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Vascular endothelial growth factor-like and its receptor in a crustacean optic ganglia: A role in neuronal differentiation?

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ABSTRACT

The neural system appears before the vascular system in the phylogenetic tree. During evolution, vascular system generation takes advantage of the pre-existing vascular endothelial growth factor (VEGF) in order to form its networks. Nevertheless, the role of VEGF in neuronal and glial cells is not yet completely understood. In order to support the hypothesis of a neural role for VEGF, we searched for VEGF- and VEGF receptor (VEGFR)-like immunoreactivities (immunohisto/cytochemistry and Western blotting) in the eyestalk of the invertebrate *Ucides cordatus* (Crustacea, Brachyura, Ucididae). Our results showed that both neurons and glial cells expressed VEGF-immunoreactivity, and that VEGFR was evidenced in neural cells. This is the first report about the VEGF/VEGFR-like immunoreactivities in the nervous tissue of a crustacean, and enables *U. cordatus* to be included in the repertoire of animal models used for ascertaining the role of VEGF in the nervous system.

1. Introduction

Vascular endothelial growth factor (VEGF) is a cytokine known for its essential roles in vasculogenesis and angiogenesis in vertebrates [1]. Indeed, VEGF has been established as a vascular permeability factor and endothelial cell mitogen [2]. Recently, VEGF has been strongly related to nonvascular functions, especially in the nervous system [3]. The influence of this cytokine in the supporting of neural cells has been extensively studied in mammals. These effects include neurogenesis, neuronal migration, neuronal survival, axon guidance, and neuronal protection [4,5].

In invertebrates the PVF–PVR system (comprised by PDGF/VEGF-like factor PVF and its receptors PVR) is considered to be ancestral to the VEGF/VEGFR system of mammals [6]. Putting together the fact that invertebrate organisms may have a vascular system with no complete lining by endothelial cells, with the fact that the vascular system evolved taking advantage of the developing nervous system net, raises questions about the ancestral function of the VEGF signaling pathway in neural development and maintenance [7–10].

None of the above mentioned studies have actually defined VEGF as originally involved in development and maintenance of the nervous system. In search for further support for this hypothesis, we tested for the presence of VEGF-like and VEGF receptor (VEGFR)-like immunoreactivity in the eyestalk of the malacostracan crustacean *Ucides cordatus*. This crab is a very particular model because it has been classified as a semiterrestrial species [11] and therefore, serves as a highly useful paradigm for studying evolutionary transition (from water to land) characteristics [12].

Our choice for studying the eyestalk of *U. cordatus* in order to explore the role of VEGF as a neuronal support factor derived from two main reasons: the accessibility of the eyestalk in these animals and because it is composed by nervous tissue and blood vessels lined by cells different from the vertebrate endothelial cells [13]. Therefore, the results presented here may constitute a relevant contribution in favor of an original role for VEGF in the adult nervous system of invertebrates.

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2. Materials and methods

2.1. Animals

Healthy adult male intermolt *U. cordatus* specimens ($n = 20$) were obtained from mangroves in Itambi, Niteroi, Rio de Janeiro State, Brazil. All procedures adopted in this study, including the location where the animals were captured, were performed after approval by the National Environmental Committee (Certificate # 14689-1/IBAMA/2008, permission to use the animals # 2440408), and by the Ethics Commission on Research Animals of the Centro de Ciências da Saúde, Universidade Federal do Rio de Janeiro (protocol DHEICB 005).

The animals were maintained in tanks with controlled laboratory conditions at temperatures from 25 to 28 °C, 12 h/12 h light/dark cycle, and fed with green leaves. They were cryoanesthetized prior to eyestalk removal and optic ganglia dissection.

2.2. Immunohistochemistry

The optic ganglia were fixed with 4% formaldehyde freshly prepared from paraformaldehyde (PF) in 0.1 M phosphate buffered crustacean saline (PBS) overnight. Then the tissues were washed in 0.1 M PBS, cryoprotected with sucrose, embedded in optimal cutting temperature compound (OCT, Tissue-Tek®), and 5 μm thick sections longitudinal to the long axis of the stalk were obtained and mounted on poly-L-lysine-coated slides. The sections were destined for the immunohistochemical reactions, using antibodies against VEGF, glial fibrillary acidic protein (GFAP), neuronal nuclei (NeuN) and VEGFR. The antigens were unavailable for preadsorption testing.

Sections were washed in PBS with Triton X-100, and treated with 10% bovine serum albumin (BSA) in PBS. Then, they were incubated with either the primary monoclonal antibody anti-VEGF (C-1, Santa Cruz, Dallas, TX, USA, cat # sc-7269) or the polyclonal antibody anti-VEGF (A-20, Santa Cruz, cat # sc-152), affinity-purified at 1:50 dilution. The sections reacted with the polyclonal antibody to VEGF were also reacted with the monoclonal antibody anti-NeuN (Millipore, Billerica, MA, USA, cat # MAB 377) at 1:50 dilution (overnight – anti-VEGF; during 48 h – anti-NeuN), at 4 °C in a humid chamber. Other sections reacted with the monoclonal anti-VEGF were also reacted with the polyclonal anti-GFAP (DBS, Pleasanton, CA, USA, cat # RP014) at 1:100 dilution. The slides were then washed in PBS 0.3% Triton X-100 and incubated with the secondary antibodies Alexa Fluor® 488 goat anti-mouse IgG (H + L) (1:300 dilution), and Alexa Fluor® 546 goat anti-rabbit IgG (H + L) (1:300 dilution) (Invitrogen). The sections were finally labeled with the fluorescent probe DAPI and mounted with Fluoromount/Plus™ (Diagnostic BioSystems). The controls consisted of omitting the incubation in the primary antibody.

Other frozen sections were prepared as above in order to be reacted with the polyclonal antibody against VEGFR (Flk-1 (S-20), Santa Cruz, Santa Cruz, CA, USA, cat # sc-48161) overnight at 1:100 dilution, at 4 °C in a humid chamber. The slides were washed in PBS 0.3% Triton X-100 and incubated with the secondary antibody Alexa Fluor® 546 Goat Anti-Rabbit IgG (H + L) (Invitrogen, Carlsbad, California, USA) at 1:300 dilution. The sections were then labeled with the fluorescent probe DAPI and mounted with Fluoromount/Plus™ (Diagnostic BioSystems, Pleasanton, CA, USA). The controls consisted of omitting the incubation in the primary antibody.

All the sections were examined and imaged using a Leica TCS SP5 confocal microscope. Serial optical sections were taken at 1-mm intervals and saved as two-dimensional projections.

2.3. Protein determination assay and Western blotting

The optic ganglia were homogenized in a potter containing RIPA buffer. The protein concentration was determined according to the Folin phenol method [14], using BSA as a standard. The total protein samples were diluted in a classical sample buffer, 1% β-mercaptoethanol, 3% SDS, and 62.5 mM Tris base. Protein aliquots of 75 μg from three different samples were separated and identified in either 12% or 7% SDS–PAGE on a Mini PROTEAN 3 system (Bio-Rad Laboratories, USA) at 60 mA/gel. The proteins were transferred at 350 mA to nitrocellulose membranes using the same system for 90 min. The membranes containing the immobilized proteins were blocked with non-fat dry milk in 0.1 M Tris buffered saline. Afterwards, the membranes were gently washed with Tween TBS. VEGF identification was performed by the incubation of the membrane with the same primary monoclonal (1:1000) antibody anti-VEGF used for immunohistochemistry, affinity-purified. VEGFR identification was performed by the incubation of the membrane with the same primary polyclonal (1:1000) antibody anti-VEGFR used for immunohistochemistry. The membranes were then washed with Tween TBS. The secondary antibodies were anti-mouse (for VEGF; Santa Cruz, Santa Cruz, CA, USA, cat # A4416) and anti-goat (for VEGFR; Santa Cruz, Santa Cruz, CA, USA, cat # A5420) IgGs conjugated to peroxidase (diluted 1:5000 in TBS). VEGF and VEGFR were immunodetected with the chemiluminescent HRP substrate (Immobilon Western, Millipore, Billerica, MA, USA).

2.4. Cell culture and immunocytochemistry

The crabs were anesthetized by chilling for 20 min, and then they were washed with 1% sodium hypochloride solution and rinsed with 70% ethanol. For culturing the cells of the visual system, the retinae were removed and the remaining optic ganglia were used. The optic ganglia were incubated in TrypLE™ Express (Gibco, Life Technologies, Grand Island, NY, USA) at 37 °C for 5 min. After removing the TrypLE™ fetal bovine serum (FBS) was added to the L-15 medium and then centrifuged for 5 min at 1800×*g*. The pellet was then resuspended in L-15 medium, in order to dissociate the cells, which were seeded in plastic Petri dishes containing collagen.

The optic ganglia were cultivated in L-15 medium with L-glutamine (Liebowitz, Sigma) supplemented with 10% FBS (inactivated, sterile, mycoplasm-free, Cultlab, Campinas, São Paulo State, Brazil) and an antibiotic mixture containing 1% penicilin/streptomycin (10,000 units/mL, Gibco), and were maintained in the oven at 28 °C for 3 days. After 3 days all the cells, except for the injured ones, adhered to both substrates. At the end of this period and also at the end of 7 days of culture, the cell cultures were fixed with 4% PF and immunoreacted with the monoclonal antibody against VEGF, using the same protocol as described above.

The cultured cells on the coverslips were then labeled with the fluorescent probe DAPI and mounted with Fluoromount/Plus™ and were evaluated under a Zeiss AxioImager.Z1/ApoTome. The images obtained were recorded using the Axiocam MRm Rev. 3 and the software Axiovision Rel 4.8Cell M™.

3. Results

The visual system of this species located in the eyestalk comprises three successively arranged optic ganglia: the *lamina ganglionaris* or lamina (La), the external medulla (EM), and the internal medulla (IM) [15].

3.1. Optic ganglia neurons and glia are labeled with anti-VEGF antibody

Neurons and glial cells were doubled-labeled for VEFG and NeuN (neuronal cells) [16] or GFAP (glial cells) [17,18] in the three optic ganglia. The immune reactions for VEGF alone, and both VEGF with GFAP and VEGF with NeuN in the *lamina ganglionaris* are shown in Fig. 1A–C.

3.2. Optic ganglia neural cells are labeled with anti-VEGFR antibody

Neural cells in the three optic ganglia were labeled for VEGFR. Fig. 1D shows neural cells in the *lamina ganglionaris* labeled for VEFGR.

3.3. VEGF-like and VEGFR-like immunoreactivity shown by Western blotting

The Western blotting using the monoclonal antibody against VEGF showed a band in ~75 kDa (Fig. 2A) in the optic ganglia lysates. The Western blotting using the polyclonal antibody against VEGFR showed a band in ~200 kDa (Fig. 2B) in the optic ganglia lysates.

3.4. Optic ganglia cultured neural cells show VEGF-like immunoreactivity

In order to check whether isolated neural cells and cells of the vascular system showed VEGF-like immunoreactivity, we cultured the optic ganglia and hemocytes. We observed that the dissociated cells from the optic ganglia cultured for either 3 or 7 days showed positive reaction for VEGF (Fig. 3A and B). However the hemocytes,

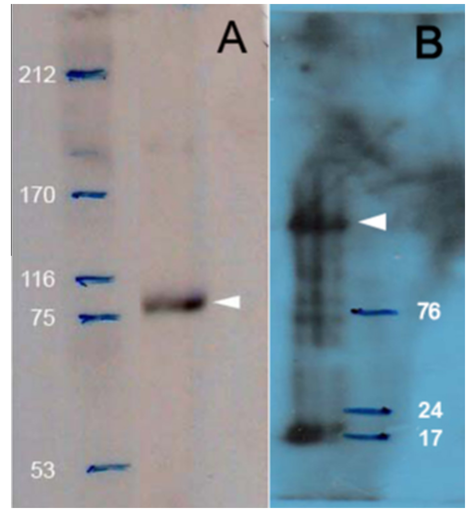

Fig. 2. Western blottings of optic ganglia proteins. (A) Shows VEGF molecular weight (arrowhead) and (B) VEGFR molecular weight (arrowhead).

also cultured for either 3 or 7 days, were not labeled with the VEGF antibody (Fig. 3C and D).

4. Discussion

Our main results are as follows: 1. Subset of differentiated GFAP-reactive glial and NeuN-reactive neurons present VEGF-like immunoreactivity in optic ganglia; 2. Neural parenchymal cells of the optic ganglia also present VEGF-R-like immunoreactivity; 3.

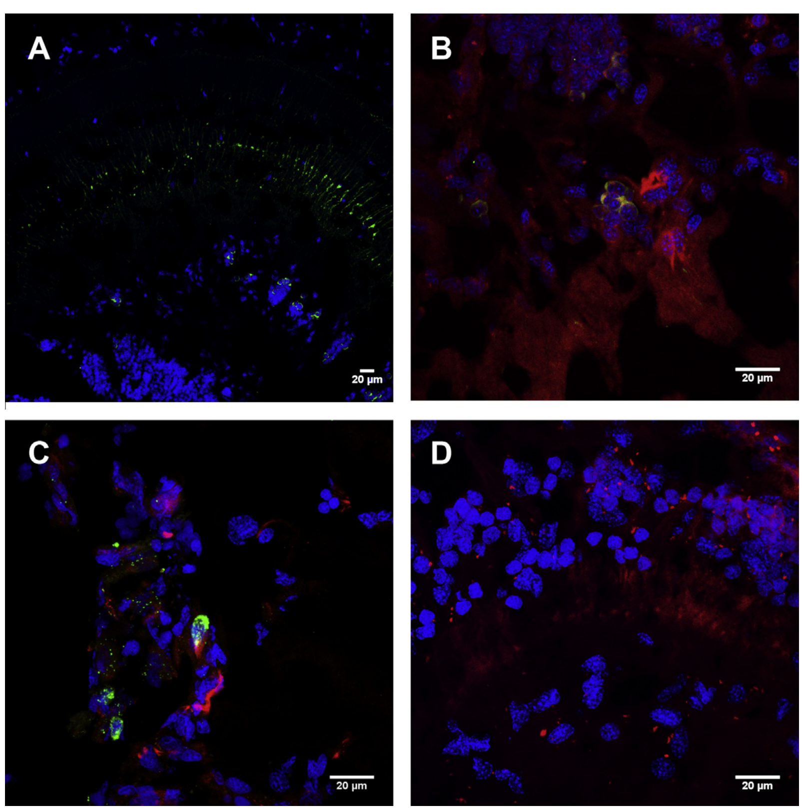

Fig. 1. Immunofluorescence of VEGF, GFAP, NeuN and VEGFR in the crustacean nervous tissue. In (A), low magnification of the VEGF expression in the *lamina ganglionaris* (green). (B) Shows the co-localization of VEGF and GFAP (yellow as a result of green – VEGF – plus red – GFAP. (C) Shows neurons evidenced by double-labeling with anti-NeuN (green) and VEGF (red). VEGFR (red) expression is observed in (D) in the neural cells. DAPI staining in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

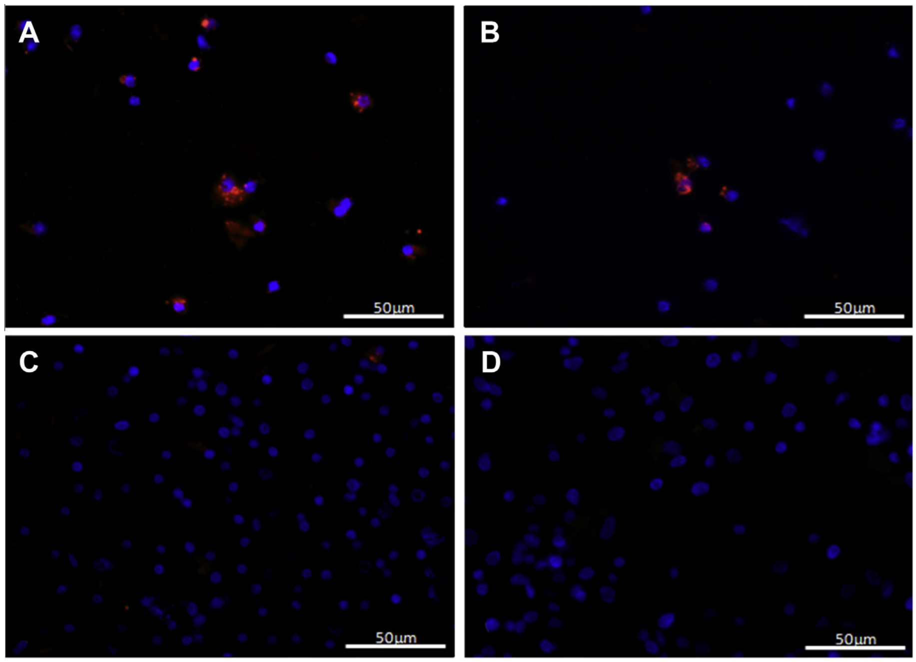

Fig. 3. VEGF-like immunoreactivity in cultured optic ganglia and hemocytes. In (A), the dissociated cells from the optic ganglia cultured for 3 days show positive reaction for VEGF (red). (B) Shows positive reaction for VEGF (red) in the dissociated cells from the optic ganglia cultured for 7 days. In (C and D), hemocytes, also cultured for either 3 or 7 days, respectively, were not labeled with the VEGF antibody. DAPI staining in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Immunoblots of optic ganglia homogenates present a band of about 75 kDa with the anti-VEGF antibody and a band of about 200 kDa with the anti-VEGF receptor antibody.

The reaction of a subset of GFAP-positive glial with the anti-VEGF antibody is not surprising since several studies in vertebrates show the same findings, while the occurrence of differentiated VEGF-positive neurons in vertebrates is not so frequent [19,20]. The most well-documented observation on this issue is that by Licht and coworkers [21] showing that VEGF is constitutively expressed by mitral cells and by tufted and periglomerular cells, close to the destination sites of incoming new neurons. More importantly, blockade of VEGF resulted in reduced dendritogenesis in developing neurons without affecting, or increasing paradoxally, dendritic spine density in already mature cells. It is interesting to notice that analogously to the olfactory bulb of vertebrates, the optic ganglia of crustaceans are sites in which new neurons are acquired in adulthood [22]. If a VEGF-like factor is responsible for a similar function in crustacean optic ganglia, it can be predicted that VEGF-R-like reactive parenchymal cells might include immature neurons.

The estimated molecular weight of the protein reacting with the anti-VEGF antibody is somewhat puzzling. First of all, it has been suggested that crustaceans may not possess a PDGF–VEGF related system [23]. In the case of another arthropod, Drosophila, the best known VEGF homologue, PVF1, has a molecular weight of about 34 kDa [24]. However, not only estimates of PVF3 isoform D molecular weight (www.encorbio.com) using the sequence provided by the Berkeley Drosophila Genome Project [25] gives a value of about 75 kDa, but also [26] reported PVF1 with 60–70 kDa in *Caenorhabditis elegans*. According to a recent work with PVF2 + PVF3 knockouts, these ligands "act locally in epithelial cells to coordinate trans-epithelial migration of hemocytes" [27] but nothing is known regarding a possible action of the PVF3 ligand in the crustacean or, at least, in the arthropod nervous tissue.

The hypothesis of an ancestral role for VEGF in development and maintenance of the nervous system is still the subject of much research. Evolutionarily, certain groups of invertebrates, which lack a true vascular system, already have a nervous system, and both VEGF and VEGFR related molecules have been shown to regulate its development [8,10,28]. In this study we present further support for the hypothesis that vascular system generation may take advantage of the pre-existing VEGF in order to form its networks [9], by showing VEGF/VEGFR-like immunoreactivity in the nervous optic ganglia.

VEGF protein is conserved from invertebrates to mammals [6,26]. Indeed, the ability of PVF to bind to human VEGFR, thereby inducing angiogenesis, has been reported confirming the conserved properties of the PVF/PVR–VEGF/VEGFR system [26]. However, because certain invertebrate organisms may lack a true vascular circulatory system, questions have been raised concerning the ancestral function of the VEGF signaling pathway [4,7,29]. In the gastrovascular system of jellyfish the expression of a PVF ortholog was reported [30], possibly acting as a guidance for the growth of the endodermal cavities. This adds to the already described functions attributed to PVF in invertebrates, another primary function, since cnidarians do not have vessels or blood cells. In line with these findings is the reported presence of VEGF/VEGFR both in the vascular and nervous systems of Drosophila [7,31]. Our findings showing VEGF/VEGFR-like immunoreactivity in neural cells of the optic ganglia of the crab *U. cordatus*, which has a relatively developed vascular system, help support the hypothesis that in addition to the role performed in the vascular tissue, VEGF is also important as a signaling molecule in the nervous system.

It has been suggested that, in more complex eukaryotes, development of a vascular system took advantage of the pre-existing VEGF/VEGFR system originally devoted to neural development. One of the mechanisms involved is related to the VEGF neuronal production during axonal sprouting, which also serves to guide blood vessels, as reviewed in [6]. In invertebrates, the presence and function of PVF is still poorly described [4], therefore our study contributes to the efforts to filling this gap. The data reported here, revealed with the help of the accessible optic lobe of *U. cordatus* is, to our knowledge, the first report showing the VEGF/VEGFR-like immunoreactivity in an adult invertebrate nervous system. More importantly, our findings add support to the hypothesis that VEGF (PVF) first appeared as factor for the development and maintenance of the nervous system.

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