Comparative analysis of antioxidative systems in malignant and benign brain tumours

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Objectives: Comparison of redox conditions in malignant and benign tumours is essential for understanding the role of reactive oxygen species in the pathophysiology of aggressive cancer profiles. Here we compare antioxidative systems in highly malignant brain tumour – glioblastoma multiforme (GBM), and in meningioma, a benign brain tumour.

Methods: Tumour tissues and blood of 67 GBM patients (mean age: 52.9 ± 11.5 years) and 67 meningioma patients (59.2 ± 10.2 years), and blood of 30 control subjects (50.8 ± 12.8 years) were analysed via biochemical assays.

Results: Components of glutathione system, which is responsible for H_2O_2 removal, showed lower activity/level in GBM: glutathione peroxidase (GBM: 9.90 ± 0.22; meningioma: 11.78 ± 0.23 U/mg of proteins; $P < 0.001$), glutathione reductase (GBM: 3.83 ± 0.13; meningioma: 4.67 ± 0.11 U/mg of proteins; $P < 0.001$), and glutathione (GBM: 6.70 ± 0.12; meningioma: 7.58 ± 0.14 μmol/g of tissue; $P < 0.001$). In contrast, the rank order of glutathione reductase activity and glutathione level in erythrocytes was: GBM > meningioma > control. Superoxide dismutase and catalase activities were lower in the blood of cancer patients compared to controls.

Discussion: Cells of malignant brain tumour show down-regulated antioxidative system which might result in increased levels of H_2O_2 compared to benign tumour tissue.

Keywords: Erythrocytes, Glioblastoma, Glutathione peroxidase, Glutathione reductase, Hydrogen peroxide, Meningioma

Introduction

Altered redox settings have been implicated in cancerogenesis and mutagenesis, tumour cell proliferation, development of metastases, and resistance to chemo- and radiotherapy.[1–5] Tumour cells show: (i) accelerated metabolism which might result in increased baseline production of reactive oxygen species (ROS); (ii) altered activity of other sources of ROS; (iii) non-physiological activity of antioxidative system (AOS); (iv) inadequate oxygen supply, which might promote ROS production; and (v) dysfunctional redox signalling (e.g. many critical mutations in oncogene proteins affect redox-sensitive regulatory moieties).[3–6] The most important ROS in tumour pathophysiology appears to be hydrogen peroxide (H_2O_2). High levels of H_2O_2 have been observed in cancer cells,[6,7] which could be related to both promoted production and inefficient removal by AOS. Hydrogen peroxide is produced in mitochondria or cytosol from superoxide via superoxide dismutase (SOD). It is removed by catalase (CAT) or via glutathione system composed of glutathione, glutathione peroxidase (GSH-Px), and glutathione reductase (GR). H_2O_2 is uncharged and relatively stable ROS that can cross nuclear/cellular membrane.[8] Inside nucleus, H_2O_2 can react with copper to produce notoriously reactive hydroxyl radical that provokes DNA alterations.[9] Another pathway for hydroxyl radical production is the reaction between ferrous iron and H_2O_2 (Fenton reaction). In this respect, a very recent study has shown that the ratio between ferrous and ferric iron increases with brain tumour malignancy grade.[10] Furthermore, it has been found that cancer cells can release H_2O_2 in order to trigger autophagy in nearby cells, which leads to the production of high-energy nutrients that are then used by cancer cells.[11] Hydrogen peroxide also acts as a signalling

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molecule targeting a number of proteins and signalling cascades.[8] Altogether, it appears that H_2O_2 could represent a multipurpose 'tool' in cancerogenesis.

Redox conditions in tumours could be assessed by determining the AOS profile of tumour tissue, but also by measuring the activity of AOS enzymes in some normal tissue that is affected by tumour, such as blood.[12–15] Large tumour masses are likely to exert oxidative pressure on erythrocytes. For example, drastic changes of AOS in erythrocytes have been reported to occur after surgical removal of brain tumours.[16] A number of studies have shown non-physiological activity of specific AOS components in tumour cells and blood of cancer patients.[17–19] However, data on brain tumours and comparative analysis of malignant and benign tumours are still scarce and inconclusive. The examination and comparative analysis of redox conditions in malignant and benign tumours could be particularly important for understanding the role of H_2O_2 and other ROS in the pathophysiology of cancers with aggressive profiles.[20–22]

Here, we aimed to compare AOS (SOD, CAT, GSH-Px, GR, and glutathione) in tumour tissue and blood of patients with two types of brain tumours that are on the opposite ends of severity scale: glioblastoma multiforme (GBM), which is the most malignant form of primary brain tumours, and benign meningioma.

Materials and methods

Patients and sample collection

We analysed tumour tissues of 67 patients with GBM (WHO grade IV) (mean age: 52.9 ± 11.5 years; range: 22–76 years; sex ratio m/f = 36/31), and tumour tissues of 67 patients with meningioma (mean age: 59.2 ± 10.2 years; range: 30–74 years; m/f = 44/23). In addition, blood samples of all GBM and meningioma patients and of 30 healthy control subjects (mean age: 50.8 ± 12.8 years; range: 23–71 years; sex ratio m/f = 14/16) were analysed. The exclusion criteria were: viral infections, antioxidative therapy and dietary supplements, and smoking. The research was performed in accordance with the Declaration of Helsinki of the World Medical Association and has been approved by the local Ethics Committee. Written informed consents were obtained from all participants.

Tissue samples were collected in the course of total or sub-total tumour resection or tumour biopsy. All specimens were obtained from tumour peripheral parts avoiding necrotic areas. The meninges, blood vessels, and damaged/cauterized tissues were removed in −20°C chamber. The tissue was cut in smaller pieces, samples were briefly washed of blood with ice-cold 0.9% NaCl solution, and then snap-frozen in liquid nitrogen. GBM and meningioma were confirmed by histopathological analysis of surgical resections in accordance with the WHO criteria.[23]

Fresh venous blood (5 ml) samples were collected from each patient two days before surgery and from control subjects using heparinized vacutainers. Erythrocytes were separated from the plasma, and washed three times with 0.9% NaCl solution by centrifugation at 3000 *g*/10 minutes/4°C. Following the procedure, erythrocytes and plasma samples were immediately frozen in liquid nitrogen. All samples were stored at −80°C until further analysis.

Biochemical assays

Frozen tumour tissue samples were thawed on ice, and 1 g of tissue was homogenized in 10 volumes (w/v) of ice-cold homogenizing buffer (50 mM Tris-HCl, 250 mM sucrose, 1 mM EDTA, pH 7.4) using homogenizer and sonication (10 kHz; 6 × 10 seconds). The homogenates were centrifuged at 100,000 *g*/90 minutes/4°C. In tissue samples, total SOD activity (manganese SOD (MnSOD) + copper-zinc SOD (CuZnSOD)) was measured according to the adrenalin method of Misra and Fridovich.[24] Previously described methods were used to determine the activities of CAT,[25] GSH-Px,[26] and GR.[27] The activity of enzymes in the tumour tissue was expressed as U/mg of proteins. Protein contents in supernatants were determined using the Lowry method. The total concentration of glutathione (reduced and oxidized) in tissue extracts was determined based on the Griffith method,[28] and presented as μmol/g of tissue.

Erythrocytes (0.5 ml) were lysed by adding 3 ml of ice-cold distilled water. SOD (i.e. CuZnSOD; erythrocytes do not contain MnSOD), CAT, GSH-Px, and GR activities were determined using the same methods as for tissue samples. Interference from haemoglobin (Hb) was eliminated by precipitation prior to the assay using ethanol/chloroform (1 : 1, v/v) that was followed by centrifugation at 3000 *g*/5 minutes/4°C.[29] The activity of enzymes in erythrocytes was presented as U/g of Hb. Hb concentration was measured by applying the Drabkin method. Finally, the total concentration of glutathione in plasma was determined based on the Griffith method and presented as μmol/l of plasma. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) or Merck (Darmstadt, Germany).

Statistical analysis

Statistical differences were evaluated by means of Student's *t*-test using STATISTICA 8.0 (StatSoft Inc., Tulsa, OK, USA). The results are presented as means ± standard error and were taken to be statistically different if $P < 0.05$.

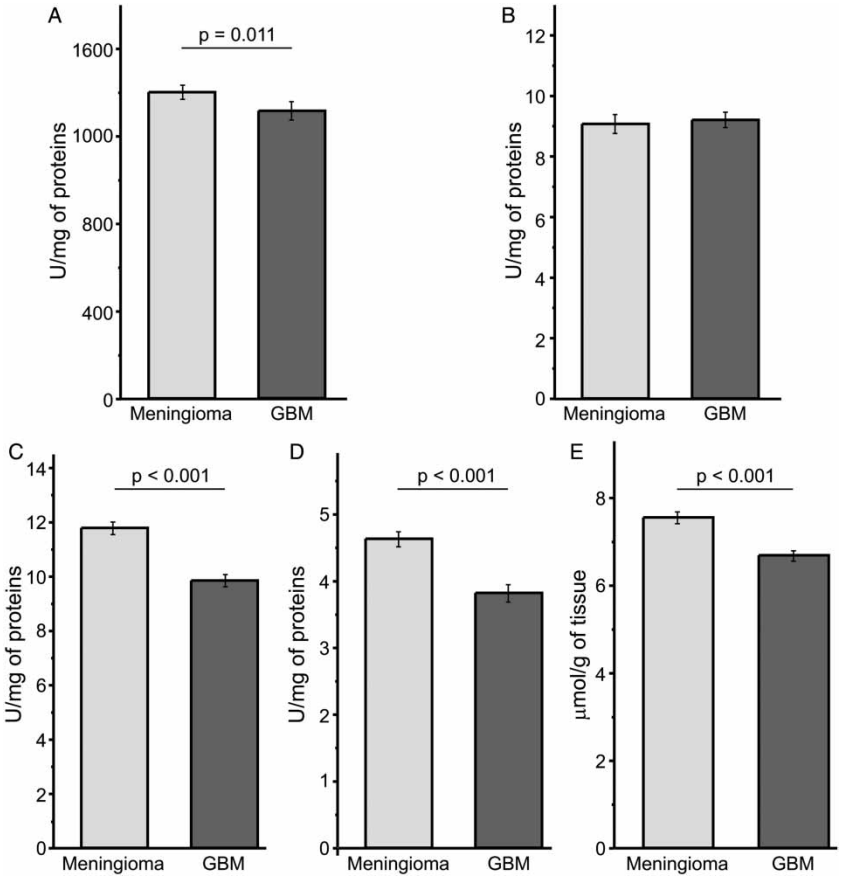

Figure 1 AOS in tumour tissues of GBM ($n = 67$) and meningioma patients ($n = 67$). (A) Total activity of SOD; (B) the activity of CAT; (C) the activity of GSH-Px; (D) the activity of GR; (E) total concentration of glutathione.

Results

It can be observed that GBM tissue showed a slightly lower total activity of SOD (CuZnSOD + MnSOD) compared to meningioma (Fig. 1A), while CAT activity did not differ between the two tumour types (Fig. 1B). On the other hand, glutathione system was down-regulated in GBM compared to meningioma, i.e. activities of GSH-Px and GR (Fig. 1C and D), and the total glutathione level (Fig. 1E) were significantly lower in GBM tissue.

SOD activity in erythrocytes of GBM patients was significantly lower compared to control values. This trend was observed in meningioma as well (Fig. 2A). CAT showed a lower activity in both GBM and meningioma compared to controls (Fig. 2B). In contrast, the glutathione system, besides GSH-Px which showed no differences between study groups (Fig. 2C), was up-regulated in the blood of cancer patients. The rank order of GR activity in erythrocytes (Fig. 2D), and of total glutathione level in the plasma (Fig. 2E) was GBM > meningioma > control.

Discussion

We compared AOS in GBM, as a model of highly malignant, aggressive brain tumour, and meningioma, which shows benign phenotype in the prevailing number of cases (~98%). Hydrogen peroxide-removing glutathione system showed a lower activity in GBM tissue compared to meningioma. This is in accordance with previous findings of one smaller study (conducted on 26 GBM and 22 meningioma patients) that found a lower GSH-Px and GR activity in GBM compared to meningioma.[30] In addition, two comparative studies on meningioma and astrocytoma found that malignant brain tumours show a lower activity of GSH-Px (the rank order was: meningioma > low-grade astrocytoma > high-grade astrocytoma),[31] as well as lower glutathione concentration and SOD activity.[31,32] It is noteworthy that Palani *et al.*[33] have shown that GSH-Px, GR, and SOD activity in gliomas gradually decrease with increasing tumour grade. In discrepancy to our findings, Pu *et al.*[31] have found higher CAT activity in meningioma compared to high-grade astrocytoma. This might be related to the fact that the high-grade astrocytoma group in that particular study included GBM and grade 3 astrocytoma patients, and potentially due to the lower number of patients (10 meningioma and 10 high-grade astrocytoma).

A lower activity of H_2O_2-removing system implies a down-regulation of enzymes that most likely allows the accumulation of H_2O_2. In relation to this, the increased level of oxidation in GBM compared to meningioma has been documented in previous

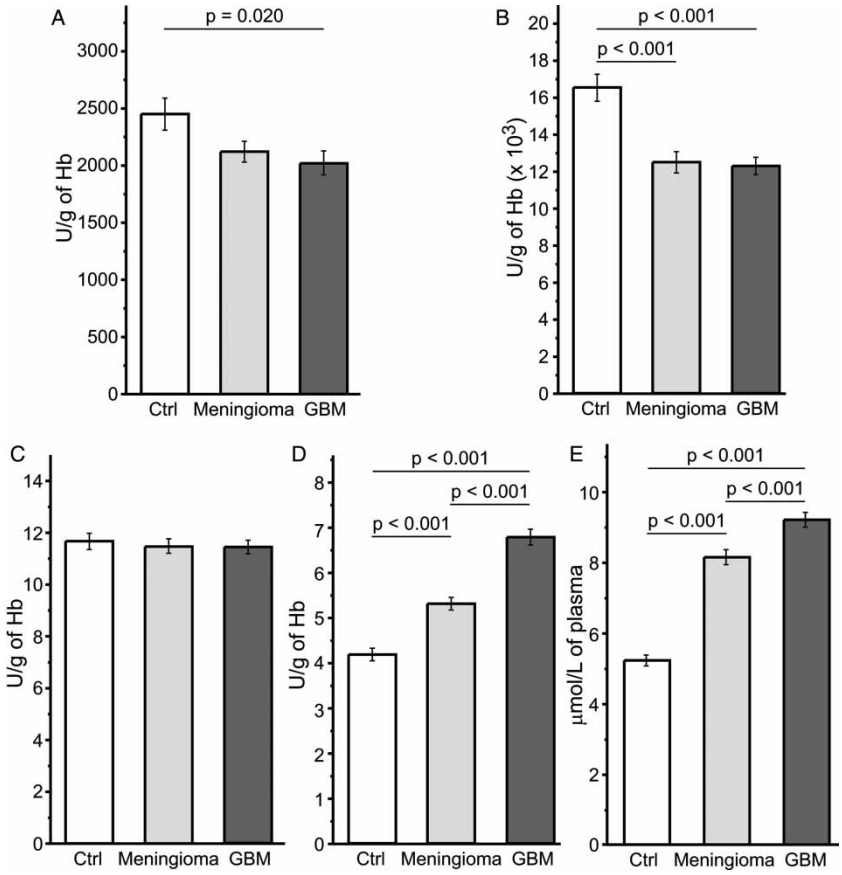

Figure 2 AOS in the blood of control subjects (Ctrl; $n = 30$), GBM ($n = 67$), and meningioma ($n = 67$) patients. (A) CuZnSOD activity in erythrocytes; (B) CAT activity in erythrocytes; (C) GSH-Px activity in erythrocytes; (D) GR activity in erythrocytes; (E) total concentration of glutathione in plasma.

studies using different oxidation markers, such as oxidized proteins which were higher in GBM,[30] and the level of reduced glutathione which was lower in GBM.[34] In addition, the level of lipid peroxidation was significantly higher in astrocytoma compared to meningioma.[32] Pro-oxidative settings in GBM, and in meningioma as well (but to a lower level), are further implicated by the results obtained on blood. GR activity in erythrocytes and glutathione level in the plasma showed the rank order: GBM > meningioma > control. These settings in the blood reflect a prolonged release of H_2O_2 from brain tumour mass into the circulation. This is in accordance with previous findings showing an increased level of markers of lipid peroxidation in the blood of glioblastoma and meningioma patients.[19,35] In addition, depletion of antioxidants has been observed in a cohort of patients with different brain tumours.[36] Furthermore, a decreased activity of CuZnSOD and CAT was observed in erythrocytes of brain tumour patients compared to healthy subjects. The former might be explained by H_2O_2-provoked inhibition of CuZnSOD.[15] On the other hand, potential mechanisms that led to the suppression of CAT activity in erythrocytes remain blur, but it is important to point up different roles of two H_2O_2-removing enzymes – GSH-Px and CAT in erythrocytes' antioxidative defence. GSH-Px is mainly located near the erythrocyte's membrane, and it is responsible for preventing H_2O_2 influx and lipid peroxidation. CAT is the primary erythrocyte enzyme responsible for coping with endogenous H_2O_2,[37] and the production of H_2O_2 in erythrocytes is decreased when CuZnSOD is inhibited. Rao *et al.*[38] have found a decreased SOD activity in erythrocytes of glioma and meningioma patients compared to control subjects. CAT was slightly decreased in patients compared to controls, whereas GSH-Px did not show any differences between groups. All this matches well with our findings. Surprisingly, their study has shown a drastically lower GR activity in cancer patients. We do not have an explanation for such discrepancy, but it is worth mentioning that control GR activity was four times lower compared to our results, whereas control values for other enzymes were similar.

A potential mechanism of down-regulation of glutathione system in tumour tissue might involve the shift of cysteine (glutathione precursor) towards a metabolic pathway that appears to be specific for glioblastoma. In brief, glioblastoma cells show an increased expression of cysteine dioxygenase 1, which catalyses the reaction of cysteine with molecular oxygen in

order to produce cysteine sulfinic acid. It has been proposed that this metabolite is involved in the development and growth of aggressive tumour phenotypes.[39] In addition, specific enzymes that are involved in glutathione biosynthesis and degradation, such as glutamate cysteine ligase and gamma glutamyl transferase, might show a non-physiological activity in tumour cells resulting in dysfunctional glutathione system.[40–42]

GBM appears to be significantly less susceptible to radiotherapy in comparison to meningioma. Radiotherapy in GBM patients results in a modest improvement of survival period.[43] On the other hand, surgical approach combined with post-operative radiotherapy in selected cases is considered to be the most appropriate for meningioma patients,[44] and radiation is highly effective in the management of skull-base meningioma, which otherwise require complex combined surgical approaches.[45] Radiotherapy is generally mediated via induction of oxidative damage. Therefore, higher radioresistance of tumour cells that show weaker AOS performance might appear paradoxical. In normal cells unaccustomed to pronounced oxidation, glutathione depletion activates cell death pathways and promotes radiosensitivity.[46,47] Unfortunately, in spite of down-regulated AOS defence and pro-oxidative conditions, GBM cells grow, proliferate, and show high radioresistance. It is plausible that further increase of oxidation via radiation might have little effect on cells that are already proficient in operating under pro-oxidative conditions and even appear to require supraphysiological ROS production. As a matter of fact, the inhibition of specific enzymes that are involved in ROS production, such as cyclooxygenase-2 (has superoxide as a by-product) or poly (ADP-ribose) polymerase 1 (promotes ROS production in mitochondria), has been reported to result in increased radiosensitivity of glioblastoma.[48,49] According to our results, the up-regulation of the glutathione system might also represent a useful strategy in fighting glioblastoma. Some efforts have been already made in this direction. For example, a recent study has shown strong *in vitro* effects of dimethyl fumarate, which is known to activate the expression of several glutathione-related enzymes, against glioma cells.[50]

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Disclaimer statements

Contributors VB – conceiving and designing the study, obtaining funding and/or ethics approval, collecting the data, analyzing the data, interpreting the data, writing the article in part; MB – conceiving and designing the study, obtaining funding and/or ethics approval, collecting the data, analyzing the data, interpreting the data, writing the article in part; IS – analyzing the data, interpreting the data, writing the article in part, revising the article.

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Conflicts of interest None.

Ethics approval The research was performed in accordance with the Declaration of Helsinki of the World Medical Association and has been approved by the Ethics Committee of the School of Medicine, University of Belgrade.

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