



## Review

# Electron microscopy morphology of the mitochondrial network in gliomas and their vascular microenvironment<sup>☆</sup>

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## ABSTRACT

Gliomas still represent a serious and discouraging brain tumor; despite of the diversity of therapeutic modalities, the prognosis for patients is still poor. Understanding the structural and functional characteristics of the vascular microenvironment in gliomas is essential for the design of future therapeutic strategies. This review describes and analyzes the electron microscopy morphology of the mitochondrial network in human gliomas and their vascular microenvironment. Heterogeneous mitochondrial network alterations in glioma cells and in microvascular environment are implicated directly and indirectly in the processes linked to hypoxia-tolerant and hypoxia-sensitive cells phenotype, effects of the hypoxia-inducible factor-1 $\alpha$ , increased expression of several glycolytic protein isoforms as well as fatty acid synthase, and survivin. The prevalent existence of partial or total cristolysis observed suggests that oxidative phosphorylation is severely compromised. A mixed therapy emerged as the most appropriate. This article is part of a Special Issue entitled: Bioenergetics of Cancer.

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## 1. Introduction

In 1857 Rudolf Albert von Kölliker (1817–1905), histologist and embryologist, first described “sarcosomes” (now called mitochondria) in muscle cells. Kölliker was among the first biologists to interpret tissue structure in terms of cellular elements. The term “mitochondrion” (meaning “threadlike granule”) was first used in 1898. In the 1930s, Warburg described increased rates of glycolysis and mitochondrial defects in cancer cells. Functionally active mitochondria were first isolated in 1948. In the 1950s, mitochondria were observed by means electron microscopy. Today, after 120 years, the study of mitochondria is a central issue in numerous human diseases.

In the last decade, advances in light and electron microscopy have led to a renewed interest in the structural diversity and dynamics of mitochondria, as well as in their interactions with other cellular components [1]. Some connections exist between cancer and mitochondria. In cancer, mitochondrial changes are associated with mitochondrial-DNA mutations, tumoral microenvironment conditions and mitochondrial fusion–fission disequilibrium. Mitochondrial structure and functions vary from tissue to tissue. The mitochondrial morphology is modified by functional requirements to adapt to different cell demands. On the other hand, in cancer cells, dysfunctional mitochondria are implicated directly or indirectly in their limitless replication, self-provision of proliferative stimuli, insensitivity to

antiproliferative signals, disable apoptosis, sustained angiogenesis, invasiveness, avoidance of the immune response, and enhance anabolic metabolism [2]. In human tumors and tumoral cell lines, mitochondria show conspicuous alterations in their ultrastructural aspects [3].

Today, gliomas still represent a serious and discouraging brain tumor; despite of the diversity of treatment modalities, generally, the prognosis for patients is still poor (i.e. fatality and sequelae). One of the major conceptual advances in oncology over the last decade has been the appreciation that all major aspects of cancer biology are influenced by the tumor microenvironment [4]. The tumor microenvironment is a mixture of extracellular matrix molecules, tumor cells, endothelial cells, fibroblasts and immune cells [5]. Angiogenesis and continuous remodeling of the tumor microvasculature are essential for adequate tumor tissue oxygenation and nutritional supply [6]. According to Vajkoczy and Menger [6], the vascular microenvironment determines pathophysiological characteristics of gliomas and the structure and function of the glioma microvasculature determine susceptibility and resistance of the tumor to specific treatment strategies [6]. Furthermore, the understandings of these aspects in gliomas are basic for the design of pharmacological treatments.

Mitochondrial structural abnormalities and dysfunction in malignant gliomas are a neglected area of research [7]. The understanding of structural and functional characteristics of the vascular microenvironment in gliomas is essential for the design of future therapeutic strategies. Electron microscopy permits the study of mitochondrial morphology and their overall organization. The aim of this review is to describe and analyze the electron microscopy morphology of the mitochondrial network in human gliomas and their vascular

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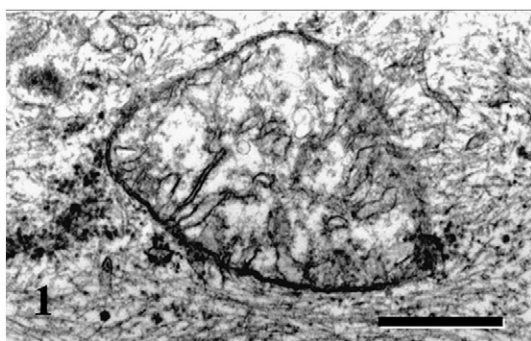
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microenvironment, and probably represent a contribution to the structural basis of several mitochondrial molecular defects reported in gliomas that would be explaining, at least in part, the resistance of astrocytic tumors to conventional chemotherapy.

## 2. Electron microscopy morphology of the mitochondrial network and functional implications in human glioma cells and vascular microenvironment

### 2.1. Mitochondrial pathology in tumoral cells

Mitochondrial swelling associated with disarrangement of cristae and partial or total cristolysis is the most constant submicroscopic alteration observed in gliomas (Fig. 1). In pilocytic astrocytomas particularly, in addition to swelling mitochondria, mitochondria with increased thickness and remarkably electron dense cristae were seen, as well as in undifferentiated neoplastic cells. On the other hand, fibrillary astrocytomas, anaplastic astrocytoma and glioblastoma multiforme show that mitochondrial swelling associated with disarrangement of cristae and partial or total cristolysis is the most constant ultrastructural finding. However, non-specific ultrastructural differences between the different grades of tumors exist. This morphological change is associated with hypoxic-ischemic conditions [8], and it is well known that hypoxic microenvironment is a characteristic of human gliomas [9,10]. Since the enzymes involved in oxidative phosphorylation are located on the inner mitochondrial membrane, its surface area and number of cristae are generally correlated with the grade of metabolic activity exhibited by a cell [11]. Gilkerson et al. [12], using immunolabeling and transmission electron microscopy of bovine heart tissue, estimated that the crystal membrane of mitochondria is the principal site of oxidative phosphorylation. Diversity of tumors exhibits an evident diminution in mitochondrial content [13,14] and in oxidative phosphorylation capacity [15,16]. Oudard et al. [13] reported a very low content of normally functioning mitochondria in 4 human xenografted gliomas and suggest that gliomas shift the energy metabolism towards a high-level glycolysis to generate their cellular ATP supply. Therefore, the prevalent existence of partial or total cristolysis observed in this study suggests consequently that the ability of human astrocytomas to generate ATP by mitochondrial oxidative phosphorylation would be severely compromised, therefore, to a low bioenergetic index. In addition, this would deteriorate the ability of astrocytoma cells to commit apoptosis. Recently, Chiche et al. [17] reported that hypoxic enlarged mitochondria protect cancer cells from apoptotic stimuli. This finding is congruent with infrequently observed ultrastructural morphologic changes suggestive of apoptosis observed in astrocytic tumors [18], in accordance to the ultrastructural criteria for apoptosis previously established [19]. This aspect is consistent with the earlier detection that human malignant glioma cells undergo necrosis rather



**Fig. 1.** Mitochondrion exhibits a cristae disarrangement, partial cristolysis and electron-lucent matrix in a tumoral cell. Method of staining: uranile acetate/lead citrate. Bar: 3.32  $\mu\text{m}$ .

than apoptosis because of energy deprivation, reported by Steinbach et al. [8]. In addition, Steinbach and Weller [20] previously point out that the amount of apoptosis is generally low in malignant gliomas. On the other hand, Cuezva et al. [14] mentioned that cancer cells with a low bioenergetic index as a result of a low mitochondrial content and/or activity would be prone to establish a transformed phenotype and become more resistant to apoptosis.

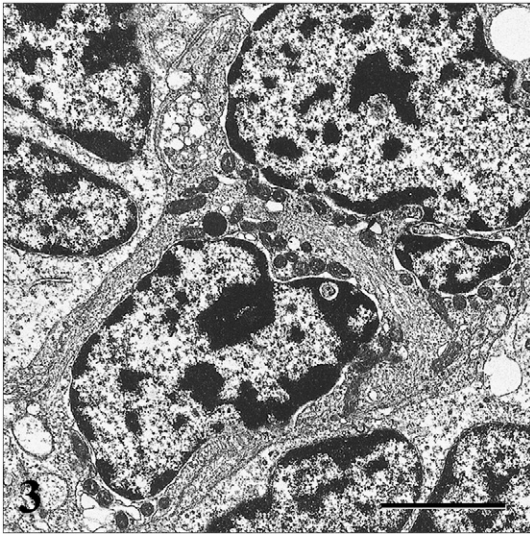
In some astrocytoma cells, a predominant presence of mitochondria with dense matrix displayed in closed groups exists (Figs. 2 and 3). In other astrocytoma cells, the principal finding is the lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis. Recently, Rossignol et al. [21] illustrated that mitochondria of HeLa cells and fibroblast adopt a condensed configuration when producing energy by oxidative phosphorylation. In the case of astrocytomas, the dense mitochondria could be capable of producing energy by oxidative phosphorylation, and lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis are incapable of generating energy by oxidative phosphorylation. Parliament et al. [22] postulated that glioma cell lines behave as “oxygen conformers” and their rate of oxygen consumption appears to vary with the availability of oxygen. Turcotte et al. [23] demonstrated variation in mitochondrial function in hypoxia-sensitive and hypoxia-tolerant human glioma cells. Possibly, the astrocytoma cells that hold dense mitochondria are hypoxia-tolerant cells, therefore, able to generate sufficient ATP concentration by oxidative phosphorylation. In contrast, the astrocytoma cells that contain lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis are hypoxia-sensitive cells, therefore, incompetent to produce adequate amount of ATP by mitochondrial respiration.

In gliomas, mitochondria show variability in number, size and shape, included in the same specimen, as well as the degree of severity of internal ultrastructural mitochondrial changes. Some mitochondria exhibited cigar, bowling-pin, ‘L’, ‘V’, ‘Y’ and irregular shapes (Fig. 4). Possibly, this is related with the cellular variability of astrocytomas and variations in microenvironment conditions, i.e., diverse degree of hypoxia, pH, and hypoglycemia, and finally if the astrocytoma cells are hypoxia-tolerant or hypoxia-sensitive. Earlier, Tandler et al. [24] suggested that the existence of extremely pleomorphism and normal



**Fig. 2.** Mitochondria with dense matrix displayed in closed group in a tumoral cell. Method of staining: uranile acetate/lead citrate. Bar: 1.62  $\mu\text{m}$ .

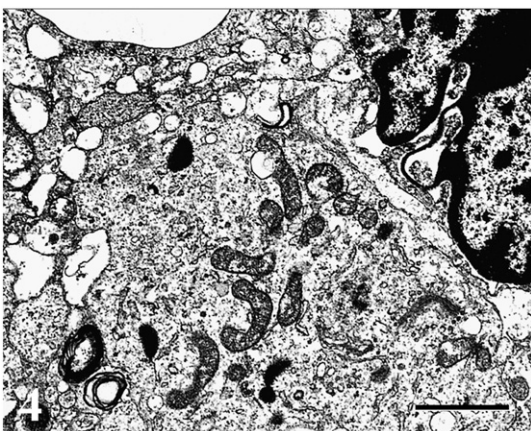




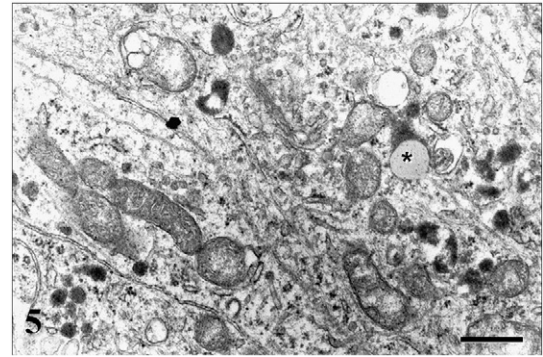
**Fig. 3.** Mitochondria demonstrate increased thickness and remarkably electron dense cristae in tumoral cells. Method of staining: uranile acetate/lead citrate. Bar: 5.68  $\mu\text{m}$ .

mitochondria represent the two extremes of a continuum in the development of the mature oncoocyte. On the other hand, the mitochondrial morphology is modified by functional requirements to adapt to different cell demands. Recently, Smolkova et al. [25] proposed the “metabolic waves hypothesis”, wherein cancer cells will exhibit differences in energy metabolism depending on oncogene activation and environmental factors, this might also explain the diverse changes in mitochondrial shape.

Lipid droplets between or near the mitochondria are present (Figs. 5 and 6). Accumulation of lipid droplets is observed in infectious, neoplastic and inflammatory conditions [26]. Apparently, the lipid droplets are inducible organelles with roles in cell signaling, regulation of lipid metabolism, membrane trafficking and control of the synthesis and secretion of inflammatory mediators [26]. In many human cancers, lipogenic pathways are activated, and the lipid droplets are the ultrastructural expression of these biochemical phenomena [27]. In gliomas, increased expression of fatty acid synthase has been observed, and the inhibition of this enzyme is associated with decreased glioma cell viability [28]. Transmission electron microscopy showed that lipid droplets were present in the necrotic and perinecrotic areas of the C6 cell-induced rat glioma [28,29]. According to Delikatny et al. [30] the lipid droplets are likely to be indicators of a reduction in mitochondrial metabolic activity. In addition, Zoula et al. [31] described that severe hypoxic C6 rat brain glioma showed accumulated small lipid droplets,



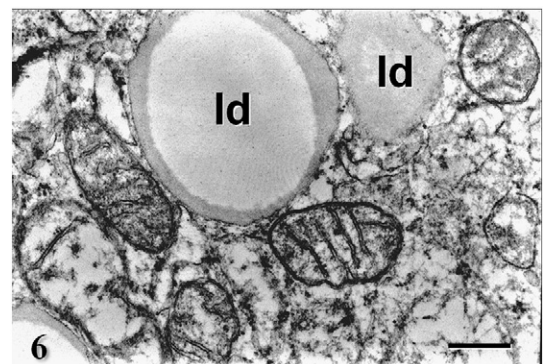
**Fig. 4.** Mitochondria show variable size and shape in tumoral cells. Method of staining: uranile acetate/lead citrate. Bar: 3  $\mu\text{m}$ .



**Fig. 5.** Lipid droplet (asterisk) is next to mitochondria with dense matrix in a tumoral cell. Method of staining: uranile acetate/lead citrate. Bar: 2  $\mu\text{m}$ .

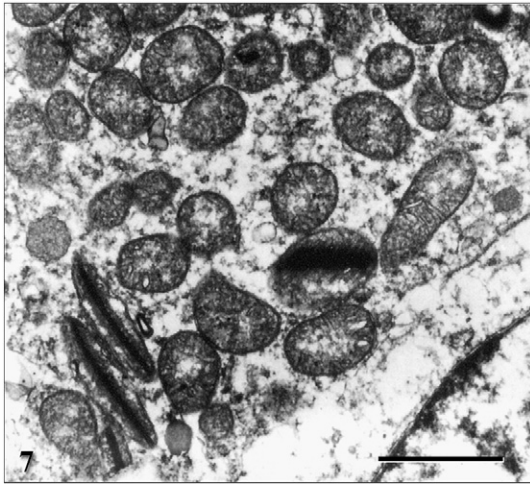
however it cannot be considered as a surrogate marker of hypoxia. C6 glioma cells proliferation arrest induced by growth factors deprivation induces an even higher accumulation of cytosolic droplets [32]. Opstad et al. [33] show in human gliomas that the distribution of the lipid droplets is independent of the tumor grade, as well as a positive correlation with the percentage of necrosis and that the formation of lipid droplets precedes necrosis.

Mitochondrial morphology is regulated by continuous fusion and fission events that are essential for maintaining a normal mitochondrial function [34]. Mitochondrial fusion–fission phenomena are scarce in gliomas [18]. These mitochondria exhibit edematous changes and cristolysis; morphological changes that suggest loss of the mitochondrial inner membrane potential and serious defect in the respiratory chain. Mitochondrial fission accompanies several types of apoptotic cell death and appears important for progression of the apoptotic pathway [35]. Different mitochondrial alterations observed in cancer cells could be linked to unbalanced mitochondrial fission or fusion events [34]. The low frequency of mitochondrial fission observed in gliomas is in concurrence with the fact that the amount of apoptosis is generally low in malignant gliomas [20]. Survivin is a molecule expressed by the most human cancers; acts as an inhibitor of apoptosis in cancer and coordinates a pathway of apoptosis inhibition [36,37]. The interference of survivin expression induces remarkable apoptosis in glioma cell line U251 [38]. Blum et al. [39] reported that the suppression of survivin expression in U87 glioblastoma multiforme cells by the Ras inhibitor farnesylthiosalicylic acid promotes caspase-dependent apoptosis. Mitochondria in astrocytomas probably produce survivin, this potentially explains the low occurrence of mitochondrial fission and ultrastructural morphologic changes suggestive of apoptosis in human gliomas [18]. In addition, growing evidence suggests that survivin is responsible for drug resistance in cancer cells [40].



**Fig. 6.** Lipid droplets (ld) in close proximity to mitochondrion with multiple alterations in a tumoral cell. Method of staining: uranile acetate/lead citrate. Bar: 1  $\mu\text{m}$ .

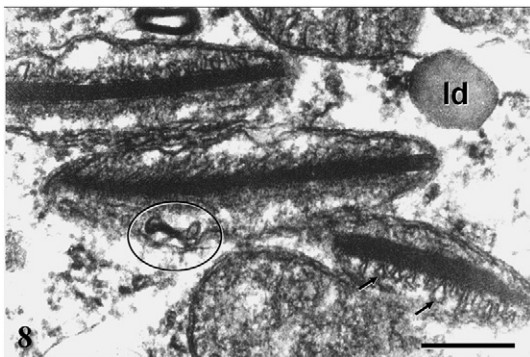




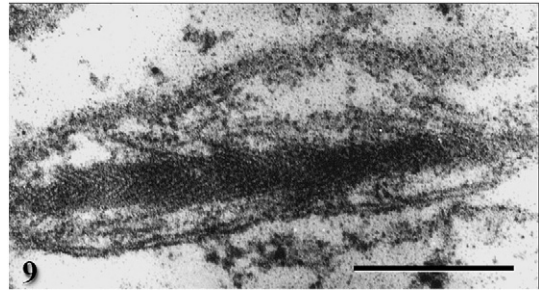
**Fig. 7.** Mitochondrion shows crystalline intermembrane inclusions in tumoral cell. Note the dense matrix in other mitochondrion. Method of staining: uranile acetate/lead citrate. Bar: 5.7  $\mu\text{m}$ .

Crystalline intermembrane inclusion bodies are unusual (Figs. 7–9). They exhibit a lattice pattern alternating electron dense and electron lucent bands. In the context of the gliomas, possibly their apparition is linked with hypoxic conditions. These kinds of mitochondrial inclusions represent crystallization of membrane proteins and a disturbance in the respiratory pathway, and are linked with tissular ischemia, inflammatory, metabolic, and neoplastic and neurodegenerative illness. Intramitochondrial lamellar bodies or paracrystalline inclusion were reported in acute myeloblastic leukemia, subependymal giant cell astrocytoma, anaplastic oligoastrocytoma, and granular cell astrocytoma [41–44]. Crystal-like intramitochondrial inclusions in sporadic Parkinson's and Alzheimer's disease hybrid cell lines were observed [45]. However, the mechanism of formation and the pathophysiologic significance of these abnormal mitochondria remain unknown.

In human gliomas the presence of mitochondria that display a few concentric layers of double leaflets of inner mitochondrial membrane contained inside a continuous envelope of outer membrane (onion ring-like structure) is scarce. This mitochondrial morphology is linked with the loss of subunit e or g of the mitochondrial ATP synthase in yeast cells [46,47]. The ATP synthase-associated subunits e and g are indispensable for the biogenesis of the mitochondrial cristae [46]. The full loss of subunit e or g decreased the mitochondrial ATPase activity by 50% [47]; however, it is not sufficient to affect growth by oxidative phosphorylation [48]. Possibly, in the case of the cancer cells, the contribution to energy metabolism of this rare mitochondrial conformation is not substantial, due to the amount and their apparently



**Fig. 8.** Higher magnification demonstrates crystalline intermembrane inclusions with mitochondrial cristae vestige (arrows). Note an adjacent lipid droplet (ld) and minuscule intramitochondrial myelin-like figure (circle). Method of staining: uranile acetate/lead citrate. Bar: 0.6  $\mu\text{m}$ .



**Fig. 9.** Mitochondrial crystalline intermembrane inclusions exhibit a lattice pattern alternating electron dense and electron lucent bands. Method of staining: uranile acetate/lead citrate. Bar: 0.83  $\mu\text{m}$ .

diminished energy production because of defective oxidative phosphorylation.

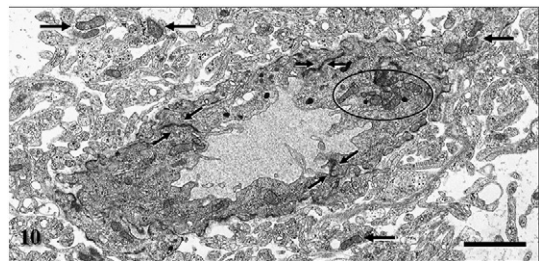
Finally, in cell processes the presence of mitochondria is scarce. This finding probably implies that at this level, the energy derived from mitochondrial respiration is marginal. Beckner et al. [49] reported that glycolytic enzymes are abundant in pseudopodia formed by U87 astrocytoma cells, and that glycolysis alone can support glioma cell migration. Recently, robust migration of glioblastoma cells has been previously demonstrated under glycolytic conditions and their pseudopodia contain increased glycolytic and decreased mitochondrial enzymes [50].

Some of the changes in the mitochondria observed in human gliomas had been reported in other human cancer [3].

## 2.2. Mitochondrial alterations and morphological changes in the vascular microenvironment components

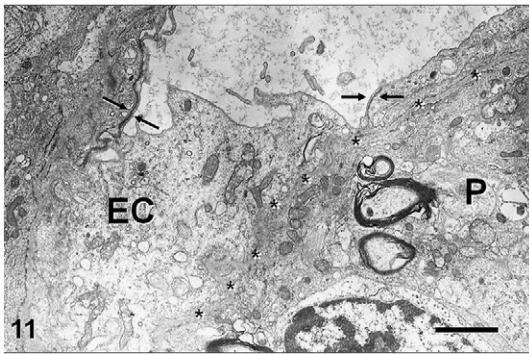
The structural characteristics of the vascular microenvironment in gliomas seem to be determined by both the tumor milieu and local tissue factors [6]. As a result the microvasculature components exhibit conspicuous changes and their morphology is highly heterogeneous when compared to normal cerebral vessels [51–58]. During development of gliomas the microvasculature becomes aberrant [59]. This aspect possibly is linked with the characteristic topographic variation in the histopathological patterns displayed by astrocytic tumors, regional angiogenic activity, constant remodeling of the tumor microvasculature, and size of the tumor [6].

In the vascular microenvironment components of gliomas, the mitochondrial network exhibit similar changes to describe in tumoral cells. The mitochondria display mainly two patterns: a) swelling associated with disarrangement of cristae and partial or total cristolysis; and b) condensed configuration (Figs. 10–14). The endothelial cells present edematous and oncotic changes in variable grade, lipid droplets, and alterations in tight junctions. Pericytes exhibit edematous changes and phagocytosed material. Astrocytic perivascular-feet show signs of



**Fig. 10.** Tumoral microvessel. Mitochondria on endothelial cells present dense matrix displayed in closed group (circle), observe four electron-dense tight junctions structurally closed (opposed arrows). Perivascular cells process exhibit mitochondria with dense matrix (bold arrows). Method of staining: uranile acetate/lead citrate. Bar: 6.66  $\mu\text{m}$ .

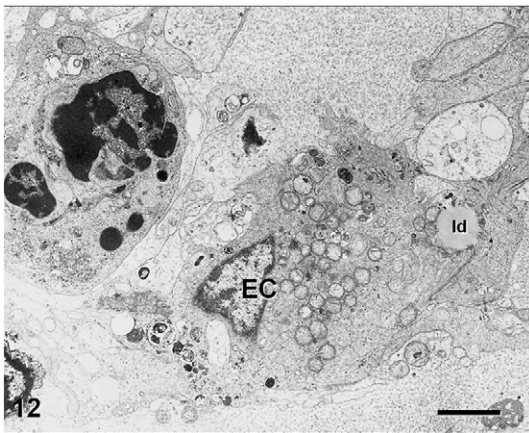




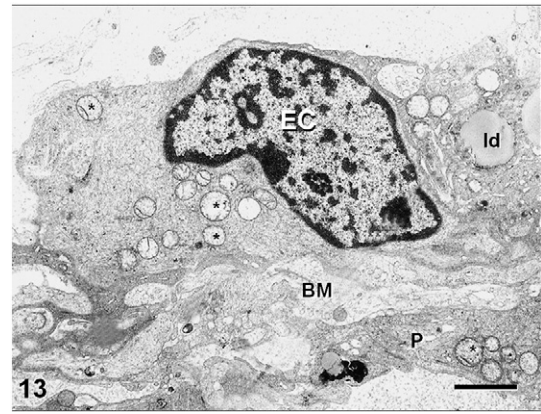
**Fig. 11.** Sector of a tumoral microvessel. Mitochondria on endothelial cells (EC) and pericyte (P) present dense and lucent matrix displayed in closed group. Note two electron-dense tight junctions structurally closed (opposed arrows). Pericyte exhibit phagocytosed material. Asterisks identify basal membrane. Method of staining: uranile acetate/lead citrate. Bar: 2.5  $\mu$ m.

oncosis with presence of glycogen-rich and glycogen-depleted processes [60]. Unger et al. [61] demonstrated the presence of large number of mitochondria in brain tumor-derived endothelial cells in culture. Caruso et al. [62] described swollen mitochondria and cytoplasmic lucency in three cases of human gastric carcinoma with coagulative necrosis.

Electron microscopic studies have identified structural alterations in tight junctions [53–55,58,60–65]. In the perspective of an intratumoral hypoxic medium, probably the anoxic–hypoxic conditions of the neoplastic tissue constitute an actin-disregulating and actin-disrupting factor that contribute with the changes in actin distribution patterns, these latter finally induce the abnormalities observed in tight junctions in astrocytic tumors. The observation of oncotic and ischemic changes in endothelial, pericytes, and astrocytic perivascular end-feet denotes the intratumoral development of hypoxic–anoxic conditions in gliomas. The capillary endothelial cells are quite vulnerable to short periods of anoxia [66]. Gliomas express the hypoxia-inducible factor-1 $\alpha$  [67–70]. The hypoxia-inducible factor-1 $\alpha$  is involved in the resistance against apoptosis, vascular remodeling and angiogenesis. In cancer cells, this factor induces overexpression and increases activity of several glycolytic protein isoforms, including transporters and enzymes [71]. Some of these glycolytic isoforms participate in survival pathways, inhibition of apoptosis and promotion of cell migration, modulate mitochondrial function and oxygen consumption by inactivating the pyruvate dehydrogenase complex in some tumors types, or by modulating cytochrome c oxidase subunit 4 expression to increase oxidative phosphorylation in other cancer cell lines [71]. In the cases of malignant



**Fig. 12.** Section of a tumoral microvessel. Mitochondria on endothelial cells (EC) exhibit cristae disarrangement, partial and total cristolysis and electron-lucent matrix displayed in closed group. Note a lipid droplet (Ld) in close proximity to mitochondria. Method of staining: uranile acetate/lead citrate. Bar: 3.32  $\mu$ m



**Fig. 13.** Region of a tumoral microvessel. Mitochondria on endothelial cells (EC) and pericyte (P) exhibit cristae disarrangement, partial and total cristolysis and electron-lucent matrix (asterisks). Note a huge lipid droplet (Ld) in close proximity to mitochondria. BM denotes a multilayered basal membrane. Method of staining: uranile acetate/lead citrate. Bar: 3.32  $\mu$ m.

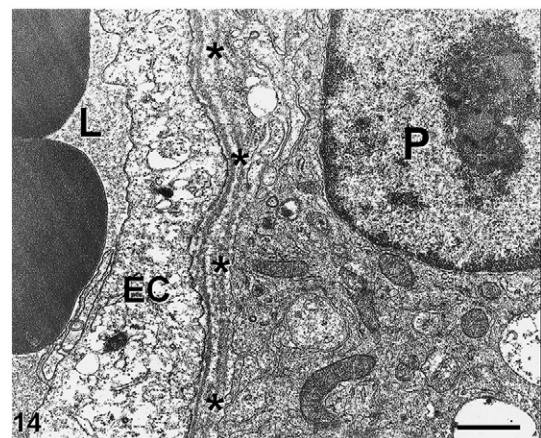
gliomas, the inhibition of the hypoxia-inducible factor-1 $\alpha$  decreases vascular endothelial growth factor secretion and tumor growth [72].

Finally, Survivin is constitutively overexpressed in the glioma vasculature [40,73], and confers chemoresistance to endothelial cells. Therefore, the inhibition of this molecule may be an effective approach to chemosensitization [40].

### 3. Perspectives for pharmacological therapeutics

Mitochondrial network alterations in glioma cells and in microvascular environment are heterogeneous, and are implicated directly and indirectly in the processes linked to hypoxia-tolerant and hypoxia-sensitive cells phenotype, effects of the hypoxia-inducible factor-1 $\alpha$ , increased expression of several glycolytic protein isoforms as well as fatty acid synthase, and survivin. Consequently, a mixed therapy emerged as the most appropriate.

An approach would be target glycolysis and/or revert the Warburg phenomenon. The glycolytic inhibitors are particularly effective against cancer cells with mitochondrial defects or under hypoxic conditions, which are frequently associated with cellular resistance to conventional anticancer treatments and radiation therapy [74,75]. Griguer et al. [76] described oxidative phosphorylation-dependent cells and glycolytic-dependent cells in human and mouse malignant glioma cell lines. Therefore, the glycolysis inhibition would be effective in hypoxia-sensitive cells. While the hypoxia-tolerant cells potentially



**Fig. 14.** Sector of microvessel. Mitochondria on pericyte (P) exhibit electron-dense matrix. EC denotes endothelial cell, asterisks basal membrane, and L vessel lumen. Method of staining: uranile acetate/lead citrate. Bar: 1.16  $\mu$ m.

represent a sensible target to inhibition or down-regulation of mitochondrial respiration, given that mitochondrial insult or failure can rapidly lead to inhibition of cell survival and proliferation [77,78].

At the vascular environment, since the numerous effects of the hypoxia-inducible factor-1 $\alpha$ , the inhibition or down-regulation in endothelial cells and pericytes is a promising alternative. For the chemosensitization, the blockage of the survivin effects also appears as important issue.

Finally, changing the metabolic environment of the glioma by means of dietary restrictions is recommended for the antiangiogenic and pro-apoptotic properties [79,80].

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