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Ultrastructural mitochondrial pathology in human astrocytic tumors: potentials implications pro-therapeutics strategies

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Abstract This study was realized to illustrate and analyze the ultrastructural mitochondrial pathology in human astrocytic tumors. Tumoral biopsies of 10 patients with pathological diagnosis of astrocytic tumors by means of transmission electron microscopy were examined. Mitochondria exhibits heterogeneous morphology in all the cases. Mitochondrial swelling with partial or total cristolysis was the most constant alteration observed. Mitochondrial fusion–fission phenomena have been demonstrated. These findings suggest that the majority of astrocytoma cells are incompetent to produce adequate amount of energy by means of oxidative phosphorylation. Ultrastructural mitochondrial pathology indicates that possibly both glycolytic inhibition and inhibition or down-regulation of mitochondrial respiration would be a potential tool for future therapeutic strategies in cases of human astrocytic tumors.

Keywords astrocytomas, gliomas, mitochondria, glycolysis, mitochondrial respiration, cancer

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Introduction

Mitochondria are implicated directly or indirectly in many aspects of altered metabolism in cancer cells [1]. Multiple mitochondrial alterations in various solid tumors and hematological malignancies have been reported [2]. It has long been believed that mitochondrial defects play a significant function in the development and progression of cancer and are important repercussions in cancer therapy. Astrocytic tumors are demoralizing in the clinical setting because they are difficult to treat and may cause significant disabilities [3–5]. In addition, with the exception of pilocytic astrocytomas, the prognosis of glioma patients is still poor, <3% of glioblastoma patients are still alive at 5 years after diagnosis [6]. Hipoxic microenvironment is a characteristic of astrocytic tumors [7–9]; they produce energy by high-level glycolysis [10]. However, what occur to mitochondria in cancer cells remain poorly understood, since no consistent pattern in several mitochondrial aspects has emerged [11]. The knowledge of mitochondrial structure and function in cancer cells offers a unique potential for the clinical use of mitochondria as markers for the early detection of cancer [12]. The aim of present paper was to analyze the mitochondrial pathology in human astrocytic tumors by means of a transmission electron microscope. The analysis of morphology of mitochondria in this kind of tumors revealed notable alterations in their structure in human specimens not been previously described. These probably represent a contribution to the structural basis of several mitochondrial molecular defects reported in cancer cells that would explain, in part, the resistance of malignant solid tumors to conventional chemotherapy.

Materials and methods

Tumor biopsies of 10 patients with pathological diagnosis of astrocytic tumors (five fibrillary astrocytoma, one anaplastic astrocytoma, two glioblastoma multiforme, two pilocytic astrocytoma) were examined with the transmission electron microscope. The neurosurgical study and the samples biopsies were obtained according to the basic principles of Helsinki Declaration.

Two- to five-millimeter-thick tumoral biopsies were immediately fixed in the surgical room in 4% glutaraldehyde – 0.1 M phosphate or cacodylate buffer, pH 7.4, at 4°C. After 2∼h of glutaralhehyde-fixation period, the cortical biopsies under a stereoscopic microscope were examined

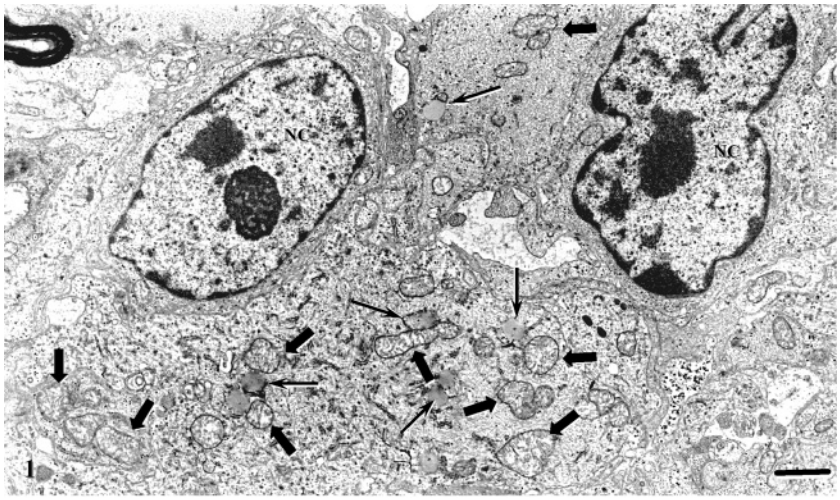

Fig. 1. Glioblastoma multiforme. Two neoplastic cells (NC) that exhibit multiple swelling mitochondria of different size and several degrees of cristae disarrangement and cristolysis (bold arrows). Note lipid droplets between or near to the mitochondria (thin arrows). Bar: 0.78 μm. Method of staining: uranyl acetate/lead citrate.

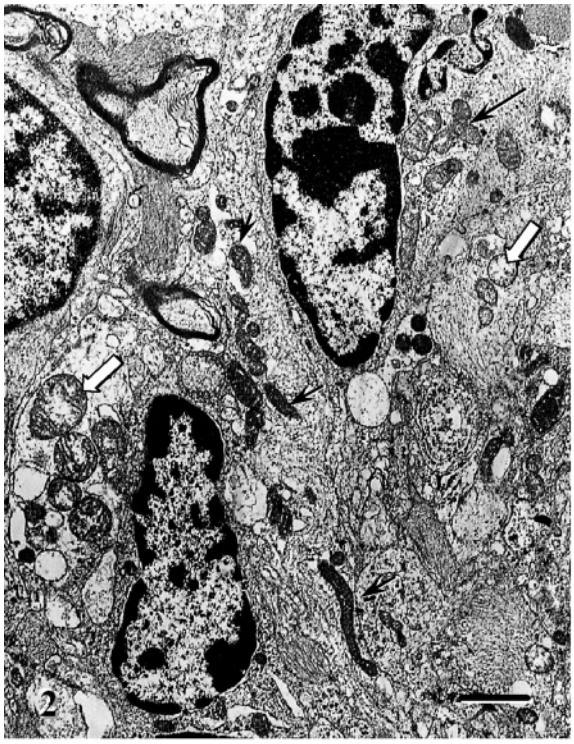

Fig. 2. Fibrillary astrocytoma. Presence of swelling mitochondria (white bold arrows), 'Y' shape mitochondrion (long arrow) and dense mitochondria (short arrows). Bar: 1.25 μm. Method of staining: uranyl acetate/lead citrate.

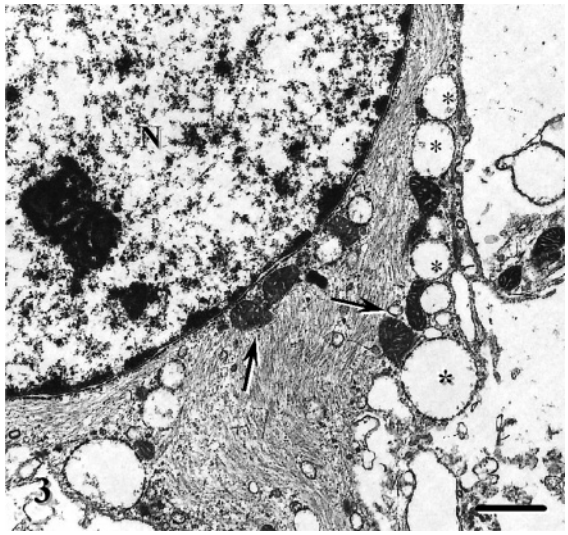

Fig. 3. Pilocytic astrocytoma. Tumoral astrocyte of pilocytic astrocytoma that shows several mitochondria characterized by increased thickness and remarkably electron dense cristae (arrows). Note their localization near to endoplasmic reticulum profiles (asterisk) and nuclei (N). Bar: 0.83 μm. Method of staining: uranyl acetate/lead citrate.

to check the glutaraldehyde diffusion rate and the brownish coloration of the surface and deeper sample regions, indicative of good glutaraldehyde fixation by an immersion technique. Immersions for 2 h in a fresh glutaraldehyde solution of 1 mm slices were done. Secondary fixation in 1% osmium tetraoxide, 0.1M phosphate buffer, pH 7.4, for 1–2 h at 4°C, was carried out. Black staining of the sample slices was also observed under a stereoscopic microscope to check the osmium tetraoxide diffusion rate and the quality of secondary fixation. The samples were washed for 5–10 min in phosphate or cacodylate buffer of similar composition to that used in the fixative solution, dehydrated in increasing concentrations of ethanol and embedded in Araldite. For proper orientation during the electron microscope study and observation of tumoral specimens, ~0.1- to 1-μm-thick plastic sections with toluidine blue and examined with a Zeiss photomicroscope

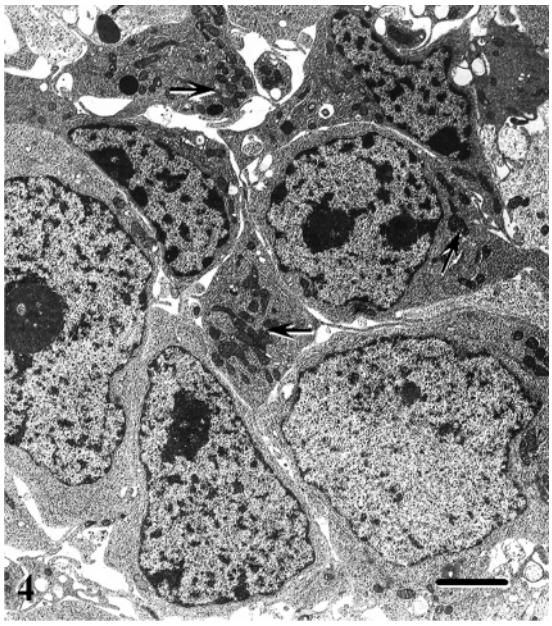

Fig. 4. Glioblastoma multiforme. Several undifferentiated neoplastic cells that show numerous mitochondria with increased thickness and remarkably electron dense cristae (arrows). Bar: 1 μm. Method of staining: uranyl acetate/lead citrate.

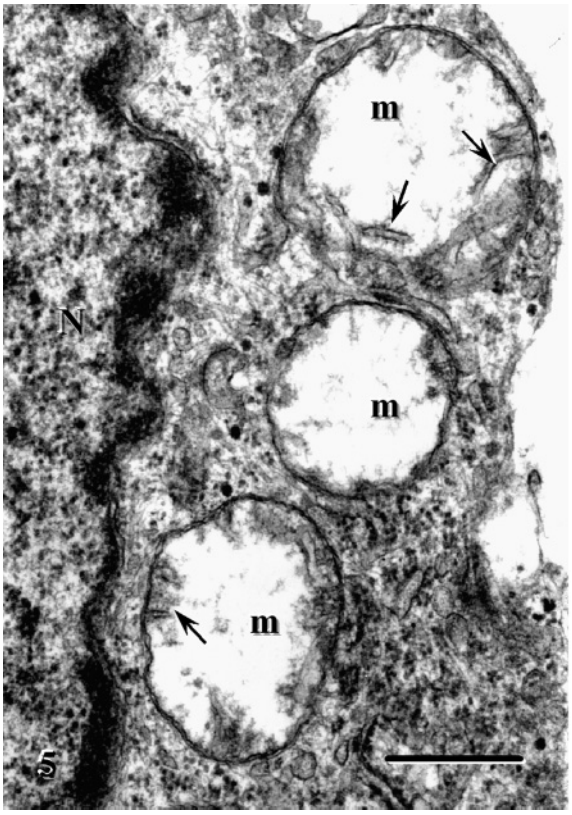

Fig. 5. Glioblastoma multiforme. A neoplastic cell with several mitochondrion (m) that exhibit cristolysis (arrows). Bar: 0.8 μm. Method of staining: uranyl acetate/lead citrate.

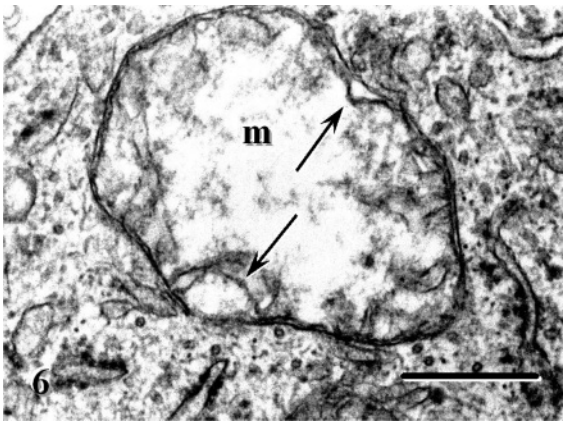

Fig. 6. Glioblastoma multiforme. Enlarged and piriform mitochondrion (m) that shows total cristolysis and electron-lucent matrix. Note the inner membrane fold (arrows). Bar: 0.33 μm. Method of staining: uranyl acetate/lead citrate.

were stained. Ultra-thin sections, obtained with Sorvall R MT 5000 ultramicrotome, with uranyl acetate and lead citrate were stained and observed in a JEOL 100B and Mitsubishi H-7000 transmission electron microscopes at magnifications ranging from ×8000 to ×80 000. Under the electron microscope, the good preservation of ultrastructure of specimens by means of classical criteria was evaluated. Digital images with Corel-Paint software were processed.

Results

No obvious or specific ultrastructural differences between the different grades of tumors were seen. In all the cases, mitochondrial swelling associated with disarrangement of cristae and partial or total cristolysis (Figs. 1, 2, 5 and 6) characterized the most constant submicroscopic alterations observed in mitochondria. However, mitochondria show variability in the abnormalities in number, size and shape, included in the same specimen, as well as the degree of severity of internal ultrastructural mitochondrial changes (Figs. 1 and 2). Some mitochondria exhibited cigar, bowling-pin, 'L', 'V', 'Y' and irregular shapes (Figs. 1, 2 and 7). Mitochondria with dense matrix were seen (Figs. 2 and 7). The mitochondria were localized predominantly in cellular bodies, close to the nuclear membrane and rough endoplasmic reticulum (Figs. 2 and 3). Lipid droplets between or near to the mitochondria were seen (Fig. 1). In contrast, in cell processes the presence of mitochondria was inconspicuous. The 'normal' rod-shaped mitochondria with clearly defined and closely apposed outer membranes and intact cristae organized perpendicular to the long axis of the mitochondrion, occasionally, were observed. In pilocytic astrocytomas particularly, in addition to swelling mitochondria, mitochondria with increased thickness and remarkably electron dense cristae were seen (Fig. 3), as well as in undifferentiated neoplastic cells (Fig. 4). In fibrillary astrocytomas, anaplastic astrocytoma and glioblastoma multiforme, mitochondrial

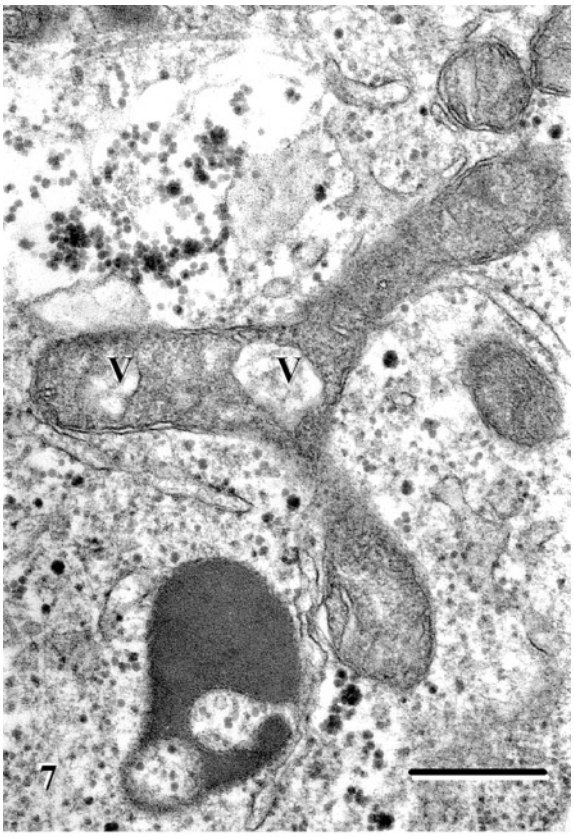

Fig. 7. Fibrillary astrocytoma. Mitochondrion display 'Y' shape that exhibits matrix condensation, subtotals cristolysis and vacuoles (V). Bar: 0.66 μm. Method of staining: uranyl acetate/lead citrate.

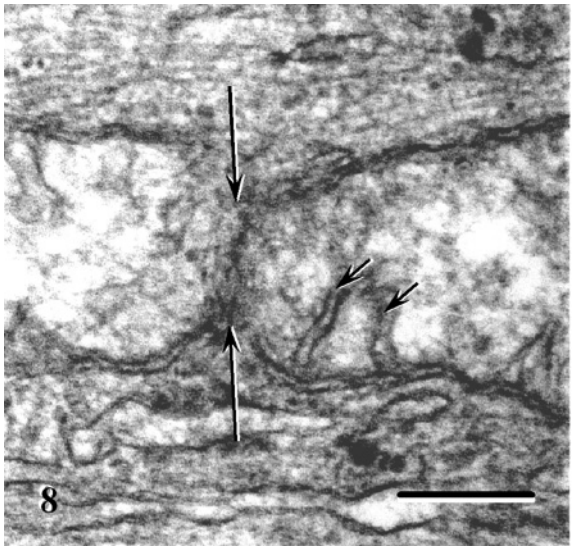

Fig. 8. Glioblastoma multiforme. Fusion–fission mitochondrial phenomena in two swelling mitochondria; both show clear matrix and cristolysis (short arrows). Note the union of mitochondrial membranes (opposed arrows). Bar: 0.25 μm. Method of staining: uranyl acetate/lead citrate.

swelling associated with disarrangement of cristae and partial or total cristolysis was the most constant ultrastructural finding (Figs. 1, 2, 5 and 6). The presence of enlarged mitochondria was characterized (Fig. 2). In a few mitochondria were seen folds of inner mitochondrial membranes (Fig. 6). Matricial condensation and existence of vacuoles were also seen (Fig. 7). Mitochondrial fusion–fission phenomena (Fig. 8) and presence of amorphous matricial densities were categorized. Ultrastructural morphologic criteria suggestive of apoptosis according to morphologic feature established previously were infrequently observed [13] (i.e. cellular shrinking, condensation and margination of the chromatin and ruffling of the plasma membrane, apoptotic bodies).

Discussion

In this investigation we observed that the mitochondria in specimens of human astrocytic tumors exhibit heterogeneous ultrastructural pathology. Some of the changes in the mitochondria observed in this study had been earlier reported in human carcinomas [14,15], Warthin's tumor [16], in human xenografted gliomas [10], in human malignant glioma cells [17], in HeLa human cancer cell lines [11] and in clones of cancer cells with mitochondrial respiration defects [18]. Mitochondria participate in a variety of cellular processes, indicating that the control of their dynamic shape is probably multifactorial and their impact on cell activity very diverse [19]. There is growing evidence of a close relationship between the energy production and the existence of a mitochondrial network in living human cells [19]. The mitochondrial network is very dynamic with typical conformations shifting between a fragmented state and tubular continuum [20, 21]. The heterogeneous ultrastructural pathology could be representing an altered mitochondrial network.

We postulate that this finding in human astrocytic tumors is agreeable with the characteristic topographic variation of cellular constituency observed in gliomas [22,23]. Another reason possibly is the frequent presence of mutations and instability of mitochondrial DNA in malignant gliomas [24], since Holmunhamedov *et al.* [25] reported that the deletion of mitochondrial DNA produces functional and morphological changes in mitochondria, and Dimitrenko *et al.* [26] reported general inactivation of the mitochondrial genome in glioblastoma. Pathological mutations in genes responsible for mitochondrial fusion or fission have been associated with alterations in the organization of the mitochondrial network and with the inhibition of energy metabolism [27,28]. Finally, the variations in intra-tumoral conditions would be an added factor. However, the leading mitochondrial change detected was mitochondrial swelling with disarrangement and distortion of cristae and partial or total cristolysis. The mitochondrial swelling with distortion of the cristae is associated with hypoxic–ischemic conditions [17]. Hypoxic microenvironment is a characteristic of human gliomas [7–9]. For these reasons, standardized methods for processing were used in this study in order to avoid any artifactual changes in specimens, because mitochondria are particularly

sensitive to environmental modifications. Gilkerson *et al*. [29], using immunolabeling and transmission electron microscopy of bovine heart tissue, established that the cristae membrane of mitochondria is the principal location of the oxidative phosphorylation process. Diversity of tumors exhibits an evident diminution in mitochondrial content [10,15] and in oxidative phosphorylation capacity [30,31]. Therefore, the prevalent existence of partial or total crystolisis observed in this study suggests consequently that the ability of human astrocytomas to generate ATP by mitochondrial oxidative phosphorylation would be compromised severely. In addition, this would deteriorate the ability of astrocytomas cells to commit apoptosis. This finding is congruent with infrequently observed ultrastructural morphologic changes suggestive of apoptosis (i.e. cellular shrinking, condensation and margination of the chromatin and ruffling of the plasma membrane, apoptotic bodies) observed in our investigation. This aspect is consistent with the fact that the apoptosis is generally low in gliomas [17,32]. On the other hand, Cuezva *et al*. [15] point out that cancer cells with a low bio-energetic index are more resistant to apoptosis. Finally, Cerruti *et al*. [33] reported resistance of human astrocytoma cells to apoptosis due to an intrinsic defect of caspase-9.

In this study, we observed astrocytoma cells with predominance of mitochondria with dense matrix displayed in closed groups (Fig. 2, 4), while in the other astrocytomas, cells lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis were the principal finding (Figs. 2, 5 and 6). Earlier, according to Ikreyi *et al*.'s [34] metabolic and electron microscopic studies, post-mortem of brain mitochondria indicates that the mitochondrial dense appearance is the functional form, while the light mitochondrial structure is a transition of the non-functioning homogenous type of brain mitochondria. Recently, Rossignol *et al*. [11] illustrate that mitochondria of HeLa cells and fibroblast adopt a condensed configuration when producing energy by oxidative phosphorylation. Parliament *et al*. [35] postulate that glioma cell lines behave as 'oxygen conformers' in that their rate of oxygen consumption appears to vary with the availability of oxygen. Turcotte *et al*. [36] demonstrate variation in mitochondrial function in hypoxia-sensitive and hypoxia-tolerant human glioma cells. We consider that possibly the astrocytoma cells with dense mitochondria are hypoxia-tolerant cells, therefore, are 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, are incompetent to produce adequate amount of ATP by mitochondrial respiration. These considerations would be implicated therapeutic targets and clinical implications. Presumably, the astrocytoma cells with dense mitochondria represent a sensible target to inhibition or down-regulation of mitochondrial respiration, with the objective of inducing ATP depletion and finally the cell death in this type of cells, because mitochondrial insult or failure can rapidly lead to inhibition of cell survival and proliferation [37]. Xu *et al*. [18] applied inhibition of glycolysis in cancer cells with mitochondrial respiratory defect. On the other hand, in the astrocytoma cells with lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis, the glycolytic inhibition perhaps appears as an effective therapeutic strategy for eradicating this variety of cells, considering that the glycolysis is the principal energetic source in glioma cells [10,38]. In accordance with Noble and Dietrich [39], tumors cannot even be treated as a homogeneous mass of identical cells.

In addition, in this study we reported the inconspicuous presence of mitochondria in astrocytoma cell processes. This finding probably implies that at this level, the energy derived of mitochondrial respiration is marginal. Beckner *et al*. [40] reported that glycolytic enzymes are abundant in pseudopodia formed by U87 astrocytoma cells and glycolysis alone can support glioma cell migration.

In addition, lipid droplets (predominantly) between or near to the lucent-swelling mitochondria with disarrangement and distortion of cristae and partial or total cristolysis were observed in this study. The lipid droplets and the reduced surface density of mitochondrial cristae are likely to be indicators of a reduction in mitochondrial metabolic activity [41]. Ultrastructural investigations of Delikatny *et al*. [42] revealed substantial damage to mitochondria and the progressive development of lipid droplets induced by cationic lipophilic chemotherapeutic agents. Quintero *et al*. [43] reported a higher accumulation of cytosolic droplets in C6 glioma cells with a state of proliferation arrest induced by growth factors deprivation. Hypoxic cell death in glioma cells resembles death induced by cytotoxic drugs [17].

In this paper, we reported mitochondrial fusion–fission phenomena (Fig. 8). We designate like fusion–fission phenomena for the static character of this study. The incidence of this event was considered infrequent for us. These mitochondria exhibit edematous changes and cristolysis; morphological changes suggest loss of the mitochondrial inner membrane potential and serious defect in the respiratory chain. To the best of authors' knowledge, this finding in astrocytomas has not been previously reported. Mitochondrial fusion in mammalian cells requires an intact mitochondrial inner membrane potential [44]; also an efficient respiration process is necessary for the maintenance of mitochondrial inner membrane potential and vice versa [45]. Consequently, we postulate that the most reasonable is that these observations correspond to mitochondrial fission. One of the steps in apoptosis is the mitochondrial fragmentation. Fragmentation of the mitochondrial network appears to occur in situations where the mitochondrial inner membrane potential is decreased or abolished [19]. Recent evidence indicates that the mitochondrial fission machinery actively participates in the process of programmed cell death [46–49]. The low frequency of mitochondrial fission observed in this study is in concurrence with the fact that the apoptosis is generally low in malignant gliomas [32]. The mitochondrial fission is

essential for normal oxidative phosphorylation function and the perturbation of mitochondrial network dynamics, via fusion or fission disruption is likely to induce the impairment of the mitochondrial energy production [19]. In addition, survivin is a recently described molecule that is expressed in most human cancers and acts as an inhibitor of apoptosis in cancer and coordinates a pathway of apoptosis inhibition [50,51]. We assume that mitochondria in astrocytomas probably produce survivin; this potentially explains in part the low occurrence of mitochondrial fission and ultrastructural morphologic changes suggestive of apoptosis observed in our investigation. Added argument is that alterations in the mitochondrial network are associated with a reduction in sensitivity to apoptosis inducers [52].

Concluding remarks

The pathological transformations exhibited by mitochondria in human astrocytic tumors are heterogeneous, probably, attributable to both the cellular variability and diversity of microenvironment conditions of this kind of tumors.

On the other hand, mitochondrial ultrastructural pathology observed in this study suggests that possibly both the glycolytic inhibition and inhibition or down-regulation of mitochondrial respiration would be a potential tool for future therapeutic strategies in cases of human astrocytic tumors. Further, molecular and experimental investigations could be done with the intention of providing evidence for this probability.

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