Introduction

Brain tumors are the most common solid tumor of childhood, second in frequency only to leukemia [1]. Astrocytomas represent the most common primary brain tumor of childhood comprising almost 50% of all brain tumors in this age group [2]. Of these, 20% are high-grade, or anaplastic lesions. Unlike adults with low-grade astrocytomas who frequently progress to higher grade lesions over time, the malignant transformation of a low-grade astrocytoma to high-grade one in a child is a rare event [3]. Medulloblastoma is another common brain tumor in childhood accounting for about 25% of pediatric brain tumors. This tumor is highly proliferative with a propensity to invade the fourth ventricle as well as the overlying subarachnoid spaces. As such, medulloblastoma is characterized by widespread dissemination along cerebrospinal fluid pathways. Unfortunately, many children who suffer from malignant astrocytoma and medulloblastoma will die from their tumors despite recent advances in surgery, radiation, and chemotherapy [4]. Improving the prognosis for these children will involve not only the identification of better treatment modalities, but a greater understanding of the pathogenetic basis for these tumors. Recently, several studies have shown that a class of genes which control cell cycle regulation play an important role in the pathogenesis of several different human tumors, including brain tumors.

Cell Proliferation Markers

The human brain is a unique organ from a cell kinetic standpoint. Neurons become incapable of cell division in the early postnatal period. Although glial cells retain their proliferative potential, as is demonstrated in the process of reactive gliosis, it is still unclear what the initiating events are that transform a normal cell into a highly proliferative malignant brain tumor cell. However, a number of studies have now been performed which have characterized the proliferative indices of human brain tumors, including pediatric brain tumors. The original cell kinetic studies conducted by Hoshino et al. [5–9] on brain tumors established the importance of measuring the growth fraction, labeling index, and cell cycle time. Early studies on brain tumor cytokinetics were performed by pulsing intra-cranial tumors with 3H-thymidine, a marker of the S phase of the cell cycle. Then, using a technique that required the preoperative administration of bromodeoxyuridine (BUdR), a thymidine analogue that is incorporated into nuclear DNA during the S phase, Hoshino et al. [8, 9] showed elegantly that the tumor-labeling index is comparable by either BUdR immunohistochemistry or 3H-thymidine incorporation.

A recent advance in cell kinetic analysis of human brain tumors came from the identification of two proliferative markers, proliferating cell nuclear antigen (PCNA) and Ki-67. Antibodies to these proteins recognize a nuclear antigen expressed exclusively in proliferating cells. Ki-67 is found in cells that are cycling in late G1, S, G2, and M phases of the cell cycle, but is absent in quiescent GO cells [10,11]. In general, labeling index values of brain tumors with Ki-67 antibodies are somewhat higher but parallel to those observed by BUdR immunohistochemistry [12]. Because G1, G2, and M phase cells are recognized by Ki-67 immunostains, it has been suggested to be an objective indicator of the biological behavior of cancer. Unlike BUdR, Ki-67 immunostaining can only be
performed on frozen sections. However, BUDR is a potential carcinogen and must be given intravenously to the patient preoperatively. Fortunately, an analogue antibody to Ki67 has been produced which enables identification of cycling cells in formalin-fixed, paraffin-embedded tissues. This antibody recognizes an antigen known as MIB-1. Today, PCNA, and MIB-1 immunohistochemistry can be performed routinely on paraffin-embedded material (fig. 1). Recent studies, however, have suggested that a certain level of PCNA immunostaining may be artificial and not truly reflective of cell proliferation. Therefore, MIB-1 has become the favored proliferation marker which correlates well with in vitro BUDR uptake.

Cell Proliferation Marker Expression in Pediatric Brain Tumors

Most of the literature on cell kinetic analyses generally pertains to adult brain tumors, although some studies have included pediatric tumors. Hoshino et al. [9] showed that the probability of survival among patients with a BUDR-labelling index under 1% was much higher than in those with indices greater than 5%. Moreover, they reported on a cell kinetic study comparing four medulloblastomas with malignant gliomas using 3H-thymidine incorporation [13]. The average labeling index was more than 10%, but the mean survival time was 6 years. In contrast, patients with malignant gliomas that had a labeling index of 5% or more died within 1 year of diagnosis. Therefore, a high labeling index was not necessarily associated with a poor prognosis in patients with medulloblastoma. This discrepancy in survival and labeling index levels can be attributed, no doubt, to the greater sensitivity of medulloblastoma to radiation therapy and chemotherapy. Ito et al. [14] reported on a BUDR-labelling analysis of 50 juvenile pilocytic astrocytomas. They found that the mean BUDR-labelling index was approximately 1%. Their results suggest that most juvenile pilocytic astrocytomas grow slowly as a result of these low BUDR-labelling indices. Some patients in their study had tumors with high labeling indices and some of their tumors recurred. However, in most cases, the clinical course was excellent regardless of the labeling index. Immunohistochemical analyses with Ki-67 have now been performed and reported for large series of human brain tumors including pediatric brain tumors [15–18]. In most studies, the Ki-67-labelling index, defined as the number of positive staining cells divided by the total number of tumor cells, has ranged from 0 to 50% and has correlated well with the histopathological grade of malignancy of the tumor. In pilocytic astrocytomas, this index was generally low with scores ranging from 0 to 1%. In primitive neuroectodermal tumors and/or medulloblastomas, the index has ranged from 5 to 50%. The mean index

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![Fig. 2. A proliferating cell passes through an orderly sequence of phases which comprise the cell cycle. Following mitosis, differentiated cells exit the cell cycle after mitosis to enter a state of quiescence called GO. If a cell is destined to continue to proliferate, it enters the first phase of interphase called Gl, which is characterized by a period of cell growth and synthesis of components necessary for DNA synthesis. During this phase the cell is sensitive to conditions in its environment. If these conditions are unfavorable, the cell can arrest in Gl. There is a point, called the restriction point (R), which is the decision-making point for cell cycle progression, and it precedes the onset of the S phase by 1–3 h. The S phase follows Gl and is the period in which chromosomal DNA is replicated. The next phase, called G2, is shorter than Gl and precedes the mitotic phase. During the G2 phase, DNA replication must be completed before the cell enters mitosis. In the mitotic phase, equal amounts of chromosomal material migrate to opposite poles of the cell. The subsequent division of the cytoplasm by cytokinesis results in two identical but separate daughter cells. The fundamental basis of many chemotherapeutic agents in use today for children with brain tumors is the blocking of tumor cell division in either a cell cycle phase-specific or nonspecific fashion.](image)
for medulloblastoma is approximately 10%. In a series of 17 pediatric brain and spinal tumors from our center [17], the mean Ki-67 index was 0.81% in low-grade astrocytomas, 0.931% in pilocytic astrocytomas, 5.02% in anaplastic astrocytomas, 6.32% in glioblastomas, 2.38% in anaplastic ependymomas, 14.26% in choroid plexus carcinomas, and up to 22% in medulloblastomas. Bodey et al. [18] suggested that the highest indices were found among the poorly differentiated classic medulloblastomas. The inherent problem of regional variability must be considered in the interpretation of Ki-67 indices, especially in tumors with extensive necrosis or in tumors like anaplastic ependymoma which show well-differentiated and poorly differentiated areas. Interestingly, two groups, Schiffer et al. [19] and Iijima and Nakazato [20], showed that there is an inherent problem of regional variability in medulloblastoma. Light-looking areas (large cell areas) and dark areas (desmoplas-tic and classic areas) may have completely different proliferative indices (PCNA and MIB-1 staining). The light areas consistently showed lower staining indices than did the dark areas. It has been suggested that the light areas consist of cells with a more differentiated neuronal lineage with a relatively low proliferation potential.

Pollack et al. [21] reported on the relationship between the MIB-1-labeling index and outcome in malignant gliomas of childhood. The median overall survival was more than 4 years in the low-MIB-1 index group compared with only 16 months in the high index group. Their results suggested the potential utility of the MIB-1-labeling index as a prognostic marker in children with malignant gliomas. Because of the results from the studies listed above, application of these proliferation indices as an adjunct to the diagnosis of brain tumors has become widespread among neuropathologists.

The Cell Cycle

An understanding of the cell cycle is critical to understanding normal cell proliferation and the altered proliferation observed in various brain tumors, such as human gliomas. A proliferating cell passes through an orderly sequence of phases which comprise the cell cycle (fig. 2). Following mitosis, a cell enters interphase. Differentiated cells exit the cell cycle after mitosis to enter a state of quiescence called GO. The proliferating cell, on the other hand, progresses into the first phase of interphase called GI, which is characterized by a period of cell growth and synthesis of components necessary for DNA synthesis. S phase follows GI and is the period in which chromosomal DNA is replicated. The next phase, called G2, is shorter than GI and precedes the mitotic phase. During the G2 phase, DNA replication must be completed before the cell enters mitosis. In the mitotic phase, equal amounts of chromosomal material migrate to opposite poles of the cell. The subsequent division of the cytoplasm by cytokinesis results in two identical but separate daughter cells.

The cell cycle phase which is most strategically positioned to control cell proliferation is GI. During the GI phase, the critical decision (restriction point) to go on to DNA synthesis and mitosis is made [22]. This restriction point is the decision-making point for cell cycle progression, and it precedes the onset of S phase by 1–3 h. Extra-

Cyclins and CDKs Act as Positive Growth Regulators

The major positive growth regulators of the cell cycle are a group of related proteins, the cyclins. Cyclins are a group of structurally similar proteins which share homology in a conserved 60-amino acid domain known as the cyclin box [23]. Cyclins are the positive regulatory sub-units of a class of related protein kinases, called CDKs. These cyclin-CDK complexes are the regulators of the major cell cycle transitions (fig. 3). When cells emerge from the GO phase and enter GI, the expression of D- and E-type cyclins is induced. At the onset of the S phase, cyclin A is first detected followed by cyclin B during the interval between the S phase and mitosis (G2) followed by rapid degradation at the end of the M phase. There are two main families of cyclins, the mitotic cyclins and GI cyclins. Mitotic cyclins consist of cyclin B and cyclin A. Cyclin A also has a second role in the S phase of the cell cycle. The GI cyclins are cyclins C, D, and E. Cyclin can interact with different CDKs.

The CDKs are believed to phosphorylate key substrates which are required to facilitate the passage of the cell through each phase of the cell cycle. Substrates of CDKs can be structural proteins, which act as the actual effectors of a cell cycle phase transition, or regulatory molecules [24–26]. The best characterized targets of D-type cyclin kinase activity are the pRB family proteins. During the GI phase, phosphorylation of pRB, by sequential activation of D-type cyclins and CDK4/CDK6 and cyclin E/CDK2 may be the single most important event in the cell cycle as it initiates the S phase [27–30].
Fig. 3. Cyclins are the positive regulatory subunits of a class of related protein kinases, called cyclin-dependent kinases (CDKs). These cyclin-CDK complexes are the regulators of the major cell cycle transitions. The CDKs are believed to phosphorylate key substrates which are required to facilitate the passage of the cell through each phase of the cell cycle. Substrates of CDKs can be structural proteins, which act as the actual effectors of a cell cycle phase transition, or regulatory molecules. Cyclin-CDK complexes have kinase activity, but if the complexes associate with CDK inhibitors (CDKIs), the complex is no longer active.

which inhibit growth. Recently it has been shown that a family of CDKIs plays a major role in the negative regulation of cyclin-CDK activity [31, 32]. These GI cyclin inhibitors are involved in the arrest in GI of cells in response to antiproliferative signals. Therefore, all CDKIs are candidate tumor-suppressor genes. The CDKIs can be subdivided into two families, those which inhibit multiple cyclin-CDK complexes, such as p21CIP1, p27KIP1, p57KIP2, and those which inhibit the GI cyclin-CDK4/6 complexes, p16INK4A, p15INK4B, p18INK4C, and p19INK4D (fig. 4). p21CIP1 plays an important role in p53-mediated GI-induced cell cycle arrest in response to DNA damage [33]. p27KIP1 is thought to mediate cell cycle arrest caused by cell contact inhibition or transforming growth factor β treatment [34, 35]. In addition CDKIs may be involved in such diverse processes as terminal differentiation, cellular senescence, repairment to DNA damage, and induction of apoptosis [36]. Loss of expression of a CDKI may have disastrous consequences for the cell. For example, loss of the p6INK4A has been linked to many human cancers.

A simplified schema which enables visualization of the cyclins, CDKs, and CDKIs in practical terms in cell cycle control is shown in figure 5.

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Fig. 4. Cyclins are the regulatory sub-units of cyclin-dependent kinases (CDKs). Cyclin-CDK partners are shown for different cell cycle phases. Cyclin/CDK activity is inhibited by a number of specific inhibitors, called CDK inhibitors (CDKIs). The CDKIs are involved in the arrest in GI of cells in response to antiproliferative signals. The CDKIs can be subdivided into two families: those which inhibit multiple cyclin-CDK complexes, such as p21CIP1, p27KIP1, p57KIP2, and those which inhibit the GI cyclin-CDK4/6 complexes, p16INK4A, p15INK4B, p18INK4C, and p19INK4D. p21CIP1 plays an important role in p53-mediated GI-induced cell cycle arrest in response to DNA damage. p27KIP1 is thought to mediate cell cycle arrest caused by cell contact inhibition or transforming growth factor β treatment.

DNA damage

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CELL CYCLE CONTROL

Cyclin-dependent kinase inhibitors p21, p27, p57, p15, p16, p18, p19

Cyclins

CDK inhibitors

Cell Cycle Control

Proliferation of normal cells is controlled by multiple growth-regulatory pathways that act to ensure proper growth regulation. It is generally thought that cancer cells ignore many growth-regulatory signals due to mutation in genes (genetic ‘hits’) that control either the growth-promoting or growth-inhibitory pathways. Therefore, transformation of glioma cells into malignant astrocytomas involves significant dysfunction of the cell cycle-control machinery. There have been a
number of recent studies which have shown alterations in cell cycle gene expression in human astrocytic tumors. Most of the studies on cell cycle gene expression in neurooncology generally pertain to adult brain tumors, although some studies include an analysis of pediatric tumors.

For human malignant gliomas, two genetic loci are particularly interesting: MST1 and CCND1 [37]. CCND1 encodes cyclin D1 while the MST1 locus controls expression of two independent but closely related tumor-suppressor genes, p16INK4A and p15INK4B. Interestingly, human cyclin D1 was cloned in a yeast complementation assay from a U18MG glioblastoma cDNA library [38]. p16INK4A is an important component of a cell cycle regulatory pathway involving cyclin D1, CDK4, and pRB. p16INK4A inhibits the kinase activity of the cyclin D1-CDK4 complex by binding to CDK4 and preventing its association with its catalytic subunit cyclin D1. Cyclin D1-CDK4 phosphorylates pRB during the G1 phase of the cell cycle to inhibit its growth-restraining activity. As a result, phosphorylated pRB is no longer able to bind to and prevent the activity of the E2F transcription factors. Phosphorylation of pRB releases E2F transcription factors to enter into the S phase. This event is believed to be an important checkpoint regulating the transition from G1 into the S phase during cell cycle progression.

Derangements in both the positive cell cycle regulators, cyclin D1 and CDK4, and the negative regulators, p16INK4A and p15INK4B, have been found previously in malignant astrocytomas [41, 45, 46]. Interestingly, in glioma cells that do not have p16INK4A homozygous deletions, CDK4 overexpression and amplification are common, suggesting that a further mechanism for uncontrolled glioma cell growth is the overriding of a negative regulator by a positive pathway. p16INK4A point mutations are only rarely observed in malignant astrocytic tumors that retain a wild-type p16INK4A allele [46]. There is a correlation between the frequency of homozygous deletion and malignancy, since low-grade astrocytomas do not have homozygous p16INK4A deletion [46, 56]. The majority of these studies have detected a p16INK4A or p15INK4B mutation or loss of expression in the astrocytic tumors, while these molecular changes have not been detected in ependymomas or neuroectodermal tumors [45, 63]. In addition, loss of p16INK4A protein without p16INK4A gene deletion was reported in pituitary tumors [64].

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Fig. 6. p16INK4A is an important component of a cell cycle regulatory pathway involving cyclin D1, CDK4, and retinoblastoma protein (pRB). p16INK4A inhibits the kinase activity of the cyclin D1-CDK4 complex by binding to CDK4 and preventing its association with its catalytic subunit cyclin D1. Cyclin D1-CDK4 phosphorylates pRB during the G1 phase of the cell cycle to inhibit its growth-restraining activity. As a result, phosphorylated pRB is no longer able to bind to and prevent the activity of the E2F transcription factors. Phosphorylation of pRB releases E2F transcription factors to enter into the S phase. This event is believed to be an important checkpoint regulating the transition from G1 into the S phase during cell cycle progression.

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With regard to pediatric brain tumors, most investigators have found rare instances of p16INK4A homozygous deletions and no point mutations in medulloblastomas [45, 65, 66], Petronio et al. [60] detected a single case of p16INK4A homozygous deletion among 20 primitive neuroectodermal tumors. In addition, p16INK4A homozygous deletions or CDK4 amplification were restricted to adult cases of gliomas, since neither was seen in pediatric high-grade gliomas [60]. Interestingly, Sure et al. [66] reported that loss of p16INK4A expression was observed in 61% of pediatric glioblastomas using immunohisto-
proteins play an important role in the regulation of cell proliferation. Cell proliferation consists of a balance between expression of genes which stimulate proliferation of brain tumor cells by blocking specific cell cycle proteins or by enhancing the function of CDKIs. For example, we have recently shown that p21CIPl expression is diminished in several astrocytic tumor cell lines with wild-type p53 status [67].

Infection of the p53-deficient astrocytoma cell line, U373 MG with a replication-defective adenoviral vector containing p21WAF1/CIP1 led to G1 cell cycle arrest and inhibition of accumulation of cells with aneuploidy [70]. In addition, an alteration in the malignant phenotype of cells was evidenced by the loss of anchorage-independent growth in soft agar and the failure to induce tumorigenesis in both peripheral and intracranial xenograft models [70]. Another group has shown that infection of a pCCL-based retroviral vector carrying p21CIPl in rat glioblastoma cells showed a 91% reduction in colony-forming efficiency and a 66% reduction in growth rate [71]. In addition, intracranial implantation of these infected cells showed complete disappearance of the p21CIPl-infected cells by day 10 and long-term survival compared to controls [71]. Interestingly, the ectopic expression of cyclin D1 in asynchronously growing cells (human glioma and rodent fibroblast cell lines) was accompanied by increased levels of the p53 protein and p21CIPl. Despite the induction of these cell cycle-inhibitory proteins, cyclin D1-associated CDK kinase remained activated and the cells grew essentially like that of the parental cell lines [72].

Other CDK Inhibitors

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Studies in Transgenic Mice

Recently, molecular techniques have advanced remarkably so that the effect of deregulated overexpression or the absence of expression of a gene can be studied in the whole organism, such as a transgenic mouse. A number of important tumor-suppressor genes have been 'knocked out' in mice, and the phenotype of the mice has supported the candidate gene's tumor-suppressive function. E1a-eyzogenous knockout of p53 leads to spontaneous development of many different tumors, although none in the brain [90]. Double RB and p53 knockout mice develop endocrine tumors, including pituitary tumors and pene-

blastosomas [90, 91]. p66NKK4A transgenic mice are susceptible to multiple tumors, although none in the brain [92]. p21CIPl knockout has very little phenotypic effect without predisposition to tumor formation [93]. Homozygous p21CIPl knockout results in a dramatic phenotype, with much larger sized mice with multiorgan hyperplasia and spontaneous development of intermediate lobe tumors of the pituitary gland [94–96]. p57KIP2 transgenic mice have delayed growth and genes which inhibit growth, such as Rb or p53 or CDKIs. We may now be in a position to design new biotherapeutic strategies to reduce the proliferation of brain tumor cells by blocking specific cell cycle proteins or by enhancing the function of CDKIs. For example, we have recently shown that transfection of U343 astrocytoma cells with a p66 retrovirus in addition to treatment with retinoic acid leads to signals which cause full differentiation of these malignant astrocytoma cells [99]. In the future, one can envision applying such combination therapy with cell cycle inhibitors and differentiation agents to
patients with malignant astrocytomas. Finally, with future advancements in targeting cells with gene therapy and with advances in molecular diagnosis, we believe that much of our knowledge about the cell cycle will be translated into new and effective therapies for children harboring intracranial neoplasms.

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References


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