MODULATED CELL PROLIFERATION, CELL DEATH AND CELL SURVIVAL SIGNALS IN HUMAN AND n-ETHYL n-NITROSOUREA (ENU) INDUCED RAT GLIOMAS

Thesis submitted for the Degree of

DOCTOR OF PHILOSOPHY

By

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Dedicated to My
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ABBREVIATIONS

AIF: Apoptosis inducing factor
ALP: Alkaline peroxidase
APAF-1: Apoptosis protease activating factor-1
ARF: Alternate reading frame
BCIP: 5-Bromo-4-chloro-3'-indoylphosphate p- toluidine salt
Bcl-2: B-cell lymphoma protein-2
bdNTP: Bromo-deoxy nitro triphosphate
BMK1: Big MAP kinase-1
CDK4: Cyclin-dependent kinase 4
CDKN2A: Cyclin-dependent kinase inhibitor 2A
CNS: Central nervous system
CNTF: Ciliary neurotrophic factor
Cox-2: Cyclo-oxygenase-2
CTL: Cytotoxic T-Lymphocytes
Cyc C: Cytochrome C
dATP: deoxy-Adenosine triphosphate
EDTA: Ethylene diamine tetra acetate
EGF: Epidermal growth factor
EGFR: Epidermal growth factor receptor
ENU: n-Ethyl n-nitrosourea
ERK[1]/2: Extracellular signal regulated kinase ½
FAK: Focal adhesion kinase
FGF-2: Fibroblast growth factor-2
GBM : Glioblastoma multiforme
GFAP: Glial fibrillary acidic protein
HNGC-1: Human neural glial cell line-1
HNGC-2: Human neural glial cell line-2
JNK: c-Jun NH_2 -terminal kinase
LOH: Loss of heterozygosity
MAPK: Mitogen activated protein kinase
MCM-2: Minichromosome maintenance protein 2
MDM2: Mouse double minute-2 protein
MEF: Mouse embryo fibroblasts
MEK/MKK/MAPKK: Mitogen activated protein kinase kinase
MKKK/MAPKKK: Mitogen activated protein kinase kinase kinase
MNU: n-Methyl n-nitrosourea
NAD: Nicotinamide adenine dinucleotide
NBT: Nitro-blue tetrazolium chloride
NMDA:N-Methyl-D-aspartate

NPC: Neuronal precursor cells
NSE: Neuron-specific enolase
P180: Postnatal day-180
P90: Postnatal day-90
PARP: Poly (ADP-ribosyl) polymerase
PBS: Phosphate buffered saline
PDGF: Platelet derived growth factor
PDGFR: Platelet derived growth factor receptor
PGE2: Prostaglandins E2
PI3K: Phospho-Inositol-3-Kinase
PKB: Protein kinase B
PMSF: Phenyl methyl sulphonyl fluoride
PTEN: Phospho-tensin homologue
Rb1: Retinoblastoma 1 protein
RIPA: Radio immuno precipitation assay
RTK: Receptor tyrosine kinase
SAPK: Stress activated protein kinase
SVZ: Sub-ventricular zone
TBS: Tris-buffered saline
TBST: Tris-buffered saline Tween 20
TEP1: TEnsin-like phosphatase
TUNEL: Terminal deoxynucleotidyl transferase
WHO: World health organization

INTRODUCTION

CHAPTER - I

CHAPTER 1

INTRODUCTION

"Glioma" is the term first coined by Rudolf Virchow, in 1860. Gliomas account for more than 50% of all brain tumors, and are by far the most common brain tumors in adults. Gliomas may further include three histopathological subgroups representing different levels of aggressiveness and malignancy: eppendymomas (<10%), oligodendrogliomas (10 to 30%) and astrocytomas (60 to 70%) (Kleihues et al, 2000). The majority of brain tumors, based on histology, includes tumors of neuroepithelial tissue, tumors of cranial and spinal nerves, tumors of meninges, lymphomas and hematopoietic neoplasms, germ cell tumors and cysts, tumors of sellar region and local extensions from regional tumors. Among the different tumor types, the incidence rate is highest for tumors of neuro-epithelial tissue, followed by tumors of the meninges, tumors of cranial and spinal nerves and tumors of sellar region (Doolittle, 2004).

World Health Organization (WHO) – Classification system

Under the auspices of the World Health Organization (WHO), the classification of astrocytomas was made based on survival and histopathological features and refers to the degree of malignancy (Gonzales, 2001). The WHO classification system has

been widely adopted throughout the world. According to the WHO classification system, astrocytic brain tumors are divided into four different grades reflecting different malignancy and histological characteristics (Lopes et al., 2001). Low grade tumors generally do not posses a metastatic tendency, whereas high grade tumors are highly metastatic. Among all the four grades, glioblastoma multiforme (GBM) is the most common, most aggressive and highly invasive form. Prognosis of these tumors remains dismal, despite of improved treatment procedures such as the use of heavy particles for irradiation and the discovery of new chemotherapeutic drugs. A major impediment to the success of therapy is that astrocytomas can infiltrate normal brain tissue at an early stage of development; thereby the specific targeting of tumor cells is difficult. Histopathological features corresponding to the pathological grade are given in the Table 1.

GBM can arise in two different ways. Some of the astrocytic brain tumors, except for pilocytic astrocytomas, posess an intrinsic tendency to progress towards a more malignant phenotype and may ultimately acquire malignancy of glioblastoma. Increasing evidence showing that astrocytoma progression reflects the sequential accumulation of genetic alterations. GBMs that evolve in this way have been termed secondary glioblastomas. These tumors often develop over months or years and typically affect young adults. GBMs developing *de novo* from glial cells are called primary glioblastomas and account for the vast majority of glioblastomas. Primary glioblastomas generally occur in elderly

WHO Grading System and Histopathological Characteristics of Gliomas

WHO Grade	Tumor Type	Histological Features
Low – Grade		
I	Pilocytic Astrocytoma	Cellular atypia
II	Astrocytoma	Cellular atypia, varying degrees of cellularity may be present
High – Grade		
III	Anaplastic astrocytoma	Increased cellularity, cellular pleomorphism and mitoses may be present
IV	Glioblastoma multiforme	All grade III characteristics and with psuedopalisading cells, endothelial cell proliferation or necrosis may be present

Table 1

patients and clinical history is usually less than six months (Ohgaki, 2005). Primary and secondary glioblastomas constitute two distinct sub-types in their clinical presentation and molecular alterations. Genetic pathways leading to the development of a primary glioblastoma include epidermal growth factor receptor (EGFR) gene amplification and loss of chromosome 10 (Von Deimling et al., 2000). The phosphatase and tensin (PTEN) homologue gene (also called NMAC1 or TEnsin-like Phospatase-1(TEP1)) has been cloned from 10q23. The PTEN gene is deleted or mutated in about 30% of glioblastomas (Li et al., 1997). In contrast, secondary GBM shows platelet derived growth factor receptor (PDGFR) over-expression along with P53 mutation. Molecular alterations associated with primary and secondary glioblastoma formation are elucidated in Fig 1. Identification of genetic alterations and assessment of their roles in malignant transformation are essential for understanding the evolution of gliomas and their biological characteristics, such as migration and invasion (Ohgaki et al., 2004). Histopathological variations among the different grades of gliomas are presented in Fig 2.

Clinical symptoms

Glioma symptoms observed among the patients are highly variable. Among which, headaches are early complaints in 70% glioma patients. The headache may be severe and occur either persistently or recurrently. The mechanism of headache is not known, but presumably it is a consequence of traction pressure on the pain-

Multiple Sequential Molecular Alterations Associated with Gliomagenesis

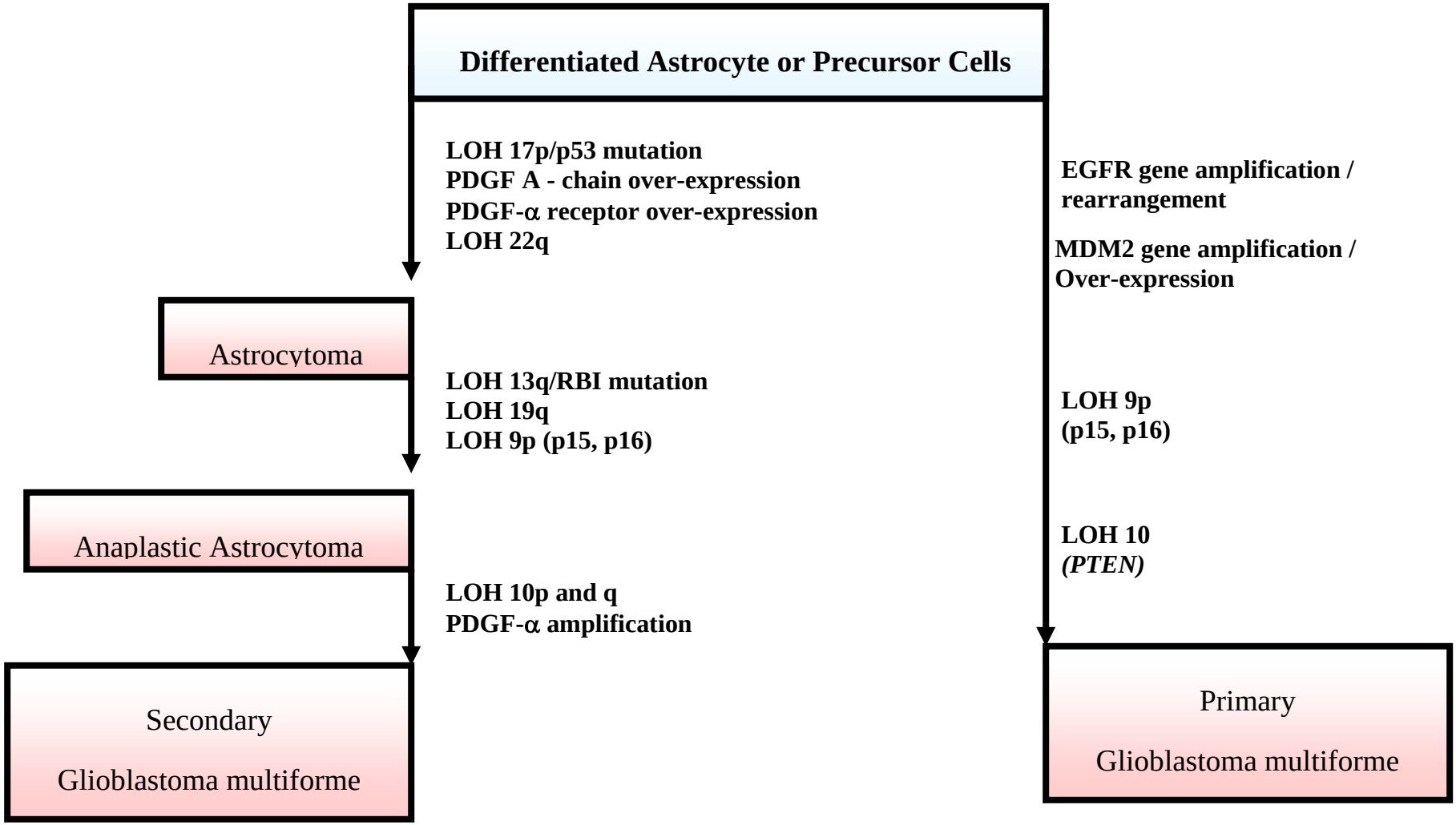
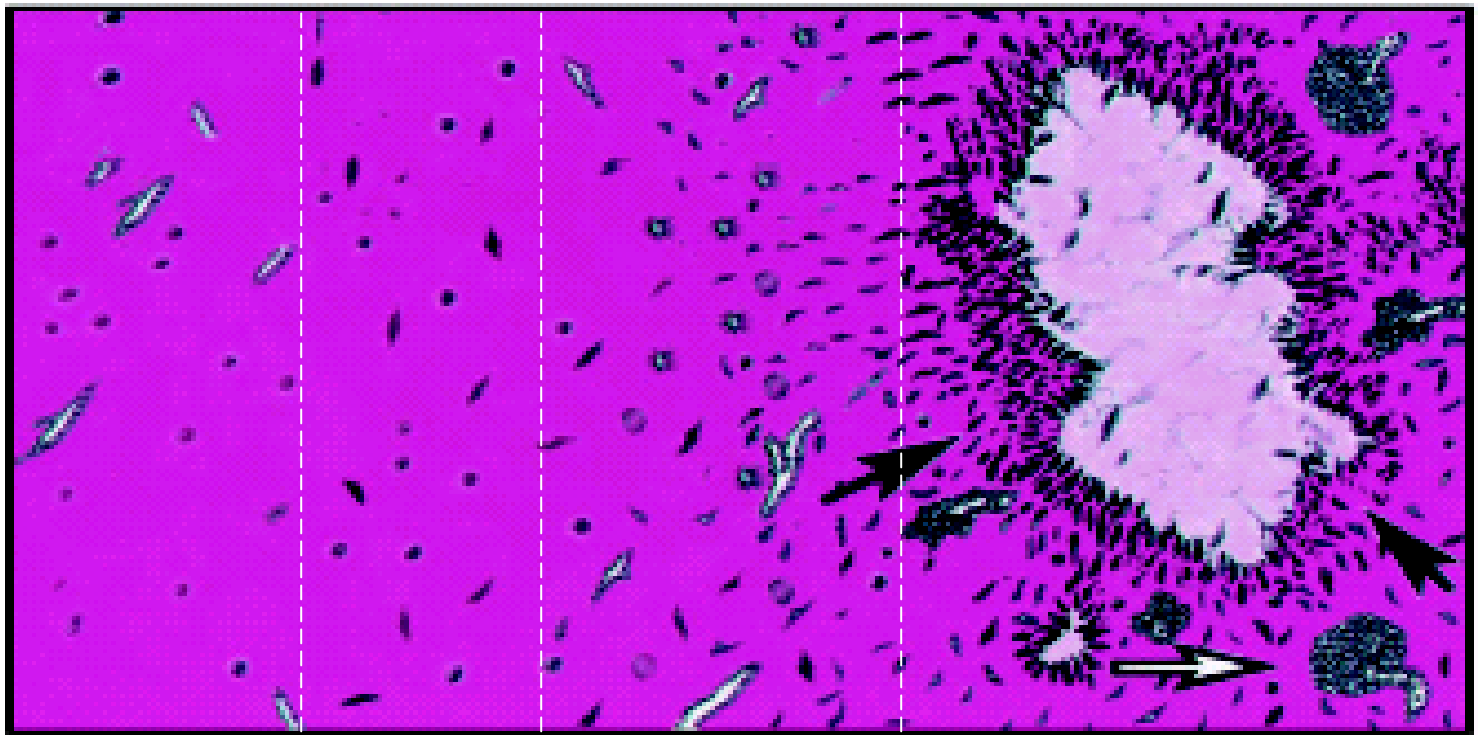


Fig 1

Fig 2
Illustration shows histopathological malignancy corresponding to glioma grade. WHO grade II shows infiltration of tumor cells. WHO grade III shows actively dividing tumor cells (mitoses). WHO grade IV characterized by presence of all the lower grade glioma features along with its hall-mark feature necrosis.

Histopathological Malignancy Corresponding to the Glioma Grade



Progression	Normal Brain	Infiltrating Astrocytoma (WHO grade II)	Anaplastic Astrocytoma (WHO grade III)	Glioblastoma multiforme (WHO grade IV)
Biology		Infiltration	Proliferation/expansion	Hypoxia/necrosis

Fig 2

producing intra-cranial structures such as dura, blood vessels or nerves. Due to elevated intracranial pressure, bi-frontal and bitemporal headache occur regardless of the tumor location. Normally 20-60% of all patients with cerebral tumors have seizures, either as initial symptom or occurring before tumor diagnosis. Seizures are more commonly associated with tumors in the anterior portion of the cerebral hemispheres. These may affect up to 50% of the patients. Other symptoms, including vomiting and visual failure along with impaired consciousness are also observed.

Glioma Therapy

The overall incidence of malignant gliomas may be increasing, particularly among adults, and hence lot of attention is paid for effective treatment procedures. Though brain tumor therapy is targeted to cure the patients, it is limited to betterment of prognosis and complete cure still remains a great challenge. Treatment for gliomas is mainly decided based on the tumor type and location, and on the condition of the patient. Surgical resection of the tumor tissue from brain is the first procedure done in the sequence of treatment. However, lack of clear-cut borders for the tumor is the major problem in removing the complete tumor mass. The extent of resection depends on the location and range of infiltration of the tumor and whether the tumor is benign or malignant. Though surgical resection of tumor tissue may not remove all the malignant cells, it offers considerable benefit for a limited time, especially

when tumor bulk *per se* is the problem, by giving relief from the increased intracranial pressure and other associated symptoms. Radiotherapy generally follows by surgical resection of tumor tissue to remove the post-operative remnants of tumor. This therapy is associated with major disadvantages, producing both systemic and local side effects. Acute reactions are usually caused by edema whereas early delayed radiation injury is mainly due to myelin loss. The delayed reaction will represent the most serious complication through an irreversible damage accompanied by lethal necrosis. Mostly a large portion of neoplastic cells are present at the center of the tumor mass. These hypoxic cells are relatively more resistant to irradiation. The best recovery of patients undergone radiotherapy is observed in tumors which are well localized, well circumscribed, and less than 5 cm in diameter. Chemotherapy is of limited use in the treatment procedure, due to heterogeneity of the cells in the tumor, presence of blood-brain barrier, poorly understood cell kinetics, variations in tumor vasculature and structure, blood flow, permeability and variations in the type and or extent of necrosis. Chemotherapy has to overcome the considerable difficulties of drug delivery and systemic toxicity. Permeability of the blood-brain barrier is a crucial factor in regulating the effective dosage to kill the tumor cells. Permeability alteration is unevenly distributed throughout the tumor. This leads to a considerable number of tumor cells present at periphery, and those invading the surrounding brain singly or in clusters, escaping the chemotherapeutics and forming a resistant clonogenic population. Recently, increased understanding of the immuno-evasion properties of brain tumor cells has a

tremendous improvement in the field of immunotherapy. Quantitative differences exist between the immune response of the brain and that of other parts of the body. Broadly, three types of immunotherapies have been employed. Non-specific immunotherapy will boost the host's general immune system. This may further increase lymphocyte infiltration of tumors and thereby clear the tumor cells. In active specific immunotherapy, treated neoplastic cells, tumor-antigen preparation or cross-reacting viral or bacterial antigens are noted to augment selectively certain specific immune reactions. Though the results have been promising, unfortunately it creates experimental allergic encephalomyelitis, caused by an immune response to cross-reactive antigen components shared by brain and injected analogous tumor tissue. Passive or adoptive immunotherapy is the third form of immunotherapy, in which immunity is transferred by administration of immune cells or sera from an immunized donor to cancer patients.

Recently, the promise for glioma therapy has been further extended by novel regional and molecular targeted therapies. Many molecular genetic abnormalities associated with phenotypic features are being increasingly characterized. They may include multiple abnormalities that span several critical regulatory areas, such as genomic instability, cell cycle control, and intracellular communication via signal transduction pathways (Rich et al., 2004). The simultaneous development of

multiple advanced therapies based on specific tumor biology may finally offer glioma patients improved survival rates (Reardon et al., 2006).

Experimental glioma models

Experimental demonstrations of genetic and molecular alterations that contribute to the etiology of central nervous system (CNS) neoplasm require precise and accurate modeling of tumorigenesis. Experimental study of nervous system tumors involves the use of established cell lines, primary cultures and human or animal material. *In vitro* analyses of gliomas mainly include, using established cell lines or primary cultures, which harbor different genetic profiles that are responsible for malignant transformation. *In vitro* analyses have the advantage that the observations are made under homogeneous conditions. Accurate animal models of glioma are essential to understand the morphological and functional properties of tumors in a multi-factorial environment. Alterations can be studied at different stages of tumor development. Tumor properties such as angiogenesis, metastasis and trials involving chemotherapeutics essentially need animal models. Different strategies followed for modeling gliomas explained in the Table 2.

Chemical carcinogenesis

N-Nitroso compounds were first described (Magee and Barnes, 1967) as important environmental carcinogens. Nitroso compounds and particularly

Different Strategies of Glioma Models

In vitro *glioma models*	
(i) Migration assays	
a. Monolayer migration assay b. Spheroid migration assay	To quantify glioma cell motility on coated plates (protein solution or specific ECM components)
(ii) Invasion assays	
a. Matrigel invasion assay b. Co-culture system	
In vivo glioma models	
Mutagens	DNA alkylation
Transplantation	Xeno or allograft, immunodeficient animals
Germline genetic modification	Transgene or gene targeting
Somatic genetic modification	Replication competent / deficient retrovirus

nitrosamides are not only carcinogenic but also toxic, teratogenic and mutagenic (Magee, 1969). Highest exposure occurs in certain occupational situations including rubber industries, contaminated cutting fluids used in metal industries, life-style exposures such as smoking, tobacco chewing, certain cosmetics, household rubber products and drugs have shown to contain low concentration of nitrosamine impurities. Nitrosoamines display organ-specific and species-specific carcinogenesis, and this organotropic action is predominantly governed by the chemical structure of the compound, strain of the animal used, mode of application, dosage, and duration of exposure (Preussmann and Wiessler, 1977). Some of the agents were found to be relatively specific for tumors of the nervous system (Druckrey et al., 1972, 1973). Using the carcinogens, it was reported that simultaneous multiplication of cells of different origin would lead to glioma formation (Zimmerman, 1962). Among the different carcinogens, methyl nitrosourea (MNU), ethyl nitrosourea (ENU) and piperdine are most commonly used carcinogens to induce the nervous system tumors. Metabolism of these oncogens may ultimately result in carbonium ion and nitrogen ion formation (Druckrey et al., 1972). The sites of action may be upon – SH, - OH or – NH groups of proteins or nucleic acids. Alkylation of guanine in DNA has been observed with ethyl and methyl nitrosourea and observed to persist longer in the brain than in the liver (Wechsler et al., 1969; Goth and Rajewsky, 1974; Kleihues and Margison, 1974). Neoplasms produced by MNU and ENU have morphological and biological similarities to naturally occurring neural tumors in human beings and animals. An

ethyl group attached to the nitrosourea results in a relative specificity of nervous system tumors by the transplacental route of administration, whereas a methyl side chain results in high yields of these tumors after intravenous administration in the mature animal. MNU has to be given in small repeated doses to adults to obtain the highest incidence, whereas ENU exerts its maximal carcinogenic action transplacentally or in neonatal application.

Transplacental administration of ENU

The transplacental route has been first used by giving intravenous injection of 7, 12-dimethyl benz (a) anthracene into 10-11 day pregnant hamsters. In a study of 232 *wistar* rats, the age at treatment had a critical role on the incidence and location of neurogenic tumors. Transplacental administration of ENU during the late mid and late gestational stages significantly influenced the morphological maturation of tumor cells. Histologically macro tumors obtained in the ENU administered rats on the 11^{th} day showed a poorly differentiated pattern, corresponding to primitive spongioblastomas, whereas rats injected on the 18^{th} day of gestation presented with macro tumors resembling human mature oligodendrogliomas, astrocytomas or mixed gliomas (Yoshino et al., 1985; Yoshino, 1985). The neonatal administration of ENU to *wistar/furth* (WF), *Long-Evas* (F1) and *Fischer* 344 (F344) rats led to the development of tumors of the nervous system with highest incidence (97-100%) during six months of observation. All the rats developed tumors in the temporal and frontal cortex and sub cortex, in all strains (Nalto et al., 1982). Transplacental ENU-

induction will lead to the development of the brain tumors composed of sub-eppendymal plate cells, glioblasts and various glial cells at different stages of maturation in 16, 18 and 20 week old rats. The altered cells may die or remain in the interphase, or may divide after a variable latency and ultimately will form the early lesion. The most vulnerable site is the angle of the lateral ventricles between the corpus callosum and caudate nucleus, located at lateral aspects of the ventricles (Pilkington and Lantos, 1979).

Among the nitrosourea-induced transplacental glioma rat models, weight loss is one of the earliest signs. Other symptoms reported include shaggy hair, limb paresis, corneal erosion, and head tilt. Rats may be either aggressive or depressive and have dyspnea (Allen, 1972). Characterization of early pre-neoplastic changes that occur during latency is highly significant for understanding specific drug targets and to know the cellular origin of gliomas. Further, neoplastic cells attain their morphological identity in the form of early neoplastic proliferation (ENP) centers after 56 days (Schiffer et al, 1978). These lesions may maintain their potential to differentiate into various types of glia, producing gliomas of mixed cellularity (glioblastoma multiforme and mixed gliomas) or they may follow a single line of maturation, resulting in astrocytomas, oligodendrogliomas and eppendymomas (Pilkington and Lantos 1979).

Cellular origin of gliomas

In spite of considerable speculation, the types of cells responsible for gliomagenesis are not yet clearly known. Forced expression of oncogenes either in neural precursors or mature cells in mice resulted in tumors similar to human gliomas (Holland, 2001). Glioma cells most frequently resemble immature astrocytes, immature oligodendrocytes or mixtures of the two cell types in their morphology and gene-expression characteristics. Central nervous system stem cells can further differentiate into neuronal and glial progenitors, which subsequently give rise to mature cell types found in the brain, including neurons, oligodendrocytes and astrocytes. Specific signal transduction pathways control the selective differentiation of precursor cells into mature glia. Engineering either a mature astrocyte or a neural stem cell may give rise to a typical oligodendroglioma, suggesting that specific combinations of gene disruption, rather than the origin of cells, determines the final tumor phenotype (Dai et al, 2001). *In vitro* analysis revealed that PDGF causes the oligodendroglial progenitor population to proliferate (McKinnon et al., 1990) and cooperates with fibroblast growth factor-2 (FGF-2) preventing its further differentiation into mature oligodendrocytes. Epidermal growth factor (EGF) and CNTF force glial progenitors towards astrocytic and oligodendrocytic differentiation (Mayer et al., 1994). However, the identification of significant numbers of neural progenitor cells in the adult brain tumor suggests that either a precursor or mature glial cell might constitute a cell-of-origin of gliomas. Better understanding of cellular origin for gliomas takes an added importance in

considering the possible future use of neural stem cells as a potential therapeutic strategy. The cellular origin of astrocytomas is illustrated in the Fig 3.

Molecular pathology associated with gliomagenesis

Alterations in the expression of genes controlling apoptotic signaling will ultimately be responsible for the malignant transformation of normal cells (Kabore et al., 2004). In gliomas most of the genetic alteration will result in the disruption of three main cellular systems: Retinoblastoma-1 (Rb1) regulation (by inactivation of Rb1 or p16/CDKN2A or amplification of CDK4), P53 pathway (by inactivation of p53 or p14/ARF) or amplification of mouse double minute-2 (MDM-2) and tyrosine kinase receptor signaling (by activating EGFR or PDGFR, or inactivating PTEN). Transgenic glioma models have shown that disruption of all three pathways increases development of gliomas dramatically (Hesselager et al., 2003; Bachoo et al., 2002; Sanson et al., 2004). Abnormalities in the two major types of pathways, those regulating the cell cycle and those mediating growth factor responses, contribute to gliomagenesis. Thus, elucidating the mechanisms by which abnormalities in the cell cycle and growth factor signaling pathways trigger glial transformation is an important goal.

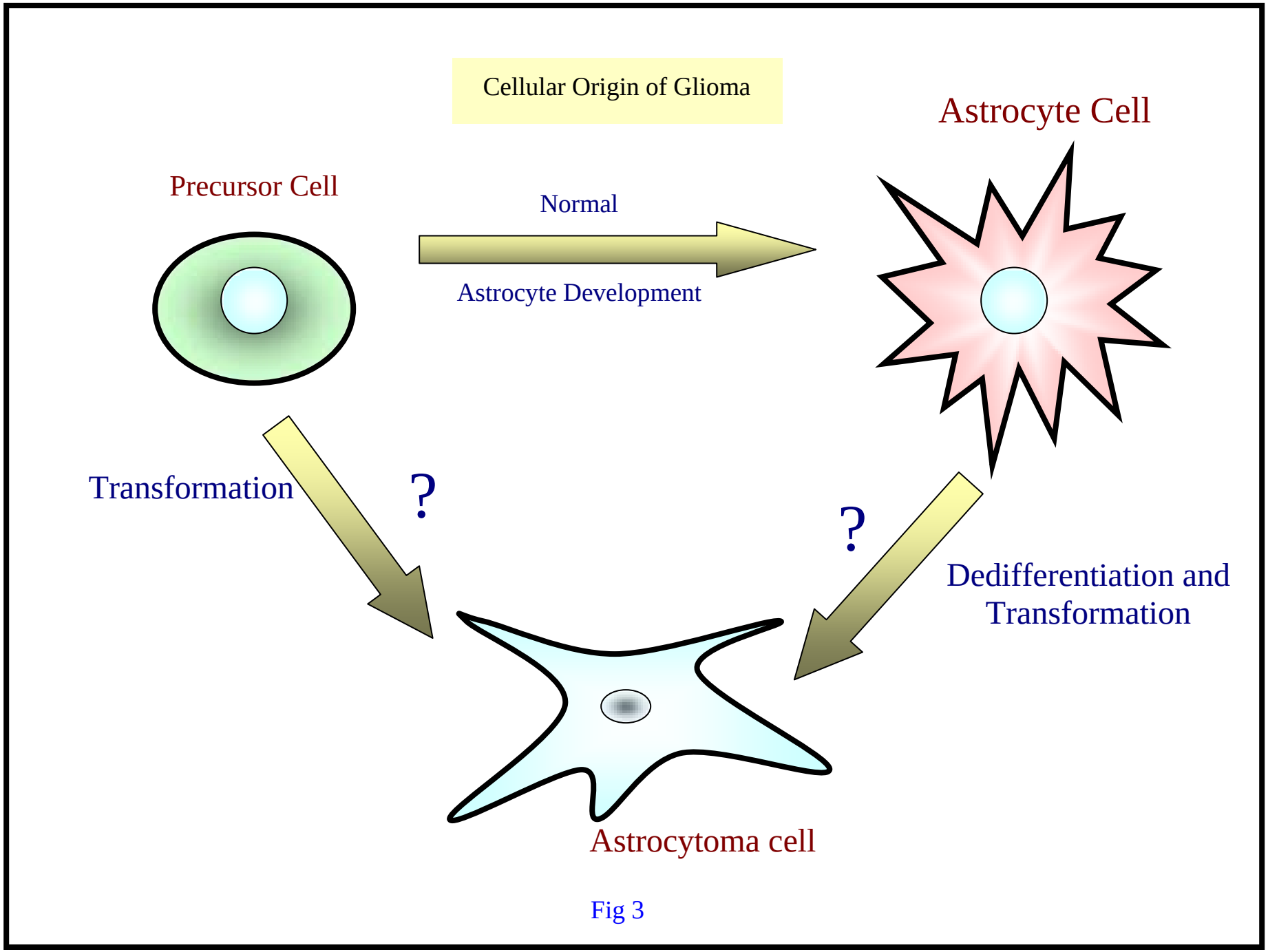

Cellular Origin of Glioma
Astrocyte Cell
Precursor Cell
Normal
Astrocyte Development
Transformation
?
?
Dedifferentiation and
Transformation
Astrocytoma cell

Fig 3

Scope of the present study

Gliomas, despite tremendous improvement in the various techniques, remain an unresolved problem. Furthermore, the incidence of malignant gliomas may be increasing, particularly among the elderly patients. Elucidation of pathogenic or predisposing factors remains a high priority, as these are largely unknown. Malignant gliomas, like other forms of cancer, exhibit aberrant proliferation, diminished apoptosis, avoidance of both external growth control and immunoregulation, and striking rates of *de novo* and acquired resistance to therapeutic intervention.

Glioma progression is due to accumulation of inherited and/or adopted genetic mutations. Different pathological grades may represent the corresponding malignancy status. In the recent past, mitogen activated protein (MAP) kinase signaling was observed to have a significant role in tumorigenesis, tumor progression, and metastasis. Distinct MAPK pathways have been characterized in detail: extracellular signal-regulated kinase 1 and 2 ($ERK^1/2$), c-Jun N-terminal kinase / stress activated protein kinase (JNK/SAPK) and p38. Among these pathways, the $ERK^1/2$ pathway is activated by growth factors and well known to be responsible for increased cell proliferation, differentiation and cell survival during the invasion and metastasis of glioma cells. Over-activation of $ERK^1/2$ may ultimately relate to the extent of cellular proliferation. The present study was

therefore carried out to find the relation between the levels of activated ERK[1]/2 and glioma malignancy.

Two forms of cell death classified broadly include apoptosis (programmed cell death) and necrosis (random cell death). Though removal of cells by cell death in cancer cells may be a desirable process in the cancer treatment, the extent of necrosis progression was reported to be inversely related to patient prognosis. However, the exact mode and mechanism of cell death in gliomas is not clearly understood. Increasing evidence, obtained from both *in vitro* and *in vivo* experiments, suggest there could be a variety of cell death programs that may be triggered in distinct circumstances. This study was aimed to estimate different cell death proteases activated during the apoptotic and / or necrotic forms of cell death. Identification of alternative cell death programs may provide novel therapeutic targets, with important consequences for attempts to treat glioma associated with the induction of apoptosis and inhibition of necrosis progression.

ENU-induced transplacental glioma rat model produces lesions in the brain, which have morphological and biological similarities to naturally occurring neural tumors in human and animal. ENU-induced tumor model can be used to study the tumors that arise from multipotent neural stem cells, which persists in the sub-ventricular zone (SVZ) regions of the brain. These tumors may develop in three stages after induction. The initiation stage may represent irreversible or stable

damage to DNA. The promotion stage is an epigenetic process bringing about a clonal expansion of initiated cells. The progression stage results when genetic instability leads to further mutagenic and epigenetic changes. This model may represent the pathological and molecular changes due to the developing tumor but not due to the continued exposure to mutagens.

In the present study, two important pathways that control cell proliferation and sustained cellular survival have been detected at early and progressive stages of tumor development in an ENU-induced transplacental glioma rat model.

Objectives of the study

- ERK ^{1}/2 pathway activation in different grades of human glioma biopsies related to their malignancy.
- Detection of different cell death protease activation that ultimately cleaves full Length poly ADP-ribosyl polymerase (PARP) into different molecular weight fragments indicating the possible existence of multiple forms of cell death.
- The possible role for ERK^{1}/2 and pAkt pathway in ENU induced tumor model was assessed at early and progressive stage of tumor development.

MATERIALS & METHODS

CHAPTER - II

CHAPTER II

Materials

Reagents for SDS-PAGE, agarose (molecular grade) for DNA gel electrophoresis, n-ethyl n-nitrosourea (ENU), and 5-bromo-4-chloro-3-indoylphosphate/nitroblue tetrazolium chloride (BCIP/NBT) were obtained from Sigma Chemicals Co. The immunohistochemistry kit was obtained from DAKO Cytomation, USA. Nitrocellulose sheets were from Millipore pvt. Ltd., USA. TUNEL kit was obtained from Bio-Rad, USA. All other chemicals were of analytical grade and obtained from standard commercial suppliers.

Human surgical tumor samples

Glioma samples from five different patients were collected from Nizam's Institute of Medical Sciences (NIMS) operating theatre, Punjagutta, Hyderabad (AP), India. Samples were collected as core and peripheral tumor tissues. The samples were divided into two parts. One part of each sample was processed for pathological studies. The second parts of each sample immediately snap frozen in liquid nitrogen and stored at -80^0C for biochemical analyses. Subjects used in the present study had not undergone any treatment prior to the surgery. Informed consent was obtained from the patient/relatives before collecting the samples.

Methods

Sample preparation and analysis of proteins

Deep-frozen human glioma biopsies and cerebral cortex regions of control and glioma rat brains were thawed gradually and homogenized using a Dounce homogenizer in Radio Immuno Precipitation Assay (RIPA) buffer containing 50 mM tris-HCl pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.4% deoxy cholate, 1% nonidet p-40 including the protease inhibitor PMSF 10 mM and the phosphatase inhibitors of β-glycerophosphate 10 mM, NaF 10 mM, Na_3VO_4, 0.3 mM. Lysates were sonicated (two times for 2 min each) and further centrifuge (at 14,000 rpm) for 15 min at 4^0C. The supernatant was collected into pre-chilled appendorfs tubes. Protein was estimated by Lowry's method (Lowry et al., 1951). Equal amounts (50 μg) of protein were separated by 10% SDS-PAGE. Protein pattern was visualized by staining with coomassie blue.

Western immunoblot analyses

After separating equal amount (50 μg) of proteins from the tissue lysates, using 10% SDS-PAGE proteins were transferred onto nitro-cellulose membranes (Towbin et al., 1979). After blocking in non-fat skimmed milk (5%) in tris buffered saline (TBS; 10 mM tris pH 7.5, 150 mM NaCl) for 1 hr, membranes were incubated for 12-14 h with primary antibodies (Monoclonal anti-GFAP from Sigma Aldrich, USA; monoclonal anti-PDGFR from Santa Cruz-Gifted by Dr. Nupam Mahajan;

polyclonal anti-Mcm2 from Santa Cruz-Gifted by Dr. Laura Calza (Italy), monoclonal anti-phospho Tyr, polyclonal anti-ERK[1]/2 and anti-phospho ERK[1]/2, monoclonal anti-Bax, monoclonal anti-phospho BAD, monoclonal anti-Bcl_2, monoclonal anti-caspase-3, polyclonal anti-full length PARP, polyclonal anti-Cox 2, and polyclonal anti-phospho Akt obtained from Cell Signaling Technology Ltd, USA; monoclonal anti-calpain I and II obtained as a gift from Dr. Panaiyur S. Mohan, Nathan Kline Institute, USA; monoclonal anti-granzyme B and anti-AIF obtained from Oncogene,USA). Membranes were washed with TBS and TBST (Tris-buffer saline containing 0.1% Tween 20) for three times 5 min each. After washings, the blots were then incubated with secondary antibodies conjugated to alkaline peroxidase (ALP) (anti-rabbit and anti mouse IgG conjugated to ALP obtained from Bangalore Genei Pvt Ltd., Bangalore, India), for 1 hr at room temperature. After incubation with secondary antibodies the membranes were washed with TBS and TBST (Tris Buffer Saline containing 0.1% Tween20) for three times 5 min each. Immunoreactivity was visualized by incubating the blots with 33 μl of 5-bromo4-chloro3-indoyl phosphate (BCIP; 5%) and 66 μl of nitro blue tetrazolium chloride monohydrate (NBT; 1%) in 10 ml of ALP buffer. Images were scanned and further analyzed quantitatively using Scion-image software.

Morphological studies

The part of human glioma biopsies and glioma rat brain samples were fixed in 4% parafarmaldehyde in phosphate buffered saline (NaCl: 400 mg, KCl: 10 mg, Na_2HPO_4: 780 mg and KH_2PO_4: 10 mg in 50 ml of double distilled water pH, 7.4) and subsequently tissues were dehydrated in a series of graded alcohol and cleared by using chloroform. Finally tissues were infiltrated and fixed in paraffin to make tissue blocks. Sections of 6 micron thickness were taken and transferred onto poly-L-lysine (Obtained from Sigma Aldrich; Bangalore, India) coated slides.

Haematoxylin and eosin (H & E) staining

Sections were first deparaffinized by using xylene (two washes for 7 min each) and cleared in absolute acetone for 2 min. Sections were then washed with 95% ethanol for 2 min followed by tap water and double distilled water for 5 min each. Sections were incubated in haematoxylin (Harries) for 5 min and then washed with tap water and double distilled water for 5 min each. Then the sections were incubated with the eosin reagent for 1 min. Washed the sections with 95% ethanol two times for 1 min and followed by acid alcohol wash (1% acid in 70% alcohol) for few seconds. Acetone wash followed by acetone and xylene mixture for 2 min each. Finally, stained sections were cleared in xylene two times for 2 min each. Mounting was done by using cover-slips and DPX mount to view the section under microscope.

Immunohistochemical (IHC) analyses

Serial sections of confirmed astrocytoma and glioblastoma human biopsy sections, and tumor-containing rat brain sections were deparaffinized in xylene, passed through graded alcohols, and rehydrated in phosphate-buffered saline (PBS). Antigen unmasking was carried out by microwaving the sections in 10 mM citrate buffer, pH 6.0. Sections were incubated with 1% hydrogen peroxide in PBS to quench non-specific peroxidase. Non-specific binding was blocked with diluted goat serum for 1 hr at room temperature in a humid chamber. Primary antibodies (Polyclonal anti-phospho $ERK^{1}/2$, polyclonal anti-Cox 2, polyclonal anti-cleaved caspases-3 obtained from Cell Signaling Technology Ltd, USA; polyclonal anti-nestin, polyclonal anti-neuron-specific enolase, polyclonal anti-Ki67 gifted by Dr. Laura Calza, Italy; monoclonal EGFR gifted by Dr. Nupam Mahajan, USA; monoclonal GFAP obtained from Sigma Aldrich, USA; monoclonal anti-granzyme B, and anti-AIF obtained from Oncogene, USA) were prediluted as per the data sheet in blocking solution, and incubated with the sections overnight at 4^{0}C. After washing in the PBS (3 X 5 min each), sections were incubated with peroxidase-conjugated secondary antibody for 1 hr at room temperature followed by PBS washes (3 X 5 min each). Diaminobenzidine (DAB) was applied until the section developed colour. Sections were then counterstained in haematoxylin, then washed with dH_2O followed by dehydration in graded alcohols, xylene and mounting was done using DPX according to manufacturer's instruction (DAKO Cytomation, USA).

DNA Agarose gel electrophoresis

Astrocytoma (WHO grade II) and GBM (WHO grade IV) patient core tumor and peripheral tissues were collected from the operating theatre and immediately snap-frozen in liquid nitrogen. Samples were homogenized in normal saline and then centrifuge (at 1000 rpm) for 10 min. The pellet was washed twice with normal saline. The washed pellet was resuspended in ten volumes of digestion buffer (containing 100 mM Tris-HCl pH 8, 5 mM EDTA, 0.2 M NaCl, 0.4% SDS, proteinase-k 0.2 mg/ml) and incubated for overnight at 37^0C. DNA was extracted with phenol: chloroform: isoamylalcohol (24:24:1), and precipitated by the addition of two volumes of absolute ethanol. DNA extracts were analyzed for purity and electrophoresed on 1.2% agarose gels in TBE buffer. The DNA fragmentation pattern was visualized by ethidium bromide staining.

In situ **TUNEL labeling**

In situ DNA end labeling (TUNEL staining) was performed on paraffin embedded tissue sections of astrocytoma and glioblastoma. Sections were deparaffinized, washed in different grades of alcohol and then with buffered saline (PBS), further incubated for 15 min at room temperature with 20 μg/ml of proteinase-k and washed in dH_2O. Sections were then incubated with terminal deoxy-nucleotidyl transferase (TdT) and bromo-deoxyribonucleotide triphosphate (BdNTP) for 60 min at 37^0C in a humidified chamber. The reaction was stopped by incubating the sections with stop buffer for 5 min, followed by a wash in PBS and

incubation in PBS with anti-bromo-horseradish peroxidase for 20 min. After two PBS washes, the sections were treated with the chromagen diaminobenzidine (0.07 mg/ml) that turned the labeled DNA fragments brown. Sections were washed in dH_2O, counter-stained with 0.3% methyl green in PBS, again washed with dH_2O and ethanol, and visualized under light microscopy.

Screening vaginal smears for time-specific gestation in rats

Institutional ethical clearance was obtained and the national ethical committee guidelines (CPCSEA - 2003) were followed for all the procedures. *Wistar* rats maintained in the animal house of University of Hyderabad were used for experimentation. Female rats weighing 250 g were screened regularly using the methyl-blue dye method for the pattern of vaginal smears to detect the different stages of the estrus cycle. When the rats showed the estrus smear pattern, they were allowed to mate. On the day following mating, the appearance of the diestrus pattern of vaginal smear indicated day-zero of pregnancy.

Induction of cerebral tumors using transplacental administration of n-ethyl n-nitrosourea (ENU) in *Wistar* rats

Pregnant *Wistar* rats on the eighteenth day of gestation were treated with ENU (Sigma Aldrich, USA) at a rate of 75 mg/kg body weight intra-peritoneally (Schiffer et al., 1978). Twenty-four rats with corneal erosion and other neurological symptoms were used for the study. A set of eight rats, after three months of extra

uterine life, were used for histopathological and western analysis. Twelve rats were used for the same experimentation after six months. Rats of the same age group treated with saline, were used as controls.

Status of mitogen activated protein (MAP) kinase–1/2 or Extracellular signal regulated Kinase (ERK)-1/2 among the human gliomas

CHAPTER - III

CHAPTER. III

INTRODUCTION

One of the characteristics of solid tumors, including brain tumors, is the deregulated cell growth observed due to perturbed signal transduction as a result of an accumulation of inherited and / or acquired defects in the genes for certain classes of signaling proteins and pathways that are targeted much more frequently by the oncogenic mutations than others (Hunter; 1997). Studies of astrocytomas have led to the identification of two major groups of signaling proteins, whose abnormalities contribute to gliomagenesis: the cell cycle pathways and the growth factor-regulated signaling pathways. Among the cell cycle proteins, the p16-cdk4-pRb and ARF-MDM2-p53 cell cycle arrest pathways play a prominent role in glial transformation (Konopka and Bonni; 2003).

The role of growth factors in gliomagenesis has increasingly been studied over the last few years. Growth factor ligands may stimulate their cognate receptors through endocrine, paracrine, autocrine or internal autocrine mechanisms (Feldkamp et al., 1997). Ligand-receptor interactions can initiate subsequent downstream signaling cascades, which involve a large number of intermediate signaling proteins

and eventually result in a deregulated cellular proliferation and cell death, aberrations of which are key features for the malignancy. The receptor protein tyrosine kinase (RTK) class of receptors has been found to be clearly linked with the pathogenesis of astrocytomas. Phosphorylation of a tyrosine residue on the intracellular domain of RTK will result in its activation. Activated RTKs then transautophosphorylate subsequent signaling proteins. Constitutive activation of RTKs is one of the important features of aberrant signaling leading to the malignant transformation and tumor proliferation by different mechanisms (Kolibaba et al., 1997). The two major RTKs implicated in the pathogenesis of human astrocytomas are the PDGFR and the EGFR. In human GBM, amplification of the EGFR gene on chromosome 7p was reported, however its rate appears to be lower in the anaplastic astrocytoma (Smith et al., 2000). Further, over-expression of another important growth factor receptor, PDGFR-α, appears to be an early event in glioma pathogenesis and is present in the all grades of tumors. Accumulating evidence suggests an important role for PDGF ligands and receptors in the vascularization of gliomas (Bogler et al., 1995; Morrison; 1999). PDGF/PDGFR alterations are most commonly observed in the secondary GBM or those malignant gliomas that arise from lower-grade tumors (Heldin et al., 1990). Though there were mixed results obtained in glioblastoma patients by inhibiting EGFR, several encouraging observations have been reported.

Gliomas may activate receptor-mediated pathways by different hypothetical mechanisms:

(i) Over-expression of both ligands and receptors leading to an autocrine loop (eg. Platelet derived growth factor (PDGF)/Platelet derived growth factor receptor (PDGFR), (Lokker et al., 2002)).

(ii) Genomic amplification and /or mutation of the receptor leading to constitutive activation even in the absence of ligand (eg. Epidermal growth factor receptor (EGFR) amplification is a late event that occurs in the pathogenesis of gliomas, unlike PDGFR. Mutated EGFR (p140EGF-R) is over-expressed in nearly 40% of glioblastomas (Holland et al, 1998; Ekstrand et al., 1994).

(iii) Activation of intracellular signal transduction due to mutated protein kinases (eg. PTEN which control focal adhesion kinase (FAK) has been shown to inhibit glioma cell migration; thereby, PTEN inactivation is associated with glioma dissemination (Kato et al., 2004).

Components of mitogen activated protein (MAP) kinase pathway

Following the activation of receptor tyrosine kinases, multiple signaling pathways are activated in the cytosol. Among different pathways, accumulating evidence shows a significant role for mitogen activated protein (MAP) kinase pathways in vital cellular activities. MAP kinase pathways are highly conserved and form central mediators to propagate the signals from the membrane to the nucleus. MAP kinases include a group of serine/threonine kinases activated through different

multiple protein kinases by undergoing dual phosphorylation (Chang et al., 2001). There are four major subgroups of MAP kinases in mammalian cells: extracellular signal regulated kinase (ERK), c-Jun-N-terminal kinase (JNK), p38 and extracellular signal regulated kinase-5, 9 (ERK-5 is also referred to as big MAP kinase 1 or BMK1) (Lee et al., 1995). The MAP kinase module is composed of at least three kinases that establish a sequential activation pathway. The top kinase of the module is a MAP kinase kinase kinase (MKKK). The MKKKs are serine/threonine kinases that, when activated will phosphorylate the next kinase in the module, a MAPK kinase (MKK). The MKKs are kinases which recognize and phosphorylate a Thr – X – Tyr motif in the activation loop of MAP kinase (Boulton et al., 1991). At present there are combinations of twelve MAPKs, seven MEKs (MAPKK) and fourteen MKKKs (MAPKKK) with an apparently redundant function which seems an extraordinarily complex system (Zhang et al., 2002). The majority of defined substrates may include protein kinases, phospholipases and cytoskeleton associated proteins such as tau-proteins (Kyriakis et al., 2001).

The role of the extracellular signal regulated kinase (ERK) pathway

The ERK pathway is the best-characterized pathway, activation of which results in transmission of mitogenic signals from the extracellular environment to the nucleus. The ERK pathway may play a critical role in both brain physiology and pathology. There is now compelling evidence that over-expression of this pathway,

secondary to oncogenic constitutive activation, plays a role in malignant transformation in many cancers. Regulation of cell proliferation is the best-studied function of ERK[1]/2. The other most-explored function is regulation of gene expression. ERK 1 and ERK 2 are 42 and 44 kDa proteins respectively (Steelman et al., 2004). They are both serine/threonine kinases, and appear to be functionally equivalent. Activation of ERK occurs by dual phosphorylation of T^{182} and Y^{184} residues in its activation loops (Crews et al., 1992). This reaction is catalyzed by MEK, which can phosphorylate at two different aminoacid residues. Following the phosphorylation, dimerization of ERK occurs, which may play a role in the nuclear translocation of this protein kinase. Activation of ERK inhibits apoptosis, induced in response to stimuli such as hypoxia, growth factor withdrawal and chemotherapeutic agents (Buckley et al., 1999). However, the exact mechanisms of how various MAP kinases can modify the rate of apoptosis are not clear. Mutations have been identified in the genes coding for several components of the ERK/MAP kinase pathway in different cancers. Mutated and constitutively activated forms of Ras are found in approximately 50% of all human metastatic tumors. However, in gliomas, specific mutations affecting Ras have not been detected, although higher levels of Ras-GTP have been documented in high-grade astrocytomas (Guha et al., 1997). Activation of other small G-proteins such as Rap1 is also known to activate the Raf1/MEK/MAP kinase pathway and may result in increased cell proliferation in astrocytomas (Gutmann et al., 1997). Over-expression of transforming ErbB receptor complexes in murine fibroblasts and human glioblastoma cells leads to

constitutive activation of ERK and c-Jun N-terminal kinase (JNK), suggesting their role in the enhanced transformation and resistance to apoptosis seen in primary glioblastoma cells (Wu et al., 1999). Sphingosine-induced C6 glioma cell death and apoptotic blebbing was dependent on $ERK^1/2$ signaling and occurred only when $ERK^1/2$ activity was decreased or abolished (Krzeminski, 2005). A growing number of recent studies have revealed that differential accessibility of $ERK^1/2$ to downstream targets, which is dictated by the persistent activation of $ERK^1/2$ within the distinct sub-cellular compartments, underlies the neurotoxin responses that are driven by this kinase (Chu et al., 2004). Inhibition of $pERK^1/2$ by using synthetic inhibitors (U0126) reduced invasion of tumor cells in the xenografted melanoma tumor in nude mice (Bedogni et al., 2004).

Bcl_2 and Bax

Neoplastic cells can persist in hostile niches (where cytokines or oxygen is limiting), escape the death that is often imposed as a fail-safe mechanism by other oncogenic changes and evolve into more aggressive derivatives. The Bcl_2 family includes both pro- and anti-apoptotic members that play a central regulatory role in apoptosis. Commitment to apoptosis is typically governed by opposing factions of the Bcl_2 family of cytosolic proteins. Initiation of the proteolytic cascades requires assembly of certain caspase precursors on a scaffold protein, and the Bcl_2 family determines whether this complex can form. Its pro-survival members can act by sequestering the scaffold protein and/or preventing the release of apoptogenic

molecules like cytochrome C (cyt C) from mitochondria. On the contrary, its pro-apoptotic members act as sentinels for cellular damage as cytotoxic signals induce their translocation to the organelles where they bind to their pro-survival relatives, promoting organelle damage and triggering apoptosis.

The Bcl_2 gene was first identified as a part of the most common translocation in human B cell follicular lymphoma (Vaux et al., 1988) and the over-expression of Bcl_2 in transgenic animal models mimics the human disease (McDonnell et al., 1989; McDonnel and Korsmeyer, 1991). Bcl_2 has since been shown to enhance cell survival by inhibiting apoptosis induced under a wide variety of circumstances although not by all cytotoxic insults suggesting that it is a ubiquitous inhibitor of cell death triggered by multiple routes.

Bcl_2 possesses a putative transmembrane domain at its carboxyl terminus and is associated with mitochondria, endoplasmic reticulum and nuclear membranes. Bcl_2 is expressed widely during embryogenesis (Le Burn et al., 1993; Novach and Korsmeyer 1994); its expression becomes more restricted in adult tissues to those components requiring long-term survival, such as stem cells, post-mitotic neurons, and cells in proliferating zones (Hockenbery et al., 1991).

The first identified Bcl_2 homologue was Bax. In fuctional assays Bax suppresses the ability of Bcl_2 to block apoptosis (Oltvai et al., 1993). One hint that Bax may be a death effector that is functionally sequestered by Bcl_2 comes from the observation that BH1 mutants of Bcl_2 both fail to associate with Bax and suppress apoptosis but are still capable of forming Bcl_2 homodimers (Yin et al., 1994).

RESULTS

Case history & Haematoxylin and eosin staining of glioma biopsies

Samples were analyzed from 4 males and 1 female patients. The patients presented with a wide variation of clinical features. Detailed case histories are presented in Table 3.

Haematoxylin and eosin staining of biopsies was used for pathological grading. Increased cellularity along with mitoses was observed in the core tumor tissues of cases #1, #2 and #5. Core tumor tissues of the cases #3 and #4 showed coagulative necrotic features. In some microscopic fields, the samples showed low-grade features. Peripheral tissues of the tumors with brain parenchyma showed reactive gliosis (Fig 4).

Case History of Glioma Patients

S.No.	Label	Gender	Age (Years)	Clinical Features	Histopathological Diagnosis
Case – 1	C1T	Male	60	**One episode of seizure, no focal deficits.**	Astrocytoma (WHO grade II)
Case – 2	C2P C2T	Female	37	**Headache from past four years of surgery olfactory hallucinations, bilateral papilloedema, no focal deficit, uncinate seizures.**	Astrocytoma (WHO grade II)
Case – 3	C3P C3T	Male	48	**Generalized tonic clonic seizures.**	Secondary GBM (WHO grade IV)
Case – 4	C4P C4T	Male	27	**Headache, vomiting from past 15 days of the surgery, bilateral papilloedema, no focal deficits.**	Secondary GBM (WHO grade IV)
Case – 5	C5T	Male	19	**Headache and vomiting from past 3 months of the surgery.**	Astrocytoma (WHO grade II)

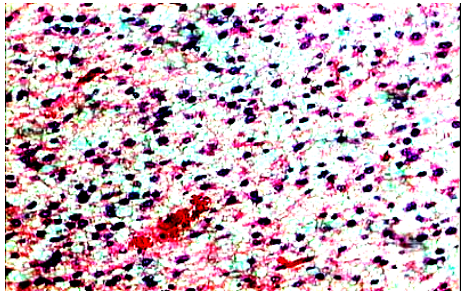
Table 3

Fig 4

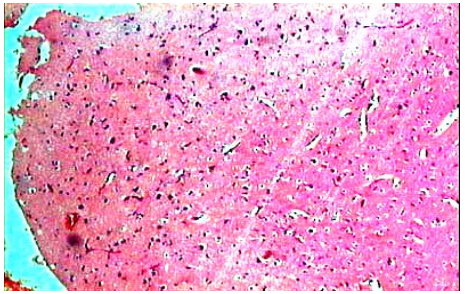
Haematoxylin and eosin staining of surgically resected glioma tissues.

(a) Case #1: Astrocytoma WHO grade II showing increased cellularity and atypia (b) Case #2: Periphery of the tumor with brain parenchyma showing reactive gliosis (c) Case #2: Lower grade astrocytoma features, WHO grade II (d) Case #3: Periphery of the tumor with brain parenchyma showing reactive gliosis (e) Case #3: GBM with low grade areas (f) Case #3: GBM with areas of coagulative necrosis and high grade morphology (g) Case #4: GBM with low grade areas (h) Case #4: GBM with high grade areas (i) Case #5: Astrocytoma, WHO grade II.

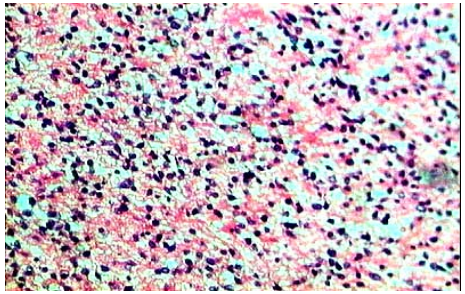
Haematoxylin and Eosin (H & E) Staining of Surgically Resected Glioma Tissues



(a) Objective: 10X

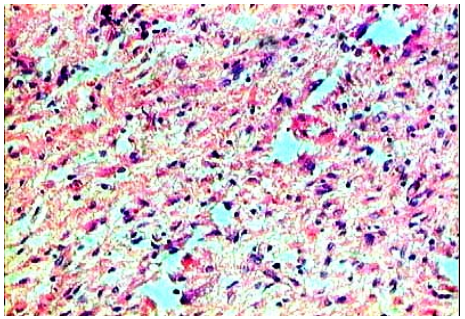


(b) Objective: 4X

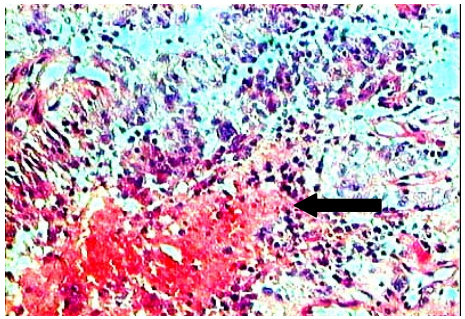


(c) Objective: 10X

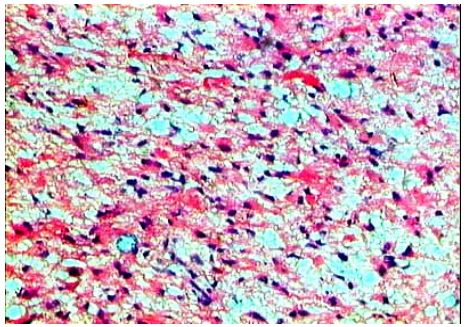
(d) Objective: 4X



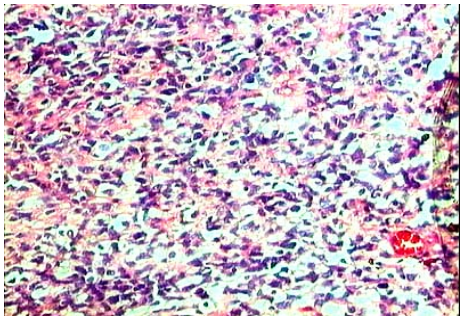
(e) Objective: 10X



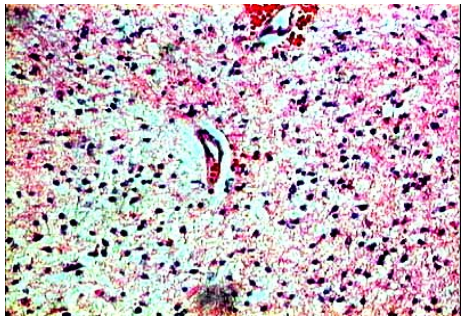
(f) Objective: 10X



(g) Objective: 10X



(h) Objective: 10X



(i) Objective: 10X

Fig 4

Differential protein pattern among the different glioma tissues

Proteins were resolved on 10% SDS-PAGE. There were different protein patterns in peripheral tissues and core tumor tissues. Similar patterns were observed in low-grade and high-grade tumor tissues. An increased expression of a protein around 66 kDa molecular weight was observed in glioblastoma core tumor tissues of cases #3 and #4 (Fig 5).

Analysis of Glial Fibrillary Acidic Protein (GFAP) and receptor activation in glioma biopsies

GFAP was observed to be present at a higher level in core tumor tissues than peripheral tissues. Among the samples analyzed higher expression levels were observed in astrocytoma tumor tissue of case #2 (Fig 6). Tissue samples were analyzed for the levels of PDGFR and EGFR. From the result it is clear that an increased level of PDGFR was observed in all the tumors of glioma tissues (Fig 7).

Mitogen activated protein (MAP) kinase ^{1}/2 or extracellular signal regulated kinase ^{1}/2 (ERK^{1}/2) pathway in human gliomas

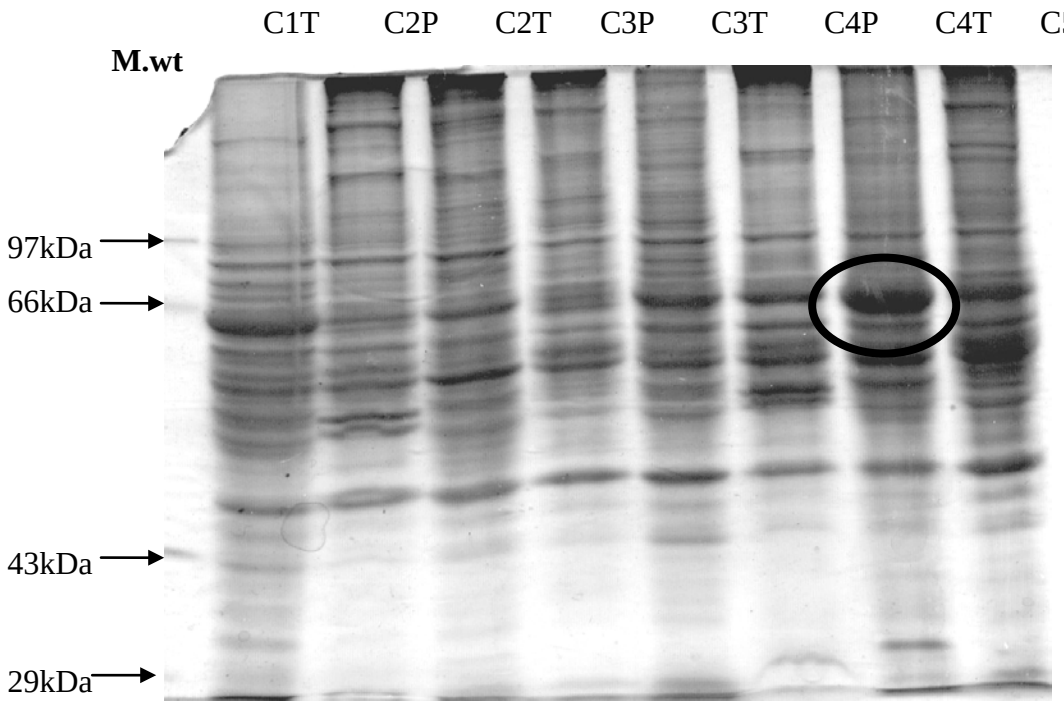
Extracellular signal regulated kinase (ERK^{1}/2) is the ultimate effector molecule in the MAP kinase pathway it undergoes dual phosphorylation at threonine or serine and tyrosine residues for its complete activation. Activated ERK^{1}/2 will then translocate into the nucleus from cytosol to activate the corresponding transcription

Fig 5

Resolution of whole tissue lysate samples on 10% SDS-PAGE showing differential protein pattern between low grade and high grade glioma tissues.

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

Differential Protein Patterns observed in SDS-PAGE among different glioma tissue samples



C1T: Case #1 core tumor sample;
C2P: Case #2 peripheral tumor sample; C2T: Case #2 core tumor sample;
C3P: Case #3 peripheral tumor sample; C3T: Case #3 core tumor sample;
C4P: Case #4 peripheral tumor sample; C4T: Case #4 core tumor sample;
C5T: Case #5 core tumor sample

Fig 5

Fig 6

Glial fibrillary acidic protein (GFAP) western immunoblot showing increased levels in the core tumor tissues.

Fig 7

Platelet derived growth factor receptor (PDGFR) western immunoblot showing the increased levels in the core tumor glioma biopsies.

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

Glial Fibrillary Acidic Protein (GFAP) Platelet Derived Growth Factor Receptor (PDGFR) in human gliomas

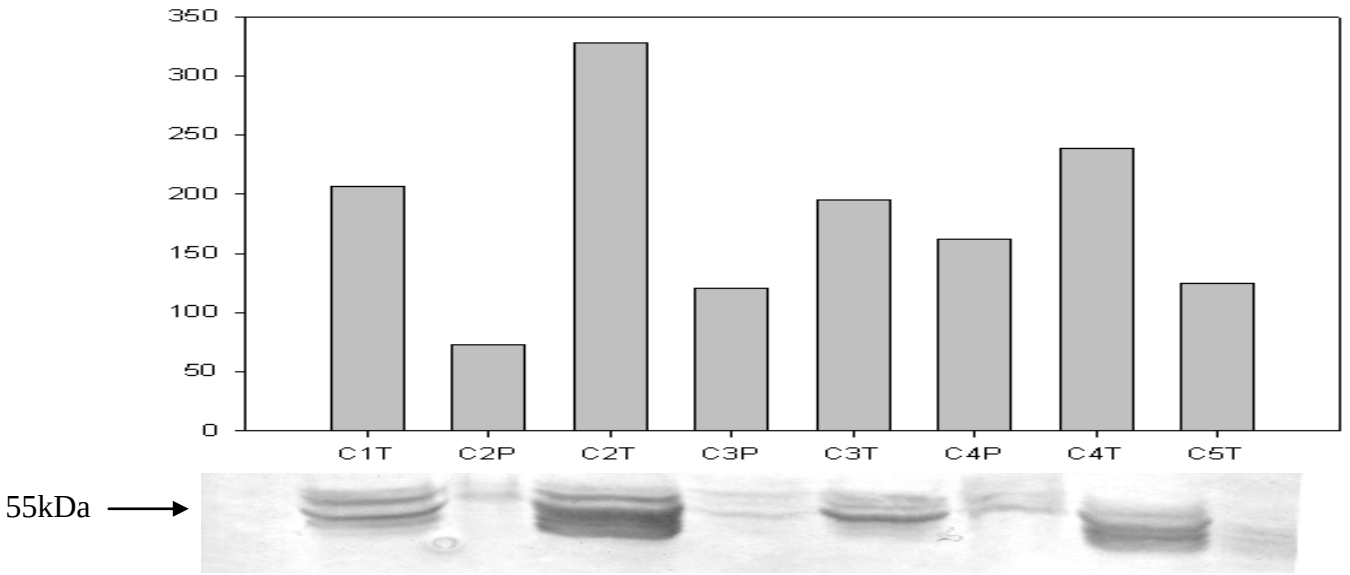


Fig 6 (GFAP)

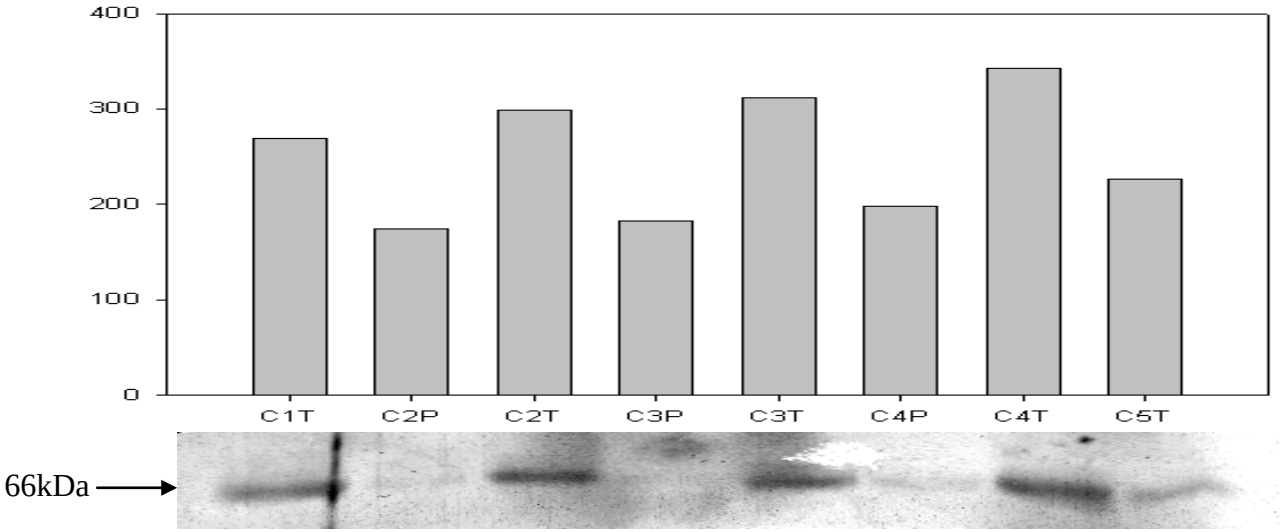


Fig 7 (PDGFR)

factors. In the present study we observed increased levels of phospho-tyrosine in all the core tumor tissues and relatively more levels among glioblastoma core tumors (cases #3 & #4) (Fig 8). Higher expression of total ERK[1]/2 was observed in core tumor tissues than peripheral tissues. Relatively higher levels were observed in case #2T (Fig 9a). However, increased levels of pERK[1]/2 was observed in case #3 both peripheral and core glioblastoma tumor samples (Fig 9b). Immunohistochemical analysis of astrocytoma and glioblastoma paraffin embedded tissue sections showed nuclear positive signals for pERK[1]/2 dispersed randomly throughout the section (Fig 10a). Increased cytoplasmic and nuclear positivity for pERK[1]/2 was observed in glioblastoma tumor sections than astrocytoma tissue sections (Fig 10b). Cytoplasmic and nuclear positivity for pERK[1]/2 was clearly observed under higher magnification (Fig 10c, 10d and 10e).

Bcl_2 and Bax status in human gliomas

An elevated level of Bcl_2 over the Bax is an indication of a predominance of cell proliferation over cell death. In the present study, western immunoblot analysis showed increased levels of Bcl_2 (Fig 11a) and Bax (Fig 11b) in the core tumor tissues than the peripheral tissues. Relatively higher levels were observed in glioblastoma core tumor tissues. Bcl_2 and bax densitometric representation showed higher levels of bcl2 over bax in all the human glioma biopsies (Fig 11c).

Fig.8 and 9

Western immunoblot analysis of p-Extra cellular signal regulated kinase ^{1}/2 (ERK^{1}/2) pathway among the human glioma biopsies. Increased levels of phospho-tyrosine observed in the core tumors and relatively more levels are observed in GBM tumors (Fig 8). Higher levels of ERK^{1}/2 observed in the core tumor tissues (Fig 9a). Case #3 patient sample, which is WHO grade IV (GBM) has shown relatively, increased levels of the ERK^{1}/2 (Fig 9b).

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

Extra cellular signal Regulated Kinase ½ (ERK[1]/2)
Status in human gliomas

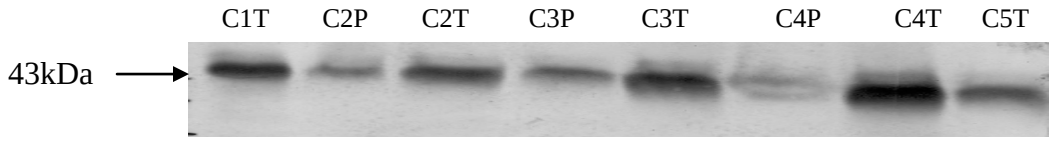


Fig 8 (Monoclonal pTyr)

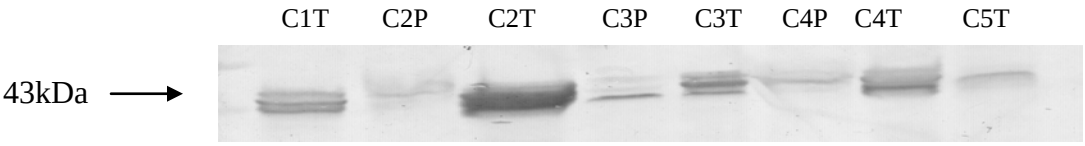
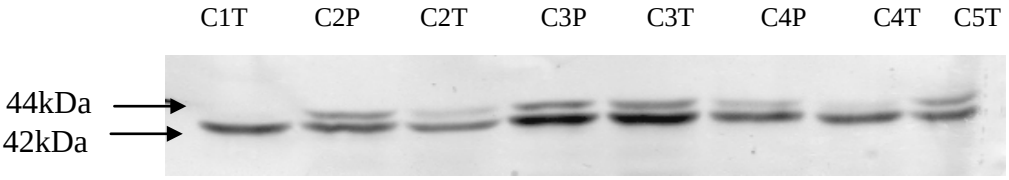


Fig 9a (ERK[1]/2)



Phospho – ERK1/2

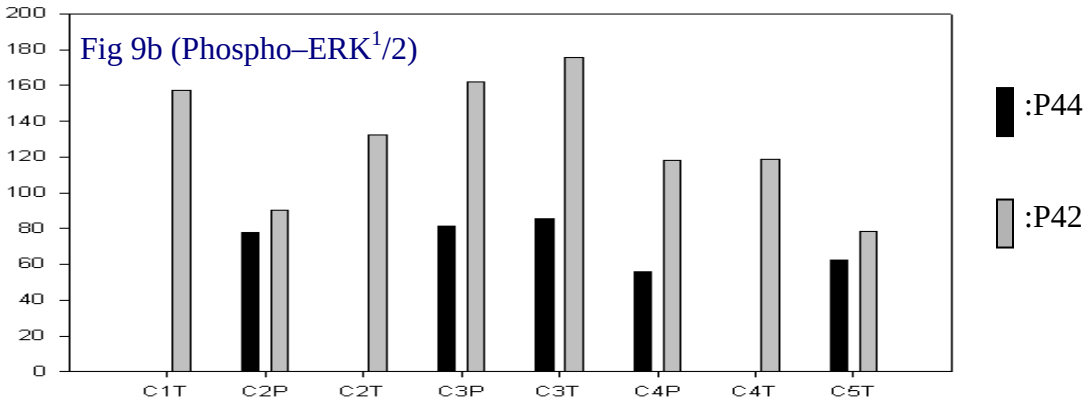


Fig.10

Immunohistochemical analysis of paraffin embedded astrocytoma and glioblastoma tumor tissues sections. Astrocytoma sections showing randomly distributed cytoplasmic positive pERK[1]/2 cells (Fig 10a). Strong cytoplasmic and nuclear positive pERK[1]/2 cells were localized around the necrotic areas of GBM tissue sections (Fig 10b, Fig c, Fig d and Fig e).

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

Immunohistochemistry – pERK ^{1}/2

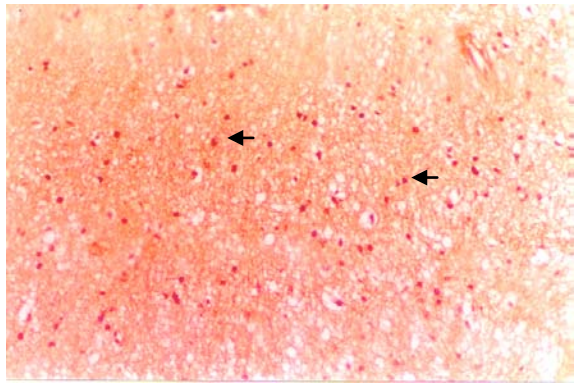


Fig.10a (Astrocytoma (4X) (++))

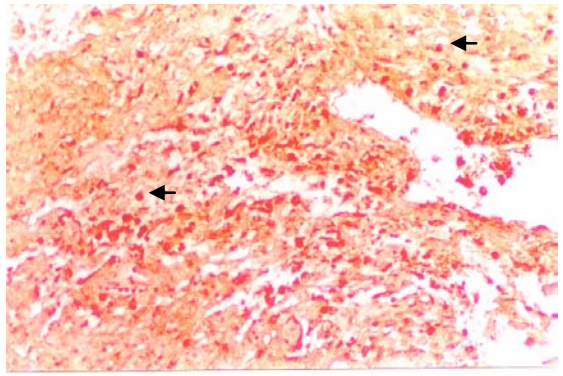
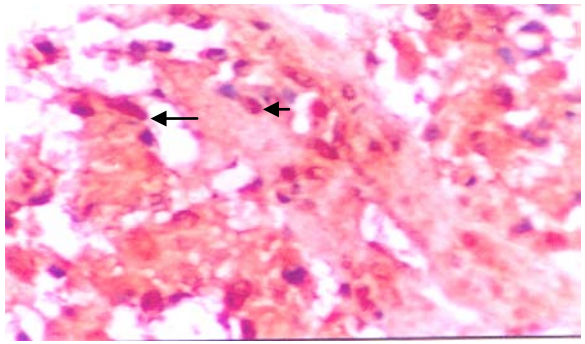


Fig.10b (Glioblastoma multiforme (4X)) (+++)



GBM: Fig. 10c (10X)

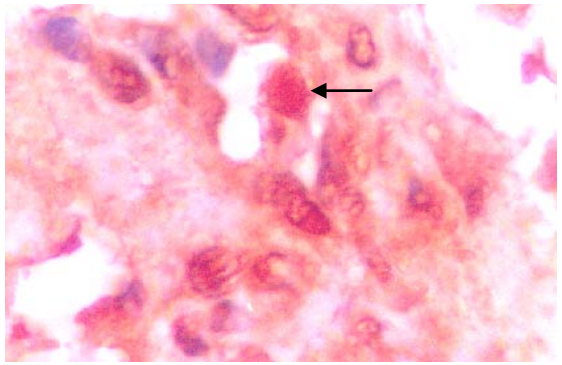


Fig.10d
Showing nuclear positivity
(40X)

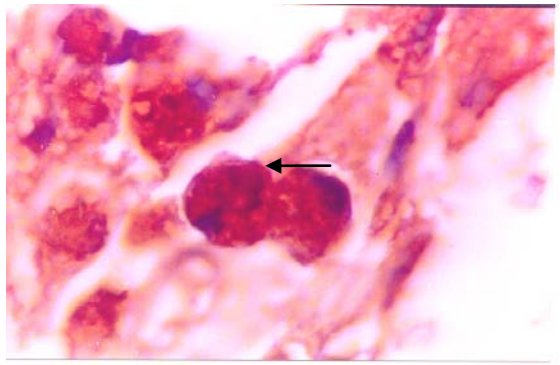


Fig. 10e
Showing cytoplasmic positivity
(100X)

Fig.10

Fig 11

Bax and Bcl-2 immunoblots (Fig 11a and Fig 11b) showing increased levels in the core tumor tissue lysates. Densitometric presentation depicts increased levels of Bcl-2 over the Bax.

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

BCl-2 and Bax in human gliomas

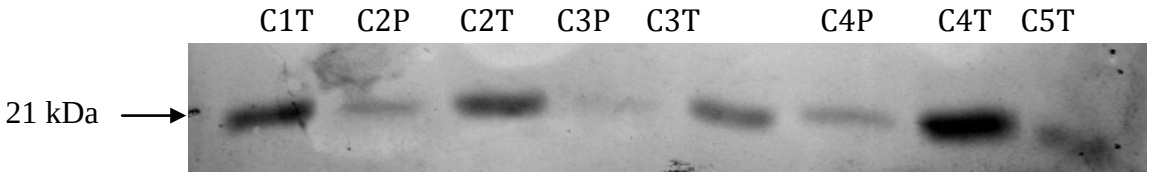


Fig 11a (Bax)

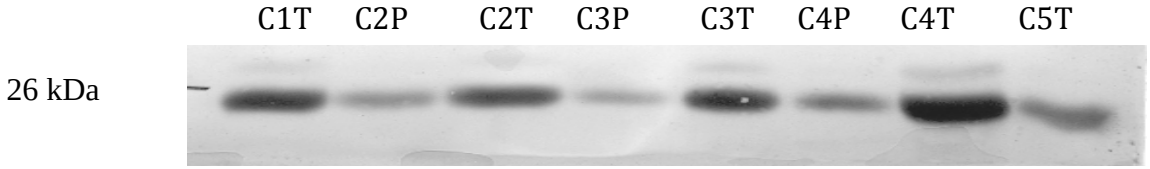


Fig 11b (Bcl-2)

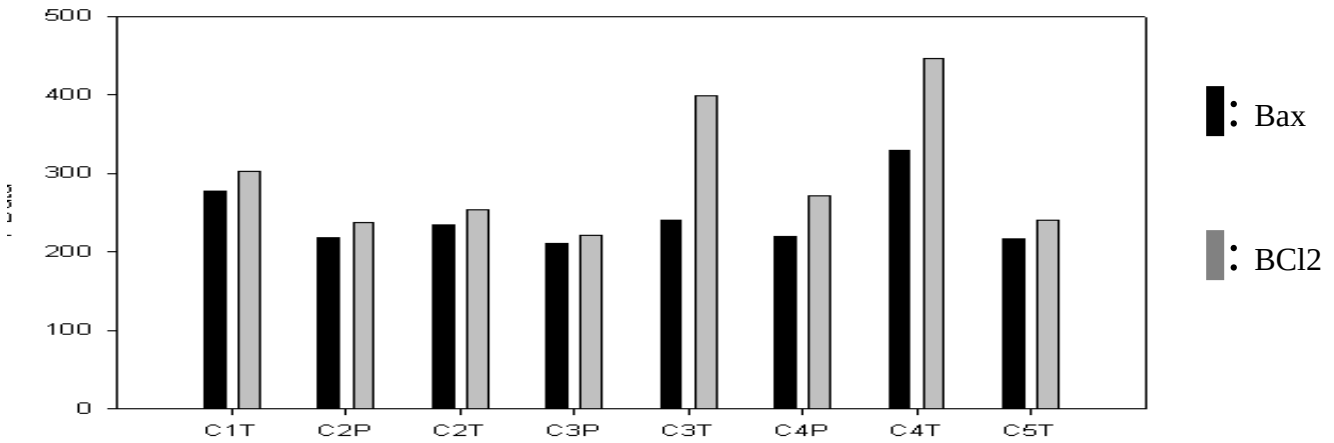


Fig 11c (Bax/Bcl-2)

DISCUSSION

Gliomas are the most common type of primary intracerebral neoplasm, and have a poor prognosis. The main prognostic factors are patient age, and malignancy grade. Because patients with the same histologic diagnosis have variable outcomes, there is a need to develop more precise prognostic tests with the aim of predicting tumor behaviour and response to therapies. In the WHO grading system, significant indicators of anaplasia to classify gliomas include atypia (coarse nuclear chromatin, nuclear pleomorphism, and multinucleation), mitotic activity, cellularity, microvascular proliferation and necrosis (Kleihues et al., 2000). Necrosis with microvascular proliferation or endothelial cell proliferation is the histological hallmark of glioblastoma (Gudinaviciene et al., 2004). All GBM tumor sections used in the study had areas of both low-grade and high-grade morphology. This may indicate that the glioblastoma samples used in the present study shall be classified as secondary glioblastoma (Lantos et al., 1996). In the recent past, there has been a lot of interest identifying the unique protein expression during gliomagenesis and its progression. Differential protein expression was reported in human glioblastoma cells lines and tumors, with four proteins expressed at higher levels compared with non-malignant brain lesions (Zhang et al., 2003; Chumbalkar et al., 2005). However, their function is unknown, and there is a need to understand their therapeutic value for controlling gliomas. Molecular and biochemical studies of gliomas have revealed abundant evidence for aberrant growth factor signaling, thus implicating downstream intracellular signaling pathways in glioma pathogenesis.

RTKs play a critical role in normal cell proliferation and differentiation. Increased levels of PDGFR in the core tumor tissues observed in the present study may be in consistent with earlier reports indicating PDGF/PDGFR alterations as a common observation in low-grade and in secondary GBM (Heldin et al., 1998). Glial fibrillary acidic protein is known to be specific for normal, reactive and neoplastic astrocytes, and thus it reveals the glial origin of the tumors. The intensity of immunostaining in the gliomas was inversely related to the degree of anaplasia: the strongest reactions were observed in well-differentiated neoplasms. Quantitative assessments confirmed the results of immunohistochemical reactions: lower levels of GFAP were consistently measured in more malignant astrocytomas; thus the amount of this protein could provide a biochemical index of malignancy (Jacque et al., 1979). ERK undergoes dual phosphorylation on both the tyrosine and threonine / serine residues for complete activation. In the present study, an elevated level of phospho-tyrosine was observed in all the core tumors, indicating its possible role in ERK activation, consistent with earlier reports (Cha et al., 2001). However, consistent patterns of selective activation of the ERK pathway in tumor cells were reported to be due to sustained activation that contributes to the neoplastic glial phenotype (Mandell et al., 1998). Lower levels of GFAP were consistently measured in more malignant astrocytomas (Yujiri et al., 1998). The barrier function of the mitochondrial membrane is perturbed early during the apoptotic process when pro-apoptotic members of the Bcl_2 family such as Bax translocate to the outer membrane of mitochondria upon the induction of the apoptosis (Gross et al., 1998).

Over-expression of Bcl_2 or Bcl-Xl induces complex changes in glioma cell phenotype, both by protecting cells from various pro-apoptotic stimuli (Glaser et al., 2001) and by enhancing their motility (Wick et al., 1998). In contrast to expectation, Bcl_2 expression was observed to increase with the malignancy, stronger staining in astrocytoma and anaplastic astrocytoma compared with glioblastoma has been observed in most (Krajewski et al., 1997) but not all the studies (Weller et al., 1995). Once docked, Bax initiates increased permeability of the outer mitochondrial membrane, leading to cytosolic release of cyt C from within the intermembrane space (Jurgensmeir et al., 1998). Formation of trimeric activation complex between cyt-c, APAF-1 and dATP triggers the stereotypical activation of cystein proteases (caspases) (Li et al., 1997). Changes in outer and inner mitochondrial membrane permeability are inhibited by the anti-apoptotic Bcl_2 (Kluck et al., 1997; Yang et al., 1997). The exact mechanism of antagonism between pro- and anti-apoptotic members of the Bcl_2 family remain unelucidated, although heterodimerisation with Bcl_2 is known to inhibit Bax function, possibly through effecting the conformation of Bax and its ion channel function. In the present study, irrespective of the glioma grade all samples showed elevated levels of Bcl_2 in relation to Bax. Altered Bcl_2/Bax ratio was reported to be associated with apoptosis induction and deregulated cell proliferation observed in human testicular tumors (Chresta et al., 1996).

Differential PARP cleavage pattern and status of cell death proteases among the human gliomas

CHAPTER - IV

CHAPTER IV

INTRODUCTION

Gliomas, like other forms of solid tumors, cannot grow larger than 1 to 2 mm without forming new blood vessels (Folkman, 1990). Cell death is prominent in cells farthest from the tumor vasculature, where hypoxia stimulates both apoptosis and necrosis. Tumors that possess low-oxygen regions have a poorer prognosis than well-oxygenated tumors, independent of treatment (Symonds et al., 1994). Earlier studies have indicated that most spontaneous cell deaths in malignant gliomas are due to necrosis rather than apoptosis (Kleihues et al., 2000). However, under the hypoxic conditions in the tumor cells, oncogenic changes can modulate the susceptibility of cells to undergo hypoxia-induced apoptosis (Graeber et al., 1996). The appearance of necrotic lesions is a distinguishing indicator of a glioblastoma; it is incompatible with a diagnosis of anaplastic astrocytoma, though it may be present in anaplastic oligodendrogliomas and oligoastrocytomas and is a sign of poor prognosis (Kleihues et al., 2000). A lucid discussion regarding the appearance of necrosis in gliomas and its possible prognostic significance has been presented (Alvord, 1992), but whether a higher level of apoptosis predicts a favorable outcome or not has remained controversial (Kuriyama et al., 2002). It was also hypothesized that the rate of tumor growth depends on the balance between the rates of cell proliferation and cell death. Thus, tumors have their own mechanisms to

accommodate the extra growing mass (Nakajima et al., 1998). Hence, there have been several clinical trials aimed at understanding the relationship between cell proliferation and apoptosis (Heesters et al., 1999; Vaquero et al., 2004). However, this relationship remains controversial (Schiffer et al., 1995).

Wide ranges of apoptosis rates have been reported in different brain tumor types. The overall basal rate determined using *in situ* DNA break labeling has been reported to be 0–1.4% in 83 glial tumors (Kordeck et al., 1996). A lower mean apoptosis was reported in low-grade glial tumors compared with malignant tumors and tumors of embryonal origin, and a trend towards low apoptotic index/mitotic index in tumors with a high labeling index and short survival (Schiffer et al., 1995). Similarly, other workers have reported that the apoptotic index assessed by haematoxylin and eosin in paraffin sections increases with increasing anaplasia in a series of astrocytomas and correlated with growth rate (Ellison et al., 1995). Specific vulnerability to apoptosis (and also necrosis) in the CNS may depend on the state of differentiation of the particular cell type and stage in the cell cycle (Ross, 1996). The pattern of apoptosis in a tumor cell population also depends on the apoptotic stimulus (Williams et al., 1997). Hence, the apoptotic index in astrocytic tumors is not a reliable prognostic factor.

Yet, to date, there is a major emphasis in the literature on the apoptotic form of cell death, and insufficient attention has been paid to elucidating the physiological

and molecular events that lead to necrosis (Raza et al., 2002). Two main types of necrosis are encountered in glioblastomas. (i) Wide necrosis of the central part of the tumor, due to insufficient blood supply to the tumor. Such necrosis is observed by neuroimaging and is characteristic of primary glioblastomas. (ii) Small irregularly–shaped foci of necrosis with pseudopalisading structures around them are found in both primary and secondary glioblastomas. In a clinical study it was reported that the extent of necrosis was inversely related to the survival probability of the patients (Hammoud et al., 1996). Though it was thought that a tumor develops necrosis when its growth rate outstrips its blood supply there is increasing evidence that the genesis of necrosis is a more complex process. Moreover, tumor cell proliferation and tumor angiogenesis may be independently controlled (Vartanian et al., 1994). Hence, it is important to determine whether the appearance of necrosis in these tumors is an epiphenomenon, or the result of intrinsic molecular pathways. There is growing evidence indicating the possible existence of different intermediary forms of cell death between apoptosis and necrosis. It remains to be determined whether the molecular mechanisms that elicit different modes of cell death are interrelated.

Identification of DNA fragmentation by TUNEL *in situ* labeling method and DNA agarose gel electrophoresis in human gliomas

The fragmentation of DNA in cells can be visualized by agarose gel electrophoresis following DNA extraction and also by *in situ* DNA nick end

labeling. In the DNA nick end labeling method, labeled nucleotides are incorporated into the free 3' OH ends of the fragmented DNA using the terminal deoxy nucleotidyl transferase enzyme (TdT) followed by detection of the labeled molecules by adding a chromagenic substrate. This is referred to as the TUNEL assay. It allows monitoring of apoptosis in cell cultures and tissue sections, providing histological localization of apoptotic cells. TUNEL assays were performed in astrocytoma and glioblastoma tissue sections. Earlier reports indicated that the quantitative apoptotic index is enhanced in glioblastomas compared with low-grade astrocytomas and anaplastic astrocytomas, and correlates with the proliferative activity of gliomas and glioblastomas (Korkolopoulou et al., 2001). Detection of DNA fragmentation method is a widely accepted assay for apoptosis (Wyllie, 1980).

Role of cell death proteases: caspase – 3, calpains and granzyme B

Proteases are important triggers for cell death, first evident in cytotoxic granules of cytotoxic T lymphocytes and natural killer cells, both of which induce cell death by binding to target cells. The granules were found to contain a series of serine proteases, including granzyme B. Further evidence for the involvement of proteases in cell death comes from studies in the nematode *Caenorhabditis elegans*. A third line of evidence comes from studies of specific protease inhibitors (Sarin et al., 1993). The participation of multiple proteases in the cell death process is further evident by the degradation of a number of proteins, including PARP, lamin B1, α-

fodrin, topoisomerase I, β-actin, and the 70kDa protein component of the U1 small nuclear ribonucleoprotein, in association with the cell death process (Martin et al., 1995).

Caspase-3

Caspases (cysteine aspartate-specific proteases) are a family of intracellular proteins involved in the initiation and execution of apoptosis. Caspase-3 was also known as yama and apopain (Tewari et al., 1995). Analysis of cell death in multiple cell types isolated from caspase-3 knockout animals revealed the complete inhibition of certain apoptotic hallmarks, such as membrane blebbing, DNA degradation and nuclear fragmentation (Zheng et al., 1998). On the other hand, caspase-3-/- mouse embryo fibroblasts (MEF) were not protected from eventual death and displayed multiple apoptotic features. Caspase-3, 6 and 7 are effector caspases which are essential for the execution of apoptosis and also for the cleavage of multiple substrates, including the cytokeratins, PARP, α-fodrin, NuMA and other. Caspase-3 inactivates proteins that protect living cells from apoptosis (Enari et al., 1998). Similarly, depletion of caspase-3 in a cell-free apoptotic system inhibited most of the downstream events, including various substrate cleavages, DNA fragmentation, chromatin marginalization, etc., whereas elimination of either caspase-6 or -7 had no effect (Slee et al., 2001). Thus, caspases-3 forms a common effector molecule for both the external and intrinsic pathways. Among the caspases, caspases-3 (p32: 32 kDa fragment) has the highest homology to CED-3 in terms of

both amino acid sequence and substrate specificity (Xue et al., 1996). Caspase-3 is activated by cleavage into p20 and p12 fragments (Nicholson and Thornberry, 1997). Caspase-3 - deficient mice show grossly abnormal brain development and die within three-weeks of birth (Kuida et al., 1996).

Calpain I (μ) and II (m)

Calpains are cytosolic calcium dependent cysteine proteases (EC3.4.22.17) with two major isoforms (μ and m-calpains, also known as calpain-1 and calpain- 2) that are ubiquitously expressed in mammalian cells (Sorimachi et al., 1997) and which were first reported in the CNS (Guraff, 1964). There are two classes of calpains: one (comprising calpains 1, 2, 5, 7, 10, 13, and 15) is ubiquitous in cytosol; the other (comprising calpains 3, 6, 8, 9, 11, and 12) occurs only or mainly in certain tissues. The terms μ-calpain and m-calpain refer to the μ-molar (2-80 μM) and milli-molar (0.2-1 mM) calcium requirement for their activity (Suzuki et al., 1995). Both enzymes are heterodimers, consisting of a large 80 kDa catalytic subunit that shares 55-65% sequence homology between the two proteases and a small identical 30 kDa regulatory subunit (Sorimachi et al., 1997). The 80 kDa subunit has four domains: domain I is the N-terminal anchoring α-helix domain and is important for regulating the activity and dissociation of the subunit; domain II, a catalytic domain, has two sub-domains in the absence of Ca^{2+}; domain III binds Ca^{2+} and phospholipids; and domain IV, also called the penta–EF-hand domain (an EF-hand unit consists of two

peptide helixes connected by a Ca^{2+}-binding loop), is important for dimer formation. The 30 kDa regulatory subunit consists of two domains: domain V is the N-terminal, glycine rich, hydrophobic domain and domain VI the penta–EF-hand domain, which is similar to domain IV of the catalytic subunit (Suzuki et al., 1995). The mechanisms by which calpains are activated and identify their protein targets are complex and poorly understood. The two isoforms share similar substrate specificities (Molinari & Carafoli, 1997). Many putative calpain substrates are cytoskeletal proteins, especially those involved in cytoskeletal/plasma membrane interactions such as spectrin, tau, actin, tubulin and glial fibrillary acidic neurofilaments and microtubule-associated proteins, MAP1B and MAP2, kinases, phosphatases (calcineurin) and phospholipases, membrane associated proteins including some receptors and ion-channel proteins, and some transcription factors (Goll et al., 2003). How calpain activity is regulated in cells is still unclear, but the calpains ostensibly participate in a variety of cellular processes, including remodeling of cytoskeletal/membrane attachments, different signal transduction pathways, cell differentiation, cell-cycle, regulation of gene expression, neuronal long-term potentiation, apoptosis and necrosis, embryonic development, and vesicular trafficking (Perrin & Huttenlocher, 2002; Goll et al., 2003). Several oncogenes and tumor-suppressor gene products are substrates for members of the calpain family (Liu et al., 2004). Calpains are implicated in apoptosis based on two types of observation: (1) the activation of calpains during cell death; and (2) the inhibition of apoptosis in the presence of calpain inhibitors. Calpains have mainly

been implicated in excitotoxic neuronal injury and necrosis (Spencer et al., 1995). However, neuronal calpains appear to be activated uncontrollably by sustained elevation of cytosolic calcium levels under pathological conditions (Zatz and Starling, 2005) such as ischemia and spinal cord injury (Banik et al., 1997) as well as in neurodegenerative diseases like Alzheimer's disease (Chiu et al., 2005), Parkinson's disease, Huntington's disease, Amyotrophic Lateral Sclerosis, induction of fatal murine Cerebral Malaria (Meena et al., 2006) and also during the ethanol consumption (Rajgopal et al., 2002). Proteolysis of structural proteins and metabolic enzymes is a common feature of Ca^{2+}-dependent neuronal death in both *in vitro* and *in vivo* models of excitotoxicity and stroke (Wang & Yuen, 1994). *In vivo* studies in brain ischemia (Chiu et al., 2005) and spinal cord injury (Ray et al., 2003) showed that calpain inhibitors consistently reduced proteolytic fragments and neuronal cell death.

Granzyme

Granzymes are a family of serine proteases that can induce apoptosis via two mechanisms. One involves the stimulation of cell-surface death receptors such as Fas to achieve cell death following extrinsic pathways, and the other, "granule exocytosis", involves the transfer of the contents from an effector cell cytoplasm to the target cell (Liou et al., 2003). Cytotoxic T lymphocytes can use, in addition to Fas signaling, the granzyme B/perforin system to kill target cells. Granzyme B is a serine protease termed 'granule enzyme', and perforin (a molecule capable of

forming pores in intracellular membranes) are taken up by the target cell. It is evident that tumors are highly infiltrated with immune cells, including natural killer (NK) cells and cytotoxic T lymphocyte cells (CTL), which are known to initiate cell death in the tumor cells. Studies using perforin knockout mice have shown the importance of the granule exocytosis pathway for controlling viral-infected and tumor cells. After a killer cell recognizes an appropriate target, the cytotoxic granules will move along the microtubules under the direction of the microtubule-organizing center to the immumnological synapse. Granzyme B is one of the important components known as a serine protease. Cytotoxic effector molecules are then delivered into the target cell via perforin. The key role of perforin in delivering the lethal hit for protection against intracellular pathogens and tumors is highlighted by studies in the perforin knockout mouse and by the identification of perforin mutations in a subset of patients with familial hematophagocytic lymphohistiocytosis (FHL). In the target cells, granzymes are further activated or processed by the action of lysosomal proteases. Recent studies suggest that granzyme B has a central role in activating the mitochondrial pathway by cleaving BID to initiate the release of cyt C together with Htr A2 / Omi and Smac/DIABLO. The granzyme B is known to initiate apoptosis by cleaving different pro-apoptotic proteins including caspases-3, PARP, BID and ICAD (Alimonti et al., 2001). The granzyme B can directly activate the target cell's caspases and can induce apoptosis (Krammer, 2000).

PARP fragmentation: may indicate co-existence of multiple forms of cell death in human gliomas

From observations in Jurkat cells, switch in the decision between apoptosis and necrosis was reported to be mainly due to the availability of intracellular ATP concentrations (Leist et al., 1997). Enhanced activation of PARP-1 is a major cause of neuronal death after brain ischemia (Garnier et al., 2003). In response to DNA damage, PARP activity is rapidly increased up to 500-fold upon binding to DNA strand nicks and breaks. Poly (ADP-ribose) polymerase enzyme transfer ADP-ribose groups from NAD^+ to form branched ADP-ribose polymers on acceptor proteins in the vicinity of DNA strand breaks or kinks. Although PARP-1 normally functions to facilitate DNA repair, extensive PARP-1 activation promotes cell death through processes involving energy depletion and the release of apoptosis-inducing factor (Ha et al., 1999; Yu et al., 2002). Genetic or pharmacological inhibition of PARP-1 activity reduces infarct size by up to 80% in brains subjected to transient or permanent ischemia (Garnier et al., 2003). PARP inhibitors attenuate necrosis but not apoptotic neuronal death in experimental models of cerebral ischemia (Moroni et al., 2001). Similarly, inhibition of PARP-1 in cultured neurons substantially increases resistance to oxygen–glucose deprivation and to NMDA toxicity (Ying et al., 2001). PARP-1 can be cleaved and inactivated by caspase-3, caspase-7, caspase-8, calpains, cathepsins (Gobeil et al., 2001) and granzyme B (Froelich et al., 1996). Thus, the cleavage of PARP could be a valuable indicator of cell death. However,

its biological relevance, if any, is not clear, since PARP-null mice develop normally (Wang et al., 1995). There are no reports of the role of PARP in glioma pathology.

AIF role in the induction of mitochondrion-mediated caspase-independent form of cell death in gliomas

In addition to the classically defined cell death programs of necrosis and apoptosis, it is becoming clear that there are death programs that are unique to cell type and death stimulus, and that use a common death machinery in specially defined, carefully choreographed programs. AIF is a 57 kDa flavoprotein that resembles bacterial oxidoreductase and resides in the mitochondrial intermembrane space (Susin et al., 1999). Upon induction of apoptosis, AIF translocates from the mitochondrion to the nucleus and causes chromatin condensation and large-scale DNA fragmentation, it thus lead to cell death primarily in a caspases-independent manner (Susin et al., 2000). This delayed form of cell death takes place independently of the cyt-C/Apaf1/caspase-9 apoptosome complex (Green et al., 1998). Embryonic stem cells lacking AIF are resistant to cell death after vitamin K3 treatment and serum starvation (Joza et al., 2001). The cell death pathway initiated by PARP-1 activation appears to be mediated by AIF, by inducing its translocation from the mitochondrion to the nucleus. Inhibition of PARP-1-initiated cell death by PARP inhibitors reduced AIF-mediated toxicity (Virag et al., 2002). The exact biochemical mechanism responsible for PARP-mediated AIF cell death remains

unelucidated, but AIF might bind to DNA and recruit proteases and nucleases that cause chromatin condensation.

Cyclo-oxygenase 2 (Cox-2)

Cox-2 has a well-known function in arachidonic acid metabolism during the process of inflammation. In malignant gliomas, necrosis is generally associated with inflammation of the cells. Cox-2-dependent prostaglandin E_2 (PGE2) production is responsible for a significant induction of brain edema and microglia represent a major component of the CNS immune response to brain tumors (Roggendorf et al., 1996). Macrophages and microglia represents a major component of the infiltrating cells in glial tumors (Badie, 2000). The invasive nature of glioma cells is responsible not only for local tumor recurrence, but also for the breakdown of the blood-brain barrier and cerebral edema formation in patients with malignant gliomas. In experimental animal models, dexamethasone suppresses the CNS inflammatory response to gliomas (Brunda et al., 1980). This has further suggested that in addition to neoplastic cells, tumor-associated inflammatory cells play a role in peri-tumoral edema formation. Hence, it was hypothesized that the microglial infiltration of brain tumors may contribute to brain edema formation in gliomas through the expression of cox-2 (Badie et al., 2003).

RESULTS

Localization of TUNEL positive cells

In the GBM tumor tissue sections, TUNEL positive cells were distributed around the regions of necrosis (Fig 12a). Non-specific positive signals were observed in the regions of necrosis (Fig 12b). Only mild positivity was observed in astrocytoma tissue sections and was distributed randomly throughout the sections.

DNA fragmentation patterns

Peripheral tissues of astrocytomas showed a clear band pattern, whereas tumor core tissues showed a smear pattern. Core tumor tissues of glioblastoma have shown smear pattern whereas peripheral tissues of glioblastoma showed a partial smear and band pattern (Fig 13).

Status of pro- and cleaved caspase-3, calpains (I & II), and granzyme B

In the different human glioma samples, procaspase-3 was observed as multiple fragments of around 32 kDa molecular weight (Fig 14a). Increased intensity of binding was observed in the GBM tumor core (C3T). Immunohistochemical analysis for cleaved caspase-3 showed pronounced cytoplasmic and peri-nuclear positivity localized mostly around the regions of necrosis in GBM sections (Fig 14b). Randomly distributed caspase-3 cells were observed in the astrocytoma tissue

Fig 12

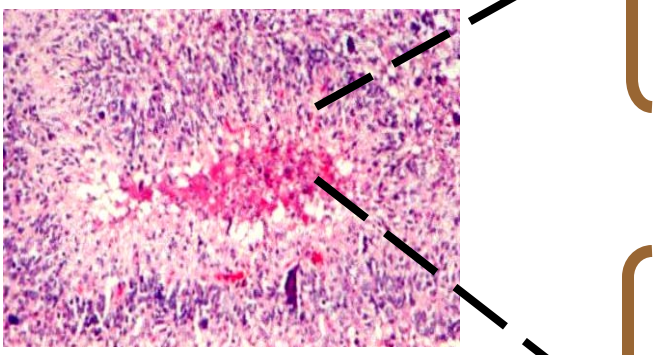
(a) *In situ* DNA nick end-labeling analysis showing TUNEL positive cells in GBM tissue section nearer the necrotic areas.

(b) *In situ* DNA nick end-labeling analysis showing non-specific TUNEL positivity in the necrotic areas of the GBM tissue sections.

Fig 13

DNA agarose gel electrophoresis showing smear pattern in the core tumor tissues and clear band pattern in the peripheral tumor tissues of low grade glioma. Partial smear pattern is observed in the peripheral tumor tissue of GBM.

In Situ DNA nick end labeling (TUNEL)



Necrosis in GBM (10X)

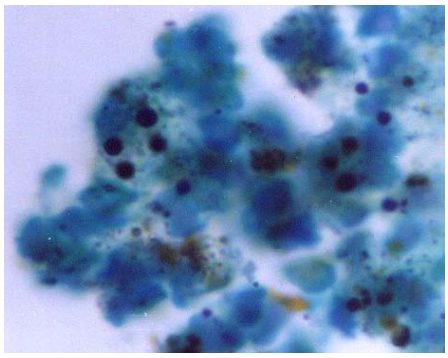


Fig 12a (100X)

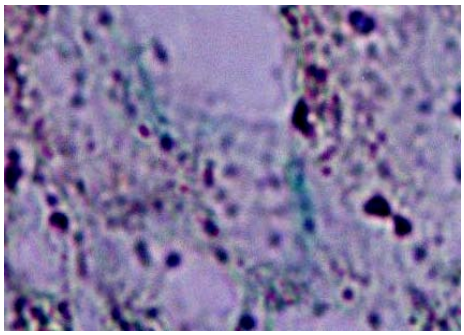


Fig 12b (100X)

DNA Agarose Gel Electrophoresis

Lane # **1 2 3 4 5 6**

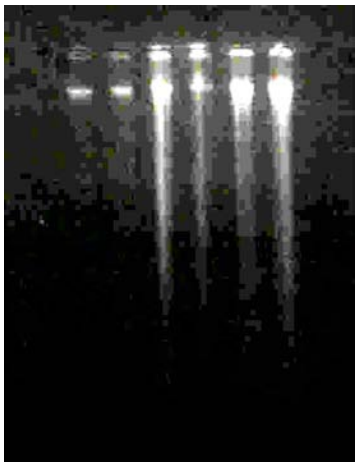


Fig 13

Lane 1,2: Peripheral tissue DNA sample histopathologically confirmed astrocytoma (WHO grade II)
Lane 3: Tumor tissue DNA sample histopathologically confirmed astrocytoma (WHO grade II)
Lane 4: Peripheral tissue DNA sample histopathologically confirmed glioblastoma (WHO grade IV)
Lane 5,6: Tumor tissue DNA sample histopathologically confirmed glioblastoma (WHO grade IV)

sections (Fig 14c). Calpain I was observed as larger 80 kDa and smaller 30 kDa regulatory subunits. An increased level of the smaller regulatory subunit of calpain I was observed in core tumor samples of glioblastoma, whereas a fragmented band pattern of the smaller regulatory unit of calpain II was observed at increased levels in the core tumor tissues. Surprisingly, astrocytoma core tumor tissue (C2T) was observed at higher level (Fig 15a & b). Granzyme B showed increased levels in all the core tumor tissues and comparatively more in glioblastoma core tumors (Fig 16a). Immunohistochemical analysis for granzyme B in the astrocytoma and glioblastoma tissue sections was done. Localized cytoplasmic positive cells for granzyme B were observed consistently around the regions of necrosis (Fig 16b & c).

Differential fragmentation of full length PARP by different cell death proteases

In the present study an elevated level of full-length PARP around 116 kDa was observed in GBM core tumor tissue (C4T). The elevated levels of cleaved caspase-3, calpain I & II, along with granzyme B, observed in tumors might have cleaved full-length PARP into 89 kDa and 24 kDa; 40 kDa and 54 kDa fragments respectively (Fig 17). Hence, it could be possible that the formation of different molecular weight fragments might originate from different cells.

Fig 14

(a) Western immunoblot analysis of caspase-3 showing multiple fragments around the 32 kDa molecular weight.
(b) Immunohistochemical analysis for cleaved caspase-3 showing localization of positive cells nearer the areas of necrosis.
(c) Immunohistochemical analysis for cleaved caspase-3 showing cytoplasmic positivity at a higher magnification**.**

Fig 15

(a) Western immunoblot analysis of calpain-I or μ-calpain showing increased levels of regulatory subunit in the core tumor tissues.
(b) Western immunoblot analysis of calpain-II or m-calpain showing increased level regulatory subunit in the astrocytoma (C2T) in fragmented pattern.

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

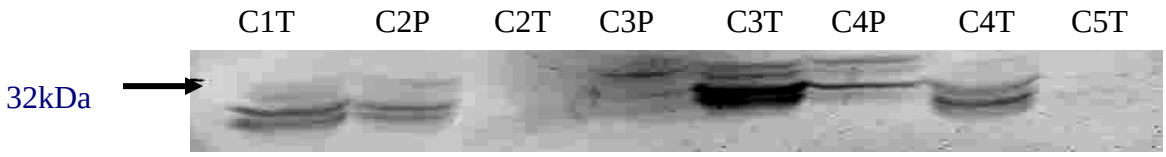


Fig 14a

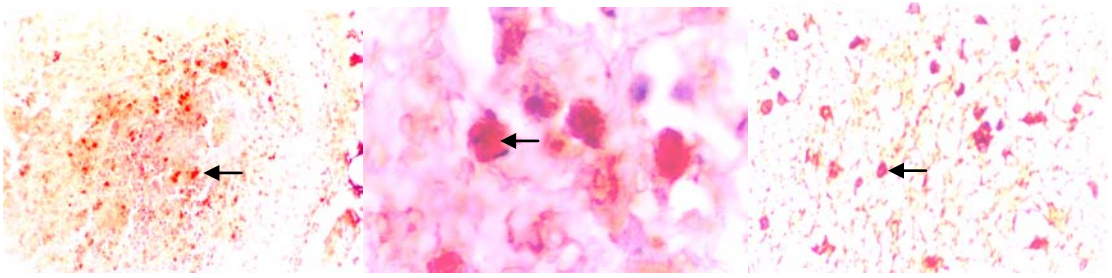


Fig 14b GBM: (10X) **(100X)** **Fig 14c Astrocytoma** (10X)

Fig 14 (Caspase blot and cl. Caspase-3 immunohistochemistry)

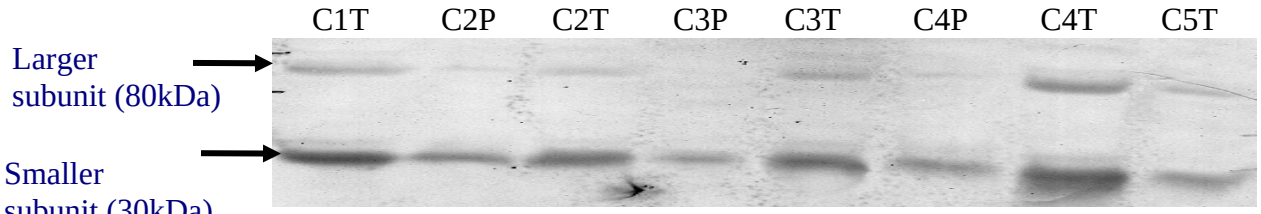


Fig 15a Calpain I (μ-Calpain)

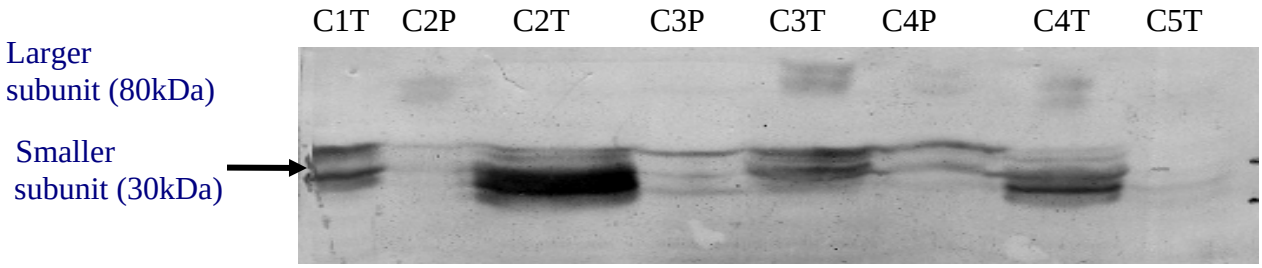


Fig 15b Calpain I I (m-Calpain)

Fig 16

(a) Western immunoblot analysis of granzyme B in whole tissue lysates of glioma biopsies, showing increased levels in the core tumor tissues.
(b) Immunohistochemical analysis of granzyme B showing strong positivity nearer the necrotic areas of GBM.
(c) Immunohistochemical analysis of granzyme B showing granular cytoplasmic positivity in the GBM sections.

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

Granzyme B in human gliomas

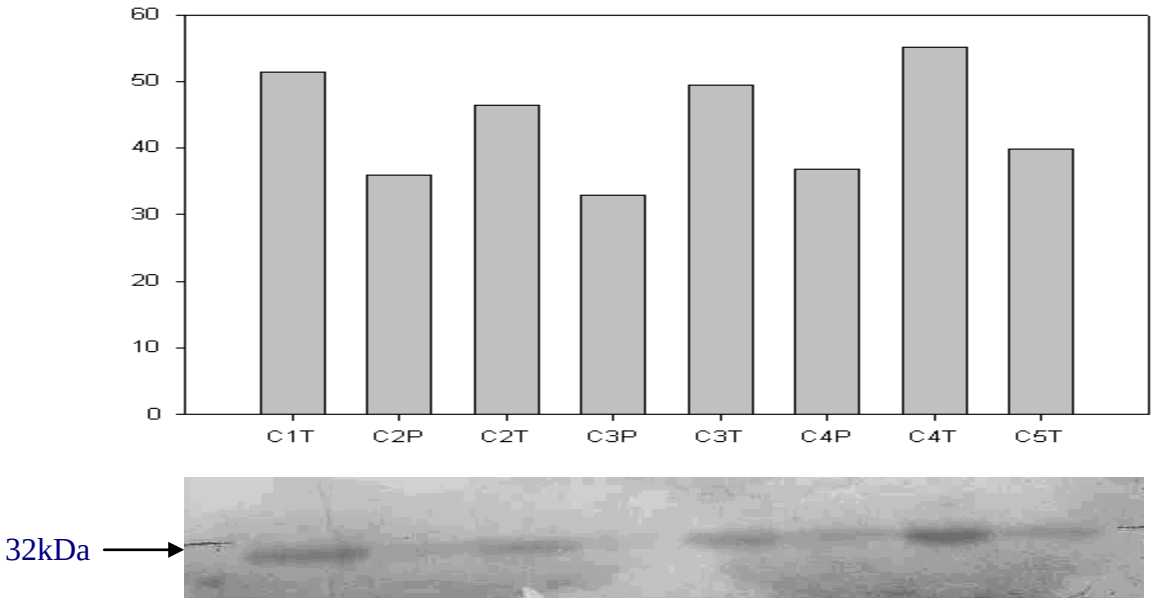


Fig 16a (Granzyme B)

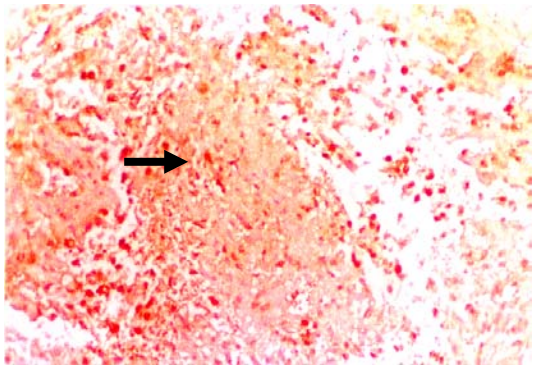


Fig 16b GBM (10X), +++

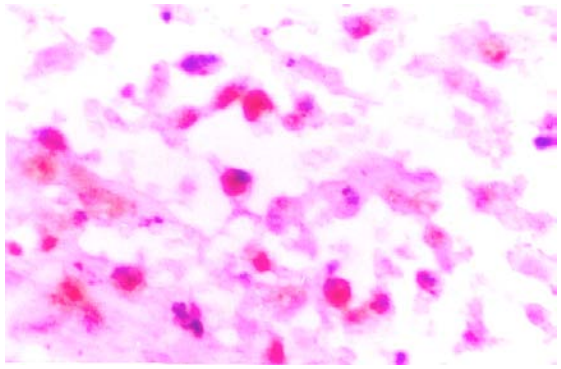


Fig 16c GBM (40X)

Fig 16

Status of AIF

AIF immunoblot analysis of glioma tissue samples showed increased levels in core tumor tissues and relatively greater levels in glioblastoma core tumor tissue (C3T) (Fig 18a). Immunohistochemical analysis for AIF among the astrocytoma and glioblastoma tissue sections showed the distribution of nuclear positivity around the regions of necrosis (Fig 18b & c).

Immunohistochemical analysis of Cox-2

Strong cytoplasmic positivity for Cox-2 was observed around the areas of necrosis in glioblastoma tumor sections (Fig 19a, b & c). Astrocytoma tissue sections did not show any significant positivity for Cox-2.

Fig 17

Western immunoblot analysis of poly (ADP-ribosyl) polymerase (PARP) showing differential cleavage pattern along with usual 89 kDa and 24 kDa molecular weight fragments.

Fig 18

(a) Western immunoblot analysis of AIF showing increased intensity of fragmented band around the molecular weight of 57 kDa.
(b) Immunohistochemical analysis of AIF showing pronounced positivity around the necrotic regions in GBM tissue sections.
(c) Immunohistochemical analysis of AIF showing nuclear positivity at higher magnification.

C1T: Case #1 core tumor tissue; C2P: Case #2 peripheral tumor tissue; C2T: Case #2 core tumor tissue; C3P: Case #3 peripheral tumor tissue; C4P: Case #4 peripheral tissue; C4T: Case #4 core tumor tissue; C5T: Case #5 core tumor tissue

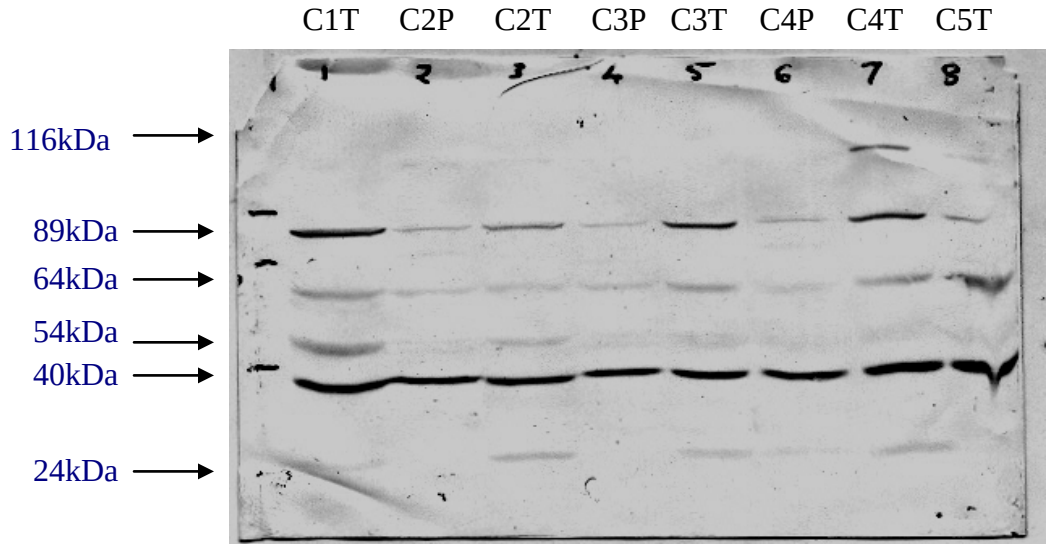


Fig 17 (Poly (ADP-ribosyl) Ribose Polymerase)

Apoptosis Inducing Factor (AIF) in human gliomas

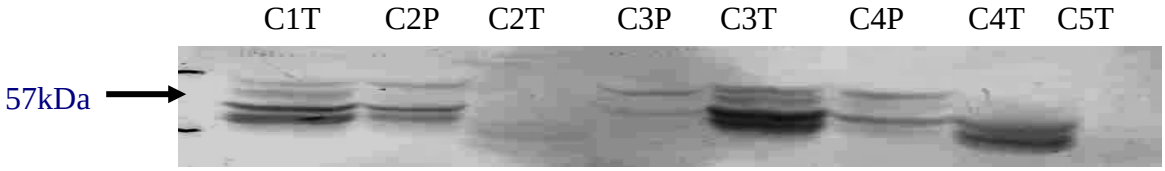


Fig 18a

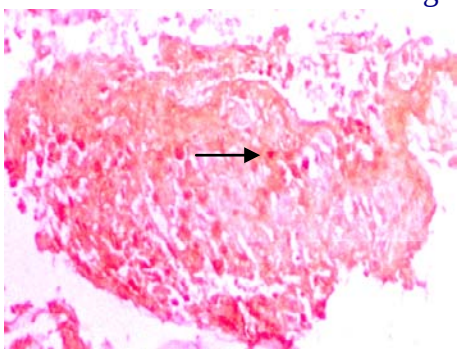


Fig 18b GBM (10X), +++

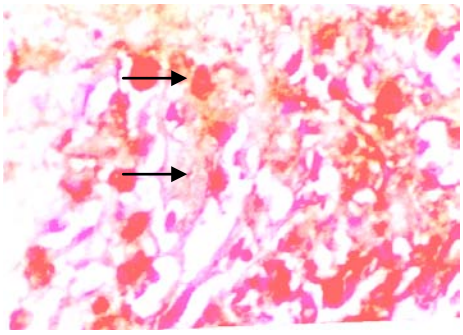


Fig 18c GBM (100X)

Fig 19

Cyclo-oxygenase–2 (Cox-2) immunohistochemical analysis in glioblastoma multiforme tumor tissue sections (a & b) Intensified cytoplasmic positive cox-2 cells observed near the areas of necrosis (c) Cytoplasmic positivity for cox-2 under higher magnification.

Cyclo-oxygenase – 2 (COX-2) signals in gliomas

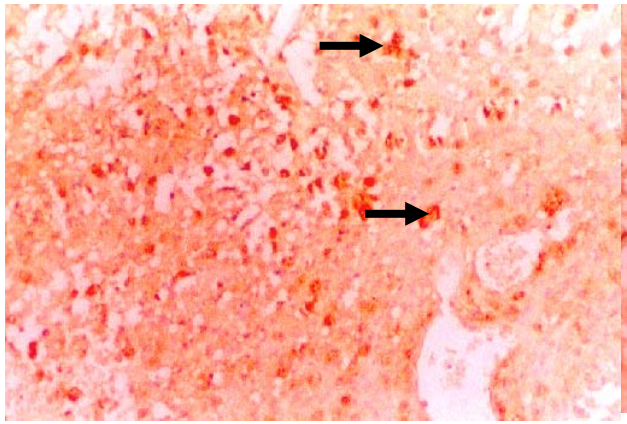


Fig 19a

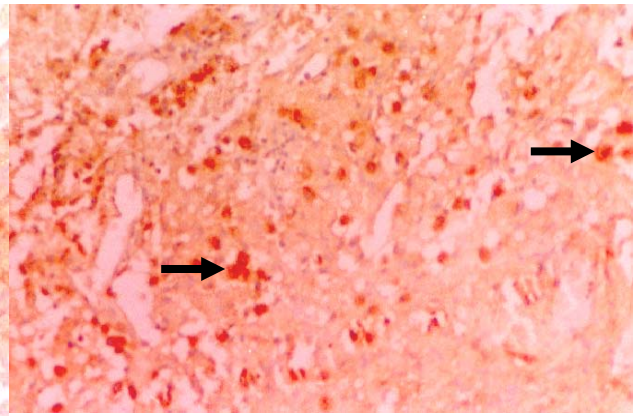


Fig 19b

(Cox 2 in GBM: 10X)

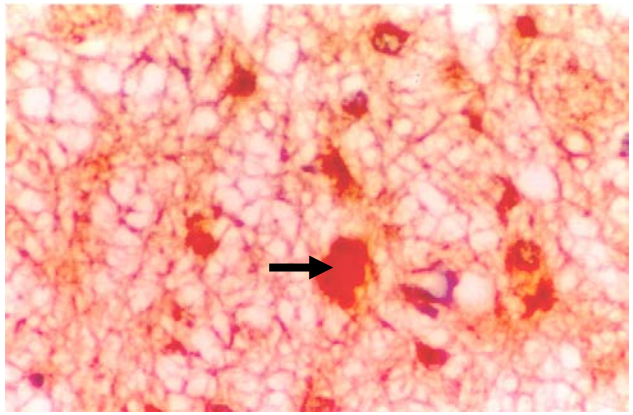


Fig 19c (Cox 2 in GBM: 100X)

Fig 19

DISCUSSION

Increasing evidence obtained in many *in vitro* and *in vivo* model systems supports the hypothesis that a variety of cell death programs may be triggered in distinct circumstances. Hence, cell demise could be the result of the multiple or alternate apoptosis-like pathways, or due to the partially programmed necrosis. A better understanding of the signaling mechanism involved in gliomagenesis and apoptosis induction in glioma cells has therapeutic importance (Arends et al., 1994). It is well known that mitochondria have a crucial role in the induction of apoptosis, but accumulating evidence suggests that other organelles, including the endoplasmic reticulum (ER), lysosomes, and the Golgi apparatus also act as major points of integration of pro-apoptotic signaling or damage sensing (Ferri et al., 2001).

Estimation of volume doubling time and cell loss in an experimental glioma rat model indicated that cell death might be an inherent property of the growing mass of tumor cells, which allows it to accommodate the extra mass (Nakajima et al., 1998). A similar observation elucidating the relationship between apoptosis and proliferation in brain tumors was assessed (Vaquero et al., 2004), but a firm conclusion could not be made. Different studies have shown that most spontaneous cell deaths in malignant gliomas are due to necrosis rather than apoptosis (Kleihues et al., 2000). Molecular evidence has shown that apoptosis and necrosis are the two major forms of cell death in gliomas, but the exact mechanism remains poorly

understood (Ray et al., 2002). Induction of a hypoxic insult in rat fibroblastic cells leads to several distinct cell-death programs on the basis of the insult, which were observed to have intermediate features between apoptosis and necrosis which is termed as apo-necrosis (Fomiglie et al., 2000).

As reported earlier, pseudopalisading regions of malignant gliomas may represent a mixture of cells showing TUNEL positivity with diminished apoptotic potential (Kordek et al., 1996). DNA agarose gels can provide a qualitative analysis of the DNA pattern in the tissues, which is mostly used to characterize the mode of cell death (Gavrieli et al., 1992). Generally, cell death in the core tumors and proliferation in the periphery are seen in the human tumors (Bigner et al., 1988). Caspas-3 activation is a hallmark of apoptosis. In our experiments we observed a band pattern of procaspase-3 around a molecular weight of ~32 kDa. Consistent with our observation, earlier studies reported single, double and triple band patterns for procaspase-3 around 32 kDa that may be characteristic multiple apoptotic fragments observed in lysates of different tissues like brain, heart, breast, stomach and others. Immunohistochemistry for cleaved caspase-3 in the present study showed a distribution of positive cells around areas of necrosis. Calpains get activated in relation to the intra-cellular calcium levels. We observed increased levels of both calpain I and II in the core tumor tissues. Increased m-calpain (calpain II) was reported in dystrophin-deficient muscle and was responsible for necrosis (Spencer et al., 1995). Increased granzyme B observed in core tumors in the present

study is consistent with earlier reports of cathepsin in brain tumors, which may be an indication of the autophagy (type II) form of cell death (Guicciardi et al., 2004). Elevated levels of pro- and activated caspase-3, calpain I (μ) and II (m) and granzyme B observed in the present study might have cleaved full length PARP (~116 kDa) into 89 kDa and 24 kDa; 40 kDa; 64 kDa and 54 kDa fragments respectively. The significance of differential PARP cleavage pattern in identifying the different forms of cell death was reported earlier by different workers in different experimental conditions (Shah et al., 1996; Aikin et al., 2004). AIF can thus induce cell death in a caspases-independent manner (Joza et al., 2001). In this study, increased nuclear positivity for AIF was observed in GBM tissues around necrotic areas. Over-activation of PARP initiates a nuclear signal that propagates to mitochondria and triggers the release of AIF. This translocates from mitochondria to the nucleus and thereby induces cell death (Hong et al., 2004). Cox-2 is an inducible enzyme that can get rapidly activated in response to inflammation. Increased positivity for Cox-2 was observed around the areas of necrosis in glioblastomas. Increased expression of Cox-2 has been reported in breast, colon, prostate and lung carcinomas and only weak expression in normal colon epithelium (Masferrer et al., 2000; Badie et al., 2003).

Thus, our experimental evidence may indicate the existence of intermediate forms of cell death along with the well-known apoptotic and necrotic forms. Further understanding of the alternate or multiple forms of cell death programs may provide novel therapeutic targets, with consequences for attempts to treat gliomas by modulating cell-death programs.

pERK, pAkt, and pBad: A Possible role in cell proliferation and sustained cell survival in n-ethyl n-nitrosourea (ENU) induced rat gliomas

CHAPTER - V

CHAPTER V

INTRODUCTION

Gliomas are diverse and range widely in their presentation, biological aggressiveness, histological differentiation and response to therapy, which ultimately poses a major problem to understand and for the therapeutic intervention. The greatest hope for controlling these aggressive tumors will be to counteract the mechanisms that drive their molecular engine. Hence, a requirement for accurate and suitable glioma models exists. Animal models of glioma are powerful tools to investigate aspects of glioma biology that cannot be studied in cell culture systems, such as angiogenesis, invasion, metastasis, and the multifactorial effects of therapeutics.

ENU-induced rat glioma model

For many decades, gliomas were considered to arise from dedifferentiation of glial cells (Rubinstein, 1987). Recently, however, much interest has been directed toward the possibility that these tumors arise from multipotent stem cells, including cells that occur in the sub-ventricular zone (Seyfried, 2001; Conover and Allen, 2002). Expression of stem cell-like characteristics in a novel model system generated from a human malignant glioma, comprising two cell lines including human neural glial cell lines #1 and #2 (HNGC 1 and HNGC 2) has been reported recently (Shiras et al., 2003). Intrauterine exposure of neuronal precursor cells to

ENU, together with specific genetic abnormalities (eg. P53 deficiency) produce glial neoplasm in the adult brain. These results have led to the hypothesis that glial tumors in the adult brain arise from mutated precursor cells that have escaped apoptotic deletion during the course of development (Leonard et al., 2001). Higher expression of a laminin receptor α6, β4 integrin proteins and mRNA in an ENU-induced model was reported to be crucial in controlling migration, proliferation, and other neoplastic transformations (Previtali et al., 1999).

The ENU-induced glioma model presents a sequential tumor progression, which may help in delineating the steps involved in neoplastic transformation. Little attention has been paid to the promotion stage of glioma development, because gliomas are virtually impossible to diagnose before they are fully developed (i.e have already entered the progression stage). To address this question, the ENU-induced model is suitable as it presents pathological changes due to the developing tumor and not from continued exposure to mutagens. Osteopontin expression in the intratumoral astrocytes can mark the tumor progression in ENU-induced glioma rats, indicating the transition phase from promotion to the progression stage in the glioma development (Jang et al., 2006).

Different tumoral stages, particularly of early proliferation in transplacental ENU-induced glioma rats

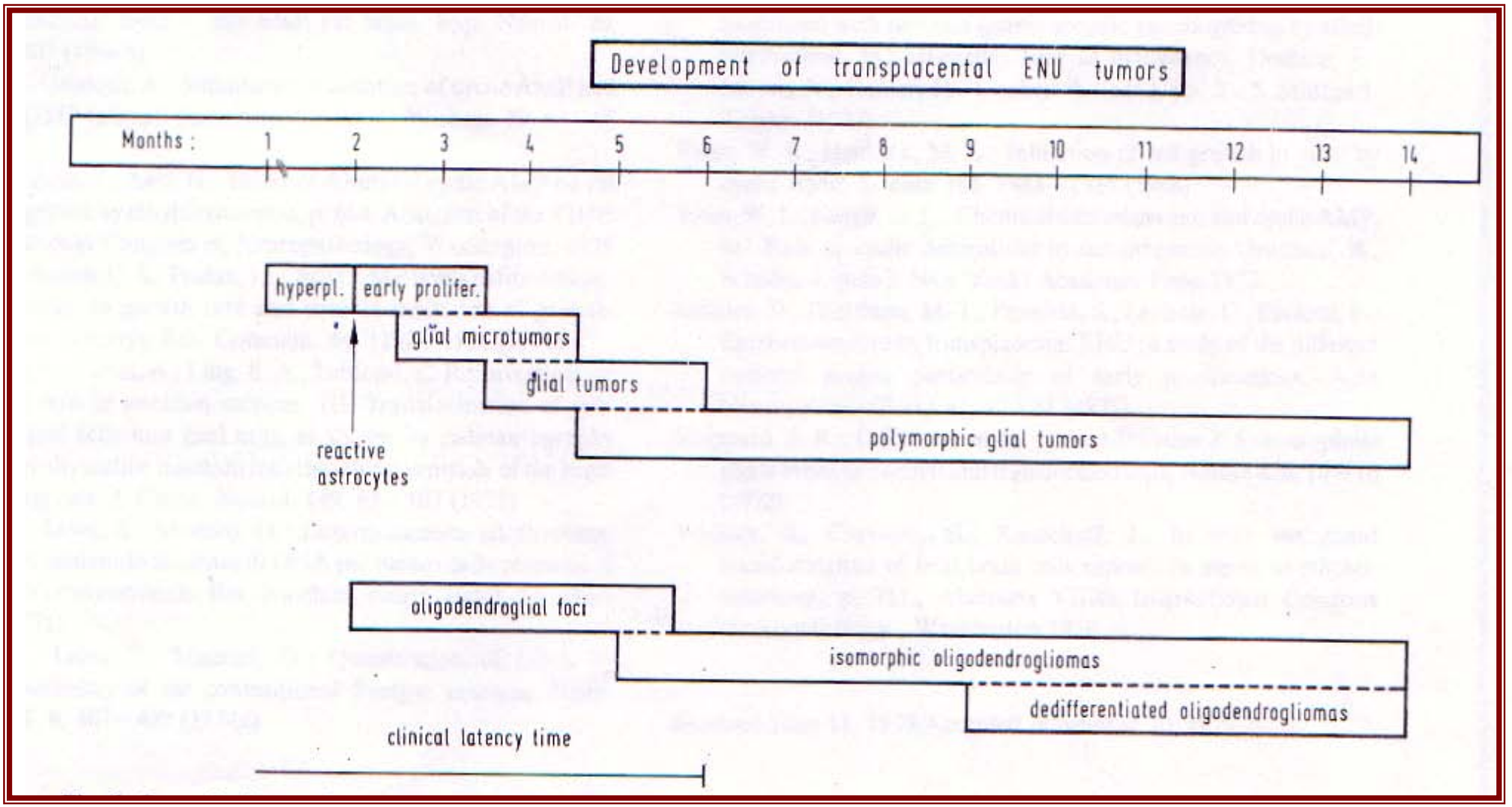
The interaction between the carcinogen and the sub-ependymal plate may occur at the time of birth, when mitotic activity is at its peak, or later in life. The altered cells may die, may remain in interphase, or may divide after a latency of varying length. This proliferation will result in small groups of abnormal cells (early lesions): these may become stationary or even regress, or further mitotic activity will lead to gliomas. The dividing cells, being multi-potent, maintain their potential to differentiate into various types of gliomas of mixed cellularity (GBM and mixed gliomas), or they may follow a line of maturation resulting in astrocytomas, oligodendrogliomas and eppendymomas (Pilkington et al., 1979). Foulds, characterized tumor growth as a process of sequential neoplastic development extending over a long period of time which, in man, might amount to several decades and be manifested by a wide variety of lesions that might emerge contemporaneously or consecutively at various times and places (Foulds, 1975). Similar observations were also made in the neoplastic transformation of fetal rat brain cells in culture after exposure to ethyl nitrosourea *in vivo*. These cells show gradual morphological changes with increased cell proliferation, loss of anchorage dependence, and potent tumorigenic tendency after subcutaneously re-implantation (Laerum and Rajewsky, 1975). In the ENU-induced transplacental glioma rat model, it is very difficult to identify the phenotypic alterations *in vivo* of presumptive tumor cells, due to the polymorphic cellular composition of nervous

tissue, and due to only a minor fraction of its constituent cells undergoes changes leading to a “malignant” phenotype (Laerum et al., 1977). However, much work has already been done on tumor cell type and site of occurrence. Short-term effects have been observed within 6-48 h of ENU administration, consisting of necroses, nuclear pyknosis and temporary cell cycle arrest (Bosch, 1977a, b). Tumor induction by alkylating agents depends on their chemical reactivities, and particularly their abilities to react *in vivo* in the target organs, thymus and bone marrow, at the O-6 position of guanine residues in DNA. Formation and persistence of O-6 alkylated guanine residues in DNA may be necessary, but not sufficient, conditions for the induction of the neoplastic state by alkylating carcinogens (Frei et al., 1978). Among early changes, immuno-electron microscopy of DNA isolated from fetal rat brain 1 hr after exposure to ENU *in vivo* showed that (O-6 EtdGuo)-antibody binding sites (ABS) are either co-isolated or identical with a small fraction of DNA fragments associated with specific, tightly bound polypeptide adducts or clusters (Nehls et al., 1984). In an experiment following transplacental ENU exposure, glial tumorigenesis and progression might have involved neuronal precursor cells (NPCs) and several apoptosis-associated molecules. Hence, the persistence of mutated NPCs in the adult brain provides the necessary time for the accumulation of additional genetic alterations such as those that occur in secondary GBMs, and the development of apoptosis resistance, which are required for tumor progression for which p53, caspase-9 and Bcl-X_L were shown to be important regulators (Leonard et al., 2001). The first morphological appearance of malignant cells are in the form

of abnormal nodules, named as early neoplastic proliferation (ENP) centers, during the 2nd month of extrauterine life; they continue to appear until 3[1]/2 months. These nodules are localized in the paraventricular white matter, sub-eppendymal region, from the genu of the corpus callosum to the commissural fornicis dorsalis and tapetum. The cells in these nodules may show unregulated proliferation (Schiffer et al., 1980). Full-fledged tumors in the ENU-induced glioma model are lesions more than 500 μ in diameter (Koestner et al., 1971). Glial tumors showing circumscribed necrosis and blood-vessel proliferation appear from 5[1]/2 months. These tumors are sharply delimited towards the hemispheric cortex and hippocampus, and grow diffusely in the paraventricular white matter (Schiffer et al., 1978). A proposed longitudinal scheme of tumor development in the transplacental ENU-induced glioma rat model is presented in the Table 4.

To better characterize the role of ERK[1]/2 in tumor behavior, we used an animal model of spontaneous human glioma by transplacental ENU administration in rats. In this study, we have analyzed a number of factors involved in signaling pathways that may contribute to tumor malignancy. We have analyzed the role of ERK[1]/2, Akt, Bcl-2 and Bad proteins in an ENU-induced transplacental glioma rat model at early postnatal (P90) and progressive postnatal (P180) stages of tumor development in relation to their malignancy.

Longitudinal Scheme of glioma tumor development in ENU induced transplacental glioma rat model



David Schiffer et al., Cerebral tumors induced by transplacental ENU: Study of the different tumoral stages, particularly of early proliferations. *Acta neuropath.* **41**, 27-31 (1978)

Table 4

RESULTS

Identification of vaginal smear patterns for the determination of different stages of the estrus cycle

Time-specific gestation is essential for the administration of ENU during the critical gestation time period of the rats. Appearances of different patterns of vaginal cells follow a cyclic manner in rats. Estrus stage was indicated by the presence of large cornified (degenerative cells which lost nuclei) epithelial cells with few nucleated cells. Metestrus was observed after ovulation and consists of large cornified epithelial cells mixed with polymorphonuclear leukocytes. The presence of polymorphorphonuclear leukocytes exclusively, along with a few nucleated epithelial cells was classified as the diestrus stage. A mixture of round nucleated epithelial cells and the presence of a few leukocytes in the smear indicated the pro-estrus stage. The pro-estrus stage is the preparatory period of the next estrous cycle. Vaginal cell patterns of different stages are presented in the Fig 20.

Neurological symptoms indicating growth of brain or spinal cord tumors

Clinical examination should include observation on the state of consciousness and behaviour, grooming state, gait, head posture, visual and tactile placing reactions, bladder function, appearance of eyes, response of limbs to stimuli, and palpation of cranium and spine. In the present study, rats were suffering due to corneal erosion from birth, weight loss, shaggy and loss of fur along with discoloration of skin. The rats were also observed to be aggressive or depressive (Fig 21). Animals with neurological symptoms indicating growth of brain tumors were used for the present experimentation.

Fig 20

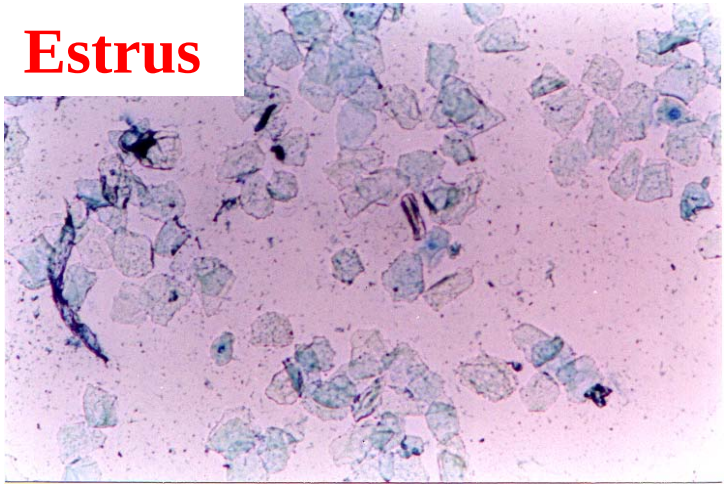
(a) Rat vaginal smear showing mixture of round nucleated epithelial cells and with few leukocytes, which lasts for 12hr. This pattern is refered to as proestrus stage of the cycle.
(b) Rat vaginal smear showing presence of exclusively large cornified epithelial cells with few nucleated epithelial cells, which lasts for 12hr. This pattern is refered to as estrus stage of the cycle.
(c) Rat vaginal smear showing presence of large cornified epithelial cells mixed with polymorphonuclear leukocytes, which lasts for 21hr. This pattern is refered to as met-estrus stage of the cycle.
(d) Rat vaginal smear showing presence of exclusively a large number of polymorpho nuclear leukocytes through the smear, which lasts for 60–70 hr. This pattern is refered to as diestrus.

Screening vaginal smear for estrus detection in rat

Proestrus

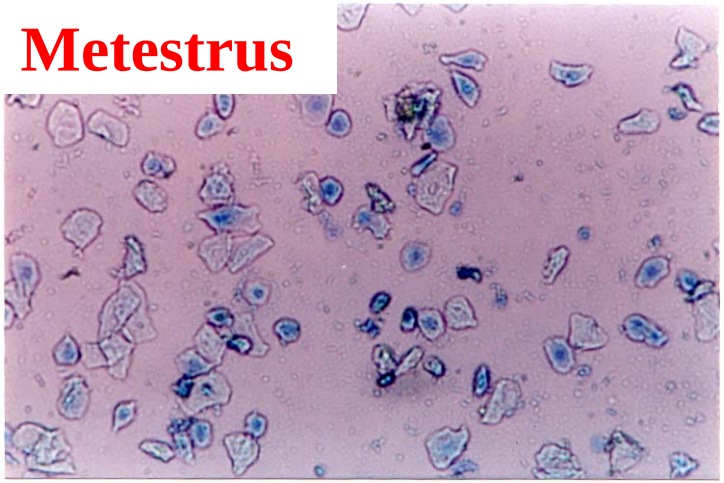
(a; Objective 10X)

Estrus



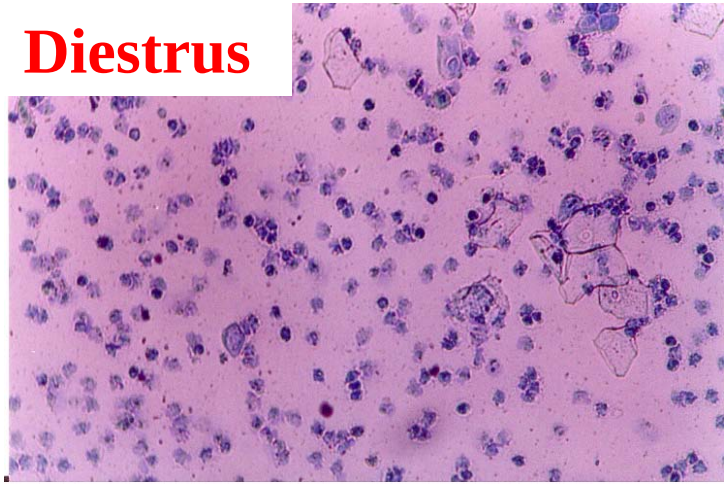
(b; Objective 10X)

Metestrus



(c; Objective 10X)

Diestrus



(d; Objective 10X)

Fig 20

Histopathological studies by using haematoxylin and eosin (H & E) staining of brain tissue sections of glioma rats

Haematoxyin and eosin staining of paraffin-embedded brain sections of early (P90) stage glioma rats showed the presence of nodular lesions. These lesions were observed in peri-ventricular white matter of the cerebral hemispheres (Fig 22a) (n=8). A slight increase in cellularity was also observed in the SVZ of the forebrain (Fig 22b). At a higher magnification, cells in the nodule resembled oligodendroglial foci (Fig 22c). There was variation in the number of tumors present in the sections. Tissue sections of progressive (P180) stage glioma rats showed an increased cellularity and angiogenesis in the lesion (Fig 23a & b). At a higher magnification, cells in the tumor showed development of primitive neuropil and astrocytic processes (Fig 23c). We did not observe necrotic features in the present study.

Malignant proliferative tendency of cells in the lesions of glioma rats

Ki 67 is a nuclear proliferation marker used to identify the labeling index of malignant cells in relation to normal cell proliferation rates. Though there was variation in the number of tumors observed in early-stage (P90) glioma rats, nodules showed similar patterns of Ki67 labeling (mild positivity of ~10%). Further, significant nuclear positive cells for Ki67 were observed in the lesions of progressive stage (P180) glioma rats (~30% nuclear positivity) (Fig 24a & b). The malignancy of the cells in the lesion was further evidenced by the increased expression of mini chromosomal maintenance-2 (mcm-2) protein observed in western blot analysis (Fig 24c).

Fig 21

N-Ethyl n-nitrosourea (ENU) induced transplacental *Wistar* rat glioma model showing signs of neurological symptoms.

Fig 22

Brain sections of ENU induced glioma rats after three months of extra uterine life (a) Presence of early neoplastic proliferation centers (ENP) in the form of nodules (b) Increased cellularity in the sub-ventricular zone (c) Cells under higher magnification showing oligodendroglial morphology with scarce cytoplasm and small dark nuclei.

ENU induced transplacental Wistar rat glioma model

Fig 21

Clinical Symptoms

- Corneal erosion (by birth)
- Weight loss & Fur loss associated with discoloration of skin
- Depressive/Aggressive

Haematoxylin and Eosin (H&E) staining of Three months old glioma rats

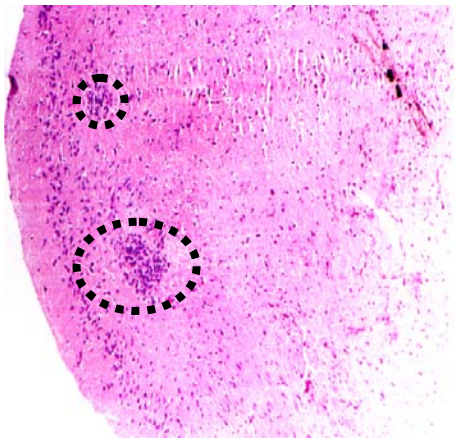


Fig 22a (10X)

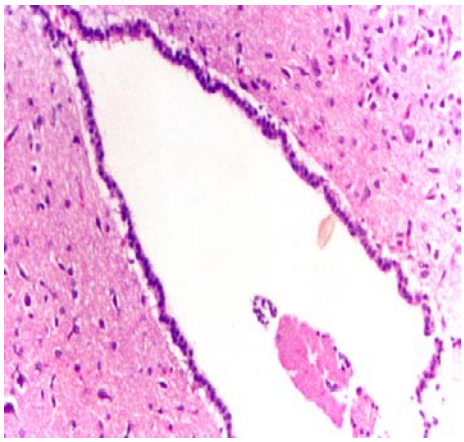


Fig 22b (10X)

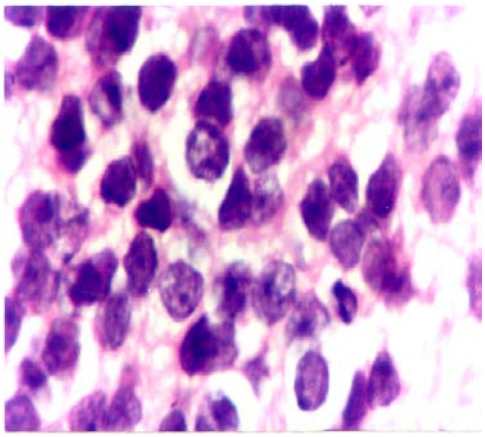


Fig 22c (100X)

Fig 23

Brain sections of ENU induced glioma rats after six months of its extra uterine life (a) A bulk tumor mass with increased tumor mass (b) Tumor showing the profound increase in angiogenesis (c) Higher magnification showing the formation of neuropile and primitive astrocyte processes.

Haematoxylin and Eosin (H&E) staining of Six month old glioma rats

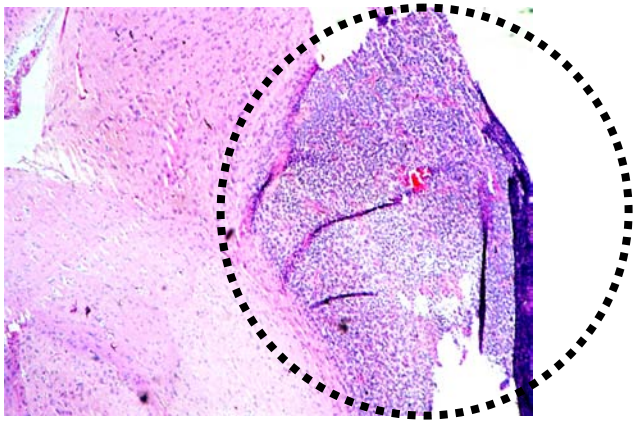


Fig 23a; 4X

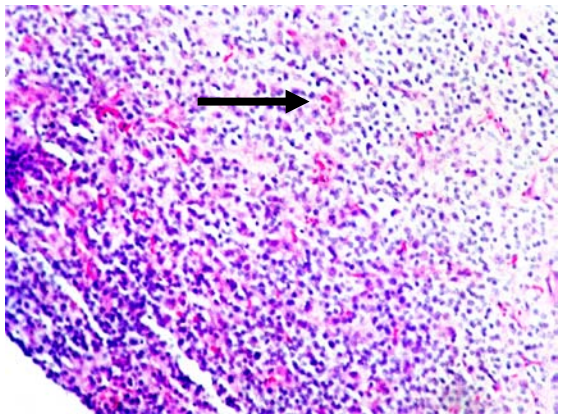


Fig 23b; 10 X

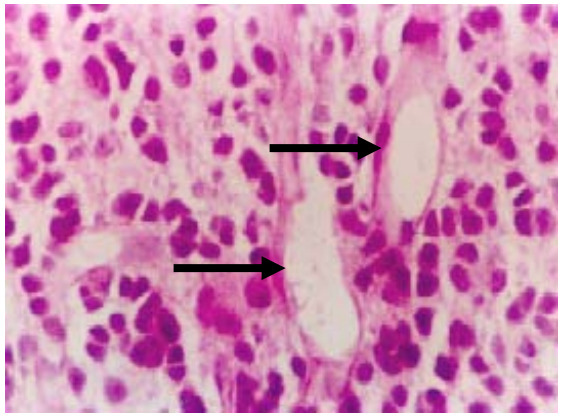


Fig 23c; 100X

Fig 23

Fig 24

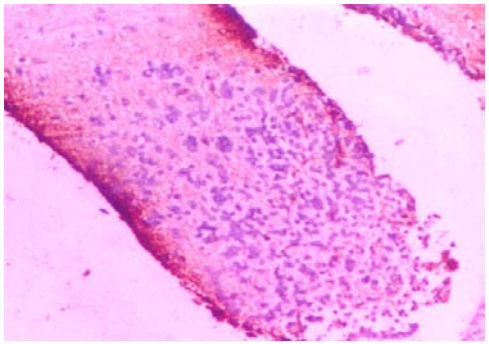
Proliferation index by Ki 67 labeling showing (a) Mild increase (~10%) in proliferation index among three month old glioma rats (b) Increased (~30%) proliferation index among the six month old glioma rats (c) Western immunoblot analysis showing significant increase in mcm-2 protein expression in glioma rats.

Western analysis of Mcm-2 protein showing significant increase in the levels among progressive stage glioma rats.

C - set of age matched saline treated control rats (n=4) S #1 - set of ENU induced glioma rats after three months of (n=6); and S #3 – set of ENU induced glioma rats after six months (n=9)

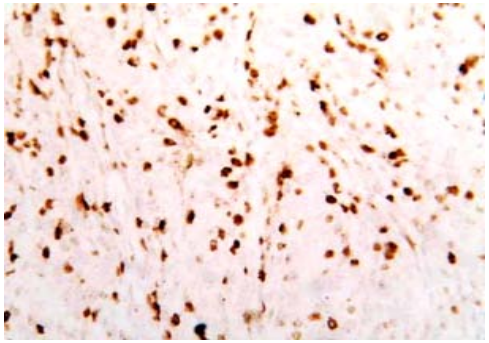
Malignant proliferative tendency of the cells in the lesions of glioma rats

a) Ki67 Labeling index among the ENU induced glioma rats



Ki67 positivity in three month old glioma rat (4x) (Proliferation Index: ~10%)

Fig 24a (4x)



Ki67 positivity in Six month old glioma rat (10x)
(Proliferation Index: ~30%)

Fig 24b (40x)

b) Minichromosomal maintenance protein –2 (Mcm-2) levels in glioma rats

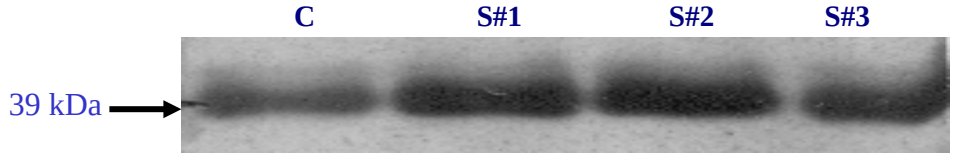
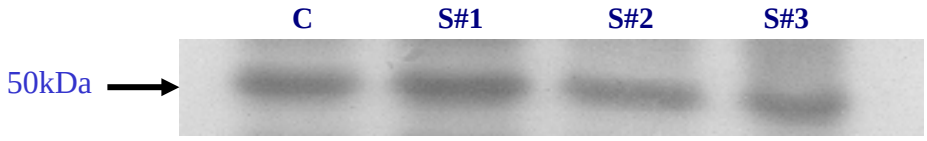


Fig 24c (Mcm-2)



β-Tubulin

C: Saline treated control rat
S#1: Set of glioma rats after three months (n=6)
S#2 & 3: Set of glioma rats after six months (n=9)

Fig 24

SDS-PAGE showing differential protein patterns in early- and progressive-stage glioma rats, in comparison to control rats

Resolution of rat glioma protein samples was performed on 10% SDS-PAGE, which showed differential protein patterns. Some of the proteins were present only in glioma rats, not in controls. Proteins showing progressive increases in level in progressive-stage (P180) glioma rats were also observed (Fig 25). This study did not characterize these proteins. It may be relevant to know the physiological roles of these proteins and to exploit their therapeutic potential for controlling tumor growth.

Types of glial tumors observed in the ENU-induced transplacental rat gliomas

Glial tumors induced by ENU are highly heterogeneous with respect to their cell type, genetic alterations, number of tumors and distributions in different rats, but signal transduction is specific to the type of cell. Among the early stage (P90) glioma rats, nodules contained neither GFAP nor neuron-specific enolase (NSE). Mild cytoplasmic positivity for nestin was observed in the tumors in ~90% of glioma rats (Fig 26). Though there was variation in the number of tumors observed in early-stage glioma rats, nodules showed similar patterns of Ki67 positivity, which may indicate a proliferative tendency. At a higher magnification, cells in the tumor showed morphological signs of transformation. Consistent with this observation, cytoplasmic-positive GFAP cells were found in the lesions of progressive-stage (P180) rat gliomas (Fig 27). Lesions also exhibited significant membrane positivity for EGFR in progressive-stage (P180) glioma rats (Fig 28).

Fig 25

Differential protein expression observed among the glioma rats in 10% SDS-PAGE showing increased levels of a protein (encircled) in glioma rats, and appearance of protein bands only in glioma rats (indicated with an arrow).

Fig 26

Immunohistochemical analysis of nestin showing strong cytoplasmic positivity observed in the nodules of three month old glioma rats.

Fig 27

Immunohistochemical analysis of glial fibrillary acidic protein showing mild increase in cytoplasmic positivity observed among the different rats of progressive stage glioma rats.

Fig 28

Immunohistochemical analysis of epidermal growth factor receptor showing strong cytoplasmic and membrane positivity observed among the different rats of progressive stage glioma rats.

Differential protein pattern among the glioma rats

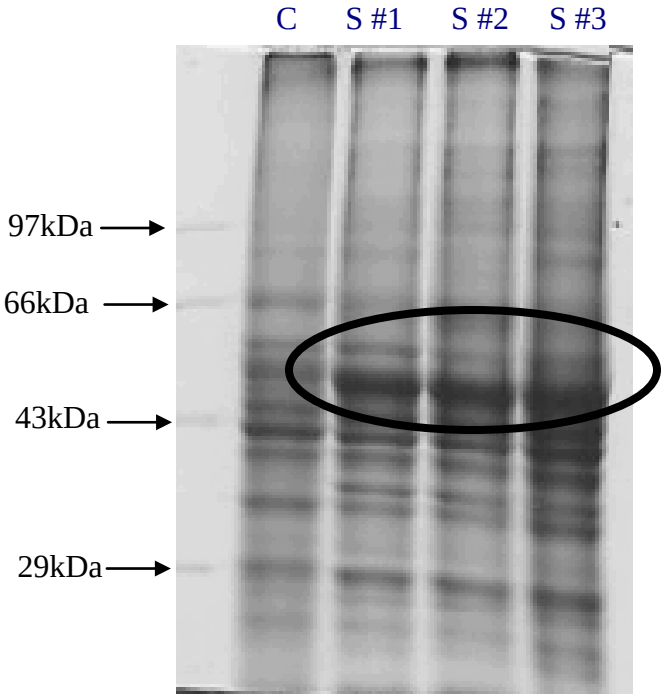


Fig 25 (10% SDS -PAGE)

Glial tumor composition

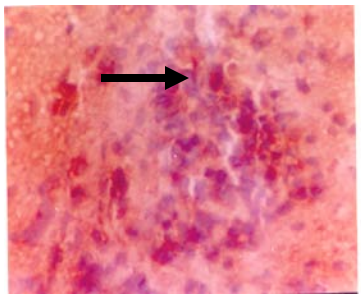


Fig 26 (Nestin) 100x; ++

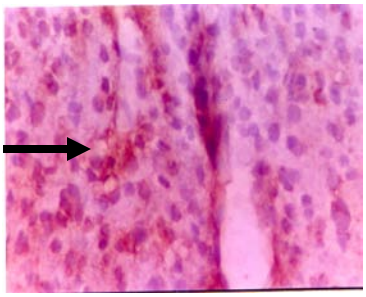


Fig 27 (GFAP) 100x; ++

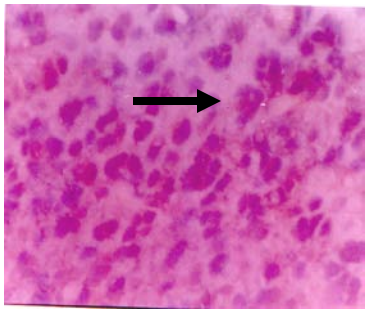


Fig 28 (EGFR) 100x; +++

Analysis of mitogen-activated protein kinase 1/2 (MAP1/2) or extracellular signal regulated kinase (ERK[1]/2) pathway activation in ENU-induced glioma rats

Among early-stage (P90) and progressive-stage (P180) glioma rats, monoclonal phospho-tyrosine blots did not show any significant difference (Fig 29). Western immunoblot analysis showed increased levels of ERK in glioma rats compared to controls (Fig 30). An increased level of pERK[1]/2 was found in progressive-stage (P180) glioma rats (Fig 31). Immunohistochemical analysis using anti-phospho ERK[1]/2 on rat brain sections with lesions showed mild increase in nuclear positivity (Fig 32).

Activation of sustained cellular survival pathways through Akt and other anti-apoptotic molecules in ENU-induced rat gliomas

An increase in pAkt levels was observed in glioma rats. Densitometric analysis depicted elevated levels of pAkt in six-month-old glioma rats (Fig 33). This may indicate the successful survival of malignant cells in the growing tumor. Elevated levels of Bcl_2 and phosphorylated Bad in six-month-old glioma rat may further indicate the inappropriate execution of apoptotic signals (Fig 34 &35). Quantification of Bcl-2 blots clearly showed increased levels in six-month-old glioma rats. In all glioma rats, pBad levels were elevated almost equally, which may indicate inhibition of apoptotic signals from the beginning stages of glioma development. Bad is one of the pro-apoptotic family members in its de-phosphorylated state, which will link upstream with cell survival signals and down-stream with anti-apoptotic signals.

Fig 29

Western analysis of phospho-tyrosine among the glioma rats.

Fig 30

Western analysis of ERK1/2 showing increased levels in glioma rats.

Fig 31

Western analysis of phospho-ERK1/2 showing significant increase in the levels among progressive stage glioma rats.

Densitometric analysis (arbitrary units) of pERK1/2 immunoblots; data are shown as the mean ± SE of independent densitometric measurements among the different glioma rats represented by C - set of age matched saline treated control rats (n=4) S#1 - set of ENU induced glioma rats after three months of (n=6); and S#3 – set of ENU induced glioma rats after six months (n=9). Statistical significance was calculated using paired t-test between control and glioma rats of three months (P*) and six months (P**) : pERK1 (P_{44}) - P_{44}*: 0.0337, P_{44}**: 0.0029; pERK2 (P_{42}) - P_{42}*: 0.042, P_{42}**: 0.002.

Fig 32

Immunohistochemical analysis of phospho-ERK[1]/2 showing mild increase in cytoplasmic and nuclear positivity in the tumors of progressive stage glioma rats.

Extra cellular signal Regulated Kinase (ERK) 1/2 pathway in glioma rats

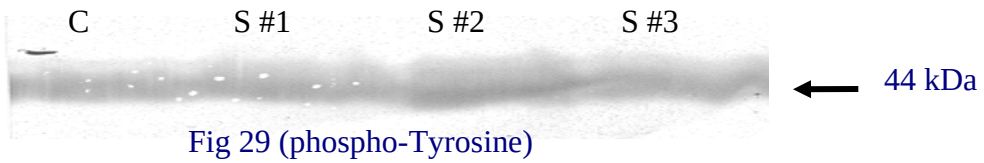


Fig 29 (phospho-Tyrosine)

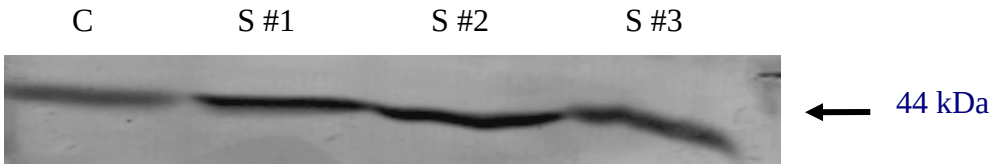


Fig 30 (ERK^1/2)

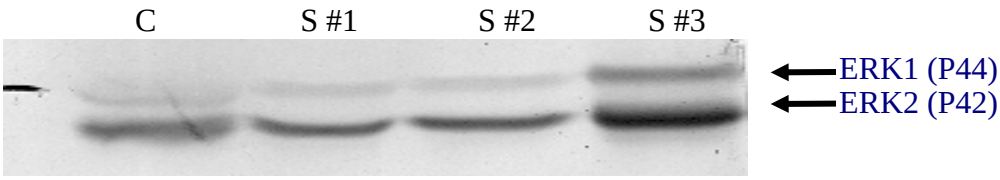
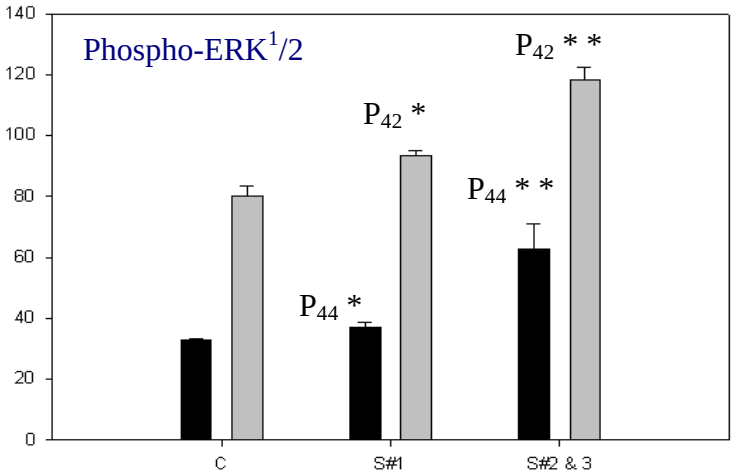


Fig 31 (phospho-ERK^1/2)



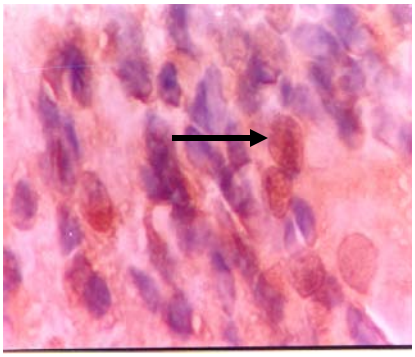


Fig 32 (pERK^1/2) 100x; ++

β-Tubulin

Fig 33

Western analysis of phospho-Akt showing significant increase in the levels among progressive stage glioma rats.

Densitometric analysis (arbitrary units) of pAkt immunoblots; data are shown as the mean ± SE of independent densitometric measurements among the different glioma rats represented by C- set of age matched saline treated control rats (n=4) S #1 - set of ENU induced glioma rats after three months of (n=6); and S #3 – set of ENU induced glioma rats after six months (n=9). Statistical significance was calculated using paired t-test between control and glioma rats of three months (P*: 0.013) and six months (P**: 0.001).

Fig 34

Western analysis of phospho-Bad showing significant increase in the levels among the glioma rats.

Densitometric analysis (arbitrary units) of pBad immunoblots; data are shown as the mean ± SE of independent densitometric measurements among the different glioma rats represented by C - set of age matched saline treated control rats (n=4) S #1 - set of ENU induced glioma rats after three months of (n=6); and S #3 – set of ENU induced glioma rats after six months (n=9). Statistical significance was calculated using paired t-test between control and glioma rats of three months (P*: 0.0009) and six months (P**:0.004).

pAkt or Protein Kinase B (PKB) status in glioma rats

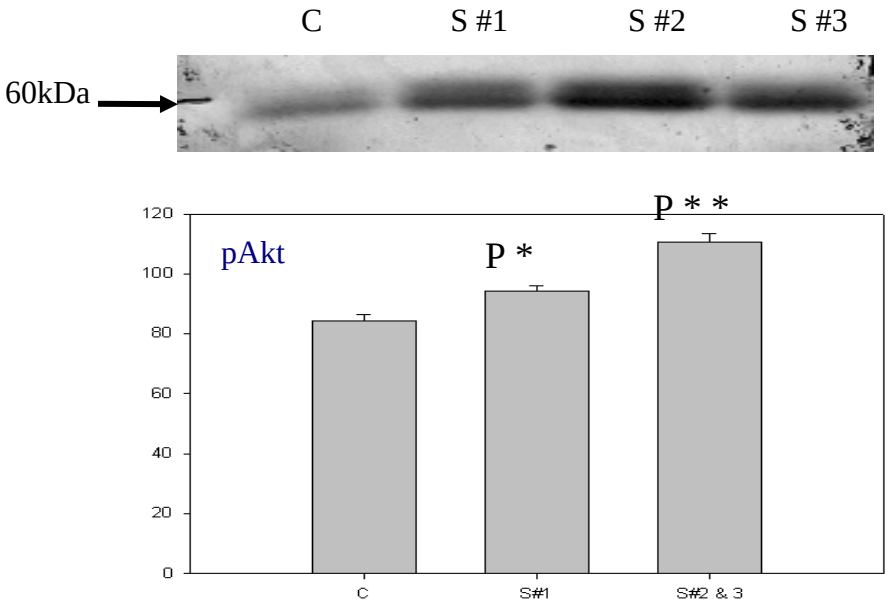


Fig *33*

pBad status in glioma rats

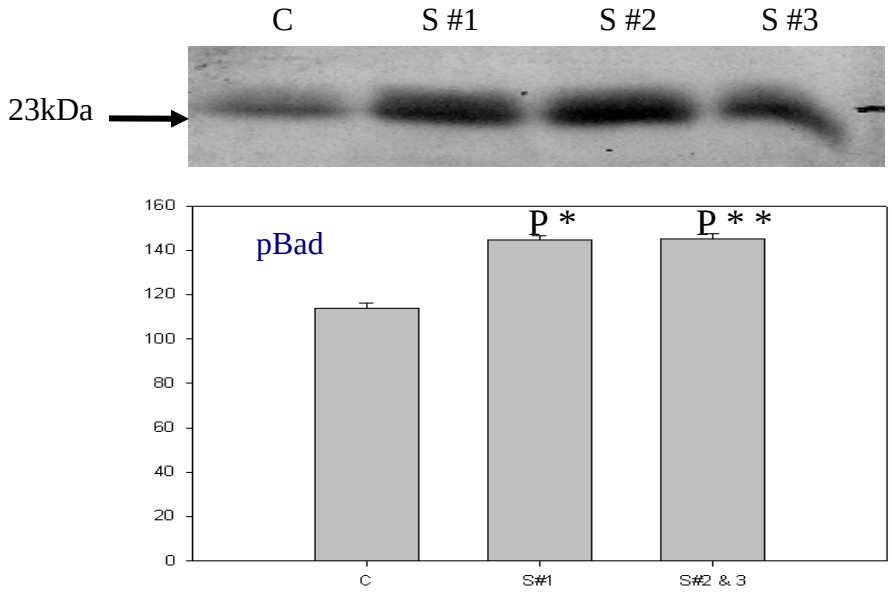


Fig *34*

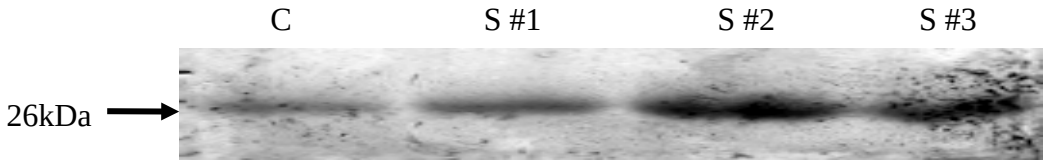
β-Tubulin

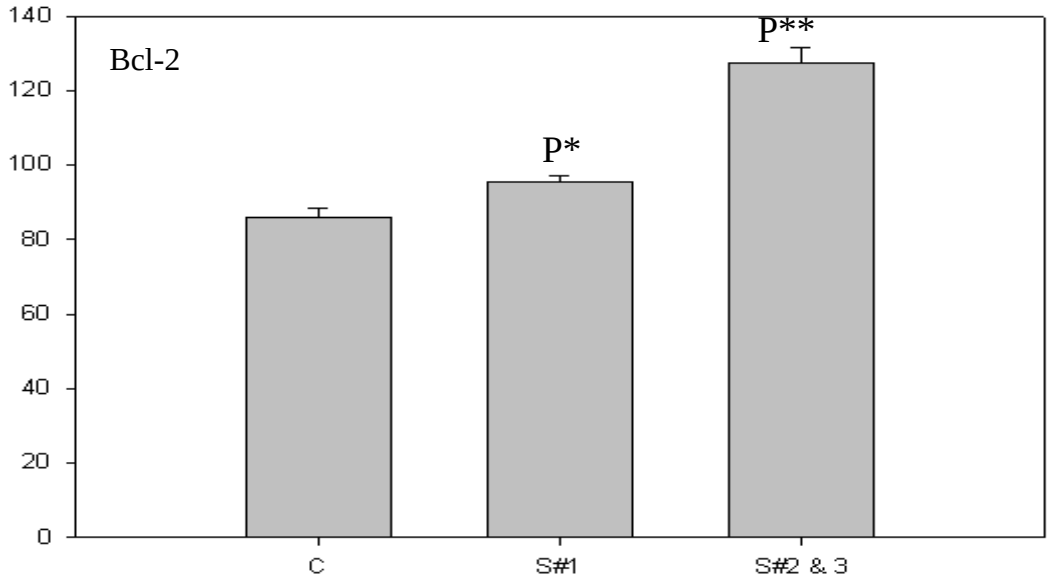
Fig 35

Western analysis of Bcl-2 showing significant increase in the levels among progressive stage glioma rats.

Densitometric analysis (arbitrary units) of Bcl-2 immunoblots; data are shown as the mean ± SE of independent densitometric measurements among the different glioma rats represented by C - set of age matched saline treated control rats (n=4) S #1 - set of ENU induced glioma rats after three months of (n=6); and S #3 – set of ENU induced glioma rats after six months (n=9). Statistical significance was calculated using paired t-test between control and glioma rats of three months (P*: 0.0005) and six months (P**:0.0018).

BCl-2 status in glioma rats





β-Tubulin

Fig 35

DISCUSSION

Gliomas have been considered to arise from the dedifferentiation of glial cells (Rubinstein, 1987). Malignancy progression may be due to the further accumulation of genetic and /or inherited mutations. Hence, a multistage cancer model is essential to understand the molecular alterations associated during the initiation, promotion and progression phases that are found in epithelial cancer formation (Berenblum et al., 1947). In the induction of brain tumors by using ENU, the carcinogen is cleared within minutes, after which a several-week latency period occurs before gliomas are noted. Hence, this model represents an epigenetic phenomenon of glioma progression *in situ*, where the tumors must develop in an environment that is not continuously exposed to mutagenic stimuli. The transplacental ENU-induced glioma model is perhaps the only one in which it may be possible to observe the transition from a preneoplastic state to a fully developed tumor.

Tumors in ENU-induced glioma rats appear first as ENPs or oligodendroglial foci, which further progress to form microglial tumors. ENPs subsequently progress to “microtumors” and then “tumors” at different stages of tumor development (Koestner et al., 1971), which corresponds to lesions less than 300 μ in diameter, between 300 and 500 μ, and more than 500 μ, respectively. ENPs appear at the end of the 2^{nd} month of extra-uterine life and continue to appear till $3^1/2$ months. Circumscribed necrosis and blood-vessel proliferations appear at $5^1/2$ months. Variable nestin expression is observed in ENU-induced glioma rats between 30 and 90 days and nestin-expressing cells of the nodules represent the early stage of the neoplastic process (Jang et al., 2004). Mcm-family proteins and their expression have attracted much attention recently for their role as markers to identify the proliferative

properties of malignant cells. Mcm-2 forms a part of the pre-replicative complex during the G1 to S phase transition of the cell cycle (Freeman et al., 1999). Mcm-2 is a proliferation marker in oligodendrogliomas, and its expression was correlated to disease prognosis (Wharton et al., 2001; Ishimi et al., 2003).

Recent reports have stressed the importance of understanding molecular signals associated with cell proliferation and cell death in finding effective drug targets against glial tumors (Raza et al., 2002; Steinbach et al., 2004; Tysnes et al., 2001). MAP kinase is primarily regulated by external growth factors that play a role in controlling the cellular activities during tumor progression and invasion (Reddy et al., 2001; Zhang et al., 2002). Increased $ERK^1/2$ activation in human GBM has already been documented (Schlegel et al., 2002). Immunohistochemical analysis of human astrocytomas showed constitutive $ERK^1/2$ activation irrespective of pathological grade (Mandel et al., 1998). Elevated $pERK^1/2$ levels during late stages of tumor development may suggest its role in cell proliferation and associated malignancy. Similar observations regarding $ERK^1/2$ role in cancer cell proliferation, differentiation, survival, angiogenesis, metastasis and cell death have been reported (Krzeminski, 2005; Lobner et al., 2004). The oncogenic role of Akt is responsible for the sustained cell survival observed in many solid tumors during tumor progression (Aoki et al., 1998). The PI3-K-Akt pathway plays a central role in the development and progression of prostatic cancer (Li et al., 2005). The cell survival activity of human gliomas is largely due to the cross-talk between the Ras and PI-3-K-Akt pathways. This cross-talk could be a potential therapeutic target (Sakata et al., 2002). Bcl_2 functions as an anti-apoptotic protein by preventing mitochondrial changes, including cyt C release and loss of membrane

potential. Over-expression of Bcl_2 can protect various cell types both *in vitro* and *in vivo* from undergoing apoptosis (Vaux et al., 1988). Elevated levels of Bcl_2 observed in the glioma rats in this study may indicate predominance of the anti-apoptotic mechanism. Bad is a member of Bcl_2 family involved in the regulation of apoptosis and gets phosphorylated by protein kinase-B (PKB) or Akt (Datta et al., 1999). The phospho-BAD cannot inhibit the anti-apoptotic function of Bcl_2 family members. In this study, increased levels of pAkt may correspond to the increased levels of pBad observed in glioma rats. Hence, PKB-dependent Bad phosphorylation leads to cellular survival and could be an important factor in the development of drug resistance in human malignant gliomas.

This work may reflect a possible role for the $ERK^1/2$ and Akt pathways in glioma malignancy. Anti-apoptotic mechanisms and sustained cellular survival signals may also be operational, and may accompany the enforced proliferation leading to full-fledged malignancy observed in ENU-induced glioma rats.

SUMMARY

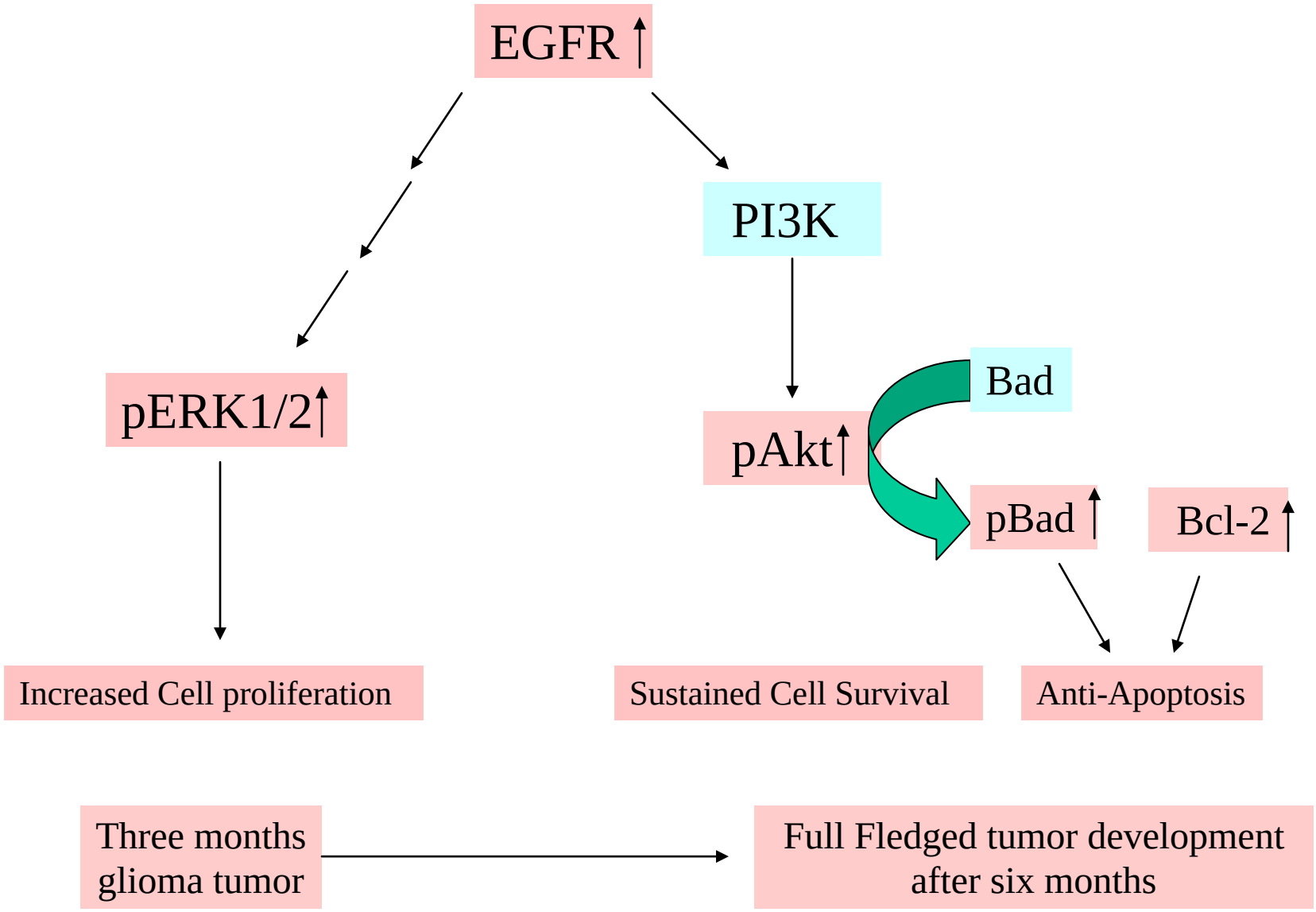
The current understanding of glioma biology reveals effective targets for anti-invasive therapy, which include manipulations of the extracellular matrix and receptors, growth factors and cytokines, proteases, cytoskeletal components, oncogens and tumor suppressor genes. A better understanding of the complex regulation and the signaling molecules involved in glioma invasion is still needed in order to design new and effective treatments for invasive tumor cells. In this study, mitogenic signaling responsible for cell proliferation, and different cell-death proteases that can be responsible for differential PARP cleavage in surgically resected human glioma biopsies were analyzed. Concomitant activation of the MAP kinase and Akt pathways is responsible for sustained cellular survival through anti-apoptotic mechanisms. In this study we report activation of MAP kinase and Akt pathways in the transplacental ENU induced glioma rat model in early (P90) and progressive (P180) stages of tumor development.

1. The increased pERK^{1}/2 observed in glioblastoma (WHO grade IV) samples by western immunoblotting analysis and immunohistochemistry may indicate its possible role in glioblastoma malignancy (Bhaskara et al., 2005).

2. Differential PARP cleavage patterns observed in glioma biopsies may be due to the activity of different proteases, including caspase-3, granzyme B, calpain I and II during the process of cell death. Strong nuclear positivity for AIF may also indicate a caspases-independent form of cell death. Tumors may contain a mixture of cells showing not only apoptosis and necrosis, but also different intermediate forms of cell death programme. A better understanding of alternate or multiple forms of cell death programs taking place in glial tumors may lead to novel therapeutic targets.

3. MAP kinase and Akt pathways were observed to play a critical role in glioma development in an ENU-induced rat model. Increased Bcl-2 and pBad levels in the progressive stage of tumor development may indicate anti-apoptotic mechanisms are also operational. Sustained cellular survival pathways, including MAP kinase and Akt pathways activated by the EGFR along with the interaction of anti-apoptotic molecules, will lead to fully-fledged tumor formation from the early neoplastic proliferation centers (Bhaskara et al., 2006).

Cell proliferation and sustained cell survival signals in ENU induced glioma rats



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