A Study on the Biochemical and Cytogenetic Status in the Blood of Glioma Patients

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Abstract: Malignant gliomas, the most common subtype of primary brain tumors, are aggressive, highly invasive and neurologically destructive tumors, considered being the deadliest of human cancers. As an attempt to understand the biology of glial tumor, a study on macromolecules like proteins, matrix metalloproteases, lipids and deoxyribonucleic acid, in the blood of glioma patients was made. Biochemical assessment of significant pathological enzymes, antioxidants and marker enzymes was performed. MMP expression was determined using gelatin zymography. Karyotyping analysis was done to determine chromosomal aberrations. A marked rise was observed in the proteins and lipids of glioma patients as compared to the normal cases. The antioxidant status of the patients was found to be lowered. Karyotypic analysis of the peripheral blood chromosomes presented various chromosomal aberrations in glioma patients. The biochemical parameters were significantly increased in the patient population (p<0.01, p<0.001) when compared to those of normal. Zymographic analysis showed the presence of MMP-2 and MMP-9 in the patient sample. Karyotypic investigation showed alterations in the chromosomal pattern of the glioma patients. The study provides baseline information on the biochemical alterations in the blood of glioma patients which can be further exploited for detailed investigations.

Key words: Glioma, DNA, karyotyping, antioxidants, zymography

INTRODUCTION

Glioma is the most common primary brain tumors occurring in adults and is highly aggressive and invasive in nature (Behin et al., 2003). Various phenotypic changes associated with gliomas include rapid growth, high glucose consumption, intratumoral necrosis and hypoxia, abundant microvascular proliferation, blood-brain barrier breakdown etc. (Hayashi et al., 2007). Increasing evidence from epidemiologic studies suggests that oxidative stress may play a role in adult glioma (Tedeschi-Blok et al., 2006). Technological advances in immunohistochemistry, molecular biology, genetics and chromosomal and nuclear analysis have influenced the effortless and rapid detection of various tumor markers (Emmenegger and Wechsler-Reya, 2008). However, despite these advancements, a reliable tumor marker is yet to be identified for diagnosis of malignant glioma (Maher et al., 2001). Recent progress in molecular cytogenetic techniques has facilitated the identification of complex chromosomal abnormalities in malignant gliomas (Padma et al., 2003).

It is accepted that plethora of reports exist to prove the production of ROS/antioxidants. Comparatively glioma is a relatively rare disease and observation of samples for many years is required to build up a therapeutic strategy. Further, reasonable numbers of reports provide contradictory results about the levels of enzymatic antioxidants in glial tumor patients. None of the related results paved the best yet, for a successful therapy. Thus, collection of related information adding to the existing knowledge becomes essential. In the present study, the antioxidant status was analyzed in human glioma blood samples in order to predict the extent of free radical damage caused by the tumor. The activities of general marker enzymes were also determined which will help in understanding the basic nature of the disease. The study also made a leap to investigate the chromosomal aberrations in the peripheral blood of the glioma patients, to assess the genetic alterations.

MATERIALS AND METHODS

The study was conducted between September 2005 to August 2006. The patient population for this study included about 36 individuals diagnosed and confirmed histopathologically of glioma from the Institute of Neurology and Neurosurgery, Madras Medical College (MMC), Chennai, India. Informed consent regarding the use of the blood samples for research purpose was obtained from the patients recruited in the study. In the inclusion criteria, patients who have been categorized...
under the World Health Organisation (WHO) classification of brain tumor and untreated for the disease, alone were considered. The exclusion criteria involved patients who are alcoholic, smoking, pregnant and lactating women, patients with any previous illness/history and postoperative cases. The karyotyping study included here was carried out at the department of Cytogenetics, Adyar Cancer Research Institute (WIA), Chennai under proper training and guidance.

**Protein estimation:** The protein content in the plasma was determined by the method of Lowry et al. (1951). The protein content in the plasma was expressed in g dL\(^{-1}\).

**Estimation of lipid peroxide:** The lipid peroxides in plasma was estimated by the method of Ohkawa et al. (1979). The amount of peroxide was expressed in TBA formed/mg protein.

**Estimation of reduced glutathione:** The level of reduced glutathione (GSH) in plasma was estimated using the method of Moron et al. (1978). The content of GSH was expressed in nmoles of μg mg\(^{-1}\) protein.

**Estimation of antioxidant enzymes**

**Superoxide dismutase activity:** The activity of Superoxide Dismutase (SOD) in plasma was estimated using the method of Misra and Fridovich (1972). The activity was expressed in units/min/mg of protein.

**Catalase activity:** The activity of catalase in plasma was assayed using the method of Beers and Sizer (1952). The activity of catalase was expressed μ moles of H\(_2\)O\(_2\) decomposed/min/mg of protein.

**Glutathione peroxidase:** The activity of Glutathione Peroxidase (Gpx) was estimated using the method of Rotruck et al. (1973). The activity of Gpx was expressed in nmoles of GSH oxidised/min/mg protein.

**Glutathione-s-transferase:** The activity of Glutathione-s-transferase (GST) in plasma was estimated using the method of Habig et al. (1974). The activity of GST was expressed in nmoles of CDNB conjugated/min/mg protein.

**Estimation of ascorbic acid (Vitamin C):** The activity of ascorbic acid in plasma was estimated using the method of Omaye et al. (1979). The activity was expressed in mg dL\(^{-1}\).

**Estimation of pathophysiological enzymes**

**Aspartate transaminase:** The activity of aspartate transaminase in plasma was estimated using the method of King (1965a). The enzyme activity was expressed in IU L\(^{-1}\).

**Alanine transaminase:** The activity of alanine transaminase in plasma was estimated using the method of King (1965b). The enzyme activity was expressed in IU L\(^{-1}\).

**Lactate dehydrogenase (LDH):** The activity of LDH in plasma was estimated using the method of Hall and DeLuca (1976). The enzyme activity was expressed in IU L\(^{-1}\).

**Estimation of marker enzymes**

**Creatine kinase:** The activity of creatine kinase in plasma was estimated using the method of Hall and DeLuca (1976). The enzyme activity was expressed in IU L\(^{-1}\).

**5' nucleotidase:** The activity of 5' nucleotidase in plasma was estimated using the method of Essner et al. (1958). The enzyme activity was expressed as IU L\(^{-1}\).

**Gelatin zymography for identification of matrix metalloproteinases:** Gelatin zymography was performed by the method of Jung et al. (2001). The gel was electrophoresed at 4°C and once run, it was treated with incubation buffer at 37°C overnight. If gelatin is degraded white bands appear.

**Karyotyping of peripheral blood chromosomes:** The peripheral blood was subjected to karyotyping by the method of Kallioniemi et al. (1992) with slight modifications. 10.0 mL RPMI 1640 medium was mixed with 2.0 mL bovine serum albumin along with 1.0 mL of peripheral blood. The mixture was incubated for 48 h. After incubation, about 0.5 mL of colchicine was added and again incubated for 25 min. The mixture was centrifuged at 1000 rpm for 10 min. To the pellet added 10-12 mL of fixative solution containing methanol: Glacial acetic acid in the ratio 3:1 and centrifugation was repeated till a white pellet was obtained. The pellet was then drawn over a clean slide and blown slowly for even distribution of cells. The slides were stained and viewed under microscope.

**Statistical analysis:** Results were expressed as Mean±SD differences between groups were analyzed by the
Student's t-test for unpaired observations, p<0.05 were considered significant.

The statistical analysis was performed using SPSS software V10.0.

RESULTS AND DISCUSSION

The antioxidant status in the normal subjects and glioma patients is shown in Table 1. The lipid peroxide level was substantially increased in glioma samples as compared to the normal (13.23±0.007 TBA formed/mL plasma; p<0.001). Different regions of the brain express different antioxidants (Dhanasekaran and Ganapathy, 2011). Free radicals are toxic agents created as by-products of metabolic activity (Zadeh et al., 2007). Reactive moieties produced during stressful conditions like cancer, cause the oxidation of polyunsaturated fatty acids in membrane lipid bilayers. Without sufficient levels of defense mechanisms such as free radical scavengers or antioxidants, increasing levels of lipid hydroperoxides and peroxides can be produced by self-perpetuating chain reactions. These lipid peroxides can directly damage cells and tissues through interaction with cellular macromolecules, including proteins, lipids and nucleic acids. The ROS are detoxified by sequential and simultaneous action of a number of enzymes including GST, GPX and GSR and metabolites including GSH and thiols (Nemat Alla et al., 2007). In the present study, the production of lipid peroxides in glioma patients showed high levels and is in line with the observations made by Soh et al. (2000).

Under normal conditions, excessive formation of free radicals and concomitant damage at cellular and tissue concentrations is controlled by enzymatic or non-enzymatic mechanisms, including vitamin E and glutathione (Ceylan et al., 2009). Glutathione (GSH) is a significant detoxicant and provides protective effect against various types of free radical damage (Mark et al., 1997). Cell death in distinct brain cells can occur due to depletion in GSH levels which leads to increased oxidative stress with subsequent rise in the levels of excitotoxic molecules (Bains and Shaw, 1997). In the present study, the GSH levels were concomitantly reduced in glioma patients (6.11±0.13 μg mg⁻¹ protein), thus proving the incapability of the free radical scavenging system to counteract the damage caused by free radicals which is in line with the above reference (Table 1).

Superoxide Dismutase (SOD) appears to play a key role in protection against free radicals especially CuZnSOD which plays an important role in protection against neuronal injury (Kajita et al., 1994) and vasospasm after subarachnoid hemorrhage (Kamii et al., 1999). SOD also protects the cells against superoxide radical which can damage the membrane (Ramachandran et al., 2008). In the present investigation, the lowered levels of SOD in glioma patients suggest the lack of protection against free radical induced damage.

It is widely accepted that ROS is responsible for the sole damage caused to macromolecules and ultimately to cellular structure (Khataibeh et al., 2006). Catalase primarily causes decomposition of hydrogen peroxide (H₂O₂) to H₂O at a much faster rate (Ramachandran et al., 2008). Overexpression of catalase results in reduction of intracellular H₂O₂ concentration, inhibition of cancerous cell DNA synthesis and proliferation and induction of apoptosis. The present study shows the decrease in catalase in glioma, thus indicating suppression of the enzymatic antioxidants.

GSTs are primary detoxifying enzymes involved in phase II detoxification and protect cells from attack by reactive electrophiles (Strange et al., 2001). They catalyze the conjugation of glutathione to electrophilic species (such as chemical carcinogens and cytotoxic chemotherapeutic agents) which is the first step that leads to the elimination of toxic compounds. Numerous studies have been shown that vitamin C exhibit a protective role (Horky et al., 2001) in certain types of cancer. Rat glial

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group I (Control)</th>
<th>Group II (Glioma)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxide in nmol of TBA formed/mL plasma</td>
<td>5.50±0.02**</td>
<td>13.23±0.007</td>
</tr>
<tr>
<td>Reduced Glutathione in μg mg⁻¹ protein</td>
<td>13.50±0.007***</td>
<td>6.11±0.13***</td>
</tr>
<tr>
<td>Superoxide dismutase in units/min/mg protein</td>
<td>10.04±0.26</td>
<td>5.96±0.04***</td>
</tr>
<tr>
<td>Catalase in H₂O₂ decomposed/min/mg protein</td>
<td>9.80±0.02</td>
<td>3.46±0.03***</td>
</tr>
<tr>
<td>Glutathione-S-transferase in nmol of CDNB conjugated/min/mg protein</td>
<td>17.70±0.14</td>
<td>5.50±0.01***</td>
</tr>
<tr>
<td>Glutathione peroxidase in nmol of glutathione oxidized/min/mg protein</td>
<td>121.70±0.30</td>
<td>2.75±0.01***</td>
</tr>
<tr>
<td>Vitamin C in mg dL⁻¹</td>
<td>0.23±0.008</td>
<td>0.77±0.01***</td>
</tr>
</tbody>
</table>

Values are as Mean±SD. Statistical significance are provided in group I and group II. **p<0.01, ***p<0.001 (n = 36)
tumor cells also have been shown to have N-acetyltransferase activity. In the present investigation an overall decrease in the activity of GPX, GST and ascorbic acid was observed in glioma cases when compared to normal, suggesting poor antioxidant defence against oxidative stress in glioma.

The level of protein activities of transaminases and lactate dehydrogenase were compared between the glioma and normal groups and were is represented in Table 2. The expression of a protein called vitronectin, is found to be most abundant in gliomas of high-grade histology (glioblastoma), less in intermediate-grade gliomas and virtually absent in low-grade lesions and correlates with the degree of glioma (Ulm et al., 1999). Glioma patients have four times the normal level of transaminases as compared to normals (Horn et al., 2008). Also, increased glucose metabolism at the tumor periphery may provide a scenario by which upregulation of AQPI, LDH and cathepsin B contributes to acidification of the extracellular milieu and to invasive potential of glioma cells in perivascular space. An elevation in ALT and aspartate aminotransferase (AST) activities is the most consistent findings in cancer. ALP and lactate dehydrogenase (LDH) activities may also be increased (Mosallanejad et al., 2011). In the present investigation, the increase in the activities of all of the above enzymesatic and non-enzymatic parameters in glioma samples (protein 12.45±0.07g dL⁻¹; AST 32.00±0.01***IU L⁻¹; ALT 42.00±0.01**IU L⁻¹; LDH 240.00±0.01**IU L⁻¹; *p<0.05, ***p<0.001) UoM Biochemistry thus supporting the results obtained from the above studies.

The activities of creatine kinase and 5′nucleotidase in the plasma of glioma and normal group is shown in Table 3. The activities of both the marker enzymes were found to be increased in glioma samples (CK 276.00±0.01***IU L⁻¹; 5′nucleotidase 17.18±0.01**IU L⁻¹; p<0.001) as compared to the normal. Serum or plasma enzyme levels have been employed as markers for monitoring chemically induced tissue damages (Samudram et al., 2008). These enzymes are more unique and changes in their activities reflect the effect of proliferation of cells with growth potential and its metabolic turnover is dramatically different from those of normal cells (Jahan et al., 2011). It was reported in primary rat brain cell cultures, that CKB mRNA levels in neuronal cerebral astrocytes and oligodendrocytes are much higher (15 to 17 fold) than in embryonic neurons. This suggests that various reactions in glial cells require abundant scores of CKB (Kuzhikandathil and Molloy, 1999). Degradation of nucleotides in the presence of an ecto-nucleotidase cascade includes ecto-ATPase, ecto-ADPase, apart from ecto-5′-nucleotidase (Cho et al., 2001) and this leads to the generation of extracellular adenosine. Adenosine, acting through G-protein coupled receptors, has been known to exert a multitude of physiological effects that are cardioprotective and cerebroprotective, including vasodilation, stimulation of angiogenesis, cytoprotection and immunosuppression. Hence, an elevation in the activities of the marker enzymes suggests more ATP consumption by the tumor cells which in turn supports tumor growth and development.

Figure 1 shows the gelatin zymography pattern in glioma and normal cases. MMPS are a large family of zinc-dependent neutral endopeptidases and are involved in the degradation of many different components of the extracellular matrix. MMP-9 specifically targets type IV collagen, a major component of the basement membrane and plays a critical role in glioma invasion across this barrier (Kongdangrit et al., 2000). Activation of MMP-2 and its proteolytic activity localized to the cell surface could differentially modulate tumor cell migration in response to particular matrix proteins by altering both composition of the extracellular matrix and expression of adhesion receptors on the cell surface. Gelatin zymography performed in the study showed the upregulation of matrix metalloproteinases 2 and 9 in the glioma samples, whereas the normal sample showed no presence. This demonstrates the impact of tumor invasion and its aggravation in glioma patients.

Figure 2-5 show the karyotypic pattern in normal and various glioma cases, respectively. Tumor initiation and progression are believed to result from a series of genetic events that cause gains and/or losses of normal cellular function. Earlier studies suggest that
Karyotype: Normal male, 46, XY

Karyotype: Ependymoma, 46, XY, Spindloidy +8, - 20

Karyotype: High grade glioma, 42, XY, Hypodiploidy, +15, -5, -9, -13, -18, -19, del (3p)

Karyotype: Grade II Astrocytoma, 45, XY, Hypodiploidy, -12

Genetic aberrations can occur at multiple sites in malignant astrocytomas (Collins, 1995). In our study, three different cases of glioma- High grade glioma, GradeII astrocytomas and Ependymoma were analysed for the presence of chromosomal aberrations.

Amplifications at 7p11.2-12 (EGFR) occurred in 2/35 primary and 4/45 recurrent AA and at 4q12 (PDGFAR) in 5/45 recurrent AA (Steck et al., 1999). In the present study a Grade II Astrocytoma male possessing 45 chromosomes showed Hypodiploidy due to loss of chromosome 12, suggesting genetic damage. The most frequent regions of LOH in high grade gliomas were located at 4q (54%), 6q (46%), 9p (38%), 10q (38%), 11p (38%), 12 (38%), 13q (69%), 14q (54%), 17 (38%), 18p (46%) and 19q (Harada et al., 1998). The high grade glioma male included in the study had 42 chromosomes and was found to be hypoploidy, with the following karyotype +15, -5, -9, -13, -18, -19, del (3p). The results are in line with the observations made by Wong et al. (2006). Cytogenetic studies on a supratentorial ependymoma showed
t(10;11;15) (p12.2; q13.1; p12) and loss of one X chromosome (Nishizaki et al., 2001). An Ependymoma case showing 46 chromosomes showed pseudoploidy with a gain in chromosome 8 and a loss in chromosome 20, suggesting genetic damage.

CONCLUSION

The present study thus provides a clue to understand the antioxidant status in the glioma patients apart from other enzyme parameters indicating the severity of the disease in the patients. Also the study gives a clear picture of the chromosomal deformities in the patients, a focus on which in future will pave a pathway for the better understanding of the tumor development.

REFERENCES


