Differences in Mitochondrial Function and Antioxidant Systems between Regions of Human Glioma

Francisca M. Santandreu¹, Marta Brell³, Alexandra H. Gene², Rocío Guevara¹, Jordi Oliver¹, Marta E. Couce² and Pilar Roca¹

¹Grup de Metabolisme Energètic i Nutrició, Departament de Biologia Fonamental i Ciències de la Salut; Institut Universitari d'Investigació en Ciències de la Salut (IUNICS), Universitat de les Illes Balears, Palma de Mallorca; and Ciber Fisiopatología Obesidad y Nutrición (CB06/03), Instituto de Salud Carlos III, ²Servicio de Anatomía Patológica, Hospital Universitario Son Dureta (HUSD), Palma de Mallorca, ³Servicio de Neurocirugía, Hospital Universitario Son Dureta (HUSD), Palma de Mallorca

Key Words

Reactive oxygen species • Human glioma • Redox behaviour • Oxidative metabolism • Manganese-superoxide dismutase • Fresh tissue • Intratumoral regions • Isolated mitochondria

Abstract

Metabolic features and oxidative stress have been extensively studied in cancer cells. However, comparative studies between cancer cell populations that coexist in human neoplastic tissue are not frequently available. The aim of the present study was to characterize markers of oxidative status and mitochondrial function in center vs. periphery of human fresh glioma samples; therefore, antioxidant systems, oxidative stress and mitochondrial parameters were assessed in gross total resections of gliomas. Mitochondrial protein and mitochondrial DNA content, enzymatic activities of mitochondrial oxidative and phosphorylative system, antioxidant mechanisms, mitochondrial H₂O₂ production, oxygen consumption and cellular oxidative damage were measured in human gliomas. Concentric regions of human glioma tissue showed similar mitochondrial structural markers; conversely, the functionality of their isolated mitochondria was significantly different. In this way, the tumor

periphery exhibited higher respiratory rate and fewer antioxidant systems than tumor center. Our results have expanded previous investigations, which report the presence of cell populations with different oxidative susceptibility in human brain tumor samples. This is, to our knowledge, the first study to investigate metabolic differences in concentric regions of gross total resections of glioma. Interestingly, the cancer cell population that exhibits an increased antioxidant capacity within the tumor mass might be responsible for tumor resistance to chemotherapy and radiotherapy.

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Introduction

Mitochondria are involved in a strikingly diverse range of disease processes. In this sense, mitochondrial dysfunction is increasingly recognized as a contributor to common diseases, such as cancer; they have multifactorial pathogenesis and a clear relationship to oxidative stress of mitochondrial origin. The mitochondrion is the main source of cellular ATP but it is much more than this; this organelle is intimately involved in the life and death of the cell, capable of integrating pro- and anti-apoptotic

signals and committing the cell to apoptosis. Mitochondrial research has contributed to two paradigm shifts in oncology: the first was the pioneering research by Warburg showing that cancer cells often rely heavily on glycolytic metabolism, even in the presence of an adequate oxygen supply [1]; the second paradigm is the appreciation that the ability of cancer cells to avoid apoptosis contributes to their limitless replicative potential and limits the efficacy of cancer chemotherapy [2]. On the other hand, this organelle is the only one apart from the nucleus to contain DNA. Mitochondrial mutations and profound mitochondrial ultrastructure alterations have been reported to exist in gliomas [3, 4]. Besides, primary mutations in mitochondrial housekeeping genes such as fumarate hydratase and succinate dehydrogenase have been described to play an important role in cancer pathogenesis [5, 6]. Additionally, recent studies have shown that tumors frequently bear homoplasmic mitochondrial DNA (mtDNA) mutations that may contribute to tumor promotion.

The ability of certain tumor cells to sustain either metabolic alterations, chronic hypoxia or chronically elevated oxidative stress are factors that help define the hallmarks of cancer [7, 8].

Although tumor invasion has been intensely investigated, many aspects of cancer biology remain poorly understood [9]. In this way, tumor growth is a complex process dependent on tumor cells proliferating and spreading in host tissues. Interestingly, it has been described that cell growth in human cancer is limited to the tumor border [10].

During cellular metabolism reactive oxygen species are produced by mitochondrial electron transport, by the cellular redox system and by immune responses. At concentrations actually compatible with the physiology of the cell, reactive oxygen species (ROS) appear able to exert, as intracellular messengers, a large variety of biochemical effects which may contribute to modulation of cell viability and function [11]. However, long-term exposure of a human cell to ROS may elicit serious consequences of enhanced oxidative damage and cell death in affected tissues [12].

It is worth noting that most primary brain tumors derive from glial cells and are collectively called gliomas. Malignant gliomas are rapidly growing tumors. The ones with highest WHO grade (grade IV) generally have vascular proliferation with malformed vessels and/or areas of necrosis and hypoxia. These tumors are among the most challenging cancers to treat and the high grade ones have a particularly poor prognosis due to their excep-

tional ability to infiltrate normal brain [13]. This feature makes complete surgical resection virtually impossible [14]; and even after complete surgical excision, high grade gliomas almost always grow back. However, it has been reported that an aggressive surgical procedure for this primary brain cancer can result in increased survival for selected groups of patients [15]. Treatment for most brain tumors depends on the location and the grade and is often a combined approach, using surgery, radiation therapy, and chemotherapy. On the other hand, radiotherapy and anticancer drugs increase reactive oxygen species in cancer cells or can lead to an inhibition of antioxidant enzymatic activities [16]. Furthermore, cancer cells may respond to the oxidative stimuli and allow biochemical adaptations to oxidative stress. In this sense, the increase in antioxidant systems has been postulated as a mechanism by which tumoral cells become resistant to antineoplastic agents.

Taking this into account, the specific aim of this study was to compare in fresh tissues the mitochondrial metabolism features and oxidative status between cells in the tumor center and tumor periphery from maximal surgical resections of human gliomas.

Materials and Methods

Materials

Routine chemicals used were supplied by Sigma-Aldrich (St. Louis, USA), Panreac (Barcelona, Spain) and Amersham Pharmacia Biotech (Little Chalfont, UK). Real-time PCR reagents and oligonucleotide primer sequences were supplied by Roche Diagnostics (Basel, Switzerland) and Amplex Red reagent by Molecular Probes (Paisley, UK).

Subjects and human brain tumor samples

The study protocol was approved by the Ethics Committee of the Son Dureta University Hospital and all subjects gave their written consent to participate in the present study. All human tumors evaluated had a signed patient consent form on file for the study. These tumor specimens were surgically removed in one block and evaluated for intraoperative diagnosis. After the diagnosis was established, concentric tissue samples were removed, therefore representative sections from central and peripheral regions were taken fresh for mitochondrial and oxidative studies. Another part was frozen, and fixed in buffered formalin for histological evaluation; the purity of each area was estimated by using hematoxylin and eosin staining to ensure that each region contained > 65% of neoplastic cells. This was carried out by a pathologist (MEC) at Son Dureta University Hospital (Palma de Mallorca, Spain). Maximal surgical resections of gliomas were obtained from 6 patients aged 20-79 years (4 men and 2 women) as shown in Table 1. Mitochondria from the 12 glioma samples were rapidly isolated after

surgical procedure. Specifically, mitochondrial isolation was started at the hospital 15 minutes after neurosurgery and was completed within 35 minutes of tumor removal.

Mitochondrial isolation

Isolation of glioma mitochondria was performed at 4 °C using a procedure based on differential centrifugation. After neurosurgery, gross total resection specimens were immediately transported, on ice, to the pathology lab and were rapidly dissected on ice-cold surface. Each region of the tumor was then weighed, finely minced, and diluted 20 times with ice-cold isolation medium (250 mM sucrose, 10 mM Tris-HCl, 0.5 mM EDTA-K⁺, pH 7.4). Each sample was homogenized using 24 up-and-down strokes in a manual homogenizer with a glass pestle (clearance 0.1 mm). An aliquot of homogenate from each region was stored at 4 °C until functional measurements were carried out. The remaining homogenate was centrifuged for 3 min at 2000xg; then, the supernatant was collected and centrifuged again for 3 min at 2000xg; the supernatant obtained at this time was centrifuged for 8 min at 12,500xg; finally, the supernatant was discarded and the pellet was resuspended in ice-cold isolation buffer at a final concentration of 5-10 mg mitochondrial protein per milliliter, determined by Bradford's method [17]. The crude mitochondrial fractions were stored at 4 °C until mitochondrial functionality was assayed (H₂O₂ production, respiration and enzymatic activities). The homogenate was used to determine lipid peroxidation, glutathione levels, lactate dehydrogenase (LDH) activity, total protein content and also total citrate synthase (CS) and total cytochrome *c* oxidase (COX) activities.

Enzymatic activities

All spectrophotometric and fluorimetric determinations were assayed in microtiter plate and were performed as previously described protocols with some modifications. LDH activity was an adaptation of Vassault method [18]. The CS assay was based on the technique described by Nakano et al. [19]. CS activity was measured in homogenates and mitochondrial fractions in order to calculate the mitochondrial recovery. Oxidative phosphorylation system (OXPHOS) activities were assessed in resuspended mitochondrial pellet. COX activity was determined by Chrzanowska-Lightowlers et al. protocol [20]. Complex V or F1F0-ATP synthase (ATPase) was assayed by an adaptation of Ragan et al. method [21]. Manganese-superoxide dismutase (MnSOD) activity was assayed by Quick et al. method [22]. Glutathione peroxidase (GPx) and glutathione reductase (GRd) were assayed by modified Smith et al. method [23] and Wen et al. method [24], respectively. Antioxidant enzymatic analysis (GPx, GRd, MnSOD and total superoxide dismutase activities) were performed in the supernatant of samples previously disrupted by sonication (10,000 g for 10 min).

Measurement of mitochondrial respiration

Mitochondrial respiration was measured polarographically, with minor modifications as described previously by Lopez-Torres et al. assay [25]. Briefly, mitochondria were incubated in a water-thermostatically regulated chamber with a com-

Surgical cases	Sex	Age	Diagnosis	Glioma recurrence
1	F	69	glioblastoma (astrocytoma grade IV)	no
2	M	75	glioblastoma (astrocytoma grade IV)	no
6	M	60	anaplastic oligodendroglioma (grade III)	yes
7	F	79	glioblastoma (astrocytoma grade IV)	no
9	M	23	astrocytoma grade II	no
10	M	20	astrocytoma grade III/IV	no

Table 1. Human brain tumor samples

puter-controlled Clark-type O₂ electrode (Oxygraph; Hansatech, Norfolk, UK) at a concentration of 0.5-1 mg mitochondrial protein/ml in respiration buffer containing 145 mM KCl, 30 mM HEPES, 5 mM KH₂PO₄, 3 mM MgCl₂, 0.1 mM EGTA, and 0.1% bovine serum albumin free fatty acids (pH 7.4 at 37 °C). State 4 respiration (absence of ADP) was measured using pyruvate-malate (5 mM/2.5 mM) and succinate (5 mM) as substrates. State 3 respiration was initiated by adding ADP (500 μM). The ratio [state 3 rate]:[state 4 rate] of mitochondrial preparations was also checked; this respiratory control index (RCR) indicates the tightness of the coupling between respiration and phosphorylation.

Detection of mitochondrial H₂O₂ production capacity

The rate of H₂O₂ production in mitochondria was determined by the fluorescent probe Amplex Red as described previously [26]. Mitochondria (0.4 mg protein/ml) were incubated at 37 °C in respiration buffer containing 0.1 U/ml horseradish peroxidase and 50 μM Amplex Red. Modulation of H₂O₂ production capacity by rotenone (2 μM), antimycin (5 μM), ADP (500 μM) or GDP (500 μM) was also assessed. H₂O₂ production was initiated by adding the substrate (succinate (5 mM) or pyruvate-malate (5 mM/2.5 mM)); background fluorescence was measured in parallel in wells containing all reactants except substrate. Fluorescence was measured in a microplate reader (FLx800; Bio-Tek Instruments, Winooski, VT) with 530 nm excitation and 590 nm emission wavelengths. Fluorescence rate was expressed as H₂O₂ production using a standard curve generated with known concentrations of H₂O₂ stabilized solution.

Glutathione assay

Glutathione levels were measured in the supernatant of deproteinized tissue lysates by a modified 5,5'-dithiobis(2-nitrobenzoic) acid (DTNB) - GSSG reductase recycling assay [27]. Reduced-/oxidized glutathione ratio (GSH/GSSG) was calculated.

Quantification of human mitochondrial DNA

mtDNA was extracted by digestion with proteinase K (100 μg/μl) in a buffer containing 50 mM KCl, 10 mM Tris-HCl, 2.5 mM MgCl₂ and 0.5% Tween 20. Mitochondrial samples were incubated overnight at 37 °C and then boiled for 5 min in order to inactivate the enzyme. mtDNA was linearised by digestion with Bcl I restriction enzyme for 3 h at 50 °C and then boiled for 5 min. Samples were centrifuged at 10,000 rpm for 5 min and the resulting supernatant was used for amplification. A quantitative PCR assay was adapted to the LightCycler technology from previous methods [28, 29]. PCR was performed to amplify

Table 2. Biochemical parameters and mitochondrial markers. Values are the means $\pm$ s.e.m. from 5-6 regions per group; data are expressed per gram of fresh tissue. No significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test). a The low LDH activity in mitochondrial fractions indicates the purity of isolated mitochondria.

	Center	Periphery	n	p
Total protein (mg/g)	54.2 $\pm$ 8.3	54.5 $\pm$ 8.9	6	0.466
Mitochondrial DNA (a.u./g)	21.8 $\pm$ 9.8	20.0 $\pm$ 8.8	5	0.184
Mitochondrial protein (mg/g)	23.2 $\pm$ 3.3	22.9 $\pm$ 2.9	6	0.362
Mitochondrial protein/mtDNA (a.u.)	8.20 $\pm$ 3.81	9.69 $\pm$ 4.36	6	0.342
Total COX activity (a.u./min·g)	11.3 $\pm$ 1.8	12.2 $\pm$ 1.8	5	0.307
Total CS activity (IU/g)	2.59 $\pm$ 0.68	2.87 $\pm$ 0.67	6	0.319
Total LDH activity (IU/g)	23.9 $\pm$ 3.0	22.3 $\pm$ 4.0	6	0.324
Mitochondrial LDH activity ^a (IU/g)	0.71 $\pm$ 0.13	0.78 $\pm$ 0.15	6	0.284

Table 3. OXPHOS activities. Values are the means $\pm$ s.e.m. from 6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test).

	Center	Periphery
COX activity (a.u./min·mg mt protein)	455 $\pm$ 106*	596 $\pm$ 129
ATPase activity/COX activity	0.85 $\pm$ 0.21*	0.66 $\pm$ 0.18
ATPase activity (mIU/mg mt protein)	318 $\pm$ 44	324 $\pm$ 39
ATPase activity (IU/g)	8.19 $\pm$ 1.67	10.0 $\pm$ 2.75

a 192-nts fragment of the human mitochondrial NADH dehydrogenase subunit 4 gene. The primer sequences were 5'-CCT GAC TCC TAC CCC TCA CA-3' and 5'-ATC GGG TGA TGA TAG CCA AG-3'. The concentration of the purified template was determined spectrophotometrically. Increasing amounts of template were amplified in parallel reactions to obtain a standard curve. Amplification was carried out in a LightCycler rapid thermal cycler system (Roche, Switzerland) using a total volume of 10 μ l containing 0.375 μ M of each primer, 3 mM MgCl₂, 1 μ l LightCycler-FastStart DNA Master SYBRTM Green I and 2.5 μ l of sample prepared as described above. The PCR reactions were cycled 32 times after initial denaturation (95 °C, 10 min), with the following parameters: denaturation at 95 °C for 10 s, annealing at 68 °C for 12 s, extension at 72 °C for 12 s.

Quantification of human mtDNA oxidative damage by realtime PCR

Many mtDNA lesions are located in the mitochondrial displacement loop region (D-loop) and in particular in a polycytidine stretch (C-tract). There are large differences in the susceptibility to damage of various regions of mtDNA. Moreover, the D-loop is highly susceptible to mutations because of its vulnerability to DNA damage and inefficient repair mechanisms. The purpose of this assay was to examine the ROS-induced DNA damage in the mitochondrial genome, based on previous methods [30-33]. The human mtDNA primer sequences were: 5'-AGT GCA TAC CGC CAA AAG AT-3' and 5'-GGC CAC AGC ACT TAA ACA-3' for a 99-nts fragment that we called sF; 5'-AGT GCA TAC CGC CAA AAG AT-3' and 5'-AGC CAC TTT CCA CAC AGA CA-3' for a 162-nts fragment that we called lF. The large fragment (lF), which includes the C-tract region, is more sensitive to oxidative damage than the small fragment (sF); in previous experiments, we observed that its amplification was delayed when compared with sF after exposure to DNA-damaging agents (H₂O₂) in a dose-dependent manner. Pre-treatment of mitochondrial pellet suspensions to obtain mtDNA was carried out as described above for the quantification of mtDNA. Amplification was performed in a LightCycler rapid thermal cycler system using a total volume of 10 μ L containing 0.375 μ M of each primer, 3 mM MgCl₂, 1 μ l LightCycler-FastStart DNA Master SYBRTM Green I and 2.5 μ L of sample. PCR reactions were cycled 32 times after initial

	Center	Periphery
H ₂ O ₂ S4 succinate (pmol/min·mg)	15.8 $\pm$ 11.1	21.4 $\pm$ 9.0
H ₂ O ₂ S4 succinate (pmol/min·g)	456 $\pm$ 269	603 $\pm$ 220
H ₂ O ₂ S4 GDP+succinate (pmol/min·mg)	15.4 $\pm$ 9.9	32.3 $\pm$ 16.6
H ₂ O ₂ S4 GDP+succinate (pmol/min·g)	442 $\pm$ 243	911 $\pm$ 373
H ₂ O ₂ S4 succinate+R+AA (pmol/min·mg)	20.9 $\pm$ 8.9	22.2 $\pm$ 5.8
H ₂ O ₂ S4 succinate+R+AA (pmol/min·g)	551 $\pm$ 196	625 $\pm$ 139
H ₂ O ₂ S4 pyr-mal+R (pmol/min·mg)	7.76 $\pm$ 3.35	17.2 $\pm$ 5.4
H ₂ O ₂ S4 pyr-mal+R (pmol/min·g)	264 $\pm$ 100	525 $\pm$ 147

Table 4. Mitochondrial H₂O₂ production capacity was measured in absence of ADP (S4). Succinate 5 mM was used as substrate with or without GDP 500 μ M, rotenone 2 μ M and antimycin A 5 μ M; rate of H₂O₂ production with pyruvate-malate (5 mM/2.5 mM) was measured in the presence of rotenone 2 μ M. Values are the means $\pm$ s.e.m. from 4 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test). R, rotenone; AA, antimycin A; pyr-mal, pyruvate-malate; S4, state 4.

	Center	Periphery
O ₂ consumption S4 pyr-mal (nmol/min·mg)	4.29 $\pm$ 1.25	5.21 $\pm$ 0.79
O ₂ consumption S4 pyr-mal (nmol/min·g)	77.4 $\pm$ 27.8	106 $\pm$ 26
O ₂ consumption S3 pyr-mal (nmol/min·mg)	9.29 $\pm$ 5.83	11.2 $\pm$ 5.49
O ₂ consumption S3 pyr-mal (nmol/min·g)	189 $\pm$ 137	250 $\pm$ 141
O ₂ consumption S4 succinate (nmol/min·mg)	8.19 $\pm$ 2.82*	9.35 $\pm$ 2.55
O ₂ consumption S4 succinate (nmol/min·g)	165 $\pm$ 47	193 $\pm$ 40
O ₂ consumption S3 succinate (nmol/min·mg)	13.0 $\pm$ 5.7	14.9 $\pm$ 4.6
O ₂ consumption S3 succinate (nmol/min·g)	269 $\pm$ 123	320 $\pm$ 107

Table 5. Mitochondrial oxygen consumption. Mitochondrial oxygen consumption was assessed under state 4 (absence of ADP) or state 3 (500 μ M ADP); succinate 5 mM or pyruvate-malate (5 mM/2.5 mM) were used as substrates. Values are the means $\pm$ s.e.m. from 4-5 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test). S3, state 3; S4, state 4; pyr-mal, pyruvate-malate.

denaturation (95 °C, 10 min), with the following parameters: 95 °C for 10 s, annealing at 59 °C for 12 s, extension at 72 °C for 12 s. After the amplification of mitochondrial specific regions,

IF/SF ratio for each sample was calculated by dividing the Ct of its large fragment by the Ct of its small fragment. There is a positive correlation between the increase in this ratio and mtDNA oxidative damage in the analysed sample. All oligonucleotide primer sequences were obtained from Primer3 and tested with IDT OligoAnalyser 3.0. Finally, a basic local alignment search tool (NCBI Blast) revealed that the primer sequence homology was obtained only for the target genes.

Measurement of thiobarbituric acid-reactive substances (TBARS)

Lipid peroxidation was determined in homogenates as malondialdehyde-thiobarbituric acid adducts according to Buege et al. [34]. The assay was performed spectrophotometrically at 532 nm, using a molar extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Protein carbonyl derivatives determination

Protein carbonyl groups were measured by an adaptation of previous methods [35, 36] using the precipitates of deproteinized samples. Protein pellets were resuspended with 2,4-dinitrophenylhydrazine (DNPH) 10 mM, and incubated for 60 min at room temperature. Then, 20% trichloroacetic acid was added, and samples were centrifuged 10 min at 10,000 g at 4 °C. After the final wash, protein pellets were resuspended in 300 µl of 3 M guanidine hydrochloride in 2 mM phosphate buffer (pH 2.3) by vortexing. Finally, the absorbance of the supernatant was measured at 360 nm. The molar absorption of 22,000 $\text{M}^{-1} \cdot \text{cm}^{-1}$ was used to quantify total protein carbonyl levels. Samples were analyzed against a blank of guanidine solution and protein content was determined after the washing steps.

Statistics

Data were expressed as mean $\pm$ s.e.m. ($n = 6$ regions/group). Intratumoral differences were analyzed by paired Student's t-test using the Statistical Program for the Social Sciences software for Windows (SPSS, Version 15.0). Statistical significance was set at $P < 0.05$.

Results

Glioma tissue composition and mitochondrial content

The isolation procedure was chosen due to the need for a rapid method to obtain sufficient volume of crude mitochondrial pellet to immediately assess ROS production and oxygen consumption, as well as to ensure the same mitochondrial recovery between the tumor regions studied (center (32.5 ± 5.0 %) vs. periphery (33.9 ± 5.1 %)). All glioma tissues were highly vascular in both regions; similar cellularity was observed in both areas by using hematoxylin and eosin staining of contiguous sections. Tissue protein expressed as milligram of total protein per gram of fresh tissue was almost the same in the central and peripheral regions (54.2 ± 8.3 vs. 54.5 ± 8.9 ,

	Center	Periphery
TBARS content (nmol/mg total protein)	390 ± 92	263 ± 47
TBARS content (µmol/g)	23.4 ± 6.7	14.5 ± 3.1
Oxidized mtDNA (a.u./mg mt protein)	1.51 ± 0.01	1.52 ± 0.00
Oxidized mtDNA (a.u./g)	35.1 ± 4.9	34.8 ± 4.4
Protein carbonyl groups (µmol/mg total protein)	250 ± 95	221 ± 39
Protein carbonyl groups (µmol/g)	6.26 ± 2.76	5.33 ± 1.27

Table 6. Oxidative damage markers. Values are the means $\pm$ s.e.m. from 6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test).

	Center	Periphery
GSH/GSSG	$5.23 \pm 1.23^*$	2.55 ± 0.61
GSSG (nmol/mg total protein)	1.42 ± 0.41	1.46 ± 0.33
GSSG (nmol/g)	86.8 ± 26.9	89.7 ± 25.5
GSH (nmol/mg total protein)	5.84 ± 1.68	3.35 ± 0.84
GSH (nmol/g)	$333 \pm 104^*$	181 ± 53

Table 7. Glutathione content. Values are the means $\pm$ s.e.m. from 6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test).

	Center	Periphery
MnSOD activity (mIU/mg mt protein)	$10.9 \pm 3.7^*$	8.61 ± 3.65
MnSOD activity (mIU/g)	$306 \pm 136^*$	242 ± 126
GPx activity (µIU/mg mt protein)	28.6 ± 12.8	21.9 ± 8.2
GPx activity (µIU/g)	695 ± 373	487 ± 219
GRd activity (µIU/mg mt protein)	5.93 ± 0.69	5.74 ± 0.43
GRd activity (µIU/g)	130 ± 25	123 ± 19
Total SOD activity (mIU/mg total protein)	10.1 ± 2.8	7.03 ± 1.90
Total SOD activity (mIU/g)	532 ± 176	343 ± 95

Table 8. Antioxidant activities. Values are the means $\pm$ s.e.m. from 5-6 regions per group; data are expressed per milligram of mitochondrial protein and per gram of fresh tissue. *Significant differences were found between central and peripheral regions of gliomas ($P < 0.05$, Student's paired test).

respectively). As shown in Table 2, no differences were observed per gram of fresh tissue in regards to mitochondrial protein content, mtDNA levels, total COX activity, total CS activity (all of which are markers of mitochondrial abundance) or in protein content per mitochondrion; the mitochondrial protein adjusted to mtDNA levels was the parameter assessed that gave the closest approximation to mitochondrial mass.

To further exclude the possible influence of the number of mitochondria, mitochondrial function measurements (expressed per gram of fresh tissue) were corrected for CS recovery. It is important to highlight that DNA content is not a good marker of cell number in glioma cells due to the presence of aneuploidy, thus, we expressed our results per protein content, an index of cellular mass.

Fig. 1. Mitochondrial functionality. Oxygen consumption and OXPHOS activities of tumor periphery compared to tumor center. Data show relative percentage (central values compared to peripheral values). A negative percentage represents higher mitochondrial function in peripheral region than in central one. Mitochondrial oxygen consumption was assessed under state 4 (S4; absence of ADP) or state 3 (S3; 500 μ M ADP); succinate 5 mM or pyruvate-malate (5 mM/2.5 mM) were used as substrates. n = 4-6. Mean difference between center and periphery, *significant differences $P < 0.05$, Student's paired test.

Oxygen consumption

The actively respiring state is sometimes referred to as “state 3” respiration, while the slower rate after all the ADP has been phosphorylated to form ATP is referred to as “state 4”. Mitochondria from the periphery consumed significantly higher amounts of oxygen in State 4 than mitochondria from the tumor center (Fig. 1, Table 5). Under energy demand (ADP presence), there was an increase in mitochondrial respiration from both tumor regions; the differences found in mitochondrial oxygen consumption were reduced under active state respiration. Although there was a considerable stimulation of oxygen consumption with substrates pyruvate-malate and succinate, the RCR observed was quite low in mitochondria from the center (1.45 ± 0.39) and in mitochondria from the periphery (1.61 ± 0.29). These RCR values could suggest poor quality of the isolated mitochondria, however, suitable mitochondrial recovery and mitochondrial purity were obtained. Mitochondrial recovery measured as CS activity and COX activity in mitochondrial fractions was about 30% with respect to homogenate (as shown in the Glioma tissue composition and mitochondrial content section). Furthermore, the low LDH activity detected in crude mitochondrial pellets from the central and peripheral regions ($2.97\% \pm 0.41$ vs. $3.63\% \pm 0.58$, with respect to the homogenate) proved the low presence of contaminating cytosolic components (Table 2). In addition to this, when the same methodology was applied to other tissues, such as rat brain, RCR values ranged from 5 to 7 could be obtained. In fact, low RCR values were expected in glioma tissue since RCR of isolated mitochondria from rat brain is higher than RCR of isolated mitochondria from human brain (parahippocampal cortex) according to the results of Kudin et al. [37]; additionally, human glioma tissue compared to non-tumoral human brain tissue show attenuated mitochondrial bioenergetics and enhanced glycolysis dependency; thus, decreased RCR values, which are associated to mitochondrial dysfunction in other pathological conditions, might be caused by an increased basal respiration (state

Fig. 2. Mitochondrial pro-oxidant production capacity. Bars show relative percentage (central values compared to peripheral values). A negative percentage represents higher mitochondrial capacity to produce H₂O₂ in peripheral region than in central region in absence of ADP. Succinate 5 mM was used as substrate with or without GDP 500 μ M, rotenone 2 μ M and antimycin A 5 μ M; rate of H₂O₂ production with pyruvate-malate (5 mM/2.5 mM) as substrates was measured in the presence of rotenone 2 μ M. n = 4. Mean difference between center and periphery, *significant differences $P < 0.05$, Student's paired test.

4) coupled to a diminished state 3 respiration in glioma tissue.

OXPHOS activities

Cytochrome *c* oxidase or complex IV and ATPase are key electron transport chain complexes in oxidative and phosphorylative capacities, respectively. Under basal state, specific COX activity was significantly higher in mitochondrial fractions from the periphery compared to those from the center (Fig. 1). The results obtained in specific COX activity were in concert with our data on oxygen consumption. This observation points to a higher oxidative capacity of isolated mitochondria from the periphery than central ones in basal state. Whereas COX activity was significantly decreased in the central region,

Fig. 3. Oxidative damage markers. Data show relative percentage (central values compared to peripheral values). A positive percentage represents higher oxidative injury in central region than in peripheral one. $n = 6$. Mean difference between center and periphery, *significant differences $P < 0.05$, Student's paired test.

ATPase activity was not more compromised in central areas when compared to the outer regions of the tumor (Table 3). Statistically higher ATPase/complex IV activity ratio in the central region was in accordance with a decreased COX activity in the tumor center (mean difference between central and peripheral regions was 0.186 ± 0.058 ; $P = 0.012$, Student's paired test). Given that basal respiration rate was statistically higher in mitochondria from the outer region of the tumor than in mitochondria from the tumor center, our data agree with the role of this mitochondrial complex which limits the rate of electron flow through mitochondrial electron transport chain.

Mitochondrial H_2O_2 production capacity

Superoxide anion, a product of one-electron reduction of oxygen, is the by-product of normal functioning of the mitochondrial respiratory chain. This radical has been reported to be generated by complexes I and III (CI and CIII) of the mitochondrial respiratory chain and readily converted to H_2O_2 by mitochondrial Mn-SOD. We approached this question using Amplex Red, a specific reagent to measure H_2O_2 , which does not form resorufin in the presence of other reactive oxygen and nitrogen species [38]; therefore, we determined the H_2O_2 released by isolated mitochondria from the tumor in a spectrofluorimetric microtiter plate. Our results showed a trend towards an increase in H_2O_2 production capacity of peripheral mitochondria when compared to central mitochondria ($P = 0.067$). The rate of H_2O_2 production showed a differential response by adding GDP: higher in mitochondria from the periphery (Fig. 2). GDP could act as an inhibitor of uncoupling proteins, which are located in the inner mitochondrial membrane and are thought to

Fig. 4. Antioxidant capacity. Bars show relative percentage (central values compared to peripheral values). A positive percentage represents higher antioxidant systems in central region than in peripheral one. $n = 5-6$. Mean difference between center and periphery, *significant differences $P < 0.05$, Student's paired test.

Fig. 5. Immunohistochemical stain with Ki-67 antibody. The cell proliferation index is higher at the peripheral regions of the tumors (A) compared to central regions (B)

be involved in the modulation of mitochondrial ROS production in human brain [39, 40]. In all conditions tested (Table 4), this trend was maintained, but standard error of the mean was too high to reach significance. Moreover, there was a significant decrease in rate of H_2O_2 production (data not shown) under State 3 (ADP presence) and under the presence of an hydrogen peroxide scavenger such as pyruvate [41]. While brain tumor mitochondria in the presence of pyruvate+malate alone generated only small amounts of H_2O_2 , a substantial production was observed after the addition of the complex I inhibitor rotenone (7.76 ± 3.35 and 17.2 ± 5.4 nmol $H_2O_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ central regions vs. peripheral regions, respectively)

or in the presence of the respiratory substrate succinate alone in mitochondria from the center ($15.8 \pm 11.1 \text{ nmol H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$) and in mitochondria from the periphery ($21.4 \pm 9.0 \text{ nmol H}_2\text{O}_2 \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$). The maximal rate of H_2O_2 generation was observed in complex III under the presence of antimycin A and rotenone; the addition of both inhibitors specifically makes it possible to measure the production of H_2O_2 in complex III since rotenone inhibits H_2O_2 generation caused by reversed electron flow through complex I. High rates of production of reactive oxygen species were features of respiratory chain-inhibited mitochondria and of reversed electron flow, respectively, in isolated mitochondria from both regions of glioma tissue. Similar observations have been described in mitochondria isolated from human parahippocampal gyrus [37].

Markers of oxidative damage

In order to estimate the profile of oxidative damage as possible consequence of free radical attack and modification of subcellular components (nucleic acids, lipids and proteins), we determined lipid peroxidation (TBARS), mitochondrial DNA oxidative damage and protein carbonylation in opposite regions of glioma samples (Table 6). For mitochondrial DNA damage and protein oxidative damage no significant differences were found between center and periphery (Fig. 3). However, there was a trend towards an increase in lipid peroxidation per gram of fresh tissue in the central area in comparison with the peripheral one, which only marginally failed to reach significance ($P = 0.054$).

Glutathione content

The GSH/GSSG couple is thought to be the major redox buffer in the cell. In the present experiments, GSH/GSSG values in central region were two-fold higher than in peripheral region (5.23 ± 1.23 vs. 2.55 ± 0.61). Mean difference of GSH/GSSG ratio between central and peripheral region was 2.68 ± 0.71 ; $P = 0.007$, Student's paired test. There were similar GSSG levels per gram of fresh tissue (mean difference between central and peripheral region was -0.0029 ± 0.0101 ; $P = 0.392$, Student's paired test), however, a statistically significant rise in GSH levels per gram of fresh tissue was found in the tumor center (Fig. 4, Table 7).

Mitochondrial antioxidant activities

There was a higher specific activity of manganese superoxide dismutase in mitochondria from tumor center compared to mitochondria from periphery in all the cases

subjected to study (Fig. 4, Table 8). Moreover, GPx activity showed a trend towards an increase in mitochondria from central region in comparison with mitochondria from periphery. Conversely, glutathione reductase activity was only slightly higher in the tumor center compared to tumor marginal zone of resection.

Discussion

Gross total *en bloc* resections of primary brain tumors are not a common practice worldwide. The rapid availability of these rare samples at our Institution gave us the unique possibility of studying the relationship between functional characteristics (bioenergetics and oxidative status) of cancer cell populations and their spatial distribution within a solid tumor. Measurements were achieved in central and peripheral regions of fresh tissue samples from patients with brain tumors. It is important to point out that our data were not affected by a possible contaminating effect of residual non-tumoral tissue in the periphery compared to the center. Histological analysis reveals that glioma cells grow within the healthy brain parenchima, therefore, there is residual non-tumoral tissue both at the central and the peripheral regions. However, in all the cases subjected to study the percentage of non-neoplastic brain tissue was minimal at both locations. Our data show phenotypic heterogeneity between cancer cell populations, given that similar density of neoplastic cells was identified in each region of the tumor. In accordance to our results, other studies have proved that there is a correlation between the karyotypic pattern and certain phenotypic features of cancer cell populations from human glioma [42]. In the present experiments glioma cell populations inside the neoplastic tissue exhibited differences in mitochondrial oxidative capacity as well as in the activation of their cellular and mitochondrial antioxidant mechanisms. This fact is in concert with other studies in human glioma that show heterogeneity in basal oxidative activity of their cell populations [43]. Keeping these observations in mind, our study in human glioma tissue corroborates the phenotypic heterogeneity observed in malignant glioma cells and suggests that functional nonuniformity may be related to particular regions of the tumor.

As has been reported, these functional differences could be related to an increase in tumor size: malignant cells are often exposed to heterogeneous environment, with some regions of solid tumors containing microenvironmental niches that create a gradient of criti-

cal metabolites, including oxygen, glucose, other nutrients and growth factors [44]. Additionally, histological and magnetic resonance imaging studies have described that brain tumors show a structural heterogeneity, with different zones from the periphery, which is thought to be the most proliferative area of the tumor, to the center, which could be necrotic in particular cases [45]. Moreover, this structural heterogeneity seems to be correlated with heterogeneous vascularization [46]. In relation to this, we determined cell proliferation in adjacent sections of these glioma samples using ki-67 immunohistochemical technique. In our study, the proliferative index was higher in the peripheral tumor sections (representative images are shown in figure 5). In our cases, immunostaining for p53 showed equal intensity in the center and in the periphery of the glioma mass (data from an on-going parallel study); the histological pattern did not seem to reveal an increased resistance to apoptosis in the tumor's center compared to the tumor's periphery. However, complementary studies in primary cultures are needed to be able to assess the possible differences in apoptosis resistance between both regions under specific stimuli.

Brain tumors suffer from reduced respiratory capacity coupled to increased glycolysis and lactic acid production [47]. Given that our results showed a higher respiratory rate in isolated mitochondria from the outer regions of the tumor compared to central regions, we thought that this could be related to the utilization of a preferential pathway to obtain energy. Nevertheless, we did not find significant differences between both regions in cellular LDH activity, a key enzyme of glycolytic metabolism.

We did not find statistically significant differences in mtDNA content between central and peripheral regions of the analysed samples; however, some studies report high amplification as the most common alteration in mitochondrial DNA of gliomas [3]. Considering well-established markers of mitochondrial number (mtDNA content, total CS activity and total COX activity), our results pointed to similar mitochondrial abundance between the center and periphery [4, 48, 49]. In contrast to the apparent structural homogeneity observed between mitochondria populations in central and peripheral areas of human glioma, we obtained an important heterogeneity in mitochondrial function measured in each region. In the present study, the drop in mitochondrial functionality from tumor periphery to the center consisted of diminished COX activity, decreased respiration in basal state and a lack of maximal functional capacity of CI and CIII (which

was assessed as mitochondrial capacity to produce H_2O_2 from both complexes). This article describes for the first time, differences in oxidative capacity and oxidative stimuli in concentric distant regions within the same tumor mass.

It is of further interest to note that the observed decline in mitochondrial oxidative capacity in the glioma center compared to glioma periphery could be better explained on the basis of underlying greater hypoxia and oxidative stress in tumor center than alterations in mtDNA content or differential mtDNA oxidative damage between both regions. Previously, the decline in COX activity has been attributed to a peroxidative damage in mitochondrial inner membrane [50], moreover, experimental hypoxia results in significantly lower COX activity and vastly increased ROS production by this complex [51]. Interestingly, mitochondrial function is unlikely to be altered in spite of mitochondrial mutations in gliomas (which are closely linked to mtDNA oxidative damage) [52]. Hence, our data suggest that mitochondrial functional alterations between central and peripheral regions might be related to oxidative injury of the most susceptible macromolecules.

In our study, the greater hydrogen peroxide production capacity observed in mitochondria from periphery is consistent with the notion that increased oxygen consumption may result in elevated reactive oxygen species production [53]. With regards to oxidative injury in glioma cells, we did not find significant differences in total protein oxidation between both regions. On the other hand, an important trend towards a higher lipid damage per gram of fresh tissue was identified in the central regions of the glioma. This finding is interesting since end products from lipid peroxidation could be used as markers for increased oxidative stress in these regions (as a result of the balance between total peroxide production, antioxidant defenses and repairing systems).

Paradoxically, the observed tendency towards a decreased capacity of production of mitochondrial H_2O_2 was coupled to a higher cellular lipid peroxidation in the central region of the neoplasia. This fact can be explained on the basis that the rate of mitochondrial H_2O_2 production measured *in vitro* indicate the fraction of hydrogen peroxide released by isolated mitochondria, thus it reflects mitochondrial capacity to produce H_2O_2 but not the intracellular levels (or ROS variations over time) to which cancer cells are exposed. The greater ROS production capacity in tumor periphery was in agreement with its higher mitochondrial functionality. Although we could not measure intracellular ROS levels, their effects are the best markers of long-term exposure of cells to ROS [54]:

- The tendency towards an accumulation of oxidative injury in tumor center
- The impairment in mitochondrial function of tumor center
- The increase in central region of soluble antioxidants, such as glutathione, and enzymes that detoxify free radicals and reactive oxygen species to cope with high intracellular levels of ROS.

On the other hand, we have to consider cancer cell divisions: the less proliferative activity in the central region would make it easy to accumulate oxidative damage; if cells inside tumor center had a lower proliferation rate than cells in tumor periphery, energy demand in tumor mass would be restricted mainly to the periphery. In this sense, it is well known that cells under high energy demand operate a “respiratory control” by stimulation of respiration by ADP that stops after conversion of ADP into ATP; it is assumed that ROS production within the cell is low in this situation [55, 56].

The present study suggests a dysregulation of the redox homeostasis in glioma center. In all the studied tumors, we found significantly higher cellular levels of reduced glutathione (expressed per gram of fresh tissue), MnSOD activity and GSH/GSSG ratio in central glioma areas when compared to peripheral ones. In this way, the increase in antioxidant systems in central region could be considered an adaptative mechanism by which cancer cells counteract chronic oxidative stress [57-59]. Interestingly, antioxidant systems could have primary implications in management of malignant gliomas [60]. On the one hand, overexpression of MnSOD (an essential primary antioxidant enzyme that converts superoxide radical to hydrogen peroxide and molecular oxygen within the mitochondrial matrix) causes tumor suppression [61] and growth inhibition which may correlate with the intracellular redox status in glioma cells [62]. On the other hand, several studies have provided evidence for the role of increased levels of antioxidant systems in resistance to anticancer treatments. Cross-resistance to cisplatin and radiation may be mediated by increased content of detoxifying thiols such as glutathione. Other studies have also proved that in glioblastoma cell lines highly coordinated activation of antioxidant enzymes such as superoxide dismutase, catalase, GPx and GRd is related to radioresistance and cross-resistance to chemotherapeutic agents [63]. Moreover, the higher content in tumor center of reduced glutathione may improve its efficiency of drug detoxifying metabolism [64]. Sublines resistant to doxorubicin, a widely used antitumoral drug, show a decrease of cytochrome *c* oxidase activity and cytochrome *aa3* content [65]. Recently, a cellular metabolic strategy

that supports the connection between mitochondrial activity and cellular response to chemotherapeutic agents has been identified [66]. Considered together, there is clearly a similarity between the presented changes in metabolic and antioxidant capacities in glioma center and the key features of drug resistance.

Ideally, regional differences in mitochondrial behaviour and antioxidant systems of cancer cell populations inside the glioma mass should be considered in therapeutic strategies. Standard therapies for malignant gliomas (such as radiation) may retard glioma growth over the short term, but they can facilitate glioma recurrence and enhance growth rate over the longer term through alterations in morphogenetic fields [67, 68]. The presence of cancer cells in the tumor mass with different susceptibility to therapy may help to explain the different response of patients to standard therapies even when a same histological grade is diagnosed. Radiotherapy and certain chemotherapeutic agents might establish a selective pressure *in vivo* by which they might select the most resistant cancer cell population.

In this study, we have proved that differences in energy metabolism and oxidative status within glioma mass are not random but follow a characteristic pattern; differences in cancer cell behaviour are associated to macroscopically distant concentric regions of human glioma tissue. The highest antioxidant capacity is localized in the tumor center whereas the greatest mitochondrial functionality is identified in the tumor periphery. Finally, our study supports the primary role of mitochondrial function and redox reactions as targets to modulate in new anticancer therapeutic strategies [69, 70] and points to the need for more profound knowledge about oxidative mitochondrial metabolism in human brain tumors. Especially, further studies about the different behaviour of cancer cell populations inside the fresh neoplastic tissue are required given that it could have primary therapeutic implications.

Abbreviations

COX (cytochrome *c* oxidase); CI (complex I or NADH-ubiquinone oxidoreductase); CIII (complex III or ubiquinol-cytochrome *c* oxidoreductase); CS (citrate synthase); LDH (lactate dehydrogenase); TBARS (thiobarbituric acid-reactive substances); ROS (reactive oxygen species); OXPHOS (oxidative phosphorylation system); mtDNA (mitochondrial DNA); mt protein (mitochondrial protein); MnSOD (manganese-superoxide

dismutase); GPx (glutathione peroxidase); GRd (glutathione reductase); GSH (reduced glutathione); GSSG (oxidized glutathione).

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