Role of the Cell Cycle in the Pathobiology of Central Nervous System Trauma

Ibolja Cernak
Bogdan Stoica
Kimberly R. Byrnes
Simone Di Giovanni
Alan I. Faden*

Laboratory for the Study of CNS Injury; Department of Neuroscience; Georgetown University Medical Center; Washington D.C. USA

*Correspondence to: Alan I. Faden; Department of Neuroscience; Georgetown University Medical Center; 3970 Reservoir Road, NW; Washington D.C. 20057 USA; Tel.: 202.687.0492; Fax: 202.687.4143; Email: faden@georgetown.edu

Received 06/12/05; Accepted 06/15/05

This manuscript has been published online, prior to printing for Cell Cycle, Volume 4, Issue 9. Definitive page numbers have not been assigned. The current citation is: Cell Cycle 2005; 4(9). http://www.landesbioscience.com/journals/cc/abstract.php?id=1996

Once the issue is complete and page numbers have been assigned, the citation will change accordingly.

KEY WORDS

cell cycle, apoptosis, neuronal injury, traumatic brain injury, astrocyte proliferation, microglial proliferation, neuroprotection, flavopiridol

ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health (NIH) Grant 36537-03 and Contract NIH-NINDS-01 (NS-1-2339) (to A.I.F.) and NIH Grant HD4-0677.

INTRODUCTION

Traumatic brain injury (TBI) is a major cause of morbidity and mortality in humans, particularly young adults.1 It is well established that much of tissue damage and related neurological deficits induced by TBI result from delayed biochemical mechanisms.2 These secondary injury processes contribute to apoptotic neuronal cell death, and stimulate astrocyte and microglial proliferation, which collectively contribute to post-traumatic neurological dysfunction. Neurons are post-mitotic cells that usually do not engage in cell cycle progression once they differentiate; therefore, cell cycle proteins are generally down-regulated in these cells.3 However, certain stimuli such as trauma can cause mature neurons to reenter the cell cycle, leading to apoptosis rather than proliferation.4,5 Progression through the cell cycle is a complex process regulated by a variety of proteins6 including cyclins7 and cyclin-activated kinases (CDKs),8,9 as well as inhibitors of cyclin-dependent kinases (CDKIs)10 such as p21 and p27.11

Astrocytes are important for maintaining normal brain physiology during development and in adulthood, but they also can also serve a pathological role in response to various insults.12 Swelling and/or hypertrophy/hyperplasia of astrocytes (astrogliosis), as well as proliferation (astrocytosis) have been described in numerous acute and chronic neurodegenerative diseases including traumatic brain injury,13 ischemia/hypoxia,14 Alzheimer disease,15 and aging.16 Among others. The glial scar resulting from astrogliosis and astrocytosis may limit regeneration in the CNS after injury17 and contribute to post-traumatic epilepsy.18 Additionally, reactive brain microglia also play important roles in brain injury, CNS inflammation and neurodegenerative disease.19 Under the influence of neurotoxic stimuli, the resting microglia transforms into the activated form20,21 producing factors that are potentially detrimental to neurons, such as monocyte chemoattractant protein-1 (MCP-1),22 osteopontin,23 major histocompatibility complex (MHC) class I and class II molecules,24,25 and complement component C1q,26,27 among others.

A causal relationship has been suggested between cell cycle proteins and accumulation of proliferating microglia and astrocytes activated by CNS injury.28,29 Thus, parallel expression of cyclin D1 and microglial-specific CR3 complement receptor beta-subunit

ABSTRACT

Upregulation of cell cycle proteins occurs in both mitotic and post-mitotic neural cells after central nervous system (CNS) injury in adult animals. In mitotic cells, such as astroglia and microglia, they induce proliferation, whereas in post-mitotic cells such as neurons they initiate caspase-related apoptosis. We recently reported that early central administration of the cell cycle inhibitor flavopiridol after experimental traumatic brain injury (TBI) significantly reduced lesion volume, scar formation and neuronal cell death, while promoting near complete behavioral recovery. Here we show that in primary neuronal or astrocyte cultures structurally different cell cycle inhibitors (flavopiridol, roscovitine, and olomoucine) significantly reduce upregulation of cell cycle proteins, attenuate neuronal cell death induced by etoposide, and decrease astrocyte proliferation. Flavopiridol, in a concentration dependent manner, also attenuates proliferation/activation of microglia. In addition, we demonstrate that central administration of flavopiridol improves functional outcome in dose-dependent manner after fluid percussion induced brain injury in rats. Moreover, delayed systemic administration of flavopiridol significantly reduces brain lesion volume and edema development after TBI. These data provide further support for the therapeutic potential of cell cycle inhibitors for the treatment of clinical CNS injury and that protective mechanisms likely include reduction of neuronal cell death, inhibition of glial proliferation and attenuation of microglial activation.
The supernatants form dissociated cortices. Cultures of rat brain astrocytes were prepared by aspiration. An MTS assay (Cell Titer 96 Aqueous One Solution) was measured by LDH release assay using CytoTox-96 cells and cells that did not receive LPS. The viability and cell proliferation. Control cell samples included non-treated and 50 M etoposide for 24 h and the extent of cell death was evaluated by detection of LDH release into the medium. LDH values were normalized to control (100%) and represented as mean ± SD. The data were analyzed by one-way ANOVA followed by Tukey post hoc test. **p < 0.01 and ***p < 0.001 compared to etoposide-treated cell cultures.

Figure 1. Inhibitors of cyclin-dependent kinases attenuate etoposide-induced cell death in primary rat cortical neurons (n = 6 per condition) as evidenced by reduction in lactate-dehydrogenase (LDH) release. Cortical neurons were pretreated with various concentrations of flavopiridol, roscovitine, or olomoucine, exposed to 50 M etoposide for 24 h and the extent of cell death was evaluated by detection of LDH release into the medium. LDH values were normalized to control (100%) and represented as mean ± SD. The data were analyzed by one-way ANOVA followed by Tukey post hoc test. **p < 0.01 and ***p < 0.001 compared to etoposide-treated cell cultures.

Figure 2. Inhibitors of the cyclin-dependent kinases flavopiridol and roscovitine reduce in dose-dependent manner the etoposide-induced caspase-3-like activity in primary rat cortical neurons. Cortical neurons were pretreated with various concentrations of flavopiridol or roscovitine, and exposed to 50 M etoposide for 24 h; caspase-3-like activity was measured using the fluorescence of Ac-DEVD-AMC substrate, which values were normalized to control and represented as mean ± SD. The data were analyzed by one-way ANOVA followed by Tukey post hoc test. **p < 0.01 and ***p < 0.001 compared to etoposide-treated cell cultures n = 6 per condition).

In vivo studies

Rat primary cortical neurons. The supernatants from dissociated cortices were combined and centrifuged through a 4% BSA layer. The cell pellet was resuspended in neuronal seeding medium (NSM) consisting of neurobasal medium (Life Technologies) and supplemented with 1.1% 100 antibiotics, 25 mM sodium-glutamate, 0.5 mM L-glutamine, and 2% B27 Supplement (Life Technologies). Cells were seeded at a density of 2% B27 Supplement (Life Technologies). Cells were seeded at a density of 5 x 105 cells per ml onto poly(d)-lysine-coated (70–150 kDa) 96-well plates (Corning) or 60-mm Petri dishes (Falcon). All experiments were performed on cultures at seven days in vitro.

Rat primary astrocytes. Cultures of rat brain astrocytes were prepared according to standard procedure.32 Astrocytes were plated in a 96-well plate for proliferation assay. They were cultured in triplicate in the following conditions. All were stimulated with 10% FBS for 48 h, starved for 24 h in DMEM with no serum, and then pretreated 1 h with various concentrations of cell cycle inhibitors followed by 10% FBS mitotic stimulation for 48 h. Control cell samples included nontreated cells and cells treated with no serum. Cells were then harvested and used for MTS cell proliferation assay (Promega), or immunoblotting.

Rat primary microglia. Rat brain microglia were dissected, and put in culture according to standard procedures.33 Microglia were plated in a 96-well plate and cultured in triplicate for proliferation assay. Flavopiridol was added to microglia 1 hour before proliferation was induced with lipopolysaccharide (LPS; 0.01 µg/ml). Cells were then assayed for both viability and cell proliferation. Control cell samples included non-treated cells and cells that did not receive LPS.

Cell viability: was measured by LDH release assay using CytoTox-96 nonradioactive cytotoxicity assay kit (Promega, Madison, WI, USA) according to manufacturer’s protocol. Relative absorbance was measured at 490 nm using a Multiskan Ascent microplate reader (Labsystems Inc., Helsinki, Finland). We have previously shown that changes in LDH release accurately reflect neuronal cell death in this model, as shown using other markers such as trypan blue or ethidium homodimer.

Proliferation assay. An MTS assay (Cell Titer 96 Aqueous One Solution Cell Proliferation Assay, Promega) was employed to measure modulation of cell proliferation in astrocyte and microglia cultures.
In vivo studies

Rat lateral fluid percussion injury. Rats were anesthetized with sodium pentobarbital (60 mg/kg ip), intubated and mechanically ventilated. After achieving the surgical level of anesthesia, an arterial catheter (25-gauge) was surgically implanted into caudal artery for blood pressure monitoring. The animals were placed in a stereotaxic frame, the scalp and temporal muscles were surgically implanted into caudal artery for blood pressure monitoring. The animals were placed in a stereotaxic frame, the scalp and temporal muscles were reflected, and a small craniotomy (5 mm), located midway between the lambda and bregma sutures over the left parietal cortex, was made. A Leur-Loc adapter was then cemented in place. To induce a traumatic brain injury, a fluid percussion head injury device was used, which consists of a plexiglas cylindrical reservoir filled with isotonic saline; one end includes a transducer mounted and connected to a 5-mm tube that attaches to Leur-Loc adapter. A pendulum strikes a piston at the opposite end of the device, producing an approximately 22-millisecond pressure pulse, which causes a deformation of underlying brain. The degree of injury is related to the pressure pulse expressed in atmospheres - 2.5 atm in our laboratory produces a moderately severe injury with regard to neurologic and histologic deficits.35 Body (core) temperature and blood gas were monitored and maintained within normal physiological limits throughout the procedure. Brain temperature was assessed indirectly through a thermistor in the temporal muscle. The animals were kept to recover on a 37˚C heating pad under close observation for 4 h.

Nuclear magnetic resonance imaging. At designated time points, the animals were anesthetized using isoflurane (4% for induction and 1.5% for maintenance of anesthesia, in a 70% oxygen and 30% nitrous oxide mixture). Anesthetized animals were placed in the heated plexiglas holder and a respiratory motion detector positioned over the thorax to facilitate respiratory gating. The plexiglas holder was then placed in the center of the 7 Tesla magnet bore (Bruker Medical Inc., Billerica, MA, USA) where a 72 mm diameter 3H birdcage coil is positioned. Field homogeneity across the brain magnet bore (Bruker Medical Inc., Billerica, MA, USA) where a 72 mm diameter 3H birdcage coil is positioned. Field homogeneity across the brain magnetic field is optimized and a sagittal scout image is acquired (RARE [rapid acquisition with relaxation enhancement pulse sequence], field of vision, 4 x 4 cm; TR 1,500/10 milliseconds, slice thickness 2 mm and 4 echoes). A 256 x 256 matrix was used with a 4 cm field of view, TR 0.5 s, TE 52.7 msec, slice thickness of 2 mm and 4 echoes. The DWI images were converted to diffusion maps by applying the Stejskal-Tanner equation in association with a Marquart algorithm using the commercially available Paravision software (Bruker, Billerica, MA, USA) so as to highlight changes in MR signal associated with changes in molecular diffusion. Apparent diffusion coefficients (ADCs) were calculated and expressed as 10^-3 mm²/sec ± S.E.M.

Ex vivo studies

Immunoblotting. The protein concentration of the cell lysate was determined with the Bio-Rad protein assay reagent. A portion of the lysate (30–50 µg of protein) was then fractionated by SDS-PAGE, and the separated proteins were transferred to a nitrocellulose filter. The filter was stained with Ponceau S to confirm equal loading and transfer of samples and was then probed with specific antibodies. Rabbit polyclonal anti-rat cyclin D1 (Research Diagnostics, Cleveland; and Abcam, Cambridge, MA; diluted 1:1000) were used as primary antibodies. Immune complexes were detected with appropriate secondary antibodies and chemiluminescence reagents (Pierce). β-Actin (Sigma) and GAPDH (Cell Signaling Technology) were used as controls for gel loading and transfer.

Caspase activity assay. Assay for caspase-3-like activity was performed as previously described.38 Briefly, aliquots of cytosolic extracts (25 µg of protein in 100 µl of extraction buffer) were preincubated at 37°C for 30 min, and then mixed with equal volume of 40 µM fluorescent tetrapeptide substrate Ac-DEVD-AMC (Bachem, Torrance, CA) in the same buffer solution. Free and/or tetrapeptide AMC bond was measured continuously in each sample over 30 min in 96-well microtiter plates, using a CytoFluor II fluorometer (PerSeptive Biosystems, Framingham, MA) at 360 nm excitation and 460 nm emission wavelengths. Data were expressed as a percentage of the caspase activity in samples from sham-treated control animals.

RESULTS

Cell cycle inhibition reduces neuronal apoptosis and astrocyte proliferation. We compared the effects of three structurally different cell cycle inhibitors (flavopiridol, roscovitine, olomoucine), which modulate different components of cell cycle regulation, on neuronal cell death and astrocyte proliferation in primary neuronal or astrocyte cultures, respectively. Flavopiridol inhibits all CDKs including CDK9, reduces cyclin D1 mRNA transcription, and leads to cell cycle arrest in G1 or at the G1/S transition.39,40 Roscovitine is a purine analogue, which prevents activation of CDK2 and CDK5, and at higher concentrations may inhibit the activity of extracellular regulated kinase 1 (ERK1) kinase.41,42 Olomoucine (2-(2-hydroxyethylamino)-6-benzylamino-9-methylpurine) has been recently described as a competitive inhibitor (ATP-binding site) of the cell cycle, regulating p34cdc2/cyclin B, p33CDK2/cyclin A and p33CDK2/cyclin E kinases, the brain p33CDK5/p35 kinase and the ERK1/2 MAP-kinase.44 DNA damage and cell death were induced in cultures of rat primary cortices by addition of 50 µM etoposide;35 effects of cell cycle inhibitors on cell viability were measured by lactate dehydrogenase (LDH) release44 24 hours after treatment (Fig. 1). Although all three cell cycle inhibitors significantly increased cell viability, flavopiridol was considerably more potent than the other compounds (Fig. 1). Both flavopiridol and roscovitine also reduced the DNA-damage induced caspase-3-like activity (Fig. 2), and attenuated cyclin D1 protein expression (Fig. 3). Exposure to etoposide for more than 15 h led to cyclin D1 induction; pretreatment with 1 µM of flavopiridol or 50 µM of roscovitine significantly reduced the activation of cell cycle (Fig. 3).

Proliferation of rat brain astrocytes was induced with 24 hours of serum stimulation (10% FBS), and flavopiridol (100 nM, 1 or 10 µM), roscovitine (1, 10, or 100 µM), or olomoucine (1, 10, or 100 µM) were added to either serum-treated or nontreated (basal level) samples and left in media for 48 hours. The effects of treatments on cell proliferation after serum stimulation were estimated using a cell viability assay as previously described.54 Although all drugs significantly reduced astrocyte proliferation as a function of their increased concentrations, flavopiridol was the most effective, even at lower doses (Fig. 4). Indeed, cell number after addition of 10 µM flavopiridol...
was comparable to nonstimulated cells, demonstrating flavopiridol's ability to completely block astrocyte proliferation in this model (Fig. 4).

Inhibition of cell cycle reduces proliferation of microglia. We also tested whether cell cycle inhibition could attenuate proliferation of microglia. Proliferation of rat brain microglia was induced with lipopolysaccharide (LPS; 0.01 µg/ml), whereas various concentrations of flavopiridol were added to the microglia cultures one hour before the onset of proliferation. Flavopiridol reduced cell proliferation in a concentration-dependent manner and at low concentrations inhibited proliferation without inducing cellular toxicity (Fig. 5).

Cell cycle inhibitor reduces brain lesion in a dose-dependent manner. To examine dose-response relationships between cell cycle inhibition and reduction of brain lesion volume, we used lateral fluid percussion injury (LFP) in rats, a highly reproducible model of TBI that has been extensively characterized with regard to its biochemical, physiological, morphological, and behavioral correlates. Different concentrations of flavopiridol (100, 250, or 500 µmol/L dissolved in vehicle consisting of 10% DMSO in saline; 5 µL for 5 minutes) were administered intracerebroventricularly (icv) 30 minutes after injury; the volume of the lesion was measured using T2-weighted magnetic resonance imaging (MRI) at 21-day post-trauma. At a concentration of 500 µmol/L, flavopiridol appeared to be toxic (LD30/24 h), although previous studies using an even higher dosing regimen (500 µmol/L, 15 µL for 10 minutes) were reported to show significant neuroprotective effects in rats subjected to global brain ischemia. Flavopiridol at 250 µmol/L significantly reduced the trauma-induced brain lesion, whereas a concentration of 100 µmol/L of the compound produced more limited effects on brain lesion volume that did not reach statistical significance (Fig. 6).

Delayed and systemic administration of a cell cycle inhibitor is capable of reducing brain lesion. To address whether delayed administration of flavopiridol may be neuroprotective, which is a more clinically relevant paradigm, we compared single dose administration of 250 µmol/L flavopiridol icv at 30 min (results acquired using a smaller set of experimental animals has been previously shown) or 4 h post-trauma, and measured brain lesion volume 21 days after TBI. There was significant reduction of brain lesion volumes in injured animals receiving flavopiridol compared to vehicle (Fig. 6); this effect appeared somewhat smaller at 4h than with the earlier treatment. Additionally, we examined the effects of cell cycle inhibition when flavopiridol was administered at a later time point systemically; significant (p < 0.001) reduction in brain lesion volumes was found in animals subjected to LFP and treated with 5 mg/kg flavopiridol intraperitoneally 24 h post-injury (Fig. 7). Intraperitoneal injection of flavopiridol also prevented post-traumatic brain edema measured by diffusion-weighted MRI 24 hours following TBI (Fig. 8).

DISCUSSION
Our data further confirm that TBI induces cell cycle reentry of mitotic (astroglia, microglia) and non-mitotic (neurons) cells of the brain,
leading to caspase-dependent cell death in neurons as well as proliferation of astroglia and microglia. In this study, proliferation of microglia induced by LPS in vitro was inhibited by flavopiridol, a cell cycle inhibitor, in a dose-dependent manner. LPS, a gram-negative bacterial component, has been shown to upregulate cytokine and chemokine expression, increase production of reactive oxygen species, and intensify nitric oxide formation mediated by activation of transcription factors such as NFkB and p38 MAP. Expression of certain cytokines can enhance cyclin D1 expression and stimulate cell proliferation. They may also contribute to neuronal and/or oligodendroglial damage as well as inhibition of neurite outgrowth. Cytokine-dependent inflammation has been implicated in neurodegenerative disorders such as Alzheimer’s disease, amyotrophic lateral sclerosis, Parkinson’s disease and multiple sclerosis, whereas inhibiting these inflammatory processes may be neuroprotective against CNS injury. In contrast, it has been suggested that inflammation may improve functional recovery after CNS injury and that stimulated microglia can produce trophic factors, such as GDNF, which may enhance axonal growth. Our in vitro data show that flavopiridol at concentrations 0.1 and 1 μM inhibited microglial activation/proliferation without causing cell death.

Numerous pharmacological treatments have been examined for their ability to interrupt or inhibit specific factors implicated in secondary injury. However, despite encouraging experimental reports, clinical trials have failed to show significant neuroprotective effects. One of the more likely explanations for such failures is the multifactorial nature of post-traumatic injury mechanisms. TBI is a complex and heterogenous pathological entity that includes mechanical injury, ischemia, hemorrhage and hypoxia. Many factors appear to contribute to secondary tissue damage after trauma including proteases, lipases, neurotransmitters/neuromodulators, ion changes and inflammatory and immune factors, among others. Therefore, effective clinical strategies should probably include either combinations of selective inhibitors of secondary injury factors or use single drugs that modulate multiple injury components. Flavopiridol and other cell cycle inhibitors have pluripotential actions, which include reduction of injury-induced neuronal apoptosis, attenuation of microglial activation and associated inflammatory factors, and reduction of astroglial proliferation/glial scar formation. It has also been shown that flavopiridol prevents kainic acid-induced apoptosis in neurons and improves neuronal survival and functional outcome after global as well as focal ischemia in the rat. Here we show considerable neuroprotective actions of flavopiridol after systemic as well as central administration, with a therapeutic window of at least 24 hours. Phase I and II clinical trials, which used this drug as a potential anti-cancer agent confirmed that flavopiridol is nontoxic at doses required for cell cycle inhibition in animal models. Taken together, our findings suggest that this agent or other cell cycle inhibitors may be useful for the treatment of clinical head injury.

References
Figure 6. Effect of flavopiridol on lateral fluid percussion-induced lesion volume 21 days after injury in the rat. Data are from rats treated 30 min or 4 h after injury with flavopiridol (5 µL of 100 or 250 µmol/L solution for 5 minutes) or vehicle. (A) Lesion volumes (n = 12; mean ± SEM) and (B) representative T2-weighted magnetic resonance imaging of a vehicle or flavopiridol-treated rats. Regions of marked hyperintensity are evident in the cortex and hippocampus. ***p < 0.0001.
Figure 7. Effect of flavopiridol on lateral fluid percussion-induced lesion volume 21 days after injury in the rat. Data are from rats treated 24 h after injury with flavopiridol (5 mg/kg ip) or vehicle. (A) Lesion volumes (n=5; mean ± SEM) and (B) representative T2-weighted magnetic resonance imaging of a vehicle or flavopiridol-treated rats. Regions of marked hyperintensity are evident in the cortex and hippocampus. ***p < 0.0001.

Figure 8. Apparent diffusion coefficient (ADC) in ipsilateral cortex (ILCx), ipsilateral sub-cortex (ILSCx), contralateral cortex (CLCx) and contralateral sub-cortex of rats subjected to lateral fluid percussion injury and treated with flavopiridol (5 mg/kg) given intraperitoneally 24 h after injury. ADC was calculated on the basis of diffusion-weighted nuclear magnetic resonance image (MRI) at 48 h post-trauma. Diffusion-weighted MRI is a sensitive method for distinguishing between vasogenic and cytotoxic edema after traumatic brain injury, whereas ADC significantly correlates with the changes of extracellular water. Increased ADC indicates vasogenic edema, while reduced ADC suggests cytotoxic edema. Flavopiridol significantly (*p < 0.05) reduced the TBI-induced vasogenic edema compared to vehicle-treated injured animals.


