 and 150 KDa, and 5.2 and 7.2, respectively). In the control blots, in which plasminogen incubation was omitted, the anti-plasminogen antibody did not reveal any spots (not shown). In the competition experiments, the inclusion of 50 mM εACA inhibited

plasminogen binding to DiES demonstrating the specificity of the reaction (Fig. S1).

The matching of spots revealed by ligand-blotting with their homologous spots in the silver-stained 2-D gels allowed to select a total of 81 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 NCBI accession number, the sequence coverage and the Mascot score. Seventeen of 81 spots were identified (21%) and corresponded to 10 different proteins. The proteins identified were chaperonine protein HSP60, actin-1/3, actin, actin 4, transglutaminase precursor, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), Ov87, hypothetical protein LOAG_14743, galectin and P22U. Between 1 and 4 isoforms of each protein were identified. Most proteins were identified by their similarity to homologous proteins from other species of filarial worms. Thus of the 17 spots identified 9 corresponded to other filarial proteins (*Brugia malayi*, *Onchocerca volvulus* and *Loa loa*), while 6 spots corresponded to 3 proteins of *D. immitis* deposited in databases (transglutaminase precursor, galectin and P22U). The 2 remaining spots corresponded to proteins from the nematode *Caenorhabditis elegans*.

4. Discussion

D. immitis infections are typically characterized by the persistence of adult worms in vascular location for years in which they are exposed to multiple aggression mechanisms from the host and where their presence causes severe and sometimes fatal pathological changes. One of these mechanisms is the generation of thromboembolisms. This process is physiologically regulated by the fibrinolytic system which is able to lyse fibrin clots. Its activation by molecules of *D. immitis* could have beneficial effects for the survival of the parasite in the circulatory system.

In this study we demonstrate in an in vitro system that DiES antigens bind plasminogen. This binding is dependent on the presence of lysine residues, as it is inhibited by ε-aminocaproic acid. We also demonstrate that DiES extract activates plasminogen and generate plasmin in a t-PA-dependent manner. All this is consistent with experimental studies carried out in bacteria, protozoa and helminths [17,19,27,28].

We also demonstrated that DiES antigens significantly stimulate the basal production of t-PA by vascular endothelial cells cultured in vitro. This is consistent also with the key role of vascular endothelium in the regulation of haemostasis [7] and with the intravascular niche of *D. immitis*. We have previously shown that DiES antigens interact with the vascular endothelium, stimulating vasodilatation and reducing leukocyte transmigration, which highlights the importance of the endothelium in the activation of mechanisms that likely promote parasite survival, also limiting the damage to the host [22].

On the other hand, plasmin produced by plasminogen activation is also involved in the lysis of extra-cytoplasmic matrices [29], which is interpreted as a mechanism related to cell invasion and intra-organic migration of different parasites [12,14]. As many molecules involved in the binding of plasminogen are multifunctional, we cannot rule out that though the activation of the fibrinolytic system has predictable beneficial effects, both for the parasite and for the host, some of the molecules involved in the process can exert other activities, contributing to the generation of damage on the host. A consequence of the arrival of the *D. immitis* adult worms into the pulmonary arteries is the appearance of proliferative endarteritis caused by the proliferation and migration of smooth muscle cells of the vascular wall into the lumen [3]. It has been shown that the over-expression of t-PA in damaged
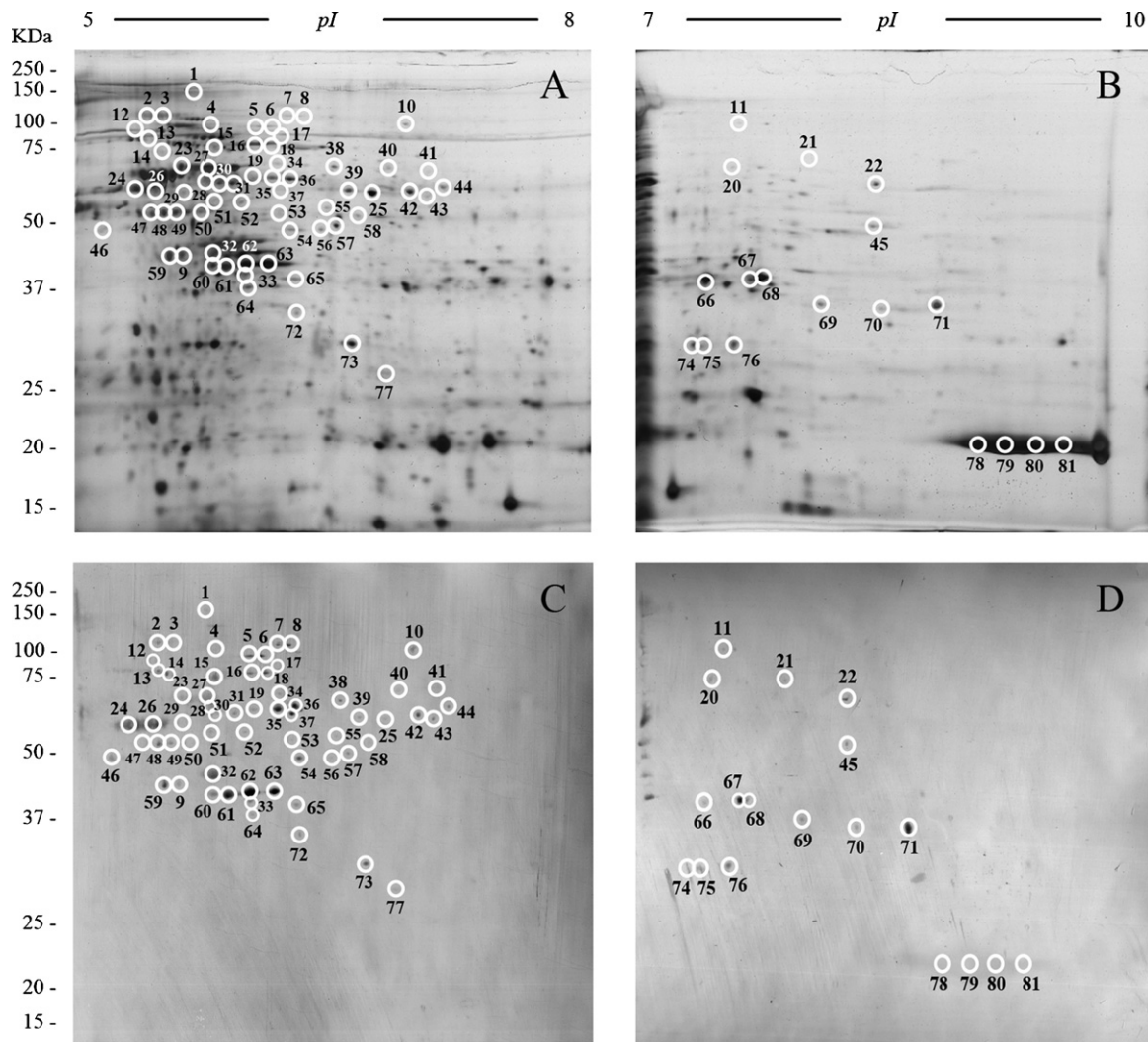
Fig. 4. Representative 2-DE of 60 μg of the DiES extract from adult *D. immitis* worms. The gels were in the 5–8 and 7–10 pH ranges, 12% polyacrylamide and silver-stained (A and B). Plasminogen-binding spots revealed on ligand blots from gels A and B (C and D). Reference molecular masses are indicated on the left. The plasminogen-binding spots analyzed by MS are circled and numbered.

endothelium induces proliferation and migration of smooth muscle cells in humans [30]. Since we have observed an over-expression of t-PA by vascular endothelial cells stimulated by DiES, it will be necessary to study in the future if the proliferative endarteritis is associated with over-expression of t-PA by endothelial cells and therefore the activation of the fibrinolytic system of the host by *D. immitis* adult worms.

The proteomic analysis of DiES extract also allowed us to identify 17 plasminogen-binding spots by MS, which corresponded to 10 proteins. Their identification was possible by the existence of a significant amount of available information on the filarial worms. Of the proteins identified in the DiES, HSP 60, different proteins of the family of actins and GAPDH are among the best characterized binding-plasminogen molecules. The HSP 60 is a binding protein belonging to the family of heat shock proteins. Its plasminogen-binding activity has been demonstrated in bacteria [31,32], in which this activity has been associated with disruption of the extracellular matrix of tissues and invasion [32].

Three proteins of the actin family binding plasminogen (actin-1/3, actin and actin-4) have been identified in the DiES extract. The interaction between actin and plasminogen is well known, as well as the fact that specific binding occurs through lysine residues which stimulate the tPA-dependent plasmin generation [33]. In

addition, its function as plasminogen receptor has been demonstrated on the surface of endothelial cells [34] and in the tegument of *Schistosoma bovis* [17]. The glycolytic enzyme GAPDH is a multifunctional molecule whose plasminogen-binding activity has been observed in different pathogens such as bacteria and fungi [35,36], blood helminths such as *S. bovis* [17] and tissue helminths like *O. volvulus* [16].

The other proteins identified in our study (transglutaminase precursor, Ov87, galectin, P22U and LOAG_14743) have been related to plasminogen binding for the first time. However, there is evidence that some of them are associated with nearby or related fibrinolysis processes. It has been demonstrated that galectin-1, which belongs to the family of galectins (like galectin and Ov87 proteins identified here) acts as receptor of t-PA and is the responsible for the increase of catalytic activity that occurs in the pancreatic cancer [37]. Moreover, the LOAG_14743 is a hypothetical protein of the annexin family. Within this family, annexin-A2 is one of the best-characterized plasminogen receptors on endothelial cells [38].

To conclude, we have demonstrated that DiES antigens in vitro activate the plasminogen binding and plasmin production, involving the vascular endothelium in the regulation of this process through the stimulus of the expression of t-PA by vascular endothelial cells. These facts demonstrate the interaction of *D. immitis* with

Table 1
Plasminogen-binding protein spots of DiES extract identified by MALDI-TOF MS. Exp, experimental; theo, theoretical.

Spot number	Accesion code	Protein definition	Species	MW (kDa) theor/exp	pI theor/exp	Sequence coverage (%)	Mascot score
23	ACY25666	Chaperonin-like protein HSP60	*Brugia malayi*	61.4/67.1	5.7/5.6	11	130
27	AF121264_1	Chaperonin protein HSP60	*Onchocerca volvulus*	64.5/67.0	5.7/5.8	17	145
28	AF121264_1	Chaperonin protein HSP60	*Onchocerca volvulus*	64.5/65.2	5.7/5.8	16	130
31	ACT1_CAEEL	Actin-1/3	*Caenorhabditis elegans*	42.1/65.2	5.3/5.9	11	41
32	XP_001894819	Actin	*Brugia malayi*	42.1/43.3	5.3/5.8	4	64
33	NP_508842	ACTin family member (act-4)	*Caenorhabditis elegans*	37.5/39.4	5.4/6.0	17	142
37	AAC24752	Transglutaminase precursor	*Dirofilaria immitis*	57.6/61.0	5.7/6.3	19	91
66	XP_001899850	Glyceraldehyde 3-phosphate dehydrogenase	*Brugia malayi*	32.1/40.8	8.5/7.5	20	207
67	XP_001899850	Glyceraldehyde 3-phosphate dehydrogenase	*Brugia malayi*	32.1/40.7	8.5/7.8	25	292
69	AAD00843	Ov87	*Onchocerca volvulus*	36.7/36.4	8.9/8.2	24	161
71	AAD00843	Ov87	*Onchocerca volvulus*	36.7/36.5	8.9/9.0	16	157
72	XP_003150284	Hypothetical protein LOAG_14743	*Loa loa*	13.3/33.7	6.7/6.3	11	94
73	AAF37720	Galectin	*Dirofilaria immitis*	32.2/30.1	6.0/6.6	11	118
78	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.2	66	499
79	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.4	62	458
80	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.6	62	489
81	AAD11968	P22U	*Dirofilaria immitis*	24.4/22.0	8.9/9.8	54	201

its vascular environment through his metabolic products, promoting mechanisms for its own survival. Ten plasminogen binding molecules of the DiES extract have been identified by proteomic analysis and MS, suggesting that *D. immitis* adult worms use different molecules to maintain the balance of the vascular environment. Future studies are needed to obtain a complete understanding of this process during HD and to elucidate if molecules of the plasminogen binding process are involved in other mechanisms related to the occurrence of pathological changes in the pulmonary arteries.

Acknowledgements

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.molbiopara.2011.10.010.

References

[1] Genchi C, Kramer LH, Prieto G. Epidemiology of canine and feline dirofilariasis: a global view. In: Simón F, Genchi C, editors. Heartworm infection in humans and animals. España: Ediciones Universidad de Salamanca; 2001. p. 121–34.
[2] Quiroz-Romero H. Parasitología y enfermedades parasitarias de los animales domésticos. México: Dimusa S.A.; 1984, pp.620–627.
[3] Venco L. Heartworm (*Dirofilaria immitis*) disease in dogs. In: Genchi C, Ronaldi L, Cringoli G, editors. Dirofilaria immitis *D. repens* in dog cat human infections. Italia: Rolando Editore; 2007. p. 117–25.
[4] McCall JW, Genchi C, Kramer LH, Guerrero J, Venco L. Heartworm disease in animals and humans. Adv Parasitol 2008;66:193–285.
[5] Dzik JM. Molecules released by helminth parasites involved in host colonization. Acta Biochim Pol 2006;53(1):33–64.
[6] Bennuru S, Semnani R, Meng Z, Ribeiro JM, Veenstra TD, Nutman TB. *Brugia malayi* excreted/secreted proteins at the host/parasite interface: stage and gender specific proteomic profiling. PLoS Negl Trop Dis 2009;3:e410.
[7] Becker BF, Heindl B, Kupatt C, Zahler S. Endothelial function and hemostasis. Z Kardiol 2000;89(3):160–7.
[8] Cesarman-Maus G, Hajjar KA. Molecular mechanisms of fibrinolysis. Br J Haematol 2005;129(3):307–21.
[9] Goggs R, Benigni L, Fuentes VL, Chan DL. Pulmonary thromboembolism. J Vet Emerg Crit Care (San Antonio) 2009;19(1):30–52.
[10] Stephens RW, Vaheri A, Kreis T, Vale R, editors. Guidebook to the extracellular matrix and adhesion proteins. New York: Oxford University Press; 1995. p. 81–2.
[11] Jong AY, Chen SH, Stins MF, Kim KS, Tuan TL, Huang SH. Binding of *Candida albicans* enolase to plasmin(ogen) results in enhanced invasion of human brain microvascular endothelial cells. J Med Microbiol 2003;52:615–22.
[12] Bernal D, de la Rubia JE, Carrasco-Abad AM, Toledo R, Mas-Coma S, Marcilla A. Identification of enolase as a plasminogen-binding protein in excretory-secretory products of *Fasciola hepatica*. FEBS Lett 2004;563(1–3):203–6.
[13] Bergmann S, Hammerschmidt S. Fibrinolysis and host response in bacterial infections. Thromb Haemost 2007;98(3):512–20.
[14] Jolodar A, Fischer P, Bergmann S, Büttner DW, Hammerschmidt S, Brattig NW. Molecular cloning of an alpha-enolase from the human filarial parasite *Onchocerca volvulus* that binds human plasminogen. Biochim Biophys Acta 2003;1627(2–3):111–20.
[15] Almeida L, Vanegas G, Calcagno M, Concepción JL, Avilan L. Plasminogen interaction with *Trypanosoma cruzi*. Mem Inst Oswaldo Cruz 2004;99(1):63–7.
[16] Erttmann KD, Kleensang A, Schneider E, Hammerschmidt S, Büttner DW, Gallin M. Cloning, characterization and DNA immunization of an *Onchocerca volvulus* glyceraldehyde-3-phosphate dehydrogenase (Ov-GAPDH). Biochim Biophys Acta 2005;1741(1–2):85–94.
[17] Ramajo-Hernández A, Pérez-Sánchez R, Ramajo-Martín V, Oleaga A. *Schistosoma bovis*: plasminogen binding in adults and the identification of plasminogen-binding proteins from the worm tegument. Exp Parasitol 2007;115(1):83–91.
[18] Vanegas G, Quiñones W, Carrasco-López C, Concepción JL, Albericio F, Avilán L. Enolase as a plasminogen binding protein in *Leishmania mexicana*. Parasitol Res 2007;101(6):1511–6.
[19] Mundodi V, Kucknoor AS, Alderete JF. Immunogenic and plasminogen-binding surface-associated alpha-enolase of *Trichomonas vaginalis*. Infect Immun 2008;76(2):523–31.
[20] Marcilla A, Pérez-García A, Espert A, Bernal D, Muñoz-Antolí C, Esteban JG, et al. *Echinostoma caproni*: identification of enolase in excretory/secretory products, molecular cloning, and functional expression. Exp Parasitol 2007;117(1):57–64.
[21] Plow EF, Herren T, Redlitz A, Miles LA, Hoover-Plow JL. The cell biology of the plasminogen system. FASEB J 1995;9(10):939–45.
[22] Morchón R, González-Miguel J, Mellado I, Velasco S, Rodríguez-Barbero A, Simón F. Adult *Dirofilaria immitis* excretory/secretory antigens upregulate the production of prostaglandin E2 and downregulate monocyte transmigration

in an "in vitro" model of vascular endothelial cell cultures. Vet Parasitol 2010;170(3–4):331–5.

[23] Carretón E, Corbera JA, Juste MC, Morchón R, Simón F, Montoya-Alonso JA. *Dirofilaria immitis* infection in dogs: cardiopulmonary biomarker levels. Vet Parasitol 2011;176(4):313–6.

[24] Maizels RM, Blaxter ML, Robertson BD, Selkirk ME. Parasite antigen parasite genes: a laboratory manual for molecular parasitology. Cambridge: Cambridge University Press; 1991.

[25] González-Miguel J, Rosario L, Rota-Nodari E, Morchón R, Simón F. Identification of immunoreactive proteins of *Dirofilaria immitis* and *D. repens* recognized by sera from patients with pulmonary and subcutaneous dirofilariosis. Parasitol Int 2010;59(2):248–56.

[26] Suckau D, Resemann A, Schuerenberg M, Hufnagel P, Franzen J, Holle A. A novel MALDI LIFT-TOF/TOF mass spectrometer for proteomics. Anal Bioanal Chem 2003;376:952–65.

[27] Avilan L, Calcagno M, Figuera M, Lemus L, Puig J, Rodriguez AM. Interaction of *Leishmania mexicana* promastigotes with the plasminogen–plasmin system. Mol Biochem Parasitol 2000;110(2):183–93.

[28] Xolalpa W, Vallecillo AJ, Lara M, Mendoza-Hernandez G, Comini M, Spallek R, Singh M, Espitia C. Identification of novel bacterial plasminogen-binding proteins in the human pathogen *Mycobacterium tuberculosis*. Proteomics 2007;7(18):3332–41.

[29] Vassalli JD, Sappino AP, Belin D. The plasminogen activator/plasmin system. J Clin Invest 1991;88(4):1067–72.

[30] Yang Z, Eton D, Zheng F, Livingstone AS, Yu H. Effect of tissue plasminogen activator on vascular smooth muscle cells. J Vasc Surg 2005;42(3):532–8.

[31] Schaumburg J, Diekmann O, Hagendorff P, Bergmann S, Rohde M, Hammerschmidt S, et al. The cell wall subproteome of *Listeria* monocytogenes. Proteomics 2004;4(10):2991–3006.

[32] Dudani AK, Mehic J, Martyres A. Plasminogen and angiostatin interact with heat shock proteins. Mol Cell Biochem 2007;300(1–2):197–205.

[33] Lind SE, Smith CJ. Actin accelerates plasmin generation by tissue plasminogen activator. J Biol Chem 1991;266(26):17673–8.

[34] Dudani AK, Ben-Tchavtchavadze M, Porter S, Tackaberry E. Angiostatin and plasminogen share binding to endothelial cell surface actin. Biochem Cell Biol 2005;83(1):28–35.

[35] Crowe JD, Sievwright IK, Auld GC, Moore NR, Gow NA, Booth NA. *Candida albicans* binds human plasminogen: identification of eight plasminogen-binding proteins. Mol Microbiol 2003;47(6):1637–51.

[36] Bergmann S, Rohde M, Hammerschmidt S. Glyceraldehyde-3-phosphate dehydrogenase of *Streptococcus pneumoniae* is a surface-displayed plasminogen-binding protein. Infect Immun 2004;72(4):2416–9.

[37] Roda O, Ortiz-Zapater E, Martínez-Bosch N, Gutiérrez-Gallego R, Vila-Perelló M, Ampurdanés C, et al. Galectin-1 is a novel functional receptor for tissue plasminogen activator in pancreatic cancer. Gastroenterology 2009;136(4):1379–90.

[38] Dassah M, Deora AB, He K, Hajjar KA. The endothelial cell annexin A2 system and vascular fibrinolysis. Gen Physiol Biophys 2009;28:20–8.