

Expression of Cyclin-Dependent Kinase 5 mRNA and Protein in the Human Brain Following Acute Ischemic Stroke

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Neuronal cell death after brain ischemia may be regulated by activation of cyclin-dependent kinase 5 (Cdk5). In this study, expression of Cdk5 and its activator p35/p25 was examined in human post-mortem stroke tissue and in human cerebral cortical fetal neurons and human brain microvascular endothelial cells exposed to oxygen-glucose deficiency and reperfusion. The majority of patients demonstrated increased expression of Cdk5 and p-Cdk5 in stroke-affected tissue, with about a third showing increased p35 and p25 cleaved fragment as determined by Western blotting. An increase in Cdk5-, p-Cdk5- and p35-positive neurons and microvessels occurred in stroke-affected regions of patients. Staining of neurons became irregular and clumped in the cytoplasm, and nuclear translocation occurred, with colocalization of p35 and Cdk5. Association of Cdk5 with nuclear damage was demonstrated by coexpression of nuclear Cdk5 in TUNEL-positive neurons and microvessels in peri-infarcted regions. *In vitro* studies showed up-regulation and/or nuclear translocation of Cdk5, p-Cdk5 and p35 in neurons and endothelial cells subjected to oxygen-glucose deficiency, and strong staining was associated with propidium iodide positive nuclei, an indicator of cellular damage. These results provide new evidence for a role of Cdk5 in the events associated with response to ischemic injury in humans.

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INTRODUCTION

Controlled reperfusion and re-establishment of the local microcirculation, together with reduction in both immediate and delayed neuronal apoptosis, after stroke, could improve neuronal survival and organization, and ultimately patient recovery (28, 29).

Cyclin-dependent kinase 5 (Cdk5) is the 35-kDa catalytic subunit of a neuronal cell division cycle 2-like protein kinase involved in the regulation of the dynamic neurocytoskeletal changes seen in the brain after ischemia (16). Cdk5 is activated via binding to its regulatory subunits p35 and p39 and their subsequent cleavage to the more stable fragments p25 and p29, respectively, by calpain (21).

Cdk5 is critical for the normal function of the brain and is involved in corticogen-

esis during development (1, 31). Cdk5 has been implicated in neuronal apoptosis in neurodegenerative diseases, including Alzheimer's disease and amyotrophic lateral sclerosis (11), and its role in both brain development and disease, as well as in mediation of neuronal death and survival, has been reviewed by a number of authors (3, 5). Cdk5 activation modulates a plethora of signaling pathways involved in neuronal migration and synaptic transmission, and Love (17) has suggested that apoptotic or necrotic death of neurons after brain ischemia may be associated with Cdk5 activation.

Increased Cdk5 activity occurred in rat brain after global ischemia (9), while strong Cdk5 and p35 immunoreactivity was detected in neurons after middle cerebral artery occlusion (MCAO) in the rat

(10). Increased expression of p25 occurred after focal MCAO in a rat model of stroke, while pharmacological inhibition of Cdk5 activation with indolinone D significantly reduced the infarct volume after 24 h compared with nontreated rats (34).

Cdk5 is also involved in modulation of cell growth and survival in non-neural cells. Angiostatin inhibited cell growth in association with Cdk5 down-regulation, while Cdk5 inhibition increased the number of apoptotic cells, suggesting a role for Cdk5 in vascular endothelial cell (EC) growth and survival (25). Here we aimed to identify in detail the expression and localization of Cdk5 in the human brain during the acute phase of ischemic stroke.

METHODS

Patient samples. Brain tissue samples were obtained from 15 patients who died from acute ischemic stroke, with the approval of the local Ethics Committee. All patients were admitted with large middle cerebral artery strokes. The patients, 13 male and 5 female, were aged between 51 and 86 years and survived between 2 and 37 days following ischemic stroke. Age-matched control brain samples were also obtained from two males (died from cardiac sudden death/traffic accident) and one female (died from acute pneumonia) who had no evidence of brain disease or cerebral infarction.

Routine blood parameters were determined on admission. Full clinical examinations were carried out on admission

Patient no.	Age/sex	Survival after stroke	SSS on admission	SSS final	Hypercholesterolemia*	Obesity†	Hypertension‡	Coronary disease	Atrial fibrillation	History of TIA	Smoking
1	86/M	2	0	0	Yes	No	Yes	Yes	No	No	No
2	84/M	3	18	0	No	No	Yes	Yes	No	No	Yes
3	68/M	3	6	0	Yes	No	Yes	Yes	No	No	No
4	71/M	5	20	0	No	No	Yes	Yes	Yes	No	No
5	85/F	5	8	0	No	Yes	Yes	Yes	No	No	No
6	84/M	6	10	0	No	No	Yes	Yes	No	No	No
7	84/M	9	56	0	Yes	No	Yes	Yes	No	No	Yes
8	51/M	14	4	0	No	No	Yes	No	No	Yes	No
9	86/M	15	40	0	Yes	No	Yes	Yes	No	No	No
10	58/M	17	22	2	Yes	No	Yes	Yes	No	No	Yes
11	74/M	20	34	0	No	No	Yes	Yes	No	No	No
12	73/M	26	35	0	Yes	Yes	Yes	Yes	No	No	No
13	84/F	29	34	0	Yes	No	Yes	Yes	No	No	No
14	60/F	34	16	16	Yes	No	Yes	Yes	No	No	Yes
15	69/F	37	30	0	No	Yes	No	Yes	No	No	No

* Serum cholesterol levels greater than 5.0 mmol/L.

† Body mass index greater than 30 kg/m².

‡ Blood pressure greater than 140/90 mm Hg.

M = male; F = female; SSS = Scandinavian Stroke Scale; TIA = transient ischemic attack.

Table 1. Clinical details of patients used in the study.

and, where possible, at 1, 7, 14 and 30 days after stroke. Clinical examination was scored according to the 58-point Scandinavian Stroke Scale (23). All patients were evaluated by CT or MRI and classified as having a large infarct (diameter >4 cm), a moderate infarct (>1.5 cm < diameter <4 cm) or a small infarct (diameter <1.5 cm) (Table 1).

Tissue was collected from grey and white matter of infarct and peri-infarcted zones, and controls were obtained from the contralateral hemisphere within 6 h of death. The peri-infarcted areas were defined as the area of tissue immediately surrounding the infarct which showed evidence of tissue disorganization confirmed by histology. Sections from each piece of cut tissue were stained with 2-, 3- and 5-triphenyltetrazolium, which stains active mitochondria pink; therefore, unstained areas represented stroke-affected cortical regions (data not included). Tissue was frozen in liquid nitrogen, kept at -70°C, and a sample of each was processed and stained with hematoxylin and eosin to determine tissue morphology (6).

In vitro oxygen-glucose deprivation (OGD) model. Human brain microvascular endothelial cells (HBMEC) were

obtained from TCS CellWorks (Buckingham, UK) and cultured according to the manufacturer's instructions. Human fetal (cerebral cortical) neurons (HFN) were extracted, purified and cultured with permission from the local ethics committee. Brain tissue from fetal specimens of 14–19 weeks gestational age, legally aborted, were, after written consent from donors, collected in cold preservation medium. Cells were isolated and cultured as previously described (4).

For OGD experiments, the culture medium was replaced by glucose-free medium containing 2% fetal bovine serum and cells were placed at 37°C into a humidified chamber perfused with 94% N₂, 1% O₂ and 5% CO₂ for 8 h (HBMEC) or with 95% N₂ and 5% CO₂ for 14 h (HFN), followed by 24-h reperfusion in fresh medium containing 4.5 g/L glucose (resulting in approximately 30% of cells undergoing apoptosis after OGD and 60% following reoxygenation, as determined from our pilot studies). Cell pellets were stored at -80°C. Cells cultured in normoxic conditions without glucose deprivation served as control. In some experiments, propidium iodide (10 µg/mL) was added to the cultures 1 h before the end of the experiment to stain dead and dying

cells (4). Experiments were carried out three times.

RNA isolation. RNA extraction was performed using standard Clontech procedures (BD Biosciences, Hertfordshire, UK). RNA quality was measured using a spectrophotometer.

Reverse transcription-polymerase chain reaction (RT-PCR). Gene expression of normal cultures as well as those exposed to OGD and reperfusion was examined by semiquantitative RT-PCR. PCR conditions were an initial denaturation step of 10 minutes at 95°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C, and a final extension step of 10 minutes at 72°C. The products were visualized by agarose gel electrophoresis. Samples without cDNA were used as negative controls. Primers designed by Primer3 Output Program (Version 0.2) (Whitehead Institute for Biomedical Research, MIT, MA, USA) were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (forward: 5'-ATG ATC TTG AGG CTG TTG-3'—reverse: 5'-CTC AGA CAC CAT GGG GAA-3'); Cdk5 (forward: 5'-GCA CCT ATG GAA CTG TGT TCA

AGG CTA-3'—reverse: 5'-CTT CAC AAT CTC AGG GTC CAG GTC ACC-3') and p35 (forward: 5'-AAG AAC GCC AAG GAC AAG CC-3'—reverse: 5'-TTG ATG ACA GAG AGG CAA CG-3').

All experiments were carried out twice and at 20, 30 and 40 cycles to ensure the semiquantitative nature of the results. Scion Imaging Software was used for analysis (<http://www.scioncorp.com>).

Western blotting. Briefly, tissue samples (50 mg) were lysed and protein separation carried out using sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described previously (27). Blots were stained overnight at 4°C with antibodies to Cdk5 (1:500), p-Cdk5 (Ser 159; 1:1000), p35 (1:200) (Autogen Bioclear, Wiltshire, UK) and α -actin (Sigma, Dorset, UK) (1:1000) used as a loading control. Protein concentration was estimated from the band intensity by densitometry. Results are semiquantitative and are given as a numerical (fold) change compared with the control (contralateral tissue), which was given an arbitrary value of 1.0. All experiments were performed twice and a representative example is shown.

Immunohistochemistry. The Avidin-Biotin-Peroxidase method (ABC Vectastain kit, Vector Laboratories, Peterborough, UK) was used for the qualitative demonstration of antigens in tissues. Antibodies to Cdk5, p-Cdk5 and p35 were used at 1:50 dilution. Deparaffinized 5- μ m sections were treated for 10 minutes in a boiling solution of concentrated citric acid (pH 6.0; Vector Laboratories) in a pressure cooker to unmask the antigens. Sections were stained with primary antibodies for 2 h at room temperature (RT), with the appropriate horseradish peroxidase-conjugated secondary antibody for 1 h at RT, and then counterstained with hematoxylin. Negative control slides had the primary antibody replaced with phosphate buffer saline (PBS) or the appropriate IgG preimmune serum. Matching control peptides were also used to absorb the Cdk5 and p35 antibodies to check for nonspecific binding (data not included). Cultured cells were fixed with 4% paraformaldehyde for 20 minutes, permeabilized with 0.2% Triton X100 for 8 minutes and blocked with normal serum before staining over-

night at 4°C, followed by 60-minute incubation with Alexafluor-green conjugated dye at RT. Stained images were obtained with a confocal microscope.

Double immunofluorescence. Following primary antibody staining, slides were incubated with normal serum and then with a second primary antibody (diluted in normal serum) for 30 minutes, followed by 30-minute incubation with Alexafluor-red conjugated dye.

Analysis of apoptosis in tissue sections. The Promega DeadEnd Colorimetric TUNEL System was used according to the manufacturer's instructions (Promega, Southampton, UK). Positive control sections pretreated with DNase I were stained in parallel (data not included). Negative control slides were performed, where terminal deoxynucleotidyl transferase enzyme was replaced with PBS and processed as above (data not included).

RESULTS

Cdk5, p35/p25 and p-Cdk5 protein levels were up-regulated in human brain tissue

Cdk5 expression. Western blotting showed that Cdk5 was up-regulated by 1.5- to 3.4-fold in gray matter peri-infarcted tissue in 10 out of 15 patients and by 1.5- to 3.6 fold in gray matter infarct in nine out of 15 patients. In white matter, Cdk5 was up-regulated by 1.5- to 2.2-fold in peri-infarcted tissue in 11 out of 15 patients and by 1.5- to 3.2 fold in infarcted regions in 10 out of 15 patients (Figure 1Ai and ii—Table 2).

In contralateral gray matter, neurons showed a regular expression of Cdk5 with cytoplasmic and axonal staining, while blood vessels were not stained in either the gray or white matter (Figure 1Bi and Ci, respectively, Table 3). A similar pattern was observed in control brain tissue (Figure 1E). In gray matter peri-infarcted and infarcted regions, nuclear staining and intracellular clustering of Cdk5 occurred in neurons, while numerous microvessels demonstrated strong positive staining. These changes were most apparent in patients who survived for 2 to 14 days after stroke (Figure 1Bii and iii and Cii and iii, respectively). In white matter, some

microvessels were also Cdk5-positive. Increased Cdk5 staining remained in microvessels and abnormal-looking neurons from gray matter peri-infarcted tissue in patients surviving up to 29 days after stroke. Colocalization of Cdk5 with TUNEL-positive neurons and microvessels was demonstrated (Figure 1Di and ii, respectively).

p-Cdk5 expression. Western blotting showed that p-Cdk5 was up-regulated in four out of 15 patients (1.5- to 4.2-fold) in peri-infarcted gray matter, and in nine out of 15 patients (1.5- to 4.3-fold) in infarcted tissue. In white matter, p-Cdk5 was up-regulated in the peri-infarcted tissue of four out of 15 patients (1.5- to 3.0-fold) and 1.5- to 3.9-fold in infarcted tissue in eight out of 15 patients (1.5- to 3.8-fold; Figure 2A—Table 2). Cdk5 phosphorylation occurred mostly near to the infarcted core. p-Cdk5 was not expressed in normal-looking neurons and microvessels from the contralateral hemisphere (Figure 2Bi and Ci, respectively), nor in control brain tissue (Figure 2D). p-Cdk5 was up-regulated in stroke-affected neurons, with observable clumping and nuclear translocation, and in microvessels in patients who survived from 3 to 37 days after stroke (Figure 2Bii and Cii, respectively, Table 3).

p35 expression. In gray matter, p35 was up-regulated in peri-infarcted tissue regions in six out of 15 patients (1.5- to 4.2-fold) and in infarcted areas in four of 15 patients (1.5- to 3.4-fold). In white matter, p35 was up-regulated in peri-infarcted tissue in three out of 15 patients (1.5- to 1.8-fold) and, in infarcted zones in four out of 15 patients (1.5–3.3 fold; Figure 3A—Table 2). p25 was not observed in tissue from the contralateral hemisphere, but was observed in five out of 15 patients in either peri-infarcted or infarcted tissue.

Normal-looking neurons from the contralateral hemisphere showed weak intracellular staining for p35 while blood vessels were unstained (Figure 3Bi and Ci, respectively). A similar staining pattern was observed in control brain tissue (Figure 3D). p35 was translocated to the nucleus of neurons and was strongly expressed in microvessels in stroke-affected regions in patients who survived from

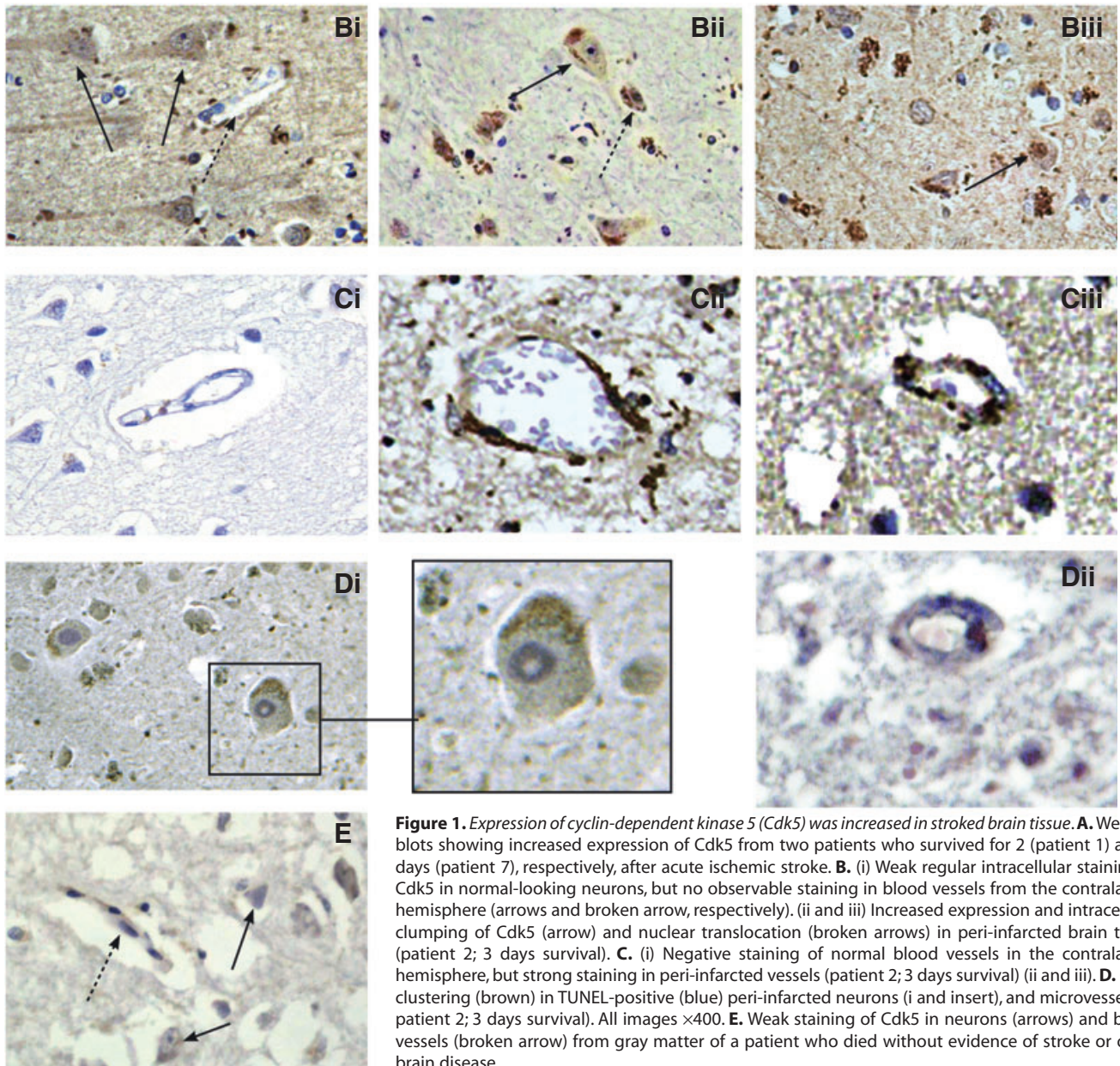
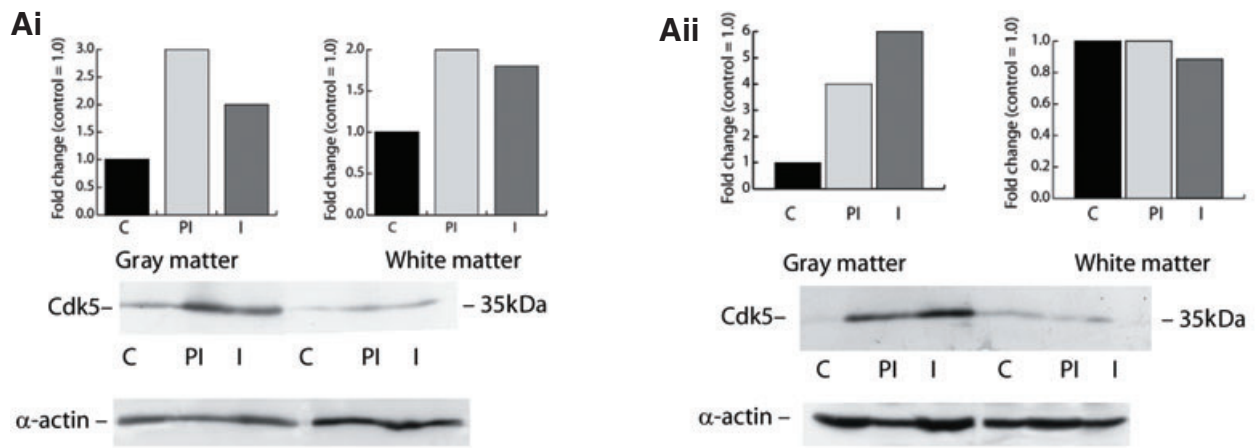


Figure 1. Expression of cyclin-dependent kinase 5 (*Cdk5*) was increased in stroked brain tissue. **A.** Western blots showing increased expression of *Cdk5* from two patients who survived for 2 (patient 1) and 9 days (patient 7), respectively, after acute ischemic stroke. **B.** (i) Weak regular intracellular staining of *Cdk5* in normal-looking neurons, but no observable staining in blood vessels from the contralateral hemisphere (arrows and broken arrow, respectively). (ii and iii) Increased expression and intracellular clumping of *Cdk5* (arrow) and nuclear translocation (broken arrows) in peri-infarcted brain tissue (patient 2; 3 days survival). **C.** (i) Negative staining of normal blood vessels in the contralateral hemisphere, but strong staining in peri-infarcted vessels (patient 2; 3 days survival) (ii and iii). **D.** *Cdk5* clustering (brown) in TUNEL-positive (blue) peri-infarcted neurons (i and insert), and microvessels (ii; patient 2; 3 days survival). All images $\times 400$. **E.** Weak staining of *Cdk5* in neurons (arrows) and blood vessels (broken arrow) from gray matter of a patient who died without evidence of stroke or other brain disease.

Patient no.	Survival (days)	Cdk5				p35				p-Cdk5				p25			
		Gray		White		Gray		White		Gray		White		Gray		White	
		PI	I	PI	I	PI	I	PI	I	PI	I	PI	I	PI	I	PI	I
1	2	2.8	3.1	0.3	2.2	4.2	3.4	2.3	3.3	0.4	0.4	1.0	1.0	ND	ND	1.8	ND
2	3	3.4	3.6	1.5	0.3	2.6	2.5	1.5	0.5	0.5	0.5	0.5	0.5	2.2	2.2	2.3	ND
3	3	1.5	1.6	1.5	3.7	1.0	1.0	1.0	1.0	4.2	4.3	3.0	2.6	1.9	2.0	1.9	2.0
4	5	0.3	1.5	1.5	1.7	1.0	1.0	1.0	1.5	2.3	2.1	2.6	2.5	ND	ND	1.8	1.8
5	5	1.5	0.5	2.3	1.0	1.0	1.0	1.0	1.0	1.0	2.4	1.0	1.0	ND	ND	ND	ND
6	6	0.4	1.5	2.7	2.5	1.0	0.3	1.0	1.0	1.5	3.1	1.0	2.8	ND	ND	ND	ND
7	9	1.5	2.8	0.5	1.0	1.0	0.3	1.0	1.0	1.0	1.7	1.0	3.6	ND	ND	ND	ND
8	14	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0	1.0	1.0	1.5	2.9	ND	1.7	ND	ND
9	15	1.0	1.0	1.5	1.5	0.4	1.0	1.0	1.0	2.5	2.4	2.6	2.9	ND	ND	0.5	0.5
10	17	2.4	3.0	1.7	2.7	2.2	1.0	1.0	1.0	1.0	3.8	1.0	1.0	ND	ND	ND	ND
11	20	1.0	1.0	2.3	1.6	1.0	2.5	1.0	2.2	1.0	1.0	1.0	1.0	ND	1.7	1.8	1.8
12	26	2.3	0.3	1.5	1.5	1.0	0.3	1.0	1.0	1.0	2.4	1.0	3.9	ND	ND	1.8	ND
13	29	2.8	1.0	0.2	0.3	2.5	1.5	1.0	1.0	1.0	2.8	1.0	2.5	ND	ND	ND	ND
14	34	2.3	2.3	1.5	1.5	1.5	1.0	1.0	1.0	1.0	1.0	0.4	0.4	1.5	1.5	ND	ND
15	37	2.3	2.3	1.5	1.5	2.5	1.0	1.6	3.3	1.0	1.0	1.0	1.0	ND	ND	ND	ND
No. of patients	Increased	10	9	11	10	6	4	3	4	4	9	4	8	3	5	6	3
	Decreased	3	3	4	3	1	3	0	3	2	2	2	2	0	0	1	1
	No change	2	3	0	2	8	8	12	8	9	4	9	5	0	0	0	0
	Not detected	0	0	0	0	0	0	0	0	0	0	0	0	11	9	8	11

Table 2. Expression of cyclin-dependent kinase 5 (Cdk5), p-Cdk5 and p35 in stroke patients demonstrated by Western blotting. Numbers show fold increase (control = 1.0): PI is peri-infarcted and I is infarcted region; ND = not detected.

Patient no.	Cdk5			p35			p-Cdk5		
	Neurons	Glia	EC	Neurons	Glia	EC	Neurons	Glia	EC
Contralateral hemisphere	Weak 1/2 + (cytoplasmic)	–	1/2 +	Weak 1/2 + (cytoplasmic)	–	–	Weak 1/2 + (cytoplasmic)	Weak 1/2 +	–
2 (3-day survival)	+++ (nuclear)	++	++	+++ (nuclear)	+	+	+++ (nuclear)	+++	+
4 (5-day survival)	+++ (nuclear)	+++	++	++ (nuclear)	++	+++	+	++	++
5 (6-day survival)	+++ (nuclear)	++	++	++ (nuclear)	++	+++	++ (nuclear)	++	+++
6 (6-day survival)	++	+	++	+++	++	++	++	+	+
9 (15-day survival)	++	++	++	+++	++	++	++	+	++
12 (26-day survival)	+++ (nuclear)	++	+++	++	++	+	+++	++	+
14 (34-day survival)	+	–	+	+	+	+	–	–	–
15 (37-day survival)	+++ (cytoplasmic)	+	++	+	+	+	+	+	+++

Table 3. Semiquantitative analysis of immunohistochemical data for cyclin-dependent kinase 5 (Cdk5), p35 and p-Cdk5 expression in human brain tissue. –, no staining; +, some staining; ++, strong staining; +++, very strong staining; EC = endothelial cell.

3–6 days after stroke (Figure 3Bii and Cii, respectively, Table 3). Increased p35 staining remained up to 26 days after stroke in peri-infarcted tissue.

Double immunofluorescence showed coexpression of Cdk5 and p35 in neuronal nuclei and microvessels from peri-infarcted regions (Figure 4Ai–iii and Bi–iii respectively).

Cdk5 was increased in Propidium Iodide (PI)-positive HFN following

OGD. RT-PCR showed up-regulation of Cdk5 in HFN exposed to OGD (12 h), which diminished during reperfusion compared with untreated cells (Figure 4A). Up-regulation of hypoxia-inducible factor-1- α and heat shock protein 72 were used as controls to monitor the effects of OGD (data not included). Western blotting showed that Cdk5 protein expression was increased in OGD-treated HFN (2.5-fold; Figure 4B), while immunohistochemistry (IHC) demonstrated notable increase in

the intensity of staining in the soma, and coexpression in cells positively stained for propidium iodide (Figure 5Ci–iii). Much stronger Cdk5 staining was seen in propidium iodide-positive cells, suggesting an association with cellular damage.

p35 gene expression was increased in HFN exposed to OGD for 12 h and remained increased during reperfusion (Figure 6A). Protein expression was increased as measured by Western blotting, although p25 was not observed (Figure 6B)

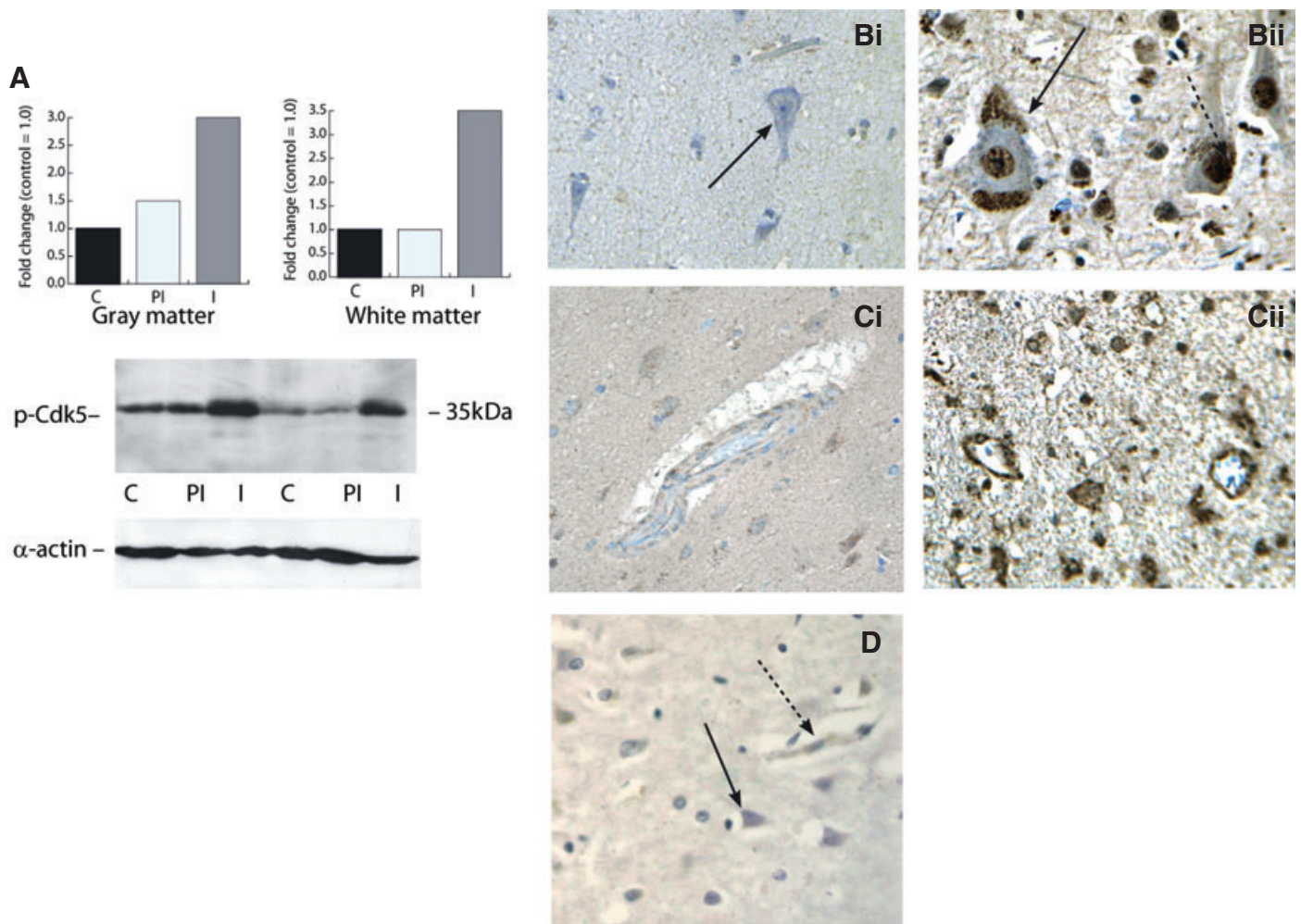


Figure 2. *p*-Cyclin-dependent kinase 5 (*p*-Cdk5) staining was observed in neurons and microvessels in peri-infarcted tissue. **A.** Western blot showing increased expression of *p*-Cdk5 in peri-infarcted and infarcted regions, from a patient who survived for 6 days (patient 6) after acute ischemic stroke. **B.** Normal-looking neurons from the contralateral hemisphere showed no observable staining of *p*-Cdk5 (i, arrow), while both intracellular (arrow) and nuclear (broken arrow) staining was observed in those from peri-infarcted areas (ii; patient 5; 6 days survival). **C.** Similarly, normal-looking blood vessels from the contralateral hemisphere were not observably stained (i); however, microvessels from peri-infarcted regions were strongly *p*-Cdk5-positive (ii; patient 6; 6 days survival). All images $\times 400$. **D.** Weak cytoplasmic staining of *p*-Cdk5 in neurons (arrows) and blood vessels (broken arrow) from gray matter of a patient who died without evidence of stroke or other brain disease. PI = peri-infarcted; I = infarcted; C = control.

and strong staining, particularly in the nuclei of cells exposed to OGD, was seen (Figure 6Ci and ii). Again, p35 staining was strongest in, and overlapped with, propidium iodide-positive cells (Figure 6Ciii).

p-Cdk5 was notably expressed in HFN cultured under normal conditions, as identified by Western blotting. IHC revealed its presence in distinct intracellular/cytoplasmic localizations. Although Western blotting failed to demonstrate an increase in expression in HFN exposed to OGD (14 h; Figure 7A), IHC showed that expression became spread throughout the nuclei and intracellular domain (Figure 7Bi and iii). Damaged propidium iodide-positive HFN stained strongly for *p*-Cdk5 and showed colocalization,

suggesting a link to cellular damage (Figure 7Biii). Colocalization of *p*-Cdk5 and p35 also occurred in the nuclei of OGD-treated cells (data not shown).

Cdk5 up-regulation was associated with cellular damage in HBMEC following OGD. RT-PCR showed up-regulation of *Cdk5* and p35 mRNA in HBMEC exposed to OGD and reperfusion compared with untreated cells (Figures 8A and B and 9A and B, respectively). Up-regulation of hypoxia-inducible factor-1- α and heat shock protein 72 were used as controls to monitor the effects of OGD (data not included). Similarly, *Cdk5* and p35 protein levels were increased after OGD (Figures 8B and 9B, respectively).

Increased nuclear staining of *Cdk5*, p35 and *p*-Cdk5 occurred in HBMEC treated with OGD (Figures 8C, 9C and 10A, respectively).

DISCUSSION

This study has identified increased *Cdk5*/p35/p25 up-regulation and *Cdk5* phosphorylation in stroke-affected tissue from patients in the acute phase of ischemic stroke. Intensely positively stained neurons, with evidence of nuclear translocation, occurred in stroke-affected regions of patients who survived for up to 1 month after stroke. Colocalization of *Cdk5* with TUNEL-positive neurons provided evidence of involvement of *Cdk5* in cell death pathways. Because of the limited

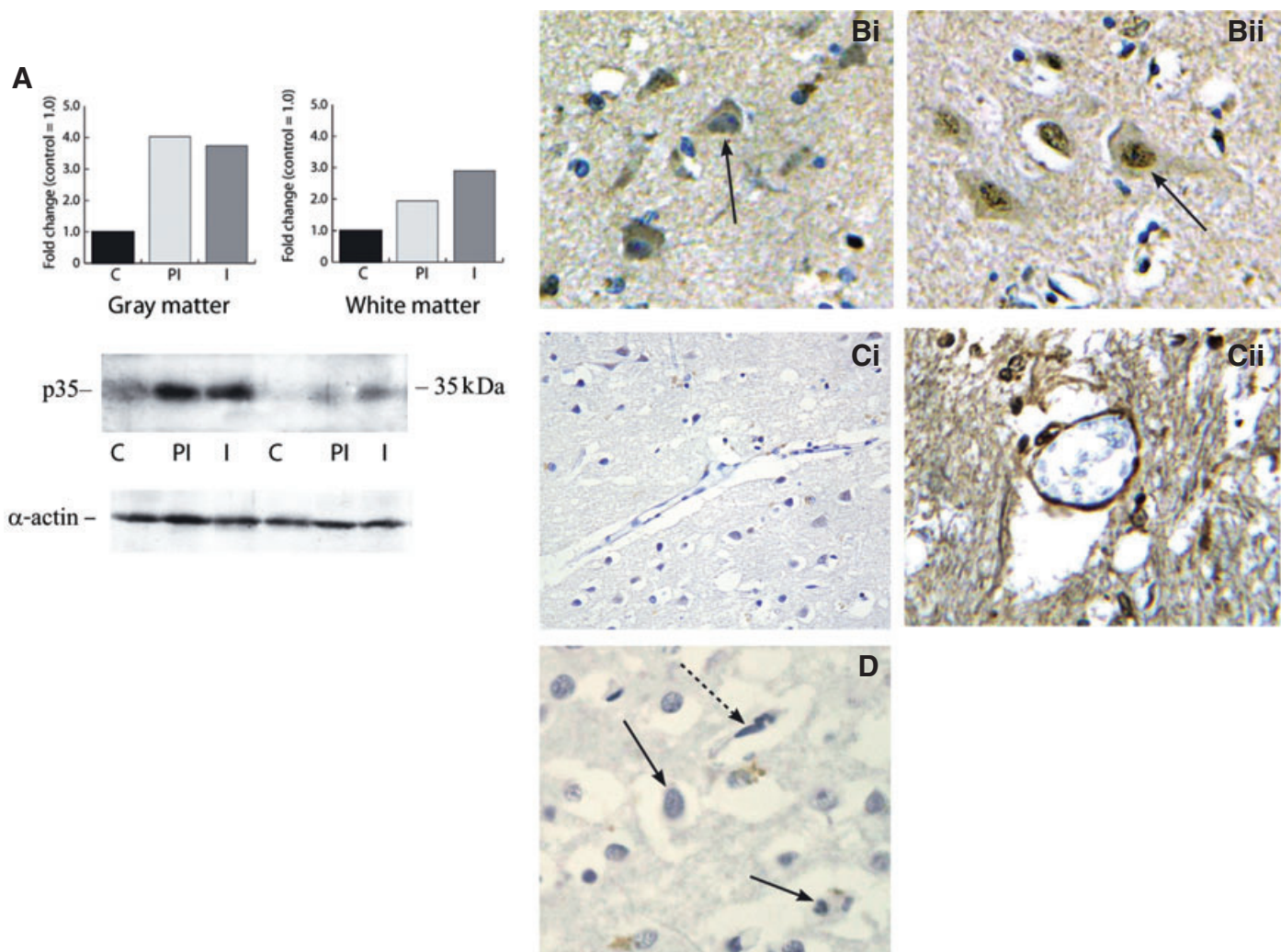


Figure 3. p35 was translocated to the nucleus of neurons and overexpressed in microvessels from stroke-affected regions. **A.** Western blot showing increased expression of p35 in peri-infarcted and infarcted regions, from a patient who survived for 5 days (patient 4) after acute ischemic stroke. **B.** Normal looking neurons from the contralateral hemisphere showed no observable staining of p35 (i, arrow showing hematoxylin positive nucleus), while nuclear staining (arrow) was observed in those from peri-infarcted areas (ii; patient 1; 2 days survival). **C.** Similarly, normal looking blood vessels from the contralateral hemisphere were not observably stained (i), however microvessels from peri-infarcted regions were strongly p35 positive (ii; patient 1; 2 days survival). All images $\times 400$. **D.** Weak staining of p35 in the cytoplasm of neurons (arrow) and blood vessels (broken arrow) from gray matter of a patient who died without evidence of stroke or other brain disease. PI = peri-infarcted; I = infarcted; C = control.

numbers of patients examined, it was not possible to determine the statistical correlation between expression levels of Cdk5/p35 and patient survival. Also, the Western blotting data are only semiquantitative. It is also worth noting that during tissue dissection, individual pieces of tissue possessed variable areas of tissue damage, ranging from severe to less severe, with occasional normal-looking segments. This is inevitable irrespective of the expertise of the neurologist. Western blotting results therefore are merely an indicator of changes in protein expression within broad areas of damaged tissue, which might explain the fact that some patient samples showed only a weak up-regulation or none at all, while the more sensitive IHC

enabled us to define the specific areas in more detail and of course to identify cell-specific changes.

Cdk5 may have a role in the events associated with neuronal response to ischemic injury, because increased Cdk5 tau protein phosphorylating activity was seen in the rat postdecapitative model of brain ischemia (5). Hayashi et al, (10) demonstrated increased Cdk5 and p35 immunoreactivity and nuclear translocation in peri-infarcted tissue, in neurons following MCAO in a rat model of stroke, in agreement with our unpublished data using a rat model of MCAO. Using the same model, Osuga et al (20) reduced the infarct volume by intracerebroventricular infusion of the pan-Cdk5 inhibitor flavopiridol, indicat-

ing a protective effect of Cdk5 down-regulation. Ischemic brains of rats showed increased expression of p25, 3 h after temporary MCAO followed by 6 h of reperfusion (18), or 2 h after transient MCAO (33).

HFN exposed to OGD and reperfusion also showed phosphorylation and nuclear translocation of Cdk5, particularly in propidium iodide-positive nuclei, indicating cell damage. Previous *in vitro* studies showed that inhibition of Cdk5 activity using indolinone A and D protected the mitochondrial integrity of lesioned rat cortical neurons and reduced caspase activation. Interestingly, Cdk5 may modulate the *N*-methyl-D-aspartate receptors as their direct phosphorylation by Cdk5 resulted

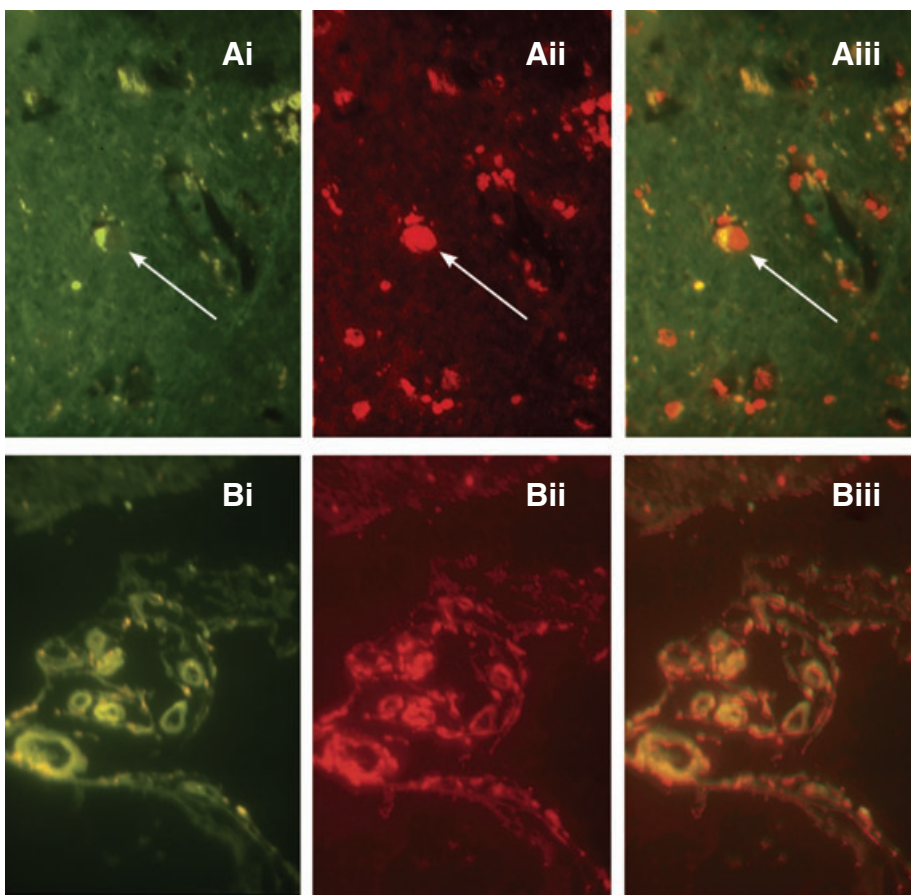


Figure 4. Double fluorescent labeling showed colocalization of p35 and cyclin-dependent kinase 5 (Cdk5) in stroke-affected tissue. **A.** Nuclear colocalization in a neuron from the peri-infarcted region (patient 1; 2 days survival; arrows) (i) p35 (green), (ii) Cdk5 (red), (iii) overlaid images ($\times 400$). Identification of neurons was by light microscopy of hematoxylin-stained slides (data not shown). **B.** Increased expression of (i) p35 (green) and (ii) Cdk5 (red) and (iii) overlaid images of an infarcted blood vessel (patient 1; 2 days survival; $\times 400$).

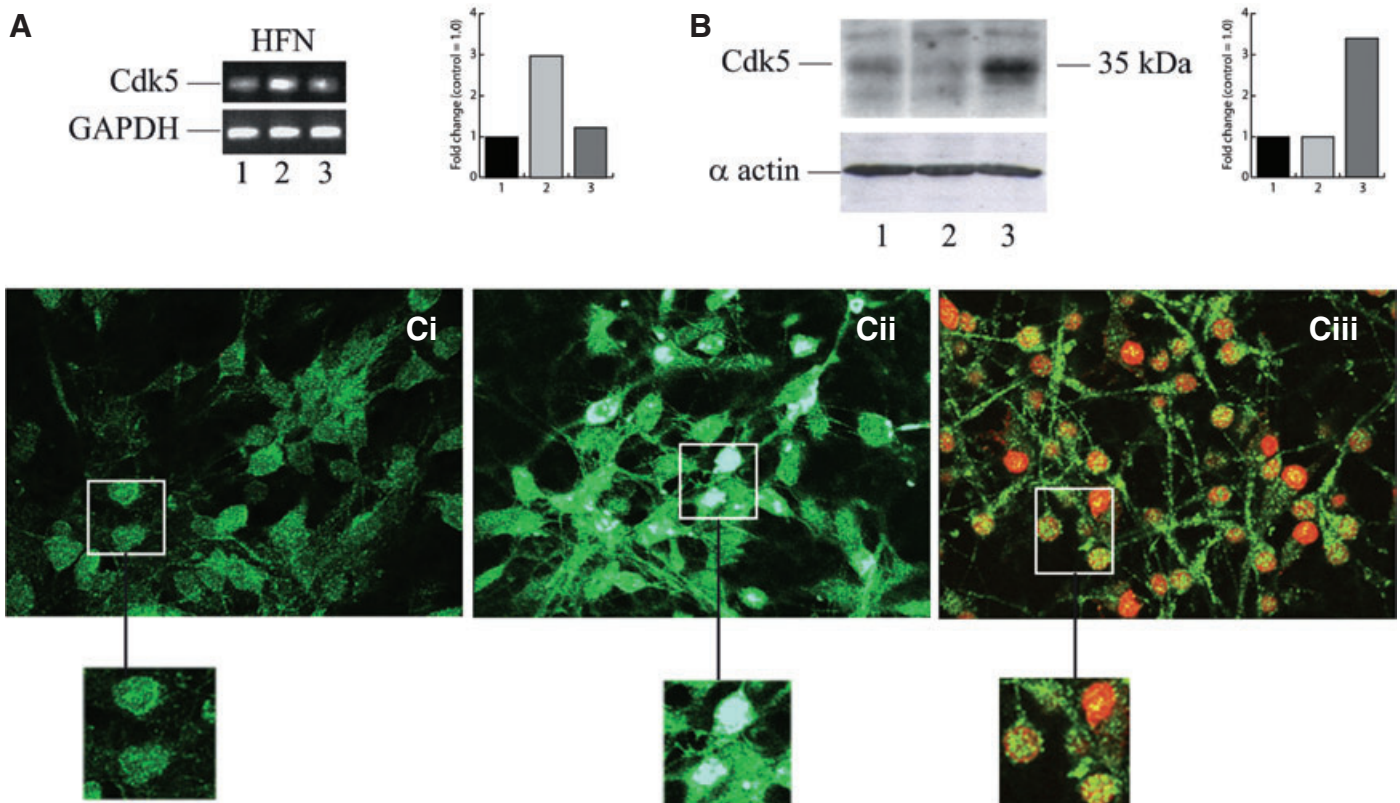


Figure 5. Cyclin-dependent kinase 5 (Cdk5) expression was increased in human fetal (cerebral cortical) neurons (HFN) exposed to oxygen-glucose deprivation (OGD). **A.** Reverse transcription-polymerase chain reaction and **B.** Western blotting showing Cdk5 up-regulation in HFN exposed to OGD and/or reperfusion. 1: control, 2: OGD 14 h, 3: OGD 14 h and reperfusion 24 h. **C.** Immunohistochemistry Cdk5 staining of cultured human fetal (cerebral cortical) neuron (HFN). (i) Weak perinuclear staining for Cdk5 in normal untreated HFN. (ii) Shows dramatically increased intensity of Cdk5 staining following OGD. (iii) Nuclear coexpression of Cdk5 and propidium iodide after OGD. (Photographs $\times 250$, inserts $\times 500$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

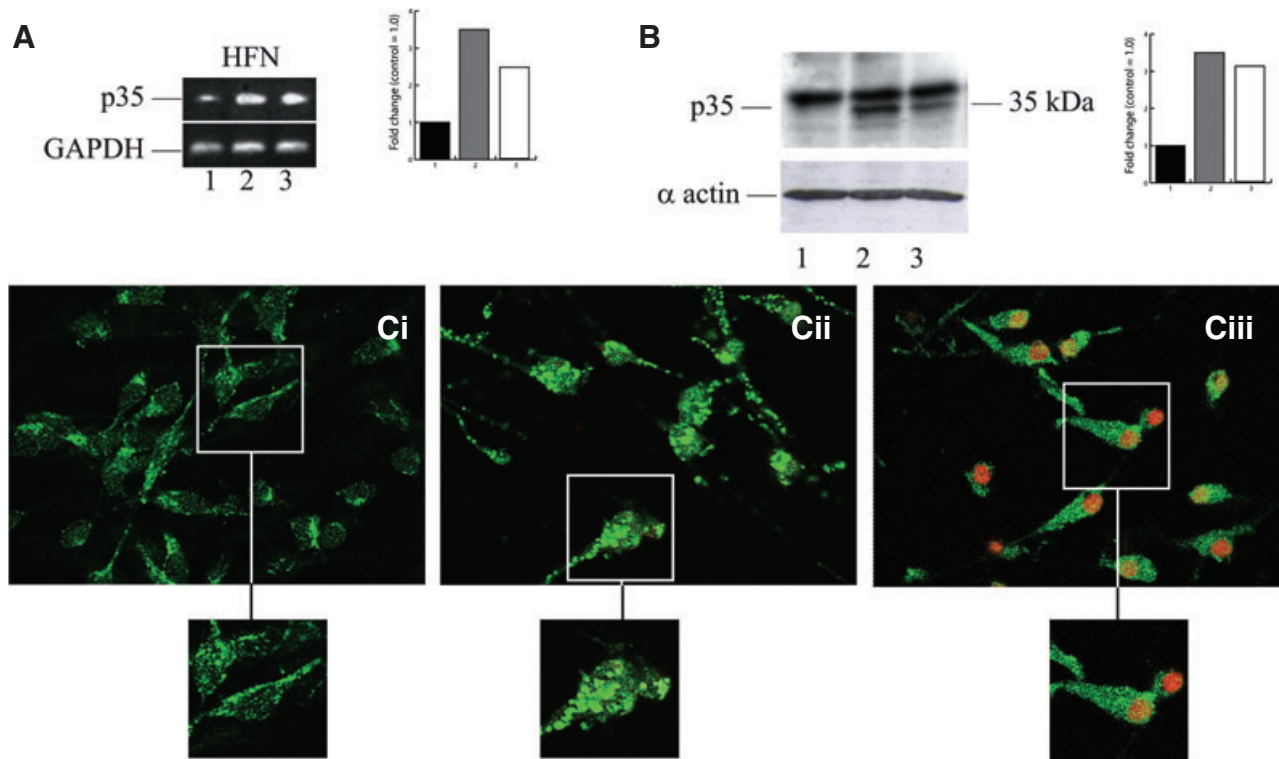


Figure 6. *p35* expression was also increased in human fetal (cerebral cortical) neurons (HFN) exposed to oxygen-glucose deprivation (OGD). **A.** Reverse transcription-polymerase chain reaction and **B.** Western blotting showing *p35* up-regulation in HFN exposed to OGD and/or reperfusion. 1: control, 2: OGD 14 h, 3: OGD 14 h and reperfusion 24 h. **C.** Immunohistochemistry *p35* staining of cultured HFN. (i) Weak intracellular staining for *Cdk5* in normal untreated HFN. (ii) Shows increased intensity of *p35* staining following OGD. (iii) Nuclear co-expression of *p35* and propidium iodide after OGD. (Photographs $\times 250$, inserts $\times 500$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

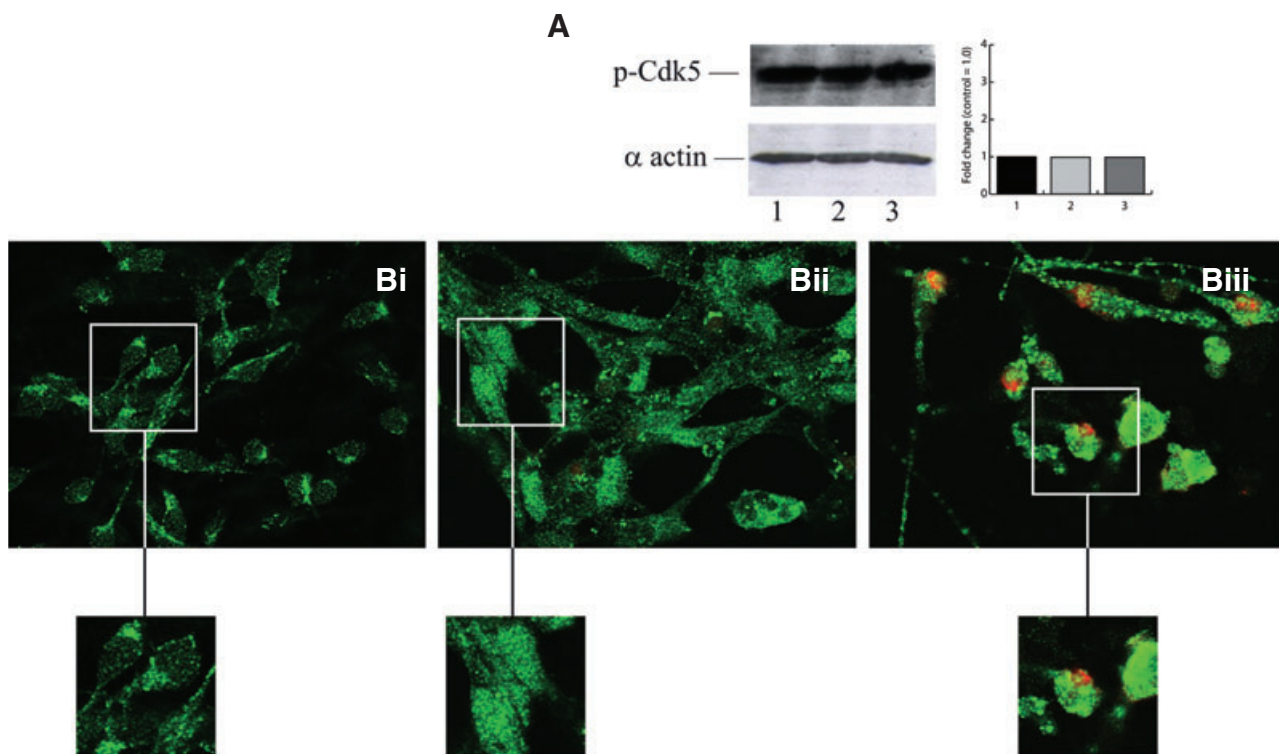


Figure 7. Increased intracellular *p-cyclin-dependent kinase 5* (*p-Cdk5*) expression was seen in damaged human fetal (cerebral cortical) neurons (HFN) exposed to oxygen-glucose deprivation (OGD). **A.** Western blotting showing *p-Cdk5* expression before and after OGD/reperfusion. 1: control, 2: OGD 14 h, 3: OGD 14 h and reperfusion 24 h. **B.** (i) Perinuclear staining of *p-Cdk5* was seen in normal untreated HFN. (ii) Shows spreading of *p-Cdk5* throughout intracellular regions and in the nucleus following OGD. (iii) Nuclear coexpression of *p-Cdk5* and propidium iodide after OGD. (Photographs $\times 250$, inserts $\times 500$).

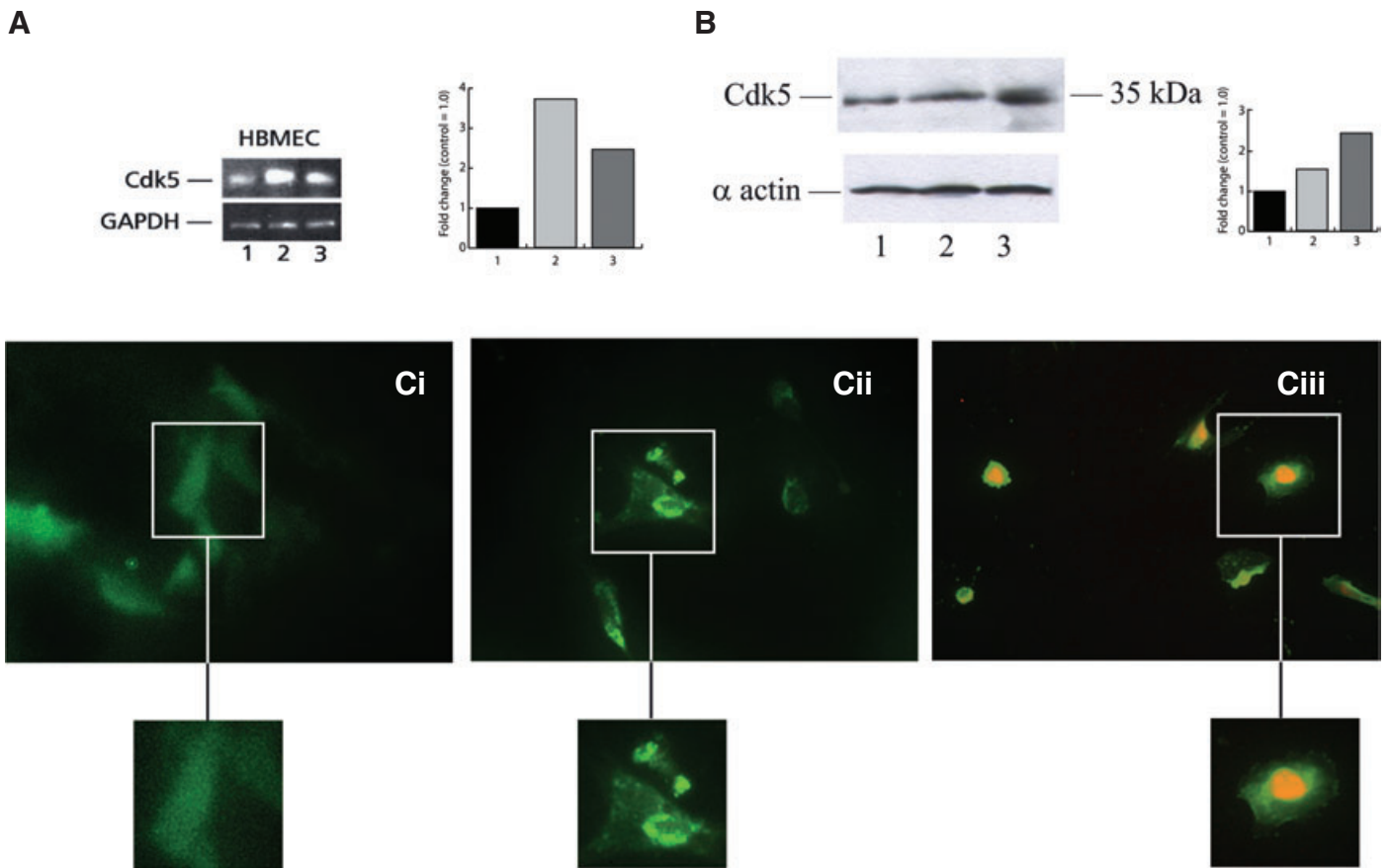


Figure 8. Cyclin-dependent kinase 5 (*Cdk5*) expression was increased in human brain microvascular endothelial cell (HBMEC) exposed to oxygen-glucose deprivation (OGD). **A.** Reverse transcription-polymerase chain reaction and **B.** Western blotting showing *Cdk5* up-regulation in HBMEC exposed to OGD and/or reperfusion. 1: control, 2: OGD 12 h, 3: OGD 12 h and reperfusion 24 h. **C.** Immunohistochemistry *Cdk5* staining of cultured HBMEC. (i) Weak diffused staining for *Cdk5* in untreated cells. (ii) Shows nuclear translocation of *Cdk5* following OGD. (iii) Shows nuclear coexpression of *Cdk5* and propidium iodide after OGD. (Photographs $\times 250$, inserts $\times 500$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

in ischemic injury in rat CA1 pyramidal neurons following forebrain ischemia (32). Weishaupt et al (33, 34) showed that *Cdk5* activated neuronal cell death pathways upstream of mitochondrial dysfunction, while Zheng et al showed that inhibitory activity against *Cdk5* conferred neuroprotection, potentially promoting functional long-term rescue of injured neurons (35). During glutamate-induced excitotoxic cell death of murine neurons, both p25 and *Cdk5* localized to the nuclei and were involved in mediation of cell death pathways, although not apoptosis. Cytoplasmic *Cdk5* contributed to survival signaling, demonstrating that both the localization of *Cdk5* and type of stimulation could affect the cell fate (19). Rashidan et al recently demonstrated that *Cdk5* was important in mediation of excitotoxic damage in murine cerebellar granule neurons induced by glutamate; however, *Cdk5* mutants did not protect against hypoxia/ischemia, rather, other Cdk such as *Cdk4* appeared to par-

ticipate in these delayed death pathways (22). Nuclear translocation and phosphorylation of *Cdk5* also resulted in neurotoxin-induced cerebellar granular neuronal apoptosis (30), suggesting that up-regulation, activation and nuclear translocation of *Cdk5* via p35/p25 are important features of the ischemic response in stroke and may be a key mediator of neuronal cell death. Phosphorylation of *Cdk5* at either Ser¹⁵⁹ and/or Tyr¹⁵, together with its association with p35, is a requirement for its efficient activation (26, 36). Tyrosine phosphorylation of *Cdk5* was first reported by Lazaro et al in 1996 (15) in rat cerebellar lysates, and more recently, was shown to be important in mediation of axonal lengthening of developing neurons following activation by cables and c-Abl (36), while Ser¹⁵⁹ p-*Cdk5* appears to be the main target for *Cdk5* phosphorylating kinases and is known to be activated via casein kinase I in PC12 cells (26). In our studies, Tyr¹⁵ p-*Cdk5* was not notably altered, while

Ser¹⁵⁹ p-*Cdk5* was increased in some patients after stroke, but more importantly, its nuclear translocation occurred both *in vivo* and *in vitro*. As we found that *Cdk5*, p-*Cdk5* and p35/25 were translocated to the nucleus in the acute phase of human stroke, and that this shift in localization occurred even in patients where the total amount of protein remained similar to the control areas, this may be an important indicator determining which cells ultimately die and which are subjected to *Cdk5* cytoplasmic prosurvival signaling.

Collateral revascularization and reperfusion of potentially viable tissue in part determines patient recovery after stroke (29). Angiogenesis correlates with patient survival, and survival of neurons is greatest in areas of tissue undergoing angiogenesis (12, 13). Following MCAO in a rat model, new blood vessels initiated through vascular buds formed regular connections with intact microvessels within 1 week of ischemia, the patterns being similar to

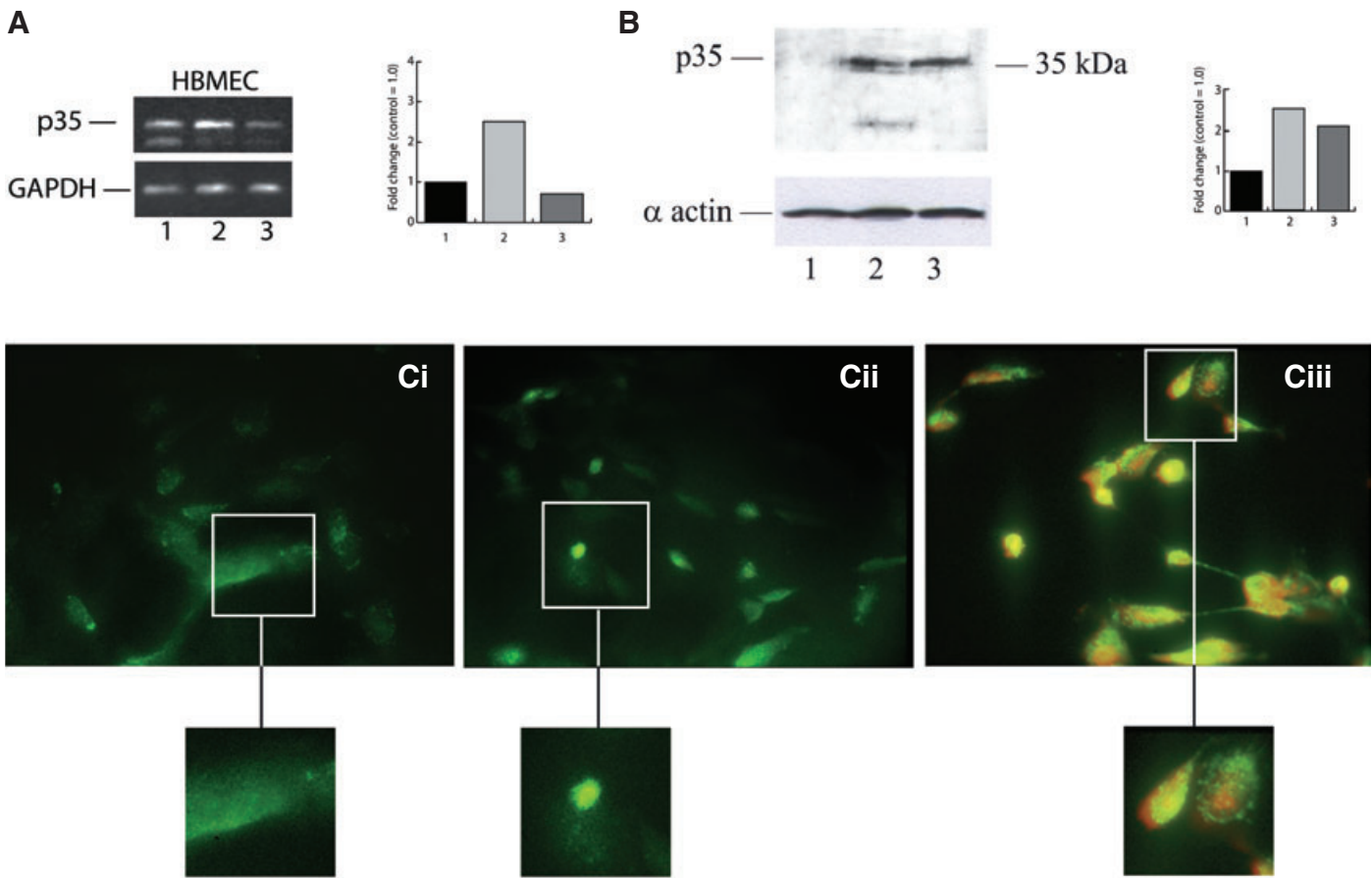


Figure 9. *p35* expression was also increased in human brain microvascular endothelial cell (HBMEC) exposed to oxygen-glucose deprivation (OGD). **A.** Reverse transcription-polymerase chain reaction and **B.** Western blotting showing *p35* up-regulation in HBMEC exposed to OGD and/or reperfusion. 1: control, 2: OGD 14 h, 3: OGD 14 h and reperfusion 24 h. **C.** Immunohistochemistry *p35* staining of cultured HFN. (i) Diffuse staining for Cdk5 in normal untreated HFN. (ii) Shows nuclear translocation of *p35* following OGD. (iii) Shows nuclear expression of *p35* with propidium iodide counterstain after OGD. (Photographs $\times 250$, inserts $\times 500$). GAPDH = glyceraldehyde-3-phosphate dehydrogenase.

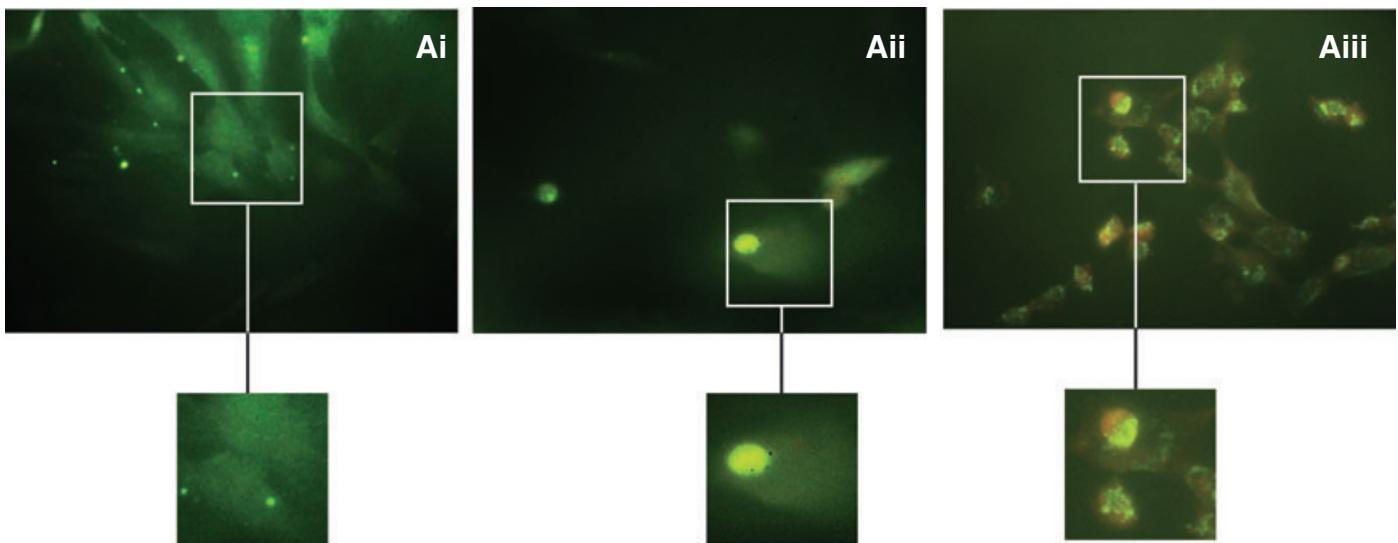


Figure 10. Nuclear translocation of *p-cyclin-dependent kinase 5* (*p-Cdk5*) expression was seen in human brain microvascular endothelial cell (HBMEC) exposed to oxygen-glucose deprivation. **A.** (i) Weak intracellular staining of *p-Cdk5* was seen in normal untreated HBMEC. (ii) Shows increased intensity of *p-Cdk5* staining following OGD. (iii) Nuclear expression of *p-Cdk5* with propidium iodide counterstain after OGD. (Photographs $\times 250$, inserts $\times 500$).

those seen in the normal brain. Apoptosis occurred within damaged EC in the “penumbra”, which resulted in collapsed lumina and a lack of reflow (14, 24). In this study, we have shown for the first time that Cdk5 may have a role in the microvessel/EC response to ischemic stroke.

Chen et al (2) isolated a novel gene IC53 from human aorta cDNA library, which had structural similarity to the Cdk5 binding protein precursor C53, and mediated proliferation of ECV304 cells. Inhibition of Cdk5 by olomoucine accelerated wound healing in corneal epithelium of organ cultured transgenic mice overexpressing Cdk5 and increased expression of activated c-Src along the wound edge (7, 8). High levels of Cdk5 expression were associated with FGF-2-induced proliferation in bovine aortic EC (25).

Here, an increase in Cdk5 staining was demonstrated in microvessels from stroke-affected regions of patients who survived up to 1 month after stroke. Cdk5-stained microvessels also colocalized with TUNEL-positive cells, suggesting that Cdk5 up-regulation in ischemia-affected EC may be associated with cell death pathways. This suggests a previously undiscovered role for Cdk5 in the inhibition of angiogenesis/revascularization within perinfarcted regions after stroke. Further studies should examine the role of Cdk5/p35/25 in modulating EC survival and angiogenesis, in detail, and identify the cell signaling pathways both *in vitro* and *in vivo*.

REFERENCES

1. Chae T, Kwon YT, Bronson R, Dikkes E, Li E, Tsai LH (1997) Mice lacking p35, a neuronal specific activator of Cdk5, display cortical lamination defects, seizures, and adult lethality. *Neuron* 18:29–42.
2. Chen J, Liu B, Liu Y, Han Y, Zhang Y, Lu L, Zhen Y, Hui R (2002) A novel gene IC53 stimulates ECV304 cell proliferation and is up-regulated in failing heart. *Biochem Biophys Res Commun* 294:161–166.
3. Cheung ZH, Ip NY (2004) Cdk5: mediator of neuronal death and survival. *Neurosci Lett* 361:47–51.
4. Cristofol RM, Gasso S, Vilchez D, Pertusa M, Rodriguez-Farre E, Sanfeliu C (2004) Neurotoxic effects of trimethyltin and triethyltin on human fetal neuron and astrocyte cultures: a comparative study with rat neuronal cultures and human cell lines. *Toxicol Lett* 152:35–46.
5. Cruz JC, Tsai LH (2004) A Jekyll and Hyde kinase: roles for Cdk5 in brain development and disease. *Curr Opin Neurobiol* 14:390–394.

6. Eke A, Conger KA, Anderson M, Garcia JH (1990) Histological assessment of neurons in rat models. *Stroke* 21:299–304.
7. Gao C, Negash S, Guo HT, Ledee D, Wang HS, Zelenka P (2002) CDK5 regulates cell adhesion and migration in corneal epithelial cells. *Mol Cancer Res* 1:12–24.
8. Gao CY, Stepp MA, Fariss R, Zelenka P (2004) CDK5 regulates activation and localization of Src during corneal epithelial wound closure. *J Cell Sci* 117:4089–4098.
9. Green SL, Kulp KS, Vulliet R (1997) Cyclin-dependent kinase 5 activity increases in rat brain following ischemia. *Neurochem Int* 31:617–623.
10. Hayashi T, Warita H, Abe K, Itoyama Y (1999) Expression of cdk5 and its activator p35 in rat after middle cerebral artery occlusion. *Neurosci Lett* 265:37–40.
11. Kesavapany S, Li BS, Amin A, Zheng YL, Grant P, Pant HC (2004) Neuronal cyclin-dependent kinase 5: role in nervous system function and its specific inhibition by the CDK5 inhibitory peptide. *Biochim Biophys Acta* 697:143–153.
12. Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM (1993) Prognostic value of blood vessel density in ischaemic stroke. *Lancet* 342:742.
13. Krupinski J, Kaluza J, Kumar P, Kumar S, Wang JM (1994) Role of angiogenesis in patients with cerebral ischaemic stroke. *Stroke* 25:1794–1798.
14. Krupinski J, Stroemer P, Slevin M, Marti E, Kumar P, Rubio F (2003) Three-dimensional structure of newly formed blood vessels after focal cerebral ischaemia in rat. *Neuroreport* 14:1171–1176.
15. Lazaro JB, Kitzmann M, Cavadore JC, Muller Y, Clos J, Fernandez A, Lamb NJC (1996) Cdk5 expression and association with p35^{necksa} in early stages of rat cerebellum neurogenesis; tyrosine dephosphorylation and activation in post-mitotic neurons. *Neurosci Lett* 218:21–24.
16. Lew J, Winkfein RJ, Paudel HK, Wang JH (1992) Brain praline-directed protein kinase is a neurofilament kinase which displays high sequence homology to p34cdc2. *J Biol Chem* 267:25922–25926.
17. Love S (2003) Neuronal expression of cell-cycle related proteins after brain ischemia in man. *Neurosci Lett* 353:29–32.
18. Nath R, Davis M, Probert AW, Kupin NC, Ren X, Schielke GP, Wang KKW (2000) Processing of Cdk5 activator p35 to its truncated form (p25) by calpain in acutely injured neuronal cells. *Biochem Biophys Res Commun* 274:16–21.
19. O'Hare MJ, Kushwaha N, Zhang Y, Aleyasin H, Callaghan SM, Slack RS, Albert PR, Vincent I, Park DS (2005) Differential roles of nuclear and cytoplasmic cyclin-dependent kinase 5 in apoptotic and excitotoxic neuronal death. *J Neurosci* 25:8954–8966.
20. Osuga H, Osuga S, Wang F, Fetni R, Hogan MJ, Slack RS, Hakim AM, Ikeda JE, Park DS (2000)

- Cyclin-dependent kinases as a therapeutic target for stroke. *Proc Natl Acad Sci USA* 97:10254–10259.
21. Patzke H, Tsai LH (2002) Calpain-mediated cleavage of the cyclin-dependent kinase-5 activator p39 to p29. *J Biol Chem* 277:8054–8060.
22. Rashidan J, Iyirhiaro G, Aleyasin H, Rios M, Vincent I, Callaghan S, Bland RJ, Slack RS, During MJ, Park DA (2005) Multiple cyclin-dependent kinases signals are critical mediators of ischaemia/hypoxic neuronal death in vitro and in vivo. *Proc Natl Acad Sci USA* 102:14080–14085.
23. Scandinavian Stroke Study Group (1985) Multi-center trial of haemodilution in ischemic stroke—background and study protocol. *Stroke* 16:885–890.
24. Segura I, Serrano A, De Buitrago GG, Gonzalez MA, Abad JL, Claveria C, Gomez L, Bernad A, Martinez-AC, Riese HH (2002) Inhibition of programmed cell death impairs in vitro vascular-like structure formation and reduces in vivo angiogenesis. *FASEB J* 8:833–841.
25. Sharma MR, Tuszynski GP, Sharma MC (2004) Angiostatin-induced inhibition of endothelial cell proliferation/apoptosis is associated with the downregulation of cell cycle regulatory protein Cdk5. *J Cell Biochem* 91:398–409.
26. Sharma P, Sharma M, Amin ND, Albers RW, Pant HC (1999) Regulation of cyclin-dependent kinase 5 catalytic activity by phosphorylation. *Proc Natl Acad Sci USA* 96:11156–11160.
27. Slevin M, Kumar S, Gaffney J (2002) Angiogenic oligosaccharides of hyaluronan induce multiple signalling pathways affecting vascular endothelial cell mitogenic and wound healing responses. *J Biol Chem* 277:41046–41059.
28. Slevin M, Krupinski J, Kumar P, Gaffney J, Kumar S (2005) Gene activation and protein expression following ischaemic stroke: strategies towards neuroprotection. *J Cell Mol Med* 9:85–102.
29. Slevin M, Gaffney J, Kumar P, Kumar S, Krupinski J (2006) Can angiogenesis be exploited to improve stroke outcome? Mechanisms and therapeutic potential. *Clin Chem* 111:171–183.
30. Tang X, Wang X, Gong X, Tong M, Park D, Xia Z, Mao Z (2005) Cyclin-dependent kinase 5 mediates neurotoxin-induced degradation of the transcription factor myocyte enhancer factor 2. *J Neurosci* 25:4823–4834.
31. Tsai LH, Takahashi T, Caviness VS Jr, Harlow E (1993) Activity and expression pattern of cyclin-dependent kinase 5 in the embryonic mouse nervous system. *Development* 119:1029–1040.
32. Wang J, Liu SH, Fu YP, Wang JH, Lu YM (2003) Cdk5 activation induces hippocampal CA1 cell death by directly phosphorylating NMDA receptors. *Nat Neurosci* 6:1039–1047.
33. Weishaupt JH, Kussmaul L, Grottsch P, Heckel A, Rohde G, Romig H, Bahr M, Gillardon F (2003) Inhibition of Cdk5 is protective in necrotic and apoptotic paradigms of neuronal cell death and prevents mitochondrial dysfunction. *Mol Cell Neurosci* 24:489–502.
34. Weishaupt JH, Neusch C, Bahr M (2003) Cyclin-dependent kinase (CDK5) and neuronal cell death. *Cell Tissue Res* 312:1–8.

35. Zheng YL, Kesavapany S, Gravel M, Hamilton RS, Schubert M, Amin N, Albers W, Grant P, Pant HC (2005) A CDK5 inhibitory peptide reduces tau hyperphosphorylation and apoptosis in neurons. *EMBO J* 24:209–220.

36. Zukerberg LR, Patrick GN, Nikolic M, Humbert S, Wu CL, Lanier LM, Gertler FB, Vidal M, Van Etten RA, Tsai LH (2000) Cables links Cdk5 and c-Abl and facilitates Cdk5 tyrosine phosphorylation, kinase upregulation and neurite outgrowth. *Neuron* 26:633–646.