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Chapter 3

Tumor Microenvironment — Perivascular and Perinecrotic Niches

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Additional information is available at the end of the chapter

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1. Introduction

Tumor microenvironment is a dynamic concept that includes, beside tumor cells, everything is not tumor cells. It consists of cells, soluble factors, signaling molecules, extracellular matrix (ECM), and mechanical cues that can promote neoplastic transformation, support tumor growth and invasion, protect the tumor from host immunity and foster therapeutic resistance [1]. It is organ-specific and in the brain it is not yet fully understood. In addition to cancer cells, it contains different stromal cells mainly represented by endothelial cells, microglia/macrophages, and reactive astrocytes [2], but other cell types should be considered such as fibroblasts, pericytes, immune cells, *etc.* (Figure 1). These cells are heterogeneously distributed in the tumor, according to its different phenotypes and relevant biological significances.

2. Microenvironment cell components

2.1. Microglia/macrophages

Malignant gliomas are rich in microglia/macrophages that are classified as ramified or resident microglia, ameboid or activated microglia, macrophages and perivascular microglia [3]. They are called tumor-associated macrophages (TAM) and lack apparent phagocytic activity [4].They are considered as both intrinsic to the central nervous system (CNS) and blood-borne arrived, subjected to the local production of chemoattractant factors [5]; they share surface markers [6], but it has been demonstrated that microglia are chemokine (C-X3-C motif) receptor 1 (CX3CR1)+/chemokine (C-C motif) receptor 2 (CCR2)– and monocytes are CCR2+/

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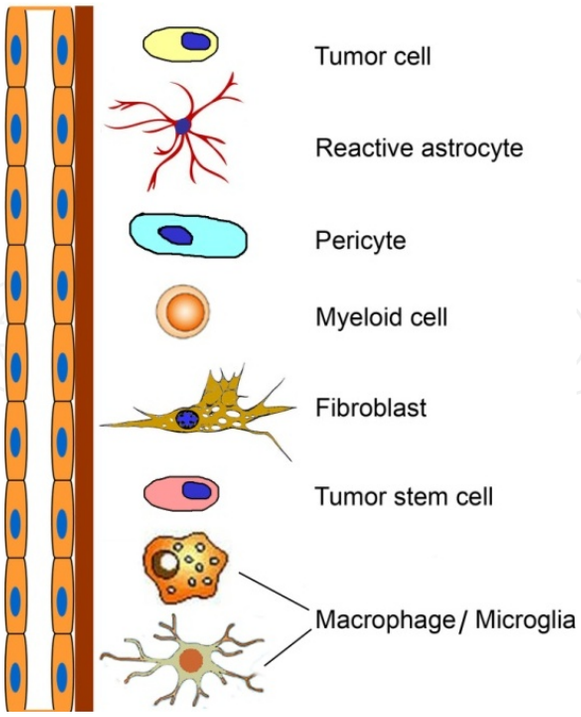


Figure 1. Scheme of the relationship between vessels/endothelium and microenvironment cells.

CX3CR1– [7]. In acute conditions microglia is blood-derived, from adult haematopoietic stem cells (HSCs), even though macrophages in adult renew independently from HSCs. Their majority derives from Tie2+ pathway generating eritro-myeloid progenitors, distinct from HSCs, from the yolk sac they migrate in the various organs [8].

It is still debated whether they are included in or they are distinct from pro-inflammatory cells. They increase both in the center and at the periphery of the tumors [9] and it has been calculated that up to one third of the cells in glioma biopsies are represented by macrophages [9,10] (Figure 2A,B). In the tissue, microglia/macrophages are found as small or large clusters around vessels or necroses, whereas at the periphery or around the tumor they are more regularly distributed. Undoubtedly, they proliferate in response to tumor growth and they have a cytotoxic defense function [11], as well as the capacity for antigen presentation [12], but they can also promote tumor infiltration and proliferation [13,14]. An inverse correlation between TAM infiltration and glioblastoma multiforme (GBM) prognosis [15] and promotion of tumor progression have been found [16].

Together with fibroblasts, pericytes, neutrophils, mast cells, lymphocytes, dendritic and endothelial cells, macrophages belong to the category of stromal cells that interact with the tumor, as discussed before, *via* cell-cell or by cytokine or chemokine-mediated signaling. Tumor cells may influence stromal cells to produce growth factors such as vascular endothelial growth factor (VEGF), tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), interleukin 1 (IL-1) or CXC ligand 2 (CXCL2), CXCL8, CXCL12 that promote angiogenesis and tumor growth. Conversely, tumor cells are stimulated to produce chemokines that influence angiogenesis [16] and growth. There is both an autocrine and a paracrine tumor

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growth stimulation [17]. The enrichment in stromal cells, especially microglia/macrophages, in the brain adjacent to tumor (BAT) strongly influences immunoregulation and tumor growth on the one side, and it represents a defense from the tumor on the other side.

The existence of a positive relationship between microglia/macrophages and tumor-initiating cells (TICs) in the two opposite directions is relevant to the problem [18]. The vessels can be associated or not with macrophages (Figure 2C,D) even with only one (Figure 2E). They occur obviously in circumscribed necroses (Figure 2F). Basically, any glioma-associated monocytic cell with macrophage characteristics has been called "tumor associated microglia". It shows a functional phenotype different from the inflammatory one and promotes glioma cell migration and tumor growth [19]. Migration promotion is accomplished through matrix metalloproteinases (MMPs) released by microglia [20,21] and CX3CL1 with its receptor (CX3CR1) [22]. The demonstration that microglia/macrophages promote glioma progression means that their inhibition can be a useful therapeutic tool [23]. Macrophages have long been recognized as critical components of immunity against tumors, because, when appropriately stimulated, they can attack tumor cells by contact interaction or by secreting cytotoxic and cytostatic factors [24]. However, they can also contribute to tumor development, by secretion of growth factors such as angiogenic factors, proteinases, which degrade the matrix, and immunosuppressor factors [25]. Their dual function is mainly exerted through TNF that demonstrates both an anticancer [26] and a tumorigenic activity [27]. However, it has also been shown that TNF can reduce glioma growth and prolong patient survival [28].

One specific question is the role of immune cells in the tumor microenvironment. These cells through cytokines, growth factors, chemokines and cerebrospinal fluid (CSF) interfere with tumor initiation, angiogenesis, proliferation and invasion [29]. IL-1β is the primary factor of microglia that enhances TGF-β, that, in turn, inhibits lymphocyte proliferation by suppressing antiglioma responses [30]. IL-1β also stimulates VEGF, epidermal growth factor receptor (EGFR) and MMP9 for angiogenesis, proliferation and invasion [31].

Macrophages can be subdivided into M1 and M2 subtypes, according their polarization status, supporting tumor suppression or progression, respectively [32]. As shown by the marker MHCII [33], they are strongly M2 in GBM [34]. In summary, it can be stated that macrophages support tumor progression and that tumor recruit macrophages [35]. There is an interrelationship between glioma stem cells (GSCs) and TAMs in GBM and it was shown that the former express Periostin, a member of the Fasciclin family (POSTN) [36] that has a supportive role in various tumors. TAM density correlates with POSTN in GBM and disrupting it TAM density is reduced so that GSCs secrete POSTN to recruit M2 that support tumor growth [37]. It was then showed that POSTN is highly expressed in high grade in comparison with low grade tumors [38]. How POSTN acts in potentiating tumor progression in niches has been widely discussed [39].

2.2. Reactive astrocytes

Reactive astrocytes can be sometimes confused with tumor cells, mainly because their phenotype changes over time until their complete maturation. There are analogies between glial reaction and physiological maturation of astrocytes during embryogenesis. In the initial

phases, the fine processes originate directly from the cell soma and then from the thick and long processes [40]. Nestin and Vimentin would be the main markers of immature astrocytes whereas glial fibrillary acidic protein (GFAP) is the main marker of mature astrocytes [41,42].

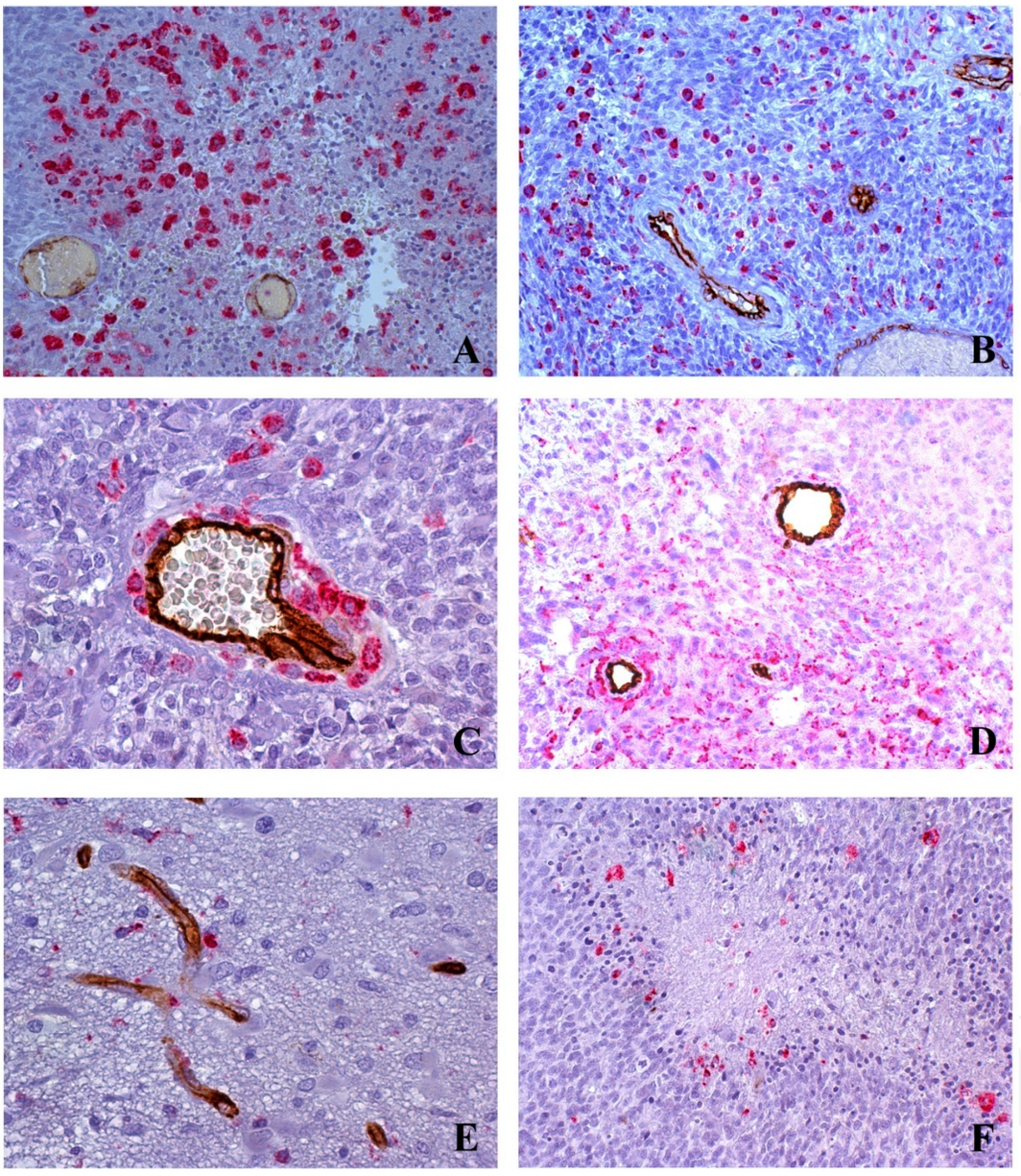


Figure 2. Glioblastoma. Macrophages/microglia. A – Cluster of macrophages in a proliferating area, not in relation with vessels; x200. B – Regular distribution of macrophages/microglia in a proliferating area not in relation with vessels; x200. C – Cluster of macrophages around a middle size vessel; x400. D – One vessel is surrounded by a crowd of macrophages, the other has none; x200. E – Capillaries with a macrophage adherent to the wall; x200. F – Macrophages in a perinecrotic palisade; x400. All double staining CD68-CD34, Alkaline Phosphatase Red and DAB, respectively.

It is still a debated question whether tumor infiltration can be recognized by magnetic resonance imaging (MRI), not only when adjacent to tumor, but also at distance. It has been observed, for example, that low grade gliomas, which preferentially locate in the *insula* and in

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the supplementary motor area, spread along distinct subcortical *fasciculi* [43]. By analyzing different peritumor areas with different MRI methods, it has been shown that fractional anisotropy and not apparent diffusion coefficient can be used to evaluate glioma cell invasion. An attempt to classify different peritumoral tissues by a voxel-wise analytical solution using serial diffusion MRI has been made [44].

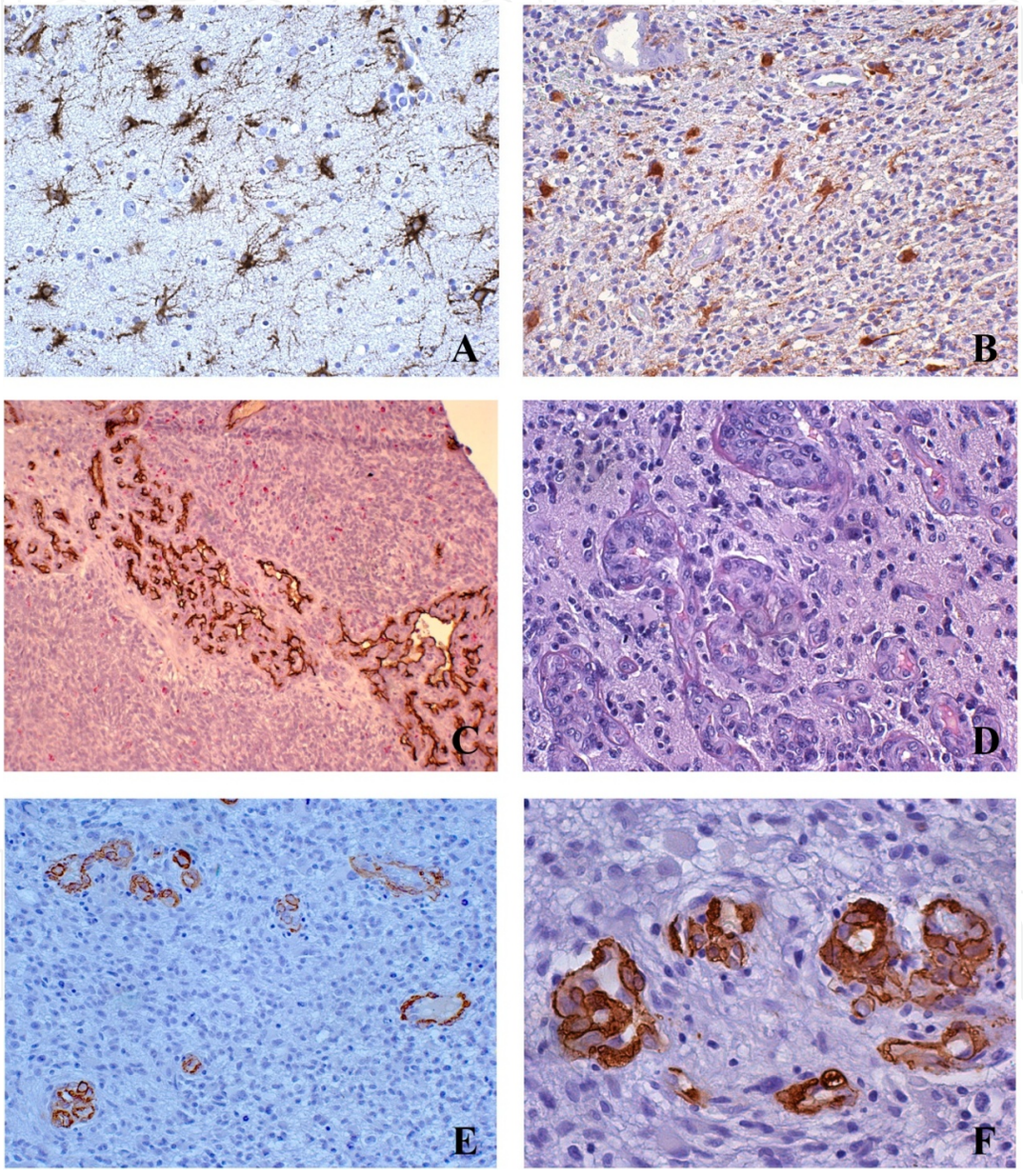


Figure 3. Glioblastoma. Reactive astrocytes. A – Regular distribution of reactive astrocytes in a cortex with mild infiltration. Some adhere to small vessels; GFAP, x200, DAB. B – Proliferating area with reactive astrocytes entrapped; almost only the large cytoplasm is visible; GFAP, x200, DAB. C – *Glomeruli*: multi-channel formations; double staining CD68-CD34, x100, DAB and Alkaline Phosphatase Red, respectively; D – Microvascular proliferations; x200, H&E. E – Pericytes; α-SMA, x200, DAB. F – *Id.* x400.

Peritumoral reactive gliosis (Figure 3A) has a particular importance because of three main characteristics: reactive astrocytes divide by mitosis as tumor cells do, they progressively lose Nestin and increase GFAP expression, as during development, and they may regionally exert a series of metabolic and molecular influences [45]. The most important point is that reactive astrocytes may be included in the advancing tumor (Figure 3B), in which they progressively become no more recognizable from tumor cells. The question is whether they disappear suffocated by the high tumor cell density, or if they remain, unrecognizable from tumor cells, to contribute to the pleomorphic aspect of gliomas, or if they even can be transformed into tumor cells [46]. There are evidences that reactive astrocytes support tumor progression [2].

3. The glioma origin and the stem cell theory

The existence of a similarity between cancer cells and embryonic stem cells is known since Virchow [47]. Glioma cells may derive through tumor transformation from immature glia cells [48,49], or primitive neuroepithelial cells or neural stem cells (NSCs) and many experimental demonstrations are available on this matter [50,51]. Glioma-initiating cells (GICs) and GSCs [52,53] share with NSCs some properties, *i.e.* proliferation and self-renewal, and GSCs share with malignant gliomas similar genetic alterations. In contrast to the hypothesis of the transformation of NSCs or neural progenitor cells (NPCs) into GSCs [54], either occurring *in situ* during embryogenesis or during migration and their relationship with GICs, the origin of GSCs has also been referred to dedifferentiation.

Dedifferentiation may refer to two distinct biological processes. The first one is represented by a multi-step process accompanied by genetic alterations that lead to the progressive transformation of normal cells into highly malignant cells. They require self-sufficiency growth signal, insensitivity to anti-growth signals, escape from apoptosis, proliferation potential, angiogenesis and invasion [55]. By combining activation of specific oncogenes and loss of tumor suppressor genes, it is possible to induce GBM from cortical astrocytes [56]. Examples are the combination of p16(INK4a)-p19(ARF) loss with K-Ras and Akt activation [57], p16(INK4a) and p19(ARF) loss with EGFR activation [58] and p53 loss with myr-Akt and c-Myc overexpression in mature astrocytes [59]. Basically, the capacity of transformation inversely correlates with differentiation. It is easier to get transformation from Nestin+ progenitors than from mature astrocytes by Ras and Akt activation [60].

A second meaning of dedifferentiation refers to tumor cells that would acquire stemness properties instead of reflecting the nature of the primitive cells [50,61].

Today, the existence of cell subpopulations, called cancer stem cells (CSCs) or GSCs, with stem cell-like properties such as multipotency, ability to self-renewal or to form neurospheres *in vitro*, is generally accepted, also for gliomas [62].

The origin of gliomas from NSCs has been repeatedly demonstrated by the experimental induction of brain tumors by nitrosourea derivatives [63]. Moreover, NSCs have been accepted as the source of gliomas, also because the signaling that regulates their self-renewal, prolifer-

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ation and differentiation occurs, altered, in gliomas. Several studies demonstrated that GBM may arise from the subventricular zone (SVZ) [64,65] that is the source of stem cells and progenitors in adults [66,67]. The latter are represented by neuroblasts (type A cells) and oligodendrocyte precursor cells (OPCs), by quiescent type B cells that give origin to highly proliferative cells, and by transit-amplifying progenitor cells (type C cells), that differentiate into two lineage-restricted progenitor cells [68,69]. These cells accumulate mutations up to give rise to gliomas [70], not excluding the intervention of human Cytomegalovirus (HCMV) [71].

GBM is a heterogeneous tumor and its heterogeneity might be explained by either the hierarchical model mechanism [72] or the stochastic mechanism of development [73]. Progenitor cells are at risk of malignant transformation since they show the activation of the adequate cell machinery, represented by telomerase activity, promitotic and antiapoptotic genes [54]. Abnormal developmental patterns are Sonic hedgehog (Shh) pathway, EGFR and phosphatase and tensin homologue (PTEN) signaling. Although their clonal origin is from a small fraction of transformed NSCs, gliomas are heterogeneous as a consequence of an anomalous tumor cell differentiation [74]. The diversity within gliomas is due to changes of the subclones, being all of them generated by multipotent tumor cells, but also through an arrest of the differentiation process.

Recently, other cells have been supposed to give origin to GBM.

4. Origin of GSCs and glioma heterogeneity

The hypothesis of GSCs is based on the concept that a rare subset of cells within GBM may have significant expansion capacity and the ability to generate new tumors [72]. The remainder of tumor cells, which predominantly resemble GBM, may represent partially differentiated cells with limited progenitor capacity or terminally differentiated non-tumorigenic cells. A possible origin of gliomas is also from mature astrocytes by acquiring stemness properties through a dedifferentiation process, as above mentioned [54,75] or from NG2 cells that fit better with tumors arising far from the ventricles or with secondary GBMs [76]. Also reactive astrocytes can be candidate for gliomas [77,78], since they can acquire a stem-like phenotype [79].

In spite of the great similarity between SVZ NSCs or progenitors and GICs, the relationship with GSCs remains unresolved. Are they equivalent, or the latter have nothing to share with the former, if not the stemness properties? An answer can be that over time GICs can acquire sufficient alterations to engender GSCs. GICs are the first genetically aberrant cells that can initiate tumor development and that are responsible for the bulk of tumor cells. OPCs, the major dividing cell population in the adult brain that gives origin to oligodendrocytes, distributed in the SVZ and in the gray and white matter, remain a further unresolved problem. The EGFR and prostaglandin-endoperoxide synthase 2 (PTGS2) inhibition prevents the tumorigenesis of transformed OPCs and GICs for anaplastic oligodendroglioma but not the tumorigenesis of transformed NSCs or GICs for GBM, suggesting that the latter can arise from OPCs or NSCs [80].

In mice models, by using the retrovirus replication-competent avian sarcoma-leukosis virus long terminal repeat with splice acceptor (RCAS) [81], OPCs expressing 2', 3'-cyclic-nucleotide 3'-phosphodiesterase (CNP) could be targeted later in their development or in the adult. Low grade oligodendrogliomas were obtained by RCAS-platelet-derived growth factor subunit beta (PDGF-β) expressing OPC markers such as sex-determining region Y (SRY)-box2 (SOX2), oligodendrocyte transcription factor 2 (OLIG2), NG2 and PDGF receptor (PDGFR), interpreted as indicating a slight dedifferentiation of tumor cells [82]. OPCs could serve as cells of origin of gliomas [83]. According to the already mentioned experiments by mosaic analysis with double markers (MADM), aberrant growth of precancerous lesions could only be found in cells differentiated along the oligodendrocyte lineage to become OPCs but not in any other lineage or in NSCs [84]. These demonstrations, however, do not exclude that aberrant growth can occur in NSCs, responsible for a direct origin of malignant tumors.

Heterogeneity in gliomas is not due to the occurrence in the same tumor of different non-tumor cells of various species, but to the cellular complexity formed by tumor cells that differ among themselves for a series of phenotypic and molecular characteristics affecting cell proliferation, invasion, *etc.* [85,86]. Cells are at risk of transformation only when demanded to proliferate, such as progenitors, opposite, for example, to B cells of SVZ that are protected [87]. The passage from B cells to amplifying cells implies a chromatin rearrangement from a quiescent to a proliferating status where genetic lesions, if not repaired, pass to the following dividing cells. There are interactions among DNA repair, epigenetics and stem cells. In the niche a homeostatic regulation of stem cells occurs, with a balance between self-renewal and differentiation, and with proliferation starting in response to a stimulating signal. Uncontrolled proliferation would take place when stem cells become independent of growth signal, because of mutations, or they resist anti-growth signals [88]. The homeostatic balance would be regulated by the interaction between Wnt/β-catenin pathway, that promotes cell growth, and bone morphogenetic protein (BMP) signaling that inhibits it. This can be the starting point of heterogeneity, largely dependent on the microenvironment. Gliomas with different genetic signatures may as well originate from different cell subtypes [89].

The same molecular mechanisms of NSCs regulate gliomas [90] that can undergo epigenetic changes and genetic mutations favoring evolution toward malignancy. During their lifespan, they can be exposed to genotoxic stress, to which they respond through repair mechanisms [76]. GBM has many molecular signatures depending on its polyclonality, and the events themselves may have an effect on the clonality. The greater is the potency of stem cells, the more anaplastic is the tumor.

The molecular profile of malignant gliomas has led to the distinction of proneural, proliferative and mesenchymal types associated to NSC profiles [91] or to the distinction of proneural, classic and mesenchymal types, the former expressing genes associated to progenitors and the latter two to stem cells [92]. The stemness would reflect the cell of origin, but it could also be acquired in the niche in adult gliomas [93]. On this basis, the contrasting results obtained on GBM can be explained by the finding of different series of TICs characterized by different phenotypic and molecular profiles [86,94].

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5. Migration of NSCs or NPCs toward tumors

NPCs can migrate from the SVZ toward a tumor and target it [95]. Today, this migration may represent a new goal for therapeutic purposes. NSCs exhibit tumor-homing capability. In mice experiments, immortalized murine NSCs, implanted into glioma-bearing rodents, distributed within and around tumors, even migrating to the contralateral hemisphere [96]. Genetically engineered NSCs show a tropism for gliomas, on which may have an adverse effect [97-100], especially if they are also transduced with herpes simplex virus-thymidine kinase (HSVtk) gene and followed by the administration of systemic Ganciclovir [101-103]. Human NSCs implanted in rat brains containing a C6 glioma, migrated in the direction of the expanding tumor [104]. The same properties are shown by mesenchymal stem cells (MSCs) injected either into carotid arteries or intracerebrally [105,106] and by hematopoietic progenitor cells [107]. Endogeneous progenitor cells have been observed to migrate from the SVZ toward a murine experimental GBM [108]. The migrated Nestin+ cells were also actively cycling, as shown by Ki-67/MIB.1 positivity, and 35% of them expressed Musashi-1 [109]. In transgenic mice, virally labeled proliferating cells of the SVZ demonstrated that NPCs accumulate around gliomas, diverted from their physiological migratory pathway to the olfactory bulb [110].

Chemokines, angiogenic cytokines and glioma-produced ECM can play a role in the NSC tropism [111]. It is possible to take advantage of the natural capacity of chemokines to initiate migratory responses and to use this ability to enhance the tumor inhibitory capacity by NPCs to target an intracranially growing glioma [112]. The therapeutic possibilities offered by NSCs are continuously increasing. For example, they can be engineered as sources of secreted therapeutics, exploiting their mobility toward CNS lesions. They could function as minipumps [113].

Rat embryonic progenitor cells, transplanted at distance from a glioma grown in the *striatum*, migrate and co-localize with it. They modify their phenotype, express Vimentin and reduce the tumor volume, demonstrating that a cross-talk exists between them and the tumor [114]. It has been shown that hypoxia is a key factor in determining NSC tropism to glioma by stromal-derived factor 1 (SDF-1) and its receptor (CXCR4), urokinase-type plasminogen activator (uPA) and its receptor (uPAR) and VEGF and its receptor (VEGFR) [115]. It could be interesting to try to enhance motility of adult NSCs toward CNS injury or disease and to take into account that EGFR could play a role, because of its participation to malignant transformation [116]. It has also been recognized that a limitation exists to the possibility of migration of neural precursors from SVZ to an induced cortical GBM in mice. The limitation is caused by the age and the proliferation potential of the SVZ. Adult mice supply fewer cells than younger mice, depending on the expression of D-type Cyclins, because with aging Cyclin D1 is lost and only Cyclin D2 is expressed [110]. Recently, novel treatment strategies using NSCs have been proposed, for example the suicide gene therapy using converting enzymes [117]. New strategies will emerge from further NSC and brain tumor stem cell (BTSC) studies [118]. Is it possible that tumors grow from transplanted NSCs [119]?

6. Perivascular niches (PVN). Relationship between NPCs/GSCs and endothelial cells

GBM is composed of three concentric zones: a central necrotic area, surrounded by an intermediate zone containing large vessels with thrombosis or altered walls; a surrounding proliferation zone that abruptly or progressively flows into the normal tissue and invades it [63]. Neo-angiogenesis takes place in the proliferating zone or in normal surrounding tissue after tumor cell invasion. In the latter, new capillaries are formed from the pre-existing venules. Basically, new capillary formation is due to the endothelial proliferation that mimics angiogenesis in normal embryonic conditions, with buds and new tubule formation. In comparison with normal angiogenesis, tumor angiogenesis is often dysregulated until the formation of *glomeruli* (Figure 3C). In the invasion zone, tumor cells wrap around vessels (co-option). In invaded cortex, the vascular tree coming down from the meningeal vessels is assailed by advancing and invading tumor cells and it progressively deforms through endothelial cell hypertrophy and hyperplasia. It becomes less adequate to perfuse the increased mass of tumor cells coming up from the white matter, because transformed into a lumpy tree with irregular lumina. The generated microvascular proliferations (MVPs) (Figure 3D) are mainly found at the transition from central necrosis to the proliferation zone, where circumscribed necroses with pseudopalisading develop [120]. As a consequence, areas very rich in capillaries and small vessels, produced by an intense angiogenesis, coexist in tumors beside areas poorly vascularized where necroses develop.

Vasculogenesis is a mechanism of tumor neovascularization that has been also attributed to circulating bone marrow (BM)-derived cells known as endothelial progenitor cells (EPCs). Its importance is debated [121,122], but it was shown that mesenchymal progenitors from bone marrow can differentiate into proliferating endothelial cells [123,124]. Also BM-derived TAMs, including TIE-2 expressing monocytes/macrophages (TEMs), circulate in the blood, home at sites of pathological neovascularization and differentiate into endothelial cells or macrophages [125,126].

Another type of vascularization is represented by the "vascular mimicry" due to the capacity of tumor cells to form a functional net of channels coated by themeselves. Two types of vascular mimicry have been described. The patterned matrix type is composed of a basement membrane, lined by tumor cells, forming channels with flowing blood [127]. Vasculogenic mimicry of the tubular type may be morphologically confused with endothelial cell-lined blood vessels. In both types, cells express endothelium-associated genes, as in embryonic vasculogenesis [128,129]. These properties are associated with CSCs [130]. By fluorescent *in situ* hybridization (FISH) and immunophenotyping, these non-endothelial cell-lined vessels have been demonstrated to be primary tumor cells. I*n vitro* CD133+ GSCs are vasculogenetic even with vascular smooth muscle-like cell differentiation. The cells do not express CD34 and show EGFR gene amplification [131]. It must be remarked, however, that usually cells of tumor vessels and MVPs never show either isocitrate dehydrogenase 1 and 2 (IDH1/2) mutations or EGFR gene amplification, never exceeding 1 or 2 copies [132]. They do not share with tumor cells genetic alterations and this is in line with the lack of TP53 mutations in MVPs [133].

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In tumors transplanted into mice and irradiated, recruitment through hypoxia of BM-derived cells occurs, able to restore circulation through SDF1 and CXCR4 [134]; among these cells, EPCs prevail [121,135-137]. Vasculogenesis can be blocked by pharmacological inhibition or antibodies toward SDF1 and CXCR4 [134].

Interestingly, also a GBM-endothelial cell transdifferentiation is considered to contribute to tumor vascularization, favored by hypoxia [138], independently of VEGF [139]. It has been observed that a quota of GBM CD31+ endothelial cells shares with tumors cells chromosomal aberrations [140] and that a quota of GBM CD105+ endothelial cells harbours the same somatic mutations identified within tumor cells, such as amplification of EGFR and chromosome 7 [141]. In a GBM model, it was demonstrated that the tumor-derived endothelial cells originated from TICs [138]. This finding is of paramount importance because of the possibility to use an anti-VEGF antibody (Bevacizumab) for therapy [142]. Unfortunately, the effects of the drug are only transient [143] and the reason of the failure is the activation of other pro-angiogenic pathways, the recruitment of BM-derived cells and the increase of pericyte protection and tumor invasion [144,145]. The possibility that the tumor becomes more aggressive after therapy has been contemplated [146].

The problem of transdifferentiation and the role that CSCs play in this process are still under discussion [139]. All the observations have been made in animals or from animal models, and *in vivo* experiments of transdifferentiation have been challenged [85]. In human pathology, the contribution of tumor cells to the GBM vasculature has never been demonstrated and vessel cells with typical genetic changes of tumor cells, such as those of EGFR, PDGFR, PTEN, TP53, IDH1/2 have never been found in GBM [132].

By intussusception, pre-existing brain capillaries can be multiplied by transluminal endothelial bridges and by lumen partitioning; it is an early phenomenon [147].

As NPCs or NSCs reside in the normal SVZ niche at close contact with endothelial cells, in GBM, GSCs and/or NPCs are located in PVN. In the latter, a strict similarity exists with what happens in the normal SVZ niche, where the intimate association between normal NSCs and endothelial cells regulates self-renewal and differentiation of the former. In PVN, angiogenesis is activated by VEGF produced by NSCs/GSCs [88,148], whereas their stemness is maintained by Notch produced by endothelial cells through nitric oxide [19,149,150]. Notch is constitutively active in high grade gliomas and conditions their progression [104]. PVNs are strictly correlated with tumor progression. There would be a bidirectional communication between endothelial cells and TICs or GSCs [151].

The PVN composition has been carefully described in GBM, with the inclusion, beside cells of the environment, of ECM, integrins, cell adhesion signaling, cadherin family, *etc.* [152]. In GBMs, strong evidence for the existence of an endothelial mesenchymal transition (EMT) process is still lacking, but this process is increasingly reported as instrumental to tumor growth and diffusion [153,154]. It is defined by the possibility that differentiated epithelial cells establish stable contacts with neighbor cells, assume a mesenchymal cell phenotype with loss of cell-cell interactions, reduce cellular adhesion, active production of ECM proteases, increase cytoskeletal dynamics and changes in transcription factor expression, and acquire a

stem cell program, all of them leading to increased migration and invasion ability [155]. The three major groups of transcription factors, the SNAI, Twist-related protein 1 (TWIST1) and Zinc-finger enhancer binding (ZEB) family members, have been reported to be altered in GBM. Their over-expression follows the activation of Wnt/β-catenin pathway and results *in vitro* in an increased cell migration and invasion [156,157]. It is likely that the high expression of mesenchymal genes in the mesenchymal subset of human GBMs [91] can be considered to be reminiscent of the EMT program [92] or that the aberrant activation of EMT factors during gliomagenesis can trigger the mesenchymal shift in GBM [158].

The influence that GSCs can exert on BM-derived endothelial cells has been summarized as follows [159]: to elicit angiogenesis, to home at the tumor the BM-derived EPCs and to promote their differentiation into blood vessels that incorporate into the existing vasculature. Trans-differentiation into endothelial-like cells contributes to the formation of blood vessels [140,160].

The PVN concept was substantiated by the demonstration of Nestin+ and CD133+ cells on capillaries, forming a microvasculature in which the microenvironment that maintains CSCs and their renewal is given by endothelial cells that, in turn, are stimulated by CSCs [149]. A positive correlation was found between the CD133+ niches and CD133+ blood vessels, similar to the correlation between the Nestin+ niches and Nestin+ blood vessels [161]. A good PVN demonstration has been given [2] and beautiful and useful schemes have been provided [159, 162]. It can be added that angiogenesis and self-renewal would represent a resistance to chemo- and radio-therapy.

The location of GSCs in PVN was confirmed by several studies using either CD133 positivity [163] or side population signature genes, such as aspartate beta-hydroxylase domain-containing protein 2 (ASPHD2) or nuclear factor erythroid 2-related factor 2 (NFE2L2) or hypoxia-inducible factor 2 (HIF-2) [164]; they increase with malignancy [161]. By comparing xenografts of C6 glioma with a high or low fraction of GSCs, it was observed that the former exhibit an increased microvessel density and an increased recruitment of BM-derived endothelial progenitors [123]. The relevance of the hypoxia will be discussed later.

6.1. Pericytes

Pericytes, the last PVN component, are perivascular cells that support blood vessels [165], control blood vessel stability, function through paracrine factors and direct cell-cell contacts, and promote vascular maturation (Figure 3E,F). They express different markers including PDGFR-β, α-smooth muscle Actin (α-SMA), Desmin, and NG2. They originate from mesoderm-derived MSCs or from neuroectoderm-derived neural crest cells, depending on their location within the brain. Pericytes are an essential element of the neurovascular unit and contribute to the function of blood-brain-barrier (BBB) [166]. Gliomas can induce the differentiation of MSCs into pericytes [167]. MSCs injected into brain tumors in mouse models have been shown to closely associate with the tumor vasculature and also with up-regulation of the expression of pericyte markers [168].

Pathology observations show that pericytes increase in number in GBM and wrap around vessels with endothelial hyperplasia.

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7. PVN neuropathology

The description of the niches must be obviously a survey of the different vascular structures in GBM with their surrounding cell components. The first question to give an answer is: does each vascular structure represent a niche, or are they distributed in the tumor and how? The second question is: is the cell composition of the niches a constant one or does it vary from one another? In the literature, GSCs have been demonstrated in perivascular position [149], as well in perinecrotic niches [164,169] as discussed later. Good schemes of PVN are provided including all the cells that can be encountered in such position [62,159,162]. Such schemes, obviously, are not encounted as real occurrences in the histological examination of GBMs.

By examining the vascular structures in the different tumor zones, in infiltration areas capillaries, arterioles, venules or penetrating vessels from the meninges occur. Around them, there are tumor stem cells/progenitors, often forming cuffings (co-option), or Nestin+ cells adherent to the walls, or reactive GFAP+ astrocytes (Figure 4A–D). Scattered in the tissue, microglia/macrophages occur rather regularly, occasionally distributed in perivascular position (Figure 2). Reactive astrocytes continue to be present also in more intense infiltration, recognizable for their GFAP positivity and for what remains of their long and thick processes; however, they are regularly distributed in the tumor tissue and occasionally they can be found in perivascular position.

In areas of intense tumor cell proliferation, many small vessels can be found either with or without endothelial hyperplasia, sometimes forming a dense net. Around them, tumor cells, mostly Nestin+ and SOX2+, crowded, that can easily be considered as undifferentiated and containing sometimes cells with stemness properties, associated with occasional CD68+ cells. In proliferating areas, larger vessels can be found, with walls thicker than in capillaries, surrounded by a dense cuffing of cells that are Nestin+ in the inner part and GFAP+ outside (Figure 4E,F). In most vessels, pericytes appear wrapping the channel outside the endothelial cell layer; they are well evident in MVPs or in *glomeruli* that, on the other hand, do not appear to be surrounded by other cell types, if not tumor cells.

In intermediate areas or near the central necrosis, many vessels of different size and nature are associated with edema or tissue dissociation and they do not show to be surrounded by any special cell kind. Scattered in the tumor, myeloid cells can be found in variable quantity, associated or not with other types of cells among which macrophages seem to be the most frequent. Microglia/macrophages are distributed in small or large clusters around necroses or around vessels where they can be associated or not with some of the other cell types. The association with myeloid cells is the most striking.

The neuropathological study provides the information that PVN represents a theoretical picture where the different cell types can be represented and where cross-talks occur among the different signalings that support some tumor activities such as invasion, growth, *etc.* Of course, the most important dialogue in these structures occurs between GSCs and endothelial cells, and this is feasible around capillaries and small vessels, even though the thickness of the vessel walls could not in absolute be an insuperable obstacle.

8. Hypoxia and necroses – Perinecrotic niches

In GBM, there are two main types of necrosis: large necroses, usually at the tumor center, of thrombotic origin, and circumscribed necroses, occurring in the proliferative areas and representing a hallmark of the tumor. Hypoxia is, therefore, a tumor characteristic [170], mediated by HIF-1/2 composed of two subunits, an oxygen insensitive HIF-β subunit and an oxygen regulated HIF-α subunit [171]. Under normoxic conditions, HIF-α is rapidly degraded following hydroxylation by the oxygen-dependent prolyl-hydroxylase domain proteins (PHDs), that mark it for ubiquitination and proteasomal degradation [172]. Hypoxia stabilizes HIF-1α by preventing its hydroxylation and degradation; together with HIF-2α, it is critically involved in the regulation of GSCs [164]. Hypoxia directly promotes the GSCs expansion. In human GBM biopsies, GSCs are enriched in perinecrotic regions, where the oxygen tension is reduced and HIF-1α and HIF-2α are activated [164,173]. HIF-2α remains elevated under chronic hypoxia, while HIF-1α is only transiently upregulated [174].

Hypoxia through HIF-1α promotes the expansion of GSCs through the phosphatidylinositol 3-kinase (PI3K)/Akt and ERK1/2 pathways, the inhibition of which reduces the fraction of CD133+ GSCs [175]. In perinecrotic regions hypoxia regulates many properties [159]. In GSCs under hypoxic conditions, it activates Notch by inducing its ligands and the activation of target genes Hes1 and Hey2 [164,176]. Blockade of Notch signaling with γ-secretase inhibitors depletes the GSC population, reduces the expression of GSC markers such as CD133, Nestin, Bmi1 and OLIG2 and inhibits the growth of tumor neurospheres and xenografts [177].

GSCs can be demonstrated to lie around circumscribed necroses or scattered in the tissue by CD133 positivity [169] or other specific antigens [164].

Hypoxia is generally realized when tumor growth exceeds neovascularization, and it would not only regulate tumor cell proliferation, metabolism, differentiation, but also induce key stem cell genes such as Nanog, Oct4 and c-Myc [178].

Necroses are the place where hypoxia occurs, but it must be taken into account that usually its occurrence is histologically deduced from its pathologic effects, *i.e.*, necrosis in the tissue. Hypoxia at its very beginning could not yet be visible as necrosis, but already efficient for other signs. It is possible that tissue features in an area not suspected to be hypoxic, are indeed due to hypoxia. An example is given by apoptosis. Apoptotic nuclei are found in proliferating tumor areas due to an intrinsic or transcriptional pathway *via* mitochondria and focused on TP53 [179], or in hypoxic areas through an extrinsic pathway or TNF [180].

It is, however, possible that isolated apoptotic nuclei in a proliferating area are not due to the first type of apoptosis, *i.e.* the intrinsic one, but to the extrinsic type, consequence of a not yet morphologically evident hypoxia [181]. As a matter of fact, HIF-1α expression can be mainly demonstrated around circumscribed necroses, but also in scattered cells in proliferating areas (Figure 5A,B) [45].

Circumscribed necroses in GBM are the hallmark of the tumor, but their origin and development are still discussed. They have been carefully described and codified [139,182,183] as due to an ischemic process following a vascular occlusion or to a pathology of the endothelium.

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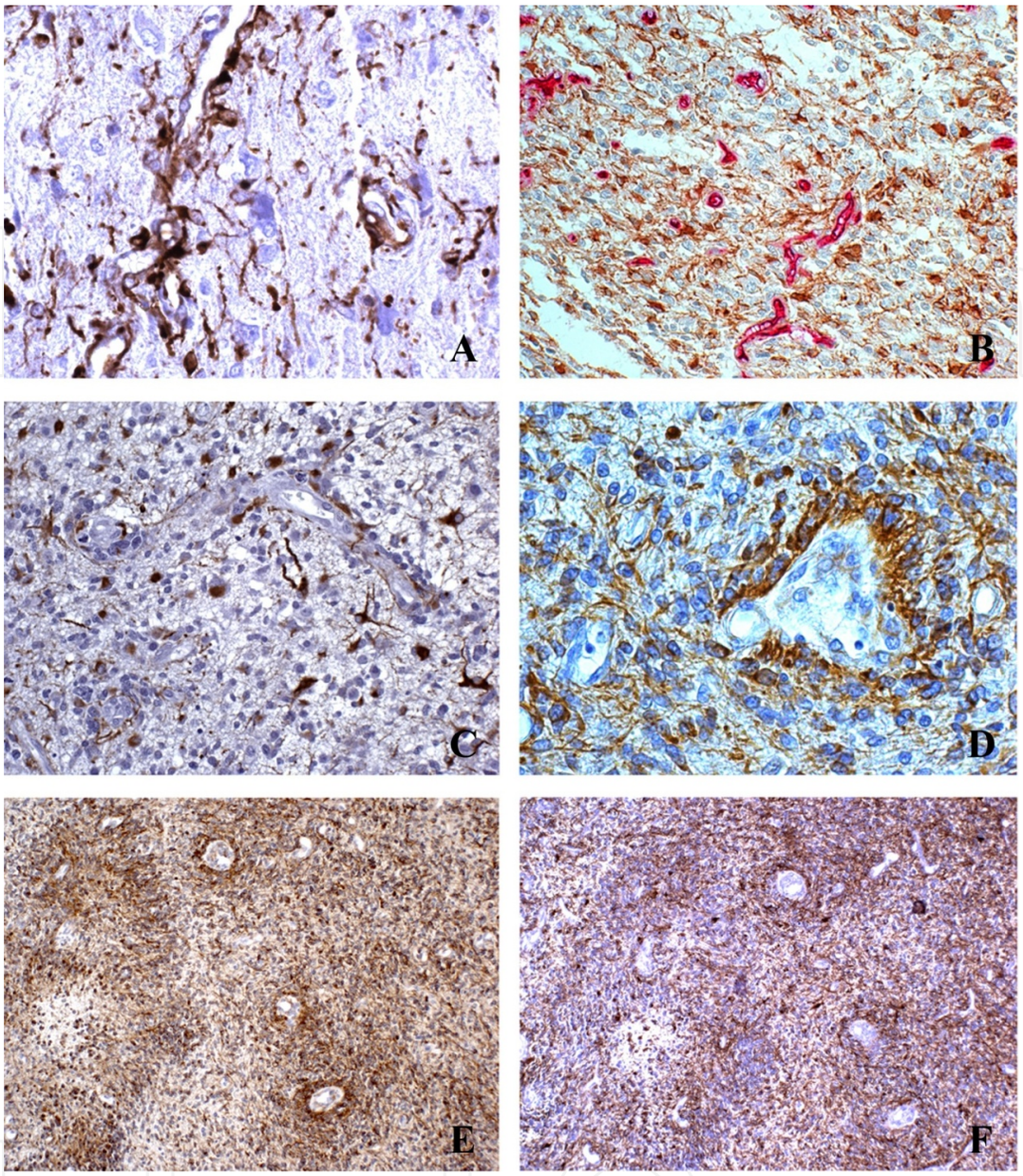


Figure 4. Glioblastoma. D – Nestin+ tumor cells adhere to small vessels; Nestin, x200, DAB. B – GFAP+ cells scattered in the tissue or in relation with vessels directly or by vascular feet: tumor cells or reactive astrocytes; double staining GFAP-CD34, x200, DAB and Alkaline Phosphatase Red, respectively. C – *Id.* with a cuffing of GAFP+ cells in the outer layer; GFAP, x200, DAB. D – Cuffing of Nestin+ cells on a medium size vessel; Nestin, DAB, x200. E – Infiltrative area with Nestin+ cells on small vessels; Nestin, x200, DAB. F – *Id.* Some GFAP+ tumor cells adhere to vessels; GFAP, x200, DAB.

The consequent hypoxia would stimulate angiogenesis, through HIF-1 and VEGF. Another interpretation can be given: necroses develop in hyperproliferating areas, with a high Ki-67/MIB.1 labeling index (LI) and a high Nestin expression in comparison with GFAP, due to the focal insufficiency of angiogenesis to feed a very large number of tumor cells, because of the imbalance between the high tumor cell proliferation capacity and the low one of endothelial cells (Figure 6A–F and Figure 7) [184-186].

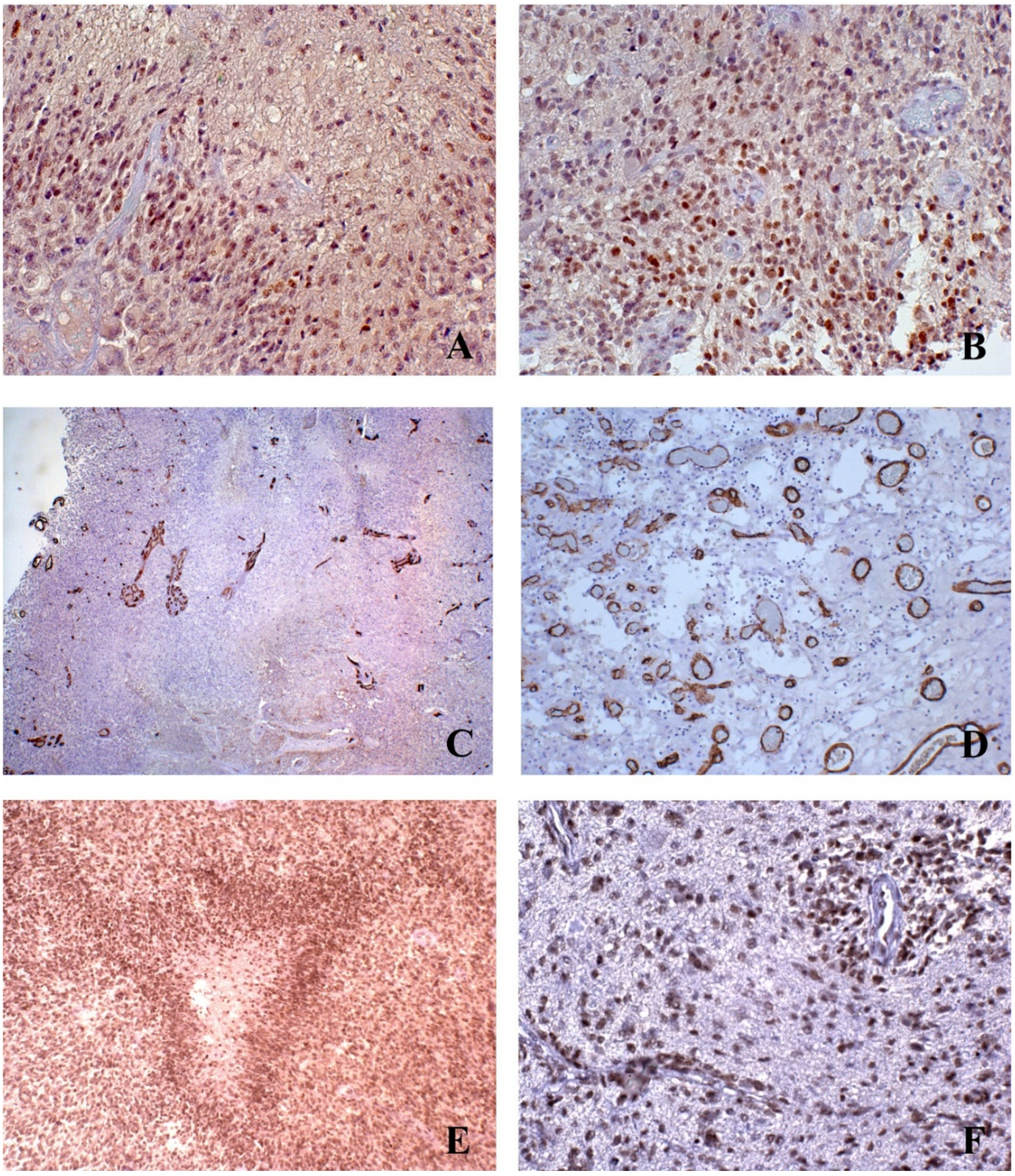


Figure 5. Glioblastoma. A – HIF-1+ cells in perinecrotic position; HIF-1, x200, DAB. B – HIF-1+ cells scattered in a proliferative area; HIF-1, x200, DAB. C – A large avascular area; CD34, x25, DAB. D – High vessel density; CD34, x100, DAB. E – Perinecrotic palisade with high density of SOX2+ cells; SOX2, x200, DAB. F – Cuffing of SOX2+ cells around vessels; SOX2, x200, DAB.

This observation does not exclude that inside necroses regressive pathological vessels occur [183]. In GBMs, beside areas with a high vessel density due to an active neoangiogenesis, large avascular areas occur where necroses develop (Figure 5C,D).

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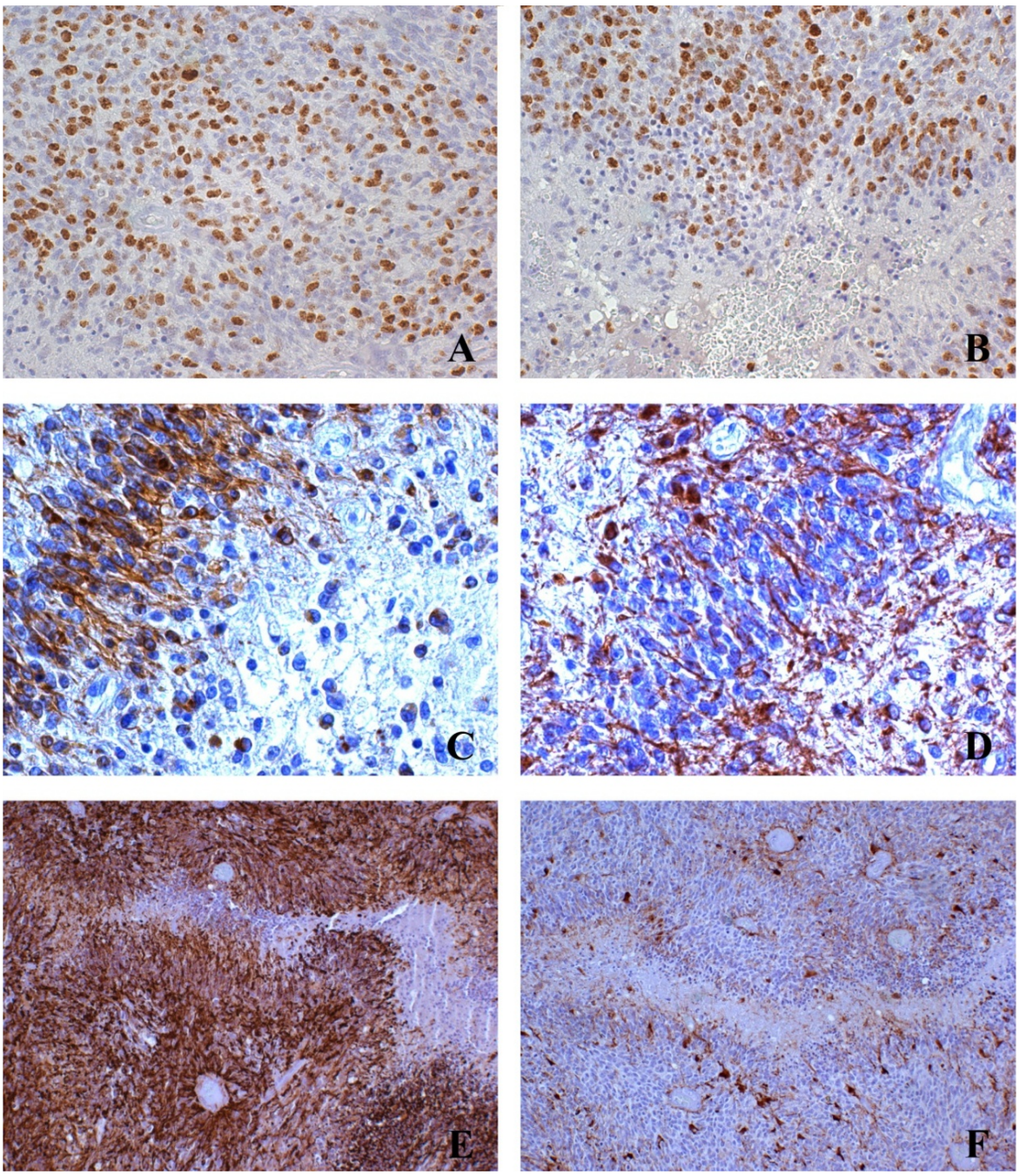


Figure 6. Glioblastoma. A – High Ki-67/MIB.1 labeling index (LI) in a hyperproliferating area; Ki-67/MIB.1, x200, DAB. B – High Ki67/MIB.1 LI in the perinecrotic palisade; Ki-67/MIB.1, x200, DAB. C – Perinecrotic palisade with high density of Nestin+ cells; Nestin, x400, DAB. D – *Id.* with only rare GFAP+ cells; GFAP, x400, DAB. E – A circumscribed necrosis in hyperproliferating Nestin+ area; Nestin, x200, DAB. F – *Id.* only rare GFAP+ tumor cells; GFAP, x200, DAB.

The palisades would be the remnants of the hyperproliferating area after necrosis development. Both are composed of a high number of cells positive for stemness markers such as CD133, Nestin, SOX2, and RE-1-silencing transcription factor (REST), and have a high proliferation index (Figure 5E,F) [163,187,188]. GSCs can be conceived as deriving from dedifferentiated tumor cells that acquired stemness properties [189]; they would be concentrated in the above mentioned malignant tumor areas where circumscribed necroses develop because of the vascular insufficiency. It is likely that GSCs around necroses represent the quota of GSCs that populated the hyperproliferating areas and remained unaffected by necrosis development. The palisadings themselves would be the remnants of hyperproliferating areas, spared by necrosis [45,189].

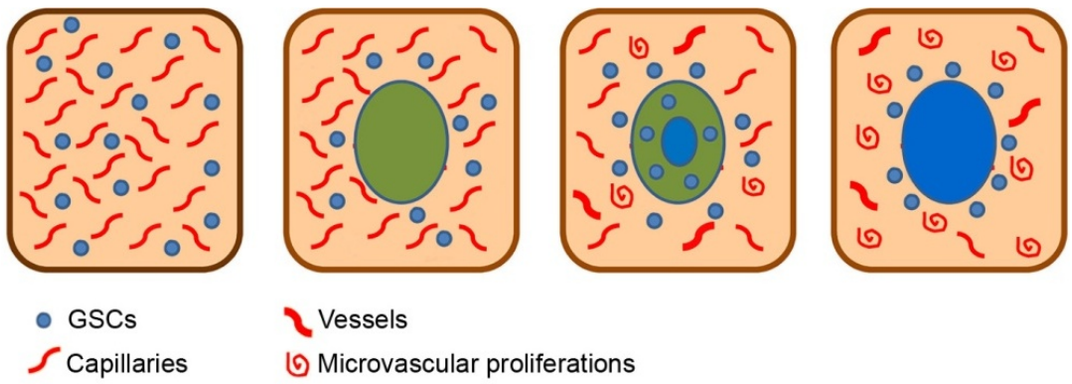


Figure 7. Progression from hyperproliferating areas with GSCs/progenitors → development of avascular area → appearance of necrosis → necrosis surrounded by GSCs/progenitors.

9. Functions of the niches in the tumor and their interdependence

The GSC maintenance is provided by the signalings that occur in the niches; they can expand and form new ones that, in turn, drive the tumor growth [190]. Signalings involved in the GSC regulation are Oct4, c-Myc, Notch, TGF-β, Wnt/β-catenin pathways. Genes associated with shorter patient survival, as already observed [91], are overexpressed in the side population found by Seidel *et al* [164] and, *viceversa*, downregulated in those associated with longer survival. The overexpression concerns more primary than secondary glioblastomas that show a reduced CSC component [191] that, however, may still support tumor growth.

Perivascular and perinecrotic niches are not separated entities, first of all for temporal reasons. Hypoxia is the main cause of angiogenesis, but this is realized through factors, such as VEGF, Angiopoietin 1 and 2, SDF1 produced by GSCs and, at the same time, the imbalance between the high proliferation rate of tumor cells and the low one of endothelial cells makes angiogenesis insufficient and causes necrosis development. Moreover, besides tumor areas rich in small neo-formed vessels and capillaries, MVPs due to a dysregulated angiogenesis do not show sufficient exchanges with tumor cells and are responsible for hypoxia [63]. Another important question, widely discussed [172], is how hypoxia and the vasculature regulate macrophages

and immune cells through HIFs and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) [192,193] and CXCR4 [194].

It cannot be established whether GSCs of perivascular and perinecrotic niches belong to the same population, because, although showing the same stemness antigens, they still represent progenitors that can be in different state of differentiation. The significance conferred to GSCs in perinecrotic position as dedifferentiated tumor cells which reached stemness beyond a certain point of dedifferentiation, cannot be recognized to GSCs in perivascular niches [45,189]. Letting aside the transdifferentiation of tumor cells into endothelial cells, which did not received sufficient support, another point of link between the two microenvironments is represented by cell migration through EMT that is promoted by hypoxia and bound to GSCs [155]. GSCs can migrate along newly formed vessels and favor tumor diffusion.

The most important question in this topic is the occurrence of circumscribed necroses in the tumor areas with the highest malignant phenotype including both avascular districts and districts with a high vessel density, so that perinecrotic niches appear to be associated with perivascular niches to characterize these malignant areas [45,189,195].

10. Conclusions

The origin of gliomas has been outlined as traceable back to the transformation of primitive neuroepithelial cells or NSCs, capable of self-renewal and proliferation, *i.e.* endowed with stemness properties, or to the dedifferentiation of adult glia to reach stemness properties. The CSC responsibility for tumor proliferation, recurrence and resistance to therapies falls today into the most credited hypothesis. Many experiments have shown that GSCs derive by transformation of NSCs or they represent a simple functional stemness status. Some aspects of the problem remain unresolved, for example, the relationship between TICs and CSCs or the CSCs location in the tumor, as well as the existence in the tumor of NSCs that continuously renew the CSCs quota.

Recently, a new concept arose to indicate everything in the tumor, outside cancer cells, that regulates tumor proliferation, invasion, differentiation, resistance to therapies as the microenvironment, with its innumerable molecular pathways and numberless signalings and crosstalks. Major expressions of the microenvironment are in GBM the perivascular and the perinecrotic niches. The former are important for the endothelial cell/CSC relationship that, on one side, maintains the stemness status of CSCs and, on the other side, gives origin to angiogenesis. The latter are important for the occurrence of hypoxia through HIF-1/2 that can induce CSC formation.

The neuropathological study of GBMs with the final goal to find a concrete expression to the perinecrotic niche concept, provides an alternative interpretation to that considering perinecrotic CSCs as induced by hypoxia. They can be the remnants of CSCs that crowded the hyperproliferating and malignant areas of the tumor in which necrosis developed for insufficient vascularization. Perivascular niches are usually very well depicted as schemes that

contemplate all the cells that can be in contact with vessels/endothelial cells. This event, however, is not observed to be realized with all the identified cells in all the vessels, going from capillaries or small vessels to larger vessels, MVPs or *glomeruli* in the different tumor areas. The cells described in the schemes never occur concurrently in one or all the vessels, so that the schemes themselves remain as a theoretical indication of possible relationships that can be established between tumor cells and vessels as a consequence of a general molecular regulation that is realized in the microenvironment.

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