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DOI: 10.2478/fv-2021-0040

FOLIA VETERINARIA, 65, 4: 72—78, 2021

THE ROLE OF LYMPHATIC MARKER *PROX-1* IN RELATION TO BRAIN TUMOURS

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ABSTRACT

The homeobox gene, *Prox-1* is a transcription factor essential for lymphatic development (lymphangiogenesis) during embryogenesis. It also performs different functions in various tissues such as: retina, lens, liver, pancreas and the central nervous system. Intense expression of *Prox-1* has been demonstrated in the developing spinal cord and brain. In adulthood its expression continues in the hippocampus and cerebellum. In adult tissues the process of lymphatic vasculature formation is accompanied under certain pathological conditions such as inflammation, tissue repair and tumour growth. *Prox-1* expression is typical for lymphatic vessels; thus it belongs to one of the most specific and widely used mammalian lymphatic endothelial marker in the detection of lymphangiogenesis and lymphatic vessel invasion in oncogenesis. It has been shown that *Prox-1* is involved in cancer development and progression. It's tumour suppressive and oncogenic properties are proven in several human cancers, including brain tumours. Among all body cancers the brain tumours represent the most feared tumours with very limited treatment options and a poor diagnosis. The aim of this paper was to show the current knowledge of the gene *Prox-1* with an emphasis on brain tumours, especially in gliomas.

Key words: brain lymphatic system; brain tumours; gliomas; lymphatic markers; *Prox-1*.

INTRODUCTION

The brain represents a significant part of the central nervous system (CNS), and since it controls all of the body functions, it is considered as an exceptional organ of the body [5]. Despite its small size, it is characterized by high metabolic activity and surprisingly consumes 25% of the total body's energy. Thus it produces metabolic waste at a higher rate than any other organ. For this reason, the brain drain is a critical factor for its proper functioning. The transport is necessary for the delivery of nutrients and other materials needed for cell repair and renewal. The transport of molecules is an integral part of cerebral waste

clearance, which is an essential link in many physiological and pathological processes of the brain. The transport is necessary for the delivery of nutrients and other materials needed for cell repair and renewal. Furthermore, the fluid flow in the brain has great impact in delivery of therapeutics and the movement of biomarkers to the peripheral body for detection [29].

In peripheral organs, lymphatic vessels are those, which eliminate waste products from the tissues [27]. The brain parenchyma does not contain lymphatics, however it has its own lymphatic drainage system. This unique system of lymphatic drainage has been developing over the last 50 years. It includes communication between brain parenchyma, extracellular space, perivascular spaces, perineural space, subarachnoid space, meningeal lymphatic vessels (MVL) and cervical lymph nodes (CLNs). The existence of lymphatic drainage routes presents an effective tool for cleaning the brain of harmful substances. Dysfunction of the brain lymphatic system has a crucial impact on brain function and the pathogenesis of neurovascular, neurodegenerative, neuroinflammatory diseases, as well as brain injury and tumours [33].

The aim of this paper was to show the current knowledge of the gene *Prox-1* with an emphasis on brain tumours, especially in gliomas.

Prox-1

The homeobox gene, *Prox-1*, was cloned in humans by homology with the Drosophila gene Prospero and mouse *Prox-1* [36, 40]. In vertebrates, *Prox-1* expression is fundamental for organ development during embryogenesis. It plays an essential role in a variety of tissues, including: the lens, cochlea, heart, liver, pancreas, and skeletal muscle. Intense expression of *Prox-1* has been reported in developing spinal cord and brain [26, 31]. During the CNS development, *Prox-1* regulates progenitor cell differentiation and starting neurogenesis, and is found in some regions of the brain like in the cerebral cortex, cerebellar cortex, in lateral ventricle wall and dentate gyrus as a part of hippocampal formation [7]. In adulthood the expression of *Prox-1* continues in hippocampus and cerebellum. Wherein, the hippocampus represents a significant amount of adult neurogenesis and exhibit one of the radical pathways in this process [19, 35].

Moreover, *Prox-1* is necessary for the induction and development of the lymphatic system by regulating the proliferation of lymphatic endothelial cells [17]. Its overexpression in blood endothelial cells will transform these cells to lymphatic endothelial cells and is identified as a master switch in the program specifying lymphatic endothelial cell fate [12]. Therefore, *Prox-1* belongs to reliable and widely used mammalian lymphatic endothelial marker in the detection of lymphangiogenesis and lymphatic vessel invasion in oncogenesis.

The molecules which were recently established, such as vascular endothelial growth factor receptor 3 (*VEGFR-3*), lymphatic vessel endothelial hyaluronic acid receptor 1 (*LYVE-1*), prospero-related homeobox-1 (*Prox-1*) and podoplanin (*PDPN*) [37] have facilitated key scientific advances and brought novel data into the molecular mechanisms that control lymphatic development and function. The following breakthrough findings include the identification of specific genetic defects in certain hereditary diseases that are associated with lymphatic hypoplasia and dysfunction, and evidence that malignant tumours can directly activate lymphangiogenesis and lymphatic metastasis [16, 32]. These four specific markers of lymphatic endothelial cells (LECs) are important for the detection of lymphangiogenesis and lymphatic vessel invasion in variety of cancer types and provided modern insights into the biology of malignant tumours [17].

Lymphangiogenesis is understood as a formation where the new lymphatic vessels are form from pre-existing lymphatic vessels [9]. It is a dynamic process during embryogenesis but is largely absent under normal physiological postnatal conditions [14]. In adult tissues is associated with some pathological conditions such as inflammation, tissue repair and tumour growth [38]. Generally, it has been found that the solid tumours frequently induce angiogenesis and lymphogenesis [28].

It has been demonstrated that *Prox-1* is involved in cancer development and progression and has both oncogenic and tumour-suppressive functions. The function of *Prox-1* in tumour metastatic growth is caused by its impact on migration and invasion of cancer cells. *Prox-1*'s contribution to cancer cell metastasis presumably resides on its role as a main regulator of the lymphatic system [7]. Tumour progression involves the phase of tumour initiation and promotion, and is characterized by increased proliferation and/or invasiveness of tumour cells [30]. Decreased expression of *Prox-1* has been found in some tumours, signifying that it has a possible role as a tumour suppressor

gene [39]. Accordingly, high expression of *Prox-1* mRNA was found in: neuroblastoma, glioma, small cell lung carcinoma, colon cancer, small intestine adenocarcinoma, liver carcinoma and rhabdomyosarcoma [7]. The expression of other lymphangiogenesis markers was detected by J i a n g et al. in primary and recurrent glioma tumours [22]. Consequently, its functions in different types of tumours depends on cell type [39].

Prox-1 is differentially expressed in human gliomas of different malignancy grades. A positive correlation was demonstrated between *Prox-1* protein expression and a higher degree of glioma malignancy [7]. Particularly, *Prox-1* is abundantly expressed in high-grade malignant astrocytic gliomas, what is related with its effects on cell growth, tumourigenesis and invasiveness [39]. Raising levels of *Prox-1* protein in malignant gliomas may reveal the presence of a subset of cancer cells with the phenotype of early neural progenitors. Even though intense expression of *Prox-1* is in high-grade gliomas, on the other hand it is also up-regulated in a subset of diffuse low-grade gliomas with worse prognosis and probably to progress more rapidly to high-grade tumours. It has been shown that low-grade gliomas with a high proportion of *Prox-1* immunoreactive cells represent a more advanced, less differentiated phenotype than their counterparts with low *Prox-1* expression. These low-grade gliomas seem to represent tumours that are further advanced on the evolution to anaplastic gliomas [7].

According to available study, where they did immunohistochemical staining of *Prox-1* in cell of astrocytomas, they investigated that *Prox-1* expression in this type of gliomas reveals a stepwise increase correlating with tumour grade. *Prox-1* expression was limited to the cell nuclei and was the most evident in areas of tumour cell crowding. The researchers found out in peritumoural normal-appearing tissue, which was close to the high-grade tumour areas much more *Prox-1* expressing cells than in non-neoplastic control samples. The *Prox-1* cells were randomly scattered in peritumoural tissue with no preference for perivascular areas. It was also proven that overexpression of *Prox-1* significantly increased the proliferation and colony formation of glioblastoma cells. For high-grade malignant glioma is typical tumour induced angiogenesis [6].

Even if, *Prox-1* was highly distributed in endothelial cells of blood vessels and capillaries within glioma tissues, the glioma vasculature itself does not express *Prox-1* protein [22]. Tumour vessels in malignant gliomas have a lymphatic-like phenotype. During lymphangiogenesis *Prox-1* mediated podoplanin and *VEGFR-3* expression that elevated levels of *Prox-1* in aggressive gliomas which are responsible for the apparent illegitimate induction of target genes involved in the process of lymphatic vessel invasion [7]. Vascular endothelial growth factor C (*VEGF-C*) and vascular endothelial growth factor D (*VEGF-D*), which are ligands for *VEGFR-3*, may promote tumour lymphangiogenesis and lymphatic metastasis. In addition, *VEGF-C* induces protection againts glioblastoma and requires lymph drainage to the deep cervical lymph nodes [17].

Definition of brain tumours

Brain tumours are kind of intracranial neoplasm and represent a heterogeneous group of tumours whose common feature is the formation of abnormal tissue mass. The cells in this mass grow and multiply uncontrollably, seemingly unchecked by the mechanisms that control normal cells [18]. More than 150 different brain tumours have been documented. The world Health Organization (WHO) classified the brain tumours into two main groups: primary and metastatic. Primary are those that originate from the tissues of the brain, they are categorized as glial or non-glial, benign and malignant. Glial tumours called gliomas are malignant brain tumours. They arise from the cells of glia (supporting cells of the brain) and include: astrocytomas, ependymomas, glioblastomas, medulloblastomas and oligodendrogliomas. Astrocytomas are distinguished into 4 grades that are correlated with clinical outcome: Grade I pilocytic astrocytoma, Grade II astrocytoma (low-grade diffuse), Grade III anaplastic astrocytoma and Grade IV glioblastoma. Glioblastoma (GBM) has the highest incidence rate, and there is a considerable heterogeneity among the different types of glioma [22]. They diffusely infiltrate the brain parenchyma and are badly differentiated tumours with proven cellular polymorphism, high proliferative activity, necrosis and neovascularization [7]. Metastatic brain tumours arise in other organs of the body and spread to the brain usually through bloodstream or lymph system. They are considered as malignant. Both groups of brain tumours are among the most feared of all forms of cancer with very limited treatment options and a poor prognosis. It has been known that brain tumours can invoke massive antitumour immune responses. The most prevalent type of adult primary brain tumours are gliomas,

which account for 80% of the malignant brain tumours [13]. They occur in the cerebral cortex with the highest percentage developing in the frontal lobe (26%). The other brain tumours locations are in the temporal lobe (19%), parietal lobe (12%), cerebellum (5%), brainstem (4%) and the occipital lobe (3%) [11]. The survival time is different between patients with glioma, depending on grades of glioma, and the prognosis is worst for patients with GBM. In addition, GBM has a high probability of recurrence even in the case successful initial surgical resection [22].

Although the brain tumours comprise only 2% of all cancers of the body, they represent a significant source of morbidity with a high mortality rate worldwide [11]. In past decades, the incidence of brain tumours has been increasing. Generally, the lowest incidence is in Africa, and the highest is in northern Europe. It is reported, that in 2016 at the global level, there were 330 000 incident cases of CNS cancer, and 227 000 deaths [8].

Nevertheless, the exact causes of brain tumours are still unclear, one of the causes that leads to their occurrence is the dysfunction and impairment of the brain lymphatic system [33].

The brain lymphatic drainage

The lymphatic system plays a crucial role in waste drainage pathway of the body and is the essential component of the immune system [25]. It is considered as a body's sewer system and first line of defence against disease [1]. It's discovery was preceded by a long and fascinating history. Although literary sources date its investigation to the 17th century, when the lymphatic circulation was correctly described by Olof Rudbeck, the first insights were contributed by the Hippocratus School [15]. The ancient anatomist (Hippocrates, Aristoteles) reported the presence of lymphatic vessels and lymph nodes without any accurate knowledge of their true functions [34].

The lymphatic system develops parallel with the blood vascular system, but unlike the cardiovascular system, the lymphatic system is not a closed system. In the body, a network of lymphatic vessels starts as blind-ended capillaries that drain excess fluid and protein from the interstitial fluid surrounding tissues and most organs. The interstitial fluid containing molecular waste is delivered from lymphatic vessels to lymph nodes for filtration and then to the venous circulation where it is carried to the liver and kidneys for further processing [3]. Moreover, the key function of lymphatics is to maintain fluid homeostasis, lipid absorption and perform the important role in the proper functioning of the immune system and the immune surveillance of the whole body [2, 28]. The lymphatic vessels are not normally present in avascular structures such as epidermis, hair, nails, cartilage and cornea [10].

The researchers always wondered how the brain effectively removes its waste, mainly because of its high metabolic rate. The questions about the existence of lymphatic vessels in CNS has been studied for many decades, but the specific markers for lymphatic endothelial cells (LECs) were discovered about a decade ago [24]. The brain was very long time considered as organ lacking lymph vessels. Even if, the first evidence of lymphatics in the brain meninges was published in 1787 by Paolo Mascagni an Italian physician, but his claim was discredited and forgotten [23]. New findings utilizing cellular, molecular and neuroimaging techniques show that functional lymphatic drainage does exist in the brain and its failure is a central feature of neurodegenerative disease and post traumatic brain injury [33]. The brain lymphatic drainage assists in maintaining the water and ion balance of the interstitial fluid (ISF), removing the metabolites, reabsorption of macromolecular solutes and modulate immune surveillance and responses of the brain [20]. Insufficient and reduced clearance from the brain could cause the accumulation of protein aggregates into insoluble clumps, which is a common feature in patients with amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson's disease and other neurodegenerative diseases [29]. Contemporary scientists indicate that some of the brain's waste products enter the cerebrospinal fluid, before being disposed of via the bloodstream [1]. The first who discovered how the CSF and brain tissue fluids are exchanged and named the lymphatic pathways, was N e d e r g a a r d et al. in 2012 [22]. CSF circulates around the brain and spinal cord, fills the brain ventricles and subarachnoid spaces and are mainly produced by choroid plexus; a collection of special endothelial cells that filter blood plasma to create the CSF. The composition of the CSF can be modulated by brain parenchymal cells. It is composed of about 99% water with several dissolved ions, and has a much lower protein concentration than plasma [23]. The content of protein in CSF increases with aging and it was proven that is higher in patients with aging-associated dementia. The CSF is produced daily, its volume is approximately 500 ml, but only 100—150 ml fills the fluid spaces of the

brain and spine at any time, ensuring constant circulation and reabsorption [29]. The total volume of CSF is renewed about 4 times a day in healthy humans. CSF flow within the ventricles is pulsatile, being influenced by cardiac and respiratory pulsation.

The traditional concept of CSF homeostasis has been challenged by the latest reports when the existence of a network of true lymphatic vessels in the brain meninges was after more than 200 years reconsidered [23]. The meningeal lymphatic vessels (MLV) found within the mouse dura mater were rediscovered in 2015 by two independent groups using novel, specific lymphatic endothelial markers [24]. They declared that the MLV which are located both dorsally and basally beneath the skull have a direct access to the CSF and, under healthy conditions continuously drain both soluble molecules and immune cells from the subarachnoid space into the cervical lymph nodes (CLNs) [4]. The dorsal MLV undergo extensive remodelling in mice with intracranial gliomas or metastatic melanomas. Disruption of the dorsal MLV alone impaired intratumour fluid drainage and the dissemination of brain tumour cells to deep cervical lymph nodes. In contrast, the last study point that the MLV situated at the basal part of the skull appeared to be morphologically distinct and have stronger draining functions than dorsal MLV.

Like conventional LECs, MLV express all of the classic markers of LECs like as *CD31*, *VEGFR3*, *Prox-1*, *PDPN*, *LYVE-1* and *CCL21* [13]. On the other hand, the meningeal lymphatics in comparison with peripheral lymphatic vessels have a less ramified network of thin-walled initial lymphatic vessels that converge and exit the cranium along particular anatomical structures, such as along the retroglenoid vein and sigmoid sinus and along the meningeal portions of the pterygopalatine artery [23]. Overall, these observations show that the lymphatic vessels of the meninges are important players in the draining lymphatic route for the clearance of molecules from the brain. [13]. The most recent study of lymphatic elements in the human brain brought new knowledge, that the fine lymphatic structure was presented in membranes covering the brain, walls of vessels, perivascular spaces and among nerve fibres. Lymphatics in and around the cranial and spinal nerves are important in transporting neuronal end products out of the brain [24].

New era brought novel and extended discoveries in the field of lymphatic system in the brain, when many detailed and intensive research was realized, using modern techniques. The presence of unique brain lymphatic drainage pathways have been confirmed by using the different types of tracer dyes of Indian ink, Evans blues, radioactive protein tracers and various fluorescent tracers with different molecular structure [33]. Finally, according to all available studies the cleaning system of the brain can be divided into two compartments. The first one, described as glymphatic system is draining the interstitial space of the brain tissue and between ISF and CSF. The second compartment is responsible for cleaning CSF towards to extracranial lymphatic structures [21]. These functions of the brain lymphatic drainage system are regulated by aging, genetic phenotypes, sleep-wake cycle, cardiac pulsations, respiratory and body posture and the effectiveness of such cerebral removal is also influenced by different pathological conditions or diseases [33].

CONCLUSIONS

The homeobox gene *Prox-1* is essential in developmental processes such as the progenitor cell regulation in various mammalian organs. Apart from its role during embryogenesis, *Prox-1* expression is associated with a number of human cancers. It performs tumour suppressive or an oncogenic role in a variety of cancer types. *Prox-1* is involved in the different stages of tumour development. Moreover, it participates in metastatic tumour growth by its impact on migration and intervention of cancer cells.

Regarding the brain tumours, it has been demonstrated, that *Prox-1* was found in the tumour cells in all diffuse astrocytic tumours, and its expression increases with the tumour grade. For that reason, *Prox-1* can be used as a diagnostic marker to distinguish low-grade from high-grade gliomas. Its oncogenic role in GBM promotes cell proliferation and invasiveness. Therefore, *Prox-1* may represent a potential target for the treatment of GBM. Also it has been an independent prognostic factor for survival in patients with World Health Organization grade II gliomas.

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Received October 4, 2021
Accepted November 5, 2021