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Role of VEGF in an experimental model of cortical micronecrosis

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Summary. Vascular endothelial growth factor (VEGF) is a major mediator in angiogenesis and vascular permeability. In central nervous system (CNS) it plays a pivotal role as: 1. inductor of endothelial cell proliferation, migration and inhibition of apoptosis, and 2. mediator of vascular permeability and subsequently of brain edema. This ubiquitous epiphenomenon is a major complication in several CNS pathologies, including head trauma and stroke.

After brain injury the expression of VEGF is increased contributing to disruption of the blood brain barrier (BBB). VEGF increase the permeability of BBB via the synthesis/release of nitric oxide and subsequent activation of soluble guanylate cyclase. The immunohistochemistry shows an increase of stained astrocytes and endothelial cells around cortical micronecrosis. VEGF immunopositivity distribution shows some correspondence with the blood brain barrier breakdown following a cortical micronecrosis.

Keywords: VEGF – Cortical micronecrosis – Brain edema – BBB – Vascular endothelium

Introduction

Vascular endothelial growth factor (VEGF) is a major mediator in angiogenesis and vascular permeability. In central nervous system (CNS) it plays a pivotal role as: 1. an inductor of endothelial cell proliferation, migration and inhibition of apoptosis, and 2. a mediator of vascular permeability and subsequently of brain edema. This ubiquitous epiphenomenon is a major complication in several CNS pathologies, including head trauma and stroke.

VEGF exists in a number of isoforms in human tissue, i.e., $VEGF_{206}$, $VEGF_{189}$, $VEGF_{165}$, $VEGF_{145}$, and $VEGF_{121}$, that differ in their molecular masses and biological activities. The VEGF isoforms bind with two tyrosine-kinase receptors, KDR/flk-1 and flt-1. In addition, $VEGF_{165}$ binds with a newly identified co-receptor, neuropilin-1, which is expressed in human endothelial cells and several types of non-endothelial cells including tumour cells. These VEGF isoforms are differentially expressed in brain tumours suggesting differences among tumour entities in the mechanisms of up-regulation as well as their employment of distinct isoforms for neovascularization (Nishikawa et al., 1998).

This angiogenic peptide released in response to hypoxia, acts on endothelial cells to promote the sprouting of new capillaries from existing blood vessels (Neufeld et al., 1999). Its angiogenic actions also involve an antiapoptotic effect that promotes the survival of endothelial cells mediated by the VEGF receptor 2 (VEGFR-2) via PI3k-dependent signalling pathways (Jin et al., 2000).

Severe reduction of blood flow to a sector of brain cortex results in a lack of oxygen and nutrient transportation, which lead to tissue hypoxia and cell death. The organism tries to recover the homeostatic situation by increasing the oxygen delivery in this zone. For this, vasodilatation is triggered during a first step. Although its precise mechanism is not yet clearly understood, it depends on the dual activity of the vascular endothelial growth factor (VEGF): VEGF acts as an endothelial-specific proliferation and differentiation factor, and as a vasculature permeabilization factor, as stated before. Therefore, it's also called vascular permeability factor (VPF). VEGF/VPF is thought to play a major role in the regulatory mechanism involved in brain tumour angiogenesis and the genesis of consequent peritumoral edema (Machein et al., 2000).

It has been described that during brain injury (trauma or infarction) it seems that the expression of

VEGF is increased, thus contributing to disruption of the blood-brain barrier (BBB). VEGF increases the permeability of BBB via the synthesis/release of nitric oxide and subsequent activation of soluble guanylate cyclase. VEGF induces the formation of fenestration in blood vessels and the formations of vesiculovacuolar organelles that form channels through which blood borne proteins goes extravasated. This functional pathway, through NO and guanylate cyclase open a treatment possibility inhibiting their synthesis or release.

About the role of VEGF in stroke, it has been found that expression of this cytokine correlates with infarction volume and clinical disability. It has been demonstrated that antagonists of VEGF reduce ischemia/reperfusion-related brain edema and injury, implicating VEGF in pathogenesis and eventually in treatment of stroke and related disorders (van Bruggen et al., 1999).

Here we show, that VEGF expression is induced temporarily by cerebral micronecrosis in a rat experimental model. The expression was most prominent in the border zone surrounding the area of infarction, and appeared to be associated primarily but not exclusively with astroglia.

Material and methods

The cerebral cortex of 60 anaesthesized Sprague-Dawley rats (weighing 200–250 gr) was directly exposed to an ultraviolet beam for 6 min through a 2 × 2 mm left parietal craniotomy (for anaesthesia, Ketamine hydrochloride 15 mg/100 g and Xylazine hydrochloride 2 mg/100 g body weight were i.p. injected). An Osram-HBO-200 ultraviolet lamp was used after a warm-up period of 10 minutes. The lamp was placed 15 cm above the cortex. The skull was covered with reflective material in order to avoid the heat effect. Animals were monitored and vital parameters during the experiments remained constant. After several survival times (30 min, 1 h, 3 h, 6 h, 12 h, 24 h, 48 h, 72 h, 1 week, 2 weeks and 1 month) animals were transcardially perfused with 4 % fresh paraformaldehyde solution in saline buffer phosphate solution preceded by a washout with a 0.9% saline solution. Five rats with a left parietal craniotomy were not irradiated and were used as controls. Pain and discomfort were minimized according to the European Community Council Directive of 24 November 1986 (86/609/EEC).

After postfixation of the whole brain for 24 h at 4°C, histoprocessing of coronal sections including the lesion was performed. Routine 4 μm paraffin slices were obtained for histology (HE, PAS and PAH staining) and immunohistochemistry. Slices were incubated with antibodies against albumin (1 : 50 Dako, Denmark) and $VEGF_{165}$ (1 : 1,000, Oncogene, USA) using the avidin-biotin complex (Vector-Elite) and diaminobenzidine (DAB) as chromogen.

Results

The animals recovered from scalp surgery and exposure with any complications. No gross signs of functional deficits could be observed.

Injury evolution was examined by neuropathological changes showed by aniline staining. Vascular permeability was evidenced by immunohistochemistry demonstrating extravasation of endogenous serum proteins and the findings correlated to VEGF expression.

Control animals displayed little evidences of VEGF immunoreactivity in some neurones and endothelial cells and no reaction at all to albumin.

Cortical lesions with minimal focal changes were observed in animals with short survival time. Lesion showed variable tissue necrosis, sponginess and glial reaction depending on the survival time.

30 min after brain exposure a pale aspect of the tissue and a light diffuse staining of the irradiated tissue with both antibodies were seen.

Albumin deposits were immunohistochemically demonstrated diffusely in the neuropil from 30 min to 12 hr after injury and in the vessels wall from 6 hr to 3 days. Stained vessels were mainly arterioles but permeable venules and capillaries were also present. Immunohistochemistry demonstrate extravasation of endogenous serum proteins from vessels in the lesion area with spreading into the underlying white matter and other adjacent regions including the white matter of the opposite hemisphere as early as in the first 48 hr.

After 3 h, the core showed fewer stained cells than perilesioned areas; this cell lost was progressive with the evolution of the microinfarct (Fig. 1). Sponginess still does not include the underlying white matter. An immunopositive rim around the lesion involving the extracellular space was delineated with albumin. $VEGF_{165}$ antibody in brain sections from injured animals already showed fine delineated cells and their processes with brown DAB-deposits at the vessel wall in the border between gray and white matter (Fig. 2). In prior periods no stained structures were found.

At 6 hours postirradiation a generally widespread, longlasting edema develops within the injured hemisphere, resulting in increased cortical thickness compared to the uninjured side, in longer survival periods this edema was most pronounced in perilesioned areas. The underlying white matter was reached and serum proteins moved following nerve fibers directions. A remarkable immunopositivity for VEGF was

Fig. 1. Lesion 3 hr after injury, phosphotungstic hematoxilin staining

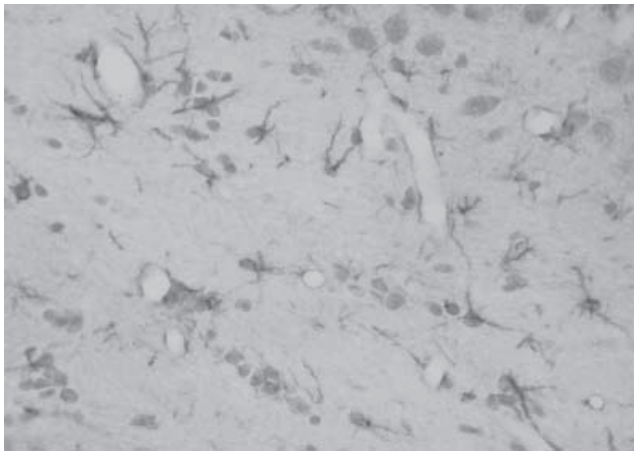

Fig. 2. Transition field among cortex and white matter, 3 hr postinjury. Immunohistochemistry against VEGF 165 some cell fine processes are delineated resembling astrocytes

present in the affected areas displaying the endothelium of small and middle vessels (Fig. 3).

At 12 h, injured brains showed a cortical micronecrosis about 1 mm diameter and 0.2 mm in depth. The micronecrosis stained brown and stood out clearly, with blood vessels full of erythrocytes, in contrast to the perfused microvessels in the surrounding areas. Remarkable spongiosis extending to the corpus callosum was found and the extravasated proteins moved following nerve fibers direction mainly to the contralateral hemisphere, in addition to the deposits in the vessel walls with both antibodies.

At 24 hr, the staining in the perilesioned region increased and involved the white matter, moving laterally. Immunopositivity was observed in the white matter of both hemispheres including the corpus callosum. In coronal sections exudate reached the contralateral hemisphere.

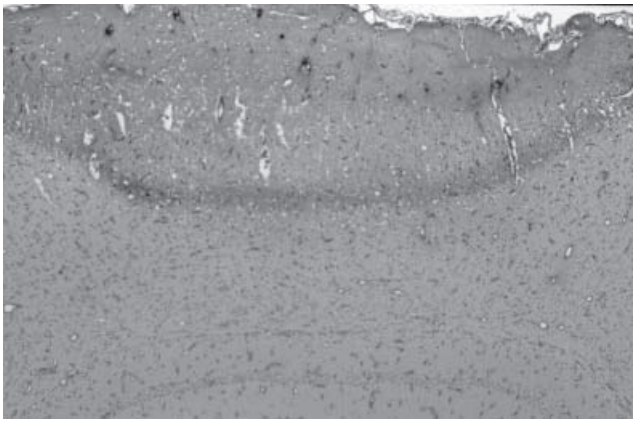

Fig. 3. Immunohistochemistry against VEGF 165. The lesion 6 hr after injury appears strongly positive. The endothelium of the most surrounding vessels is also positive

The distribution of the immunopositivity was dissimilar, either in extracellular space, perivascular or between the myelin fibers. The endogenous proteins lying in a first step in the vascular wall of middle and small vessels, they were found in a second step in perivascular lakes and finally they could be observed in astrocytes and eventually also in neurons.

By VEGF immunohistochemistry, an increase in the number of stained cells around cortical micronecrosis was observed. Morphologically, these cells looked mainly like astrocytes but some neuron bodies were always positive.

After 48 hr the front of exudate spread on the contralateral hemisphere.

In perilesioned areas, a diffuse brown staining was prominent around empty microvessels at 48 h, when the white matter was almost completely involved. Antigen-antibody conjugates were conspicuous in neurons and astrocytes near the lesion. In the underlying white matter astrocytes were strongly positive for albumin as well as for VEGF (Fig. 4).

The maximal spreading of the immunostaining with albumin was reached 72 hr after the injury (Fig. 5). At this time hypertrophic reactive astrocytes, intensely positive for both antibodies, were found in the subcortical white matter far away from the lesion. In remote areas only the glial cells and the basement membranes of small microvessels were positive for the albumin antibody. With VEGF-antibody, astrocytic gliosis was not observed in the contralateral hemisphere at any survival time, but strong positive astrocytes were shown in the perilesioned cortex and in the underlying white matter.

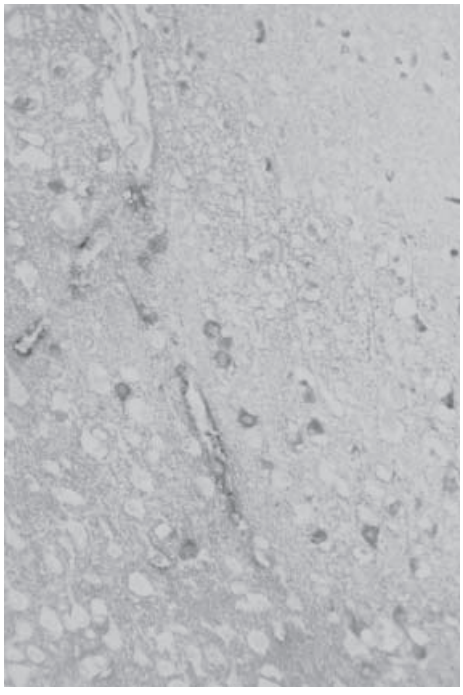

Fig. 4. Perilesional region 48 postinjury. Some vessels and neuron bodies are positive for VEGF

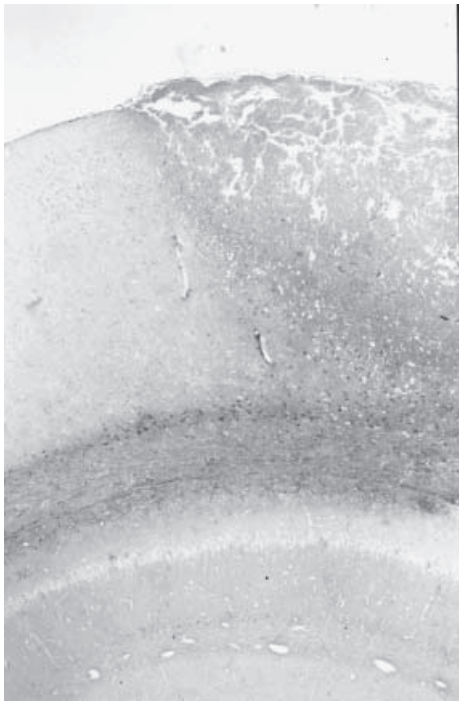

Fig. 5. Lesion and underlying white matter 72 hr after the injury. Albumin spread on gray and white matter, perivascular cumulates and positive cell bodies can be seen

At 1 week after exposure none vessel showed permeability for serum proteins. Around the micronecrosis the number of astrocytes positive to both antibodies were increased, and remained more than 1 week after the lesion.

A variable degree of albumin immunostaining, in neuropil, vessel wall or cellular structures remained up to 2 week after exposure. Positivity for VEGF returned to the basal distributions.

After 3 weeks and 1 month rats showed a depressed area on the cortical surface covered by thickened meninges and no differences to control cases in immunopositivity of any type could be seen.

Our findings indicate that VEGF expression is induced temporarily by cerebral micronecrosis and keeps some parallelism to the BBB breakdown. The expression was most prominent in the border zone surrounding the area of infarction, and appeared to be associated primarily but not exclusively with astroglia.

Discussion

Vascular endothelial growth factor, a key regulator of vasculogenesis and embryonic angiogenesis, was recently found to be up-regulated in an animal model of stroke (Marti et al., 2000). Forty-eight to seventy-two hours after permanent middle cerebral artery occlusion a strong increase in the number of newly formed vessels at the border of the infarction was evidenced. VEGF was strongly up-regulated in the ischemic border, at times between 6 and 24 hours after occlusion. In addition, both VEGF receptors were up-regulated at the border after 48 hours and later in the ischemic core.

Furthermore, in the ischemic rat brain, reduced tissue oxygen tension not only triggered VEGF expression, but also increased protein and mRNA levels for VEGF and its receptors KDR/Flk-1, Flt-1 (van Bruggen et al., 1999).

VEGF participates in the response of the CNS to injury probably in a dose-dependent way (Hofman et al., 2000) but simultaneously as a citoquine related to the inflammatory response that recruits proteins from the extracellular matrix, thus facilitating the progress of the secondary cerebral damage.

Prior studies have demonstrated a biphasic pattern in the spreading of edema during the first 24 hr after the UV-I model (Lafuente et al., 1992). Other authors using different experimental models (Nag, 1996; Cobbs et al., 1998) reported a maximal peak of edema at 48 hr after injury. These data suggest that the blood brain barrier disruption initiated at 6 hours correlates with VEGF expression. However, BBB-disruption develops further until 48 hr after irradiation, when

VEGF expression decreases after reaching the maximal level.

As edema resorption takes place when VEGF expression decreases, this result suggests that postinfarct edema has multiple origins and that resorption mechanisms play an important role at this time. The proliferation of astrocytes (which achieve a maximal development at 72 hr) and the topographical edema distribution are two factors involved in the resorption of brain edema.

Recent evidence has been provided that Src kinases regulates VEGF-mediated vascular permeability in the brain following stroke and that suppression of Src activity decreases vascular permeability thereby minimizing brain injury. This was associated with reduced edema, improved cerebral perfusion and decreased infarct volume 24 hours after injury as measured by magnetic resonance imaging and histological analysis (Paul et al., 2001). Most importantly, Src represents a key intermediate and novel therapeutic target in pathophysiology of cerebral ischemia where it appears to regulate neuronal damage and related edema.

Morphological findings demonstrated that edema in the contralateral hemisphere is due to spreading of edematous fluid from the ipsilateral one. These data show the advantages of a model inducing the spreading of brain edema through the white matter from a small cortical area of necrosis to involve distant areas of the brain. Edematous fluid transport proteins have been found far away from the micronecrosis. As a consequence, the border of the edema is always richer in proteins than the normal edematous areas (Lafuente et al., 1994).

Although pathophysiological mechanism could be different between several species or organs, the explanation pointed by some authors (Hofman et al., 2000; Sawada et al., 2000) is perfectly consistent and complementary with the paracellular pathways proposed in the eightieth's decade (Artigas et al., 1983; Nakagawa et al., 1985). Their findings show how it could be possible to explain the increase of vesicles of various sizes across the endothelial cytoplasm. This mechanism unifies both theories, i.e., the formation of vesiculovacuolar organelles as a morphological feature and the alteration of TJ involving VEGF participation as the structural background for them.

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