Decreased cyclin B1 expression contributes to G₂ delay in human brain tumor cells after treatment with camptothecin¹

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DNA damage produces delayed mitosis (G₂/M delay) in proliferating cells, and shortening the delay sensitizes human malignant glioma and medulloblastoma cells to cytotoxic chemotherapy. Although activation of the cyclin-dependent kinase CDC2 mediates G₂/M transition in all tumor cells studied to date, regulation of CDC2 varies between tumor types. Persistent hyperphosphorylation of kinase and reduced cyclin expression have been implicated as mediators of treatment-induced G₂ delay in different tumor models. To evaluate regulation of G₂/M transition in human brain tumors, we studied the expression and/or activity of CDC2 kinase and cyclins A and B1 in U-251 MG and DAOY medulloblastoma cells after their treatment with camptothecin (CPT). Synchronized cells were treated during S phase, then harvested at predetermined intervals for evaluation of cell cycle kinetics, kinase activity mRNA, and protein expression. CPT produced G₂ delay associated with decreased CDC2 kinase activity and cyclin B1 expression. Kinase activity was associated with CDC2 bound to cyclin B1, not cyclin A, in both cell lines.

Cyclin A mRNA and protein expression were reduced after CPT treatment; however, decreased protein expression was short lived and moderate in the glioma and primitive neuroectodermal tumor/medulloblastoma cells, respectively. We conclude that G₂ delay is a common response of brain tumor cells to chemotherapy with topoisomerase I inhibitors and that a mechanism of this delay may be reduced expression of cyclin B1. *Neuro-Oncology* 3, 11–21, 2001 (Posted to *Neuro-Oncology* [serial online], Doc. 00-025, November 8, 2000. URL <neuro-oncology.mc.duke.edu>)

Signals that regulate transition between cell cycle phases in proliferating cells represent potential targets for new cancer treatment strategies because they modulate cell responses to DNA injury as well as progression through the cell cycle (Johnson and Walker, 1999). Inhibition of cellular division by delaying the transition from the second gap phase of the cell cycle (G₂) to mitosis (M) is an important cellular response to DNA-damaging agents (Lock and Ross, 1990; McKenna et al., 1991; O'Connor et al., 1993). G₂ delay may be necessary to complete DNA repair before damaged, potentially lethal replicons are conferred to the next generation of cells through mitosis. Cytotoxicity of radiation and chemotherapy is inversely associated with the duration of radiation- or chemotherapy-induced G₂ delay in human lymphoma (O'Connor and Kohn, 1992) and transformed rat embryo cells (McKenna et al., 1991). Furthermore, pharmacologic abrogation of radiation- or drug-induced G₂ delay with caffeine, staurosporine, or pentoxifylline increases cell death in HeLa cells (Bernhard et al., 1994), human lymphoma cells

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³Abbreviations used are as follows: CPT, camptothecin.

(O'Connor et al., 1993), and rat brain tumor cells 9-L and 9-L2 (Aida and Bodell, 1987). We recently published studies demonstrating the ability of caffeine and staurosporine to abbreviate chemotherapy-induced G₂ delay in U-251 MG cells and DAOY medulloblastoma, 2 well-characterized human brain tumor cell lines. Shortening of G₂ delay produced by the platinating agent, cisplatin, and the topoisomerase I inhibitor, CPT³ increased cytotoxicity and apoptosis (Janss et al., 1998b). However, doses of caffeine, staurosporine, and pentoxifylline, necessary to overcome G₂ delay in vitro, would be lethal in vivo (Nehlig et al., 1992; Stewart et al., 1997). Strategies that selectively alter signals for commitment to mitosis and increase the cytotoxicity of currently available DNA-damaging agents need to be identified; however, it appears that regulation of G₂ delay varies between tumor cell types.

G₂ to M transition in eukaryotic cells is regulated by the cyclin-dependent kinase, CDC2 (synonyms cdk1, p34^{CDC2}; Nurse, 1994; Ohi and Goud, 1999). Kinase activity of CDC2 requires activating phosphorylation at threonine 161 (Desai et al., 1995; Gould and Nurse, 1989) and complex formation with either cyclin A or cyclin B1 protein (Pagano et al., 1992). Inhibitory phosphorylations at threonine 14 and tyrosine 15 positions are imposed by Wee1Hu (human analog to yeast wee1 kinase) or Myt1 (membrane-associated and tyrosine/threonine-specific CDC2 inhibitory kinase 1) (Liu et al., 1997, 1999). At G₂/M transition, the inhibitory phosphorylations are removed from CDC2 by CDC25C phosphatase. CDC2 is inactivated as cyclin dissociates and is rapidly degraded during mitosis (Dunphy, 1994).

G₂ delay induced by treatment with DNA-damaging agents may be regulated by different mechanisms in various cell lines. In HeLa cells, decreased CDC2 kinase activity after radiation is mediated by reduced expression of cyclin B1 protein (Kao et al., 1997) due to shortened cyclin B1 mRNA half-life (Maity et al., 1995). In contrast, treatment-induced G₂ delay in human lymphoma, human leukemia, and Chinese hamster ovary cells results from persistent hyperphosphorylation, hence inactivity of CDC2 kinase (Lock, 1992; O'Connor et al., 1992; Shimizu et al., 1995). These mechanisms may be redundant or may differ between tumors. Thus, understanding regulation of G₂/M transition in brain tumor cells is necessary if strategies that exploit abrogation of G₂ delay are to be applied to increase efficacy of current therapy in neuro-oncology.

To evaluate regulation of chemotherapy-induced G₂ delay in human brain tumor cells, we used CPT to study the impact of DNA-damaging therapy on cell cycle kinetics, activity and phosphorylation of CDC2 kinase, and expression of mitotic cyclin A and cyclin B1 in U-251 MG and DAOY primitive neuroectodermal tumor/medulloblastoma cells. CPT was chosen because of its documented toxicity to both U-251 MG and DAOY cells (Janss et al., 1998a, 1998b), its ability to produce prolonged G₂ delay in these cells, and the observation that its cytotoxicity is increased by staurosporine, a pharmacological agent that shortens CPT-induced G₂ delay (Janss et al., 1998b). Use of 2 different brain tumor cell lines permitted identification of cell-specific

differences in G₂/M regulation and may enable selection of target (targets) with broad activity as adjuvant therapy. CPT produced prolonged G₂ delay associated with decreased CDC2 activity and cyclin B1 expression. Although cyclin A protein expression decreased after CPT treatment, the decrease was short lived and moderate in glioma and medulloblastoma cells, respectively. CDC2 existed in both inactive (hyperphosphorylated) and active (hypophosphorylated) forms throughout the cell cycle; shift of kinase to active form correlated with times of low cyclin expression rather than G₂/M transition. These data suggest that reduced cyclin B1 expression contributes to chemotherapy-induced G₂ delay in human brain tumors.

Materials and Methods

Cell Culture

Two human brain tumor cell lines were used: DAOY primitive neuroectodermal tumor/medulloblastoma and U-251 MG (Friedman et al., 1988). Both cell lines were grown in Richter's zinc option medium (GIBCO BRL, Rockville, Md.) with 10% fetal calf serum (Sigma, St. Louis, Mo.) and 50,000 units streptomycin and penicillin per liter of medium and were harvested as previously described (Friedman et al., 1988).

Synchronization

Subconfluent cultures were harvested and plated at 6×10^5 cells/100-mm culture dish in the medium described above. A thymidine-aphidicolin block similar to that described by Heinz et al. (1983) was used to synchronize the cells at the G₁/S boundary (Maity et al., 1996), then to release them from the G₁ block. Cells were harvested for flow cytometric evaluation and extraction of RNA or protein at predetermined times throughout the cell cycle after release from G₁ block. Additional plates were harvested for assessment of the toxicity of treatment in synchronized cells treated during S phase.

Treatment

After release from the aphidicolin block, cells were allowed to progress from G₁ to S phase, then treated with CPT (100 nM, 1-h exposure; SmithKline Beecham Pharmaceuticals, Philadelphia, Penn.) or serum-free medium control (1-h exposure). U-251 MG and DAOY cells were exposed to CPT or serum-free medium 4 h after their release from aphidicolin to maximize treatment of tumor cells in S phase. (Three hours after release from aphidicolin, 70% [SE 2.3%] of U-251 MG cells were in S phase and 18.5% [SE 2.3%] DAOY cells were in S phase. Four hours after release from aphidicolin, 71% [SE 3.0%] U-251 MG cells and 62% [SE 7.0%] DAOY cells were in S phase.) Cells were incubated with CPT-100 nM in serum-free medium for 1 h at 37°C then rinsed and incubated in medium with 20% fetal calf serum. The dose of CPT was chosen because it approximates the ID₅₀ (inhibitory dose resulting in 50% growth

suppression) for unsynchronized DAOY and U-251 MG cells (Janss et al., 1998a). Control treatment consisted of 1-h exposure of cells to serum-free medium.

Assay for Cytotoxicity in Synchronized Cells

Cells were harvested and plated (300 cells per 10-mm petri dish). Six replicate plates were prepared for each treatment group (control, CPT). After 7 days, cells were stained and colonies were counted. Fractional survival was calculated by dividing mean colony count for the 6 replicate plates treated with CPT (100 nM, 1-h exposure during S phase) by the mean colony count for 6 replicate control plates (cells treated with serum-free medium for 1 h during S phase).

Flow Cytometry

Cells were harvested and immediately frozen in liquid nitrogen in 1 ml of 40-mM citrate buffer and were stored at -70°C . At time of analysis, cells were thawed and stained with propidium iodide as described by Vindelov and Christensen (1990). Flow analysis was performed on the Becton-Dickinson FACScan flow cytometer, and the data were interpreted using the Cell-FIT cell cycle analysis program (Version 2.01.2; Becton Dickinson, Mansfield, Mass.). At least 10,000 cells were used for each sample.

Protein Extraction and CDC2 Kinase Activity

For each time point, more than 100,000 synchronized cells were lysed with 300 μl of buffer, then frozen at -70°C (Prasad et al., 1995). Protein concentration was determined using the BCATM protein assay kit (#23225; Pierce, Rockford, Ill.). Kinase activity was quantified with SignaTECTTM CDC2 Protein Kinase Assay System (Promega, Madison, Wis.) using (^{32}P)ATP 10 $\mu\text{Ci}/\mu\text{l}$. Details of the procedure can be found in the technical bulletin provided by the company. Briefly, reactions were performed with cell lysate (10 $\mu\text{g}/5 \mu\text{l}$ volume), positive control (CDC2 kinase 5 U/ μl , Cat. # V2891) or negative control (no substrate). Total reaction volume was 25 μl at 30°C for 10 min. Reaction was terminated using 100 μM olomoucine. Samples (15 μl) of each terminated reaction were spotted onto the SAM² Membrane, washed, dried, then separated and analyzed by measuring radioactivity in a scintillation counter.

Immunoprecipitation

To immunoprecipitate CDC2 bound to a specific cyclin, cell lysate (50 μg of protein) was added to 1 μg mouse antihuman cyclin B1 (Upstate Biotech, Lake Placid, N.Y.) or mouse antihuman cyclin A (Upstate Biotech) and lysis buffer to a final volume of 200 μl and incubated at 7°C . After 2 h, 20 ml 50% Protein A-Sepharose CL-4B (Amersham Corp., Arlington Heights, Ill.) was added. This mixture was washed with lysis buffer and centrifuged (1000 RPM, 2 min), and supernatant was stored at -70°C until CDC2 kinase activity could be determined using the assay described above.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted as previously described (Chirgwin et al., 1979). RNA was quantified by spectrophotometer at a wavelength of 260 nm. RNA (10 μg) was denatured with formalin and formamide, separated by electrophoresis on a 1% agarose gel, transferred to a Durulon membrane (Stratagene, La Jolla, Calif.), and cross-linked using a UV Stratalinker (Stratagene). Membranes were prehybridized with $5\times$ saline-sodium citrate, 100 mg denatured herring sperm DNA, $4\times$ Denhard's solution (2% bovine serum albumin, 2% Ficoll, and 2% polyvinylpyrrolidone), then hybridized using a cyclin B1, cyclin A, and rpL23 probe. Radioactive probes were made from a 1-kb fragment of human cyclin B1 cDNA, a 0.281-kb fragment of human cyclin A cDNA, and the 0.6-kb cDNA of the ribosomal protein rpL32, then labeled with (^{32}P)dCPT using the random primer method as previously described (Maity et al., 1995).

Immunoblotting for Cyclin B1, Cyclin A, and CDC2

For each time point, cells were lysed and protein was quantified as described above. Samples consisting of 50 μg protein were separated by electrophoresis on 10% sodium dodecyl sulfate polyacrylamide gels before transferring them to a nitrocellulose membrane (Bio-Rad, Hercules, Calif.). The membrane was probed with one of the following primary monoclonal antibodies: mouse antihuman cyclin B1 (dilution 1:1000; Upstate Biotech), mouse antihuman cyclin A (dilution 1:500; Upstate Biotech), or rabbit antihuman CDC2/cck1 (dilution 1:250; Calbiochem, San Diego, Calif.). Secondary antibody coupled to horseradish peroxidase (goat antimouse, or goat antirabbit; Amersham) was then used, and detection was performed by chemiluminescence (Amersham).

Optical Densitometry and Statistical Analysis

Durulon membranes containing mRNA were exposed with an intensifying screen using XAR-5 film at -70°C . Autoradiographs were digitized with a charge-coupled device camera, and densitometry was performed with NIH Image software (Version 1.55; National Institutes of Health, Bethesda, Md.). The relative cyclin mRNA level was calculated as the ratio of the intensity of cyclin B1 or cyclin A to the rpL23 band. Relative cyclin protein expression was quantified by subjecting exposed film to analysis using densitometry as per above. Relative cyclin protein expression per 50 μg protein was calculated as the ratio of the intensity of cyclin B1 or cyclin A to the intensity of peak expression of cyclin in samples not treated with CPT.

Results

Cytotoxicity of Camptothecin in U-251 MG and DAOY Cells

CPT treatment (100 nM, 1-h exposure during S phase) of synchronized DAOY and U-251 MG cells was compara-

ble, resulting in 6% and 9% clonogenic survival, respectively, relative to treatment with serum-free medium.

Cell Cycle Kinetics in U-251 MG and DAOY Cells in the Absence and Presence of CPT

Exposure to 100 nM CPT during S phase resulted in G₂ delay in both U-251 MG and DAOY cells. Fig. 1 illustrates flow cytometry profiles of brain tumor cells at various times after release from G₁ block. Four hours after release from G₁/S interface, 66% to 69% control and CPT-treated glioma cells were in S phase ($n = 4$). Twelve hours after release, 58% to 71% of control U-251 MG cells had cycled around into G₁, whereas only 7% to 18% of CPT-treated cells were in G₁ and 55% to 72% remained in G₂/M (for example, see Fig. 1A). There was a transient S phase delay after treatment with CPT. Note the persistence of S-phase cells 12 h, but not 16 h, after release from G₁ as illustrated in Fig. 1A. In control U-251 MG cells, the percentage of cells in G₂/M peaked at 8 to 12 h after release from G₁, then declined as cells cycled into G₁ phase. After CPT treatment, the percentage of U-251 MG cells in G₂/M peaked at 18 h because cells persisted in G₂ phase (Fig. 2A, top).

G₂ arrest was also seen in DAOY medulloblastoma cells after treatment with CPT. More than 12 h after release from G₁ block, 41% to 48% of cells treated with CPT were still in G₂/M phase, but only 8% to 13% of control cells remained in G₂/M, and 51 to 75% had cycled through to G₁ at that time (see Fig. 1B). There was a transient S-phase delay after treatment with CPT. Note the persistence of S-phase cells 9 h, but not 12 h, after release from G₁ as illustrated in Fig. 1B. The maximum percentage of untreated DAOY cells in G₂/M occurred 12 to 15 h after release from G₁ block. After CPT treatment, the peak percentage of DAOY cells in G₂/M was delayed to 18 h because cells persisted in G₂/M (Fig. 2B, top). Flow cytometry indicated that by 30 h after release from G₁, U-251 MG and DAOY cells treated with CPT had begun to cycle around into G₁.

CDC2 Kinase Activity in U-251 MG and DAOY Cells in the Absence and Presence of CPT

Peak CDC2 kinase activity in untreated U-251 MG and DAOY cells was 12 to 15 h and 12 h, respectively, coinciding with the peak percentage of cells in G₂/M phase and anticipating decline of cells in G₂/M phase as cells cycled around to G₁ phase (Fig. 2). The magnitude of peak kinase activity was consistent within cell type, but differed between tumor cell type; peak activity was 0.0337 to 0.0519 and 0.0585 to 0.0633 pmol ATP/min/μg protein in U-251 MG and DAOY cells, respectively. Treatment with 100 nM CPT during S phase decreased CDC2 kinase activity in both brain tumor cell lines, which correlated with delayed entry into mitosis. Immunoprecipitation of protein with monoclonal antibodies for cyclin A or cyclin B1 indicated that CDC2 kinase activity in both cell lines was associated with cyclin B1, not cyclin A (data not shown).

Hyperphosphorylated (inactive) and hypophosphorylated (active) forms of CDC2 were detected throughout

Fig. 1. Flow cytometric profiles of synchronized U-251 malignant glioma and DAOY medulloblastoma cells. Cells were synchronized and harvested at predetermined time points. A. Flow cytometric data for synchronized U-251 MG cells 0, 4, 9, and 12 h after release from G₁ block. Time of harvest after release from G₁ block is indicated at the top of each profile. The profile at the left lower corner is that of cells harvested at time of release from G₁ block (78% of cells remain in G₁). Regions corresponding to G₁, S, and G₂/M phases of the cell cycle are labeled in this profile and are comparable for the remaining 6 profiles. Cells were treated with camptothecin (CPT, bottom) or serum-free medium (Control, top). B. Flow cytometric data for synchronized DAOY cells 0, 5, 12, and 16 h after release from G₁ block. Time of harvest after release from G₁ block is indicated at the top of each profile. The profile at the left lower corner is that of cells harvested at time of release from G₁ block (68% of cells remain in G₁). Regions corresponding to G₁, S, and G₂/M phases of the cell cycle are labeled in this profile and are comparable for the remaining 6 profiles. Cells were treated with camptothecin (CPT, bottom) or serum-free medium (Control, top).

the cell cycle in both U-251 MG and DAOY cells (Fig. 2C). In treated control U-251 MG and DAOY cells, the shift to the hypophosphorylated form occurred at 15 and 20 h after release from G₁ block, respectively, and persisted at subsequent sampling times, corresponding to times of decreased cyclin A and cyclin B1 expression. Treatment with CPT (100 nM for 1 h during S phase) did not reduce total CDC2 expression in either U-251 MG or DAOY cells. Shift to the active, hypophosphorylated CDC2 was seen between 12 and 15 h after release from G₁ block despite persistence of G₂ delay in both cell lines.

Fig. 2. Decrease of CDC2 kinase activity in synchronized human brain tumor cells with and without CPT treatment. A. U-251 malignant glioma cell distribution through the cell cycle (top) and CDC2 kinase activity (bottom) as a function of time after release from G₁ block. In the top graph, the percentage of U-251 MG cells in G₂/M determined by flow cytometry is shown along the vertical axis; beneath, CDC2 kinase activity (in pmol ATP/min/mg protein) of U-251 MG cells harvested at the same time is shown along the vertical axis. The horizontal line indicates the time of treatment (Rx), either 100 nM CPT (□) or serum-free medium (■). B. DAOY medulloblastoma cell cycle kinetics (top) and CDC2 kinase activity (bottom). In the top graph, percentage of synchronized DAOY cells in G₂/M is shown along the vertical axis; beneath, CDC2 kinase activity (in pmol ATP/min/mg protein) of the same cell samples is shown along the vertical axis. Time of treatment, either 100 nM CPT (○) or serum-free medium (●), is indicated by Rx. C. Western blots depicting expression of CDC2 kinase in U-251 malignant glioma and DAOY medulloblastoma cells. Time of cell harvest, in h, after release from G₁ blockade is presented above each band. Treatment, either 100 nM CPT or serum-free medium for 1 h during S phase, is indicated above time of harvest.

Cyclin B1 Expression in U-251 MG and DAOY Cells in the Absence and Presence of CPT

Peak cyclin B1 mRNA expression in U-251 MG and DAOY cells occurred 15 to 20 h after release from G₁ block in the absence of DNA damaging therapy, whereas peak protein expression occurred 12 to 15 h after release from G₁ block in control cells (Figs. 3, 5, 6). Treatment with CPT reduced cyclin B1 mRNA expression to levels below baseline, and this low level persisted at least 25 h

after removal of CPT (30 h after release from G₁ block; Fig. 3). Peak cyclin B1 protein expression was reduced more than 50% by brief exposure to CPT in both U-251 MG and DAOY cells and remained below peak levels for up to 30 h after release from G₁ block (Fig. 3). In U-251 MG cells, CPT treatment reduced cyclin B1 protein expression (mean 46% of peak level in untreated cells, range 33%-60%). In DAOY cells, CPT treatment reduced cyclin B1 protein expression (mean 51% of peak level in untreated cells, range 32%-70%).

Fig. 3. Cyclin B1 mRNA and protein expression in synchronized brain tumor cells with and without CPT treatment. A. Distribution of U-251 MG cells through the cell cycle (top), expression of cyclin B1 mRNA (middle), and expression of cyclin B1 protein (bottom) as a function of time after release from G₁ block. In the top graph, percentage U-251 MG cells in G₂/M is shown along the ordinate; beneath, the fractional expression of cyclin B1 mRNA and protein from samples harvested at corresponding intervals are presented on the ordinate. These are graphic presentations of data in Figs. 5A and 6A. Cyclin protein expression was normalized to peak cyclin expression in control treated cells. The horizontal line indicates the time of treatment (Rx), either 100 nM CPT (□) or serum-free medium (■). B. Distribution of DAOY cells through the cell cycle (top), expression of cyclin B1 mRNA (middle), and expression of cyclin B1 protein (bottom) as a function of time after release from G₁ block. In the top graph, percentage of DAOY cells in G₂/M is shown along the ordinate; beneath, the fractional expression of cyclin B1 mRNA and protein from samples harvested at corresponding intervals are presented on the ordinate. The horizontal line indicates the time of treatment (Rx), either 100 nM CPT (○) or serum-free medium (●). These are graphic presentations of data in Figs. 5B and 6B.

Cyclin A Expression in U-251 MG and DAOY Cells in the Absence and Presence of CPT

In the absence of CPT treatment, cyclin A mRNA expression peaked in U-251 MG and DAOY cells 12 and 20 h after release from G₁ block, respectively (Figs. 4-6). Similarly, protein expression peaked 12 to 15 h after release from G₁ block in the control U-251 MG and DAOY cells (Fig. 4). Treatment with CPT reduced cyclin A mRNA expression to baseline levels in U-251 MG and DAOY cells (Fig. 4). Expression of cyclin A mRNA remained low at least 25 h after removal of CPT

(30 h after release from G₁ block). Cyclin A protein expression was decreased by CPT treatment in both cell lines, but the pattern of decrease differed. Three hours after removal of CPT (8 h after release from G₁ block), cyclin A protein expression in U-251 MG cells was 56% to 76% of that in untreated cells and remained low until 15 h after release from G₁ block, when expression surpassed that of untreated cells (Fig. 4A). Cyclin A protein expression in DAOY cells was not depressed 3 h after removal of CPT and was minimally decreased 4 and 7 h later (12 and 15 h after release from G₁ block; Fig. 4B).

Fig. 4. Cyclin A mRNA and protein expression in synchronized brain tumor cells with and without CPT treatment. A. Distribution of U-251 MG cells through the cell cycle (top), expression of cyclin A mRNA (middle), and expression of cyclin A protein (bottom) as a function of time after release from G₁ block. In the top graph, percentage of U-251 MG cells in G₂/M is shown along the ordinate; beneath, the fractional expression of cyclin A mRNA and protein from samples harvested at the same time are presented on the ordinate. These are graphic presentations of data in Figs. 5A and 6A. The horizontal line indicates the time of treatment (Rx), either 100 nM CPT (□) or serum-free medium (■). B. Distribution of DAOY cells through the cell cycle (top), expression of cyclin A mRNA (middle), and expression of cyclin A protein (bottom) as a function of time after release from G₁ block. In the top graph, percentage of DAOY cells in G₂/M is shown along the ordinate; beneath, the fractional expression of cyclin B1 and cyclin A mRNA from samples harvested at the same time are presented on the ordinate. These are graphic presentations of data in Figs. 5B and 6B. The horizontal line indicates the time of treatment (Rx), either 100 nM CPT (○) or serum-free medium (●).

Discussion

We recently reported that human malignant glioma and medulloblastoma cells exhibit increased sensitivity to cytotoxic agents when treatment-induced G₂ delay is abrogated (Janss et al., 1998b). High rates of treatment failure observed for patients with malignant gliomas and medulloblastomas (Packer et al., 1994; Walker and Strike, 1976) justify efforts to identify novel therapeutic strategies, including those that target G₂/M transition. In this report, we show that G₂ delay is a common response for U-251 MG glioma and DAOY medulloblastoma cells after treatment with DNA-injuring agent CPT and that the delay is associated with reduced CDC2 kinase activ-

ity. Reduction in CDC2 kinase activity in brain tumor cell lines did not correlate with a large shift of CDC2 to the hyperphosphorylated (inactive) form but rather with reduced expression of cyclin B1 mRNA and protein expression. Decrements in cyclin A protein expression that were associated with CPT-induced G₂ delay were transient or moderate in both glioma and medulloblastoma cells. Together, these findings suggest that cyclin B1 expression may be a rate-limiting mediator of treatment-induced G₂ delay in human brain tumor cells.

This is the first report of decreased cyclin B1 mRNA and protein expression associated with chemotherapy-induced G₂ delay in human brain tumor cell lines. Regulation of G₂ delay in U-251 malignant glioma cells and

A U-251 MG cyclin mRNA expression

B DAOY cyclin mRNA expression

Fig. 5. Northern blot depicting expression of cyclin B1, cyclin A, and rpl32 (loading control) in synchronized brain tumor cells with and without CPT treatment. A. U-251 malignant glioma cells. Time of cell harvest, in hours, after release from G₁ blockade, is presented above each band; and treatment, either 100 nM CPT or control (serum-free medium) for 1 h, during S phase is indicated above time of harvest. B. DAOY medulloblastoma cells.

DAOY medulloblastoma cells approximates that of HeLa cells rather than that of human lymphoma or leukemia cells. In synchronized HeLa cells, CPT and radiation-induced G₂ delay was associated with decreased cyclin B1 mRNA and protein expression (Maity et al., 1996; Muschel et al., 1991). However, in HeLa cells, decreased cyclin B1 protein expression associated with CPT treatment was transient (Maity et al., 1996), whereas in brain tumor cells the decrease was prolonged. Tsao et al. (1992) documented both decreased cyclin B1 protein expression and CDC2 kinase activity in synchronized HeLa cells treated with CPT. The importance of cyclin B1 in treatment-induced G₂ delay was supported by Kao et al. (1997), who reported that overexpression of cyclin B1 in HeLa cells abbreviated the radiation G₂ delay, whereas depression of cyclin B1 expression using antisense oligonucleotides augmented the duration of G₂.

In both U-251 MG and DAOY cells, treatment-induced G₂ delay was associated with decreased CDC2 kinase activity. In some cell lines, the decrease is associated with

persistent phosphorylation of CDC2 (Lock, 1992; O'Connor et al., 1992; Shimizu et al., 1995), whereas in others it is associated with decreased cyclin expression (Kao et al., 1997; Maity et al., 1995; Maity et al., 1996). Because both hyperphosphorylated (inactive) and hypophosphorylated (active) forms of CDC2 appeared throughout the cell cycle in synchronized brain tumor cells regardless of treatment, it is difficult to implicate persistent phosphorylation of CDC2 as the sole mediator of G₂ delay in U-251 MG and DAOY cells. Incomplete synchronization of brain tumor cells may have contributed to distribution of CDC2 in different phosphorylation states throughout the cell cycle. However, it does not account for the persistence and even predominance of hypophosphorylated CDC2 during treatment-induced G₂ delay.

Topoisomerase-I inhibitors are a class of anticancer agents that bind to DNA-topoisomerase-I complexes, stabilize single-strand DNA nicks, and facilitate double-strand breakage upon collision with the replication fork (Ryan et al., 1991), thereby interfering with DNA synthesis and cell proliferation. Efficacy of topoisomerase-I

A U-251 MG cyclin protein expression

B DAOY cyclin protein expression

Fig. 6. Western blots depicting expression of cyclin B1 and cyclin A in synchronized brain tumor cells with and without CPT treatment. A. U-251 malignant glioma cells. Time of cell harvest, in hours, after release from G₁ blockade is presented above each band. Treatment, either 100 nM CPT or serum-free medium for 1 h, during S phase is indicated above time of harvest. B. DAOY medulloblastoma cells.

inhibitors in treatment of brain tumors has been documented in *in vivo* and *in vitro* studies and in clinical trials (Slichenmyer et al., 1993). CPT, the prototypical topoisomerase-I inhibitor, was used to identify molecular events that contribute to G₂ delay in brain tumor cells because previous work had documented its cytotoxicity and ability to induce prolonged G₂ delay in U-251 MG and DAOY cells (Janss et al., 1998a, 1998b).

Shortening CPT-induced cell cycle delay in DAOY cells by coadministration of staurosporine, a protein kinase C inhibitor, increased cytotoxicity and apoptotic cell death (Janss et al., 1998a). This is consistent with the report of Shao et al. (1997) that the staurosporine analog, UCN-01, which abrogates CPT-induced cell cycle delay, augments the cytotoxicity of CPT in human colon and breast carcinoma cell lines. Interestingly, they observed that S-phase delay and G₂ arrest of HT29 colon carcinoma cells after treatment with CPT were associated with increased expression of cyclin A and no alteration of cyclin B1 16 h after removal of CPT. Cyclin A binds to cyclin-dependent kinase 2 (Cdk2) and facilitates S-phase progression, and binds with CDC2 to facilitate G₂/M transition (Desai et al., 1995; Pagano et al., 1992). The authors postulated that accumulation of cyclin A after treatment with CPT reflects the drug's interference with S phase after recruitment of cyclin A-Cdk2 complex.

Whereas synchronized malignant glioma and medulloblastoma cells, like HT29 cells, exhibited S-phase delay and G₂ arrest when exposed to CPT, cyclin A and cyclin B1 expression decreased at 16 h after removal of CPT. Discrepancies between our findings and those of Shao et al. (1997) may be due to use of different cell lines and/or technical differences (that is, treatment duration of 8 h in unsynchronized cells versus 1 h during S phase in synchronized cells). It is unlikely that the decreased cyclin B1 mRNA expression observed in malignant glioma and medulloblastoma cell lines after treatment with 100 nM CPT was due to a nonspecific effect of CPT on transcription. Although CPT is known to inhibit transcription at doses much higher than used in this study, this effect is dose dependent and reversible within 30 min following removal of the drug (Horwitz et al., 1971; Kessel, 1971). Furthermore, Maity et al. (1996) demonstrated that decreased cyclin B1 mRNA expression in synchronized HeLa cells after treatment with CPT (500 nM) is not due to generalized down-regulation of transcription.

Unsynchronized U-251 malignant glioma cells are relatively resistant to the cytotoxic effects of CPT compared with DAOY medulloblastoma cells (Janss et al., 1998a). As discussed in the introduction, some authors postulate that the duration of treatment-induced G₂ delay correlates with treatment resistance. The data presented here

do not address whether duration of CPT-induced G₂ delay contributes to relative resistance in human brain tumor cells, because both synchronized U-251 MG and DAOY cells exhibited prolonged G₂ delay and decreased cyclin B1 expression after exposure to CPT. U-251 MG cells may employ strategies of resistance to drug treatment not related to G₂ delay, or use of synchronized cells may have obscured the small difference in the relative toxicity of CPT in U-251 MG and DAOY cells.

U-251 MG and DAOY cell lines used in this study have a mutated *P53* oncogene (Bigner et al., 1989; He et al., 1991). Agents that abrogate G₂ delay induced by DNA injury, such as staurosporine or UCN-01, increase cytotoxicity more effectively in cells without intact *P53* (Bredel et al., 1999; Shao et al., 1997; Wang et al., 1996). This is likely due to the importance of intact *P53* in blocking progression through G₁ after DNA damage (Cox and Lane, 1995). Cells that progress through G₁ despite DNA injury rely heavily on S-phase and G₂-phase regulation to delay commitment to mitosis before repair can be completed. Failure to complete the repairs leads to accumulation of DNA damage and decreased cell viability. Thus, strategies that target regulation of G₂/M progression to increase the efficacy of cancer treatments are likely to be effective in tumors with impaired regulation of G₁, such as those having a mutated *P53*. Such strategies may include alteration of CDC2 activity by altering cyclin B1 expression (Innocente et al., 1999; Maity et al., 1995;

Toyoshima et al., 1998), recruitment of recently identified regulatory kinases (Matsuoka et al., 1998; Peng et al., 1997), and/or suppression of transport proteins (Hermeking et al., 1997; Toyoshima et al., 1998). We would expect such strategies to have significant impact in neuro-oncology because mutations of the *P53* gene are common in primary malignant brain tumors (Fults et al., 1992; Howng et al., 1996; Wu et al., 1993) and secondary malignancies of the CNS (Gafanovich et al., 1999). Although *P53* mutations are rare in pediatric brain tumors, including medulloblastomas (Biegel et al., 1992; James et al., 1990), abnormalities of G₁ regulatory proteins other than *P53* have been identified in brain tumors (He et al., 1995), and shortening treatment-induced G₂ delay produces modest increases in cytotoxicity in cells with intact *P53* (Shao et al., 1997).

The findings of this report indicated that G₂/M transition checkpoints, and specifically cyclin B1, represent promising targets for novel strategies to augment the cytotoxic effects of chemotherapy in human medulloblastomas and malignant gliomas.

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