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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

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Abstract

Canine and feline cardiopulmonary dirofilariosis caused by *Dirofilaria immitis* is a chronic and potentially fatal disease. Adult worms live in the pulmonary arteries of infected immunocompetent hosts for years. The aim of the present study is the identification of the influence of the metabolic products (excretory/secretory antigens, DiE/S) of *D. immitis* on the vascular endothelial cells, because the vascular endothelium interplays in a direct manner with the parasite and their products. For this purpose, HAAE-1 vascular endothelial cells were treated with DiE/S, using non-treated cultures as negative controls. Significant increases in the COX-2, 5-LO expression and PGE_2 level were detected in the treated cells compared with the control cells. Moreover, DiE/S decreases monocyte transmigrations across vascular endothelial cell monolayers. Treatment with DiE/S does not have a cytotoxic effect and do not alter apoptosis, necrosis or cell cycle of vascular endothelial cells. These results suggest that the DiE/S stimulates the production of mediators and mechanisms that favor the survival of the parasite, in vascular endothelial cells, contributing to restrict vascular and lung damages in the infected host, without altering the basic physiologic processes of endothelial cells.

1. Introduction

Dirofilaria immitis is the causal agent of canine and feline cardiopulmonary dirofilariosis. Adult parasites lodge in the pulmonary arteries and the right ventricle of infected animals, where they can live for years producing microfilariae (McCall, 2001). Cardiopulmonary dirofilariosis is a chronic inflammatory disease. Initial damages occur in the pulmonary arteries (endarteritis and perivascular inflammation). These damages are key for the following development of the disease, affecting the lung parenchyma and the right cardiac chambers (Venco and Vezzoni, 2001). Besides this usual chronic way of the disease an acute development can appear when the simultaneous death of many worms, natural or as a consequence of a filaricidal treatment

occur, cause the exacerbation of the inflammatory reactions and the appearance of massive thromboembolisms (McCall et al., 2008).
Vascular endothelium is the key in the development of the inflammation because it produces inflammatory mediators (eicosanoids), controls different processes like the vascular tone, homeostasis, platelet and leukocyte adhesion and regulates the flow of the luminal contents to the perivascular tissues (Liu and Weller, 1992). The eicosanoids are lipid mediators generated during the arachidonic acid metabolism. Prostaglandins and thromboxanes are produced by cyclooxigenases (COXs) and leukotrienes by lipoxygenases (LOs). Prostaglandin E2 (PGE2) is responsible for the activation of Th2-type anti-inflammatory response (Betz and Fox, 1991), while thromboxane B2 (TxB2) has the opposite effect, stimulating the Th1-type pro-inflammatory response and constricts blood vessels (Ball et al., 1986, Sala and Giancarlo, 1995). On the other hand, leukocytes migrate through of the wall of blood vessels. Among these, monocytes are, together with neutrophils, the key cells in inflammatory reactions stimulated by filarial parasites (Brattig et al., 2000, O'Connor et al., 2000).
During cardiopulmonary dirofilariosis the vascular endothelium is the tissue that receives directly the stimuli of the parasite antigens, nevertheless, very few studies to determine the implication of the endothelium in the pathogenic mechanisms of the vascular disease have been carried out. The consequences of the exposition of the endothelium to the *D. immitis* excretory/secretory products during the development heartworm disease was studied by Kaiser et al. (1989) and Mupanomunda et al. (1997). These authors demonstrated that the mechanisms of endothelium-dependent relaxation involve nitric oxide (NO) and a non-specified product of a cyclooxygenase activity, suggesting that *D. immitis* adult worms release pharmacological active factors that alter endothelial cell function.
Like other filarial species, *D. immitis* harbours endosymbiont *Wolbachia* bacteriae (Sironi et al., 1995). When adult worms die, massive amounts of parasite and bacteria antigens are released into the blood stream. The effects of these antigens on vascular endothelial cell cultures have been previously analyzed "in vitro" by Morchón et al. (2008).
Nevertheless, *D. immitis* adult worms are able to survive for years in immunocompetent hosts. During this time, the metabolic products of the parasites (excretory/secretory antigens) interact with the hosts tissues, modulating the immune response and the associated pathology, to achieve the parasite survival (Moreno and Geary, 2008, Bennuru et al., 2009).
The objective of the present work is to determine the effect of the excretory/secretory antigens of the *D. immitis* adult worms on the vascular endothelium, to simulate what occurs in cardiopulmonary dirofilariosis while the worms are alive in the pulmonary arteries, using the same "in vitro" model employed to test the activity of the somatic antigens of *D. immitis* on the vascular endothelial cells.

2. Materials and methods

2.1. Cell culture

Vascular endothelial cells HAAE-1 from ATCC (LGC Promochem) were grown in Ham's F12k medium (LGC Promochem ATCC) supplemented with 2 mM l-glutamine, 10% fetal bovine serum (FBS) (ATCC LGC Promochem), 50 U/ml penicillin, 50 μg/ml streptomycin, 0.1 mg/ml heparin (Sigma Chemical Co), 0.03 mg/ml endothelial cell growth supplement (ECGS) (Sigma Chemical Co). Plates were pre-coated with 0.1% pig gelatine (Sigma Chemical Co). Cells were cultured at 37 °C in a humidified atmosphere in the presence of 5% CO_2–95% air. Medium was changed every 3 days.

2.2. Reagents and stimulation of endothelial cells

Excretory/secretory antigens adult worm *D. immitis* (DiE/S) were prepared as previously described (Santamaría et al., 1995) and stored at −80 °C. In brief, live worms (25) obtained from a naturally infected dog were washed in sterile PBS pH 7.2 and incubated for 4 days in 50 ml of Eagle's minimum essential medium (MMEE) (Sigma Chemical Company, St. Louis, MO, USA) supplemented with 0.04% gentamycin and 0.01% nistatin, at 37 °C. The medium was changed daily. All fractions were mixed and concentrated by dialysis against 0.01% PBS pH 7.2 and filtered through an Amicon YC05 (Amicon Corporation Scientific System Division, Danvers, MA, USA). DiE/S was tested for the presence of endotoxin contamination using a quantitative *Limulus* amebocyte lysate test (LAL test QCL 1000; <0.4 U/mg protein; BioWhittaker, Walkersville, MD). The endotoxin quantity was under the sensitivity level of cell stimulation (<0.4 U/mg protein). Endothelial cells were treated as previously described (Morchón et al., 2008). In brief, endothelial cells (10^6 cells/plate) were plated on 100 mm culture plates and were grown for 4 days to obtain confluent cultures and treated with 1 μg/ml of DiE/S for 24 h. Non-stimulated cells were used as controls in the same conditions.

2.3. PGE_2, TxB_2 and LTB_4 assays

PGE_2, TxB_2 and LTB_4 concentration in the endothelial cells culture medium were measured by ELISA using PGE_2, TxB_2 and LTB_4 High Sensitivity Immunoassay kits (R&D Systems, Minneapolis, MN, USA) and following the commercial instructions as we previously described (Rodríguez-Barbero et al., 2006). The results are presented as the mean ± SEM of three experiments performed in duplicates.

2.4. Western blot analysis

Western blot analysis was performed as previously described (Morchón et al., 2008) for the COX2 and 5-LO expression. Treated and non-treated vascular endothelial cells were lysed in ice-cold lysis buffer (20 mM Tris–HCl (Ph 7.5), 140 mM NaCl, 10 mM ethylendiaminetetraacetic acid, 10% glycerol, 1% Igepal CA-630, aprotinin, pepstanin, pepstatin, and leupeptin at 1 μg/ml each, 1 mM phenylmethylsulfonyl fluoride, and 1 mM sodium orthovanadate). Protein samples (10 μg) were separated by SDS-PAGE under reducing conditions and blotted onto polyvinylidine difluoride membranes. Membranes were blocked before incubation with primary antibodies: anti-COX-2 (Santa Cruz Biotechnology Inc) and 5-LO (Chemicon International). After incubation with HRP-conjugated secondary antibodies, bands were visualized by a luminol-based detection system with *p*-iodophenol enhancement. Anti-α-tubulin antibody (Oncogene Research Products, San Diego, CA, USA) was used to confirm loading of comparable amount of protein in each lane. Protein expression was quantified by densitometry using Scion Image Software (Scion, Frederick, MD, USA).

2.5. Transmigration assay

Vascular endothelial cells were seeded at 10^5 cells per transwell insert (3-mm pore size; BD). Endothelial cell monolayers were stimulated with human TNF-α (10 ng/ml) 18 h before adding the monocytes. At the beginning of the assay, the lower chamber medium was replaced with serum-free RMPI medium containing 1 μg/ml DiE/S. Human monocytic cell line U937 was labeled with 2.5 mM fluorescent dye (CellTrackerOrange; Molecular Probes). Subsequently, 10^5 monocytes U937 were added to the upper chamber on top of the endothelial monolayer. After incubation for 4 h at 37 °C, the number of transmigrated cells in the lower chamber was measured using a fluorescence microplate reader at excitation/emission wavelength of 538/604 respectively.

2.6. Apoptosis, necrosis, and cytotoxicity assays

Annexin-V-FITC (Annexin-V) and propidium iodide (IP) were used to discriminate apoptotic from necrotic cells respectively with the Vibrant™ Apoptosis Assay Kit (Molecular Probes) by flow cytometry on a FACSort cytometer (Becton Dickinson Immunocytometry Systems, San Josè, CA). Cytotoxicity was assessed by the Toxilight BioAssay Kit (Cambrex, Verviers, Belgium) following the commercial instructions. This commercial kit quantitatively measures the release of adenylate kinase from damaged cells.

2.7. Statistical analysis

The results from the Western blots, ELISAs, apoptotic, necrosis and cytotoxicity measurements were analyzed with the Student's *t*-test. Cell proliferation and trans-endothelial exchange significance measurements (between-groups comparisons) were performed by ANOVA and corrected for repeated measurements when appropriate. If ANOVA revealed overall significant differences, individual means were evaluated post hoc using Bonferroni's procedure. All the results were expressed as the mean ± S.E.M. 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. The DiE/S from *D. immitis* adult worms stimulate both COX-2 and 5-LO expression, and the synthesis of prostaglandin E2 in vascular endothelial cell cultures

To determine whether DiE/S induces the synthesis of inflammatory mediators, proteins from DiE/S-treated vascular endothelial cells extracts were separated by SDS-PAGE and analyzed by Western blotting using anti-COX-2 or anti-5-LO antibodies. DiE/S induced a marked increase in COX-2 and 5-LO ($p < 0.05$) protein expression after 24 h of stimulation (Fig. 1A). Next we assessed whether DiE/S-induced COX-2 and 5-LO expression have a functional consequence such as prostanoids production. We analyzed the presence of PGE_2, TxB_2 and LTB_4 on endothelial cell supernatants stimulated with DiE/S during 24 h (Fig. 1B). DiE/S significantly increases only PGE_2 production (the arachidonic acid metabolic product through the COX-2) in endothelial cells ($p < 0.05$). No significant differences in TxB_2 and LTB_4 production in stimulated versus non-stimulated cells were observed.

3.2. DiE/S decrease the monocyte transmigration through vascular endothelial cell monolayers

To determine the effect of DiE/S on transmigration of monocytes, we used monocyte transference through a monolayer of vascular endothelial cells. DiE/S induced significant a decrease of monocyte transmigration. The values of fluorescence of monocytes transfer observed for 24 h compared with untreated endothelial cells showed a significant decrease ($p < 0.05$) (Fig. 2).

3.3. DiE/S are not cytotoxic and do not alter apoptosis, necrosis of vascular endothelial cells

To determine whether DiE/S induces cytotoxicity effect on vascular endothelial cells, cytotoxicity assay stimulated with DiE/S was analyzed by Toxilight BioAssay Kit for 24 h. DiE/S did not induce cytotoxicity effect on endothelial cells. Furthermore, to determine whether DiE/S induces apoptosis and necrotic cells on vascular endothelial cells, we employed the Vibrant™ Apoptosis Assay Kit (Molecular Probes) by flow cytometry on a FACSort cytometer (Becton Dickinson Immunocytometry Systems, San Josè, CA). We found no difference between the control cells and the ones treated with DiE/S for 24 h on apoptosis by Annexin-V and necrosis by IP.

4. Discussion

Like other helminth parasites, *D. immitis* can survive for long periods of time in immunocompetent hosts, in a location in which the worms are exposed to different types of cells and immune mediators. This form of life is possible because the parasites have developed efficient mechanisms to evade the immune response of the hosts (Hewitson et al., 2008). Related to this function, the metabolic products excreted by the worms have the capacity to modulate the host immune response and arrange the biological niches to achieve their survival (Bennuru et al., 2009).
The objective of the present work is to determine the influence of the excretory/secretory antigens of the *D. immitis* adult worms on the vascular endothelium, considering it is the tissue that first interacts with the antigenic products of the parasite and because of the importance it has in the control of the inflammatory reactions, simulating the events that happen in the pulmonary arteries while the *D. immitis* worms are kept alive.
The DiE/S antigens produce two fundamental effects on the vascular endothelial cells: an increase of two of the enzymes responsible for the synthesis of eicosanoides (COX2 and 5-LO) and a significant increase of the expression levels of PGE2, as well as a decrease of the transmigration of monocytes, without producing cytotoxic effects on vascular endothelial cells. Previously, simulating the effect of massive death of *D. immitis* adult worms (causing acute inflammatory pathology) on vascular endothelium, vascular endothelial cell cultures were treated with an antigenic extract of adult worms and with the dominant protein of the surface of *Wolbachia*. In both cases, a significant increase in the eicosanoids production, adhesion molecules and inflammatory mediators expression was observed, while a decrease in endothelial permeability was only induced by the *D. immitis* antigens (Morchón et al., 2008).
PGE2 is an eicosanoid responsible for different functions. It stimulates the T-helper 2 cells and the expression of IL-4 and IL-10, which determines the upregulation of the Th2-type anti-inflammatory response and the downregulation of the Th1-type pro-inflammatory response. In a previous work we also observed an increase of the production of PGE2 in vascular endothelial cell cultures treated with adults' somatic antigens of *D. immitis* (Morchón et al., 2008), probably because both extracts share antigenic molecules. Nevertheless, expression of PGE2 was not observed when the endothelial cells were treated with the *Wolbachia* surface protein (WSP) (Simón et al., 2008). These facts are consistent with the capacity to stimulate the Th2-type response attributed to *D. immitis* antigens and that of *Wolbachia* to stimulate the Th1-type response (Morchón et al., 2007a) in cardiopulmonary dirofilariosis.
PGE2 is also involved in the control of vascular function by producing vasodilation and reduction of permeability to molecules in vascular endothelial cell cultures (Birukova et al., 2007). PGE2 has been identified in different filarial infections and in all cases, their presence has been associated with facilitating mechanisms for the survival of the parasites. In experimental feline heartworm disease, PGE2 serum reached its maximum level 60 days pi suggesting an important role in the parasite survival during migration (Morchón et al., 2007b). Production of PGE2 has also been demonstrated in microfilariae of lymphatic filariae (Liu and Weller, 1992) and different

developmental stages of *Onchocerca volvulus* (Brattig et al., 2006). Likewise, this eicosanoid has been detected in monocytes adjacent to the worms in subcutaneous oncocercomas of hyporeactive individuals, which was interpreted as a mechanism of containment of the inflammatory reaction (Brattig et al., 2009). The fact that excretory/secretory products of *D. immitis* stimulate PGE2 production in vascular endothelial cells may be one way in which the parasite affects the environment in which he lives by promoting dilation of the arteries and inhibiting the inflammatory reaction.

In this work, in addition to the increase of PGE2 by vascular endothelial cells, a significant decrease of monocyte transmigration through monolayers of vascular endothelial cells treated with DiE/S was observed. This is consistent with the fact that the E/S antigens of *D. immitis* do not alter the expression of adhesion molecules (ICAM-1, VCAM-1, PECAM-1) in vascular endothelial cells (data not shown). Because monocytes and neutrophils are the most important cells in the host response against filarial parasites (Brattig et al., 2000, O'Connor et al., 2000), the excretory/secretory antigens could control perivascular inflammation, which is a key event in vascular pathology of the cardiopulmonary dirofilariasis.

In conclusion, we show that the excretory/secretory antigens of *D. immitis* adult worms influence vascular endothelial cells by stimulating vasodilation through the increased production of PGE2, and by limiting the transmigration of monocytes to the perivascular tissues. These activities seem to be clearly related to a facilitation of the survival of the parasites, while producing a control of the damages in the host. Future studies are needed to identify specific molecules that are responsible for these mechanisms.

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Figures

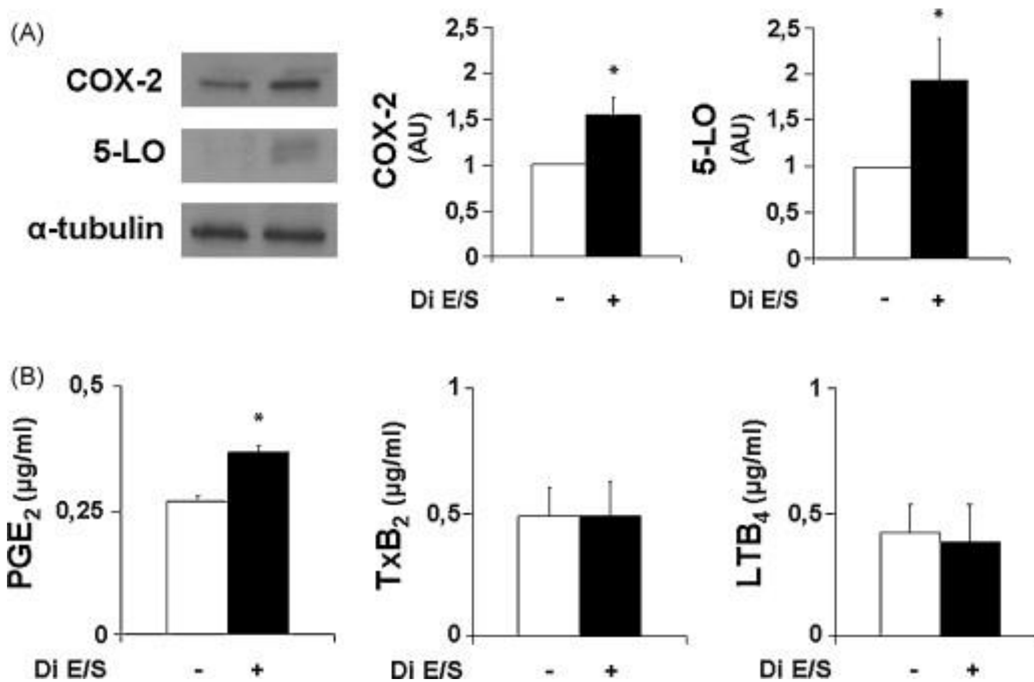

Fig. 1. Effects of DiE/S on the metabolism of arachidonic acid in human vascular endothelial cells. (A). Protein extracts from lysed DiE/S-untreated or treated confluent cell cultures were analyzed by Western blot for COX-2 and 5-LO. α-Tubulin served as a protein control. (B) Eicosanoids were measured by ELISA in the culture medium of endothelial cells untreated and treated with DiE/S. Results were expressed as the mean ± S.E.M. of, at least, three independent experiments. An asterisk (*) designates significant (p < 0.05) differences from control cells. Non-treated control cells (□). Stimulated endothelial cells (■).

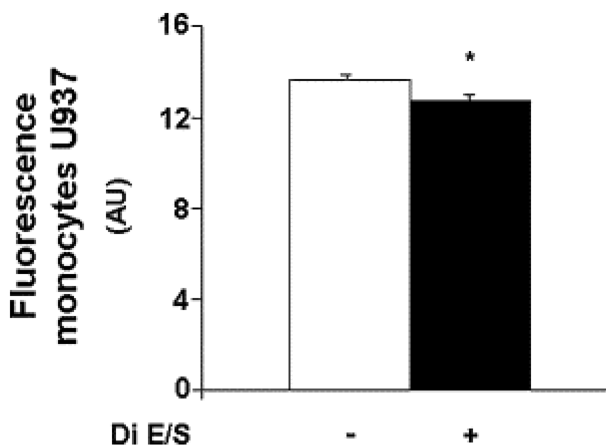

Fig. 2. Effects of DiE/S on transmigration assay to monocytes in human vascular endothelial cells. The number of transmigrated cells from DiSA-untreated (□) or treated (■) was measured using a fluorescence microplate reader at excitation/emission wavelength of 538/604 respectively. The graphic representation is expressed in arbitrary units (AU) as the mean ± S.E.M. of three different experiments in emitted fluorescence units. An asterisk (*) designates significant (p < 0.05) differences from control cells.