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Endogenous Experimental Glioma Model, Links Between Glioma Stem Cells and Angiogenesis

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1. Introduction

Glioblastomas (GBM) are the most malignant solid tumours (grade IV) of CNS. They are glial lineage neoplasias with a high proliferative and invasive capacity, reaching to occupy an entire lobe of the brain (Kleihues et al., 2007). According with their genesis, they can be differentiated between primary and secondary glioblastoma. The primary is the most common glioblastoma. This is a new generated tumour after a brief medical history (three months), with no evidence of a less malignant lesion. On the other hand, secondary glioblastoma develops from diffuse astrocytoma, anaplastic astrocytoma or oligodendroglioma and malignant progression. Its development time is about five years. It is thought that both types of glioblastomas may be generated from neoplastic cells with characteristic of stem cells (Ohgaki & Kleihues, 2009). In addition, these cancer stem cells called "glioma stem cells" (GSCs) may be the responsible for glioma recurrences due to chemo-and radio resistance (Bao et al., 2006; Rich, 2007). Glioma stem cells (GSCs) are a subpopulation of neoplastic cells identified in glioma sharing properties with neural stem cells (self-renewal, high proliferation rate, undifferentiating, and neurospheres conformation) and the capacity for leading the tumourigenesis and tumour malignancy. The proliferation and the invasion into adjacent normal parenchyma have been attributed to glioma stem cells as well. Indeed, they were related to the angiogenesis process needed for the growth and survival of the neoplasia.

The microvascular network in gliomas has to get adapt to metabolic tissue requirements (Folkman, 2000). When the vascular network cannot satisfy cell requirements (Oxygen pressure of 5-10 mm Hg) tissue hypoxia occurs. This situation triggers the synthesis of pro-angiogenic factors as matrix metalloprotease (MMP-2), angiopoietin-1, phosphoglycerate kinase (PGK), erythropoietin (EPO), and vascular endothelial growth factor (VEGF)-A (Fong, 2008).

Vascular endothelial growth factor (VEGF) is a major regulator of tumour angiogenesis (Bulnes & Lafuente, 2007; Lafuente et al., 1999; Machein & Plate, 2004; Marti et al., 2000).

VEGF acts as mitogen, survival, antiapoptotic and vascular permeability factor (VPF) for the endothelial cells (Dvorak, 2006). The increase of this pro-angiogenic factor, secreted either by neoplastic cells or by cells of the tumour microenvironment, induces the start of angiogenesis, the called "angiogenic switch" (Bergers & Benjamin, 2003). This event results in the transition from avascularised hyperplasia to outgrowing vascularised tumour and eventually to malignant progression. It has been shown in human glioma biopsies that VEGF overexpression correlates directly to proliferation, vascularization and degree of malignancy, and therefore inversely to prognosis (Ke et al., 2000; Lafuente et al., 1999; Plate, 1999). The synthesis of VEGF is mediated by the Hypoxia-Inducible Factor (HIF-1), a critical step for the formation of new blood vessels and for the adaptation of microenvironment to the growth of gliomas (Jin et al., 2000; Marti et al., 2000; Semenza, 2003). Recent researches have reported that glioma stem cells play a pivotal role inducing the angiogenesis via HIF-1/VEGF (Bao et al., 2006). By the other hand, hypoxia has been related to clones selection of tumour cells. These clones adapted to the tumour microenvironment have acquired the phenotype of tumour stem cell with increased proliferative and infiltrative capacity (Heddleston et al., 2009; Li et al., 2009). Invasion of adjacent normal parenchyma has been attributed to glioma stem cells as well.

Due to these evidences, GSCs are currently being considered as a potential therapeutic target of the tumours. Recent studies have been focused on the identification of GSCs. In human glioblastomas they have been identified using CD133 marker (Ignatova et al., 2002). However, little is known about their genesis during glioma progression, especially during the early stages.

Some authors have previously reported the induction of glial tumour in rats by transplacentary administration of the carcinogen ethylnitrosourea (ENU) as a suitable method for studying the natural development of glioma (Bulnes-Sesma et al., 2006; Zook et al., 2000). In addition to this, it has been reported that ENU glioma model is a representative model for human glioma due to its location and also to its similar cellular, molecular and genetic alterations (Kokkinakis et al., 2004). Our experience with this model has proven to be useful to study many aspects of tumourigenesis and neoangiogenesis. In previous researches we reported the progression of tumour malignancy associated with vascular structural alterations and blood brain barrier (BBB) disturbances (Bulnes & Lafuente, 2007; Bulnes et al., 2009). ENU induced glioma permitted us to identify tumour development stages following microvascular changes. In addition, it was possible to study the angiogenesis process. Recently, we have used this model to study the relationship between glioma stem cells and angiogenesis process during the neoplasia development.

Many evidences corroborate the hypothesis that "glioma stem cells" have a close relationship with angiogenesis process, intratumour hypoxia and neoplastic microvascular network. In this chapter we centred to show this relationship from early to advanced stages of glioma using ENU-model.

2. Endogenous glioma model

Over the years, different methods have been employed to induce experimental tumours in the Central Nervous System of animals. Exposure to radiation, inoculation of carcinogenic virus, xenografts of tumour cell lines or tumour fragments in nude rats or mice, administration of chemical substances (Bulnes-Sesma et al., 2006) and genetically engineered mouse models have been used to replicate CNS tumours. The administration of chemical

substances as nitroso compounds is one of the most commonly-used methods to induce experimental CNS neoplasm. There is strong experimental data showing that nitrosamides (R1NNO-COR2), a type of N-nitroso compounds (NOC), are potent neuro-carcinogens when administered transplacentally. N-nitrosoureas MNU and ENU (a class of nitrosamides) have been demonstrated to be carcinogenic in animals, and particularly related to the development of CNS tumours. N-ethyl-N-nitrosourea (ENU) acts alkylating the O_6 in the guanine (G:C---T:A transition) and the O_2 in the thymine (T:A---A:T transversion). The accumulation of these successive DNA mutations seems to be responsible of the neurooncogenic effect of ENU (Bulnes-Sesma et al., 2006; O'Neill, 2000). Recently it has been reported that ENU exposure affects primitive neuroepithelial cells of the subventricular plate (SVZ) and germinative zone (VZ). ENU prenatal exposure affects the differentiation of these cells generating glial lineage tumours (Burger, 1988; Vaquero et al., 1994; Yoshimura et al., 1998) and its exposure in adult affects the neurogenesis of the SVZ (Capilla-Gonzalez et al., 2010). In previous studies we found that gliomas induced in offspring were similar to the human gliomas (Kokkinakis et al., 2004). Therefore, ENU brain induced tumours have allowed the study of several aspects of glioma behaviour, for example, microvascular organization (Schlageter et al., 1999; Yoshimura et al., 1998); neoplastic cell dedifferentiation (Jang et al., 2004); gene mutations (Bielas & Heddle, 2000; O'Neil, 2000); microcirculation and angiogenesis process (Bulnes & Lafuente, 2007; Bulnes et al., 2009) or experimental therapeutic agents (Kish et al., 2001).

In our model, the glioma induction was performed by prenatal exposure of Sprague Dawley rats to ENU. Briefly, pregnancy rats, on the 15th day of gestation, were given a single i.p. injection of 80 mg of ENU/kg body weight (Bulnes et al., 2009; Bulnes et al., 2010). Offspring rats exposed to ENU were reared in standard laboratory conditions and the study was performed from 5 months to one year of age. The identification of ENU-Gliomas was performed by T2-w and postcontrast T1-weighted NMR images and by histopathology diagnosis from H&E staining and immunophenotypic study as previously described (Bulnes & Lafuente 2007) (Figure 1, 2). Following our results, ENU-glioma starts from the fifth month of offspring rat age and becomes GBM at 10 months of age (Bulnes-Sesma et al., 2006). ENU-glioma starts as cellular proliferation growing near ventricles in association with subcortical white matter. Over 6 months of extrauterine life, this tumour proliferation become nodular and rats display neurological signs (Figure 1). Around one year they grow as a GBM toward the contralateral hemisphere (Figure 2). Following our findings, we have identified three stages of ENU-glioma development: initial, intermediate and advanced. The advanced stage corresponds to anaplastic oligodendroglioma or glioblastoma (GBM) similar to the human. ENU-GBM may reach to infiltrate whole cerebral hemisphere, showing malignant histopathological features such as: high tissue heterogeneity, aberrant angioarchitecture, macro-haemorrhages, macrocysts or palisade necrosis (Klehiues et al., 2007). Thanks to this model we could isolate early glioma stages, which is impossible to carry out in human brain.

3. Stem cells and cancer stem cells

Stem cells are functionally defined as self-renewing and multipotent cells that exhibit multilineage differentiation (Till & McCulloch, 2011). Nowadays they have been proposed to be an important tool in regenerative therapy being used to regenerate tissue in many diseases like heart stroke, neurodegenerative diseases, etc (Nadig, 2009). However, in oncology and especially in cerebral gliomas, the presence of the stem cells has been related

to a poor prognosis. Recent investigations in glioblastomas have reported that these cancer stem cells called glioma stem cells (GSCs) have tumourigenic capacities like tumour malignant process, peripheral tissue infiltration and angiogenesis induction (Hadjipanayis & Van Meir, 2009; Rich, 2007).
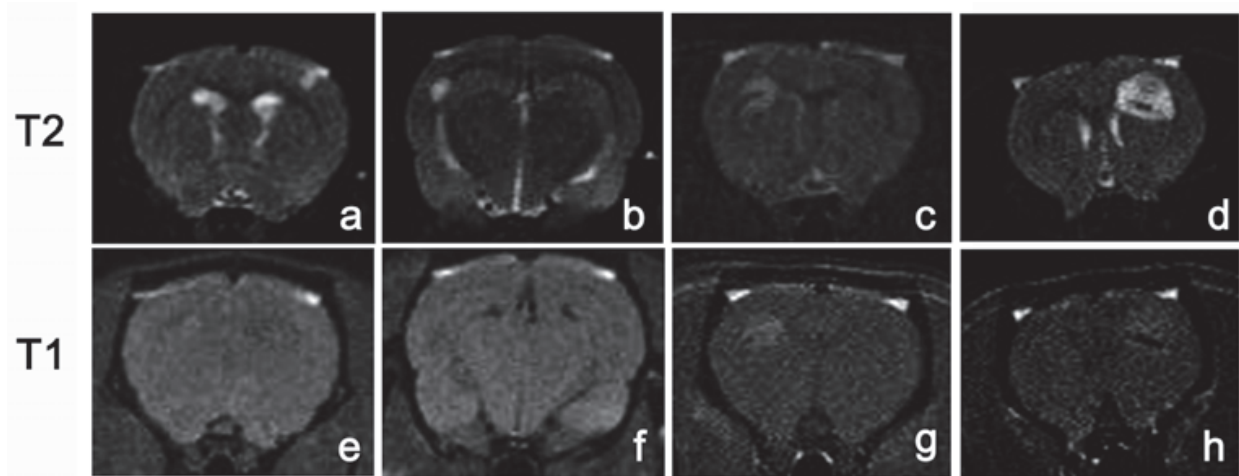

Fig. 1. Coronal sections of rat brains displaying ENU-glioma showed by MRI on T2-w and T1-w after injection of gadolinium. a, b) Small neoplastic mass growing on the cerebral cortex with an homogeneous hyperintense signal on T2-w images. These neoplastic masses correspond to initial stage of ENU-glioma. e, f) Both masses display an isointense signal on T1-w. c, d) ENU-glioma tumour with nodular shape showed on T2-w hyperintense signal that represents intermediate stage. g, h) At this stage there is a gadolinium contrast enhancement observed as homogeneous soft hyperintense signal on T1-w image.
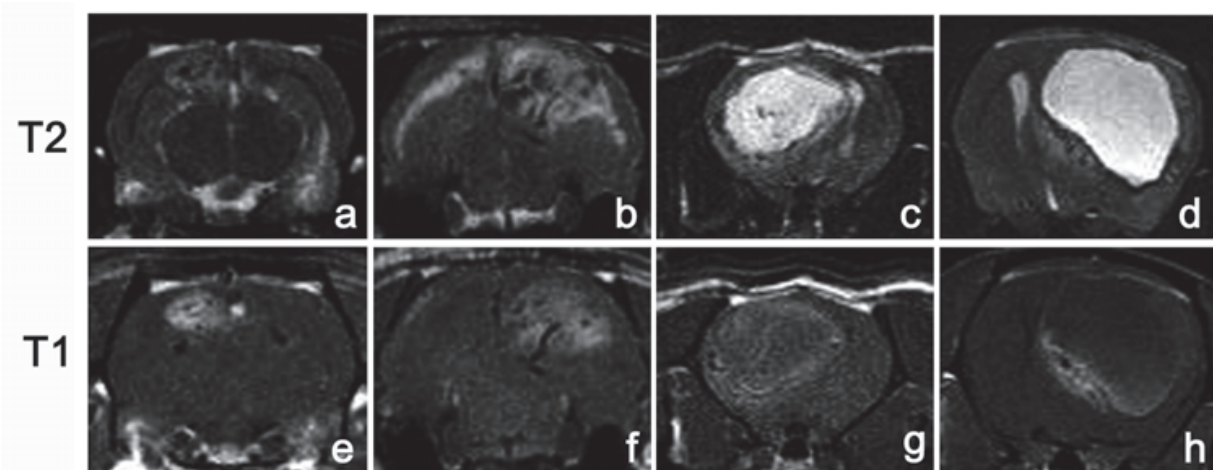

Fig. 2. Coronal sections of rat brains with ENU-glioma of advanced stage showed by MRI on T2-w and T1-w after injection of gadolinium. All of these anaplastic gliomas display heterogeneous hyperintense signal on T2 (a-d) and on T1-w (e-h). This heterogeneity is due to the presence of histopathology features of malignity. c-d) ENU-GBMs high-proliferative covering a whole cerebral hemisphere. The T2-w images reveal an intratumour hyperintense signal corresponding with intratumour oedema or macrocysts. g-h). Gadolinium enhancement of this T1-w image adopts a rim shape bordering the neoplastic mass. This rim represents the microvascular proliferation with dysfunction of Blood Brain Barrier.

In the middle of the 60s, Altman and Das reported the first evidences about stem cells in adult brain. They observed stem cells in the hippocampus and olfactory bulb of rats, and it supposed the first sign of division of stem cells. Later on they were called Neural Stem Cells (NSCs). NSCs were considered the unique population of Central Nervous System cells characterized by self-renewal and multilineage differentiation properties (Muller et al., 2006). They can form neurospheres (Reynolds & Weiss, 1992) and differentiate in vitro into the three neuroectodermal lineages astrocytes, oligodendrocytes and neurons (Alvarez-Buylla & Garcia-Verdugo, 2002). Furthermore, when they are transplanted in vivo in the cerebellum, they can generate neurons and glial cells (Lee et al., 2005). Also, after transplantation into nude mice they can differentiate into neuroblasts (Tamaki et al., 2002). NSCs reside in the germinal layers of the developing brain, initially in the early neuroepithelium, later in the ventricular (VZ) and subventricular zone (SVZ) during embryogenesis (Götz & Huttner, 2005). In adult brain, three areas are supposed to harbour neural stem cells: dentate gyrus of hippocampus, SVZ (Doetsch et al., 1999; Eriksson et al., 1998) and the fibbers connecting olfactory bulb to lateral ventricle (Lois & Alvarez-Buylla, 1994; Whitman & Greer, 2009). In recent times, they were also isolated in the subcortical white matter (Nunes et al., 2003).

In the 1960s, evidence emerged supporting the presence of stem cells in tumours. Bergsagel and Valeriote (1968) showed that only certain cells within a tumour had the capacity to generate a new tumour; they termed these cells "tumour stem cells". After this, tumour stem cells were identified in breast tumour (Al-Hajj et al., 2003), pancreatic tumour (Esposito et al., 2002) etc.

The first concept of cancer stem cell, later on also called tumour initiating cells, appeared in the beginning of the 90s. Bonnet and Dick (1977) describe how some cells, isolated from leukaemia patient´s blood, had proliferation and differentiation capacities in vivo. Fan et al. (2007) described cancer stem cells as the cellular subpopulation capable of tumour regeneration within a permissive environment. Rich and collaborators reported that cancer stem cells have tumourigenic, infiltration and angiogenesis properties as well as radio/chemo-resistance (Rich, 2007; Hadjipanayis & Van Meir, 2009).

The relation between stem cells and cancer stem cells was studied. The results explained that both cellular types share the previously mentioned characteristics, as well as many cell signalling pathways as oncogene bcl-2, Sonic hedgehog (Shh) and Wnt signalling cascade (Reya et al., 2001). Both types of stem cells also share common markers like CD133, Nestin (Dahlstrand et al., 1992) and transcription factor Sox2 (Gangemi et al., 2009). However, there are differences between stem cells and cancer stem cells, such as expression of different markers, chromosomal alterations and tumourigenic capacity. Holland et al. (2000) published that cancer stem cells could develop from modified neural stem cells. They have been described many pathways that can lead to cancer stem cell formation like Notch (Takebe & Ivy 2010), Akt (Germano et al., 2010) activation or p53 pathway alteration.

3.1 Glioma stem cells (GSCs)

Dahlstrand et al. (1992) identified a cancer stem cells subtype inside glial lineage brain tumours which were called Glioma Stem Cells (GSCs). These GSCs may be responsible for maintenance of the entire tumour and also they have the potential, when injected in immunodeficient mice, to generate gliomas similar to the original tumours (Heddleston et al., 2009).

GSCs indeed of share properties of somatic or embryonic stem cells (high proliferation rate, undifferentiating, formation of neurospheres) are chemo-and radio resistant (Bao et al., 2006, Rich, 2007). Their radiotherapy resistance may be thanks to a more efficient DNA reparation mechanism and protein kinases phosphorilation Chk1 and Chk2 (Bao et al., 2006). The resistance to chemotherapeutic drugs is through membrane transporters that bomb the drugs outside the cell (Donnenberg & Donnenberg, 2005).

The first GSCs identification was found in the tumour advanced stage corresponding with human-GBM (Ignatova et al., 2000). However, the first moment of GSCs expression remains unknown, as well as their role in early stages of tumour development. It is very important to identify and explain GSCs apparition in early glioma stages to research about future tumour therapy.

The discovery of GSCs in gliomas involved the creation of a new glioma-genesis hypothesis called "hierarchical hypothesis". Before GSCs discovery, glioma development was explained by the "stochastic theory". Stochastic theory is based on all neoplastic cells are clones from a single undifferentiated cell and they have the same genetic alterations (Hadjipanayis & Van Meir, 2009). Nowadays the "hierarchical theory" explains that only a few neoplastic cells can adapt to the tumour environment and are able to start the tumourigenic process. Even though the low proliferation of GSCs, they guide the tumour growth giving raise to more mature cells with limited proliferation capacity (Shen et al., 2008).

After the glioma stem cells finding, the research about glioma development has been centred in the identification of them. So far markers as CD133/Promonin-1, presents in glioma stem cells (Dell'Albani, 2008), Nestin, a protein found in neural stem cells in SVZ and other markers of neuroepithelial stem cells (Jang et al., 2004) including Musashi-1, Sox-2, GFAP, Map-2, Neural-tubulin, Neurofilament O4 and Noggin were used in order to identify tumour stem cells. But the lack of a specific marker makes it very difficult to identify (Hadjipanayis & Van Meir, 2009; Li et al., 2009).

Nestin is an intermediate filament protein typical for neural precursor cells. It has been extensively used as a marker for neural stem cells. It is expressed in primitive neuroepithelial cells of all regions of CNS during the development. In adult its expression is restricted to the ventricular wall (SVZ) and the central canal. In pathological conditions like brain trauma, CNS ischemia, neurotoxicity, neoplastic transformation and in response to cellular stress, the nestin over-expression was showed (Holmin et al., 1997, Jang et al., 2004). In primary malignant tumours of CNS high amounts of cells positive for Nestin have been reported. Nestin has been described as a marker of GSCs in astroglial tumours (Singh et al., 2003), indicating undifferentiating and malignance degree (Schiffer et al., 2010), but it is not specific for glioma stem cells (Hadjipanayis & Van Meir, 2009). Indeed, Nestin expression has been described to appear since the first stages in glioma models (Jang et al., 2004).

CD133 (prominin-1) was the first identified member of the prominin family of pentaspan membrane proteins which acts as a marker of hematopoietic progenitor cells. It is a cell surface marker used for the identification and isolation of stem/progenitor cells in several tissues, for instance, endothelium, brain, bone narrow, liver, prostate, pancreas and foreskin (Mizrak et al., 2008). CD133 was originally described as an hematopoietic stem cell marker and was subsequently related to number of progenitor cells including neuroepithelium (Corbeil et al., 2000) as well as cancer stem cells in various tumours such as prostate and

colon cancer (Cheng et al., 2009; Collins et al., 2005; O'Brien et al., 2007). In human glioblastoma, CD133 expression has been associated to GSCs and bad prognosis of the tumour (Germano et al., 2010).

4. Tumour angiogenesis

Gliomas proliferate in the brain, a privileged organ from the point of view of blood supply. The exchange of metabolites between blood and cerebral tissue occurs essentially in the brain capillaries. The diameter of brain capillaries in the adult human is between 5 and 7 microns. These microvessels feed to the cells that are 10-20 microns away. Although the distance between cells and microvessels is lesser than 20 μm, the growth and survival of the gliomas depend on vascular remodelling and angiogenesis (Folkman, 2006). Along the early stages of small gliomas the metabolic demand is supplied by the vast microvascular network but when the metabolic supply has been exceeded, new formation of vessels becomes necessary (Carmeliet & Jain, 2000; Yancopoulos et al., 2000). The genesis of the new vessels from pre-existing ones is called angiogenesis in opposite to vasculogenesis refereed to the formation of vessels from hemopoietic niches (Carmeliet, 2003; Risau & Falmme 1995; Risau, 1997).

Angiogenesis is a complex process that requires proteolytic and mitogenic activity of endothelial cells and interaction of these with the extracellular matrix molecules and cells of peri-endothelial support cells (pericytes and smooth muscle cells). Many molecules and pathways are involved in this process, such as VEGF, its receptors VEGFR-1 and VEGFR-2, the endothelial receptor tyrosine kinase tie-1 and tie-2 and the angiopoietin ligands 1 and 2. Many other molecules as PDGF and TGF-β, integrin receptors, are very important (Millauer et al., 1993; Neufeld et al., 1999).

Angiogenesis requires some angiogenic stimulus, such as hypoxia, new metabolic requirements or tumour growth to start. Intratumour hypoxia occurs at the time when there is an imbalance between supply and demand oxygen due to the irregular and chaotic blood flow (Jensen, 2006). The relative tissue hypoxia triggers the production of hypoxia inducible factor-1α, upregulating the expression of VEGF. In addition to this, it was reported that hypoxia plays a fundamental role in the induction of cell phenotype neoplastic to the undifferentiated state of GSCs. According to recent research, hypoxia selects tumour cell clones that have adapted to the tumour microenvironment and have acquired the phenotype tumour stem cell, with its capabilities of proliferation and infiltration (Heddleston et al., 2009; Li et al., 2009).

Heddleston et al. (2009) observed how in cultures of human glioma neoplastic cells exposed to hypoxia reverted to a state of tumour stem cells. Griguer et al. (2008) related the appearance of CD133 + cells with oxygen stress in gliomas. On the other hand, it was observed a decrease in the expression of CD133 when reverted to conditions of normoxia. Furthermore, studies of human GBM have described the relationship between the gradient of intratumour oxygen and the appearance of the phenotype tumour stem cell (Pistollato et al., 2010). As above, only a cluster of neoplastic cells resists to the conditions of hypoxia and intratumoural ischemia. This group of cells may be stem cell precursors, and after adapting to the new microenvironment, are transformed to GSCs.

4.1 Vascular endothelial growth factor (VEGF)

Vascular endothelial growth factor (VEGF) is a major regulator of angiogenesis in development (Bengoetxea et al., 2008; Ferrara et al., 2003; Ment et al., 1997) and pathological

disease (Bulnes & Lafuente, 2007; Lafuente et al., 1999; Marti et al., 2000; Plate, 1992). However, the role of VEGF in nervous tissue is even more extensive. Previous studies showed that VEGF also has strong neuroprotective, neurotrophic and neurogenic properties (Jin et al., 2002; Ortuzar et al., 2011; Rosenstein & Krum, 2004; Storkebaum et al., 2004).

Although the synthesis of this proangiogenic cytokine is associated to tumour cells and endothelial cells, it has been described in others, such as: neurons, astrocytes, pericytes, smooth muscle cells, macrophages, lymphoid cells, platelets and fibroblasts (Zagzag et al., 2000). The VEGF family consists of five different homologous factors, VEGF-A, VEGF-B, VEGF-C, VEGF-D and placental growth factor (PlGF) (Ferrara et al., 2003). VEGF-A (VEGF) is the predominant form and is a hypoxia-inducible 45 KDa homodimeric glycoprotein.

VEGF-A acts as mitogen, survival and antiapoptotic factor for the endothelial cells from arteries, veins and lymphatics. Faced with increased secretion of VEGF and its binding to receptors on the surface of endothelial cells, VEGF is a signal transduction leading to production of molecules including enzymes for the degradation of extracellular matrix and increase of vascular permeability. This will facilitate cell proliferation, survival and migration of endothelial cells. It is also known as the vascular permeability factor (VPF) (Dvorak, 2006) on the basis of its ability to induce leakage through the blood brain barrier in some pathological situations (Ferrara, 2001; Lafuente et al., 1999; Lafuente et al., 2002). Helmlinger et al. (2000) stated that in the vasodilatation process the VEGF induced the elongation of endothelial cells but not their proliferation. In the angiogenesis process, VEGF works in line with other factors such as angiopoietin and ephrins (Tonini et al., 2003). It has been shown in human biopsies that VEGF overexpression in gliomas correlates directly to proliferation, vascularization and degree of malignancy, and therefore inversely to prognosis (Ke et al., 2000; Lafuente et al., 1999; Plate, 1999).

5. ENU glioma microvascular adaptation

Along the glioma progression, there is a transition from the homogeneous capillary network to an anarchic angioarchitecture. Microvessels have to adapt in order to maintain blood perfusion and metabolic support in adverse conditions, constituting a peculiar tissular microenvironment in response to hypoxia (Blouw et al., 2003). Glioma microvascular remodelling consists in a process of vascular aberration along the neoplasia development. Vascular development process led to microvascular proliferations that are a histopathological hallmark of glioblastoma (Kleihues et al., 2007). Some authors consider the core of a high-grade glioma as an avascular zone, since it has scarce capillaries with wide lumen and a fragmented basal membrane, being rather inefficient for metabolic exchange (Vajkoczy & Menger, 2004).

Tumour blood vessels have multiple abnormalities that result in a heterogeneous environment. They are disorganized, tortuous, sinusoidal, branchy and leaky, the diameter is irregular and the walls are thinner than those found in healthy brain tissue (Bigner et al., 1998). Following our results obtained by LEA and Butyrylcholinesterase (BChE) histochemistry (Bulnes et al., 2009) we showed a transition from the homogeneous capillary network of early stages to an anarchic angioarchitecture of advanced ENU-glioma stages (Figure 3). It was found that the vessel density decreased and the vascular size increased in order to glioma malignity (Bulnes et al., 2009). The initial stage of ENU-glioma was constituted by microvessels similar to the brain capillaries, the intermediate stage by tortuous, disorganized and dilated vessels and the advanced stage by anarchic and aberrant

vessels such as: multilayered "glomeruloid tuft"; "garland" of proliferated vessels and huge dilated vessels (Klehues et al., 2007).

One result to take in consideration was the gradient from the well-oxygenated tumour periphery to the central hypoxic core of ENU-glioblastoma. Dilated intratumour vessels, expressing VEGF (Lafuente et al., 1999) increase their lumen on account of endothelial elongation but not of cell proliferation (Helmlinger et al., 2000). The intratumour area displays irregularly branching vessels, variable intravascular spaces and large avascular areas. It is also worth mentioning that perivascular cells of aberrant vessels of ENU-GBM often displayed a high activity for BChE, depicted by a strong brown staining (Bulnes et al., 2009). BChE activity is strongly related to neurogenesis and cellular proliferation (Mack & Robitzki, 2000), having a great role in tumourigenesis. These findings have led us to postulate that these perivascular cells might be stem cells proliferating around intratumour vessels (Anderson et al., 2005; Brat et al., 2004) and migrating through the vascular extracellular matrix (Ruoslahti, 2002). This could corroborate the hypothesis that stem cells adapted to hypoxic stress use the vascular extracellular matrix for migration and invasion. In addition to this, in previous work we have shown that these cells co-expressed Ki-67 and VEGF (Bulnes & Lafuente, 2007).
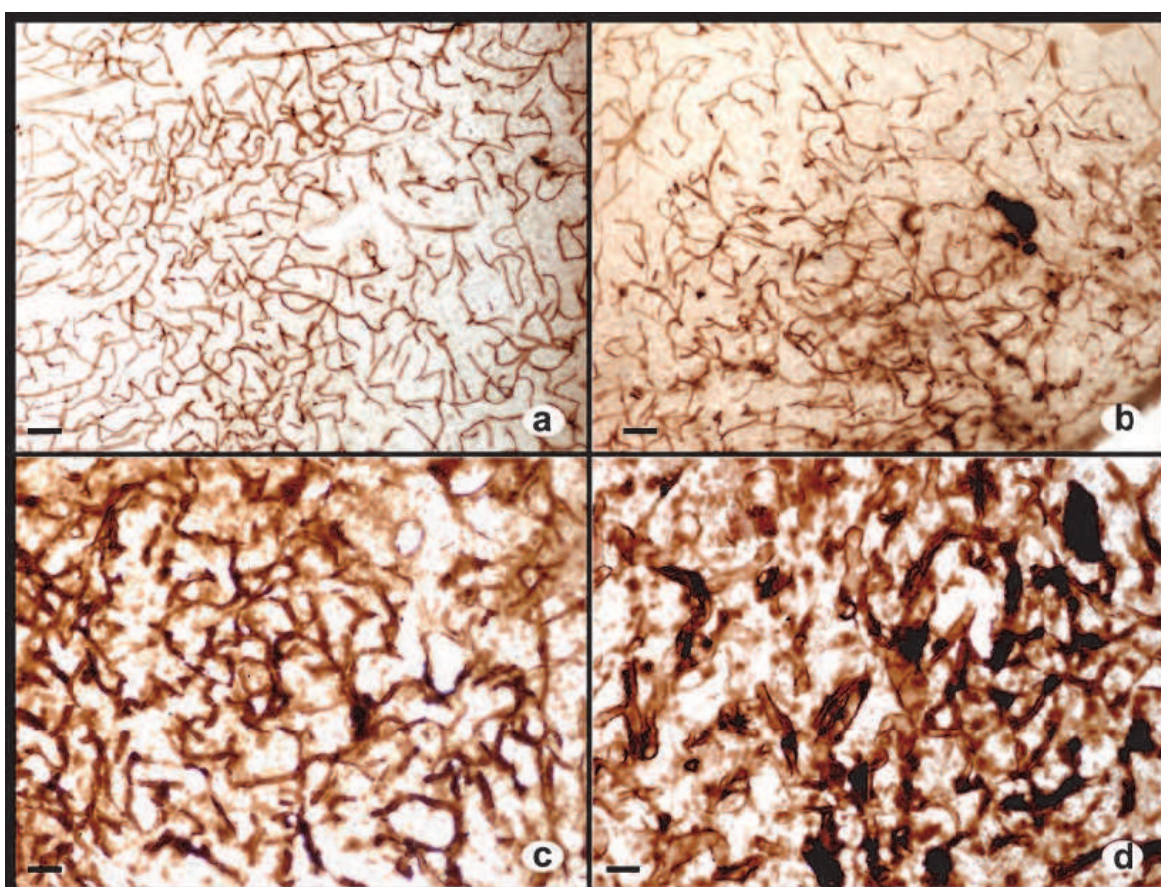

Fig. 3. Angioarchitecture study of gliomas shown by butyrylcholinesterase histochemistry. a) Angioarchitecture of the cerebral cortex of the rat brain. b) Periventricular small neoplastic mass (initial stage) showing some strongly-positive vessels for BChE. c) Intermediate ENU-glioma stage displaying a network of numerous tortuous capillaries of anarchic distribution. d) Malignant infiltrating macrotumour, with dilated vessels of the intratumour area with strongly BChE positive cells. (Scale bar of 50μm).

Glioma malignancy process is mediated by the vascular remodelling and the angiogenesis process where the blood brain barrier (BBB) function is implicated. The BBB is the set of mechanisms (physical and metabolic) that regulate the passage of elements from the blood plasma to neural tissue. This especial barrier is necessary for the cerebral homeostasis and it is associated with the hydrostatic and osmotic pressure gradients across the capillary (Hatashita & Hoff, 1986).

In pathological conditions, the increase of vascular permeability could be due to the blood brain barrier dysfunction, to a structural break-down or to its immaturity. Endothelial cells (ECs) of tumour vessels do not form a closed barrier, and pericytes are loosely attached (Baluk, et al., 2005). Defective tight junctions explain the tumour vessel leakiness which leads to blood brain barrier (BBB) breakdown and the oedema associated with brain tumours (Hashizume et al., 2000; Papadopoulos et al., 2004). Brain oedema in gliomas is an epiphenomenon related to BBB breakdown and is another cause of tumour mortality (Ballabh et al., 2004). The BBB distortion and permeability increase have been related to intravital dyes extravasation (Lafuente et al., 1994, 2004), Gd-DTPA contrast enhancement on T1-w images (Brasch & Turetschek, 2000; Cha et al., 2003; Claes et al., 2007) and to changes in the expression of BBB markers as glucose transporter-1 (GluT-1) (Dobrogowska & Vorbrodt, 1999) and structural rat specific antigen of BBB (EBA) (Argandona et al., 2005; Lafuente et al., 2006; Lin & Ginsberg, 2000; Krum et al., 2002; Sternberger et al., 1989; Zhu et al., 2001).

In our ENU model, vascular adaptations predominate over angiogenesis (Lafuente et al., 2000; Bian et al., 2006). Microvascular adaptations in early development stages are based on vasodilatation, endothelium elongation and permeability increase mediated by VEGF-A without BBB dysfunction. On the other hand, in malignant gliomas the microvascular adaptations vary according to blood flow perfusion. Permeability increase in intratumour vessels is not enough to supply the metabolic demand, and triggering of the angiogenesis process on the tumour border is necessary. When the blood flow inside and around the tumour becomes irregular and chaotic, partly due to the aberrant microvessels, the relative tissue hypoxia triggers the production of hypoxia inducible factor-1α (Chen et al., 2009; Jain et al., 2007), upregulating the expression of VEGF-A and endothelial nitric oxide synthase (eNOS). VEGF-A induces the synthesis of NO by phosphorylation of endothelial NO synthase via PI-3K/Akt kinase (Osuka et al., 2004, Ziche & Morbidelli, 2009), thus promoting BBB breakdown and increasing permeability. Although, the role of eNOS and VEGF-A in tumour induced brain oedema is still a matter of debate. Our previous studies demonstrates that eNOS overexpression in the microvasculature of intermediate and advanced ENU-gliomas correlates with the loss of immunostaining for primary BBB markers GluT-1 and EBA (Bulnes et al., 2010) (Figure 4).

Following the finding showed in human tissues, in ENU-malignant glioma astrocytic processes and pericytes were loosely attached to endothelial cells of tumour vessels without forming a continuous layer (Baluk et al., 2005) (result not published). In addition to this, defective tight junctions (TJs) without occludin protein expression, also lead to oedema associated with ENU induced brain tumours. We showed an intratumoural glioma oedema instead of peritumoural one by gadolinium contrast enhancement and intravital dyes extravasation (Bulnes et al., 2009, 2010).

6. Glioma stem cells and angiogenesis in ENU model

The moment named "angiogenenic switch", when the angiogenesis starts, is showed at ENU-glioma intermediate stage due to the presence of overexpression of VEGF and eNOS

(Bulnes et al., 2010). Because stem cells have been associated with the synthesis of VEGF (Bao et al., 2006), we focused on the identification of GSC using antibodies against the antigens CD133 and Nestin. We showed three distribution patterns of these cells (Figure 5): 1- isolated in the tumour periphery areas; 2- numerous small cells forming intratumour niches and 3- cells around the tortuous and aberrant vessel (intermediate-advanced stages).
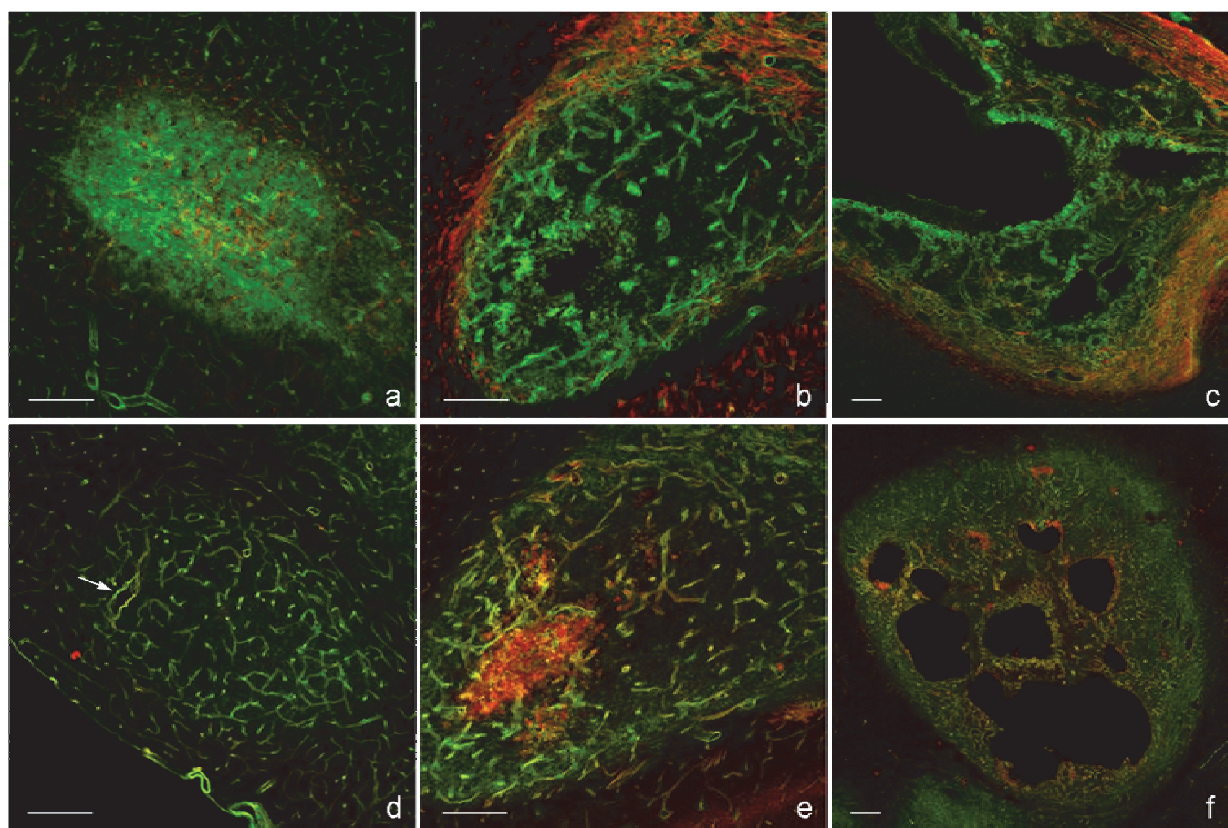

Fig. 4. Vascular endothelial growth factor and endothelial nitric oxide synthase expression during ENU-glioma development. Confocal microphotographs showing VEGF$_{165}$ (a-c, red) and eNOS (d-f, red) in different stages of glioma. Vascular network is showed by immunofluorescence for tomato lectin LEA (green). (a, d) Initial stages of gliomas display basal stain of VEGF$_{165}$ (a) and overexpression of eNOS only in dilated vessels (d, white arrow). (b, e) Anaplastic ENU-glioma corresponding with the intermediate tumour stage shows overexpression of VEGF$_{165}$ in the neoangiogenic tumour border (b) and overexpression of eNOS (e, yellow) in dilated and tortuous vessels from intratumour area. (c, f) ENU-induced glioblastomas show an heterogeneous pattern of expression for both markers. VEGF distribution is mainly showed in the peritumour neoangiogenic area (c) while eNOS overexpress as patching in vascular sections of intratumour aberrant microvessels (f). (Bar scale of 200 μm).

According to human astrocitomas, in ENU-glioma the number of positive cells for CD133 and Nestin antibodies increases with malignant grades of the tumour (Ma et al., 2008). Nestin+ cells were found in every stage of tumour development. It corroborated that the expression of Nestin is linked to the glioma grade, as stated in previous researches (Ehrmann et al., 2005).
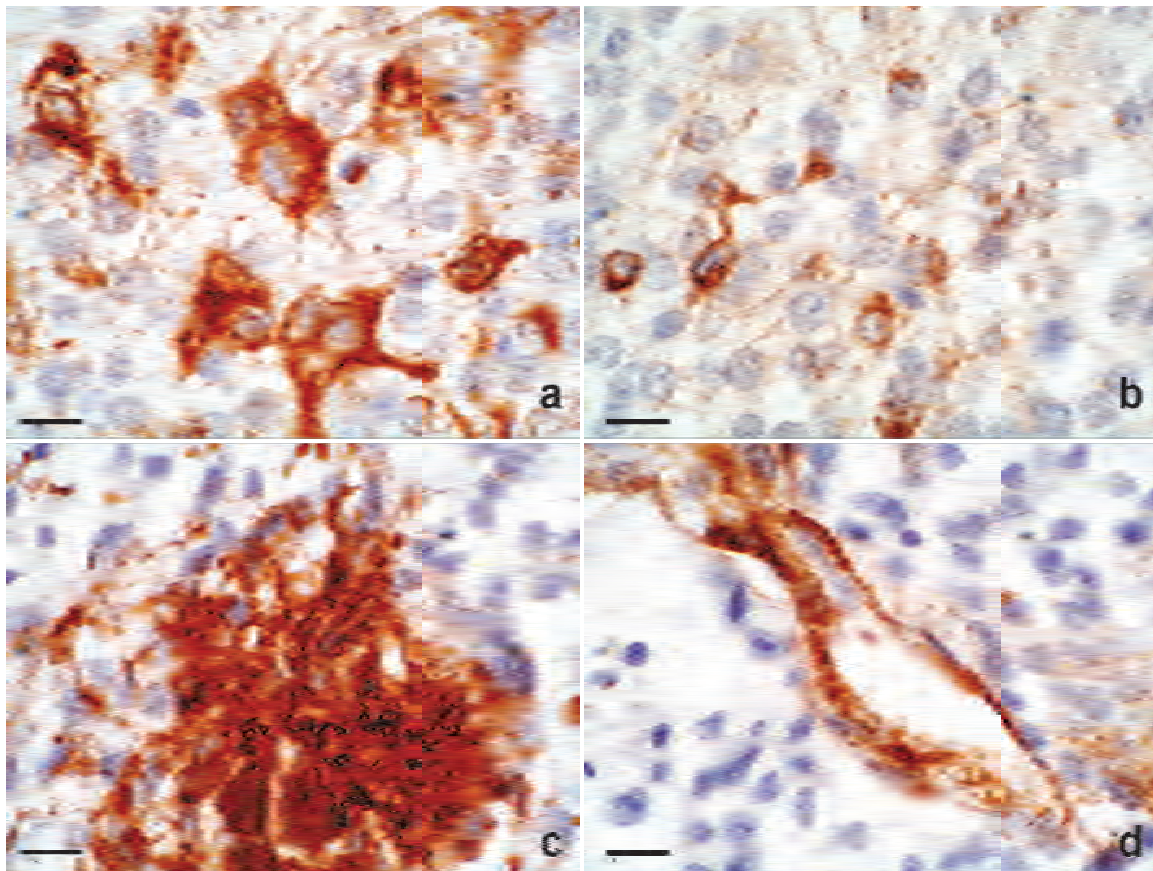
Fig. 5. Immunoexpression of Nestin antigen in 4 µm paraffin sections showed by DAB staining (Brown). a-b) Intratumour area of ENU-Glioma showing two kinds of isolated cells marked by Nestin antibody. a) Cells of big cytoplasm and nucleon distributed predominantly near the periphery of the tumour. They display an astrocyte shape and GFAP positivity. b) Small cells with scarce cytoplasm and prolongations. c-d) Two distribution of stem cells: Intratumour niches (c) and around the vascular endothelium of neoplastic microvessels (d). (Bar scale of 10µm).

By the other hand, CD133+ cells were only present since intermediate stages corresponding with "angiogenenic switch". The distribution of CD133+ cells corresponds mainly to overexpression of VEGF in neoangiogenic border and intratumour hypoxic areas of neoplasia (Bulnes & Lafuente, 2007). It has been reported that tumour stem cells over-express VEGF factor, so this cell population could be involved in the process of angiogenesis. Our results agree with the staining of CD133 described in the advanced and medium stage of human gliomas. Therefore, CD133 expression has been related to poor prognosis (Zeppernick et al., 2008).

We showed that some cells coexpress the antibodies Nestin, CD133 and $VEGF_{165}$. They were forming niches around microvessels or into hypoxic areas (Figure 6). Only cells distributed in the periphery of neoplasia were stained for GFAP and displayed astrocyte morphology.

The relationship between CD133+ cells and vessels wall was shown around the glomeruloid vessels, distributed in the neoangiogenic border of ENU-GBM, and delimiting huge dilated intratumour vessels (Figure 7). The presence of CD133+ cells near these aberrant vessels which display BBB disturbance may corroborate the pivotal role of stem cells in the

neoplasia proliferation and invasion. These cells may be use extracellular matrix of vessel wall to migrate and infiltrate the brain parenchyma (Borovski et al., 2009).
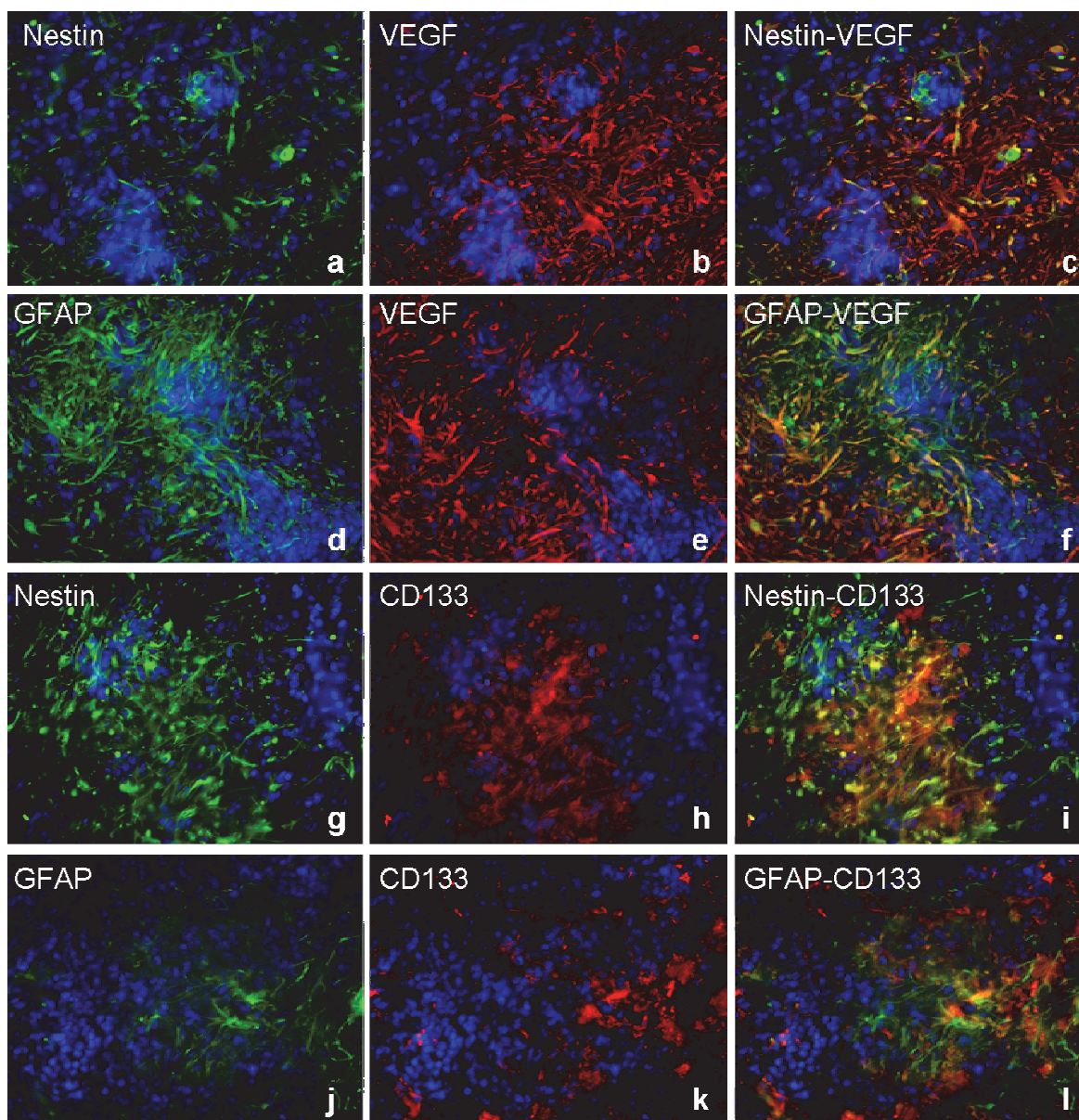

Fig. 6. Relationship between stem cell markers and proangiogenic factor VEGF in intratumour niches of advanced ENU-glioma stage. Study performed by double immunofluorescence, all tumours are counterstained with Hoechst. a-c) Microphotographs of Nestin+ cells (a, in green) and VEGF+ cells (b, in red) and colocalization (yellow, c). VEGF+ cells predominate over Nestin+ cells. Some cells with big cytoplasm are Nestin-VEGF+. Small Nestin+ cells form a cluster and lack the staining of VEGF (at the top). d-f) Colocalization (yellow) of glial fibrillary acidic protein (GFAP, green) and VEGF (red). All VEGF+ cells in this intratumour area are stained for GFAP and display the astrocyte shape. g-i) Relationship between the two markers of stem cells: Nestin (green) and CD133 (reed). This niche shows higher density of nestin+ cells (g) than CD133+ cells (h). Almost all of the CD133+ cells coexpress nestin antibody (i, yellow). j-l) Coexpression of GFAP (green) and CD133 (red). Some cells coexpress both antibodies (l, yellow). (x400 Amplification)
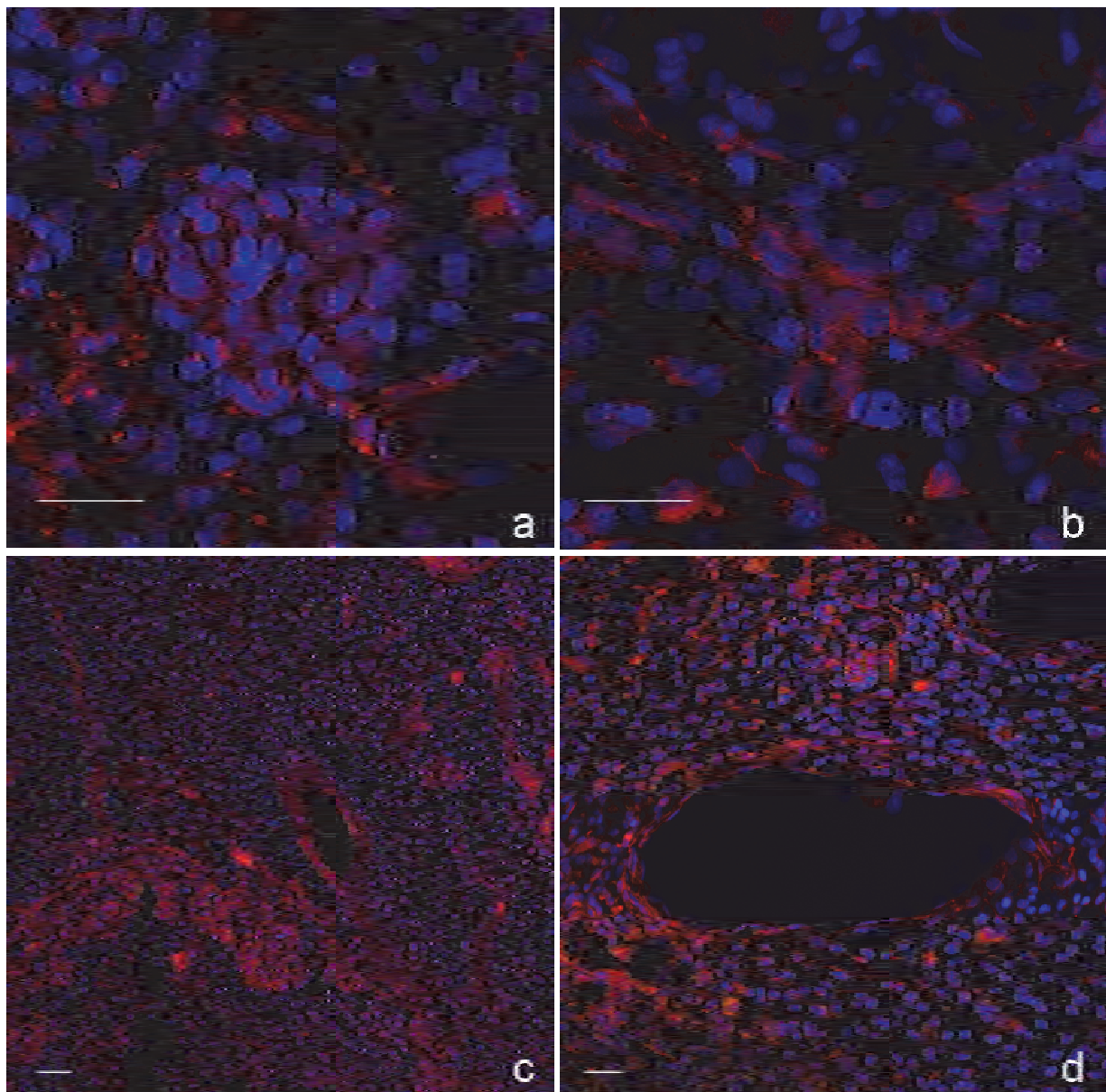
Fig. 7. Immunofluorescence confocal images of CD133 antibody (red) in ENU-glioblastoma. All sections are counterstained with Hoechst (blue). a) Intratumour niche displaying some CD133+ cells. b) Tortuous vessel of the periphery of the neoplasia with CD133+ structures attached to the vascular endothelium. c) Aberrant vessels sections demarcated by CD133+cells. d) Vessels with huge lumen display CD133+ cells around some vascular sections. (Scale bar of 20μm).

Although some authors proposed that CD133+ cells were selected cells with tumorigenic capacity (Schiffer et al., 2010), others postulated that a fraction of CD133+ cells might be related to the endothelial differentiation and could generate tumour vessels (Wang et al., 2010). Recently, Soda et al. (2011) reported that part of the vasculature of GBM was originated from tumour cells. Therefore, some researchers as Wang et al. (2010) and Ricci-Vitiani et al. (2010) were centred to describe the proportion of the stem cells that contributed to blood vessels in glioblastoma. After their results they postulated that glioblastoma microvessels were originated from tumour stem like cells.

7. Conclusion

Following evidences reported in the literature and our findings, the distribution of "glioma stem cells" close to microvascular wall during the glioma malignancy process suggests a synergistic role of both structures. Indeed, based on our results we corroborate the hypothesis that glioma stem cells may induce angiogenesis via VEGF synthesis or endothelial differentiation.

This knowledge will contribute to the generation of new antitumour therapy treatment against glioma stem cells. ENU experimental model would be considered as an useful option to check a design of treatment strategies against these cells.

8. Acknowledgment

9. References

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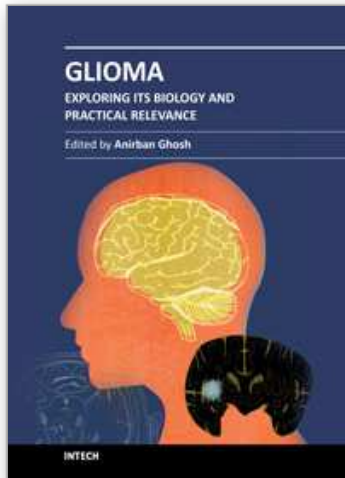
Glioma - Exploring Its Biology and Practical Relevance
Edited by Dr. Anirban Ghosh

The tittle 'Glioma - Exploring Its Biology and Practical Relevance' is indicative of its content. This volume contains 21 chapters basically intended to explore glioma biology and discussing the experimental model systems for the purpose. It is hoped that the present volume will provide supportive and relevant awareness and understanding on the fundamental advances of the subject to the professionals from any sphere interested about glioma.

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