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Review

Cell cycle machinery and stroke

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Abstract

Stroke results from a transient or permanent reduction in blood flow to the brain. The mechanisms involving neuronal death following ischemic insult are complex and not fully understood. One signal which may control ischemic neuronal death is the inappropriate activation of cell cycle regulators including cyclins, cyclin dependent kinases (CDKs) and endogenous cyclin dependent kinase inhibitors (CDKIs). In dividing cells, activation of cell cycle machinery induces cell proliferation. In the context of terminally differentiated-neurons, however, aberrant activation of these elements triggers neuronal death. Indeed, there are several lines of correlative and functional evidence supporting this "cell cycle/neuronal death hypothesis". The objective of this review is to summarize the findings implicating cell cycle machinery in ischemic neuronal death from in vitro and in vivo studies. Importantly, determining and blocking the signaling pathway(s) by which these molecules act to mediate ischemic neuronal death, in conjunction with other targets may provide a viable therapeutic strategy for stroke damage.

Keywords: Stroke; CDKs; Cyclins; Cell cycle; Apoptosis

1. Introduction

Stroke occurs primarily as a result of a transient or permanent interruption of blood supply to the brain. This condition can stem from an occluded or ruptured blood vessels and in some cases cardiac arrest. Consequently, neurons in the affected brain region, or the whole brain in the case of a cardiac arrest, are deprived of oxygen and glucose. This sets in motion a cascade of cellular activities that ultimately culminate in neuronal cell death [1–3].

Presently, stroke is a leading cause of death and permanent disability in industrialized nations. Stroke occurs on average every 45 seconds in the USA (www.strokecenter.org). The American Heart Association (AHA) estimates the cost of treating stroke related injury and disability at $58 billion for 2006 alone (www.americanheart.org). Currently the treatment of stroke is mainly reliant on the use of thrombolytics such as tissue plasminogen activator (TPA), which themselves can pose an inherent risk of intracerebral hemorrhage. This limits the use of TPA to only certain cases of stroke. Furthermore, the efficacy of TPA depends on timely presentation of <3 h which excludes 95% of stroke patients [4,5]. Thus there is a need to develop new and efficacious neuroprotective strategies for the treatment of stroke.

The development of new strategies for stroke hinges on better understanding of the complex cellular and molecular interplay that ensue following stroke. A maelstrom of dyregulated molecules and potential perpetrators of ischemic neuronal death have been recently suggested. One of these exciting new developments involves the role of cell cycle molecules such as the cyclin dependent kinases. The notion that cell cycle machinery may mediate ischemic neuronal death is not unique to stroke. Indeed research evidence from numerous labs have demonstrated correlative relationship between the dysregulation of cell cycle machinery and neuronal death models of neurodegenerative diseases such as Parkinson's disease (PD) [6,7], Alzheimer's disease (AD) [8–10], amyotrophic lateral sclerosis (ALS) [11,12], and Niemann–Pick type C disease [13,14]. Whether or not cell cycle mechanism is similar in neurodegenerative diseases and acute conditions such stroke is unknown. This review will focus only on evidence implicating cell cycle molecules in ischemic neuronal death. We will also briefly discuss the potential involvement of other members of the CDK family not directly involved in cell cycle

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control, but only as potential activators of cell cycle machinery in stroke.

2. Ischemic neuronal death

The mechanism(s) of ischemic neuronal death is complex and may be determined by factors such as the location, severity and duration of insult. For example, neuronal death in the ischemic core, the region most severely affected by the lack of blood flow, occurs within minutes to a few hours and is marked predominantly by necrotic and excitotoxic cell death. Neuronal death in the ischemic penumbra, the region less severely affected by ischemia, is marked mainly by delayed apoptotic-like death that can progress over a period of days.

The precise sequence of events leading to neuronal death following stroke is at present not fully defined. However, a core picture is developing. Following the disruption of blood flow, the affected brain region(s) undergo a period of hypoxia resulting in a decrease in cellular ATP, due primarily to impaired mitochondrial oxidative respiration, a process that generates majority of the cellular energy required to maintain proper cell functions. The reduction of cellular energy results in the impairment of vital cellular functions such as the maintenance of the Na+/K+ pump, massive depolarization and excessive release of glutamate. Over activation of the glutamate channel particularly the *N*-methyl-D-aspartate receptor (NMDAR)-type channels mediates massive influx of Ca2+ resulting in cellular excitotoxicity and neuronal death. Increase in intracellular free calcium results in the activation of Ca2+ sensitive enzymes such as calpains proteases which then act to activate other molecules and cleave cellular structures. In addition to the events describe above other stressors such as oxidative stress, and DNA damage have been demonstrated to mediate ischemic neuronal death. Furthermore, extrinsic stressors such as the activation of glial cells and inflammation may impinge on ischemic neurons to mediate their demise. These later findings are particularly relevant when it comes to therapeutic interventions. In fact, cell cycle regulation may also play a critical role with these non-neuronal cell types in brain injury [15,16]. However, we will not mention this further in this review, but will instead focus on how the cell cycle machinery may impact neurons more directly.

3. Cell cycle regulation

Cell cycle is a highly regulated process. Timing progression of cell cycle through different phases, G0, G1 S, G2, and M requires an orchestrated functions of several elements, including cyclins, cyclin-dependent kinases (CDKs), retinoblastoma protein (Rb; pocket proteins) and E2F complex proteins [17]. Different complexes of cyclin-CDK drive each phase of cell cycle [18]. In this regard, the current model is that cyclin D–cdk4/6 and cyclin E–cdk2 complexes regulate G1/S progression, cyclin A–cdk2 complexes mediate S/G2 transitions, and cyclin B–cdc2 complexes mediate M-phase progression [19,20]. In addition, a recent report has suggested that cyclin C–cdk3 complexes also regulate G0/G1 transition [21] (Fig. 1). CDKs activity is regulated by binding to activating cyclin partners [22] as well as endogenous CDK inhibitors such as members of the INK4 (p15, p16, p18, p19) and Cip/Kip (p21, p27, p57) families [23,24]. Finally, phosphorylation also plays a critical role in CDK regulation. For example, cyclin H–cdk7–Mat1 (Cdk Activating Kinase; CAK complex) by phosphorylation of a central threonine [25] and cdc25 by dephosphorylation of thr14 (on cdc2) or tyr15 (on cdk2) modulate CDKs conformation to a fully active structure so they recognize their substrates more efficiently [23]. CDKs mediate cell cycle progression by phosphorylating downstream targets [26]. For example, an important target of G1 CDKs is the tumor

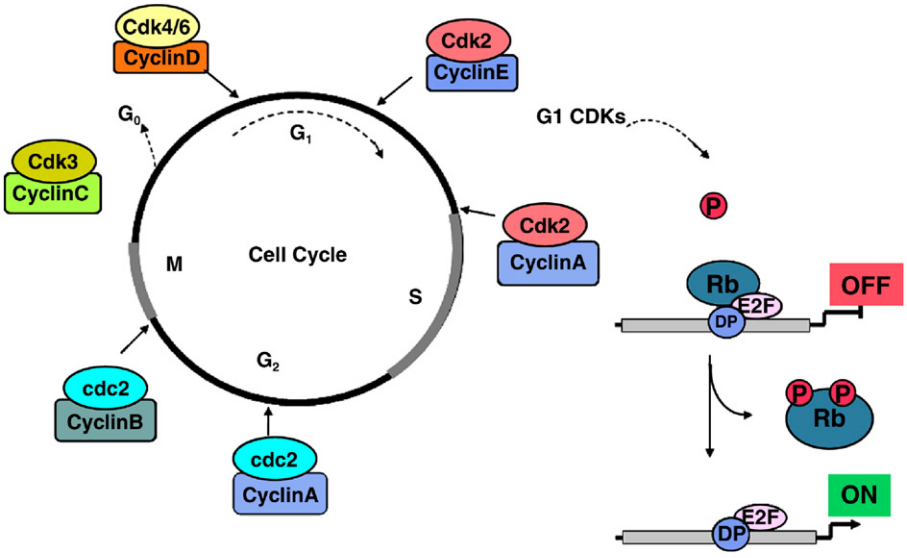

Fig. 1. Schematic representation of our current understanding of the mammalian cell cycle. The cell cycle is broadly divided into four phases culminating in cell duplication. Each phase of the cell cycle is regulated by different complement of cyclin dependent kinases together with a cognate cyclin. During the G1/S-phase, the downstream target, the retinoblastoma gene product. pRb is sequentially phosphorylated by the G1 CDKs resulting in the release of E2F transcription factor and DP. This results in the transcription of E2F responsive genes and those required for the progression thorough S-phase.

suppressor, Rb [27]. In mid- to late-G1, Rb is sequentially phosphorylated by cyclin D1–cdk4/6 and cyclin E–cdk2 complexes. Once hyperphosphorylated, Rb repression of E2F containing complexes is alleviated. E2F, along with a required binding partner DP activate genes required for S phase progression [28–30].

A growing body of exciting evidence indicates that CDKs may have a function beyond its canonical role in cell cycle regulation. Indeed, it has been suggested that neuronal death may be controlled by the inappropriate activation of some of cell cycle elements [31]. The hypothesis, compatible with the general cell cycle/neuronal death notion, is that activation of these regulatory elements, in the context of post-mitotic neurons, signals for death rather than cell cycle progression. Several lines of in vitro and in vivo evidence support this hypothesis that aberrant activation of cell cycle involves in different pathological conditions such as stroke [32–34] , AD [35], PD [6,7] , ALS [11].

In this review, we will first start our discussion with deregulation/upregulation of cell cycle regulators in in vitro paradigms of ischemic neuronal death and then will provide in vivo evidence suggesting that cell cycle might control death following ischemic neuronal injury in the adult animal. Finally, we will touch upon the role of other CDKs, in particular the neuronal cdk5 which has important roles in neuronal development and death and which might also link to cell cycle signals.

4. In vitro evidence for involvement of cell cycle elements in stroke

Generally, terminally differentiated cells such as neuron do not divide. Indeed as neurons undertake the process of terminal differentiation they irreversibly withdraw from the cell cycle. The levels and activity of key cell cycle regulators are downregulated in differentiated neurons [36,37]. For example, Cdk4, a regulator of G1/S transition becomes increasingly associated with the CDK inhibitor p27 and its activity declines in parts due to loss of phosphorylation by its activator CAK [36]. Similarly, the levels and activity of cdk2 another important regulator of the G1/S is downregulated in differentiated neurons. Consequently, the levels of hypophosphorylated Rb increases resulting in greater E2F1 sequestration [36]. Silencing E2F1 in neurons is particularly important since its overexpression promotes aberrant S-phase entry in neurons [38]. Furthermore, overexpression of E2F1 alone in the context of a post-mitotic neuron and in the absence of insult induces neuronal death [38,39]. Downregulation of cell cycle machinery and inhibition of E2F1 activity through its sequestration by Rb is thus an important feature of terminally differentiated neurons. This is underscored by observations that overexpression of Rb or the CDK inhibitor p27 is sufficient to induce neuronal differentiation [36]. In addition to downregulation of CDK levels and activity, observations by Sumrejkanchanakij et al. [40] suggests that the cell cycle may be held in check in post-mitotic neurons through cellular redistribution of cell cycle components. For example, in undifferentiated neurons cyclin D1 appears both cytoplasmic and nuclear but becomes predominantly cytoplasmic in differentiated neuroblastoma and cortical neurons [37,40]. Ectopic expression of cyclin D1 in differentiated neurons leads to cytoplasmic sequestration whereas forced nuclear expression by fusing to a nuclear localization signal induces apoptosis [37]. This suggests that cellular redistribution of cell cycle machinery is an important aspect in the maintenance of quiescent state in adult neurons. While the above evidence indicates what happens under normal developmental conditions, accumulating research evidence also suggests that cell cycle machinery are activated in adult neurons in response to numerous stressors such as DNA damage, NGF deprivation, proteosome inhibition and â-amyloid toxicity and models of ischemic neuronal death [39,41–47].

In regards to the latter type of injury, multiple lines of evidence in vitro suggest a role for cell cycle regulators as mediators of ischemic injury. Firstly, Katchanov and colleagues [42] reported lose of the CDK inhibitor p27 following oxygen glucose deprivation (OGD) of neocortical neurons. This group also reported increases in cyclin D1 protein levels and activation of cdk2 following OGD [42]. Similar increase in cyclin D1, cyclin E, and cdk2 is observed following kainic acid (KA) induced death in cerebellar granule neurons (CGNs) [48,49]. Secondly, Rb, a downstream target of cyclinD/Cdk4 is increasingly phosphorylated following hypoxia/reoxygenation and KA induced death [34,50] suggesting inactivation of Rb and activation of the cell cycle in these neurons. Additionally, increase in E2F1 (downstream effector of Cdk4) protein levels and proliferating cell nuclear antigen (PCNA) another S-phase marker (required for completion of DNA synthesis and repair) is observed following KA induced death [48,51]. Finally, increase in E2F1 mRNA transcript is also observed following OGD [52]. These observations discussed above seem to implicate the reactivation of cell cycle machinery in ischemic neuronal death and have lead to the hypothesis that inappropriate activation of the cell cycle in the context of differentiated neurons may underlie ischemic neuronal death. However, this evidence only showed the activation of cell cycle components and does not address the issue of whether this signal is required for death. In other words, does the activation of the cell cycle in adult neurons underlie neuronal death in stroke or are these observations simply an artifact of the cell death process? Studies using pharmacological inhibitors of cell cycle regulators seem to lend support to the former. For example, treatment of neurons with the CDK inhibitors olomoucine, roscovitine, 3-amino thioacridone (3-ATA) and flavopiridol has been shown to protect cortical neurons and CGNs from OGD and KA induced cell death [42,47,51,53]. While these pharmacological studies supports the involvement of cell cycle components in ischemic neuronal death, the caveat to these studies is the relatively non-specific nature of inhibitors such as flavopiridol which in addition to the cell cycle CDKs can potently inhibit non-mitotic CDKs such as cdk5 [54], cdk7 [25] as well as non-CDK-related kinases such as GSK-3β [55]. To address this, the functional relevance of cell cycle machinery in ischemic neuronal death was demonstrated by utilizing genetic manipulations of components of the cell cycle. Firstly,

Rashidian et al. [34] showed that adenoviral delivered kinase dead cdk4 protects CGNs from hypoxia mediated delayed death. Secondly, they showed that neurons derived from mice expressing kinase dead cdk4 or null for its regulator cyclin D1 are resistant to hypoxia mediated ischemic death [34]. Further support for the functional role of cell cycle pathway in ischemic neuronal death is provided by observations that cortical neurons and CGNs derived from E2F1 null mice are less susceptible to death mediated by OGD and KA induced death respectively [50,52]. Furthermore, E2F1 deficiency improves the recovering of CA1 neurons from loss of synaptic transmission following anoxic insult of hippocampal slices in vitro [52]. The above in vitro evidence is summarized in Tables 1 and 2.

Table 1
Correlative evidence for the involvement of cell cycle components in stroke models

Component	In vitro			In vivo		
	Model	Effect	References	Model	Effect	References
Cyclin D1	OGD	Increased level	[42]	MCAO	Increased level	[42,56–58]
				Focal cerebral ischemia	Increased level	[32]
				Global Ischemia	Increased level	[59–63]
				KA injection	Increased level	[61,64–66]
				Spinal cord ischemia	Increased level	[67]
				Cardiac arrest	Increased level	[86]
Cyclin E	KA	Increased level	[48]			
Cyclin A				MCAO	Increased level & nuclear localization	[70]
Cyclin H				Global ischemia	Increase level in resistant neurons	[87]
Cyclin G1				MCAO	Increased level	[71]
				Global ischemia	Increased level	[72]
				Spinal cord injury	Increased level	[92]
				CCAO and MCAO	Increased level & nuclear translocation	[93]
Cdk4				MCAO	Increased level	[68]
				Focal cerebral ischemia	Increased level	[32]
				Global Ischemia	Increased level	[63]
				KA injection	Increased level	[66]
				Spinal cord ischemia	Increased level	[67]
				Cardiac arrest	Increased level	[86]
Cdk2	OGD	Activated	[42]	MCAO	Increased activity	[42]
	KA	Increased level	[48]	Hypoxia–ischemia	Increased activity	[69]
				MCAO	Increased level & nuclear translocation	[70]
				Cardiac arrest	Increased level	[86]
p16				MCAO	Decreased level	[42]
				Hypoxia–ischemia	Decreased level	[69]
p21				MCAO and global ischemia	Increase level in surviving neurons	[71–73]
p27	OGD	Loss	[42]	Hypoxia–ischemia	Decreased level	[69]
Rb	Hypoxia	Phosphorylation	[34]	MCAO	Phosphorylation	[32,58,78]
	KA	Phosphorylation	[50]	Global ischemia	Phosphorylation	[33,34]
				Hypoxia–ischemia	Phosphorylation	[69]
				KA injection	Phosphorylation	[65]
E2F1	OGD	Increased mRNA	[52]	Focal cerebral ischemia	Increased level	[32]
	KA	Increased level	[48,51]	Global ischemia	Increased level	[81,82]
PCNA	KA	Increased level	[51]	MCAO	Increased level	[58,70]
				Global ischemia	Increased level	[63,84]
Brdu				MCAO	Incorporation in neurons	[58]
				Hypoxia–ischemia	Incorporation in neurons	[69]
Cdk5				MCAO	Increased level	[108]

Table 2
Functional evidence for the involvement of cell cycle components in stroke models

Component	In vitro			In vivo		
	Model	Method; effect	References	Model	Method; effect	References
Genetic studies						
Cyclin D1	Hypoxia	Null mutant neurons protective	[34]	KA injection	Antisense; protective	[66]
Cdk4	Hypoxia	Dominant negative expression; protective	[34]	MCAO	Synthetic inhibitor; protective	[58]
				KA injection	Antisense; protective	[66]
				Global ischemia	Dominant negative expression; protective	[34]
E2F1	OGD	Null neurons; protective	[52]	Focal cerebral ischemia	Null mice; resistant	[79,80]
	KA	Null neurons; protective	[50]			
	Anoxia	Null mutant hippocampal slices; protective	[52]			
Cdk5				Global ischemia	Dominant negative expression; protective	[34,109]
Pharmacological studies						
Flavopiridol	KA	Protective	[51]	Focal cerebral ischemia	Protective	[32]
	KA	Protective	[47]	Global ischemia	Protective	[33]
Olomucine	KA	Protective	[53]			
	OGD	Protective	[42]			
Roscovitine	KA	Partial protection	[53]			
3-ATA	KA	Protective	[47]			

Taken together these in vitro evidence strongly implicate the reactivation of cell cycle components in ischemic neuronal death. What evidence is there to implicate the involvement of cell cycle machinery in vivo? Are these observations relevant in intact physiological systems?

5. In vivo evidence for involvement of cell cycle elements in stroke

The above evidence indicates that neurons grown in culture can re-activate cell cycle signals upon an exogenous stress leading to death. This suggests that terminally differentiated, post mitotic neurons from embryonic or early post natal sources have retained their capacity to reactivate cell cycle CDK mediated signals. However, these neurons have only recently become post-mitotic and might represent a unique situation where the cell cycle machinery is still present. Indeed, reports have shown that while downregulated, multiple CDKs, cyclins as well as their regulators exist in cultured neurons [36,37]. Questions remain about the more relevant situation where ischemic injury occurs in fully matured adult neurons where there is likely very little if any cell cycle machinery present under basal conditions. Here too, there is accumulating evidence that upregulation/activation of cell cycle machinery occurs and this leads to death in neurons following stroke insult.

The first body of work linking cell cycle to ischemic injury in the adult context is the correlative data demonstrating that CDKs are changed following stroke injury in vivo. In these situations, a central player may again be the cdk4–cyclin D complex. For example, Cdk4/6 kinase activity/levels along with its activator cyclin D1 are upregulated in different models of ischemia in vivo. It has been shown that cyclin D1 is expressed in infarct region following MCAO model of focal ischemia [32,42,56–58] and in vulnerable regions of the hippocampus following transient global ischemia [59–63]. Consistent with these observations, increased levels of cyclin D1 has been demonstrated in response to an excitotoxic death induced by systemic injection of KA [61,64–66] and transient spinal cord ischemia [67]. Cdk4 itself is also changed during ischemic insult. Aberrant expression of cdk4 has been shown in MCAO [68] and clip models of focal ischemia [32]. Furthermore, other groups have reported induction of cdk4 expression in other models such as transient global ischemia [63], KA-mediated cortical death [66] and transient spinal cord ischemia [67].

Although the above evidence implicates cyclinD1–cdk4 complexes in ischemic injury, it must be kept in mind that other components of G1 phase might have potential roles in vivo as well. For instance, cdk6 is also activated by cyclin D [17]. In addition, there are some lines of data suggesting deregulation of cdk2 after MCAO and a hypoxic cerebral ischemia [42,69]. Consistent with this, Li et al. [70] provided evidence that cdk2, as well as its partner, cyclin A, are deregulated following focal ischemia.

In line with the upregulation/activation of cell cycle CDK complexes, evidence also point to a modulation of endogenous regulators of CDKs, the CDKIs. For example, Katchanov et al. [42] demonstrated that p16 is downregulated very early in dying neurons, followed by upregulation of cyclin D1 after a mild cerebral ischemia. Additional intriguing observations suggesting that CDKIs might have a functional role in ischemic death were provided by Van Lookeren et al. [71,72]. They indicated that p21 expression is enhanced in surviving neurons

surrounding dead area in both focal and transient forebrain ischemia. They suggested that elevation of p21 is actually a response to the ischemic insult to protect the neurons by arresting them in first steps of the cell cycle. In line with these findings, p21 mRNA was elevated in perifocal ischemia regions following focal ischemia, while no significant change was observed in ischemic area [73]. More importantly, Kuan et al. [69] demonstrated that p16 and p27 are depleted in dying hippocampal neurons and this may promote G1/S phase transition following hypoxia–ischemia insult.

The above mentioned in vivo evidence does not indicate whether these signals actually play a role in ischemic damage. To address this, several labs, including ours, have provided more direct data by carrying out additional functional studies. In support of a require role, inhibition of the cell cycle regulating proteins is protective in some of the ischemic models. For instance, administration of a synthetic cdk4 inhibitor, or alternatively, cyclin D1 and cdk4 antisense oligonucleotides has been shown to be neuroprotective in MCAO and KA-inducing excitotoxicity models, respectively [58,66]. We have recently shown that flavopiridol, a CDK inhibitor, administrated before or after the insult, blocks the delayed death evoked by focal or global ischemia [32,33]. Importantly, flavopiridol blocked activation of the cdk-Rb pathway, reduced E2F1 levels and improved behavioral performance [32,33]. More critically, we also investigated involvement of specific CDKs by expression of kinase dead dominant negative forms of several cell cycle CDKs, as well as cdk5, in delayed and excitotoxic types of ischemia. In this research for the first time we provided functional evidence that in adult system, cyclin D1–cdk4 complex has a key role in inducing delayed neuronal death [34]. We also showed that phosphorylation of Rb, mediated by this complex, occurs very early following global ischemia and that this activation is inhibited by DN cdk4 expression [34]. Expression of DN cdk4 also improves behavioral performance in rats suggesting that brains are not only neuroprotected, but that this leads to improved function [34]. This final point is particularly relevant to the goal of human therapy.

How do cyclin–CDK complexes mediate a potential ischemic death signal, in vivo? In other words, what is (are) the downstream target(s) for these complexes in adult systems? Recent findings suggest that inactivation of Rb leads to neuronal apoptosis, in vivo. Supporting this, overexpression of large T antigen in cerebellar Purkinje cells initially resulted in DNA synthesis but eventually caused neuronal degeneration in transgenic mice [74]. Rb deficiency is also embryonically lethal because of widespread apoptosis in CNS and PNS [75,76]. However, in this case, much of the death may be due to non-neuron autonomous events and not directly caused by Rb deficiency [77]. Observations in different in vivo models of ischemia have suggested that CDKs activity induced by apoptotic insults lead to phosphorylation and inactivation of Rb. For example, we have shown that Rb phosphorylation (on ser-795, a consensus site for CDKs) is an early that event happens a few hours after focal ischemia [32], global ischemia [33,34] and KA injection [65]. Moreover, other groups have also shown that Rb is progressively phosphorylated at CDKs consensus sites after focal ischemia induced by MCAO and hypoxic cerebral ischemia and that this might trigger apoptotic neuronal death pathways [58,69,78].

To investigate the mechanism by which Rb inactivation may promote death, E2F family proteins, as a group of proteins regulated by Rb, have been studied in vivo. In this regard, it has been suggested that E2F may play an important role in neuronal death in select conditions such as stroke. For example, E2F1 deficient mice has been shown to exhibit smaller brain injury and improved behavior after focal ischemia [79,80]. In agreement with this, upregulation of E2F1 has been implicated in animal models of ischemia. For instance, we have shown that focal ischemia induces E2F1 expression which occurs following Rb phosphorylation and administration of CDK inhibitor diminishes this effect and protects the neurons [32]. Microarray analysis also has also revealed that E2F1 is among the upregulated genes after global ischemia [81]. Finally, a very recent study demonstrated that E2F1 and its target gene, c-myb, is upregulated in hippocampus following global ischemia [82]. What factor(s) determine which responsive genes, whether cell cycle or apoptotic, be regulated? It is not clear yet, but there is an idea suggesting that there is a pool of transcriptional activity for E2F. When this activity gets to a specific threshold the genes regulating cell cycle progression are induced. But when this activity reaches a second higher threshold, apoptotic genes are induced and death mechanisms start [83]. Almost nothing is known about the targets of E2F1 in ischemic death, in vivo, and this area needs to be intensively investigated.

Although what has been discussed so far suggests that cell cycle is aborted mostly at G1/S checkpoint in ischemic death, some reports have shown S phase entry. Indeed, neurons start to replicate DNA before they die in some systems. To investigate whether DNA synthesis is induced in dying neurons, induction of PCNA and incorporation of Brdu have been used as markers for S phase. In this regard, induction of PCNA has been demonstrated in several models of focal and global ischemia [58,63,70,84]. In other reports, incorporation of Brdu in apoptotic neurons is indicative of tendency for DNA synthesis in these cells [58,69]. Interestingly, these injured, “S phase positive” neurons contained elevated G1 cyclin–CDK complexes and co-localized with apoptotic markers (TUNEL positive); suggesting that cell cycle is already triggered and they have transited to S phase to resume DNA synthesis but for some reasons they undergo apoptosis and do not replicate. Therefore, there is no in vivo evidence of G2 entry in ischemic neurons, to our knowledge. Nevertheless, expression of cdc2, as E2F target and a G2 marker has been investigated in other neuronal death systems [85]. This might be an important area of future research in the stroke field.

In spite of the accumulating in vivo experimental studies demonstrating aberrant deregulation of cell cycle in stroke, there is little known about how/whether these processes occur in humans. Recently, a group investigated cell cycle elements in brains of postmortem patients who died after cardiac arrest or focal brain infarction. They reported elevated levels of cyclin D1, cdk2 and cdk4 [86]. This study shows that results obtained

from experimental studies in animals may be relevant to the human condition.

The in vivo evidence discussed above is summarized in Tables 1 and 2.

6. Role of other CDK members including cdk5 in stroke

In addition to the core cell cycle components discussed above, other cyclins and CDK members which are not exclusively or fully linked with cell cycle regulation may also participate in ischemic response. For example, cyclin H, a component of the CAK complex is increased in CA3, dentate gyrus, and cortex after global ischemia [87]. Since these areas are resistant to this type of ischemia, it has been suggested that cyclin H might have a role in survival through its DNA repair function in ischemic injury. Cyclin G is another member of the cyclins family but its role in cell cycle is controversial. While some evidence shows its stimulating role in growth [88,89], others suggesting it inhibits growth [90,91] in proliferating cells. The function of cyclin G in neurons is also unclear. Nevertheless, it has been shown that cyclin G1 is increased following MCAO and global ischemia [71,72], as well as spinal cord injury [92]. Furthermore, Maeda et al. [93] demonstrated that cyclin G1 is translocated into the nucleus of degenerating neurons following transient common carotid artery occlusion (CCAO) and MCAO. Therefore, they suggested that the cytoplasmic cyclin G1 might be associated with cell survival while nuclear cyclin G1 might signal death.

Perhaps the strongest evidence for the involvement of a CDK member not classically associated with the cell cycle involves cdk5. Cdk5 is a unique member of cyclin-dependent kinases family. Unlike the other CDKs, it does not require a cyclin to be activated. P35 and p39, instead, are activators of cdk5 and are present mainly in neurons [94,95]. For this reason, although is ubiquitously expressed, cdk5 activity is mainly restricted to nervous system [96]. Even though there is no functional evidence for involvement of cdk5 directly in the core cell cycle machinery, some recent observations have led to the idea that it might be associated with cell cycle components and, therefore, affects its transition. Initially it was shown that cdk5 was able to bind to cyclins D1, D3 and E [97–99]. However, this binding did not lead to increased CDK activity. Further studies showed that cdk5 could phosphorylate Rb in vitro [100]. In this study, overexpression of p25 in inducible neuroblastoma cells led to deregulation of cell cycle elements such as elevation of cyclins A and B1 and cdc2, downregulation of p27 and finally apoptosis. Interestingly, Rb phosphorylation on ser807/811 sites was an early event in this study and was mediated by cdk5 activity since it was abolished by a cdk5 inhibitor [100]. Finally, a very recent report revealed that cdk5 is necessary for neuronal cell cycle arrest [101]. This group suggested that cdk5 connects and inhibits cell cycle possibly by competing with cdk4/6 for binding to cyclins or through a kind of phosphorylation of Rb that inhibits E2F or, alternatively, by phosphorylation of RNA polymerase.

Cdk5 has been implicated in different neurodegenerative disorders including brain ischemia. The generally accepted concept about cdk5 is that cdk5/p35 complexes are regulators of normal biological functions of neuron. But under some neuropathological conditions p35 is cleaved to a more potent activator, p25 [102]. Production of p25 unmasks pathological face of cdk5 since it has prolonged half life, over activates, dislocates and might change cdk5 substrate specificity. There is increasing evidence, though, that argue this model. Several studies have debated role of p25 in pathogenecity of cdk5 in some circumstances [103–107]. While an exhaustive review of this issue is not possible here, we will suggest that, depending upon different factors; both p35 and p25 are able to drive "good-normal" cdk5 to "bad-pathogenic" cdk5.

Contribution of cdk5 in stroke has not been intensively studied and our knowledge about desregulated cdk5 in stroke comes from very recent research. One of the initial reports came from Hayashi et al. study [108]. They showed that immunoreactivity of cdk5 and p35/p25 is enhanced in MCAO model. Likewise, we [34] as well as Wang et al. [109] , have recently provided evidence that cdk5 is a mediator of excitotixc neuronal death in global and focal ischemia.

Similar to classic cell cycle CDKs, the important question of the mechanism by which aberrant cdk5 causes neuronal damage remains. Several possibilities may exist. For example, it has been reported that cdk5 can phosphorylate the NMDA receptors regulating calcium influx [109]. Interestingly tau, a microtubule-associated protein which is connected to Alzheimer's disease pathology, has also been recently shown to be as a substrate for cdk5 following ischemia [110]. What this means is unknown, however. There might also be other candidates such as MEF2 (Myocyte Enhance Factor-2) or p53 transcription factors. Both have been shown to be regulated by cdk5 and both have been implicated in ischemic/excitotoxic death [111–113]. However, the link between cdk5 and these factors have not been yet addressed in in vivo stroke studies.

7. Conclusion

As summarized above, there is increasing evidence of the involvement of multiple cell cycle regulatory signals in ischemic injury. Several important questions remain however, and include: 1) What are the mechanism(s) by which cell cycle signals are actually activated following stroke injury; 2) Are there other mechanisms by which cell cycle CDKs may impact death other than through modulation of Rb; 3) Does cdk5 regulate cell cycle signals in models of stroke 4) Are cell cycle targets in and of themselves a viable therapeutic option for stroke.

The latter question is of particular importance since most pharmacotherapeutic strategies tested in the clinic for treatment of stroke have failed. One reason for this is likely that preclinical testing in animal models has not been sufficiently stringent prior to clinical trials. In this regard, vigorous benchmarks of (a) efficacy of any strategy in multiple animal models, (b) behavioral and functional improvements beyond neuroprotection and (c) long lasting efficacy have been

established. Cell cycle inhibitors appear to at least meet some of these criteria. For example, the CDK inhibition has been shown to be neuroprotective in multiple models and lead to improved behavior/function. However, the CDK inhibitor flavopiridol, at least, seems to lack long term efficacy. This may be due to the non-selective nature of flavopiridol or and indication that CDK inhibition by itself cannot completely normalize neuron function. These issues will have to be carefully addressed before CDK inhibitors can be contemplated for future clinical trials.

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