UNIVERSITY OF SALAMANCA

Angiogenic and antiangiogenic factors in diseases due to helminths

Ph.D Thesis

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Department of Animal Biology, Parasitology, Ecology and Soil Science-Agricultural Chemistry

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Factores angiogénicos y antiangiogénicos en helmintosis

Ph.D Thesis

Memoria que presenta Fariborz Shariati Sharifi
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Certify, that the present dissertation entitled “Angiogenic and antiangiogenic factors in diseases due to helminths” submitted to apply for PhD degree has been carried out by Fariborz Shariati Sharifi under our direction in the Department of Animal Biology, Parasitology, Ecology and Soil Science-Agricultural Chemistry, University of Salamanca, we considered that this dissertation comply the necessary requirements to be evaluated.

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Certifican que la presente Tesis Doctoral titulada “Factores angiogénicos y antiangiogénicos en helmintosis” que se presenta para optar al grado de Doctor por la Universidad de Salamanca ha sido realizada por Fariborz Shariti Sharifi bajo nuestra dirección el departamento de Departamento de Biología Animal, Parasitología, Ecología y Edafología-Química Agrícola de la Universidad, consideramos que reune los requisitos necesarios por lo que autorizamos su presentación para ser evaluada.

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Tesis Doctoral presentado por Fariboz Shariati Sharifi para la obtención del Grado de Doctor por la Universidad de Salamanca

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By time, Indeed mankind is in loss.
Except for those who have believed and
Done righteous deeds and advise each other
To truth and advise each other to patience.
(Holy Qur'an)

To My family

Maryam, my sons Amir and Shervin

That has been my pillar of support. I could not have completed this degree without their love, help, understanding and encouragement.

I regret my father did not live long enough to see me receive this degree.

I thank my mother for her support and for taking my answers for her favourite question "when are you going to come back?" with a good sense of humour.

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Abbreviations

Ang1	Angiopoietin 1
ANOVA	Analysis of variance
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid.
DMEM	Dulbecco's modified Eagle Medium.
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
EDTA	Ethylenediaminetetra-acetic acid
eg.	Exemple
ELISA	Enzyme-linked immunosorbent assay
F-ALK	Alkaline extract of adult *S. venezuelensis*
F-ES	Female excretory/secretory antigens of *S. venezuelensis*
FGF-2	Fibroblast growth factor-2
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HIF-1	Hypoxia-inducible factor 1
HSPG	Heparin sulfate proteoglycans
iNOS	Inducible nitric oxide synthase
i.p	Intraperitoneal
Ig	Immunoglobulin
IL	Interleukin
L3	Larval stage of nematodes
L3-ES	Excretory/secretory antigens of larvae the *S. venezuelensis*
L3-PBS	PBS-soluble extract antigen of larvae the *S. venezuelensis*
L-NAME	N^{w}-Nitro-L-arginine methyl ester
LPS	Lipopolysaccharide
lsrRNA	Large subunit RNA
mRNA	Messenger RNA
mtRNA	Mitochondrial RNA
MTT	3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide
NO	Nitric Oxide
nRNA	Nuclear RNA
OD	Optical density

PDGF	Platelet-derived growth factor
p.i.	Post-infection
PlGF	Placental growth factor
PMSF	Phenilmethysulfonylfonylfluoride
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RT-PCR	Reverse transcriptase polymerase chain reaction
Σ	Sum (statistic)
s.c.	Subcutaneous
SD	Standard deviation
SEM	Standard error of the mean
sp/spp	Specie/species
ssrRNA	Small subunit RNA
TNF	Tumour necrosis factor
tRNA	Transfer RNA
uv	Ultraviolet
VEGF	Vascular Endothelial Growth Factor

1 Introduction

Knowledge on angiogenesis control has been a major advance in the understanding of the pathogenesis of many diseases in the last decades. The studies about the role of angiogenic and antiangiogenic factors in the biology of neoplastic (tumor growth and development of metastases) are most abundant in the literature. However, both the generation and inhibition of new vessel formation plays an important role in other pathophysiological processes (eg. ischemia, infection and inflammation).

Firstly, we have divided the introduction in three sections: we briefly point out the basic concepts of angiogenesis and vasculogenesis, as well as their role in neoplastic and non-neoplastic diseases.

In a second section we study the characteristics of the two major angiogenic factors: vascular endothelial growth factor (VEGF) and fibroblast growth factor-2 (FGF-2). Specifically, the structural features of the above factors and the biological mechanism of action will be indicated. Also, antiangiogenic factors as endostatin, a potent inhibitor of endothelial cell proliferation, migration, angiogenesis and tumor growth will be studied.

Finally, we discuss the role of angiogenic and antiangiogenic factors in the pathogenesis of helminthic diseases. Until now, only very little information has been found and virtually confined to infection with *Schistosoma* spp, filarial species, *Taenia solium* and *Trichinella* spp.

1.1 Angiogenesis

Vertebrates, including mammalian species, have a closed circulation system for supplying nutrient and oxygen to the tissues in the body. The large and small blood vessels in this circulation system consist of two major cell types, one is the vascular endothelial cells lining the inside of the vessels (monolayer), and the other is the smooth muscle cells which regulate the contraction and dilation of blood vessels. The blood vessels are developed by several steps in embryogenesis, vasculogenesis (blood vessel formation from precursor cells), angiogenesis (blood vessel formation from pre-existing vessels and vascular endothelial cells), and vascular remodelling.

Angiogenesis, the process of new blood vessel formation from pre-existing ones, plays an important role in various physiological and pathological conditions, including embryonic development, wound repair, inflammation, and tumor growth (Carmeliet and Jain, 2000). Uncontrolled release of angiogenic growth factors and alteration of the production of natural angiogenic inhibitors, with a consequent modification of the angiogenic balance, are responsible for the uncontrolled endothelial cell proliferation that takes place during tumor neovascularization and in angiogenesis dependent diseases (Folkman, 2000).

Animal cells need oxygen and nutrients for their life and are there located within 100-200 µm of blood vessels with a limit for oxygen (Figure 1.1). For each organism that growth beyond this size, they must recruit new blood vessels by vasculogenesis and angiogenesis. Undoubtly blood vessels are essential for growing tumors; metastasize to another organ and also for treatment and deliver drugs to all regions of a tumor in effective quantities (Carmeliet, 2000).

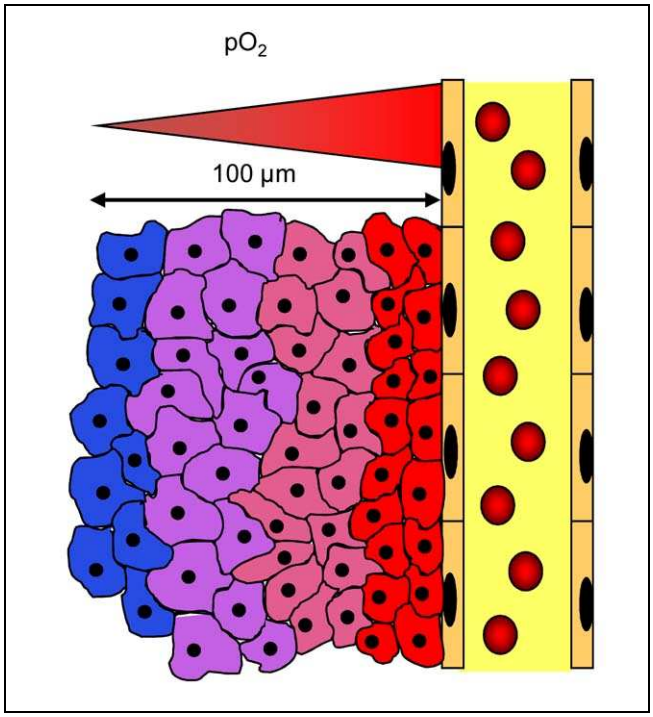

Figure 1.1: Oxygen gradient concentration in tissues

1.1.1 Angiogenesis in tumors

In 1971, Folkmann proposed that tumor growth and metastases depend on angiogenesis, and therefore blocking angiogenesis could be a strategy to stop tumor growth.

In the initial stages of malignant tumors there is a balance between the proliferation of neoplastic cells and their destruction, by both apoptosis and immune control. When the primary tumor reaches a size limit, the local concentration of oxygen decrease and then cells produce angiogenic factors. Moreover, tissue destruction leads to the next release of antiangiogenic substances. Given that the average life of anti-angiogenic factors is greater than the stimulant, initially tumor growth and metastasis development is controlled. However, when the balance is altered in favor of proangiogenic activity, takes place the formation of new vessels with the consequences noted in following sections.

Pro-angiogenic factors includes several molecules released by parenchymal or inflammatory cells in response to mechanical factors, metabolic (eg. hypoxia, acidosis) or immune response. Moreover, tissue destruction leads to generation of molecules with antiangiogenic potential. Table 1.1a and 1.1b include the main substances identified, based on the literature (O'Reilly *et al.* 1997, Sathornsumetee and Rich, 2007). The predominance of angiogenic factors leads to the formation of new blood vessels from endothelial precursors both local and mobilized from the bone marrow.

Tumor angiogenesis involves both blood vessels and the lymphatics vessels. Regarding to blood vessels can be observed differences in comparison with the normal vasculature. Thus, from a macroscopic point of view, the distribution is very disorganized with dilated tortuous vessels, an irregular diameter and excessive branching and shunts. Microscopically, the endothelium has numerous openings (fenestrate endothelial, vesicles, transcellular holes), are widened intercellular junctions and basement membrane is discontinuous or absent. The smooth muscle surrounding the endothelial cells does not contract in response to normal stimuli, which limits the use of vasoconstrictors in antitumor treatment. The main alterations of lymphatics vessels were compression of the localized within the tumor and dilation of those located in the periphery (by excess VEGF-C, see below). These enlarged lymph vessels can pick neoplastic cells derived from the surface of the tumor and thereby facilitate lymphatic metastasis.

Table 1.1a: Pro-angiogenic factor receptors their abbreviations and functions.

Factor/Receptor	**Abbreviations**	**Functions**
Vascular endotelial growth factors	VEGFs	Stimulates vasculogenesis Stimulates angiogenesis Increases vascular permeability Promotes leucocyte adhesion
Vascular endotelial growth factor receptors	VEGFRs	Integrate angiogenic and survival signal
Neuropilin-1	NRP-1	
Angiopoietin-1	Ang-1	Stabilize vessels Inhibit permeability
Angiopoietins receptor	Tie-2	
Platelet derived growth factor-BB	PDGF-BB	Recruit smooth muscle cells
Transforming growth factor β1	TGF-β1	Stimulate extracellular matrix production
Endoglin		
Fibroblast growth factor-2	FGF-2	Stimulates angiogenesis
Integrins αvβ3, αvβ5, α5β1		Receptors for matrix macromolecules and proteinases
VE-Cadherine	CD31	Endothelial junctional molecule
Ephrins		Regulate arterial/venous determination
Matrix metalloproteinases	MMPs	Remodel matrix, release and activate growth factors
Nitric oxide synthases	NOSs	Vasodilate Stimulate angiogenesis
Cyclooxygenase-2	COX-2	
Antigen 133	CD133	Angioblast (circulant endothelial precursor) differentiation
Inhibitors of differentiation 1/3	Id1/Id3	Determine endothelial plasticity

Table 1.1b: Anti-angiogenic factor receptors their abbreviations and functions.

Factor / receptor	**Abbreviations**	**Functions**
Soluble vascular endothelial growth factor 1	Soluble VEGFR-1	Sink for VEGF, VEGF-B
Angiopoietin 2	Ang-2	Natural antagonist of angiopoietin 1
Thrombospondin 1/2	TSP-1 /TSP2	Inhibits endothelial migration, growth, adhesion and survival
Angiostatin		Suppress tumor angiogenesis
Endostatin (collagen XVIII fragment)		Broad spectrum angiogenesis inhibitor
Vasostatin (Calreticulin fragment)		Inhibits endothelial proliferation
Platelet factor 4	PF-4	Inhibits binding of VEGF and FGF-2
Tissue inhibitors of metalloproteinases	TIMPs	Suppress pathological angiogenesis
Proteolytic fragment of metalloproteinase II	PEX	
	Meth-1 /Meth-2	Angiogenesis inhibitors that contain metalloprotease, thrombospondin and disintegrin domains
Cytocines	IFN-α, -β,-γ; IP-10, IL-4, IL-12, IL-18	Inhibits endothelial migration; Downregulate FGF-2
Prothrombin		Suppress endothelial growth
Prolactin		Inhibits VEGF and FGF-2
Vascular endothelial growth factor inhibitor	VEGFI	Inhibitor of endothelial growth and angiogenesis
Secreted protein, acidic, cysteine-rich	SPARC	Inhibit endothelial binding and activity of VEGF
Osteopontin		Interference with interin signaling

The vascular changes described in the previous section determine chaotic intratumoral blood flow with appearance of areas of hypoxia and acidosis, which can select cells that have lost their apoptotic response to hypoxia, which is then clonally, expand. Furthermore, the identified angiogenic factors can modulate the expression of cell adhesion molecules and other surface markers on tumor vascular endothelium. For example, VEGF and tumor necrosis factor-α (TNF-α) upregulate, while the growth fibroblast growth factor (FGF) and transforming growth factor-β1 (TGF-β1) downregulate adhesion molecules. Both phenomena (chaotic blood supply and non-uniform expression of adhesion molecules) explain the low and irregular interaction between leukocytes and endothelium in tumor vessels.

Finally, we note that the above phenomena depend on both the tumor type and the location in relation to the type of stromal cells present (Gohongi *et al*., 1999)

1.1.2 Angiogenesis in non-neoplastic diseases

Inflammation and hypoxia contribute to angiogenesis in non-neoplastic diseases. In a healthy adult, endothelial cells of blood vessels are in a quiescent state. In the presence of hypoxia or inflammation vascular remodeling occurs with formation of new vessels. One aspect of particular interest is that angiogenesis in non-neoplastic, unlike the neoplastic vessels, lead to the formation of blood vessels with a normal structure and function.

Hypoxia is therefore a very important stimulus of angiogenesis as other biological processes that attempt to restore normal tissue oxygenation (Figure 1.2). The basic mechanism is the activation of a protein called HIF-1 (hypoxia-inducible factor 1). HIF-1 is a heterodimeric protein formed by the junction of two subunits (HIF-1α and HIF-1β). While HIF-1β is constitutively expressed in all tissues, HIF-1α is activated in hypoxic conditions. HIF-1 acts on the promoter region of different genes that have a common response element (HRE, HIF-1 responsive element). The main genes activated by HIF-1 are:

- Genes encoding glucose transporters (GLUT-1) or enzymes of glycolysis. The result is a stimulation of anaerobic glycolysis and therefore an increase in lactate production.

- Erythropoietin gene in kidney. Increased release of erythropoietin stimulates the proliferation and differentiation of erythropoietic precursors.

- Gen of tyrosine hydroxylase, key enzyme in the synthesis of dopamine in the carotid bodies responsible for the circulatory and ventilatory response to hypoxia

- Genes related to angiogenesis such as VEGF, inducible nitric oxide synthase (iNOS), platelet-derived growth factor (PDGF) and angiopoietin 1 (ang1).

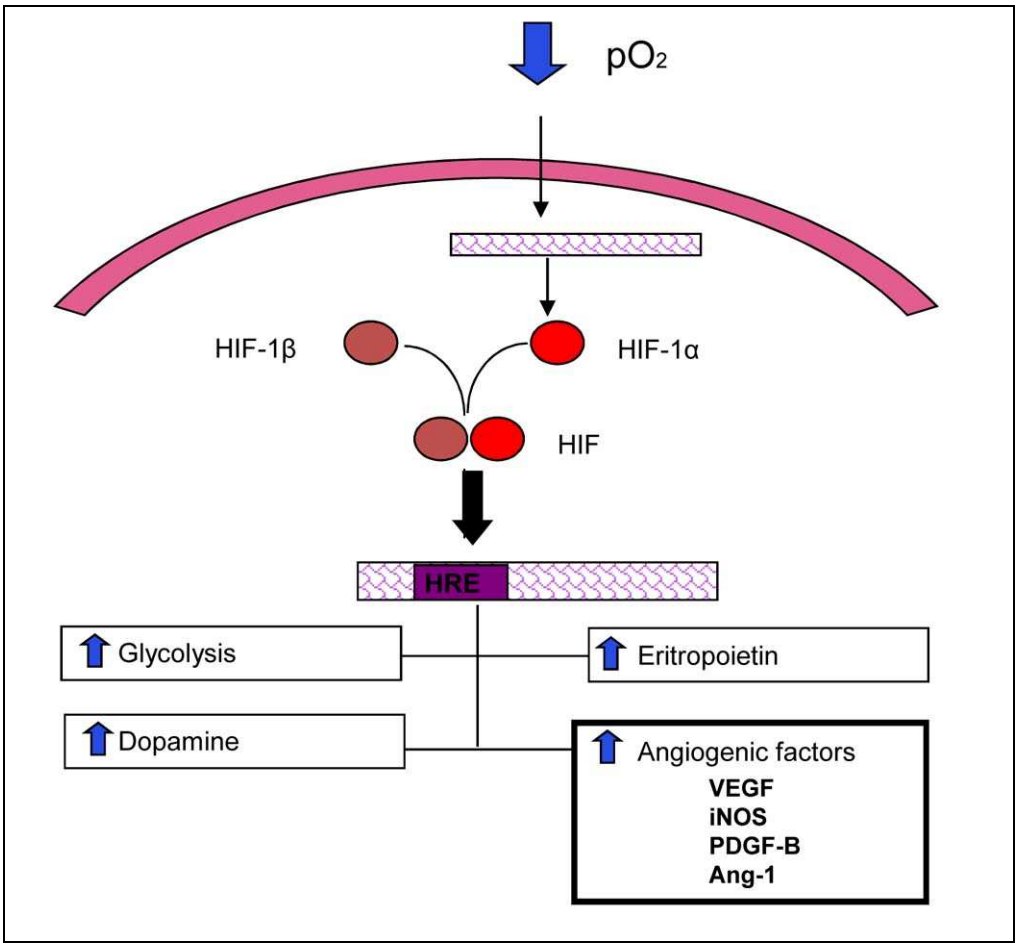

Figure 1.2. Integrative response to hypoxia.

Furthermore, during inflammation, there is recruitment and activation of blood cells to the focal lesion. Many of these cells (eg. macrophages, platelets, eosinophils, mast cells and other leukocytes) release pro-angiogenic factors summarized in Table Ia. In addition, professional phagocytes are capable of releasing proteases releasing angiogenic factors of the extracellular matrix.

As the previous data, angiogenesis changes such as excess or deficiency have been observed in multiple diseases where hypoxia or inflammation plays an important role. In particular there have been changes in angiogenesis in various types of systemic diseases, both autoimmune diseases (eg. rheumatoid arthritis, lupus erythematosus, systhemic sclerosis) (Carvalho *et al.,* 2007), metabolical diseases (diabetes mellitus) (Crawford *et al.,* 2009) or hematological diseases (Castleman´s disease) (Nishi *et al.,* 2000). Moreover, modifications in angiogenesis have seen detected in localized diseases: gastrointestinal tract (eg. Crohn's disease) (Carvalho *et al.,* 2007), eyes (age-related macular degeneration) (Bressler, 2009), central nervous system (ie. multiple sclerosis, Alzheimer's disease) (*Carvalho et al.,* 2007, Carmeliet *et al.,* 2002) and respiratory diseases (pulmonary emphysema, idiopathic pulmonary fibrosis) (Richter *et al.,* 2005) or kidney diseases (glomerulonephritis) (Foster, 2009).

1.2 Angiogenic and anti-angiogenic factors.

1.2.1 Vascular Endothelial Growth Factor (VEGF).

The vascular endothelial growth factors (VEGFs) are pleiotropic polypeptides produced by different cell types, including eosinophil (Horiuchi and Weller, 1997). From the early of 1980s that VEGF was identified independently as vascular permeability factor (VPF) and as vascular endothelial cell-specific growth factor in 1980s (Senger *et al.,* 1983*;* Leung *et al.,* 1989), more studies have been carried out about structure and function of these molecules. In this section, we review briefly the most important aspects of these molecules.

1.2.1.1 Structure of VEGFs

The VEGFs belong to the VEGF-PDGF (platelet-derived growth factor) super-gene family characterized by 8 cysteine residues are at the same position. 2 out of 8 cysteins generate intermolecular cross-linking, and other 6 cysteins make 3 intramolecular bands to form 3 loop structures (Figure 1.3) (Shibuya, 2001).

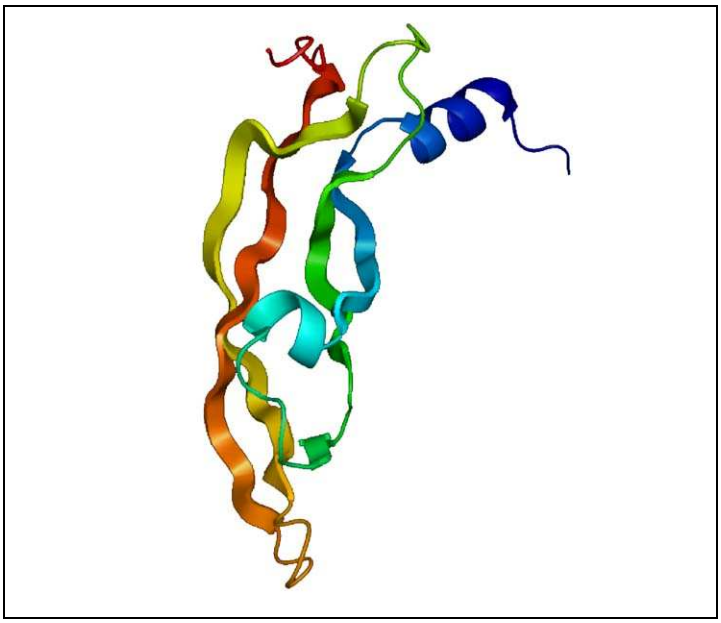

Figure 1.3: Monomer of VEGF-F

Seven molecules of VEGF family have been identified (VEGF-A, VEGF-B, VEGF-C, VEGF-D, placental growth factor (PlGF), VEGF-E and VEGF-F). In the mammals, only the first five have biological importance, VEGF-E is a viral protein [Orf-virus (open reading frame, in the genome of Orf virus)-derived VEGF] (Shibuya, 2003) and VEGF-F a protein derived from snake venom (Tokunaga *et al*., 2005).

Among angiogenic factors identified in humans, vascular endothelial growth factor-A (VEGF-A) appears to mediate the basic signalling of angiogenesis (often referred to as simply VEGF). Table 1.2 has shown genetic and functional differences of the five VEGF synthesized in mammals.

Table 1.2: Types of VEGFs.

	VEGF-A	**VEGF-B**	**VEGF-C**	**VEGF-D**	**PlGF**
Chromosome	6p23.1	11q.13	4q34	Xp22.31	14q.24
Isoforms	YES	YES	NO	NO	YES
Receptors	VEGFR1 VEGFR2 NP-1 NP-2	VEGFR1	VEGFR3	VEGFR3	VEGFR1 NP-1
Biologic function	Multiple	A little known -Coronary vessels	Lymphangiogenesis		Complementary of VEGF-A

VEGF-A exists as multiple isoforms resulting from alternative pre-mRNA splicing of eight exons (Ladomer *et al*., 2006). The isoforms are named according to the amino acid number of the monomer, generically termed $VEGF_{xxx.}$ The general nomenclature of these proteins include, in addition to the general designation (VEGF) ,the total number of amino acids ($_{xxx}$) and a suffix (b), if the alternative processing affects the distal portion of exon 8. The total number of amino acids derived mainly from the inclusion or not of amino acids encoded (totally or partially) by exons 6 and 7 (Figure 1.4).

These structural features have a clear biological significance. Thus, the inclusion or exclusion of exons 6 and 7 modulates interactions with heparan sulfate proteoglycans (HSPGs) and neuropilin receptors. Furthermore, proximal or distal processing of exon 8 has functional consequences, as $VEGF_{xxx}$ isoforms are proangiogenic and $VEGF_{xxx}$b are antiangiogenic.

The major human isoforms of VEGF-A are $VEGF_{206}$, $VEGF_{189}$, $VEGF_{189b}$, $VEGF_{165}$, $VEGF_{165b}$, $VEGF_{121}$ and $VEGF_{121b}$. In proangiogenic forms, the isoform that has a major biological importance is $VEGF_{165}$, followed by $VEGF_{121}$, $VEGF_{189}$ and $VEGF_{206}$.

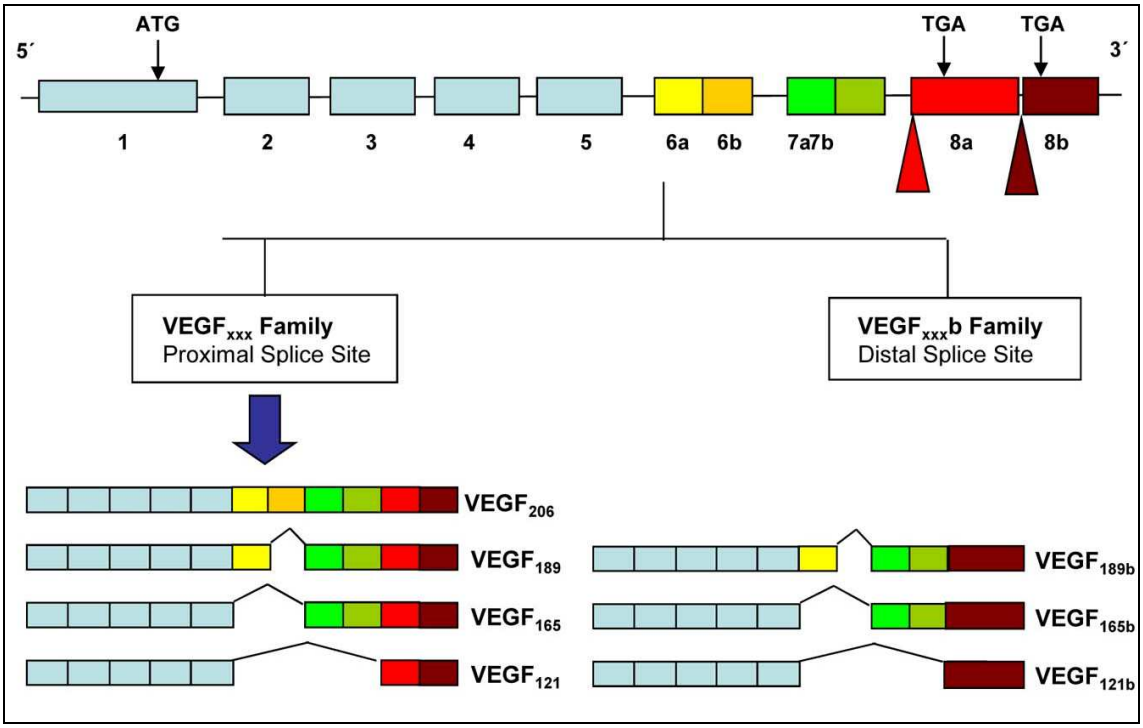

Figure 1.4. Isoforms of VEGF-A.

1.2.1.2 Biological effects of VEGF

VEGF-A exerts its biologic effect through interaction with cell-surface receptors. The receptors for VEGFs are 5 main types (Otrock *et al*., 2007): VEGF receptor-1 (*VEGFR-1; Flt-1*), VEGF receptor-2 (*VEGFR-2; Kinase insert domain-containing receptor/Flk-1*), VEGF receptor-3 (*VEGFR-3; Flt-4*), selectively expressed on vascular endothelial cells, and neuropilin receptors (NP-1 and NP-2), expressed on vascular endothelium and neurons (Figure 1.5). VEGF-A is capable of binding to all of them except for VEGFR-3.

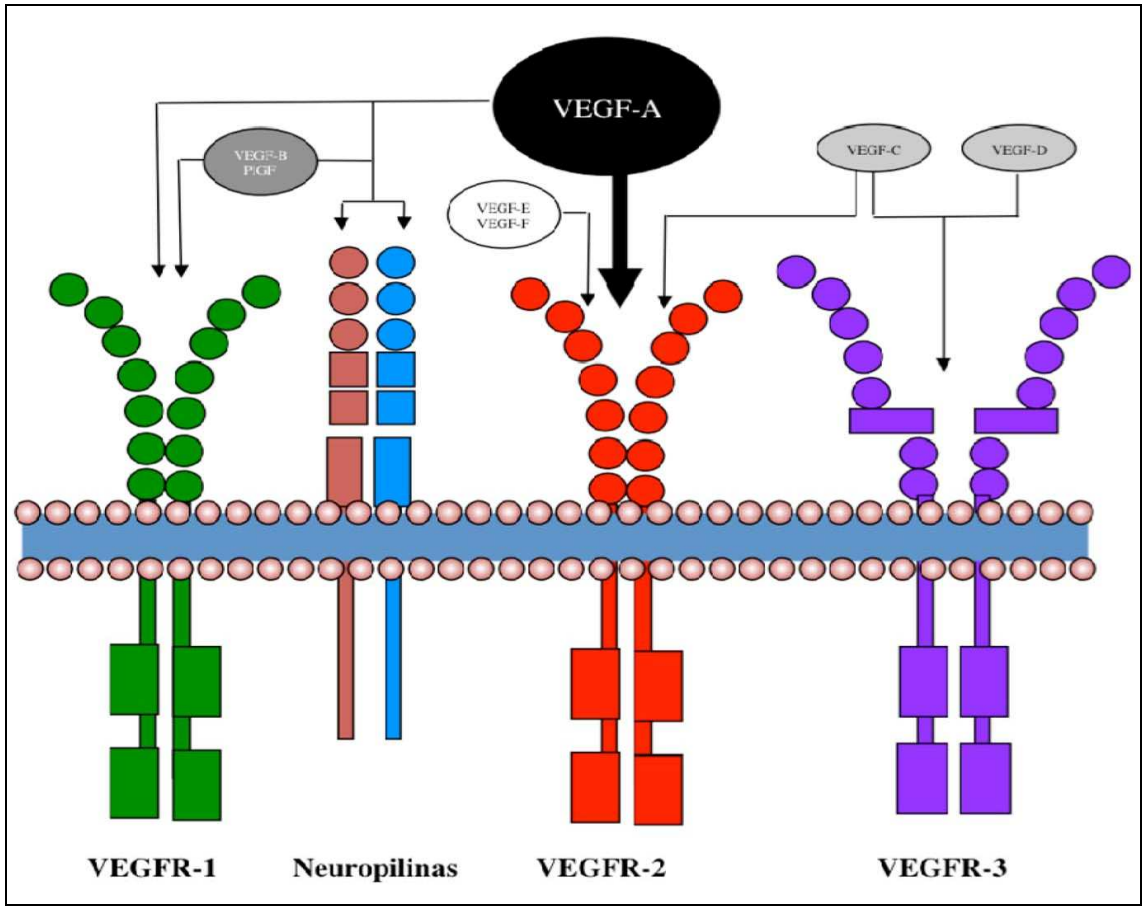

Figure 1.5. VEGFs receptors.

VEGFR-1, also called fms-like tyrosine kinase, Flt-, is a molecule constituted by 7 extracellular Ig-type domains, a transmembrane region and an intracellular tyrosine kinase domain. This molecule is expressed on endothelial cells, osteoblasts, mononuclear phagocytic system cells, placental trophoblast cells, mesangial cells and some hematopoietic stem cells (Zachary and Gliki, 2001). Although the affinity of VEGFR-1 by VEGF-A is 100 times greater than the rates for VEGFR-2, the tyrosine kinase activity of VEGFR-1 is lower. In embryogenesis, the expression of VEGFR-1 is linked to a negative effect on angiogenesis (Shibuya, 2001). In subsequent periods, the binding VEGF-A to VEGFR-1 appears to play an important role in the generation of paracrine signaling in endothelial cells.

VEGFR-2 (also called KDR: kinase-insert domain receptor or flk-1: fms-like kinase) has a structure very similar to VEGFR-1. This receptor is mainly expressed in

endothelial cells, but it has also been demonstrated in neurons, megakaryocytes, osteoblasts and hematopoietic cells. The main action of VEGF-A is exerted by interaction with this receptor.

The neuropilin 1 and 2 were identified as recipients of a family of molecules known as semaphorins/collapsin, a family of molecules regulating the development of the nervous system. Both are expressed during embryonic development in nervous tissue, cardiovascular, skeletal, while in adults its expression is very wide (Bielenberg, *et al*., 2006). The role of neuropilin, mainly of NP-1 is to act as a co-receptor for VEGFR-2 by increasing its biological action.

Multiple cell types are capable of trigger synthesis and release of VEGF-A in response to very different stimuli such as metabolic changes (hypoxia, hypoglycemia), hormonal (estrogens), cytokines and other growth factors, proinflammatory molecules and genetic disorders (Shibuya, 2003, Neufeld *et al*., 1999). One of the best characterized is hypoxia, which induces the stabilization and translocation to the nucleus of two HIF subunits. The union of HIF-1α and HIFβ interacting with a specific region of the VEGF gene (HRE, *hypoxia response element*) induces the synthesis of this molecule (Liao and Johanson, 2007). On the other hand, hypoglycemia, both in laboratory studies (Neufeld *et al*., 1999) and clinical settings (Dantz *et al*., 2002) leads to an increased synthesis of VEGF. Estrogens also play an important role in inducing VEGF production in both physiological situations (menstrual cycle, ovulation) and pathological (breast cancer) (Hyder and Stancel, 1999). Multiple cytokines and growth factors (Table 1.3) induce the production of VEGF (Neufeld *et al*., 1999).

Table 1.3. Cytokines that stimulates the production of VEGF.

Cytokine	**Abbreviations**
Fibroblast growth factor 4	FGF-4
Granulocyte-macrophage colony stimulating factor	GM-CSF
Insulin-like growth factor-1	IGF-I
Interleukin 1	IL-1
Interleukin 5	IL-5
Keratinocyte growth factor	KGF
Transforming growth factor ß	TGF-ß

Among the inflammatory mediators associated with the production of VEGF are oxygen free radicals (Brauchle *et al*., 1996) and nitric oxide (Chin *et al*., 1997). Finally, several kinds of genetic alterations present in tumour hereditary diseases or associated with overproduction of VEGF. The best characterized are associated with p53 mutations, the gene encoding the protein of von Hippel-Lindau and PTEN (phosphatase and tension homologue deleted on chromosome 10).

The interaction of VEGF-A and VEGFR-2 receptor triggers a series of initial signaling mechanisms that can be summarized into 5 phases (Schlessinger, 2000): (*i*) ligand binding to the receptor, (*ii*) receptor of dimerization; (*iii*) activation of tyrosine kinases, (*iv*) receptor of autophosphorylation and (*v*) binding and activation of adapters to autophosphorylation sites. The signalling pathway of VEGF/VEGFR-2 is different from other growth factors and is summarized in Figure 1.6.

Basically after the interaction between VEGF-A and VRGFR-2, four transduction pathways are activated: (*i*) Activation of PI3K (represented in purple/violet colours) that converts PIP2 into PIP3. This molecule acts in two ways on the effects of VEGF, first by inhibition of apoptosis (on BAD and caspase 9) and second by stimulating the nitric oxide production by endothelial isoenzyme action on the ONS. (*ii*) CDC42 activation displayed in share of green which eventually leads to a reorganization of the actin filaments, (*iii*) Diminished adhesion of endothelial cells (indicated in brown/orange) using as adapter molecule Src and (*iv*) activation of phospholipase C gamma (via represented in blue) starting from PIP2 generates DAG and IP3. The consequences of this approach are twofold: the generation of prostaglandins and activation of endothelial cell proliferation.

In summary, the four mechanisms by which VEGF leads to angiogenesis are: (i) increased cell survival, (*ii*) increase cell migration, (*iii*) stimulate of cell proliferation and (*iv*) increase of permeability.

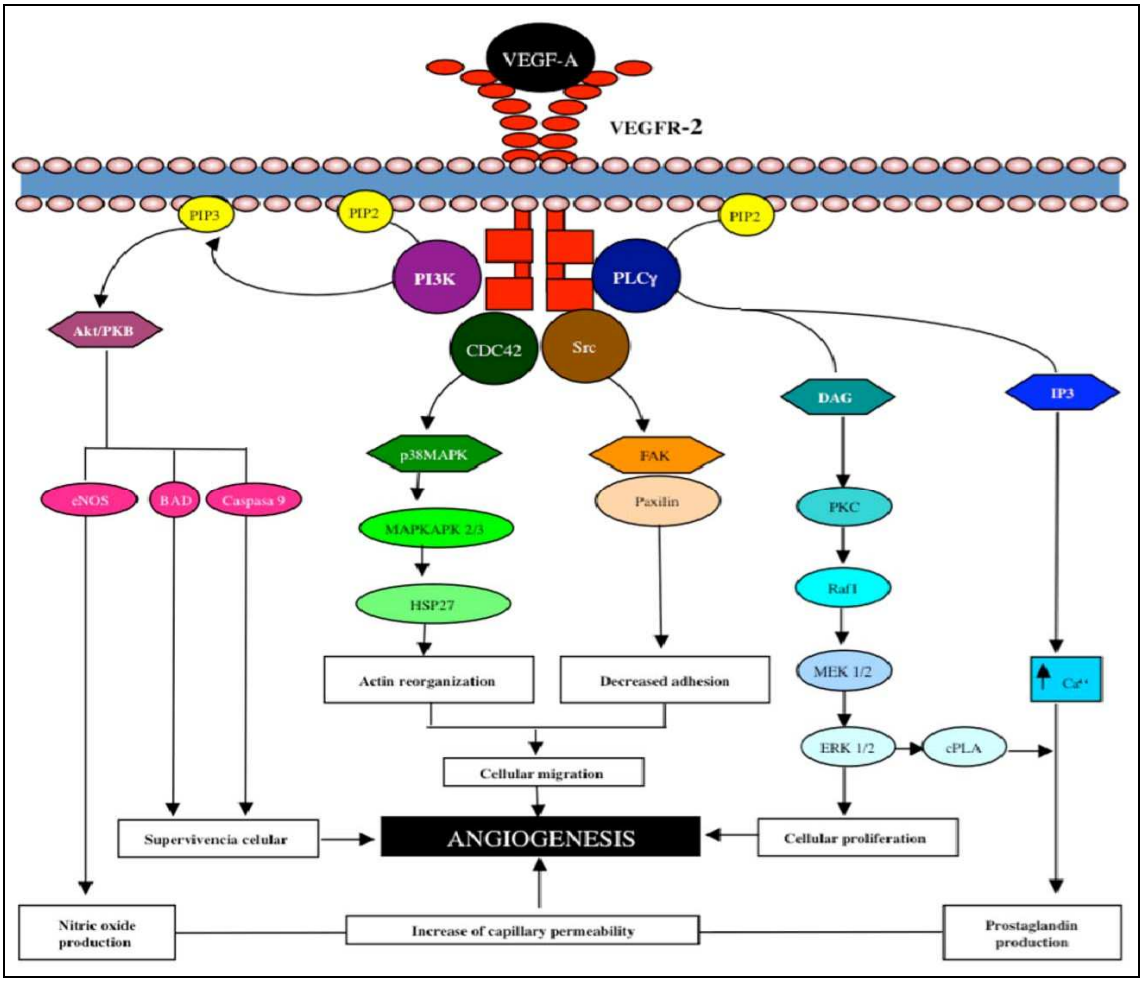

Figure 1.6. Signalling through VEGFR-2.

1.2.2 Fibroblast Growth Factor-2 (FGF-2)

FGF-2 or basic FGF (*fibroblast growth factor*) represents the best-characterized member of the larger family of heparin-binding growth factors. FGF-2 is a potent angiogenic molecule in vivo and in vitro stimulates smooth muscle cell growth, wound healing and tissue repair (Nugent and Iozzo, 2000). In addition, FGF-2 may stimulate hematopoiesis and may play an important role in the differentiation and/or function of the nervous system, the eye and the skeleton.

1.2.2.1 Structure of FGF-2

FGF-2 was firstly identified as a 146-amino acid that was later found a represent to proteolytic product of the primary 18 kDa heparin binding protein. Larger forms of FGF-2 have been identified with 22, 22.5, 24 and 34 kDa, resulting from alternate CUG-translation start sited. FGF-2 contains four cystein residues with no intramolecular disulfide bands, a large number of basic residues with an isoelectric point of 9.6 and two sites that can be phosphorylated by protein kinases A and C, respectively (Bikfalvi *et al*., 1997).

The interaction of FGF-2 with heparin protects this growth factor against heat or acid denaturation and protease cleavage. While heparin is only synthesized by connective tissue mast cells, heparin sulfate is widely distributed throughout all mammalian tissue and organs attached to core proteins as heparin sulfate proteoglycans (HSPG). HSPGs are a functionally diverse class of molecules found on cell surfaces and in the extracellular matrix where they have been shown to interact with FGF-2 and modulate its distribution and function. The specific sites on FGF-2 involved in binding to heparin and heparan sulfate were revealed from analysis of the crystal structure of FGF-2 (Figure 7).

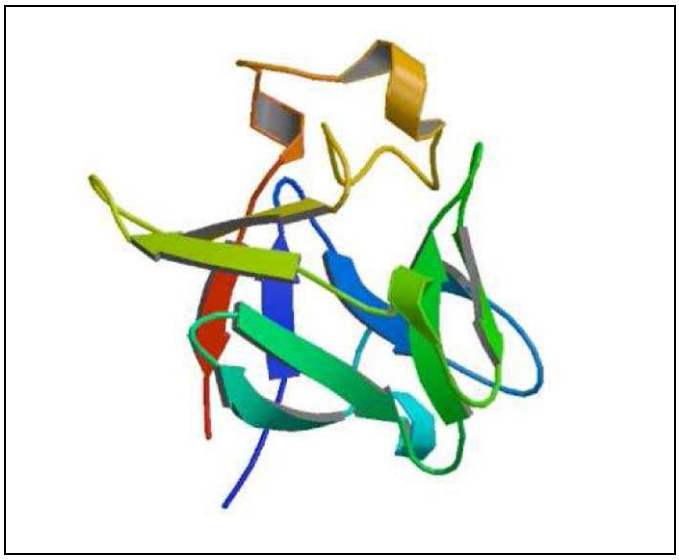

Figure 1.7: X-ray crystal structure of FGF-2

1.2.2.2 Biological effects of FGF-2

FGF-2 plays key roles in development, remodelling and disease states in almost every organ system (Table 1.4).

Table1.4: Functions of FGF-2.

Organ	**Putative functions**
Blood vessel	Angiogenesis Smooth muscle cell proliferation Blood pressure control Atherogenesis
Central nervous system	Neuronal differentiation and survival
Lung	Branching morphogenesis Fibrosis
Limb	Limb development
Muscle	Myogenesis
Bone	Stimulation of osteoblasts
Reproductive system	Spermatogenesis
Eye	Photoreceptor survival and transduction Stimulation of lens epithelial cells Proliferation of corneal
Skin	Melanogenesis Morphogenesis of the suprabasal keratinocytes

One of the best characterized activities of FGF-2 is its ability to regulate the growth and function of vascular cells such as endothelial and smooth muscle cells. FGF-2 has been implicated in the development and growth of new blood vessels (angiogenesis) and in the pathogenesis of vascular disease such as atherosclerosis (Figure 1.8). FGF-2 activity has generally been attributed to the activation of the intrinsic tyrosine kinase activity of its receptors. However, some study has indicated that FGF-2 can stimulate the dephosphylation of the cell surface HSPG syndecan-4, in the absence of its receptor.

FGF-2 has been localized in the nervous system in a variety of species. In human adult brain, strong staining for FGF-2 is observed in central nervous system and in cerebellar Purkinje cells. In the adult brain FGF-2 immunoreactivity is observed in astrocytes, in selected neural populations, and occasionally in microglial cells. In the human adult brain, all FGF-2 isoforms are found (18-, 22-, 24 and 34kDa) (Nugent and

Iozzo, 2000). Several functions for FGF-2 in the nervous system have been proposed (i.e. regeneration of the oligodendroglial lineage after demyelination or a trophic role in the catecolamin cell groups of the rat brain).

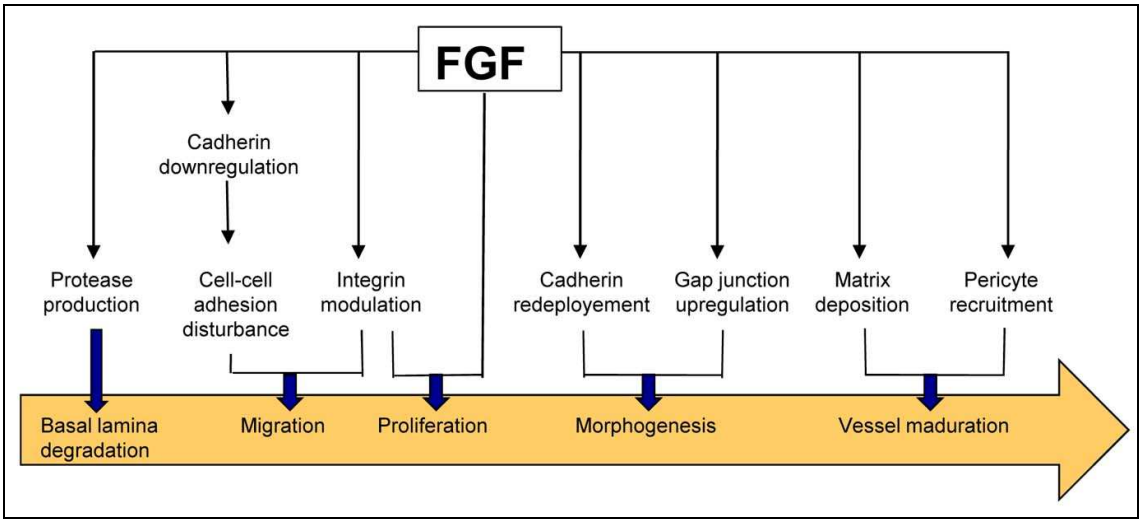

Figure 1.8: Mechanisms of FGF-2 induced angiogenesis.

In the lung, FGF-2 has been related with branching morphogenesis and development of pulmonary fibrosis **(**Han et al., 1992, Hetzel *et al.*, 2005**)**

FGF-2, as well as several other FGF family members such as FGF-4 and -8, stimulates limb development. It is, however, unlikely that FGF-2 is the prime candidate for limb formation as its expression pattern does not correlate with the tempor-spatial events occurring during limb generation. FGF-4 and -8 seem to be better candidates for endogenous limb-forming molecules (Olwin *et al*., 1994).

Development studies indicate that FGF may play an important role in muscle development. Disruption of FGF signaling by expression of a dominant negative FGFR2 result in gastrulation defects that are reflected in the lack of formation of the notochord and muscle (Utton *et al*., 2001). Even in embryos that show mild defects, muscle formation is impaired.

The proliferation, differentiation and TGF-β production of osteoblasts are stimulated by FGF-2 (Devescovi et al., 2008)

FGF-2 modulates basal and LH/human choriogonadotrphin (LN/hCG)-stimulated Leydig cell function (Laslett *et al*., 1997)

Several functions have been proposed for ocular FGF-2: (*i*) induction of retinal regeneration in vitro and protects photoreceptors from light damage, (*ii*) stimulation of lens epithelial cells to proliferate, migrate, and differentiate into fiber cells, (*iii*) proliferation of corneal endothelium (Wu et *al*., 2008).

The proliferation and differentiation of normal human melanocytes and several activities of keratinocytes are dependent on FGF-2 production (Berking *et al*., 2001).

1.2.3 Anti-angiogenic factors

Angiogenesis inhibitors are substances that inhibit the growth of new blood vessels. Angiogenesis inhibitors can be endogenous including proteins or fragments of proteins that are formed in the body (Ribatti, 2009) or exogenous such as drugs or dietary components.

1.2.3.1 Endogenous inhibitors of angiogenesis

The main endogenous inhibitors of angiogenesis are included in **Table 1.5**.

1.2.3.1.1 Angiostatin.

Angiostatin, a 38 kDa specific inhibitor of endothelial cell proliferation, is an internal fragment of plasminogen containing at least three of the kringles of plasminogen (Persano et al., 2007). Angiostatin was isolated from subclone of Lewis lung carcinoma in which the primary tumor inhibited the growth of its metastases. Angiostatin, generated by the primary tumor, was demonstrated to potently inhibit angiogenesis. Really, systemic therapy with angiostatin led to the maintenance of metastases in a microscopic dormant state defined by a balance of apoptosis and proliferation of the tumor cells.

Angiostatin inhibits EC migration and proliferation most likely through its ability to bind a cell surface ATP synthase, which may act as a receptor. Moreover, by inhibiting extracellular matrix (ECM)-stimulated plasminogen activation, it determines a decrease in endothelial cell invasion. Other studies revealed that angiostatin may induce apoptosis in EC, albeit at relatively high concentrations (Benouchan and Colombo 2005).

Table 1.5. Types o endogenous inhibitors of angiogenesis.

Origin	**Type**
Matrix derived	Anastellin
	Arresten
	Canstatin
	Endorepellin
	Endostatin
	Fibulin
	Targeting fibronectin-binding integrins
	Thrombospondin-1 and -2
	Tumstatin
Non matrix derived	Growth factors and cytokines
	Interferons
	Interleukins
	Pigment epithelium derived factor (PEDF)
	Fragments of blood coagulation factors
	Angiostatin
	Antithrombin III
	Platelet factor-4
	Prothrombin kringle 2
Others	2-Methoxyestradiol
	Chondromodulin
	Prolactin fragments
	Soluble Fms-like tyrosine kinase-1 (S-Flt-1)
	Tissue inhibitors of metalloproteinase (TIMPs)
	Troponin I
	Vasostatin

1.2.3.1.2 Endostatin.

Among the many inhibitors of angiogenesisis endostatin is one of the endogenous inhibitor that has been shown to inhibit the growth of a wide variety of tumors with no apparent toxic side effects. Of particular interest is the lack of acquired drug resistance exhibited by experimental cancers in mice when exposed to repeated doses of endostatin (O'Reilly *et al*., 1997).

Endostatin is a 20 kDa C-terminal cleavage product of collagen XVIII which belongs to the multiplexin family and is characterized by multiple triple-helix domains and interruptions (Figure 1.9).

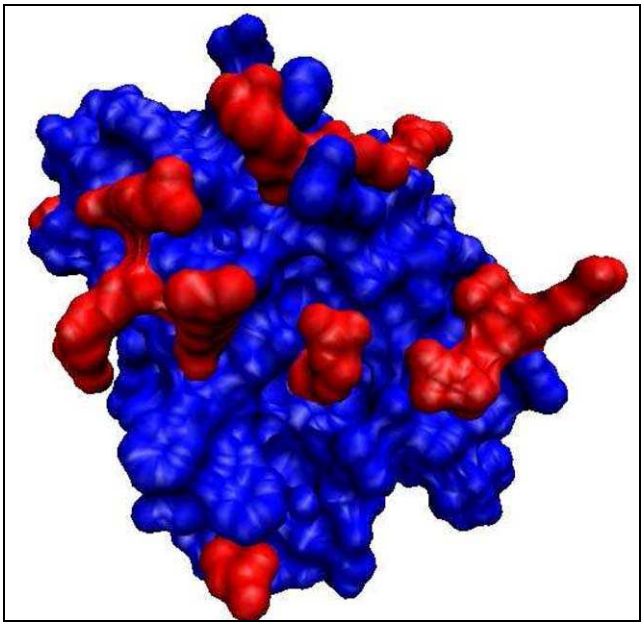

Figure 1.9. X-raycrystal structure of endostatin.

The generation of endostatin from collagen XVIII is catalyzed by proteolytic enzymes such as cathepsin L and matrix metalloproteases (Heljasvaara *et al*., 2005).

Like many other angiogenesis inhibitors, endostatin has an affinity for heparin. The crystal structure of endostatin reveals an extensive basic patch of 11 instances of arginine, which may serve as a binding site for heparin. This site has recently been shown to be involved in the inhibition of induced angiogenesis (Olsson *et al*., 2004). At the same time, heparin binding was not observed to play any role in the ability of endostatin to inhibit the migration of endothelial cells. Endostatin binds with low affinity to the heparin sulfate proteoglaycans, glypican-1 and glypican-4, and with high affinity to an unidentified molecule on endothelial cells.

Other experiment has also shown that endostatin associate with proteins such as α5 and α*v* integrins, fibulin, laminin-1 and tropomyosin. Furthermore, it has been reported that endostatin is internalized by endothelial cells, but not by mouse fibroblasts. Therefore, it is possible that endostatin may present its signaling directly in the cytoplasm without any need for a transducing member receptor.

The physiological functions of endostatin are comparably diverse. Recent studies show that endostatin inhibits renal epithelial cell branching morphogenesis, and may play a role in the regulation of ureteric arborization (Van Hensbergen *et al*., 2002).

The ability of endostatin to inhibit tumor growth and angiogenesis in vivo is demonstrated by extensive studies performed on distinct animal models in numerous laboratories throughout the world. Recently, Kalluri's laboratory could provide genetic proof that endostatin is an endogenous angiogenesis inhibitor and a tumor suppressor (Sund *et al*., 2005). When endostatin was knocked out, tumors grew 2-to 3-fold (200 to 300%) faster than wild type mice. In contrast, when endostatin was over-expressed by only 1.6-fold increase of the circulating level to mimic individuals with Down syndrome, tumors grew 3-fold slower than the wild type mice. Finally, some work has convincingly shown that endostatin-deficient mice exhibited increased angiogenesis. Endostatin levels are elevated in certain types of cancer, in intratumoral fluid and malignant ascites and in chronic inflammatory diseases such as rheumatoid arthritis and diabetic retinopathy.

1.3 Angiogenesis and helminthic diseases

Although the investigation about angiogenesis and tumor come back nearly 100 yearas ago, but there is existed a little information about the role of angiogenesis and angiogenic factors in helminthiasis, both in humans and in experimental models or laboratory studies.

Angiogenic factors are produced either by the parasite or the host can stimulate neovascularization through a number of different mechanisms (Figure 1.10). As an example, has been demonstrated that nematode parasite *C. elegans* encodes a factor capable binding mammalian VEGF receptors and inducing angiogenesis (Tarsitano *et al*., 2006). This is named *C. elegans* pvf-1 gene that codifies a PDGF/VEGF-like factor with a biochemical properties similar to vertebrate PDGF/VEGF growth factors. More important, pvf-1 binds to the human receptors VEGFR-1 (Flt-l) and VEGFR-2 (KDR) and is able to induce angiogenesis. The mechanisms related with host molecules can be divided into three categories; first of all, may be more important, potent angiogenic proteins such as vascular endothelial growth factors (VEGF) and the fibroblast growth factor (FGF-2) induce angiogenesis directly, by stimulation of endothelial cell proliferation, migration and differentiation into vascular tubes. Second, other angiogenic factors, such as interleukin-1, promote angiogenesis indirectly by stimulating inflammatory cells to produce VEGF. And third, angiogenic proteins such as heparinase are proteolytically cleaved, and the released peptides stimulate

components of the host's immune system, which in turn stimulate the production of other angiogenic factors such as VEGF and FGF-2 (Zcharia *et al*., 2001). Finally, some angiogenic factors stimulate other mechanisms involved in the final lesions. In fact, experimental data suggest that hypoxia in relation to VEGF may stimulate proliferation of synthesis of collagen type1 in activated myofibroblast-like rat hepatic stellate cells (Novo *et al*., 2007). They have shown VEGF and angiopoitin 1 (Ang-1) can operate as hypoxia-dependent, autocrine and paracrine factors able to stimulate nonoriented migration and chemotaxis of human myofibroblas-like rat hepatic stellate cells (HSC/MFs) through the activation of (Ras/Erk) signaling.

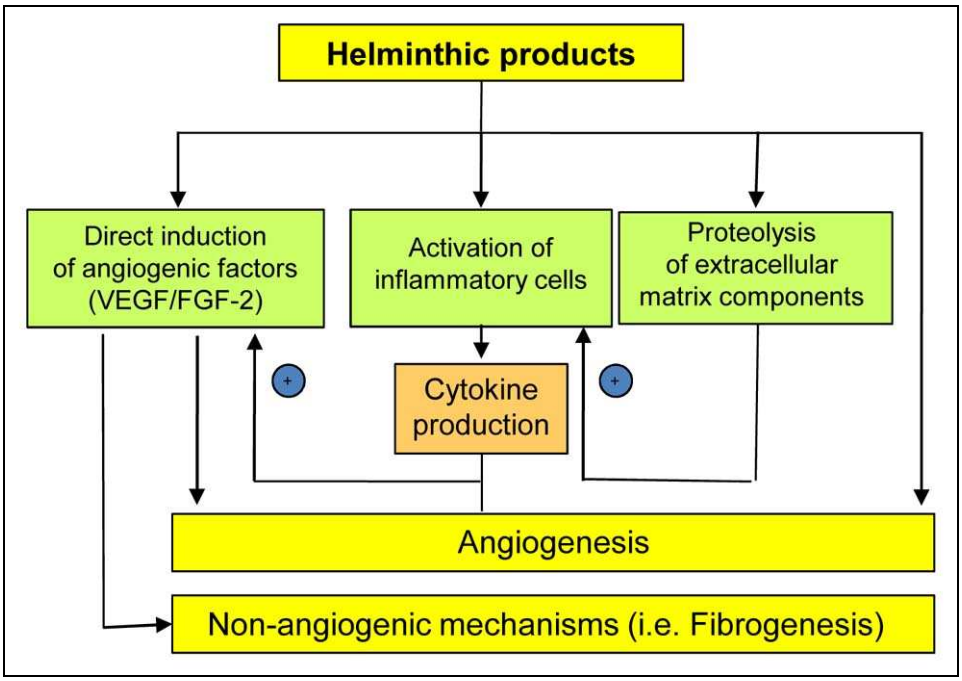

Figure 1.10. Helminths and angiogenesis

In practice, the main information about angiogenesis and angiogenic factors in helminthic diseases is limited to infections caused by *Schistosoma* spp, filarial, *Taenia solium* and *Trichinella* spp.

Schistosomiases are infections caused by different species of the genus *Schistosoma*. With the exception of cercarial dermatitis and the Katayama syndrome, the pathogenic basis of schistosomiasis is the formation of granulomas around parasite eggs.

Although information is scarce in the literature, we note that in the initial stages of schistosomiasis, there is a stimulation of angiogenesis. Thus, it has been clearly

demonstrated by histological and immunohistochemical techniques the presence of angiogenesis in several experimental models of schistosomiasis (Farah *et al*., 2000; Botros *et al*., 2008; Baptista and Andrade, 2005). In this context is interesting to note several aspects: (*i*) angiogenesis is an early phenomenon, so in evolutionated phases (such as those has been found in humans) is hard to objectify (Baptista and Andrade, 2005), (*ii*) an aspect importantly, demonstrated experimentally in the induction of angiogenesis, is the need for repeated exposure to the parasite (Farah *et al*, 2000) and (*iii*) genetic factors play an essential role in the angiogenic response to the parasite (Van de Vijver et al, 2006; Rutitzky *et al*., 2005).

The mechanisms by which *Schistosoma* spp triggers angiogenesis are of various types and have been obtained in experimental studies. Thus, several studies have identified specific *Schistosoma mansoni* egg-derived regulatory molecules that include lysophosphatidylserine and prostanoids. Also eggs secrete/excrete additional factors that are capable of stimulating endothelial cell proliferation, migration, p42/44 MAPK phosphorylation and cell sprouting directly. The effect of this factor is not restricted to endothelial cells, but also stimulated vascular smooth muscle cells (Kanse *et al*., 2005). On the other hand, *Schistosoma mansoni* soluble egg antigen (SEA) promotes proliferation, increased tube formation, decreased apoptosis and increased 2-fold messenger RNA for vascular endothelial growth factor (VEGF) of human umbilical vein endothelial cells (HUVECs) (Loeffler *et al*., 2002) These findings suggest that products secreted by schistosome eggs may promote angiogenesis within hepatic granulomas by up-regulating endothelial cell VEGF. Moreover, *Schistosome* eggs, attached to endothelium (Pearce *et al*., 2004) indirectly promote angiogenesis through hypoxia and inflammatory response. The main host-derived initiators of this inflammatory response include the early inflammatory cytokine, tumor necrosis factor-α (TNF- α) and the endothelial cell-adhesion molecule, intercellular adhesion molecule-1 (ICAM). At later stages, is developed periportal fibrosis by a mechanism that primes lymphocytes to produce increased levels of profibrotic molecules that include TGF-β and IL-4 (Farah *et al*., 2000). Finally, extravasation of the egg into the surrounding tissue and disruption of the portal stroma causes the migration of inflammatory cells to the site of egg deposition (Silva *et al*., 2006). The pre-existing portal stroma, showing normal CD34-immunoreactivity, is disrupted completely in and around the granulomas and is only preserved closely near to the portal vein and bile duct. Modulation of basement membrane components, proteoglycans, collagens and fibronectin imbalance

the connective stromal tissue matrix, has been described previously in schistosomal granulomas (Jacobs *et al*., 1998). Proteolysis of the dense extracellular matrix by collagens and matrix metalloprotinases (MMPs), a process preceding angiogenesis, seems necessary to allow the development of larger inflammatory reaction.While the endothelial cell proliferation fraction of a quiescent mature vasculature is 0.01% (Carmeliet and Jain, 2000), the presence of multiple proliferating cells in each granuloma shows the dynamic angiogenesis in schistosome induced inflammation.

At our knowledge, only two groups have studied the role of angiogenic factors in human schistosomiasis and their results are hardly comparable. Thus, Tawfeek GM et al., (2003) evaluate 90 patients with schistosomiasis related to *S mansoni* classified in five groups according to sonographic studies: infected lightly, infected heavily, intestinal, early hepatosplenic and periportal fibrosis. When compared with controls, they found that VEGF levels were significantly raised in all schistosomiasis patients groups except lightly infected and intestinal groups. Moreover, the level of VEGF correlated with disease progression from lightly infected to periportal fibrosis patients. Using a different study design, Toledo and colleagues measured serum levels of VEGF in patients with schistosomiasis with or without portal hypertension (de Toledo *et al*., 2009). These authors found no significant differences between both groups, although the average values in the group with portal hypertension were younger, a fact that coincides with low levels of VEGF detected in patients with cirrhosis from other causes (Shi *et al*., 2001).

Filariasis is diseases related with the infection of several genus and species of nematodes (*Wuchereria bancrofti, Brugia malayi, Brugia timori, Onchocerca volvulus, Loa loa, Mansonella perstans, Mansonella ozzardi* and *Mansonella streptocerca*). Altered angiogenesis has been described in only two types of filarial diseases: lymphatic filariasis (Pfarr *et al*., 2009) and onchocerciasis.

Clinical lymphatic filariases (and specifically infections related with *W. bancrofti)* are characterized with lymphoedema, lymph vessel dilation, lymph extravasation and, in some cases, the development of elephantiasis. There is some evidence of the role of angiogenic factors (and specifically of the family of VEGF) in the pathogenesis of lymphatic filariasis. Thus, in a longitudinal study in 63 Polynesian patients living in an hyperendemic focus of *W. bancrofti*, chyluria was associated with increased vascular endothelial growth factor (VEGF) levels, whereas elephantiasis presented a high

endothelin-1 (ET-1) profile **(**Esterre *et al*., 2005). On the other hand, host genetics is related with the clinical evolution of lymphatic filariasis. Specifically, in a cohort of lymphatic filariasis patients from Ghana, three VEGF-A promoter polymorphisms were examined (Debrah *et al*., 2008). The authors found that C/C genotype at -460 was significantly higher in hydrocele patients and in patients with high serum VEGF levels. Finally, in a cohort of bancroftian filariasis in Ghana, serum VEGF-C and sVEGFR-3 were elevated at basal evaluation (Debrah *et al*., 2006). Doxycycline-treated patients (for *Wolbachia* spp eradication) decrease VEGF-C and sVEGFR-3 at a level close to that of endemic normal values, with amelioration of supratesticular dilated lymphatic vessels and with an improvement of lymphatic pathology.

Onchocerciasis is characterized by two types of clinical manifestations: subcutaneous nodules (macrofilarial-related) and skin and ocular lesions (microfilarial-related). *O. volvulus* nodules in perfusion studies have different patterns of angiogenesis (Smith *et al*., 1988). Small nodules had an extensive blood supply, diffusely distributed throughout the nodule matrix, and in close association with the coils of the worms. In bigger nodules the central area appeared more dense, and intense vascularization appeared to be more peripheral; in the largest nodules the central core was not well vascularized, but a band of heavy vascularization was seen at the margin of the core, fed by superficial vessels and in close contact with worm coils. At least one derived *O. volvulus* protein (*Ancylostoma* secreted protein homologue) can contribute to abnormal angiogenesis (Higazi *et al*., 2003)

Neurocysticercosis is a common central nervous system (CNS) infection caused by *Taenia solium* metacestodes. In this infection is well-documented the importance of the granulomatous response in their pathogenesis. A small series of eight patients with neurocysticercosis subjected to craniotomy for histological and immunohistochemical analysis, shows dying parasite surrounded by a mature granuloma with associated fibrosis, angiogenesis and an inflammatory infiltrate. The most abundant cell types were plasma cells, B and T lymphocytes (Th1), macrophages, and mast cells (Restrepo *et al*., 2001). Moreover, an increased angiogenesis has been observed in animal models of neurocysticercosis (Sikasunge *et al*., 2009).

Trichinellosis is a nematode infection in which primarily affects striated muscle cells. Larvae of *Trichinella* spp initiate the transformation of myocytes into nurse cells that become surrounded by elaborate networks of blood vessels. In mice experimentally

infected by *Trichinella* spp several vessel abnormalities were detected (Baruch and Despommier, 1991). Thus, vascular complexes were found only around infected myocytes and were characterized by large circumferential vessels that give rise to the smaller channels of the retes. The secondary vessels vary widely in caliber and are distributed in a random fashion. Three types of network were found: simple, complex, and hypercomplex, and they were distributed normally, with the complex retes the most common. Comparison of the structure of the baskets with that of vessels in surrounding uninfected muscle strongly suggests that the vascular retes are the result of de novo angiogenesis induced during the infection. In this setting, excretory/secretory products of larval *T. spiralis* and *T. pseudospiralis* have been related with degenerative/ regenerative muscular changes and angiogenesis (Ko *et al*., 1994).

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2 Hypothesis and objetives

In the previous review we observed that: *(i)* angiogenesis is an important process of new blood vessel formation not only in tumor diseases but also in the pathogenesis of other diseases, specifically due to helminths *(ii)* studies on the role of angiogenesis in disesases due to helminths are scarce *(iii)* there is no bibliographical information on the production of angiogenic factors by macrophages or the use of anti-angiogenic factors in helminth infections *(iv)* although it has demonstrated the role of nitric oxide in these diseases and there is a documented relationship between nitric oxide and VEGF and other angiogenic agents, there is no specific bibliographical information about the interaction of both inflammatory mediators in these parasitic diseases.

The objectives proposed in this Thesis doctoral are:

1 .- To evaluate the expression of angiogenic factors (VEGF and FGF2) in alveolar macrophages stimulated with different antigens from different stages of the life cycle of *Trichinella spiralis, Trichinella pseudospiralis, Strongyloides venezuelensis* and *Schistosoma mansoni*.

2. - To study the relationship between the expression of angiogenic factors and nitric oxide production using specific inhibitors of inducible nitric oxide synthase.

3. - Using in vitro models of helminth infections to evaluate the effect of anti-angiogenic factors (endostatin).

4. - Using experimental models of infection the strongyloidosis *S. venezuelensis* and schistosomiasis by *Schistosoma mansoni* to evaluate the effect of anti-angiogenic factors in the development of the disease, studying the mechanisms that may be involved.

5. - To analyze the production of angiogenic factors in sera of patients diagnosed with imported diseases due to helminths.

3 Investigation articles

3.1 Article 1.

Trichinella: Differential expression of angiogenic factors in macrophages stimulated with antigens from encapsulated and non-encapsulated species

Shariati F, Pérez-Arellano JL, López Abán J, Arefi M, Martínez Fernández AR, Muro A.

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Trichinella: Differential expression of angiogenic factors in macrophages stimulated with antigens from encapsulated and non-encapsulated species

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ABSTRACT

The newborn larval stage of *Trichinella spiralis* enters the host striated skeletal muscle cell resulting in the formation of the nurse cell. Vascular Endothelial Growth Factor (VEGF) was detected in cells in the area immediately surrounding the nurse cells. However, no data are available on the antigens involved, the role of other angiogenic factors or the relationship of angiogenesis with Nitric Oxide (NO) production.

Using macrophage cell culture we study the effect of different *Trichinella* L1 antigens from one encapsulated (*T. spiralis*) and one non-encapsulated (*Trichinella pseudospiralis*) on the expression of VEGF and basic Fibroblast Growth Factor (FGF2). Also, we investigate the relationship between the production of NO and angiogenic mediators. The results show that encapsulated and non-encapsulated *Trichinella* species are different in their capacity to stimulate the expression of VEGF and FGF2 from host macrophages. Finally, there is no relationship between angiogenic factors and NO production by *T. spiralis* antigen.

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1. Introduction

Angiogenesis, the process of new blood vessel formation from pre-existing ones, plays a key role in various physiological and pathological conditions, including embryonic development, wound repair, tumour growth and inflammation (Carmeliet and Jain, 2000). Angiogenesis is a multi-step process that begins with the degradation of the basement membrane by activated endothelial cells that will migrate and proliferate leading to the formation of solid endothelial cell sprouts in the stromal space. Then, vascular loops are formed and capillary tubes develop with formation of tight junctions and deposition of new basement membrane. Numerous inducers of angiogenesis have been identified, including the members of the vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factor-α and -β (TGF-α and -β), platelet-derived growth factor (PDGF), tumour necrosis factor-α (TNF-α), cytokines, chemokines and the fibroblast growth factor (FGF) family.

Vascular endothelial growth factor A (VEGFA) is a protein which in humans is encoded by the VEGFA gene, which is organized as eight exons separated by seven introns (Houck et al., 1991; Tischer et al., 1991). Alternative exon splicing was initially shown to result in the generation of four different isoforms ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$), having, respectively, 121, 165, 189 and 206 amino acids, after signal sequence cleavage. $VEGF_{165}$, the predominant isoform, lacks the residues encoded by exon 6, whereas $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, such as $VEGF_{145}$ and $VEGF_{183}$ (Neufeld et al., 1999). The VEGF gene has also been cloned in mouse and rat (Pagès and Pouysségur, 2005). The importance of a region of 1.2 kb was demonstrated for the mouse and the rat gene, while a bigger region of 2.362 kb was investigated in the human gene.

To date, more than 1200 PubMed-referenced papers related to FGFs and fibroblast growth factor receptors (FGFRs) in endothelial cells and during neovascularization have been published. FGF is found in almost all organs of the body and has been reported to play a fundamental role in various physiological processes, including growth, differentiation and injury repair. Basic fibroblast growth factor (FGF2), in particular, has been studied extensively. A major biological effect of FGF2 is induction of proliferation in cultured cells, including fibroblasts, endothelial cells and vascular smooth muscle cells (Klagsbrun and Edelman, 1989). As stated above, FGFs exert their biological activities by binding to high affinity tyrosine kinase FGFRs on the surface of target cells. *In vitro*, endothelial cells of different origin express FGFR1 (Bastaki et al., 1997; Javerzat et al., 2002) and, under some circumstances, FGFR2

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(Dell'Era et al., 2001), whereas the expression of FGFR3 or FGFR4 has never been reported in endothelium. Only a limited number among the 22 members of the FGF family have been investigated for their angiogenic potential *in vitro* and *in vivo*, the bulk of experimental data referring to the prototypic FGF1 and FGF2.

Nematode worms of the genus *Trichinella* are one of the most widespread zoonotic pathogens in the world. Infection by *Trichinella* sp. has been detected in domestic and/or wild animals of all continents, with the exception of Antarctica, where there is no record of the parasite (Pozio and Murrell, 2006). This global distribution of *Trichinella*, together with different cultural eating habits, represents the main factor favouring human infections in industrialised and non-industrialised countries. Major political and economic changes, revolutions and wars can contribute to an increase in prevalence among the human population (Murrell and Pozio 2000; Bolpe and Boffi, 2001; Marinculic et al., 2001; Djordjevic et al., 2003). Previously, all *Trichinella* infections occurring in animals and humans were attributed to *Trichinella spiralis*. Today, eight species (*T. spiralis, Trichinella nativa, Trichinella britovi, Trichinella pseudospiralis, Trichinella murrelli, Trichinella nelsoni, Trichinella papuae, and Trichinella zimbabwensis*) and three genotypes (*Trichinella* T6, T8 and T9) within two clades (encapsulated and non-encapsulated) are recognised in this genus (Pozio and Murrell, 2006).

In the majority of situations in which a new vessel forms, a hypoxic event initiates the process (Fong, 2008). Cells in the immediate vicinity of reduced oxygen tension, e.g., inflammatory and interstitial cells in connective tissue, up-regulate and secrete angiogenic cytokines (Brindle, 1993), the most important of which is vascular endothelial growth factor (VEGF). The newborn larval stage of *T. spiralis* enters the host striated skeletal muscle cell resulting in the formation of the nurse cell. VEGF was detected in cells in the area immediately surrounding the nurse cells and the continued presence of VEGF in nurse cells could maintain the constant state of hypoxia (Capo et al., 1998). In a previous study in our laboratory, we have demonstrated that *T. pseudospiralis* and *T. spiralis* induced the basal and stimulated NO production, respectively (Andrade et al., 2007).

The aim of the present work is to examine the effect of encapsulated and non-encapsulated *Trichinella* antigens on VEGF and FGF2 expression from macrophage cells. Moreover, we study the relationship between angiogenic factors and NO production using inhibitory molecules analogues of L-arginine.

2. Material and methods

2.1. Parasites and antigen preparation

Crude somatic extracts were prepared from *T. spiralis (STs)* (MFEL/SP/62/ISS48, GM-1) and *T. pseudospiralis (STps) (MPRO/SU/72/ISS13)* L1 as described earlier (Dea-Ayuela et al., 2001). Briefly, L1 larvae of *T. spiralis* and *T. psuedospiralis* were collected after artificial digestion of the carcasses of mice experimentally infected. Once washed ten times in PBS buffer, they were disrupted in a manual glass homogeniser followed by sonication 10 s/pulse in a Virsonic 5. The homogenised product was allowed to extract over night at 4 °C and then centrifuged at 20.000*g* for 30 min. The supernatant was collected and dialysed overnight at 4 °C against PBS. Protein concentrations in the resulting extracts were estimated using the Micro-BCA protein assay reagent kit (Pierce, Rockford, Illinois, USA). The antigens were aliquoted and stored at −20 °C until use.

2.2. Isolation of alveolar macrophages

Male Wistar rats weighing 250–300 g, from the Experimental Animal Service of the University of Salamanca were used for all experiments. Principles of Laboratory Animal Care, formulated by the National Society for Medical Research were used as guidelines for the use and care of animals. Alveolar macrophages were obtained by bronchoalveolar lavage as previously described (Espinoza et al., 2002). Briefly, a total of 15 ml of pyrogen free saline solution was instilled in five aliquots (3 × 4 ml) using a Teflon catheter (Becton Dickinson SA, San Agustin del Guadalix, Spain). The recovered fluid was filtered through sterile gauze and then centrifuged at 250*g* for 10 min at 4 °C and the supernatant was separated from the cell pellet. The latter was washed twice with PBS (pH 7.4) and the cells were resuspended at a concentration of 1×10^6/ml.

2.3. Macrophage culture

Alveolar macrophages were cultured as previously described (Espinoza et al., 2002). The cells were resuspended in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum γ-irradiated (Sigma Chemical Co, St. Louis, MO) treated for 30 min at 56 °C, 2 mM glutamine (Sigma), 100 U/ml of penicillin and 100 μg/ml of streptomycin (Sigma). Alveolar macrophages (1×10^6/well) were plated on culture plates (Costar, Cambridge, MA, USA) and allowed to adhere for 2 h at 37 °C in 5% CO_2. Non-adhering cells were removed by gentle washing with complete medium and 1 ml of fresh complete medium was added for further cell culture. Alveolar macrophages were incubated alone (negative control, ø), with 10 μg/ml of lipopolysaccharide (LPS) (positive control) (Sigma) and different concentrations (0.1–50 μg/ml) of *STs*. After 18 h at 37 °C in 5% CO_2, the supernatant was collected, centrifuged at 500*g* for 10 min and stored at −80 °C until analysis. After removing the supernatant for nitrite determination, the cell viability was assessed by the mitochondrial reduction of MTT (3-[4, 5-dimethylthiazol-2-yl]-2.5-diphenyl tetrazolium bromide) (Sigma) to formazan as described by Kiemer and Vollmar (1997). Briefly, the alveolar macrophages were incubated with MTT (0.5 mg/ml) for 1 h at 37 °C and solubilised in dimethyl sulfoxide (DMSO) (Sigma). The extent of formazan production was determined photometrically at 550 nm. Following this, alveolar macrophages were also recovered, aliquoted and stored at −80 °C until analysis.

2.4. Detection of VEGF in rat alveolar macrophages by RT-PCR

Total RNA was extracted from alveolar macrophages using an RNeasy Mini Kit (Qiagen GmbH, Hilden Germany). A total of 1 μg RNA was used as template for the first-strand DNA synthesis (Roche). Primers specific for rat VEGF were designed according to (Yang et al., 2004; Liu et al., 2003). Primer sequence for VEGF was: sense, 5′-CTGCTCTCTTGGGTGCACTGG-3′ and anti-sense, 5′-CACC GCCTTGGCTTGTCACAT-3′, generating three bands of 601, 540 and 408 bp, corresponding to VEGF isoforms of 188, 164 and 120 amino acids; Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) sense, 5′-GGTCGGTGTGAACGGATTTG-3′ and GAPDH anti-sense, 5′-GTG AGCCCCAGCCTTCTCCAT-3′ generating a 452 base pairs (bp) PCR product. PCR reactions were carried out through reverse transcription incubation at 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min and a single cycle at 72 °C for 7 min. PCR products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide and quantification of RT-PCR products (VEGF and GAPDH) was carried out by densitometry with ImageJ computer program, available at http://rsb.info.nih.gov/ij/. The cDNA bands for VEGF were isolated from the gel, cloned into the pGEMT vector and sequenced in an automated sequencing system (ABI Prism 3100 Genetic Analyzer, Applied Biosystems) using universal and reverse primers. The sequences where compared with National Center for Biotechnology Information (NCBI) nucleotide BLAST searches (http://www.ncbi.nlm.nih.gov/BLAST). All experiments at least have been done by triplicate.

2.5. Detection of VEGF by ELISA

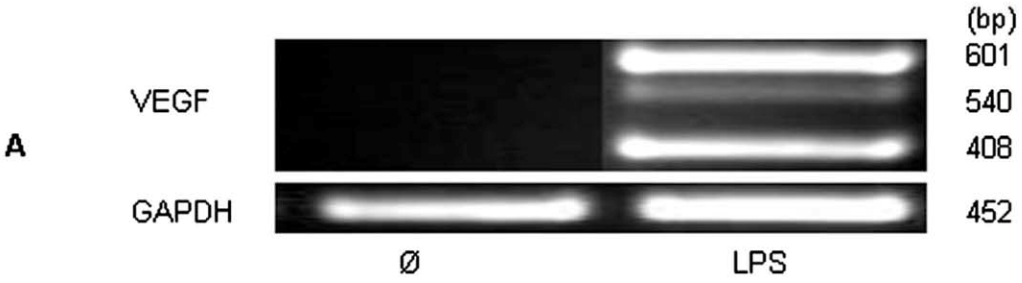
Soluble VEGF levels in supernatant of alveolar macrophage cultures were detected with the RayBio® Rat VEGF ELISA Kit (Ray Biotech, Inc, Norcross GA, USA) according to the protocol suggested by the manufacturer.These determinations were performed in duplicate culture and three fold measures. The concentration of VEGF was calculated from standard curves using known concentrations of rat recombinant VEGF.

2.6. Detection of FGF2 in alveolar macrophages by RT-PCR

Total RNA was extracted from the alveolar macrophages using an RNeasy Mini Kit (Qiagen Inc.). A total of 1 μg RNA was used as template for the first-strand DNA synthesis. Primers specific for rat FGF2 were designed according to Jyo-Oshiro et al. (1999). Primer sequence for FGF2 was: sense, 5′GCCGGCAGCATCACTTCGC T-3′ and anti-sense, 5′CTGTCCAGGCCCCGTTTTGG-3′. PCR reactions were carried out through reverse transcription incubation at 94 °C for 2 min, 50 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min and a single cycle at 72 °C for 5 min. PCR products were analysed by electrophoresis in 1.5% agarose gel stained with ethidium bromide. The quantification of RT-PCR product (FGF2) was carried out by densitometry as above. Also GAPDH was used as an internal control. The cDNA bands for FGF2 were isolated from the gel, cloned into the pGEMT vector and sequenced as above. All experiments at least have been done by triplicate.

2.7. Inhibitors of NO production

N^{w}-Nitro-L-arginine methyl ester (L-NAME; Affinity, UK) was used as an inhibitor of all nitric oxide synthase (NOS) isoforms and L-canavanine (Sigma Chemical Co) was used as a specific inhibitor of inducible nitric oxide synthase (iNOS); Both inhibitors (at a final concentration of 10^{-2} M) were used to assess the specificity of the induction of NO production by the *T. spiralis* antigen.



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VEGF-a 70    TCACCGCCTTGGCTTGTCACATTTTTCTGGCTTTGTTCTATCTTTCTTTGGTCTGCATTC 129
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VEGF-b 1455  TCACCGCCTTGGCTTGTCACATTTTTCTGGCTTTGTTCTATCTTTCTTTGGTCTGCATTC 1396

VEGF-a 130   ACATCTGCTATGCTGCAGGAAGCTCATCTCTCCTATGTGCTGGCTTTGGTGAGGTTTGAT 189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1395  ACATCTGCTATGCTGCAGGAAGCTCATCTCTCCTATGTGCTGGCTTTGGTGAGGTTTGAT 1336

VEGF-a 190   CCGCATGATCTGCATAGTGACGTTGCTCTCCGACGTGGGCACGCACTCCAGGGCTTCATC 249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1335  CCGCATGATCTGCATAGTGACGTTGCTCTCCGACGTGGGCACGCACTCCAGGGCTTCATC 1276

VEGF-a 250   ATTGCAGCAGCCCGCACACCGCATTAGGGGCACACAGGACGGCTTGAAGATATACTCTAT 309
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VEGF-b 1275  ATTGCAGCAGCCCGCACACCGCATTAGGGGCACACAGGACGGCTTGAAGATATACTCTAT 1216

VEGF-a 310   CTCATCGGGGTACTCCTGGAAGATGTCCACCAGGGTCTCAATTGAACGGCAATAGCTGCG 369
             |||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||||
VEGF-b 1215  CTCATCGGGGTACTCCTGGAAGATGTCCACCAGGGTCTCAATTGGACGGCAATAGCTGCG 1156

VEGF-a 370   CTGGTAGACGTCCATGAACTTCACCACTTCATGGGCTTTCTGCTCCCCTTCTGTCGTGGG 429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1155  CTGGTAGACGTCCATGAACTTCACCACTTCATGGGCTTTCTGCTCCCCTTCTGTCGTGGG 1096

VEGF-a 430   TGCAGCCTGGGACCACTTGGCATGGTGGAGGTACAGCAGTAAAGCCAGGGTCCAGTGCAC 489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1095  TGCAGCCTGGGACCACTTGGCATGGTGGAGGTACAGCAGTAAAGCCAGGGTCCAGTGCAC 1036

VEGF-a 490   CCAAGAGAGCAGAA 503
             ||||||||||||||
VEGF-b 1035  CCAAGAGAGCAGAA 1022
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Fig. 1. (A) Effect of lipopolysaccharide (LPS) on the VEGF production by alveolar macrophages. Ø: Non-stimulated macrophages LPS: LPS-stimulated macrophages (10 μg/ml). (B) Sequence of VEGF fragments from LPS-stimulated macrophages (VEGF-a). The homology with *Rattus norvegicus* vascular endothelial growth factor A transcript variant 3, mRNA, ACCN: NM 001110334.1 (VEGF-b) was 99%.

2.5. Detection of VEGF by ELISA

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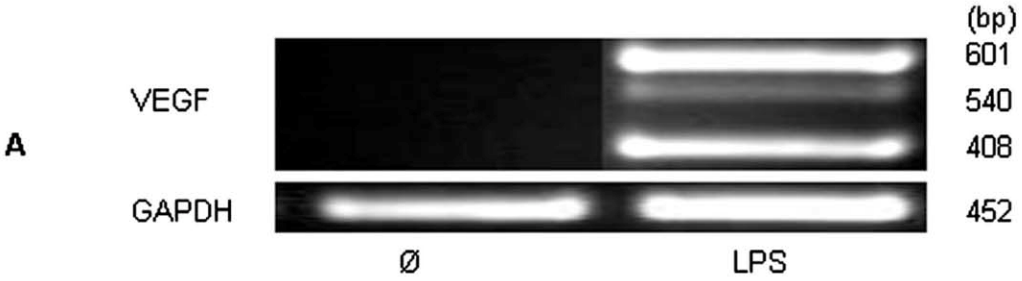
2.7. Inhibitors of NO production

N^{w}-Nitro-L-arginine methyl ester (L-NAME; Affinity, UK) was used as an inhibitor of all nitric oxide synthase (NOS) isoforms and L-canavanine (Sigma Chemical Co) was used as a specific inhibitor of inducible nitric oxide synthase (iNOS); Both inhibitors (at a final concentration of 10^{-2} M) were used to assess the specificity of the induction of NO production by the *T. spiralis* antigen.



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VEGF-a 70    TCACCGCCTTGGCTTGTCACATTTTTCTGGCTTTGTTCTATCTTTCTTTGGTCTGCATTC 129
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VEGF-b 1455  TCACCGCCTTGGCTTGTCACATTTTTCTGGCTTTGTTCTATCTTTCTTTGGTCTGCATTC 1396

VEGF-a 130   ACATCTGCTATGCTGCAGGAAGCTCATCTCTCCTATGTGCTGGCTTTGGTGAGGTTTGAT 189
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1395  ACATCTGCTATGCTGCAGGAAGCTCATCTCTCCTATGTGCTGGCTTTGGTGAGGTTTGAT 1336

VEGF-a 190   CCGCATGATCTGCATAGTGACGTTGCTCTCCGACGTGGGCACGCACTCCAGGGCTTCATC 249
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1335  CCGCATGATCTGCATAGTGACGTTGCTCTCCGACGTGGGCACGCACTCCAGGGCTTCATC 1276

VEGF-a 250   ATTGCAGCAGCCCGCACACCGCATTAGGGGCACACAGGACGGCTTGAAGATATACTCTAT 309
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1275  ATTGCAGCAGCCCGCACACCGCATTAGGGGCACACAGGACGGCTTGAAGATATACTCTAT 1216

VEGF-a 310   CTCATCGGGGTACTCCTGGAAGATGTCCACCAGGGTCTCAATTGAACGGCAATAGCTGCG 369
             |||||||||||||||||||||||||||||||||||||||||||||| ||||||||||||||
VEGF-b 1215  CTCATCGGGGTACTCCTGGAAGATGTCCACCAGGGTCTCAATTGGACGGCAATAGCTGCG 1156

VEGF-a 370   CTGGTAGACGTCCATGAACTTCACCACTTCATGGGCTTTCTGCTCCCCTTCTGTCGTGGG 429
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1155  CTGGTAGACGTCCATGAACTTCACCACTTCATGGGCTTTCTGCTCCCCTTCTGTCGTGGG 1096

VEGF-a 430   TGCAGCCTGGGACCACTTGGCATGGTGGAGGTACAGCAGTAAAGCCAGGGTCCAGTGCAC 489
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
VEGF-b 1095  TGCAGCCTGGGACCACTTGGCATGGTGGAGGTACAGCAGTAAAGCCAGGGTCCAGTGCAC 1036

VEGF-a 490   CCAAGAGAGCAGAA 503
             |||||||||||||
VEGF-b 1035  CCAAGAGAGCAGAA 1022
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Fig. 1. (A) Effect of lipopolysaccharide (LPS) on the VEGF production by alveolar macrophages. Ø: Non-stimulated macrophages LPS: LPS-stimulated macrophages (10 μg/ml). (B) Sequence of VEGF fragments from LPS-stimulated macrophages (VEGF-a). The homology with *Rattus norvegicus* vascular endothelial growth factor A transcript variant 3, mRNA, ACCN: NM 001110334.1 (VEGF-b) was 99%.

Polymyxin B, a specific inhibitor of NO production triggered by LPS, was used at 80 μg/ml to assess possible LPS derived contamination in the different antigenic preparations. All experiments at least have been done by triplicate.

2.8. Statistical analysis

The results of the VEGF protein detection were reported as arithmetic mean and standard error. Differences in groups were performed by analysis of variance (ANOVA). When global differences were detected, a post-ANOVA test using the Fisher LSD analysis was applied. Differences between means were considered statistically significant at $p < 0.05$. All statistical analyses were performed using Statworks and Statview 4.5 software packages for a Macintosh computer.

3. Results

Three VEGF isoforms were detected in alveolar macrophages stimulated with 10 μg/ml of LPS by RT-PCR (Fig. 1A). Alveolar macrophages expressed three bands of 601, 540 and 408 bp, corresponding to VEGF isoforms of 188, 164 and 120 amino acids. The PCR product was cloned in pGEMT vector and sequenced. This sequence presented 99 percent homology with VEGF from *Rattus norvegicus* (Fig. 1B).

Also FGF2 was detected in alveolar macrophages stimulated with 10 μg/ml of LPS by RT-PCR (Fig. 2A). Alveolar macrophages expressed a single band of 423 bp. The PCR product was cloned in pGEMT vector and sequenced. This fragment has 99 percent homology with FGF2 from *Rattus norvegicus* (Fig. 2B).

The effect of *in vitro* incubation of rat alveolar macrophages with different concentrations of *T. spiralis* antigens *(STs)* (0.1, 1.0, 10 and 50 μg/ml) on expression of VEGF is shown in Fig 3A. The results indicate that LPS-stimulated macrophages (10 μg/ml) and macrophages stimulated with *STs* from 1 μg/ml induce VEGF mRNA expression in a dose-dependent manner until 10 μg/ml when compared with VEGF mRNA levels detected in non-stimulated macrophages. The effects of different concentrations of *STs* (0.1, 1.0, 10 and 50 μg/ml) on expression of FGF2 in rat alveolar macrophages are shown in Fig 3B. The results

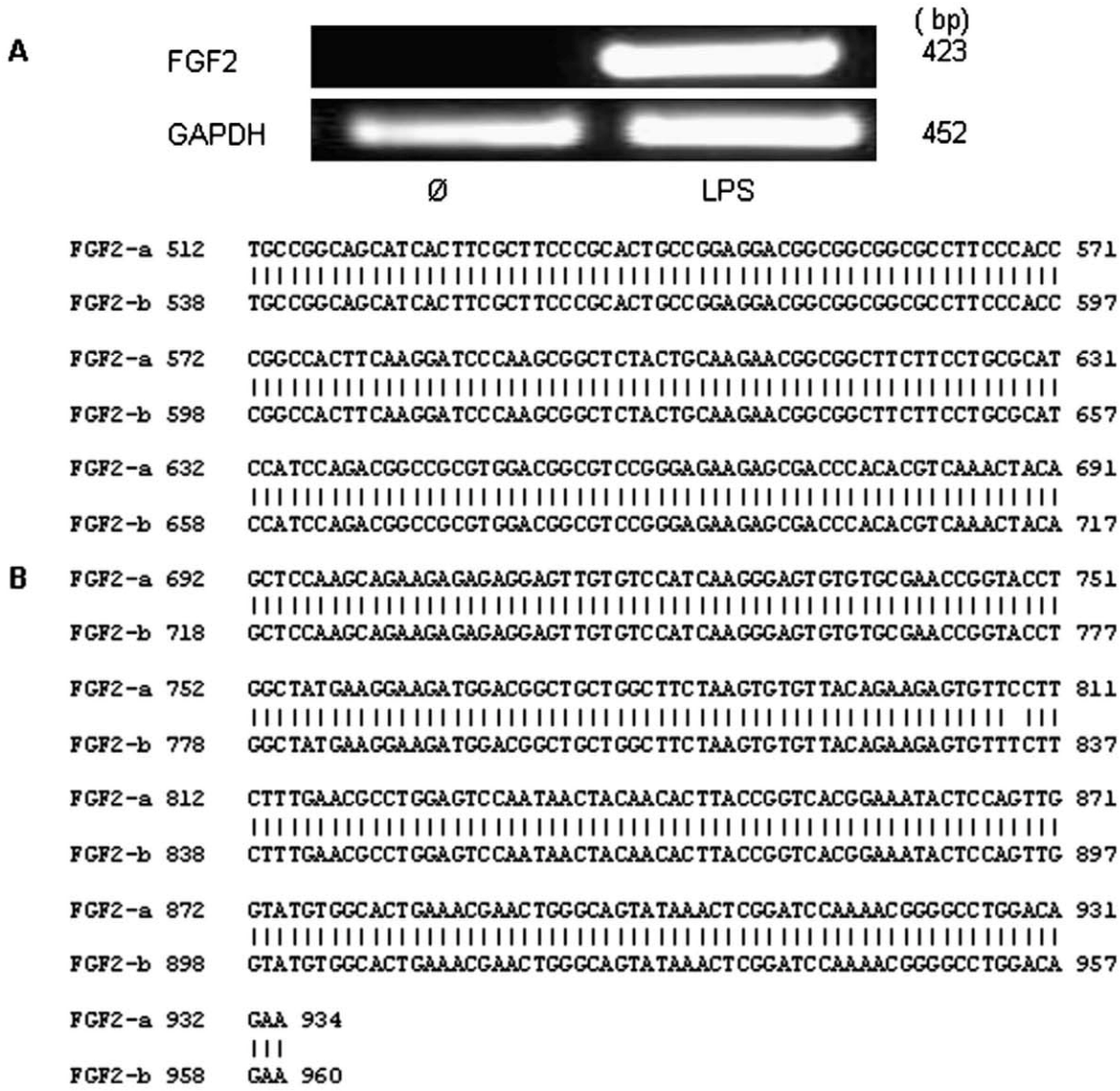

Fig. 2. (A) Effect of lipopolysaccharide (LPS) on the FGF2 production by alveolar macrophages. Ø: Non-stimulated macrophages LPS: LPS-stimulated macrophages (10 μg/ml). (B) Sequence of FGF2 fragment from LPS-stimulated macrophages (FGF2-a). The homology with *Rattus norvegicus* fibroblast growth factor 2, mRNA, ACCN: NM 019305.2 (FGF2-b) was 99%.

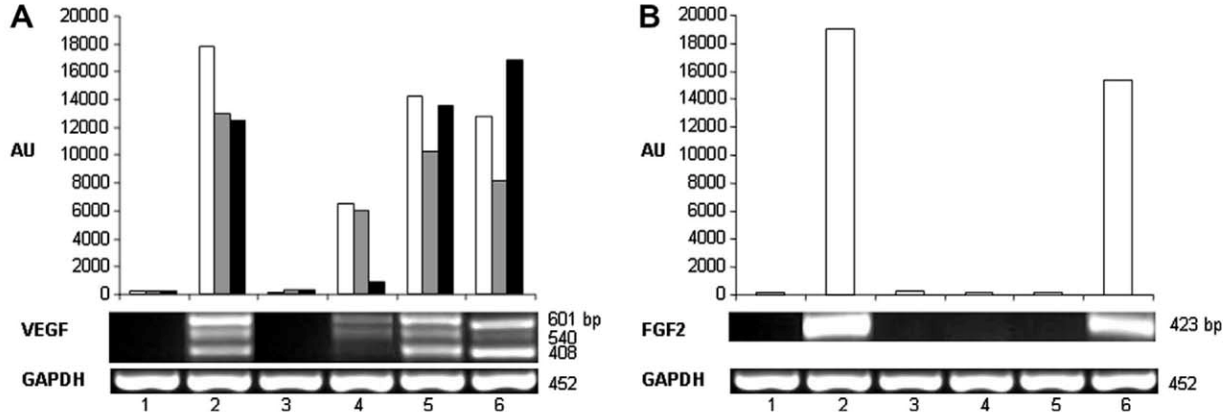

Fig. 3. (A) VEGF expression in rat alveolar macrophages induced by *Trichinella spiralis (STs)* antigen detected by RT-PCR. GAPDH mRNA expression levels from rat alveolar macrophages were used as internal control. (1) Non-stimulated macrophages (negative control), (2) LPS-stimulated macrophages (positive control), (3–6) *STs*-stimulated macrophages with 0.1, 1, 10 and 50 μg/ml. AU: arbitrary units calculated by densitometry of corresponding amplicons. 601 bp VEGF band (white bars), 540 bp VEGF band (grey bars) and 408 bp VEGF band (black bars). (B) FGF2 expression in rat alveolar macrophages induced by *Trichinella spiralis (STs)* antigen detected by RT-PCR. GAPDH mRNA expression levels from rat alveolar macrophages were used as internal control. (1) Non-stimulated macrophages (negative control), (2) LPS-stimulated macrophages (positive control), (3–6) *STs*-stimulated macrophages with 0.1, 1, 10 and 50 μg/ml. AU: arbitrary units calculated by densitometry of corresponding amplicons. 423 bp FGF2 band (white bars).

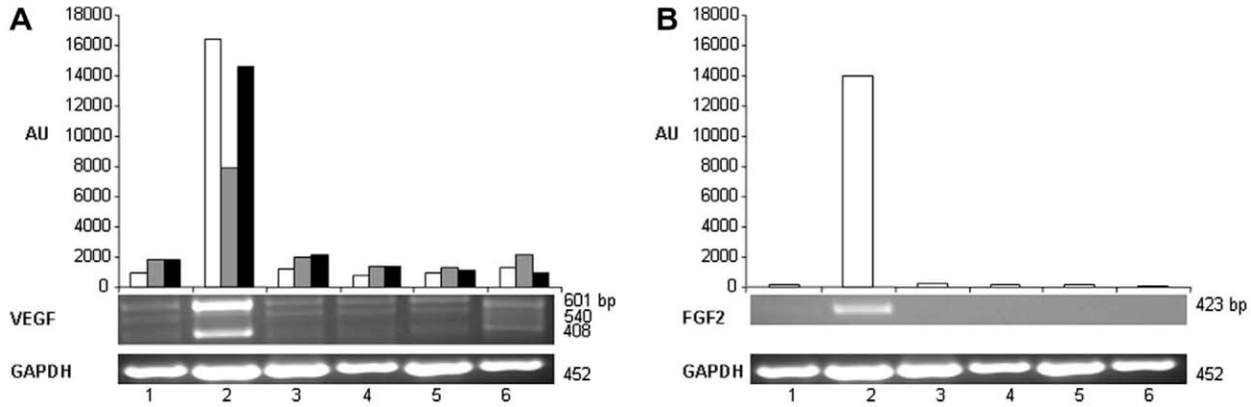

Fig. 4. (A) VEGF expression in rat alveolar macrophages induced by *Trichinella pseudospiralis (STps)* antigen detected by RT-PCR. GAPDH mRNA expression levels from rat alveolar macrophages were used as internal control. (1) Non-stimulated macrophages (negative control), (2) LPS-stimulated macrophages (positive control), (3–6) *STps*-stimulated macrophages with 0.1, 1, 10 and 50 μg/ml. AU: arbitrary units calculated by densitometry of corresponding amplicons. 601 bp VEGF band (white bars), 540 bp VEGF band (grey bars), 408 bp. (B) FGF2 expression in rat alveolar macrophages induced by *Trichinella pseudospiralis (STps)* antigen detected by RT-PCR. GAPDH mRNA expression levels from rat alveolar macrophages were used as internal control. (1) Non-stimulated macrophages (negative control), (2) LPS-stimulated macrophages (positive control), (3–6) *STps*-stimulated macrophages with 0.1, 1, 10 and 50 μg/ml. AU: arbitrary units calculated by densitometry of corresponding amplicons. 423 bp FGF2 band (white bars).

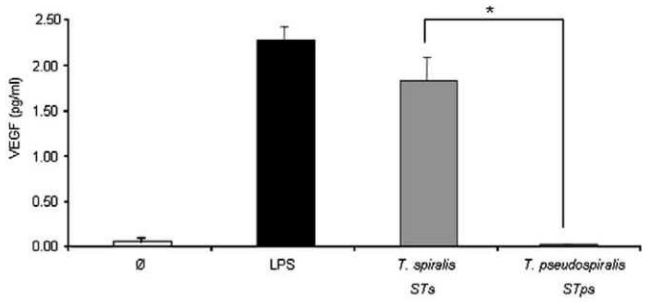

Fig. 5. VEGF protein detection in supernatant of alveolar rat macrophages cultures treated with L1 somatic antigen of *T. spiralis (STs) T. pseudospiralis (STps)* and its controls not treated macrophages (Ø) and lipopolysacharide treated macrophages (LPS). Means and SEM of six measures in duplicate culture.*$p < 0.05$.

show that LPS-stimulated macrophages (10 μg/ml) and macrophages stimulated with only 50 μg/ml *STs* induce FGF2 mRNA expression.

In vitro incubation of rat alveolar macrophages with *T. pseudospiralis* antigens (*STps*) resulted in no stimulation of VEGF production by these cells, when compared with basal levels (Fig. 4A). Also FGF2 was not detected in alveolar macrophages stimulated with *STps* antigens (Fig 4B).

VEGF protein expression was confirmed by ELISA in supernatant of alveolar macrophages stimulated with *STs* and *STps* antigens. The results show that macrophages stimulated with *STs* had 1.86 ± 0.25 pg of VEGF/μg of protein and macrophages stimulated with somatic *STps* had 0.02 ± 0.01 pg of VEGF/μg of protein, with statistical differences ($p < 0.05$). When compared with macrophages stimulated with LPS had 2.31 ± 0.15 pg of VEGF/μg of protein and 0.09 ± 0.01 pg of VEGF/μg of protein in macrophages no stimulated (Fig. 5).

We studied the relationship between angiogenic factors and nitric oxide in macrophages stimulated with *STs* antigens, using specific inhibitors of inducible nitric oxide synthase (L-NAME and L-canavanine). The results show that L-NAME and L-canavanine did not produce low expression levels of VEGF (Fig. 6A) and FGF2 (Fig. 6B).

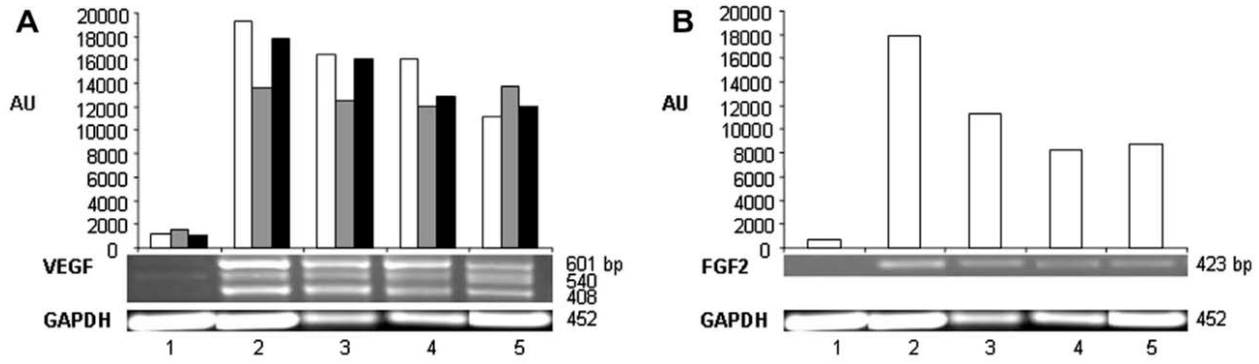

Fig. 6. (A) Effects of specific inhibitor of inducible nitric oxide synthase (iNOS) on VEGF mRNA expression of *STs*-stimulated macrophages. GAPDH mRNA expression levels from rat alveolar macrophages were used as internal control. (1) Non-stimulated macrophages (negative control), (2) LPS-stimulated macrophages (positive control), (3) *STs*-stimulated macrophages (50 μg/ml). (4) *STs*-stimulated macrophages plus L-NAME (50 μg/ml + 10^{-2} M). (5) *STs*-stimulated macrophages plus L-canavanine (50 μg/ml + 10^{-2} M). AU: arbitrary units calculated by densitometry of corresponding amplicons. 601 bp VEGF band (white bars), 540 bp VEGF band (grey bars), 408 bp. (B) Effects of specific inhibitor of inducible nitric oxide synthase (iNOS) on FGF2 mRNA expression of *STs*-stimulated macrophages. GAPDH mRNA expression levels from rat alveolar macrophages were used as internal control. (1) Non-stimulated macrophages (negative control), (2) LPS-stimulated macrophages (positive control), (3) *STs*-stimulated macrophages (50 μg/ml). (4) *STs*-stimulated macrophages plus L-NAME (50 μg/ml + 10^{-2} M). (5) *STs*-stimulated macrophages plus L-canavanine (50 μg/ml + 10^{-2} M). AU: arbitrary units calculated by densitometry of corresponding amplicons. 423 bp FGF2 band (white bars).

4. Discussion

The encapsulated species are characterised by deposition of collagen and other components of the extracellular matrix around the larvae of *Trichinella* (Wu et al., 2008). An associated key process to the fibrogenesis is the angiogenesis (Wynn, 2008). In fact, experimental data suggest that hypoxia in relation to VEGF may stimulate proliferation of synthesis of collagen type 1 in activated myofibroblast like cells (Novo et al., 2007).

Our results indicate that the macrophages are able to produce and to release not only VEGF but also FGF2 in response to antigens of encapsulated species like *T. spiralis*, in a dose-dependent manner. Gene expression of VEGF was determined by RT-PCR and protein expression measured by ELISA. Nevertheless, non-encapsulated species likes *T. pseudospiralis* were not able to induce the expression of these angiogenesis factors. The larva penetrates the muscle cell, grows in it and destroyed myofibrils. The satellite cells that surround the cell with muscle fibre undergo morphological changes transforming into nurse cells. Previously it has been demonstrated that the nurse cells are able to produce VEGF during the infection by *T. spiralis*, (Capo et al., 1998). In this paper we demonstrate that inflammatory cells that surround the injured muscle fibre contribute in the production of angiogenic factors. Probably, both cell types contributed to the development of the capsule of this species.

Previous studies of our group have demonstrated that antigens of encapsulated and non-encapsulated species of *Trichinella* were able to stimulate nitric oxide (NO) production (Andrade et al., 2007). As NO is an inductive factor for VEGF production (Namba et al., 2003), we evaluated whether the inhibition of NOS, by using the specific inhibitors L-NAME and L-canavanine, modified the effect of antigens of *Trichinella* on the production of angiogenesis factors. This study demonstrates that the effect of the antigens of *T. spiralis* does not depend on NO.

In summary, antigens from encapsulated species of *Trichinella* directly stimulated the production of angiogenic factors (VEGF and FGF2) that contributed to the pathogenesis of this nematode infection.

Acknowledgments

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3.2 Article 2.

Role of angiogenic factors in acute experimental *Strongyloides venezuelensis* infection

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Role of angiogenic factors in acute experimental *Strongyloides venezuelensis* infection

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SUMMARY

This study aims to investigate the role of angiogenic factors in the pathogenesis of experimental strongyloidiasis. Two complementary approaches were used: Firstly, CD1 mice were treated with endostatin, an angiogenesis inhibitor, and infected with Strongyloides venezuelensis. Also, the mechanisms involved in this process were studied. Parasitological examination revealed a significant decrease in egg per gram of faeces, number of collected larvae from lung tissue and number of collected adult females in mice treated with endostatin. Direct mechanisms with diminution of angiogenesis factors and an indirect mechanism with increase of eosinophil perhaps produced their effect. Secondly, the effect of the antigens responsible for stimulation of angiogenic factors [vascular endothelial growth factor (VEGF) and fibroblast growth factor 2 (FGF2)] from alveolar macrophages and the mechanisms involved in their production were investigated. Alveolar macrophage cells obtained by bronchoalveolar lavage were incubated at different concentrations of somatic and excretory/secretory antigens of S. venezuelensis. Also, mRNA levels of VEGF and FGF2 in macrophage cells were detected by RT-PCR. L3-PBS larvae antigens induced angiogenic factors. The relationship between angiogenesis factors and nitric oxide has been observed using nitric oxide synthase inhibitors.

Keywords *endostatin, fibroblast growth factor (FGF2), nitric oxide, Strongyloides venezuelensis, vascular endothelial growth factor (VEGF)*

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INTRODUCTION

Strongyloides is a genus of parasitic nematodes which includes some 50 species of obligatory parasites of vertebrates. Two species of *Strongyloides* infect humans, *Strongyloides stercoralis* and *Strongyloides fuelleborni* (1). In healthy individuals, infection with *Strongyloides* can be clinically inapparent or can lead to cutaneous, gastrointestinal or pulmonary symptoms. However, *Strongyloides* infection in immunocompromized individuals (e.g. corticosteroid use and human T lymphotropic virus type I infection) can result in disseminated strongyloidiasis, in which worms move beyond the confines of the gut into other organs (2).

The lifecycle of *Strongyloides* is complicated and available data have been mainly obtained in experimental infections (*Strongyloides ratti* and *Strongyloides venezuelensis*) (3,4). Usually, hosts become infected when free-living infective third stage larvae (L3sv) penetrate the skin and/or digestive mucosal surfaces. These larvae gain access to blood vessels and are dispersed to many organs, being passed through the lungs (3). During this migration L3sv moult to L4 stage and then the adult parasitic worms appear in the gut after a few days with reproduction commencing shortly thereafter, detected by the presence of eggs and/or larvae in the faeces. A key pathogenic clue of acute infection (and probably of hyper infection) by *Strongyloides* spp. is their dissemination through blood vessels until they reach target organs (mainly lung and gut). There are no direct data on the role of *Strongyloides* spp. infection on angiogenesis. However, both indirect evidence in experimental model (3), and human hyperinfection (demonstration of vascular anomalies by arteriography or endoscopy) (5,6) suggest the involvement of angiogenic factors in the pathogenesis of this infection.

Angiogenesis is the process of new blood vessel formation from pre-existing ones, plays a key role in various physiological and pathological conditions, including

embryonic development, wound repair, tumour growth and inflammation (7). Angiogenesis is initiated by vasodilatation and an increased permeability being regulated by a delicate balance of pro and anti-angiogenic factors. Amongst angiogenic factors, vascular endothelial growth factor (VEGF)/vascular permeability factor and fibroblast growth factor-2 (FGF-2) are the best characterized positive regulators. In particular, VEGF has distinct specificity for vascular endothelial cells (8). The biological actions of VEGF include stimulation of endothelial cell proliferation, migration, differentiation, tube formation, vascular permeability and maintenance of vascular integrity (9). FGF2 is less specific for endothelial cell proliferation, but is a potent angiogenic factor *in vitro* and *in vivo* (10). Moreover, many endogenous inhibitors of angiogenesis have been described, endostatin (C-terminal fragment of collagen XVIII) and angiostatin being the best characterized (11). Although the precise mechanism for the antiangiogenic effect of endostatin is not well known, this molecule can block endothelial cell proliferation, survival and migration through blocking VEGFR2 signalling and other mechanisms (12).

The aim of this study was to evaluate the role of angiogenic and angiostatic factors in the pathogenesis of experimental strongyloidiasis. We used two complimentary approaches: (i) an *in vivo* model of infection by *S. venezuelensis* in CD1 mice was used for the evaluation of the effect of endostatin on the parasitic infection and for the mechanisms involved in the reduction of parasite burden, (ii) an *in vitro* study of the antigens responsible for stimulation of angiogenic factors from alveolar macrophages and the mechanisms involved in their production.

MATERIALS AND METHODS

Animals

Male Wistar rats and female CD1 mice were purchased from Charles River Laboratories, Barcelona, Spain. All experiments of this work comply with current European Union law on animal experimentation. All infected and control animal strains were maintained under standard laboratory conditions in the animal experimentation facilities of the Salamanca University.

Parasites and antigens

Strongyloides venezuelensis filariform infective larvae (L3sv) and adult female were obtained from the strain of the Department of Parasitology of the University of Minas Geráis, Belo Horizonte, Brazil and their lifecycle was maintained by serial passages in male 4-week-old Wistar rats in Salamanca University. L3sv and adults were decontaminated according to Martins *et al.* (13). The larvae were suspended at a concentration of $3{\cdot}0 \times 10^5$/mL in PBS with protease inhibitors with a final concentration of 5 mM ethylenediaminetetraacetic acid, 2 mM phenylmethylsulphonyl fluoride, 1 μM pepstatin, 4 μM aproptinin and 10 μM chymostatin. PBS-soluble extract antigen (L3-PBS) was obtained according to Conway *et al.* (14). Excretory secretory antigens of larvae (L3-ES) were prepared in accordance with Northern and Grove (15). Every day cultures were observed and when motility was less than 80% they were discarded. Female adult worms were suspended in PBS with protease inhibitors as above. Alkaline extract of adult *S. venezuelensis* (F-ALK) was prepared according to Machado *et al.* (16). Female excretory secretory antigens (F-ES) were prepared in accordance with Brindley *et al.* (17). Cultures were observed day to day to monitor motility and every 2 days supernatants were collected as above.

All antigens were aliquoted and stored at −80°C. Protein concentration was determined using the Micro BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) and samples were run in a 15% sodium dodecyl sulphate–polyacrylamide gel electrophoresis to assess the antigen.

Evaluation of the effect of endostatin on *S. venezuelensis* infection in murine experimental model

In the first experiment, we used three groups of 6-week-old CD1 mice weighing 16–25 g, as follows: Group A, uninfected group; Group B, mice infected with 3000 L3 of *S. venezuelensis* per animal; Group C, mice infected with 3000 L3 and treated with 2·5 mg/kg of endostatin (Sigma Chemical Co, St Louis, MO) at days 0 and 2. On the third day of the experiment, mice were killed and the lungs were harvested. The lungs were then sliced and larvae were collected and counted. At 0 and 3 days of the experiment, we collected blood samples in EDTA anticoagulant under isoflurane anaesthesia (Isoba vet; Schering-Plough) for blood cell counts with a hemocytometer Hemavet 950 (Drew Scientific Group). Also, lungs, liver and gut were recovered for RNA extraction.

In the second experiment, we used three groups of 6-week-old CD1 mice weighing 16–25 g, as follows: Group A, uninfected group; Group B, mice infected with 3000 L3 of *S venezuelensis* per animal; Group C, mice treated with 2·5 mg/kg of endostatin at days 1, 3, 5 and 7 of the experiment and infected with 3000 L3 at day 2. All the animals were killed at day 14 of the experiment. The infection was monitored daily from day 6 of the experiment, counting eggs per gram of faeces. Animals were placed individually on clean, moist absorbent paper and allowed to defecate.

Eggs were counted using the Cornell–McMaster quantitative method. Faeces were weighed and broken up in a known volume of a 10% formalin solution in a 1·5 mL vial. The parasitological analysis was performed twice. Blood samples were obtained on days 1, 4, 7, 12 and 14 of the experiment to count blood cells in a Hemavet 950 hemocytometer as above. The animals were then killed and adult worms in intestine were recovered. Lungs, liver and small intestine were recovered for RNA collection.

Detection of VEGF and FGF2 in mice infected with *S. venezuelensis* by RT-PCR

Total RNA was extracted from the snap-frozen tissue using an RNeasy Mini Kit (Qiagen GmbH, Hilden Germany). A total of 1 μg of RNA was used as template for the first-strand DNA synthesis (Roche). Primers specific for rat VEGF were used in accordance with Yang *et al.* (18). Primer sequence for VEGF was: sense, 5′-CTGCTCTCTTGGGTGCACTGG-3′ and anti-sense, 5′-CACCGCCTTGGCTTGTCACAT-3′, generate three bands of 601, 540 and 408 bp, corresponding to VEGF isoforms of 188, 164 and 120 amino acids. Primers specific for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were: sense, 5′-GGTCGGTGTGAACGGATTTG-3′ and GAPDH anti-sense, 5′-GTGAGCCCCAGCCTTCTCCAT-3′ generating 452 bp PCR product. PCR reactions were carried out through reverse transcription incubation at 94°C for 5 min, 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a single cycle at 72°C for 7 min. PCR products were analysed by electrophoresis in 2% agarose gel stained with ethidium bromide. Primers specific for detection of FGF2 were used in accordance with Jyo-Oshiro *et al.* (19). Primer sequence for FGF2 was: sense, 5′-GCCGGCAGCATCACTTCGCT-3′ and anti-sense, 5′-CTGTCCAGGCCCCGTTTTGG-3′. PCR reactions were carried out through reverse transcription incubation at 94°C for 2 min, 50 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min and a single cycle at 72°C for 5 min. PCR products were analysed by electrophoresis in 1·5% agarose gel stained with ethidium bromide with GADPH as internal control.

Evaluation of the effect of endostatin on L3 larvae of *S. venezuelensis*

A range of endostatin concentrations between 0·1 and 50 μg/mL was applied in phosphate buffered saline (PBS pH 7·2). Ivermectin (Sigma) and was used as positive control at 10 μg/mL final concentrations. We observed the effect of endostatin on the parasite *in vitro* 300 L3 larvae of *S. venezuelensis* in each well. The experiment was performed by triplicate after incubation at 37°C in 5% CO2. The viability of the L3 was calculated by the detection of motility by the light microscope. We observed the larval motility between 1 h until 6 days.

Isolation and culture of alveolar macrophages

Alveolar macrophages were obtained from male Wistar rats of 250–300 g by bronchoalveolar lavage as previously described (20).The latter were washed twice with PBS (pH 7·4) and the cells were re-suspended at a concentration of 1×10^6/mL. Alveolar macrophages were cultured as previously described (20). Briefly, cells were re-suspended in Dulbecco's Modified Eagle Medium supplemented with 10% γ-irradiated foetal bovine serum, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma Chemical Co, St Louis, MO, USA), and maintained at 37°C in 5%CO2. Macrophages in culture were incubated alone (negative control, Ø), with lipopolysaccharide 10 μg/mL (LPS; Sigma; positive control) and/or with several concentrations, ranging from 0·1 to 50 μg protein/mL, of different parasite antigens. The LPS dose used in our studies was chosen as optimal based on previous investigations (21). After 18 h at 37°C in 5% CO2, culture supernatants were collected, centrifuged at 500 ***g*** for 10 min and stored at −80°C until analysis. Remaining cells were subjected to the MTT viability assay as described earlier (20), viability being higher than 87·5% in all cases. Following the viability assay, alveolar macrophages were collected and stored at −80°C for further analysis.

Detection of VEGF and FGF2 in alveolar macrophages by RT-PCR

Total RNA was extracted from alveolar macrophages using an RNeasy Mini Kit (Qiagen Inc.). A total of 1 μg RNA was used as template for the first-strand DNA synthesis (Roche). Primers specific for VEGF, FGF2 and GAPDH were used as above.

Effect of inhibitors of Nitric Oxide on VEGF and FGF2 expression

To determine the relationship between nitric oxide and VEGF and FGF2 on macrophage cells stimulated by *S. venezuelensis* antigen we used an inhibitor of all nitric oxide synthase (iNOS) isoforms – nitro-L-arginine methyl ester (L-NAME; Affinity) and a specific inhibitor of iNOS - L-canavanine (Sigma). Both inhibitors were used at a final concentration of 100 mM as previously described by Andrade *et al.* (20).

Polymyxin B, a specific inhibitor of LPS, was used to assess possible LPS contamination or LPS-like activity in the different parasite antigens used during our study (22). Briefly, alveolar macrophages were incubated with 80 μg/mL of polymyxin B plus LPS (10 μg/mL) and 50 μg/mL antigens parasite. *S. venezuelensis* antigens were used at different concentrations (0·1–50 μg/mL) on alveolar macrophages.

Statistical analysis

The results of the faecal egg counts, larvae and adult females were reported as arithmetic mean and standard deviation. Differences in groups were performed by ANOVA. When global differences were detected, a post-ANOVA test using the Fisher LSD analysis was applied. Differences between means were considered statistically significant at $P < 0{\cdot}05$. All statistical analyses were performed using Statworks and Statview 4·5 software packages for a Macintosh computer.

RESULTS

Evaluation of the effects of endostatin on *S. venezuelensis* infection in a murine experimental model

We evaluated the effect of endostatin on collection of larvae in mice infected with 3000 L3 of *S. venezuelensis* and mice treated with endostatin in lung. We individually observed the data of collection of larvae in lung, as well as its mean and standard error of the mean (Figure 1a). The mean number of L3 *S. venezuelensis* recovered at 2 days post-infection was 196 ± 22 in the group of infected mice, compared with 69 ± 15 in the group of mice treated with endostatin. The differences were statistically significant $P < 0{\cdot}05$.

In addition, we evaluated the effect of endostatin on collection of females in intestine. We individually observed the data of collection of females in intestine, as well as mean and standard error of the mean (Figure 1b). The mean number of female parasites recovered

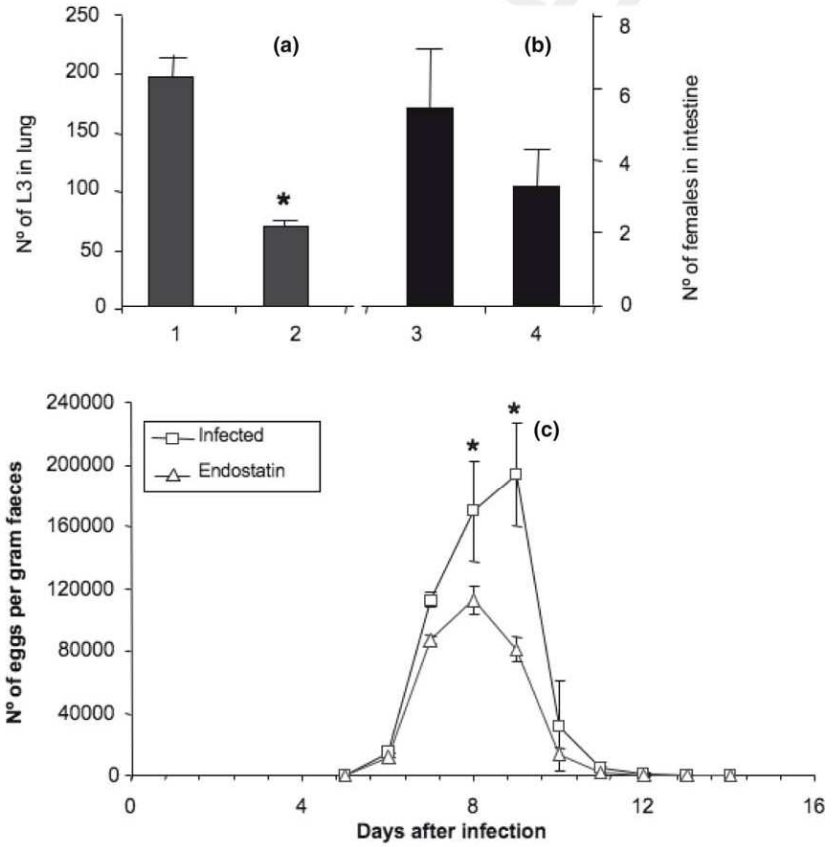

Figure 1 (a) Numbers of L3 of *Strongyloides venezuelensis* recovered from lung at 2 days post-infection: mean ± standard error in infected mice group (1). Mean ± standard error in mice treated with endostatin (2·5 mg/kg) (2); *$P < 0{\cdot}05$. (b) Numbers of females of *S. venezuelensis* recovered from intestine at 14 days post-infection: Mean ± Standard error in group of infected mice (3): mean ± standard error in mice treated with endostatin (2·5 mg/kg)(4); *$P < 0{\cdot}05$. (c) Number of eggs per gram of faeces counted from 5 to 14 days post-infection in infected mice and infected mice treated with endostatin. *$P < 0{\cdot}05$.

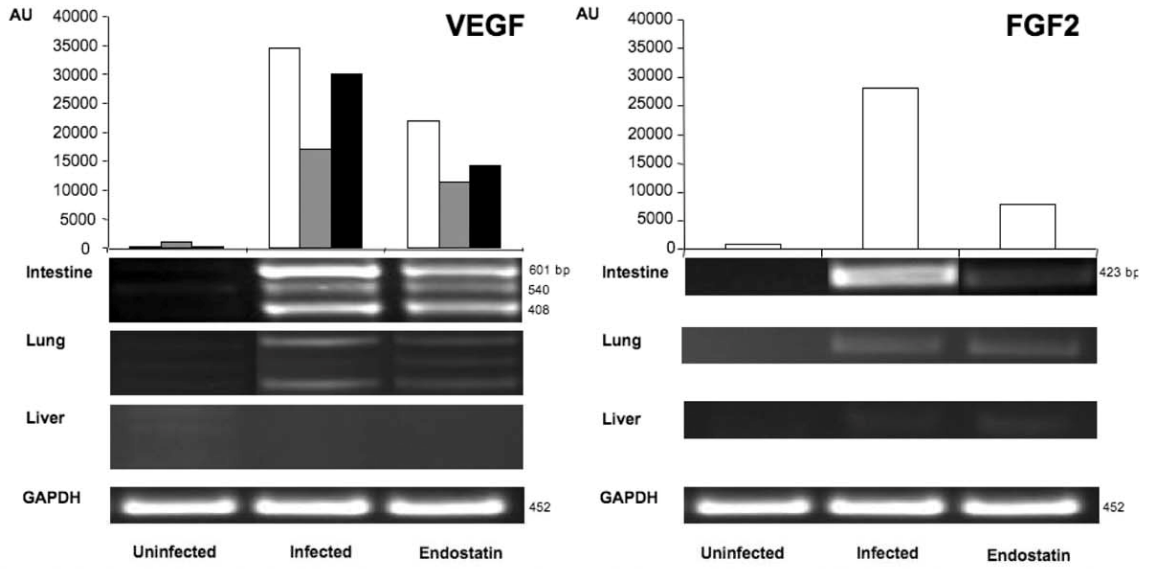

Figure 3 RT-PCR for VEGF and FGF2 detection from intestine, lung and liver at 14 days post-infection in three experimental groups: Uninfected mice, mice infected with 3000 L3 of *Strongyloides venezuelensis* and mice treated with 2·5 mg/kg endostatin and infected as above. VEGF expressed in intestine, lung and not expressed in liver. FGF2 expressed in intestine and less than in lung but not expressed in liver. AU arbitrary units calculated by densitometry of corresponding amplicons, 601 (white bars), 540 (grey bars) and 408 bp (black bars) for VEGF, a band with 423 bp (white bars) for FGF2. GAPDH expression was used as internal control

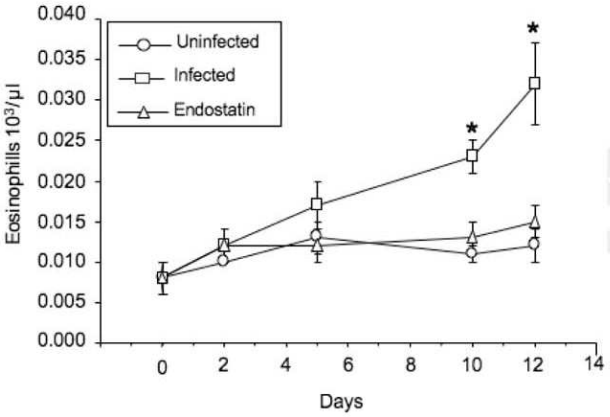

Figure 4 Numbers of total eosinophils were enumerated and identified through Hemavet. Data are expressed as mean's of the cell numbers from uninfected animals (n = 6/day), infected mice with 3000 L3 of *Strongyloides venezuelensis* (n = 6/day), infected mice treated with 2·5 mg/kg of endostatin (n = 6/day). $^*P < 0{\cdot}05$.

mectin after 48 h demonstrated 3 ± 1% of mobility. Moreover, mobility at 3 days of experiment reached to zero. On the other hand, untreated larvae presented mobility between 88 ± 2·3 and 97 ± 0·6 and larvae treated with concentrations of 0·1 to 50 µg/mL endostatin demonstrated mobility between 81 ± 3·2 and 96 ± 1. This experiment demonstrated that endostatin has not direct effect on L3 larvae of *S. venezuelensis*.

Evaluation of the antigens of *S. venezuelensis* in the stimulation of angiogenic factors (VEGF and FGF2) by alveolar macrophages

We studied the effects of different concentrations of different antigens of *S. venezuelensis* (0·1–50 µg/mL) on the expression of VEGF and FGF2 in alveolar macrophages (Figure 6). The results indicate that macrophages stimulated with larvae PBS-soluble extract (L3-PBS) from 1 µg/mL induced VEGF (601 bp isoforms) and FGF2 mRNA expression in a dependent dose when compared with other antigens of *S. venezuelensis*. Antigens from excretory secretory larvae (L3-ES), somatic and excretory secretory female (F-ALK and F-ES) antigens of S. *venezuelensis* were not able to cause the expression of either VEGF or FGF2.

Evaluation of the mechanisms involved in the stimulation of angiogenic factors (VEGF and FGF2) by alveolar macrophages

VEGF production of macrophages incubated with L3-PBS antigen from *S. venezuelensis* larvae and the nitric oxide specific inhibitors (L-NAME or L-canavanine) was completely abolished with differences between cells incubated with the antigens alone and the combination of the inhibitors plus the antigens (Figure 7). Similarly, results were obtained for the expression of FGF2 when cells incubated

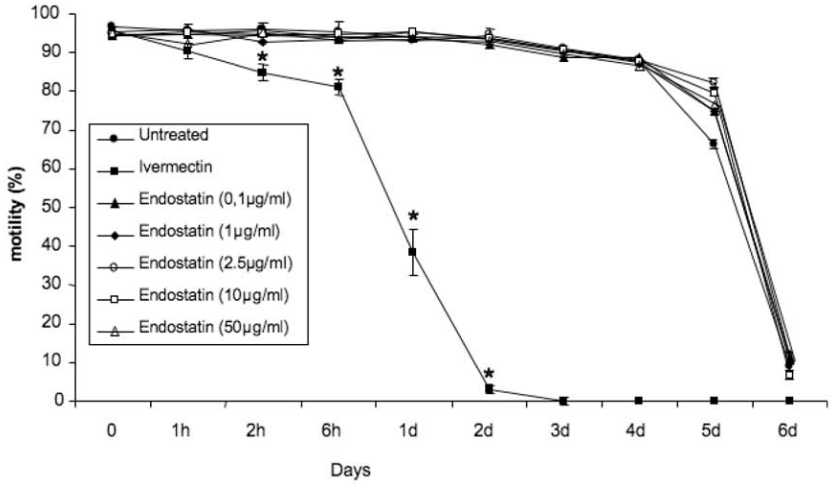

Figure 5 Effect of endostatin on culture of L3 *Strongyloides venezuelensis*, Untreated L3 of *S. venezuelensis* in PBS and L3 treated with ivermectin (10 µg/mL) were used as controls. Endostatin was used at different concentrations (0·1–50 µg/mL). Cultures were maintained during 6 days and were performed by triplicate. I6

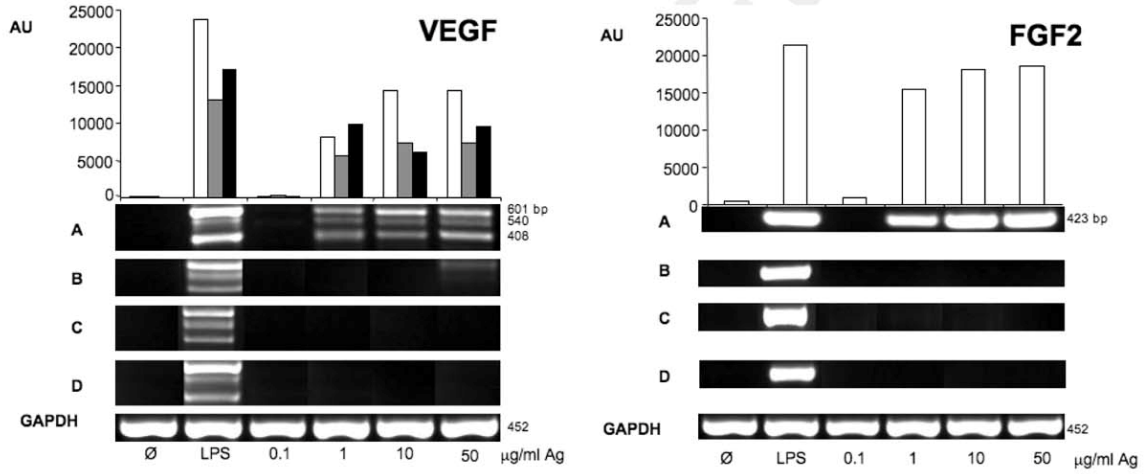

Figure 6 Effect of *Strongyloides venezuelensis* antigens on expression of VEGF and FGF2 in alveolar macrophages, (a) mRNA expression in rat alveolar macrophages by PBS-soluble extract from larvae 3 of *Strongyloides venezuelensis* (L3-PBS), (b) excretory secretory antigens of L3 of *S venezuelensis* (L3-ES), (c) Alkaline extract of female (F-ALK) and (d) female worm excretory secretory antigens (F-ES). GAPDH mRNA expression levels from rat macrophages detected by RT-PCR are used as internal positive control. Nonstimulated macrophages, negative control (Ø), LPS stimulated macrophages, positive control (LPS) and different concentrations of *S venezuelensis* antigens, 0·1–50 µg/mL (A-D) AU, arbitrary units calculated by densitometry of corresponding amplicons. 601 bp VEGF band (white bars), 540 bp VEGF band (grey bars) and 408 bp VEGF band (black bars). 423 bp FGF2 band (white bars). I7

with L3-PBS antigen and the nitric oxide specific inhibitors. In addition, a similar effect was observed with cells incubated with LPS and cells incubated with LPS plus nitric oxide inhibitors.

DISCUSSION

Strongyloidiasis is one of the major nematode infections of humans with cosmopolitan distribution in tropical and subtropical regions (23). It is estimated that some 100–200 million individuals are infected worldwide with *Strongyloides* spp., however, these infections can be difficult to detect, so these may be underestimates. *Strongyloides* infection in immunocompromized individuals, particularly following the administration of steroids, can result in disseminated strongyloidiasis (2). Some authors proposed that *S. ratti* and *S. venezuelensis* are suitable parasite models for the study of *S. stercoralis* (24).Our previous work has shown the production of nitric oxide by alveolar macrophages stimulated with larvae antigen of *S.*

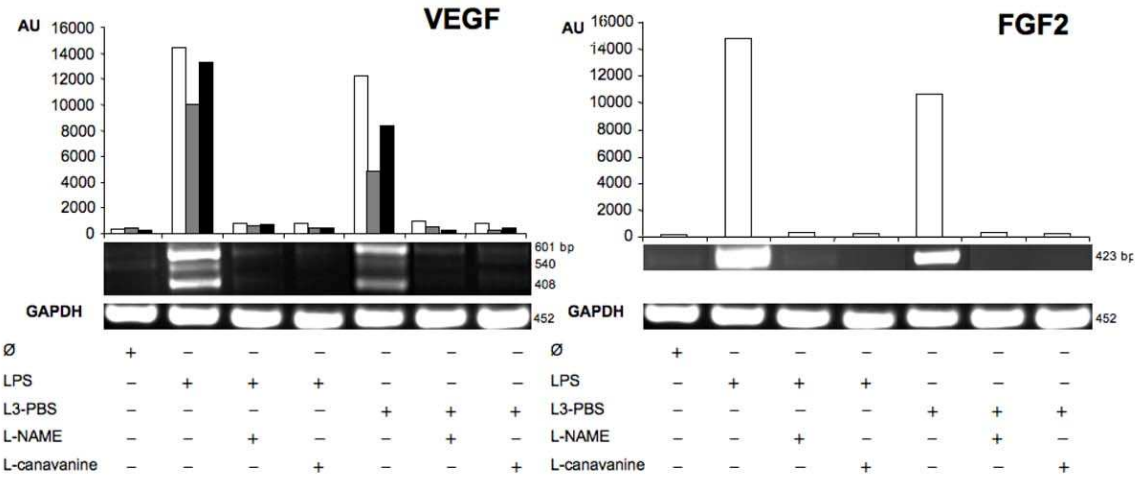

Figure 7 Effects of specific inhibitor of inducible nitric oxide synthase (iNOS) with L3-PBS antigen of *Strongyloides venezuelensis* on macrophage VEGF and FGF2 production. GAPDH mRNA expression levels from rat macrophages detected by RT-PCR are used as internal positive control. Nonstimulated (Ø), stimulated (LPS), LPS + L-NAME, LPS + L-canavanine, L3-PBS 50 μg/mL stimulated, L3-PBS 50 μg/mL + L-NAME, L3-PBS 50 μg/mL + L-Canavanine AU, arbitrary units calculated by densitometry of corresponding amplicons. 601 bp VEGF band (white bars), 540 bp VEGF band (grey bars) and 408 bp VEGF band (black bars). 423 bp FGF2 band (white bars).

venezuelensis (L3-PBS), demonstrating the participation of this inflammatory mediator in the experimental strongyloidiasis (unpublished data). Nevertheless, more studies are needed to determine the role of other inflammatory mediators and the relationship with nitric oxide in the strongyloidiasis.

Angiogenesis is a complex multi-step process that leads to neovascularization generated from pre-existing blood vessels. It is associated with inflammation, wound healing, tumour growth and metastasis. The generation of new blood vessels is regulated by proangiogenic and antiangiogenic molecules (25). VEGF and FGF2 are major mediators of angiogenesis in the development and progression of many diseases. Few studies exist about the relation between angiogenesis factors and helminthoses. A positive correlation was observed between plasma VEGF and the stage of hydrocoele in men infected with the filarial nematode *Wuchereria bancrofti* (26). Also, VEGF was found to be protective against cerebral malaria associated mortality (27). In the present work we evaluated the role of angiogenesis factors in the experimental strongyloidiasis: the modulation of the infection using a specific inhibitor of angiogenesis (endostatin), the induction of VEGF and FGF2 in alveolar macrophages stimulated with different antigens derived from different phases of the biological cycle of *S. venezuelensis* and the probable relationship between these factors and the production of nitric oxide.

Endostatin is a 20-kDa C-terminal fragment of collagen XVIII that, when added exogenously, inhibits angiogenesis (28). Our work demonstrates that the angiogenesis factors have an important function in the primary infection by *S. venezuelensis*. The endostatin diminishes both the number of larvae in lung and the number of eggs in the faeces. Is this because of direct effects of the parasite or is it indirectly via effects of the host? For answer this question, we performed *in vitro* studies on the effect of endostatin on parasite mobility. We demonstrated that endostatin has not direct effects on L3 larvae of *S. venezuelensis*. Then, indirect effects on the host could be attributed to the endostatin treatment. This can be associated to two complementary mechanisms. First, endostatin directly decreases the expression of the mean angiogenic factors. In fact, we have shown that mice treated with endostatin and infected with *Strongyloides* spp., have a reduced expression of VEGF and FGF2 both in lung and intestine. Secondly, some authors observed that eosinophil potentially participates in angiogenesis by inducing VEGF production (29). Moreover, VEGF has been associated with blood-brain barrier disruption in patients with eosinophilic meningitis caused by *Angyostrongylus cantonensis* (30). When compared with the infected group our data indicate that mice infected with *S. venezuelensis* and treated with endostatin have a significant reduction of blood eosinophil counts.

Macrophages are known to produce several potent angiogenic factors including VEGF, placenta growth factor, basic FGF2, transforming growth factor-β and IL-8 and a lot of pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α and granulocyte monocyte-colony stimulating factor (31). Studies performed by our group have demonstrated the induction of VEGF and FGF2 in alveolar macrophages stimulated with larvae antigens of *T. spiralis* (32). In the present paper, we studied the effect of somatic

and excretory/secretory antigens of larvae and females of *S. venezuelensis* on the production of VEGF and FGF2 in alveolar macrophages. Only the somatic antigen of larvae 3 of *S venezuelensis* stimulates the production of angiogenesis factors (VEGF and FGF2). In addition, we investigated whether the effect exerted by these antigens in the modulation of the angiogenesis factors was direct or through other inflammatory mediators, such as nitric oxide. iNOS is known to regulate VEGF expression, and thereby angiogenesis (33–35). As alveolar macrophages release nitric oxide in response to helminthic antigens (21), may be inhibition of iNOS could be decreased VEGF production. We confirmed the relationship between the production of nitric oxide and the angiogenesis factors by using inhibitors of the ONSi (L-NAME and L-canavanine), which inhibited the expression of angiogenesis factors.

In summary, this study demonstrated that angiogenesis factors play a role in the primary infection by *S. venezuelensis* as the inhibition by endostatin produced a decrease in the number of larvae and females. Direct mechanisms with diminution of angiogenesis factors and indirect mechanisms with decrease of the number of eosinophils could be related to the protection from the parasitic infection. Angiogenic factors are induced by somatic antigens of third stage larvae of *S. venezuelensis*. A positive relationship between angiogenesis factors and nitric oxide has been observed using nitric oxide synthase inhibitors.

ACKNOWLEDGEMENTS

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3.3 Article 3.

Angiogenic Factors expression on schistosomiasis: human and experimental infections.

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Belén Vicente, Maryam Arefi, Antonio Muro.

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Running head: Schistosomiasis and angiogenesis.

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Abstract

Background: Angiogenesis is the process of new blood vessel formation, has an important role in various processes, including cancer and inflammation. Schistosomiasis is a helminthoses that affects more than 200 million people in the world. Previous works have demonstrated that soluble antigens of *Schistosoma mansoni* induced angiogenesis by up-regulation vascular endothelial growth factor (VEGF) in human endothelial cells. The objectives are: (*i*) to study VEGF in sera of patients diagnosed of schistosomiasis and other helminthoses (*ii*) to evaluate the effect of endostatin (angiogenesis inhibitors) in mice infected with *S. mansoni* (*iii*) to detect VEGF and fibroblast growth factor (FGF2) expression in macrophages stimulated with *S. mansoni* antigens.

Methodology/Principal Findings: VGEF protein was detected in sera of healthy patients, patients diagnosed of schistosomiasis, filariasis and hookworms infections. Patient diagnosed with schistosomiasis only showed significant differences in VEGF level in

compared with healthy patients. Uninfected group, mice infected with *S. mansoni* and mice infected and treated with endostatin were used. Parasitological, analytical data and IgG, IgG1 and IgG2a specific antibodies were analyzed. VEGF and FGF2 expression were detected by RT-PCR in liver and intestine. Mice treated with endostatin showed significant differences in eggs recoveries from liver and IgG2a antibodies detection in compared with infected mice group. Finally, the effects of the cercarial and adults *S. mansoni* antigens on the VEGF and FGF2 stimulation were evaluated. Macrophages were obtained by bronchoalveolar lavage (BAL) from Wistar rats. Cells were cultured at different concentrations of antigens (0.1-50 µg/ml), and their expression were determined by RT-PCR. Cells stimulated with 50µg/ml of cercarial *S .mansoni* antigens expressed VEGF and FGF2.

Conclusion/Significance: These finding may suggest the important role of angiogenic factors in the pathogenesis of schistosomiasis.

Competing interests: The authors have declared that no competing interests exist.

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Summary:

Schistosomiasis is one disease produced by helminths which affect many people in tropical areas. Except of acute phase (cercariae dermatitis and Katayama fever), granuloma formation is the principal mechanism involved in the pathogenesis of this disease. Experimental studies have demonstrated angiogenesis (blood vessels formation from pre-existing vessels) in the initial phase of granuloma formation. In the present work, firstly the level of the main pro-angiogenic factors (vascular endothelial growth factor) were analyzed in sera from people diagnosed with schistosomiasis. We observed significantly high levels of this angiogenic factor in patients with schistosomiasis in compared with healthy people and patients diagnosed with other helminthic infections. In addition, we evaluated in experimental schistosomiasis murine model the effect of angiogenesis inhibition using anti-angiogenic factors (endostatin). A lesion decrease was observed in mice pre-treated with endostatin and infected with *Schistosoma mansoni*. Finally, mechanisms of angiogenesis induction were studied and observed that cercariae antigens stimulated the angiogenic factors (vascular endothelial growth factor and fibroblastic growth factor-2) by host alveolar macrophages.

Introduction

Schistosoma mansoni is one of the blood flukes there are to intravascular typical residence in the pelvic or mesenteric veins of the host. The female adult worms produce hundreds to thousands of eggs per day. Each ovum contains to ciliated miracidium larva, which secretes proteolytic enzymes that help the eggs to migrate into lumen of the bladder or the intestine [1]. Early pathological changes arise after mechanical occlusion of the microvasculature by the eggs causing acute vasculitis with endothelial damage and necrosis. Granuloma formation results from a delayed hypersensitivity response generated by the host against antigens secreted by the parasite eggs [2]. In initial phases the inflammatory response is intense with important neovascularization. Moreover, fibrosis was produced in later phases and it was responsible of the pathological disorders of this disease [3]. However, considerable variations in the magnitude of the disease have been described among different hosts and specifically among different mouse strains [4].

Angiogenesis, the formation of new endothelial vessels from pre-existing post-capillary venule, is a characteristic feature of inflammatory diseases, wound repair and cancer [5]. The angiogenic activity depends on the balance or imbalance between angiogenic and angiostatic mediators. Remodelling and degradation of the surrounding stroma is essential to start to an angiogenic phenotype. These stromal changes facilitate recruitment and activation of leucocytes, fibroblast and endothelial cells. While granulomas are traditionally considered to be avascular structures, schistosome granulomas should be seen as an inflammatory condition that initiates a variable degree of wound healing response in which angiogenesis and fibrosis are highly involved [6].

The aim of this work was to evaluate the role of angiogenic factors in the pathogenesis of schistosomiasis. Firstly, vascular endothelial growth factor (VEGF) detection in sera of patients diagnosed of schistosomiasis and other helminthic diseases were studied. Secondly, the effects of endostatin (angiogenesis inhibitor) in mice infected with *Schistosoma mansoni* were analyzed. Finally, VEGF and fibroblastic growth factor (FGF2) expression from alveolar macrophages stimulated with different *S. mansoni* antigens were analyzed.

Methods

Human population

The study group was formed by 53 patients from sub-Saharan areas with eosinophilia (≥450 eosinophil/µl). They had recently arrived in Canary Island of Spain. They have been

living in Spain since 6 months ago and have been diagnosed only by one parasite as shown in **Table 1**. All patients were diagnosed by direct parasitological tests included: *(i)* Coprology in 3 stool samples for ova and parasites by using Kato-Katz and Ritchie techniques, *(ii)* Identification of *Schistosoma haematobium* by examination of urine for eggs with sedimentation, *(iii)* Knott's test for detection of microfilaremia in blood and *(iv)* Immune chromatographic test (ICT Filariasis, Binax, Portland and Maine) for the detection of *Wuchereria bancrofti*.

Healthy control group was formed by immigrants from the same geographic area as patients of the study group. We have done for all clinical examination, a systemic analytical study and also parasitological tests and direct serological examination. In all cases the control group, there were not detected eosinophilia and presence of parasite.

Parasite and antigens

BALB/C mice were maintained under standard conditions in an environment with controlled temperature and humidity with 12 h light/dark cycle and free access to water and food. *Schistosoma mansoni* cercariae were obtained from *Biomphalaria glabrata* snails previously infected with *S. mansoni* miracidia [7]. Mice were sacrificed on the 7 week post-infection (pi) and adult worms were recovered from the hepatic portal and mesenteric veins by perfusion technique[8].

Adult worm antigen (AWASm) and cercarial antigen (CSm) from *S. mansoni* were used accordance with Dunne et al[9]. Briefly, *S. mansoni* worms were recovered by portal perfusion buffer. Recovered worms were washed free of erythrocytes and then snap frozen in liquid nitrogen. Frozen worms were ground into a paste, allowed to thaw and then centrifuged at 10,000*g* at 4ºC for one hour. After that 1mM N-Tosyl-1 phenylalanine chloromethyl ketone and 1mM Phenyl-methylphonylfluride (Sigma Chemical Co, St Louise, MO) were added. The soluble material was centrifuged, filtered through a 0.22 µm sterile filter and then stored at −80ºC. Cercarial antigen (CSm) was obtained from cercariae harvested within three hours of being shed from snail, snap frozen in liquid nitrogen and then processed identically to adult worm antigen.

VEGF detection in human samples

The determination of VEGF was realised in serum from human samples by ELISA technique (Vascular Biotrak Endothelial Growth Factor [(h) VEGF] human ELISA System, GE Healthcare Buckinghamshire, UK) accordance with manufacture instructions.

Mice experimental design

Three groups of six-weeks-old CD1 mice weighing 16-25 g were used as follows: uninfected group, mice infected with 150 *S. mansoni* cercariae per animal, mice infected with 150 *S. mansoni* cercariae and treated with 2.5 mg/kg of endostatin (Sigma) at 36, 38, 40 and 42 days post-infection[10]. From these animals' sera and blood samples were taken weekly from week 0 to 7 after challenged.

Animals were sacrificed at week 7 post-infection (pi) and the following parasitological parameters were assessed: *(i)* number of worms after liver perfusion, *(ii)* number of eggs per gram of liver, and *(iii)* number of eggs per gram of intestine, , and *(iv)* number of granulomas on liver surface counted under a stereoscopic microscope. In addition, from all animals liver and intestine were harvested and adult worms were collected and counted. Blood samples were obtained and these samples were used for ELISA techniques and analyzed on hemocytometer Hemavet 950 (Drew Scientific Group).

ELISA technique was performed by detection of IgG, IgG1 and IgG2a in mice infected with *Schistosoma mansoni* and treated with endostatin. Briefly, 96-well polystyrene plates (Costar) were coated with 5 µg/ml of *S. mansoni* AWA antigen diluted in carbonate buffer pH 9.6. Serum were added at 1:100 dilutions and incubated for 1 hour at 37ºC. Horseradish peroxidase rabbit anti-mouse IgG, IgG1 and IgG2a (Sigma, St. Louis, MO.) at 1:1.000 dilution was added. After incubation for 1 hour at 37ºC, substrate solution (*ortho*-phenylene diamine plus H_2O_2) was added, and the reaction was stopped at 10 minutes with 3 NH_2SO_4. Absorbances were measured at 492 on an Ear400FT ELISA reader (Lab Instruments). The results are expressed as means of the optical density from all the animals of each group plus the standard error.

VEGF and FGF2 expression in mice infected with S. mansoni by RT-PCR

Total RNA isolated from liver or intestine of all mice were used to analyze the expression of VEGF and FGF2 by RT-PCR as described previously (Shariati et al., in press)[11]. Briefly, total cellular was isolated with the Rneasy Mini Kit (Qiagen GmbH, Hilden Germany), according to manufactures instructions. Resulting RNAs were reverse

transcribed with the First Strand cDNA Synthesis kit (Roche), according to manufactures instructions. Primers specific for rat VEGF were sense, 5´-CTGCTCTCTTGGGTGCACTGG-3´ and anti-sense, 5´- CACCGCCTTGGCTTGT CACAT-3´. VEGF-PCR reaction were carried out through reverse transcription incubation at 94ºC for 5 min, 35 cycles of 94ºC for 1 min, 55ºC for 1 min, 72ºC for 1 min and a single cycle at 72ºC for 7 min. Primers specific for detection of FGF2 were sense, 5´GCCGGCAGCATCACTTCGCT-3´ and anti-sense, 5´CTGTCCAGGCC CCGTTTTGG-3´. FGF2-PCR reaction were carried out through reverse transcription incubation at 94ºC for 2 min, 50 cycles of 94ºC for 30 seconds, 60ºC for 30 seconds, 72ºC for 1 min and a single cycle at 72ºC for 5 min. PCR product were analyzed by electrophoresis in agarose gel stained with ethidium bromide with glyceraldehydes 3-phosphate dehydrogenase (GAPDH) as internal control. Primers specific for (GADPH) were sense, 5´-GGTCGGTGTGAACGGATTTG-3´ and GAPDH anti-sense, 5´-GTGAGCCCCAGCCTTCTCCAT -3´ that were used as positive control[11].

VEGF and FGF2 expression in alveolar macrophage stimulated with different antigens of Schistosoma mansoni

Macrophages were obtained from male Wistar rats of 250-300g by bronchoalveolar lavage (BAL) as previously described[12]. Rats were euthanized with an intraperitoneal of sodium pentobarbital (100 mg/kg bw). Lungs were lavaged with aliquots of 5 ml of sterile phosphate-buffered saline (PBS, pH 7.4) using sterile Teflon catheter (VYCON Code 123.06, lot 220987, Vigon, Spain) for collection of BAL fluid. The fluid was immediately withdrawn by gentle suction. Lavage fluid was collected in 50 ml tubes and centrifuged at 1000*g* at 4ºC temperature to pellet the cells.

The cells were suspended in 1 ml fresh complete medium containing 44 ml of Dulbecco's Modified Eagle Medium (DMEM), 2 mM glutamine, 10% heat inactivated fetal bovine serum (FBS) and antibiotic solution[(with 10,000 units penicillin and 10 mg streptomycin per ml) (Sigma Chemical Co, St Louise, MO)]. Viability of cells was estimated by the mitochondrial reduction of MTT (3-[4, 5-dimethyl-thiazol-2-yl]-2.5-diphenyl tetrazlium bromide) (Sigma) to formazan which was found to be more than 87.5% accordance with Andrade et al [13]. Cells were then counted, seeded in a 12-Well Cell Culture Cluster (Corning, NY 14831 USA) at the density of 1×10^6 cells/well and incubated at 37 ºC in 5% CO2 incubator (RS Biotech, Galaxy). After 2 h, non-adherent cells were washed away with sterile PBS and adherent cells (macrophages) were incubated in fresh complete medium.

Cells were treated with a dose of 10 µg/ml lipopolysaccharide (LPS; Sigma; positive control) and 0.1-50 µg/ml of *S mansoni* antigens (AWASm and CSm). Non-stimulated macrophages were used as negative control. All experiments were performed in triplicate.

Total RNA was extracted from alveolar macrophages using an RNeasy Mini Kit (Qiagen GmbH, Hilden Germany.). A total of 1µg RNA was used as template for the first-strand DNA synthesis. Primers specific and PCR reactions for VEGF, FGF2 and GAPDH were done as above.

Ethics Statement

The HUIGC (Hospital Universitario Insular de Gran Canaria)-IRB approved an oral consent process that included a written summary and a short form. The oral presentation and the short form were made in a language understandable to the subject. The interpreter (witness) was fluent in both the language of the participant and the language of the consent. The written summary was signed by the 1) witness and 2) person obtaining consent. The short form was signed by the 1) witness and 2) the participant or participant's legally authorized representative.

Mice were housed and handled under standard animal laboratory conditions according to the EU regulations. All animal procedures were approved by the University of Salamanca Animal Care and Committee.

Statistical analysis

Data of VEGF in human sera, number of eosinophils and epidemiological data of human population were reported as mean ± standard deviation (SD). Linear regression was performed with INSTAT programme. The overall differences among groups were compared by non-parametric Kolmogorov-Smirnov analysis. Data of parasite recovery, eggs per gram of liver or intestine in mice, blood cells and serological data were reported as mean ± SD. Differences in mice groups were performed by analysis of variance (ANOVA). When global differences were detected a post-ANOVA test using the Fisher least significant differences (LSD) analysis was applied. Differences between means were considered statistically significant at $p<0.05$. All statistical analyses were performed using Statworks, Statview 4.5 and INSTAT software packages for an IBM computer.

Results

Evaluation of VEGF detection protein in human population

Result showed significantly differences in VEGF detection protein between patients with schistosomiasis and healthy control patients ($p< 0.05$) (Table 1). Also, no differences were found between patients infected with *S. mansoni* and *S. haematobium*. Moreover, it was not observed any differences between patients diagnosed with other helminthoses (filariasis and hookworms infections) and healthy control patients. In addition, association between VEGF detection protein and number of eosinophils were not observed (Figure 1).

Evaluation of the effects of endostatin on Schistosoma mansoni infection in a murine experimental model

We studied the number of parasite recovery and eggs per gram in liver and intestine separately counted on day 46 post-infection. The mean number of worms recovery is 6.67 ± 0.5 in mice infected with *S. mansoni* and 5.5 ± 0.6 in mice infected and treated with endostatin. Although the parasite recovery in infected group with *S. mansoni* were higher than mice treated with endostatin, these differences were not statistically significant. The mean number of eggs per gram in liver in the group of infected animals was significantly higher ($p<0.05$) than mice treated with endostatin (Figure 2). Moreover, the mean number of eggs per gram in small intestine in mice infected was higher than mice treated with endostatin but differences were not significant (Figure 2). We also studied the number of granulomas from liver in *S. mansoni* mice infected compared with mice treated with endostatin. The results showed that the mean number of granulomas in mice infected group was significantly higher than mice treated with endostatin ($p< 0.05$) (Figure 3).

Red blood cells and platelet counts did not show any difference between groups. Only there was existed an increasing of the number of eosinophil in mice infected with *S. mansoni* (0.112±0.032) in compared with mice treated with endostatin (0.065±0.023) but differences was not significant (data not shown).

Specific IgG, IgG1 and IgG2a *S. mansoni* antibodies are shown in Figure 4. Specific IgG and IgG1 *S. mansoni* antibodies had not reduction between *S. mansoni* infected group and mice treated with endostatin. On the other hand, IgG2a antibodies detection presented statistical differences ($p< 0.05$) in mice infected and treated with endostatin.

RT-PCR in liver showed that VEGF-mRNA expression decreased in mice treated with endostatin in comparison with mice infected with *S. mansoni* (Figure 5). Similarly, FGF2

expression in liver also decreased in mice treated with endostatin in comparison with mice infected with *S. mansoni* (Figure 5). In contrary, VEGF and FGF2-mRNA expression in intestine did not show differences between *S. mansoni* infected group and mice treated with endostatin (Figure 6).

Evaluation of the effects of Schistosoma mansoni antigens on the VEGF and FGF2 production by rat alveolar macrophages

We studied the effects of different concentrations of different antigens of *S. mansoni* (0.1-50µg/ml) on the VEGF and FGF2 expression in alveolar macrophages (Figure 7). The result indicated that macrophages stimulated with 50µg/ml of cercarial antigen of *S. mansoni* (CSm) were able to induce VEGF and FGF2 mRNA expression (Figure 7A). On the other hand, macrophages stimulated with adult worm antigens of *S. mansoni* (AWASm) were not able to produce VEGF and FGF2 angiogenic factors (Figure 7B).

Discussion

The main helminthoses that affect the human population are geohelmintosis, schistosomiasis and filariasis[14]. These same worms also are responsible for parasitic infection in immigrant patients asymptomatic with absolute or relative eosinophilia [15, 16]. In the present work we evaluated VEGF concentration in the sera of patients with parasitological diagnosis of schistosomiasis, filariasis and hookworms infections. We selected patients from sub-Saharan Africa with eosinophilia who showed the presence of a single worm, not co-infection. We found that patients diagnosed of schistosomiasis had a high level of VEGF in sera compared with healthy patients group. These results are similar to those reported by other authors in *S. mansoni* infections [17]. We observed that there were no significant differences between VEGF levels in patients with schistosomiasis produced by *S. mansoni* and *S. haematobium*. Angiogenesis plays a fundamental role in many physiological and pathological processes, including the development of hepatic fibrosis. In cirrhosis, blood serum levels of VEGF are decreased[18]. However, high VEGF levels were detected in sera of patients with schistosomiasis, although controversial results were found in the VEGF detection among the different clinical phases of schistosomiasis [17, 19]. In addition, we observed that eosinophils are not involved in the expression of VEGF in humans because there was no association between number of eosinophils and VEGF detection in different helminthoses.

Secondly, we studied the effects that occur after inhibition of angiogenic factors in a experimental murine model of *S. mansoni*. We use endostatin as a specific inhibitor of angiogenesis[20]. The results showed that there was not significant reduction in the number of adult worms in mice infected with a significant reduction in the number of eggs in liver and number of granulomas in mice treated with endostatin compared with *S. mansoni* infected mice. This indicates that the inhibition of angiogenesis induced a decreasing of injury characteristics trigger hepatic formation of the granuloma and subsequent fibrosis.

Moreover, we studied the mechanisms involved in the reduction of injury. Firstly, we analyzed data of the blood cells count (red, white blood and platelet cells). No significant differences in count of blood cells (specifically in eosinophils) were found between *S. mansoni* infected mice and mice treated with endostatin. The results obtained in this experiment are consistent with observations in count of eosinophils in human population infected with schistosomiasis, filariasis and hookworm infections. This is opposite to that found by our group when we analyzed the relationship between angiogenesis and strongyloidiasis (paper in press). Secondly we proved the decreasing of the expression of VEGF and FGF2 in liver. This data showed the effective inhibition realised by endostatin. Thirdly, we analyzed the immune response produced in mice infected and treated with endostatin. We found a decreasing significantly in the detection of specific IgG2a antibodies. This is associated with down-regulated Th1 responses in animals treated with endostatin which the reduction of number of eggs and granulomas in liver were observed. These results agree with observations by other authors in which severe schistosomiasis is associated with persistently elevated pro-inflammatory T-helper-1 (Th1)-type cytokines, whereas milder pathology is present when Th2 cytokines dominate [21]. Moreover, shift toward Th1-type cytokine production by a numerically stable population of CD4 T cells correlates with severe exacerbation of immunopathology in schistosomiasis [22].

Finally, the last objective of our study was to identify *S. mansoni* antigens involved in the expression of VEGF and FGF2 angiogenic factors by alveolar macrophages. We used cercarial (CSm) and adult worms (AWASm) *S. mansoni* antigens. Our results showed that cercarial antigens were able to stimulate alveolar macrophages to produce VEGF and FGF2 factors. Our results and the data obtained by Loeffler et al[23] where products secreted by *Schistosoma* eggs may promote angiogenesis within hepatic granulomas by up-regulating endothelial cell VEGF, shown the role of angiogenesis in schistosomiasis and its participation in the granulomatous reaction associated to the injury produced.

In summary, this study show the role of angiogenesis in the pathogenesis of schistosomiasis based on three aspects: (*i*) VEGF detection in patients diagnosed of schistosomiasis, (*ii*) reduction of eggs recovery and granuloma formation in liver in mice treated with endostatin with down-regulated Th1 immune response (*iii*) cercarial antigen of *S. mansoni* stimulated VEGF and FGF2 production by alveolar macrophages.

Acknowledgment

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Table 1: Epidemiological characteristics (age and sex) and count of eosinophils in periphery blood and VEGF measured by ELISA in sera of Sub-Saharan Africa immigrant patients infected with Schistosoma, filariae and hookworms. Healthy patients from endemic area are included as control group.

	Group of Study	Number of patients	Age (Year mean ±SD)	Sex (Male %)	Eosinophil/ µl (Mean ± SD)	VEGF pg/ml (Mean ± SD)
Patients infected with *Schistosoma*						274.2 ± 213*
	S. mansoni	10	24.5 ± 4.7	90	842 ± 669	213.6 ± 164
	S. haematobium	15	19.5 ± 7.4	100	999 ± 458	314.5 ± 237
Patients infected with filariae						250.3 ± 161
	Mansonella perstans	9	27.7 ± 12.5	77.7	1243 ± 718	198.4 ± 145
	Loa loa	3	31.6 ± 8.6	33.3	1453 ± 614	417.4 ± 131
	Wucheria bancrofti	1	30	100	990	216
Patients infected with hookworms		15	23.8 ± 4.7	93.4	880 ± 429	135.6 ± 109
Healthy control group		18	28.9 ± 8.6	94.4	176 ± 107	123.7 ± 104

SD: Standard deviation % Percentage.* Statistical differences in comparison with healthy control group $p<0.05$.

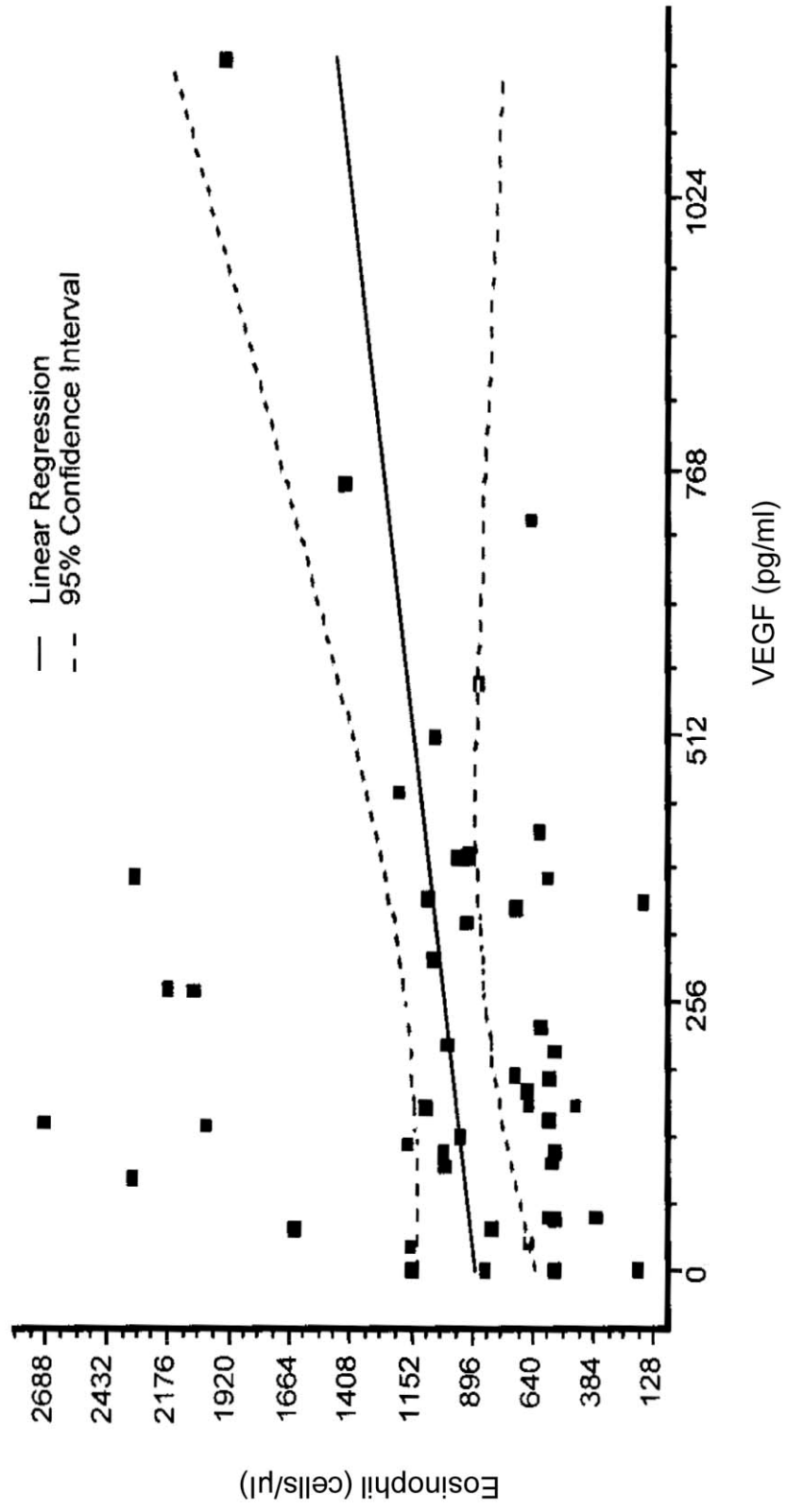

Figure 1: Lineal regression to compare VEGF protein and total eosinophil in patients diagnosed with helminthic infections. Sera of schistosomiasis, filariasis and hookworms infections from Sub-Saharan Africa immigrants were analyzed. Healthy patients from endemic area are included as control group. The major of data are out of the confidence interval. This data demonstrated no association between two variables.

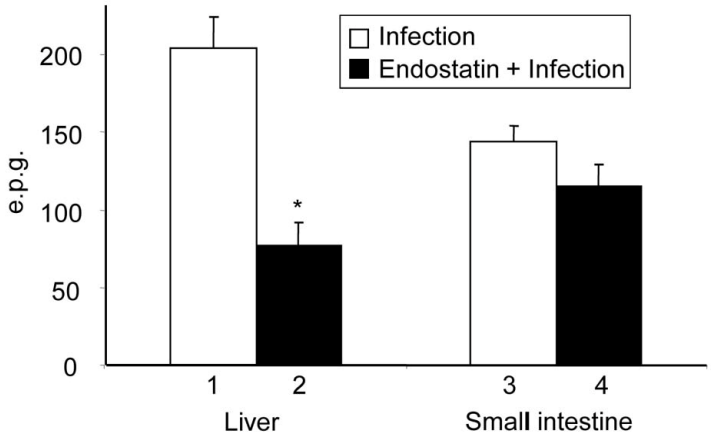

Figure 2: Determination of *S. mansoni* eggs in liver and intestine. Mice were divided in two groups. *(i)* mice infected with *S. mansoni* and necropsy 7 week pi and *(ii)* mice infected with *S .mansoni* and treated with endostatin, necropsied 7 week pi *$p<0.05$.

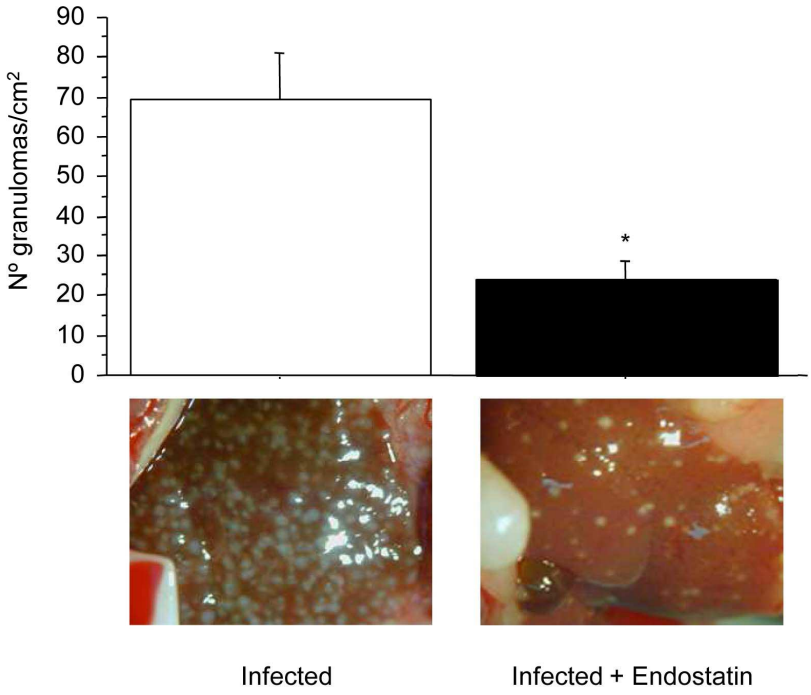

Figure 3: Number of granulomas on liver surface. Hepatic granulomas on liver surface/cm^2 were counted in two groups. *(i)* mice infected with *S. mansoni* and necropsy 7 week pi and *(ii)* mice infected with *S .mansoni* and treated with endostatin, necropsied 7 week pi. The results are expressed as mean ± standard error. An illustration of liver granulomas of treated and untreated control group is shown.* $p<0.05$.

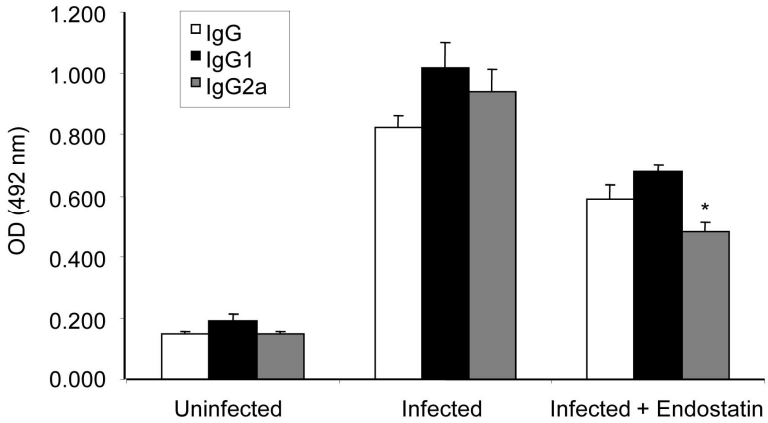

Figure 4: Detection of IgG, IgG1 and IgG2a specific antibodies by ELISA. Sera of three groups as *(i)* uninfected mice *(ii)* mice infected with *S. mansoni (iii)* mice infected with *S .mansoni* and treated with endostatin were analyzed. IgG (white colour), IgG1 (black colour) and IgG2a (grey colour) *p<0.05.

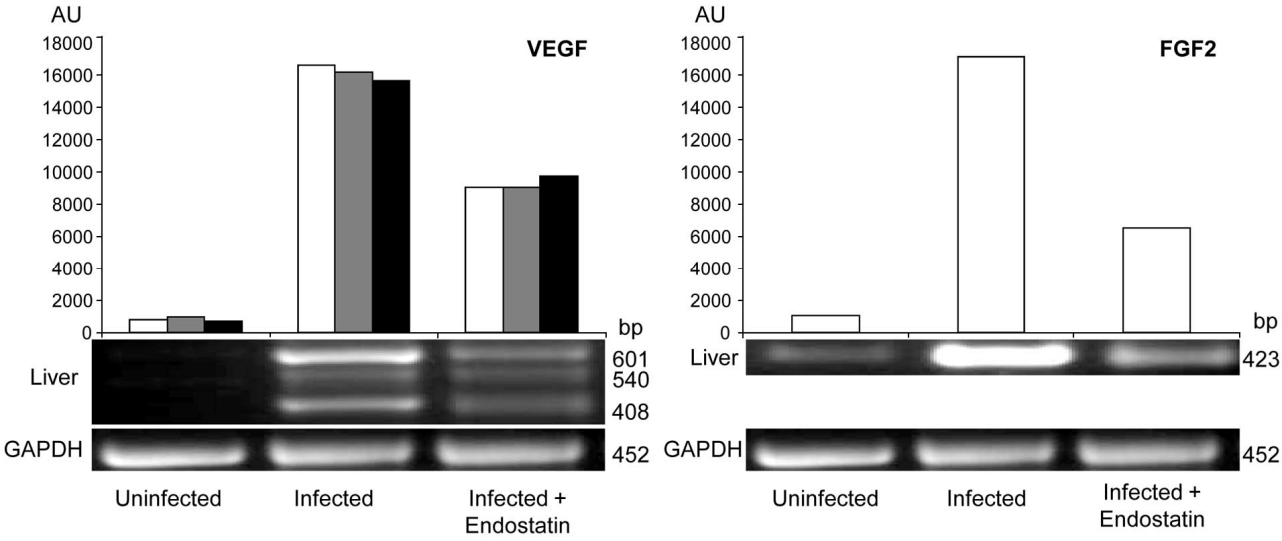

Figure 5: Detection of VEGF and FGF2 by RT-PCR in liver. Three experimental groups were used: *(i)* uninfected mice *(ii)* mice infected with *S. mansoni (iii)* mice infected with *S .mansoni* and treated with endostatin. VEGF was expressed as three bands and arbitrary units (AU) calculated by densitometry of each band 601 bp (white bars), 540 bp (grey bars), and 408 bp (black bars). FGF2 expression showed a band with 423bp (white bars) and densitometry analysis of it as arbitrary unit (AU). GAPDH expression was used as internal control.

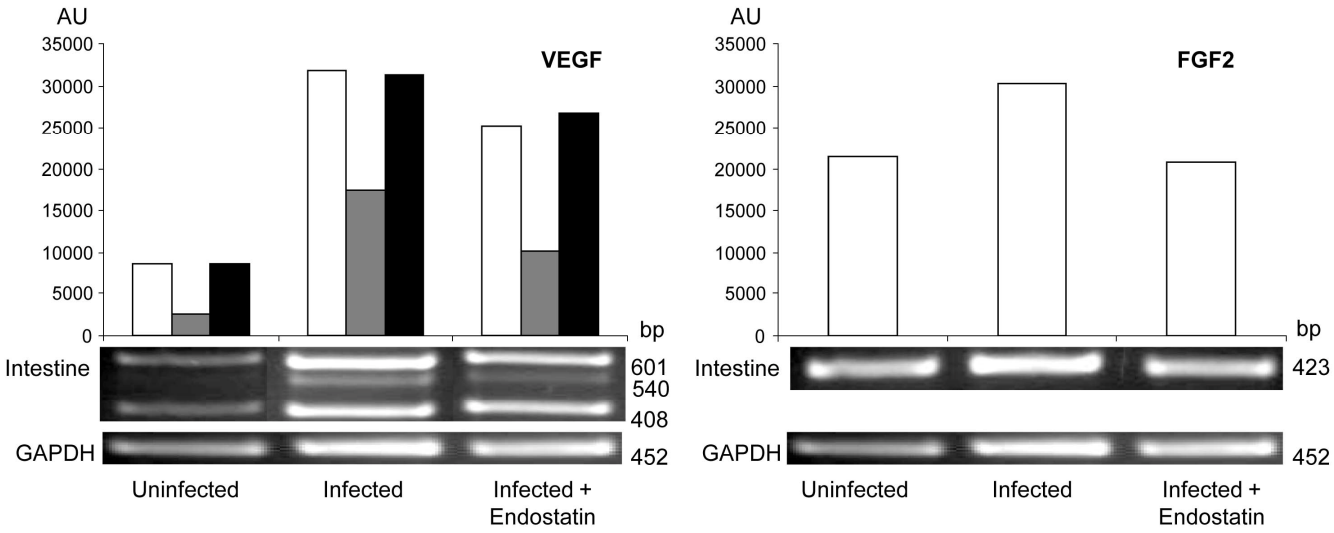

Figure 6: Detection of VEGF and FGF2 by RT-PCR in intestine. Three experimental groups were used: *(i)* uninfected mice *(ii)* mice infected with *S. mansoni (iii)* mice infected with *S .mansoni* and treated with endostatin. VEGF was expressed as three bands and arbitrary units (AU) calculated by densitometry of each band 601 bp (white bars), 540 bp (grey bars), and 408 bp (black bars).FGF2 expression showed a band with 423bp (white bars) and densitometry analysis of it as arbitrary unit (AU). GAPDH expression was used as internal control.

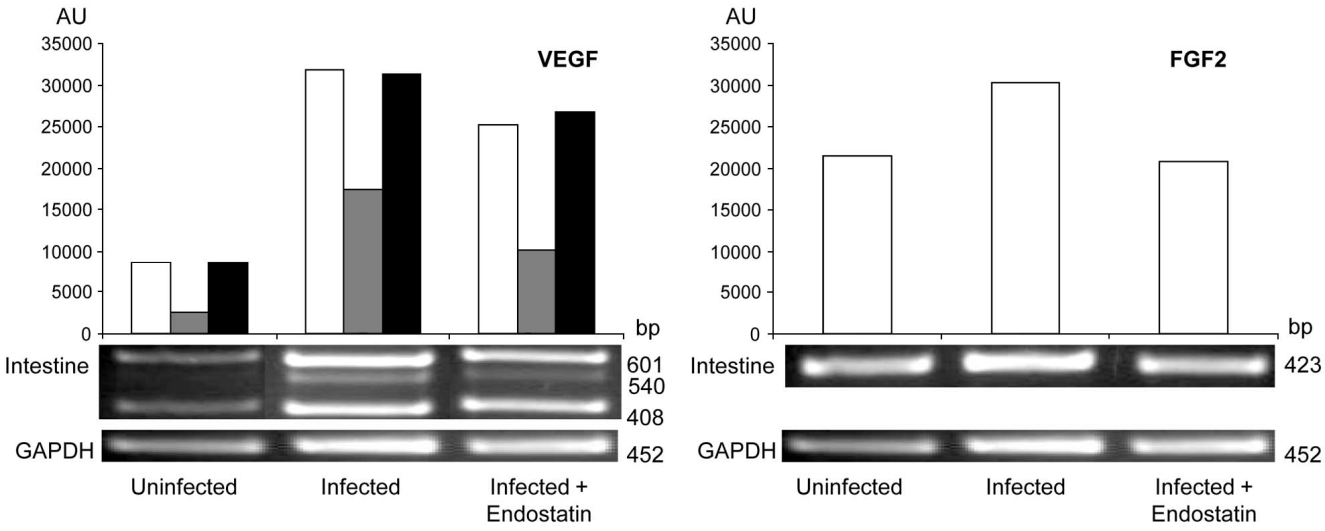

Figure 7: Effect of Schistosoma mansoni antigens on VEGF and FGF2 in alveolar macrophages. mRNA expression in rat alveolar macrophages stimulated with cercarial antigen of S. mansoni (A),, and adult worm S. mansoni antigen (B). GAPDH mRNA expression levels from rat macrophages detected by RT-PCR are used as internal positive control (C). Non-stimulated, negative control (Ø), LPS stimulated macrophages, positive control (LPS) and different concentrations of S. mansoni antigens, 0.1-50µg/ml. Arbitrary units are calculated by densitometry of corresponding amplicons.

4 Conclusions

1. - The antigens of encapsulated species *Trichinella spiralis* are able to stimulate in a dose-dependent manner, angiogenic factors (VEGF and FGF2) in rat alveolar macrophages.

2. - Angiogenesis plays an important role in primary infection by *Strongyloides venezuelensis*, as its inhibition by using endostatin causes a decrease in magnitudes of infection.

3 .- The endostatin reduces infection of *Strongyloides venezuelensis* not by direct action on the parasite but through indirect effects such as decreased production of angiogenic factors by host and / or reduction of the eosinophilic response.

4. - The larval somatic antigens of *Strongyloides venezuelensis* are responsible for the induction of angiogenic factors and their modulation is related to the generation of nitric oxide.

5. - In patients with schistosomiasis there is an increased VEGF levels in serum with significant differences compared with other diseases due to helminths.

6. - Inhibition of angiogenesis by endostatin in murine experimental schistosomiasis induces a decrease in the number of eggs in liver, as well as in the presence of granulomas and hepatic fibrosis.

7 .- The cercariae *S. mansoni* antigens induce the expression of angiogenic factors in rat alveolar macrophages.

5 Resumen en español

El conocimiento sobre el control de la angiogénesis ha sido un gran avance en la comprensión de la patogénesis de muchas enfermedades en las últimas décadas. Los estudios sobre el papel de los factores angiogénicos y antiangiogénicos en la biología del crecimiento neoplásico, crecimiento del tumor y el desarrollo de las metástasis, son los más abundantes en la literatura. Sin embargo, tanto la generación como la inhibición de nuevos vasos desempeñan un papel importante en otros procesos fisiopatológicos, por ejemplo isquemia, infección e inflamación. La revisión bibliográfica de esta Tesis Doctoral está estructurada en tres secciones: en primer lugar analizamos los conceptos básicos de la angiogénesis y vasculogenesis, así como su papel en las enfermedades neoplásicas y no neoplásicas. En un segundo apartado estudiamos las características de los dos principales factores angiogénicos: VEGF (factor de crecimiento del endotelio vascular) y FGF-2 (factor de crecimiento de fibroblastos). Específicamente se indican las características estructurales de los factores y el mecanismo biológico de acción. Además, describimos factores antiangiogénicos como por ejemplo la endostatina, un potente inhibidor de la proliferación y migración. Por último, analizamos el papel de los factores angiogénicos y antiangiogénicos en la patogénesis de las helmintosis. Al respecto, hemos encontrado escasa información y limitada al estudio de la esquistosomosis, filariosis e infecciones por *Taenia solium* y *Trichinella* spp.

Los vertebrados, incluyendo las especies de mamíferos, tienen un sistema de circuito cerrado para el suministro de nutrientes y oxígeno a los tejidos. Los vasos sanguíneos presentan dos tipos principales de células, células endoteliales que recubren el interior de los vasos y células del músculo liso que regulan la contracción y la dilatación de los vasos

sanguíneos. Desde el punto de vista embriológico, los vasos sanguíneos tienen dos etapas principales: vasculogénesis (formación de vasos sanguíneos a partir de células precursoras) y angiogénesis (formación de vasos sanguíneos a partir de vasos preexistentes y de células del endotelio vascular). La angiogénesis desempeña un papel importante en diversos procesos fisiológicos y patológicos, incluyendo el desarrollo embrionario, la cicatrización, la inflamación y el crecimiento tumoral. Tanto la liberación no controlada de los factores angiogénicos como la alteración de la producción natural de los inhibidores de la angiogénesis, con la consiguiente modificación del equilibrio angiogénico, son responsables de la proliferación incontrolada de células endoteliales que tiene lugar durante la neovascularización en la angiogénesis tumoral y en enfermedades asociadas.

Folkmann en 1971 propuso que el crecimiento de tumores y metástasis dependen de la angiogénesis y por tanto su bloqueo puede ser una estrategia para detener el crecimiento tumoral. En las etapas iniciales de los tumores malignos hay un equilibrio entre la proliferación de células neoplásicas y su destrucción, tanto por apoptosis como por control inmunológico. Cuando el tumor primario alcanza un tamaño crítico, disminuye la concentración de oxígeno y las células producen factores angiogénicos. Por otra parte, la destrucción del tejido conduce a la producción de sustancias antiangiogénicas. La vida media de factores antiangiogénicos es mayor que el crecimiento tumoral y las metástasis en desarrollo. Sin embargo, cuando el equilibrio se altera a favor de la actividad pro-angiogénica, la formación de nuevos vasos se lleva a cabo con las consecuencias que se indican en las secciones siguientes. Factores pro-angiogénicos incluyen varias moléculas liberadas por las células del parénquima o por células inflamatorias en respuesta a factores mecánicos, metabólicos (por ejemplo, hipoxia, acidosis) o respuesta inmune. Por otra parte, la destrucción del tejido conduce a la generación de moléculas con potencial antiangiogénico. El predominio de los factores angiogénicos conduce a la formación de

nuevos vasos sanguíneos a partir de precursores endoteliales locales y a partir de la médula ósea.

En la angiogénesis tumoral están implicados tanto los vasos sanguíneos como los vasos linfáticos. Los vasos sanguíneos se pueden considerar anormales con respecto a la vascularización normal. Por lo tanto, macroscópicamente la distribución es muy desorganizada con dilatación de vasos tortuosos, con un diámetro irregular y un número excesivo de ramificaciones. Microscópicamente, el endotelio tiene numerosas aberturas con las uniones intercelulares ampliadas y la membrana basal discontinua o ausente. El músculo liso que rodea las células endoteliales no se contrae en respuesta a los estímulos normales, lo que limita el uso de vasoconstrictores en el tratamiento contra los tumores. Las principales alteraciones de los vasos linfáticos son la compresión de los vasos localizados en el tumor y la dilatación de los situados en la periferia por exceso de VEGF-C . Estos vasos linfáticos pueden recoger las células neoplásicas derivadas de la superficie del tumor y facilitar así la metástasis linfática.

Las modificaciones vasculares descritas en la parte anterior determinan el flujo sanguíneo caótico intratumoral con aparición de zonas de hipoxia y acidosis, que pueden seleccionar células que han perdido la respuesta apoptótica a la hipoxia y por tanto amplian la clonación. Por otra parte, los factores angiogénicos puede modular la expresión de moléculas de adhesión celular y otros marcadores de la superficie del tumor del endotelio vascular. Por ejemplo, VEGF y el factor de necrosis tumoral-α (TNF-α) aumentan el número de moléculas de adhesión, mientras que el factor de crecimiento de fibroblastos (FGF) y el factor de crecimiento transformante β1 (TGF-β1) disminuye el número de moléculas de adhesión.

La inflamación y la hipoxia contribuyen a la angiogénesis en las enfermedades no neoplásicas. En un adulto sano, las células endoteliales de los vasos sanguíneos se

encuentran en estado de reposo. En presencia de hipoxia o remodelación de la inflamación vascular ocurre la formación de nuevos vasos. Un aspecto de particular interés es que la angiogénesis en enfermedades no neoplásicas permiten la formación de nuevos vasos con estructura y función normal. La hipoxia es un estímulo muy importante de la angiogénesis así como de otros procesos biológicos que tratan de restaurar la oxigenación de los tejidos normales. El mecanismo básico es la activación de una proteína llamada HIF-1 (factor inducible de hipoxia 1). HIF-1 es una proteína heterodimérica formada por la unión de dos subunidades (HIF-1α y HIF-1β). Mientras HIF-1 β se expresa de modo constitutivo en todos los tejidos, HIF-1α se activa en condiciones de hipoxia. HIF-1 actúa en la región promotora de genes diferentes que tienen un elemento común de respuesta. Entre los genes activados por HIF-1 se encuentran genes relacionados con la angiogénesis como los que codifican para VEGF, óxido nítrico sintasa inducible (iNOS), factor del crecimiento derivado de las plaquetas (PDGF) y angiopoyetina 1 (*ang1*). Además, durante la inflamación no hay reclutamiento ni activación de células sanguíneas circulantes hacia el foco lesional. Muchas de estas células (por ejemplo, macrófagos, plaquetas, eosinófilos, mastocitos) liberan factores pro-angiogénicos. Además, los fagocitos son capaces de producir proteasas y de estimular la liberación de factores angiogénicos de la matriz extracelular.

Los cambios en la angiogénesis (por exceso o defecto) han sido observados en enfermedades múltiples donde la hipoxia o la inflamación desempeñan un papel importante. En particular, se han producido cambios en diversos tipos de enfermedades sistémicas, tanto autoinmunes (artritis reumatoide, lupus eritematoso, esclerosis múltiple), metabólicas (diabetes mellitus) o hematológicas (enfermedad de Castleman). Por otra parte, las modificaciones en la angiogénesis se han detectado en enfermedades localizadas que afectan al tracto gastrointestinal (enfermedad de Crohn), sistema nervioso central (esclerosis

múltiple, enfermedad de Alzheimer), respiratorio (enfisema pulmonar, fibrosis pulmonar idiopática) o riñón (glomerulonefritis).

Los factores de crecimiento del endotelio vascular (VEGFs) son una familia de moléculas producidas por diferentes tipos celulares, entre los que se encuentran los eosinófilos. Desde su descripción inicial a principios de los años 80, los estudios acerca de la estructura y función de estas moléculas se han multiplicado de forma exponencial. Los VEGFs pertenecen a la superfamilia VEGF/PDGF (platelet-derived growth factor) y poseen como característica común tener ocho residuos de cisteína, dos de ellos relacionados con la formación de puentes intermoleculares y los otros seis implicados en la formación de puentes intramoleculares con formación de 3 dominios globulares. Se han descrito siete moléculas de la familia VEGF: VEGF-A, VEGF-B, VEGF-C, PlGF (placental growth factor), VEGF-E y VEGF-F. En mamíferos, únicamente los cinco primeros tienen importancia biológica, siendo VEGF-E una proteína vírica (virus Orf) y VEGF-F una proteína presente en el veneno de serpientes (víboras). Dentro de las 5 moléculas identificadas en humanos, la que reviste mayor importancia biológica es el VEGF-A, en muchas ocasiones denominado simplemente VEFG. El gen que codifica VEGF-A está formado por ocho exones. De forma característica, el ARN mensajero sufre un procesamiento alternativo, *splicing*, por el que las proteínas derivadas y englobadas con la denominación genérica de VEGF o VEGF-A son diferentes. La nomenclatura general de estas proteínas incluye, además de la denominación general (VEGF) el número de aminoácidos total ($_{xxx}$) un sufijo (b) si el procesamiento alternativo afecta a la porción distal del exón 8. El número total de aminoácidos deriva principalmente de la inclusión o no de aminoácidos codificados total o parcialmente por los exones 6 y 7. Estos aspectos estructurales tienen una clara trascendencia biológica. Así, la inclusión o exclusión de los exones 6 y 7 modula las interacciones con proteoglicanos heparan sulfato (HSPGs) y

receptores tipo neuropilina. Por otro lado, el procesamiento proximal o distal del exón 8 tiene consecuencias funcionales, ya que las isoformas $VEGF_{xxx}$ son proangiogénicas y las $VEGF_{xxx}b$ son antiangiogénicas. Las isoformas principales en el ser humano de VEGF-A son $VEGF_{206}$, $VEGF_{189}$, $VEGF_{189b}$,$VEGF_{165}$, $VEGF_{165b}$, $VEGF_{121}$ y $VEGF_{121b}$. En las formas proangiogénicas, la isoforma que posee una mayor importancia biológica es $VEGF_{165}$, seguida de $VEGF_{121}$, $VEFG_{189}$ y $VEGF_{206}$.

El VEGF-A ejerce su efecto biológico a través de la interacción con los receptores de superficie celular. Los receptores para VEGFs son de 5 tipos principales: VEGFR-1 (vascular-endothelial growth factor-1), VEGFR-2 (vascular-endothelial growth factor-2), VEGFR-3 (vascular-endothelial growth factor-3), neuropilina-1 y neuropilina-2. Cada uno de los VEGFs presenta una selectividad específica por uno o varios de los receptores indicados. VEGF-A es capaz de unirse a todos ellos con excepción de VEGFR-3. VEGFR-1 (también denominado *fms*-like tyrosine kinase; Flt-1) es una molécula constituida por 7 dominios tipo Ig extracelulares, una región transmembrana y un dominio intracelular tirosina kinasa. Esta molécula se expresa en las células endoteliales, osteoblastos, células del sistema mononuclear fagocítico, células trofoblásticas placentarias, células mesangiales y algunas células madre hematopoyéticas. Aunque la afinidad del VEGFR-1 por VEGF-A es 100 veces mayor que por VEGFR-2, su actividad tirosina kinasa es menor. En la embriogénesis, la expresión de este receptor está ligada a un efecto negativo en la angiogénesis. En periodos posteriores, la unión VEGF-A a VEGFR-1 parece que desempeña un papel importante en la generación de señales paracrinas en las células endoteliales.VEGFR-2 (también denominado KDR: kinase-insert domain receptor o flk-1: *fms*-like kinase) tiene una estructura muy similar a VEGF-1. Este receptor se expresa principalemente en las células endoteliales, aunque también se ha demostrado en neuronas, megacariocitos, osteoblastos y células hematopoyéticas. Las principales acciones de VEGF-

A se ejercen por interacción con este receptor. Las neuropilinas 1 y 2 fueron identificadas como los receptores de una familia de moléculas conocidas como semaforinas/colapsinas, una familia de moléculas reguladoras del desarrollo del sistema nervioso. Ambas se expresan durante el desarrollo embrionario en tejido nervioso, cardiovascular y esquelético, mientras que en el adulto su expresión es muy amplia. El papel de las neuropilinas, principalmente de NP-1 es actuar como correceptor de VEGFR-2 aumentando su acción biológica.

Muchos tipos celulares son capaces de desencadenar la síntesis y liberación de VEGF-A en respuesta a estímulos muy diferentes: modificaciones metabólicas (hipoxia, hipoglucemia), hormonales (estrógenos), citocinas y otros factores de crecimiento, moléculas proinflamatorias y alteraciones genéticas. Uno de los mejor caracterizados es la hipoxia, que induce la estabilización y translocación al núcleo de dos factores HIF (hypoxic inducible factor) 1α e HIF2α . La unión con el factor nuclear HIF1β con los factores HIFa, al interaccionar con una región específica del gen del VEGF (HRE, hypoxia response element) induce la síntesis de esta molécula. Por otro lado, la hipoglucemia, tanto en estudios de laboratorio como clínicos da lugar a un aumento en la síntesis de VEGF: Los estrógenos también desempeñan un papel importante en la inducción de la producción de VEGF tanto en situaciones fisiológicas (ciclo menstrual, ovulación) como patológicas (cáncer de mama). Múltiples citocinas y factores de crecimiento inducen la producción de VEGF. Entre los mediadores inflamatorios relacionados con la producción de VEGF deben destacarse los radicales libres de oxígeno y el óxido nítrico. Finalmente, varios tipos de alteraciones genéticas, presentes en enfermedades hereditarias o tumorales se asocian a hiperproducción de VEGF. Las mejor caracterizadas se relacionan con mutaciones de p53, del gen que codifica la proteína de von Hippel-Lindau, de los genes supresores PTEN (phosphatase and tensin homologue deleted on chromosome 10).

La interacción entre VEGF-A y el receptor VEGFR-2 pone en marcha una serie de mecanismos de señalización iniciales que pueden ser resumidos en 5 fases: *(i)* unión del ligando al receptor, *(ii)* dimerización del receptor; *(iii)* activación de tirosina-cinasa; *(iv)* autofosforilación del receptor y (*v*) unión y activación de adaptadores a los lugares de autofosforilación. La vía de señalización del VEGF/ VEGFR-2 es diferente a la de otros factores de crecimiento. Básicamente tras la interacción entre VEGF-A y VRGFR-2 se activan 4 vías de transducción: *(i)* Activación de PI3K que transforma PIP2 en PIP3. Esta molécula actúa de dos formas en los efectos del VEGF: inhibiendo la apoptosis (por acción sobre BAD y caspasa 9) y estimulando la producción de óxido nítrico por acción sobre la isoenzima endotelial de la ONS. *(ii)* activación de CDC42 finalmente conduce a una reorganización de los filamentos de actina, *(iii)* Disminución de la adhesión de las células endoteliales empleando como molécula adaptadora Src y *(iv)* activación de la fosfolipasa C gamma que partiendo de PIP2 genera DAG e IP3. Las consecuencias de esta vía son de dos tipos: la generación de prostaglandinas y la activación de la proliferación de las células endoteliales. En resumen, los cuatro mecanismos por los que VEGF conduce a la angiogénesis son: *(i)* aumento de la supervivencia celular, *(ii)* incremento en la migración celular, *(iii)* estimulación de la proliferación celular y *(iv)* facilitación de la permeabilidad.

FGF2 o factor de crecimiento de fibroblastos representa el mejor caracterizado de la familia de factores de crecimiento que unen heparina. FGF-2 es una molécula con efecto angiogénico potente *in vivo* e *in vitro,* que estimula el crecimiento de las células musculares lisas, la cicatrización de las heridas y la reparación de los tejidos. Además, el FGF-2 puede estimular la hematopoyesis y desempeñar un papel importante en la diferenciación y función del sistema nervioso, los ojos y el esqueleto. FGF-2 fue identificado como una proteína de 146 aminoácidos y más tarde como una forma proteolítica de 18 kDa. Contiene cuatro residuos de cisteína, sin bandas disulfuro intramolecular, un gran número de residuos

básicos y dos sitios que pueden estar fosforilados por proteínas quinasas A y C, respectivamente. La interacción de FGF-2 y la heparina protege a este factor contra el calor, la desnaturalización ácida y la fragmentación mediante proteasas. Mientras que la heparina es sólo sintetizada por las células cebadas del tejido conectivo, el sulfato de heparina se distribuye ampliamente en todos los órganos y tejidos de mamíferos, unida a proteínas básicas como la heparina sulfato proteoglicanos (HSPG). HSPGs son una clase funcional de diversas moléculas que se encuentran en la superficie celular y en la matriz extracelular, donde se ha demostrado que interactúan con FGF-2 y modulan su distribución y función.

FGF-2 desempeña un papel clave en las diferentes etapas de la enfermedad en la mayoría de los órganos. Una de las actividades mejor caracterizadas es su capacidad para regular el crecimiento y la función de las células vasculares tales como las células endoteliales y del músculo liso. FGF-2 se ha implicado en el desarrollo y crecimiento de nuevos vasos sanguíneos (angiogénesis) y en la patogénesis de las enfermedades vasculares como la aterosclerosis. FGF-2 ha sido observado en el sistema nervioso de una variedad de especies. En el cerebro humano adulto, se ha encontrado en el sistema nervioso central y en las células de Purkinje del cerebelo, en los astrocitos, en poblaciones neuronales seleccionadas y ocasionalmente en las células de la microglia, encontrándose todas sus isoformas. En el pulmón, el FGF-2 se ha relacionado con la morfogénesis de la ramificación y el desarrollo de la fibrosis pulmonar. FGF-2, así como varios otros miembros de la familia FGF tales como FGF-4 y -8, estimulan el desarrollo de las extremidades. Sin embargo, es poco probable que FGF-2 sea el principal candidato para la formación de las extremidades ya que su patrón de expresión no se correlaciona con los eventos que ocurren durante la generación de estas. Los estudios de desarrollo indican que el FGF puede desempeñar un papel importante en el desarrollo muscular.

La proliferación, diferenciación y producción de TGF-β de osteoblastos son estimuladas por FGF-2. Además estimula la función de las células de Leydig. En el ojo, FGF-2 produce la inducción de la regeneración *in vitro* de la retina y protege a los fotorreceptores del daño de la luz. Por último, la proliferación y la diferenciación de los melanocitos normales humanos y las actividades de varios de los queratinocitos son dependientes de la producción de FGF-2.

Los inhibidores de la angiogénesis son sustancias que inhiben el crecimiento de nuevos vasos sanguíneos. Pueden ser endógenos (proteínas o fragmentos de proteínas que se forman en el organismo) o exógenos (fármacos o componentes de la dieta). Uno de los principales inhibidores es la endostatina, proteína de 20 kDa, procedente de la división del colágeno XVIII y que pertenece a la familia de las multiplexinas. La generación de endostatina desde el colágeno XVIII está catalizada por enzimas proteolíticos como catepsina L y metaloproteasas. Al igual que muchos otros inhibidores de la angiogénesis, endostatina tiene alta afinidad por la heparina, con un sitio que sirve de unión a la heparina. Este sitio participa en la inhibición de la angiogénesis inducida. También se une con baja afinidad a proteoglicanos sulfato de heparina, glipicano-1 y glipicano-4 y con alta afinidad a una molécula no identificada en las células endoteliales. Además se asocia con proteínas como integrinas, fibulinas, laminina-1 y tropomiosina. Por último, se ha observado que endostatina se introduce directamente en el citoplasma a través de las células endoteliales.

Las funciones de la endostatina son diversas. La más conocida es la de ser un potente inhibidor de la angiogénesis endógena y por tanto supresor de tumores. Se ha observado en animales de experimentación que si se eliminaba la endostatina, los tumores crecían dos o tres veces más rápidos. Por el contrario, cuando la endostatina se sobreexpresaba, los tumores crecían tres veces más lentos. Algunos trabajos han demostrado que los ratones deficientes en endostatina mostraban aumento de la angiogénesis. Se han encontrado niveles

de endostatina elevados en ciertos tipos de cáncer, en líquido intratumoral, ascitis maligna y en enfermedades inflamatorias crónicas como la artritis reumatoide y la retinopatía diabética.

Existe poca información sobre el papel de la angiogénesis y los factores angiogénicos en las helmintosis, tanto en los seres humanos como en modelos experimentales. Los factores angiogénicos producidos por el parásito o por el hospedador pueden estimular la neovascularización a través de varios mecanismos. Se ha demostrado que algunos genes de *C. elegans* (PVF-1) codifican un factor con capacidad de unión a los receptores de VEGF de los mamíferos, VEGFR-1 (FLT-l) y VEGFR-2 (KDR), que inducen angiogénesis. Respecto a los mecanismos utilizados por el hospedador, en primer lugar, tanto los factores de crecimiento del endotelio vascular (VEGF) como el factor de crecimiento de fibroblastos (FGF-2) inducen angiogénesis directamente, mediante la estimulación y proliferación de células endoteliales, y a través de la migración y diferenciación en los vasos. En segundo lugar, otros factores angiogénicos como la interleucina 1 (IL-1) promueven indirectamente la angiogénesis mediante la estimulación de las células inflamatorias para producir VEGF. En tercer lugar, la fragmentación de proteínas angiogénicas como la heparinasa libera péptidos que estimulan los componentes del sistema inmunitario del hospedador, el cual a su vez estimula la producción de factores angiogénicos como VEGF y FGF-2. Por último, algunos factores angiogénicos estimulan los mecanismos implicados en las lesiones definitivas. De hecho, datos experimentales sugieren que la hipoxia en relación con el VEGF puede estimular la proliferación de la síntesis de colágeno tipo1 en miofibroblastos activados. Esto ha demostrado que el VEGF y la angiopoyetina-1 (Ang-1) pueden funcionar como factores autocrinos y paracrinos dependientes de la hipoxia, capaz de estimular la migración y quimiotaxis de los miofibroblastos a través de la activación de señales Ras/Erk.

En la práctica, las principal información sobre angiogénesis y factores angiogénicos en las helmintosis se limita a las infecciones causadas por *Schistosoma* spp, filarias e infecciones por *Taenia solium* y *Trichinella* spp.

La esquistosomosis está causada por la infección por diferentes especies del género *Schistosoma*. Con la excepción de la dermatitis cercariana y el síndrome de Katayama, la base patogénica de la esquistosomosis es la formación de granulomas alrededor de los huevos del parásito. Aunque la información de la literatura es escasa, observamos que en las etapas iniciales de la esquistosomosis existe estimulación de la angiogénesis. La presencia de angiogénesis en modelos experimentales de esquistosomiosis ha quedado claramente demostrada por técnicas histológicas e inmunohistoquímicas. En este contexto es interesante señalar varios aspectos: *(i)* La angiogénesis es un fenómeno temprano, así que en fases evolucionadas (como las que se encuentran en los seres humanos) es difícil observar. *(ii)* Un aspecto importante para que se genere angiogénesis es la necesidad de exposición repetida al parásito. *(iii)* Los factores genéticos desempeñan un papel esencial en la respuesta angiogénica frente al helminto.

Los mecanismos por los que *Schistosoma* spp desencadena angiogénesis son de varios tipos y se han obtenido en estudios experimentales. Así, se han identificado moléculas reguladoras de angiogénesis derivadas de los huevos de *S. mansoni* como lisofosfatidilserina y prostanoides. También los huevos secretan factores adicionales que son capaces de estimular la proliferación y migración de células endoteliales y la fosforilación de p42/44 MAPK. Este efecto no se limita a las células endoteliales, sino también estimula las células vasculares del músculo liso. Por otra parte, antígenos solubles de huevos de *S mansoni* (SEA) estimulan la proliferación y formación de nuevos vasos, disminuyendo la apoptosis y aumentando la expresión del gen para VEGF de las células endoteliales humanas de la vena umbilical (HUVECs). Estos hallazgos sugieren que los productos secretados por los huevos

de *Schistosoma* pueden promover la angiogénesis en los granulomas hepáticos por sobreexpresión de VEGF. Por otra parte, los huevos de *Schistosoma* que se adhieren al endotelio promueven indirectamente la angiogénesis a través de la hipoxia y de la respuesta inflamatoria, iniciada por TNF-α e ICAM-1. En etapas posteriores, se desarrolla fibrosis periportal por un mecanismo en el que participan linfocitos que producen moléculas profibroticas como TGF-β e IL-4. Por último, la infiltración de los huevos en el tejido circundante causa la migración de células inflamatorias hacia ese lugar. El sistema porta se interrumpe por completo alrededor de los granulomas y sólo se conserva muy cerca de la vena porta y de los conductos biliares. La proteolisis de la matriz extracelular mediante colágeno y metaloproteasas es un proceso anterior a la angiogénesis, pero necesario para permitir el desarrollo de una importante reacción inflamatoria. Aunque la proliferación de las células endoteliales de vasos maduros está en reposo, la presencia de múltiples células proliferativas en cada granuloma formado muestra la dinámica de la angiogénesis en la inflamación inducida por los esquistosomas.

Solamente dos grupos de investigación han estudiado el papel de los factores angiogénicos en la esquistosomosis humana y sus resultados son difícilmente comparables. Por un lado, se evaluaron 90 pacientes con esquistosomosis producida por *S. mansoni* clasificados en cinco grupos de acuerdo a los estudios ecográficos: infección leve, intensa, intestinal, hepatoesplénica y con fibrosis periportal. Detectaron que los niveles de VEGF se elevaban significativamente en los pacientes con esquistosomosis en todos los grupos excepto los grupos con infección leve e intestinal. Además, el nivel de VEGF se correlacionaba con la progresión de la enfermedad desde infección ligera hasta el desarrollo de fibrosis periportal. Utilizando un diseño de estudio diferente, otro grupo de investigación midió los niveles séricos de VEGF en pacientes con esquistosomosis, con o sin hipertensión portal. Estos autores no encontraron diferencias significativas entre ambos grupos, aunque

los valores medios en el grupo con hipertensión portal eran más bajos, un hecho que coincide con los bajos niveles de VEGF detectados en pacientes con cirrosis por otras causas.

Las filariosis son enfermedades relacionadas con la infección de varios géneros y especies de nematodos (*Wuchereria bancrofti, Brugia malayi, Brugia timori, Onchocerca volvulus, Loa loa, Mansonella perstans, Mansonella ozzardi y Mansonella streptocerca*). La alteración de la angiogénesis se ha descrito en dos tipos de filariosis: filariosis linfática y oncocercosis. Las manifestaciones de la filariosis linfática (específicamente las infecciones relacionadas con *W. bancrofti*) clinicamente se caracterizan por la presencia de linfedema, dilatación de los vasos linfáticos, extravasación de linfa y en algunos casos desarrollo de elefantiasis. Existen trabajos donde se estudia la relación entre factores angiogénicos (y específicamente de la familia del VEGF) y la patogénesis de la filariosis linfática. Así, en un estudio longitudinal en 63 pacientes polinesios que viven en un foco hiperendémico de *W. bancrofti*, se asoció la presencia de quiluria con un alto nivel del factor de crecimiento del endotelio vascular (VEGF). Sin embargo, los pacientes con elefantiasis presentaron elevados niveles de endotelina-1 (ET-1). Por otro lado se han relacionado factores genéticos con la clínica de la enfermedad y la determinación de factores angiogénicos. En una cohorte de pacientes con filariasis linfática procedentes de Ghana se examinaron tres polimorfismos de VEGF-A. Los autores encontraron que el genotipo C/C en 460 fue significativamente mayor en pacientes con hidrocele y en pacientes con niveles séricos elevados de VEGF. Por último, en una cohorte de pacientes infectados con *W. bancrofti* que tenían sobrexpresados los genotipos VEGF-C y sVEGFR-3 y que fueron tratados con doxiciclina (para la erradicación de la *Wolbachia* sp) tenían disminuidos VEGF-C y sVEGFR-3 y cuando llegaron a valores normales, presentaron mejoría clínica en sus síntomas.

La oncocercosis se caracteriza por dos tipos de manifestaciones clínicas: nódulos subcutáneos relacionados con filarias adultas y lesiones oculares relacionados con las microfilarias. Los nódulos que se originan en la infección por *Onchocerca volvulus* tienen diferentes patrones angiogénicos. Los nódulos pequeños tienen suministro adecuado de sangre con distribución difusa en toda la matriz de los nódulos, en estrecha asociación con los vermes adultos. Los nódulos mayores tienen una zona central más densa y no bien vascularizada y en las zonas más periféricas con vascularización intensa, alimentadas por vasos superficiales y en estrecho contacto con los vermes adultos. Al menos una proteína derivada de *O. volvulus* (homóloga a una secretada por *Ancylostoma*) puede contribuir a la alteración de la angiogénesis.

La neurocisticercosis es una afección del sistema nervioso central (SNC) causada por la fase larvaria de *Taenia solium*. En esta infección se conoce la importancia de la respuesta granulomatosa. Una serie de ocho pacientes con neurocisticercosis sometidos a craneotomía para análisis histológico e inmunohistoquímico, mostró que el parásito al morir se rodea de un granuloma asociado a fibrosis, angiogénesis e infiltrado inflamatorio. Los tipos de células más abundantes fueron las células plasmáticas, linfocitos B y T (Th1), macrófagos y mastocitos. Además, se ha observado aumento de la angiogénesis en los modelos de neurocisticercosis en animales.

La triquinelosis es una infección producida por distintas especies de *Trichinella* en el que existe una afectación de las células del músculo estriado. Las larvas del género *Trichinella* inducen la transformación de los miocitos en células nodrizas que se rodean de complejas redes de vasos sanguíneos. En ratones infectados experimentalmente por *Trichinella* se detectaron varias anomalías de los vasos. Así, los complejos vasculares se encontraron sólo alrededor de los miocitos infectados, caracterizándose por grandes vasos que se ramificaban en vasos pequeños formando redes. Esta redes son el resultado de la

angiogénesis *de novo* inducida durante la infección. En este contexto, los productos excretores/secretores derivados de larvas de *T. spiralis* y *T. pseudospiralis* están relacionados con cambios musculares (degenerativos, regenerativos) y angiogénesis.

De la revisión anterior podemos afirmar que: *(i)* La angiogénesis es un proceso de formación de nuevos vasos importante no sólo en las enfermedades tumorales sino también en otras enfermedades, especificamente en helmintosis. *(ii)* Los estudios sobre el papel de la angiogénesis en helmintosis son escasos. *(iii)* No existe información bibliográfica acerca de la producción de factores angiogénicos por macrófagos ni del empleo de factores anti-angiogénicos en helmintosis. *(iv)* Aunque se ha demostrado el papel del óxido nítrico en helmintosis y existe una relación documentada entre óxido nítrico y VEGF entre otros agentes angiogénicos, no se dispone de una información bibliográfica concreta acerca de la interacción de ambos mediadores inflamatorios en estas enfermedades parasitarias.

Teniendo en cuenta estos datos los **objetivos** propuestos en esta Tesis Doctoral son:

1.- Evaluar la expresión de factores angiogénicos (VEGF y FGF2) en macrófagos alveolares estimulados con diferentes antígenos procedentes de diversas fases del ciclo biológico de *Trichinella spiralis*, *Trichinella pseudospiralis*, *Strongyloides venezuelensis* y *Schistosoma mansoni*.

2.- Estudiar la relación existente entre la expresión de factores angiogénicos y la producción de óxido nítrico, utilizando inhibidores específicos de la óxido nítrico sintasa inducible.

3.- Emplear modelos *in vitro* de helmintosis para evaluar el efecto de factores anti-angiogénicos (endostatina).

4.- Utilizar modelos experimentales de infección de estrongioloidosis por *S. venezuelensis* y esquistosomosis por *S. mansoni* para valorar el efecto de factores anti-

angiogénicos en el desarrollo de la infección, estudiando los mecanismos que puedan estar involucrados.

5.- Analizar la producción de factores angiogénicos en sueros de pacientes con diagnóstico de helmintosis importadas.

Tanto los materiales y métodos como los resultados y discusión de los experimentos realizados en esta Tesis Doctoral se han dividio en tres apartados que corresponden a los estudios realizados sobre angiogénesis y tres de la pricipales helmintosis: triquinelosis, estrongiloidosis y esquistosomosis.

En el **primer trabajo** se estudia la expresión diferencial de factores angiogénicos en macrófagos estimulados con antígenos de especies encapsuladas y no encapsuladas de *Trichinella*. Este trabajo ha sido publicado en *Experimental Parasitology* 2009, 123: 347-353.

Los objetivos, metodología, resultados, discusión y conclusiones principales se exponen a continuación:

Introducción: El estadio larvario de *T. spiralis* se introduce en la célula del músculo estriado formando la célula nodriza. Se detectó factor de crecimiento del endotelio vascular (VEGF) en en el área que rodea las células nodrizas. Sin embargo, no se dispone de datos sobre los antígenos implicados, el papel de otros factores angiogénicos o la relación de la angiogénesis con el óxido nítrico (ON).

Metodología: Se usaron cultivos celulares de macrófagos alveolares para estudiar el efecto de diferentes antígenos de larva1 de *T. spiralis* (especie encapsulada) y *T. pseudospiralis* (especie no encapsulada) sobre la expresión de VEGF y factor básico de crecimiento de fibroblastos (FGF2). Además, se investiga la relación entre la producción de ON y los mediadores angiogénicos.

Resultados: Los resultados muestran que especies encapsuladas y no encapsuladas de *Trichinell*a son diferentes en su capacidad de estimular la expresión de VEGF y FGF2 por los macrófagos alveolares. *T. spiralis* estimula la producción de factores angiogénicos mientras que *T. pseudospiralis* no es capaz de estimular estas células. Por último, no hay ninguna relación entre los factores angiogénicos y la producción de ON por el antígeno de *T. spiralis*.

Discusión: Las especies encapsuladas se caracterizan por el depósito de colágeno y otros componentes de la matriz extracelular alrededor de las larvas de *Trichinella*. Un proceso clave asociado a la fibrogénesis es la angiogénesis. De hecho, los datos experimentales sugieren que la hipoxia en relación con el VEGF puede estimular la proliferación de la síntesis de colágeno tipo 1 en miofibroblastos. Nuestros resultados indican que los macrófagos son capaces de producir y liberar no sólo VEGF, sino también FGF2 en respuesta a los antígenos de las especies encapsuladas como *T. spiralis* en una manera dosis-dependiente. La expresión génica del VEGF se determinó mediante RT-PCR y expresión de la proteína medida por ELISA. Sin embargo, las especies no encapsuladas como *T. pseudospiralis* no fueron capaces de inducir la expresión de estos factores angiogénicos. La larva penetra en la célula muscular, crece en ella y destruye las miofibrillas. Las células satélite que rodean a la fibra muscular sufren cambios morfológicos transformándose en células nodrizas. Previamente se ha demostrado que las células nodriza son capaces de producir VEGF durante la infección por *T. spiralis*. En este trabajo se demuestra que las células inflamatorias que rodean a la fibra del músculo lesionado contribuyen a la producción de factores angiogénicos. Probablemente, ambos tipos de células están implicadas en el desarrollo de la cápsula de esta especie. Estudios previos realizados por nuestro grupo han demostrado que antígenos de especies encapsuladas y no encapsuladas de *Trichinella* fueron capaces de estimular la producción de óxido nítrico.

Como este mediador inflamatorio es un factor de inducción para la producción de VEGF, se evaluó si la inhibición de la óxido nítrico sintasa inducible, mediante el uso de inhibidores específicos como L-NAME y L-canavanina, modifican el efecto ejercido por los antígenos de *Trichinella* en la producción de factores angiogénicos. Este estudio demuestra que el efecto de los antígenos de *T. spiralis* no depende de óxido nítrico. En resumen, los antígenos de las especies encapsuladas de *Trichinella* estimulan directamente la producción de factores angiogénicos que pueden contribuir a la patogenia desarrollada en esta infección.

En el **segundo trabajo** se estudia el papel de los factores angiogénicos en la infección aguda experimental desencadenada por *Strongyloides venezuelensis*. Este trabajo ha sido aceptado para publicación en *Parasite Immunology* el día 11 de Enero de 2010.

Los objetivos, metodología, resultados, discusión y conclusiones principales se exponen a continuación:

Introducción: Este estudio tiene como objetivo investigar el papel de los factores angiogénicos en la patogénesis de la estrongiloidosis experimental.

Metodología: Dos enfoques complementarios fueron utilizados: En primer lugar, se trataron ratones CD1 con endostatina, un inhibidor de la angiogénesis, y posteriormente fueron infectados con *Strongyloides venezuelensis*. Además, se estudiaron los mecanismos implicados en este proceso. En segundo lugar, se investigó el efecto de los antígenos responsables de la estimulación de los factores angiogénicos (VEGF y FGF2) por macrófagos alveolares y los mecanismos implicados en su producción. Los macrófagos alveolares se obtuvieron por lavado broncoalveolar y se incubaron a diferentes concentraciones de antígenos somáticos y de excreción de secreción de *S. venezuelensis*. Además, los niveles de ARNm de VEGF y FGF2 fueron detectados por RT-PCR.

Resultados: Los ratones tratados con endostatina mostraron disminución significativa de huevos por gramo de heces y del número de larvas obtenidas del pulmón. Los

mecanismos implicados en esta disminución pueden ser tanto directos, con disminución de los factores angiogénicos, como indirectos con aumento de eosinófilos. Además se ha comprobado que únicamente los antígenos somáticos de las larvas 3 estimulan VEGF y FGF2. Por último se ha observado la relación existente entre los factores de la angiogénesis y el óxido nítrico analizada mediante el uso de inhibidores de la óxido nítrico sintasa inducible.

Discusión: La estrongiloidosis es una nematodosis de distribución cosmopolita que es especialmente relevante en individuos inmunocomprometidos, preferentemente en personas que reciben terapia con corticoides y en las regiones tropicales y subtropicales. Los mecanismos patogénicos en esta enfermedad son bastante desconocidos. Algunos autores han propuesto para el estudio de estos mecanismos modelos experimentales similares a *S. stercoralis,* utilizando especies de *Strongyloides* como *S ratti y S. venezuelensis*. El pulmón y las células que intervienen en su defensa, macrófagos alveolares, constituyen un primer elemento de estudio en la infección producida por este nematodo. Los trabajos realizados por nuestro grupo de investigación han puesto de manifiesto la producción de ON por macrófagos alveolares estimulados con antígenos larvarios de *S. venezuelensis*, demostrando la participación de este mediador inflamatorio en la estrongiloidosis experimental. Sin embargo, faltan estudios sobre el papel de otros mediadores inflamatorios y su posible relación con ON en la estrongiloidosis. La angiogénesis es un proceso complejo que conduce a la neovascularización generada a partir de los vasos sanguíneos preexistentes. Se asocia con la inflamación, la cicatrización de heridas, el crecimiento tumoral y la metástasis. La generación de nuevos vasos sanguíneos está regulada por las moléculas pro-angiogénicas y antiangiogénicas. Los factores de crecimiento vascular endotelial (VEGF) y factores de crecimiento de fibroblastos (FGF) son los principales mediadores de la angiogénesis en el desarrollo y progresión de muchas enfermedades. Existen poco trabajos

donde se estudia la relación entre factores angiogénicos y helmintosis. Una correlación positiva se observó entre el VEGF en plasma y la aparición de hidrocele en personas infectadas con *W. bancrofti*. Además, se encontró VEGF como factor protector en la malaria cerebral. En este trabajo nos planteamos evaluar el papel de factores angiogénicos en la estrongiloidosis experimental. Para ello estudiamos: *(i)* La modulación de la infección utilizando inhibidores específicos de la angiogénesis como la endostatina. *(ii)* La inducción de VEGF y FGF2 en macrófagos alveolares estimulados con antígenos derivados de distintas fases del ciclo biológico de *S. venezuelensis* y *(iii)* la relación entre estos factores y la producción de ON. La endostatina es una molécula de 20 kDa que pertenece al fragmento carbóxilo terminal del colágeno XVIII que cuando se añade de forma exógena inhibe la angiogénesis. En este estudio se demuestra que los factores angiogénicos tienen un papel relevante en la primoinfección por *S. venezuelensis*. El uso de endostatina disminuye tanto el número de larvas en el pulmón como la producción de huevos en heces. ¿Esto es debido a efectos directos sobre el parásito o indirectos vía hospedador? Para responder a esta cuestión, realizamos estudios *in vitro* sobre efecto de la endostatina sobre la supervivencia o mortalidad de los parásitos. Los resultados demuestran que la endostatina no tienen efecto directo sobre el parásito. El efecto indirecto sobre el hospedador puede ser atribuido a dos mecanismos complementarios. En primer lugar disminuyendo directamente la producción de factores angiogénicos, dato que se observa en nuestro estudio mediante la reducción de la expresión de VEGF y FGF2 en el pulmón y el intestino. En segundo lugar, algunos autores han observado que los eosinófilos tienen la posibilidad de participar en la angiogénesis mediante la inducción de la producción de VEGF. Por otra parte, el VEGF se ha asociado con la alteración de la barrera hematoencefálica en pacientes con meningitis eosinofílica causada por *Angyostrongylus cantonensis*. Nuestros datos indican que los ratones infectados con *S. venezuelensis* y tratados con endostatina tienen menor número de eosinófilos. Los macrófagos son conocidos por producir varios factores angiogénicos, incluyendo el potente

factor de crecimiento del endotelio vascular (VEGF), factor de crecimiento de la placenta (PlGF), factor básico de crecimiento de fibroblastos (FGF2), factor de crecimiento transformante β (TGF-β), interleucina-8 (IL-8) y una gran cantidad de citocinas pro-inflamatorias, como la interleucina-1β (IL-1β), la interleucina-6 (IL-6), el factor de necrosis tumoral alfa (TNF-α) y el factor estimulante de colonias de granulocitos y monocitos (GM-CSF). Los estudios realizados por nuestro grupo han demostrado la producción de VEGF y FGF2 en macrófagos alveolares estimulados con antígenos larvarios de *T. spiralis*. En este trabajo nos hemos propuesto estudiar los antígenos de *S. venezuelensis* responsables de la inducción de factores angiogénicos. Para ello utilizamos antígenos somáticos y excretores secretores de larva y hembras de *S. venezuelensis*. Solamente el antígeno somático de larva III de *S. venezuelensis* estimula la producción de VEGF y FGF2 en macrófagos alveolares y estos factores están asociados con la infección originada por este nematodo. Futuros estudios mediante análisis proteómico irán encaminados a dilucidar las moléculas procedentes del antígenos somático larvario responsables de este efecto. Estas podrán servir como dianas terapéuticas para diseñar vacunas frente a *Strongyloides*. El óxido nírico producido por los macrófagos activados y las células endoteliales es una molécula de señalización intracelular e intercelular generados a partir de L-arginina, que podría estar relacionado con las etapas de la angiogénesis. En este trabajo estudiamos la posible relación entre estos mediadores tras la estimulación macrofágica de los antígenos somáticos larvarios de *S. venezuelensis*. Utilizamos inhibidores de la iNOS como L-NAME y L-canavanina y hemos podido comprobar la asociación entre la producción de ON y la producción de factores angiogénicos, ya que estos inhibidores son también capaces de inhibir la expresión de los factores angiogénicos. Esta relación no se observó en la inducción de VEGF y FGF2 por antígenos larvarios de *T. spiralis*.

En el **tercer trabajo** se estudia la expresión de los factores de angiogénicos en la esquistosomosis humana y experimental. Este trabajo ha sido enviado para publicación a *Plos Neglected Tropical Diseases* el día 18 de Enero de 2010.

Los objetivos, metodología, resultados, discusión y conclusiones principales se exponen a continuación:

Introducción: La angiogénesis, el proceso de formación de nuevos vasos sanguíneos, tiene un papel importante en diversos procesos, incluyendo el cáncer y la inflamación. La esquistosomosis es una helmintosis que afecta a más de 200 millones de personas en el mundo. Los trabajos anteriores han demostrado que los antígenos solubles de *Schistosoma mansoni* inducen la expresión del factor de crecimiento del endotelio vascular (VEGF) en células endoteliales humanas. Los objetivos del estudio son: *(i)* Estudiar la presencia de VEGF en suero de pacientes diagnosticados de esquistosomosis y de otras helmintosis. *(ii)* Evaluar el efecto de endostatina en modelo experimental murino infectado con *S. mansoni. (iii)* Detectar VEGF y factor de crecimiento de fibroblastos (FGF2) en macrófagos alveolares estimulados con antígenos de cercaria y adultos de *S. mansoni.*

Metodología: Se han utilizado sueros de pacientes sanos y con diagnóstico parasitológico de esquistosomosis y otras helmintosis, para detectar VEGF mediante un técnica de ELISA comercial. Además se han empleado ratones CD1 divididos en tres grupos: *(i)* Sanos. *(ii)* Ratones infectados con 150 cercarias de *S. mansoni. (iii)* Ratones infectados y tratados con endostatina. Las magnitudes analizadas fueron: datos parasitológicos, hematológicos, detección de IgG, IgG1 e IgG2a específicas mediante ELISA y expresión de VEGF y FGF2 en hígado e intestino mediante RT-PCR. Por último se ha evaluado en macrófagos alveolares la expresión de VEGF y FGF2 estimulados con diferentes antígenos de *S. mansoni* mediante RT-PCR.

Resultados: En primer lugar, los pacientes diagnosticados con esquistosomosis mostraron diferencias significativas en la detección de VEGF respecto a los demás grupos estudiados. Sin embargo, no hubo diferencias entre los pacientes infectados por *S. mansoni* y *S. haematobium*. En segundo lugar, los ratones infectados y tratados con endostatina mostraron diferencias significativas en la disminución del número de huevos presentes en hígado y en la detección de anticuerpos IgG2a específicos. Por último, solo se observó expresión de VEGF y FGF2 en macrófagos alveolares estimulados con 50µg/ml de antígeno de cercaria de *S. mansoni*.

Discusión: Las principales helmintosis que afectan a la población humana son geohelmintosis, esquistosomosis y filariosis. También son estas helmintosis las detectadas en pacientes inmigrantes asintomáticos con eosinofilia absoluta o relativa. En este trabajo nosotros evaluamos VEGF en sueros de pacientes diagnósticados con una helmintosis. Para ello seleccionamos inmigrantes subsaharianos con eosinofilia donde la realización de un estudio detallado mostraba la presencia de un solo helminto, no de coinfecciones. Encontramos que los pacientes diagnosticados de esquistosomosis tenían niveles significativos de VEGF en suero respeto al grupo de pacientes sanos. Estos resultados son similares a los observados por otros autores en infecciones por *S. mansoni*. También pudimos comprobar que no había diferencias significativas entre los valores de VEGF encontrados en esquistosomosis por *S. mansoni* y por *S. haematobium*. La angiogénesis juega un papel fundamental en muchos procesos fisiológicos y patológicos, incluyendo el desarrollo de la fibrosis hepática. En la cirrosis, se reducen los niveles de VEGF en suero. Sin embargo, se detectaron altos niveles de VEGF en el suero de pacientes con esquistosomosis, aunque otros autores encontarron valores diferentes de VEGF en las distintas fases clínicas de la esquistosomosis. Además hemos comprobado que los eosinófilos no son las únicas células implicadas en la expresión de VEGF en humanos ya

que no hay asociación entre el número de eosinófilos y los valores de VEGF. En la segunda parte de nuestro trabajo, nosotros estudiamos en un modelo experimental de *S. mansoni*, los efectos que se producían tras la inhibición por factores anti-angiogénicos. Para ello utilizamos endostatina como inhibidor específico de la angiogénesis. Ratones infectados con *S. mansoni* y tratados con endostatina no presentaban reducción significativa de vermes adultos. Sin embargo, había reducciones significativas en el número de huevos en hígado, así como en el número de granulomas hepáticos. Esto indica que la inhibición de la angiogénesis induce una disminución de las lesiones hepáticas desencadenantes de la formación del granuloma y por tanto de la fibrosis hepática subsiguiente. A continuación estudiamos los posibles mecanismos implicados en la reducción de la lesión. En primer lugar analizamos datos de recuento de serie roja, blanca y plaquetas. No observamos diferencias significativas en ninguno de los datos analizados, específicamente en la reducción del número de eosinófilos. Estos resultados obtenidos en el modelo experimental concuerdan con lo observado en sueros humanos. Este dato es opuesto al hallado por nuestro grupo cuando analizamos la relación angiogénesis y estrongiloidosis. En segundo lugar pudimos demostrar la disminución de la expresión de VEGF y FGF2 en hígado. Este dato demuestra la inhibición efectiva realizada por endostatina. En tercer lugar analizamos la respuesta inmunitaria producida entre los grupos de estudio. Encontramos disminución significativa en la detección de IgG2a específicas. Esta reducción está relacionada con la disminución de respuestas Th1 efectoras en los animales tratados con endostatina, en los cuales existe reducción en el número de huevos en hígado y de granulomas observados. Estos resultados coinciden con las observaciones de otros autores en que la esquistosomosis grave se asocia con elevación persistente de citocinas pro-inflamatorias tipo Th1, mientras que la afección más leve está presente cuando aumentan las citocinas tipo Th2. Por otra parte, el cambio hacia la producción de citocinas tipo Th1 producidas por una población estable de células $CD4^+$ se correlaciona con la exacerbación grave de la inmunopatología de

la esquistosomosis. El último objetivo de nuestro estudio fue identificar los antígenos de *S. mansoni* que participan en la expresión de VEGF y FGF2 producido por los macrófagos alveolares. Usamos antígenos de cercarias y de vermes adultos de *S. mansoni.* Nuestros resultados han mostrado que los antígenos de cercaria eran capaces de estimular la producción de factores angiogénicos en macrófagos alveolares. Estos resultados y los datos obtenidos por otros autores muestran el papel de la angiogénesis en la esquistosomosis y su participación en la reacción granulomatosa asociada a la lesión. En resumen, este estudio pone de manifiesto el papel de la angiogénesis en la patogenia de la esquistosomosis debido a tres aspectos: *(i)* La detección de VEGF en pacientes con diagnóstico de esquistosomosis. *(ii)* La reducción de huevos y la formación de granulomas en el hígado de ratones tratados con endostatina con predominio de respuestas tipo Th1. *(iii)* El aumento de expresión de VEGF y FGF2 tras la estimulación de macrófagos alveolares por antígenos de cercarias de *S. mansoni*.

Finalmente las **conclusiones** de esta tesis doctoral son son:

1.- Los antígenos de la especie encapsulada *Trichinella spiralis* son capaces de estimular de forma dosis-dependiente los factores angiogénicos como VEGF y FGF2 por macrófagos alveolares de rata.

2.- La angiogénesis desempeña un papel relevante en la primoinfección por *Strongyloides venezuelensis,* ya que su inhibición mediante el empleo de endostatina produce disminución de las magnitudes de infección.

3.- La endostatina reduce la infección de *Strongyloides venezuelensis* no por acción directa sobre el parásito sino a través de efectos indirectos como la disminución de la producción de factores angiogénicos por el hospedador o la reducción de la respuesta eosinofílica.

4.- Los antígenos somáticos larvarios de *Strongyloides venezuelensis* son los responsables de la inducción de factores angiogénicos y su modulación está relacionada con la generación de óxido nítrico.

5.- En pacientes con esquistosomosis existe un aumento en los niveles séricos de VEGF con diferencias significativas con respecto a otras helmintosis.

6.- La inhibición de la angiogénesis mediante endostatina en la esquistosomosis experimental murina induce una disminución en el número de huevos en hígado, así como en la presencia de granulomas y de fibrosis hepática.

7.- Los antígenos de cercarias de *S. mansoni* inducen la expresión de factores angiogénicos en macrófagos alveolares de rata.

6 Methodological appendix

6.1 Collection of rat alveolar macrophages samples for measurement of nitric oxide.

Alveolar macrophages from male Wistar rat were obtained by bronchoalveolar lavage Espinoza *et al*., (2002) and Nurkiewicz *et al*., (2004). Lungs were lavaged with aliquots of 5 ml of sterile PBS with sterile cannula for collection of BAL fluid as order that come below. Male Wistar rats are used in all experiments and alveolar macrophages were obtained by bronchoalveolar lavage technique (BAL).

Materials:

- Male Wistar rat weighting 250-300 g.
- Sodium pentobarbital (≥100 mg/kg, i.p.).
- Teflon catheter (VYCON Code 123.06).
- Phosphate-Buffered Saline (PBS lx sterile pH 7.4)
- Ethanol (141086, Panreac).
- Thoma Pipette.
- Microspirator (258 00, Brand).
- Liquid of Turk (251390, Panreac).
- Neubauer.

Procedure:

- Rats were euthanized with an intraperitoneal injection (ip) of sodium pentobarbital ≥100 mg /kg body weight.
- A tracheal cannula (teflon catheter) was inserted.
- BAL was performed through the cannula using ice-cold PBS (each time, to inject 5 ml of PBS sterile with pH 7.4 through the cannula and collection BAL with a syringe 5 ml) and repeat 10 to 15 times, and collect BAL in a 50 ml tube.
- Centrifuge BAL at 500 g for 10 min at 4ºC and remove the supernatant.
- Macrophage cells resuspended cells in 1 ml of Dulbecco's medium.
- The isolation of acellular BAL fluid and BAL cells were counted as described Porter et al., 2004).

6.2 Rat alveolar macrophages cultures

Rat alveolar macrophages were exposed to different concentrations of extracts of *S. venezuelensis*, using the basal cells (macrophages) as a negative control and macrophages stimulated with LPS as a positive control. The procedure is based on the technique described by Evans *et al*. (2002a) and Andrade *et al*. (2005a).

Materials:

- Dulbecco's modified Eagle's medium (DMEM, D-0819 Sigma).
- Fetal bovine serum (12003C, Sigma).
- Glutamine (G-5792, Sigma).
- Complete medium (10% fetal bovine serum, 2mM of glutamine, 100U/100µg/ml penicillin-streptomycin and 44 ml of DMEM).
- Peniciline y estreptomicine (P-4333, Sigma-Aldrich, Inc., San Luis, EE.UU.).
- Culture Plates (Costar, Cambridge, MA).
- Lipopolysaccharide (LPS) (L-2880, Sigma)
- Polymixin B (PMB P-4932, Sigma)
- L-NAME: Nω-nitro-L-arginin methyl ester hydrochloride (N-5751, Sigma)
- L-canavanine (C-9758, Sigma)
- Trypsin (T9935, Sigma)
- Incubator of CO_2 (Galaxy St, BIOTECH)

Procedure:

- Macrophages (1×10^6/well) were washed with DMEM two times and then were placed on culture plates.
- Incubate 2h at 37ºC in 5% CO_2 to adhere cells on the plastic bottom of the culture plates.
- Remove non-adhering cells by gentle washing with complete medium and discard it and add 1 ml of fresh complete medium for further cell culture.
- Incubated adherent alveolar macrophages alone (as a negative control, Ø), with lipopolysaccharide (LPS) as a positive control with several concentrations, ranging from 0.1 to 50 µg protein/ml, with different antigens.
- Incubate for 18h at 37ºC in 5% CO_2, then culture supernatants collected and centrifuged at 500 *g* for 10 min.
- Collect supernatants after 18h, centrifuge at 500 *g* for 10 min and store at -80ºC as an archival material.
- Recovered macrophages and stored at-80ºC.

6.3 Determination of viability of rat alveolar macrophages

The cell viability was assessed by mitochondrial reduction of MTT after removing the supernatant according to Kiemer and Vollmar (1997). Cell viability, should be higher than 87.5% in all cases.

Materials:

- Spectrophotometer (Easy Reader EAR 400FT).
- MTT: (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl-tetrazolium bromide; M2003, Sigma).
- Dimethyl sulfoxide (DMSO, 07-4872, Sigma)
- 96wells Plates

Procedure:

- Add 200 µl of MTT (0.5 mg/ml) in each well and incubate alveolar macrophages for 1 hr at 37ºC
- Remove MTT completely from all plate and add 200 µl of DMSO or 50%ethyl alcohol with 50%DMSO
- Determine optical density immediately at 550nm in a Easy reader EAR 400 FT photometer.
- Following the measure viability of cells, alveolar macrophages were collected and store them at -80ºC.

6.4 Griess assay for measure of nitrites.

To measure nitric oxide in the cell culture supernatant was used Griess technique that is based on the property have nitrites react with primary aromatic amino acid medium, resulting diazonium compounds that give color pink (Green *et al*., 1982; Campisi *et al*., 2002). Supernatant of macrophage culture are used for nitrite determination according to Andrade et al. (2007).

Materials:

- Solution A: 1% sulfanilamide, 0.12M (S-9251, Sigma) in H3PO4 0.36 M
- Solution B: dihidrocloruro of N-(1-naftil) etilendiamine 7.7mM (N-5889, Sigma) in H3PO4 0.36 M
- Stock solution: 1 M of NaNO2 (S-2252-500G, Sigma).
- Spectrophotometer (Easy Reader EAR 400FT)

Procedure:

- Make different dilution of medium that we want to determine nitrites from 1 µM to 100 µM.
- Prepare the Griess reagent, solution A and solution B, mix them and prepare with the equal portion immediately before their utilization.

- Add 100 µl to each well of blank (only complete medium), patron and sample triplicate in 96 wells plaque.
- Add 50 µl of reagent of Griess for each well; incubate 15 min in bench top (20-30 ºC).
- Measured the absorbance of the azochromophore at 550 nm.

6.5 Extraction of RNA from rat alveolar macrophages

For the RNA extraction from macrophages was used the commercial kit RNeasy Mini Kit (Qiagen). This kit is used for purification of total RNA from animal cells and animal tissue and for cleanup of RNA from crude RNA prep and enzymatic reactions.

Materials:

- RNeasy Mini Kit (Code, Qiagen)
- Qiashreder (Code, Qiagen)
- RNase-Free DNase Set (Code, Qiagen)

Procedure:

- Determine the number of cells (macrophages, between 10^6-10^7cells) and centrifuge them for 5 min at 300 × g. Then carefully remove all supernatant by aspiration and proceed to step 2.
- Add appropriate of Buffer RLT (350-600 µl) for disruption the cells. Vortex or pipet them to mix and go to the third step.
- Homogenize the lysate for 2 min centrifuge at full speed using Qiashreder spin column.
- Add 1 volume of 70% ethanol (the same volume of lysate) to homogenized lysate and mix well by pipeting.
- Transfer 700 µl of the sample to an RNeasy spin column placed in a 2 ml collection tube. Then gently closed the lid and centrifuge them for 15 s at 8000*g* and after that discard the flow-through.
- Add 350 µl of RW1 to the RNeasy column, closed the lid gently and centrifuge 15 seconds at 8000 *g* (for washing the column membrane). Throw away the flow-through.
- Add 80 µl of DNase l incubation mix (add 10 µl DNase enzymes to 70 µl RDD buffer and mix them gently) to the spin column membrane and place on the bench top for 15 min at 20-30ºC.
- Add 350 µl RW1 to the spin column, close the lid gently and centrifuge for 15 seconds at 8000*g*. Then discard the flow-through.
- Add 500 µl of RPE buffer to the column, close the lid gently and centrifuge at 8000*g* for 15 seconds (For washing the column membrane), then throw away the flow-through.
- Add again 500 µl of RPE buffer to the column, close the lid slowly and centrifuge them for 2 min at 8000*g* to wash the column membrane.
- Place the spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.
- Finally, put the spin column in a new 1.5 collection tube and add 30-50 µl RNase-Free water directly to the column membrane. Close the lid slowly and centrifuge at 1 min at 8000 x *g* to elute the RNA.
- Store RNA at-80ºC.

6.6 RNA extraction from experimental animal tissue

It is essential to use the correct amount of stating material in order to obtain optimal RNA yield and purity from liver, intestine and lung. A maximum amount of 30 mg fresh or frozen tissue or 15-20 mg RNA later stabilized tissue.

Materials:

- RNeasy Mini Kit (Qiagen)
- Qiashreder (Qiagen)
- RNase-Free DNase Set (Qiagen)
- Mortar and pestle

Procedure:

- Weight 10-30 mg of tissue and cut it into slices less than 0.5 cm thick as quickly as possible. Then the tissue pieces completely submerge in the RNAlater (Stabilization reagent). For archival storage at -20ºC, first incubate the tissue overnight in the RNAlater at 2-8ºC. Then remove the tissue from the reagent, and transfer it to -20ºC for storage.
- Place the weighed (fresh, frozen, or RNA*later* stabilized) tissue in liquid nitrogen, and grind thoroughly with a mortar and pestle. Decant tissue powder and liquid nitrogen into an RNase-Free, liquid-nitrogen-cooled, 2 ml microcentrifuge tube. Allow the nitrogen liquid to evaporate but don't allow thawing the tissue.
- Add the appropriate volume of RLT buffer, and pipet the lysate directly into a QIAshreder spin column placed into a 2 ml collection tube and centrifuge for 2 min at full speed.
- Centrifuge the lysate for 3 min at full speed and then carefully removed the supernatant by pipeting, and transfer it to a new microcentrifuge tube. We use only this supernatant (lysate) in subsequent steps.
- Add 1 volume of 70% ethanol (the same volume of lysate) to homogenized lysate and mix immediately by pipeting (don't centrifuge it).
- Transfer 700 µl of the sample to an RNeasy spin column placed in a 2 ml collection tube. Then gently closed the lid and centrifuge them for 15 s at 8000*g* and after that discard the flow-through.
- Add 350 µl of RW1 to the RNeasy column, closed the lid gently and centrifuge 15 seconds at 8000*g* (for washing the column membrane). Throw away the flow-through.
- Add 80 µl of DNase l incubation mix (add 10 µl DNase enzymes to 70 µl RDD buffer and mix them gently) to the spin column membrane and place on the bench top for 15 min at 20-30ºC.
- Add 350 µl RW1 to the spin column, close the lid gently and centrifuge for 15 seconds at 8000*g*. Then discard the flow-through.
- Add 500 µl of RPE buffer to the column, close the lid gently and centrifuge at 8000 *g* for 15 seconds (For washing the column membrane), then throw away the flow-through.
- Add again 500 µl of RPE buffer to the column, close the lid slowly and centrifuge them for 2 min at 8000 *g* to wash the column membrane
- Place the RNeasy spin column in a new 2 ml collection tube and discard the old collection tube with the flow-through. Close the lid gently, and centrifuge at full speed for 1 min.

- Then, place the RNeasy spin column in a new 1.5 collection tube. Add 30-50 µl RNase-Free water (Depend the RNA yield) directly to the column membrane. Close the lid slowly and centrifuge for 1 min at 8000*g* to elute the RNA.
- If the expected RNA yield is 30 µg, repeat the step 13 using another 30-50µl RNase free water, or using elute from step 13 (If we want high RNA concentration).
- Store RNA at-80ºC immediately and it is essential that working with RNA fast because it's very instable and degrade rapidly.

6.7 RT-PCR for detection of VEGF and FGF2 in alveolar macrophages

It was performed with RT-PCR kit (11483188001, Roche) by RNA extracted from rat alveolar macrophages. It is the first complementary DNA strand in two steps; synthesis at 42ºC and denaturation at 95ºC (Andrade et al., 2005).

Materials:

- Sterile vials of 0.5 ml.
- Thermal cycler (Perkin Elmer Instrument, Norwalk, USA).
- Oligonucleotides OligodT (27-7858, Pharmacia Biotech).
- Mastermix (cDNA).
- Nuclease-free water (DEPC).

Table 5.1: Reagent and amount and final concentration per sample for RT-PCR.

Reagent	**Volume.1 sample**	**Final conc. 1 sample**
10× Reaction Buffer	2.0 µl	1×
25mM $MgCl_2$	4.0 µl	5mM
Deoxynucleotide Mix	2.0 µl	1mM
Oligo-p (dT)15	2.0 µl	0.04 A260 units (1.6µg)
RNase Inhibitor	1.0 µl	50 units
AMW Reverse Transcriptase	0.8 µl	≥20 units
Sterile water	variable	-
RNA sample	variable	-
Total	**20µl**	

Procedure:

- The amount of RNA sample add to sample reactions (The amount of RNA that added to sample reaction is depends on the nature of the RNA used and on the intended application; normally, ≤1µg of total RNA, 50-100 ng of poly [A] + RNA, or 6 fg-1µg of a single, purified RNA species [e.g., the control Neo pa RNA] is required according **Table 5.1.**
- Briefly vortex and centrifuge the mixture to collect the sample at the bottom of the microfuge tube.- Incubate the reaction at +25ºC for 10min; during the first incubation, primer anneals to the RNA template.

- Then incubate the reaction at +42ºC for 1hr; during the second incubation, The RNA is subsequently reverse transcribed, resulting in cDNA synthesis.
- The AMW Reverse Transcriptase denatured by Incubation the reaction at 99ºC for 5 minutes.
- Cool the reaction at 4ºC for 5 minutes.
- At this point, the reaction tube maybe stored at +2 to 8ºC for 1-2h or at −15to−25ºC for longer periods.

6.8 Chain reaction polymerase (PCR).

VEGF is determined in rat alveolar macrophages after cultivation and determination of viability. Total RNA was extracted from macrophages following the instructions of the commercial kit RNeasy Mini Kit (Qiagen GmbH, Hilden Germany).Then a total 1 µg RNA was used as template for the first-strand DNA synthesis (Roche 1483188). The VEGF insert amplification was performed with primers described by Yang *et al*. (2004) and FGF-2 insert amplification was performed with primers described by Jyo-Oshiro *et al*. (1999). Primer sequence for VEGF was: sense, 5´-CTGCTCTCTTGGGTGCACTGG-3´ and anti-sense, 5´-CACCGCCTTGGCTTGTCACAT-3´. Primer sequence for FGF2 was: sense, 5´GCCGGCAGCATCACTTCGCT-3´ and anti-sense, 5´CTGTCCAGGCCCCGTTTTGG-3´. Glyceraldehydes-3-phosphate dehydrogenase (GAPDH) was used as an internal control and GAPDH sense, 5´-GGTCGGTGTGAACGGATTTG-3´ and GAPDH anti-sense, 5´-GTGAGCCCCAGCCTTCTCCAT -3´. To reveal the reaction, the PCR product was separated in an agarose gel and ethidium bromide.

Materials:

- 10X Buffer (Promega M1906).
- $MgCl_2$ (25mM).
- Deoxynucleotide mix 1 mM (Boehringer Mannheim, 1 093 088).
- Taq DNA polymerase (Promega, M1665).
- Water DEPC.
- Primers: VEGF 20 mM, FGF-2 20mM and GAPDH 20 mM.
- Thermal cycler.
- Template cDNA.

Procedure:

- For each reaction to a container with 20 µl in each eppendorf 2 µl of buffer, 3 mM of $MgCl_2$ 50mM, 2mM of deoxynucleotide mix1 mM and 0.125 µl of Taq DNA polymerase.
- Add to each reaction 1mM of each primer in case of VEGF and GAPDH and 0.5 mM in case of FGF-2.
- Add 1µg of blank sample and complete up to final volume 20 µl of sterile water and mixed well.

- Incubate 94 ºC for 5min, 35 cycles of 94ºC for 1min, 55 ºC for 1 min, 72 ºC for 1 min. and a single cycle at 72ºC for 7 min in case of VEGF and GAPDH.
- Incubate 94 ºC for 2min, 50 cycles of 94 ºC for 30 s, 60 ºC for 30 s, 72ºC for 1 min and a single cycle at 72 ºC for 5 min. in case of FGF-2.
- At the end of cycles programmed at 4°C.

6.9 DNA electrophoresis in agarose gel with ethidium bromide

To view the products obtained by PCR is necessary to observe in an agarose gel with ethidium bromide, confirming its size and compare them with a marker. Ethidium bromide is mutagenic and carcinogenic and therefore must avoid inhaling the vapors and must be handled with gloves.

Materials:

- TAE buffer solution.
- Agarose (A9539, Sigma).
- Microwave.
- Ethidium bromide (161-0433, Bio-Rad).
- Loading buffer.
- Molecular marker (1Kb Plus DNA Ladder).
- Transilluminator UV, BioDoc-It System.
- Template cDNA

Procedure:

- Take 60 ml of TAE buffer solution and add 0.6 g agarose and heated in a microwave for two minutes for preparing the gel.
- Cool the gel up to 50°C and add 0.5 µl ethidium bromide and shake it until dissolved.
- Pour into the tray and place the molds to form the wells.- Put in a vial 10 µL of the sample and add 4 µl loading buffer.
- In the first well pour 2.5 µl of molecular marker (1Kb Plus DNA Ladder) and 2.5 loading buffer.
- Place in each well in the agarose gel so that the wells are in the negative pole of the tank and the DNA can migrate toward the positive pole.
- Allow to run the samples to a voltage between 40 and 120 V- Display the result of electrophoresis on a transilluminator UV and take a photo from gel.

6.10 Life cycle of *Schistosoma mansoni* in laboratory

A strain of S. mansoni was maintained by laboratory passage in Biomphalaria glabrata snails and BALB/C strain mice (Cheever et al., 2002). Snails were kept in glass recipients with dechlorinated tap water and fed fresh lettuce *ad libitum* (Freire et al., 2002). Cercarial shedding was induced phototropically at 30 ºC and the parasites used for infection within three hours of emergence. Anaesthetized mice were infected with cercariae administered on a shaven abdomen and worms were subsequently perfused through an incised portal vein (Doenhoff et al., 1978). Count eggs in the liver and small intestine of infected mice according to Cheever et al., (2002)

6.10.1 *Infection of* snails

Materials

- Livers of mice infected at 7 week of infection.
- Homogenizator.
- Sedimentation cup.
- Saline solution: 8.5 g NaCl in a 1 l of destilled water.

Technique:

- Fragment and homogeneize the liver tissue in saline solution.
- Add up to one liter of water and leave it for 20 min at 4ºC in darkness and remove foam and detritus.
- Perform other more sedimentation as above.
- Leave only 50 ml mineral water at the bottom of the sedimentation cup at 25-28ºC and light
- Expose every snail with 7 miracidia
- Mainain the snails at 24-28ºC during 4 weeks, then the snail put in light and 28ºC for cercariae hatching for 2 h.
- Count three times for establish the average individual dosis

6.10.2 Infection of mice

Materials

- Adhesive tape
- Anestesia (Ketamin 100-200 mg/kg and diazepam 5 mg/kg with atropine 40 μg/kg).
- Plastic ring.

Technique:

- Injecte 100 μl of the anesthesic mixture.
- Place the ring on the shaved abdomen.
- Administrate 150±10 cercariae in 1 ml maximum.
- Leave the darkness mice for 45 minutes.

6.10.3 Perfusion of mice:

Materials

- Mice infected with 150 cercariae of *S. mansoni.*
- Scissors, tweezers.
- Syringe and needle (0.5 mm).
- Heparin saline.
- Pentobarbital sodium, solution.
- Potassium hydroxide 5%.
- McMaster chamber.

Technique

- Anesthetize with sodium pentobarbital.
- Cut and remove skin of the abdomen and access to the abdominal cavity.
- Open the chest and inject heparin salin in the left ventricle.
- Cut the portal vein to 0.5 cm, collect and count the worms.
- Remove the liver and weigh it and immerse it in 50 ml of potassium hydroxide, incubate18 h a 37ºC and then count eggs per gram in a McMaster chamber.

6.11 Adult worm antigens from *S. mansoni* (AWASm).

Adult worm antigens (AWASm) from *S. mansoni* were made in accordance with Dunne *et al*. (1997).

Materials:

- Adult worm of *Schistosoma mansoni.*
- Liquid nitrogen.
- Mortar and paste.
- Proteases inhibitors [1mM N-Tosyl-1 phenylalanine chloromethyl ketone and phenylmethylsulphonyl fluoride (PMSF)].
- Sterile filter with 0.22 µm.

Procedure:

- Recovered *S. mansoni* from the portal perfusion.
- Wash the worms to be free of erythrocytes and submerge them in liquid nitrogen immediately.
- All the worms ground into a paste, and centrifuge them at 10.000*g* at 4ºC for 1h.
- Add proteases inhibitors (1mM of N-tosyl-1 phenylalanine chloromethyl ketone, 1mM of phenylmethylsulphonyl fluoride PMSF).
- Centrifuge the soluble material and filtered through a 0.22 µm sterile filter.
- Determination of protein concentration by Micro BCATM Assay.
- Store at-80ºC.

6.12 Cercarial antigen of *Schistosoma mansoni* (CSm).

Cercarial antigen (CSm) from *S. mansoni* was used accordance with Dunne *et al*. (1997).

Materials:

- Cercariae of *S. mansoni*.
- Liquid nitrogen.
- Proteases inhibitors [1mM N-Tosyl-1 phenylalanine chloromethyl ketone and phenylmethylsulphonyl fluoride (PMSF)].
- Sterile filter with 0.22 µm

Procedure:

- Recovered and harvest the cercariae of S. mansoni from intermediate host (infected snail).
- Submerge them in liquid nitrogen immediately.
- Sonicate, and centrifuge them at 10,000 x g at 4 ºC for 1h.
- Add proteases inhibitors (1mM of N-Tosyl-1 phenylalanine chloromethyl ketone, 1mM of PMSF).
- Centrifuge the soluble material and filtered through a 0.22 µm sterile filter.
- Determination of protein concentration by Micro BCATM Assay.
- Store at -80 ºC.

6.13 Life cycle of *Strongyloides venezuelensis* in laboratory

The third stage infective larvae (L3) of *S. venezuelensis* were obtained from the strain that is maintained at the Laboratory of Parasitology, Faculty of Pharmacy University of Salamanca by serial passage in Wistar rats. This line comes from the Laboratory of Taxonomy and Biology of Invertebrates, Department of Parasitology (Federal University of Minas Gerais, Belo Horizonte, Brazil) and was yield by Professor Alan L. de Melo.

The cycle was performed in accordance with the technique of Martins *et al*. (1999), where from feces of infected animals recovering eggs of *S. venezuelensis*, to allow the development of these larvae of first (L1), second (L2) and third stage (L3). Feces were mixed with an inert substrate composed of vermiculite. After incubation at 28ºC for at least 72 h, the L3 collected by a Baermann technique (Rugail *et a*l., 1954). This technique is based on thermo-hydrotropism parasitic characteristic of this phase (Figure 5.1.) Larvae 3 were counted and confirmed that at least 80% of worm have mobility. Finally 3.000 L3 in 0.5 ml saline solution or PBS sterile was injected subcutaneously to male rats of 60-120 g (Figures 5.1 and 5.2).

Materials:

- L3 of *S. venezuelensis.*
- Wistar rat (100-150g).
- Vermiculite. (Termite, Asfaltex, Sant Cugat Del Valles, Spain)
- PBS.
- Polycarbonate cages.
- Wire mesh.
- Funnel-size according to need
- Funnel stand
- Clamp or spring clip
- Cheesecloth or dental napkin
- Thin stick or metal rod
- Strainer
- Microscope
- Test tube 12 ml
- Pasteur pipette
- Small Petri dishes
- Scissors
- Disposable paper towels
- Spoon or spatula
- Rubber band or length of string
- Jug or flask
- Microscope slides and coverslips

Procedure:

- Place the infected animals in cages on the grid. Basically it was necessary to filter paper moistened to prevent desiccation of the feces.
- Collect and mix the feces of 24 hours with 20 ml of distilled water and vermiculite in sufficient quantity to maintain saturation of moisture and good aeration and incubate 3-7 days at 28 ºC with relative humidity above 80%.
- To infect male Wistar rats subcutaneously (sc) with third stage larvae of *S. venezuelensis*.
- Rats keep in polycarbonate cages that have wire mesh with opening of $0.5cm^2$ is placed 2 centimeters from the bottom.
- Place some vet papers on the bottom of the cage to keep the faeces moist.
- Collect the faeces after 48-72 hours.
- Mix and homogenize well faeces with vermiculites.
- Incubate coprocultures 3-5 days at 28ºC and 90%humidity.
- Place a double layer of cheesecloth or dental napkin on a disposable paper towel or equivalent on bench.
- Using a spoon or spatula weigh or measure approximately 5-10 g of faecal material. Then place faecal material in the centre of the cheesecloth (1).
- Form a pouch containing the faecal material by holding the four corners of the cheesecloth together and moulding the cloth around the faecal material (2, 3, and 4).
- Using a rubber band or length of string close the cheesecloth pouch and then push the stick or short metal rod under the rubber band or string so that the pouch can be worms suspended (5, 6).

- Place the pouch containing the faecal material in the funnel and trim off the excess cheesecloth (7).
- Fill the funnel with lukewarm water (42ºC) and make sure the faecal material is well covered (8, 9) and leave the apparatus to stand for 45 min.
- Draw off a few millilitres of fluid from the stem of the funnel into a test tube, and then either leave to sediment for at least 30 minutes or the fluid can be drawn into a centrifuge tube and spun at 1000 rpm for 2 minutes.
- Check sediment sample in a Petri dish for the presence of larvae and collect larvae (L3) of *S. venezuelensis*, count them and consider motility and morbidity.

Figure 5.1: Biological cycle of *S. venezuelensis* in the laboratory. Obtention of feaces from rats infected (1). Coporcultures (2). Subcutaneous infection (3). Incubator at 28ºC and more than 85% or relative humidity..

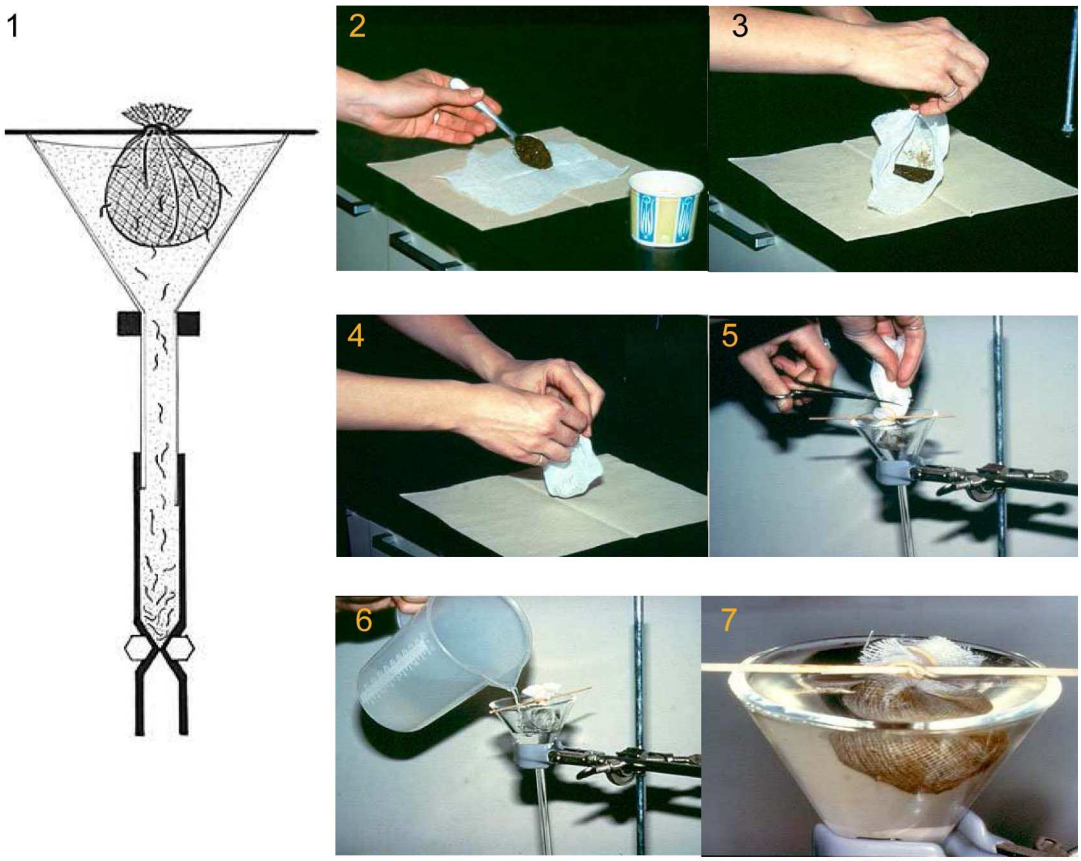

Figure 5.2 The Baermann technique is based on the active migration or movement of larvae. Baermann apparatus (1). Faeces are plased on 8 layer of gauze (2), to enveloped (3, 4), then placed in the Baermann apparatus (5) and finally water at 42-44ªC is poured. Faeces are moisted by the water, larvae moved into and we can collect them.

6.14 Decontamination of larvae and female adult of *S. venezuelensis*

Third stage of larvae and female adult of *S. venezuelensis* decontaminate according to Martins *et al*. (2000). Larvae and parthenogenetic females of *S. venezuelensis* obtained from stool culture and necropsy didn't have sufficient features to be cultivated because microbial flora of the environment in which they were obtained, could contaminate the culture media. For all this, because to remove these contaminating organisms larvae and females of *S. venezuelensis* processed with sodium hypochlorite, antibiotics and antifungal in sterile distilled water.

Materials:

- Third stage larvae of *S. venezuelensis*
- Female adult of *S. venezuelensis*
- PBS sterile
- Sodium hypochlorite 0.25 % (212297, Panreac).
- Sterile distilled water
- Sodium benzylpenicillin 180 µg/ml
- Ceftazidime 1.0 mg/ml

Procedures:

- Wash larvae and adults with sterile distilled water
- Treated with sodium hypochlorite 0.25% for 10 min
- Wash with sterile distilled water
- Exposed larvae and adults to Sodium benzylpenicillin 180 µg/ml and Ceftazidime 1.0 mg/ml for 30-60 min
- Wash with sterile distilled water
- Store at -80

6.15 Necropsy of mice infected with *Strongyloides venezuelensis*.

Necropsy was performed to obtain larvae from lungs or adults from the intestine (Vlaminck, 2010)

Materials

- Scissors, tweezers
- Saline solution: 8.5 g NaCl in a 1 l of destilled water.
- Sedimentation cup

Technique:

- Sacrifice mice with cervical dislocation
- Remove skin from the abdominal region and open the intestinal cavity.
- Remove the upper 2/3 of the small intestine, cut open it longitudinally, and incubate in PBS at 37ºC for 2 h.

- Remove the lungs, fragmente in PBS, and incubate for 2 h at 37ºC.
- Quantificate under stereo microscopy worms that emerge from each organ.

6.16 Obtanining different antigens from third stage of larvae (L3) and female parthenogenetic from *S. venezuelensis*.

Preparation of PBS-soluble extract antigen (L3-PBS) and excretory secretory of L3 from *S. venezuelensis* were done as described Conway *et al*. (1994). We obtained a total of eight proteins and metabolic products extracts from L3 and parthenogenetic females of *S. venezuelensis*.

6.16.1 Soluble antigen preparation from *S. venezuelensis* L3 (L3-PBS).

The soluble antigen in phosphate buffer solution (PBS) of L3 larvae of *S. venezuelensis* was obtained after various processes including: freezing / thawing, homogenization and application of ultrasonic. This procedure was performed in a solution containing protease inhibitors (Conway et al., 1994).

Material:

- PBS sterile
- Third stage larvae of *S. venezuelensis*
- Protease inhibitors: (5mM ethylendiaminetetraacetic acid (EDTA), 2mM phenylmethylsulphonyl fluoride (PMSF)), 1µM pepstatin, 4µM aproptinin and 10 µM chymostatin)
- NaOH 0.15 M
- Syringe filter (Millipore) with 0.2 µm pore size
- Dulbecco-modified Eagle's Medium (DMEM)
- Ethylenediaminetetraacetic acid (EDTA) in distilled water 1M (E-9884, Sigma).
- Phenilmethysulfonylfluoride (PMSF) 0.33 M in ethanol (P-7626, Sigma).
- Chemostatina in water (C-7268, Sigma).
- Aprotinin in 0.9% NaCl saline (A-6012, Sigma).
- 1M pepstatin A in ethanol. (P4265. Sigma).
- Phosphate buffer solution (PBS) 0.14 M NaCl, 2.7 nM KCl, KH2PO4 1.5 nm, Na2HPO4 6.5 nM.
- Sodium hydroxide 0.01 M (621845, Panreac).
- Hydrochloric acid 1 M (181021, Panreac).
- Semi-permeable membranes of cellophane.
- Kit BCA protein determination: Micro BCA Protein Assay Kit (23235 Pierce).
- Glass Homogenizer.
- Sonicator.
- Centrifuge.
- Chamber cold at 4 °C.
- Shaker with thermostat.

Procedure:

- Wash larvae with sterile distilled water
- Decontamination of larvae (Martins method)
- Resuspended L3 at a concentration of approximately 2×10^5/ml in PBS with 5mM EDTA, 2mM PMSF and Aproptinin 100 IU / ml, Pepstatin A 1 µM and Chemostin 1 mM.
- Homogenize the suspension in ice-water bath 30-60 minutes.
- Subsequently sonicate the suspension 8 times for 20 seconds at 70 kHz.
- Incubate 1hr at 4ºC
- Centrifuged at 13,000 g for 30 minutes at 4 ºC.
- Collect the supernatant and store at -80ºC.
- Determine the protein concentration in the supernatant by Micro BCA TM Assay.
- Check the bands in a polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) to 12.5% stained with Coomassie blue.

6.16.2 Alkaline extraction of adult female antigen of *S. venezuelensis* (F-ALK)

This extract is obtained by exposing adult female worm of *S. venezuelensis* in an isotonic solution alkalinized with sodium hydroxide according to Machado et al. (2003) and Faccioli et al. (2003).

Material:

- PBS sterile.
- Female adult worm of *S. venezuelensis.*
- Protease inhibitors: (5mM ethylendiaminetetraacetic acid (EDTA), 2mM phenylmethylsulphonyl fluoride (PMSF)), 1µM Pepstatin, 4µM Aproptinin and 10 µM chymostatin).
- NaOH 0.15 M.
- Syringe filter (Millipore) with 0.2 µm pore size.
- Dulbecco's modified Eagle's medium (DMEM, D-0819 Sigma).
- Ethylenediaminetetraacetic acid (EDTA) in distilled water 1M (E-9884, Sigma).
- Phenilmethysulfonylfluoride (PMSF) 0.33 M in ethanol (P-7626, Sigma).
- Chymostatin in water (C-7268, Sigma).
- Aprotinin in 0.9% NaCl saline (A-6012, Sigma).
- Pepstatin A in ethanol 1 M (P4265, Sigma).
- Phosphate buffer solution (PBS) 0.14 M NaCl, 2.7 nM KCl, KH_2PO_4 1.5 nM, Na_2HPO_4 6.5 nM.
- Sodium hydroxide 0.01 M (621845, Panreac).
- Hydrochloric acid 1 M (181021, Panreac).
- Semi-permeable membranes of cellophane.
- Kit BCA protein determination: Micro BCA Protein Assay Kit (23235 Pierce).
- Glass Homogenizer
- Sonicator
- Centrifuge
- Chamber cold at 4 °C
- Shaker with thermostat

Procedure:

- Wash adult female worm with sterile distilled water and decontaminate them subsequently.
- Suspended 1.000 adult worms in 20 ml of PBS
- Add EDTA 5mM, PMSF 2 mM and Aprotinin 100UI/ml, Pepstatin A 1 µm and Chymostatin 1mM.
- Add 1 ml of NaOH 0.15M/20ml of suspension of parasite.
- Agitation the suspension slowly at 4ºC 6 hours
- Add 0.3M of HCL (PH 7)
- Sonicate the suspension 8 times for 20 seconds at 70 kHz
- Centrifuge at 13,000 *g* for 30min at 4ºC
- Store supernatant at -80
- Determination of protein concentration by Micro BCA TM Assay and Check the bands in a polyacrylamide gel with sodium dodecyl sulfate (SDS-PAGE) to 12.5% stained with Coomassie blue.

6.16.3 Preparation of the excretory/secretory of L3 (L3-ES) antigen form *S. venezuelensis*

Excretory secretory antigens of third stage larvae of *S. venezuelensis* are prepared according to Northern and Grove (1990) (L3-ES).L3 *S. venezuelensis* were kept in distilled water and incubated at 28 ºC with good condition to have sufficient lighting. Due to the low feeding activity of the L3, it is necessary to be cultivated for 5 to 10 days to collect the products of excretion/secretion of the parasite according to Brindley *et al*. (1988).

Materials:

- Larvae 3 *S. venezuelensis* decontaminated.
- Culture flasks 100 ml.
- Incubator

Procedure:

- Resuspended 1500 L3/ml in 20 ml of PBS with penicillin 100 IU/ml, streptomycin 0.1 mg/ml, fluconazol 0.8 mg/ml, 5 mM EDTA, 2 mM PMSF and Aprotinin 100 IU/ml, Pepstatin A 1 µM and chymostatin 1 mM.
- Place in the incubator at 28ºC and humidity of 80%.
- Check daily that the larvae are moving. Count was conducted over one hundred mobile L3. If bacterial contamination is detected larvae must be discarding.
- Maintain the cultures until the mobile L3 are 30%.
- Centrifuge the culture at 1000 *g*, 5 minutes, and the supernatant collected and lyophilized.
- Weigh the lyophilized and then rehydrate it to obtain a concentration around 1µg/µl.
- Determine the protein concentration and check on a SDS-PAGE gel 12.5% stained with Coomassie blue. Store at - 80°C.

6.16.4 Female excretory/secretory antigens preparation of *S. venezuelensis*.

Parthenogenetic female of *S. venezuelensis* once decontaminated, can be maintained in the laboratory for about a week with cell culture media, adding glucose and carbon dioxide atmosphere according to Maruyama and Nawa (1997).

Materials

- Female parthenogenetic *S. venezuelensis* decontaminated.
- Dulbecco's modified Eagle's medium (DMEM, D-0819 Sigma).
- Glucose 50% in PBS.
- Culture flasks 100 ml.
- Incubator with 5% CO_2 at 37°C.

Procedure:

- Resuspended 30 females/ml in 20 ml PBS with 1% glucose, penicillin 100 IU/ml, streptomycin 0.1 mg/ml, fluconazol 0.8 mg/ml, 5 mM EDTA, 2 mM PMSF and Aprotinin 100 IU / ml, Pepstatin A 1 μM and chymostatin 1 mM in cell culture flasks at 37°C with 5% CO_2 and 80% humidity.
- Add sugar daily to maintain a minimum of 1%, check the mobility of 100 females and control contamination with bacteria and other micro organism by Gram's method in the culture medium.
- Remove the culture medium every 3 days until mobility reach below 70%.
- Centrifuge the culture at 1.000 *g* for 5 minutes, recovering the supernatant and freeze it at -80°C and lyophilized.
- Rehydrate the lyophilized protein to obtain an approximate concentration of 1 mg/ml of antigen.
- Remove the supernatant and store at -20 ºC.
- Determination of protein concentration by Micro BCATM Assay and check the bands in SDS-PAGE gel 12.5% stained with Coomassie blue.

6.17 Mc Master egg counting for quantitation of nematode eggs

Fecal worm egg examination methods are based on the principle of differential density. In other words, parasites eggs sink in water, but they will float in various chemical solutions that are more dense than water because the eggs are lighter than the fluid used as a flotation solution.

Materials:

- Compound microscope.
- Scale.
- Saturate sodium chloride solution mix water with salt and add more salt then heat the mixture in pan with stirring until boiling. Then let it to cool at room temperature. The solution will look cloudy and some material will precipitate and now it OK. Pour clear part of solution into a dispensing container of dome kind and store at room temperature. Do not refrigerate as additional solute will precipitate.

- 50 ml centrifuge tube with screw cap.
- Pipet (1 ml syringe or eye dropper works well)
- McMaster egg counting slide.
- Paper towels.
- A fresh fecal sample should be collected and kept refrigerated until tested.

Procedures:

- Weight out 2 g of feces into a 50 ml centrifuge tube and fill to 30 ml with salt solution.
- Pour off approximately 25 ml of the salt solution into another small container keeping feces in the tube.
- Let soak for a few minutes and mix (soft feces) or break up (fecal pellets) with tongue blade.
- Add back about ½ of the salt solution and mix well, breaking up any remaining feces and homogenize feces as best as possible.
- Add back the remaining salt solution and screw the cap back onto the tube.
- Shake tube vigorously for about 1 minute to disrupt any remaining feces as much as possible.
- Set tube aside for a few minutes to let bubbles dissipate.
- Wet McMaster chamber with water and dry top and bottom on paper towels.
- Rock tube several times to thoroughly mix solution without causing large air bubbles to form.
- Pipet immediately (using 1 ml syringe or eye dropper) a sample of the suspension and fill both sides of counting chamber. Work quickly. If it takes more than a few seconds to load the first chamber, then mix fecal solution again and refill pipet before loading the second chamber.
- Let stand for 1-2 minutes to allow eggs to float to top.
- Count all eggs inside of grid areas (greater than ½ of egg inside grid) using low power (10x) objective. Focus on the top layer, which contains the very small air bubbles (small black circles, if numerous large air bubbles are visible, remove the fluid and refill)
- Count only trichostrongyle/strongyle eggs (oval shaped, ~80-90 μm). Do not count strongyloides (oval, ~50 microns long), tapeworm eggs (triangular/D-shaped) or coccidia (various sizes). Notations are made as to the presence of other species, but only the trichostrongyle/strongyle eggs are counted.
- Once filled, the chambers can sit for no longer than 60 minutes before counting without causing problems. Longer than this and drying/crystal formation begin.
- Total egg count (both chambers) ×50=EPG (eggs per gram).

6.18 Enzyme-linked immunosorbent assay (ELISA)

ELISA is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample. The ELISA has been used as a diagnostic tool in medicine and veterinary as well as a quality control check in various industries. Detection of an experimental *Schistosoma mansoni* infection in CD1 mice serum the IgG total, IgG1 and IgG2a has done by ELISA technique

Materials:

- Microtiter plates of 96-Well.
- Eppendorf tubes.
- Twelve-channel pipettor.
- Adjustable pipettor of 1ml.
- Humid chamber.
- Wash battle or ELISA plate washer.
- Polystyrene microtiter plates (Costar, Cambridge, MA, USA)
- Peroxidase rabbit anti-mouse immunoglobulin G (Sigma).
- Peroxidase monoclonal anti-mouse immunoglobulin G1 and G2a (Nordic immunology)
- Plate reader.
- TMB.
- Carbonate buffer.
- Wash buffer.
- Blocking buffer.

Procedures:

- Coate plates with 100 µl of either *S. mansoni* AWA per well at a previously determined protein concentration of 5 µg/ml diluted in carbonate buffer pH 9.6.
- Place serum at a dilution of 1:100 was added to the wells.
- Incubate for 1 hour at 37ºC.
- Wash three times with 200 µl of PBS-Tween 20 per well.
- Add horseradish peroxidase rabbit anti-mouse IgG, IgG1 or IgG2a at a dilution of 1:1.000.
- Wash three times with 200 µl of PBS-Tween 20 per well.
- Incubate for 1 hour at 37ºC, with substrate solution *ortho*-phenylene diamine plus H_2O_2
- Stop the reaction at 10 minutes with 3 N of H_2SO_4.
- Read absorbances at 492 nm

6.19 References

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