Emerging evidence has shown that tumor suppressor p53 expression is enhanced in response to brain ischemia/hypoxia and that p53 plays a critical role in the cell death pathway in such an acute neurological insult. However, the mechanism remains unclear. Recently, it was reported that Peg3/Pw1, originally identified as a paternally expressed gene, plays a pivotal role in the p53-mediated cell death pathway in mouse fibroblast cell lines. In this study, we found that Peg3/Pw1 expression is enhanced in peri-ischemic neurons in rat stroke model by in situ hybridization analysis, where p53 expression was also induced by immunohistochemical analysis. Moreover, we found that p53 was co-localized with Peg3/Pw1 in brain ischemia/hypoxia by double staining analysis. In human neuroblastoma-derived SK-N-SH cells, Peg3/Pw1 mRNA expression is enhanced remarkably at 24 h post-hypoxia, when p53 protein expression was also enhanced at high levels. Subcellular localization of Peg3/Pw1 was observed in the nucleus. Adenovirus-mediated high dose p53 overexpression induced Peg3/Pw1 mRNA expression. Overexpression of Peg3/Pw1 reduced cell viability under hypoxic conditions, whereas that of the C-terminal-deleted mutant and anti-sense Peg3/Pw1 inhibited hypoxia-induced cell death. These results suggest that Peg3/Pw1 is involved in the p53-mediated cell death pathway as a downstream effector of p53 in brain ischemia/hypoxia.

Clarification of the molecular events underlying the cell death pathway in response to brain ischemia/hypoxia is an important step toward the development of fundamental treatment for such an acute neurological insult. However, the molecular mechanism in which neuronal cells undergo cell death in brain ischemia/hypoxia remains obscure. Previous reports have shown that p53 is activated under hypoxic conditions in vitro (1, 2) and also in peri-ischemic regions of the animal focal stroke model (3, 4). Moreover, it has also been shown that neuronal cells deficient in the p53 gene are significantly protected from excitotoxic and ischemic insult (5, 6), indicating that the p53-mediated signaling cascade contributes remarkably to neuronal cell death in such acute brain injuries. Peg3/Pw1, originally identified as a paternally expressed gene, is a large molecule containing 12 zinc finger-like domains and 2 proline-rich periodic repeat domains (7). The expression of Peg3/Pw1 mRNA was detected ubiquitously in all tissues at low levels during mouse development, and in adult mouse, Peg3/Pw1 expression was observed abundantly in central nervous system and skeletal muscle (7, 8). In human, Peg3/Pw1 mRNA was observed at high levels in ovary, testis, and placenta and at modest levels in brain and pancreas (9). Its subcellular localization was observed in the nucleus (10), and Peg3/Pw1 expression was also induced by immunohistochemical analysis. In this study, we examined the induction of p53 protein expression was also observed by immunohistochemical analysis. In this study, we examined the induction of p53 protein expression was also observed by immunohistochemical analysis. In this study, we examined the induction of p53 protein expression was also observed by immunohistochemical analysis. In this study, we examined the induction of p53 protein expression was also observed by immunohistochemical analysis. In this study, we examined the induction of p53 protein expression was also observed by immunohistochemical analysis.
Mount Sinai Medical Center, New York, NY) were digested with BanHI and XhoI and then directly subcloned into pEGFP plasmid (CLONTECH, Palo Alto, CA), which produces the N-terminal GFP-tagged protein under the control of CMV promoter. To generate antisense Peg3/Pw1 construct, Peg3/Pw1 cDNA was subcloned into pCDNA3(+) (Invitrogen) using LipofectAMINE reagent (Invitrogen) as described by the manufacturer. Stable cell lines were generated by selecting the transfected cells in neomycin (1200 μg/ml) for about 2 weeks.

Cell Culture—Rat cortex neurons or astroglial cells were prepared from Sprague-Dawley rat E17 embryo or postnatal day 1 pups, respectively as described previously (13, 14). Cortical hemispheres were dissected, and the meninges were carefully removed. Brain tissues were digested with collagenase B (Roche Molecular Biochemicals) at 37 °C for 15 min. Tissues were further dissociated by repeated trituration. The neuronal cells were seeded at a density of 1 × 10^6 cells/cm^2 in poly-l-lysine (10 μg/ml)-coated plates (Falcon Lab and Maintainware, Lincoln Park, NJ) and grown in Dulbecco’s modified Eagle’s medium supplemented with B27 supplement (Invitrogen), 30 mM glucose, and 0.5% (ν/ν) penicillin-streptomycin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% room air. In experiments using primary cultured neurons, all experiments were performed in 8- to 10-day-old cultures. Astroglial cells were seeded at a density of 1 × 10^6 cells/cm^2 in 175-cm^2 culture flasks and grown in α-modified Eagle’s medium with 10% fetal calf serum. Cells were trypsinized with 0.2% Triton X-100 for 15 min at 37 °C and counted to 0.2% Triton X-100 for 10 min at room temperature and then plated with a primary antibody in blocking buffer overnight at 4 °C. The membrane was washed four times in PBS containing 0.3% Tween 20, probed with the secondary antibody in blocking buffer for 1 h at room temperature, and washed again in PBS containing 0.05% Tween 20. Detection of signal was performed with an enhanced chemiluminescent detection kit (ECL, Amersham Biosciences, Inc.) by generating autoradiographs. The primary antibodies were used anti-p53 monoclonal antibody (DO-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-Bax polyclonal antibody (number P-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-KDEL monoclonal antibody (StressGen Biotechnologies Corp., San Diego, CA), and anti-β-tubulin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in RT-PCR. RNA isolation, Northern Blot Analysis, and RT-PCR—Total RNA derived from 2 × 10^7 cells was extracted from cells by the acid guanidium-thiocyanate/phenol chloroform method. For Northern blot analysis, total RNA (20 μg/lane) was separated by electrophoresis on 1.0% agarose/formide gels and transferred overnight onto polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA). The membrane was prehybridized for 1 h at 65 °C in hybridization buffer containing 0.9 mM NaCl, 90 mM sodium citrate, pH 7.0 containing 5× Denhardt’s solution, SDS (0.5%), and heat-denatured salmon sperm DNA (100 ng/ml). cDNA probe was radiolabeled with [32P]dCTP (NEN/PerkinElmer Science) by a random labeling kit according to the manufacturer’s manual (Technical Support, Boston, MA). The cDNA probe (5 ng/ml) filters were washed twice with 2× SSC, 0.5% SDS, and 0.2× SSC, 0.5% SDS. For 60 min, respectively, at 65 °C, exposed to x-ray film (Fuji Photo Film), and subjected to autoradiography. For RT-PCR, 5 μg of total RNA was reverse-transcribed using oligo(dT) by reverse transcriptase from Mornoly murine leukemia virus (Invitrogen) in a volume of 25 μl. For PCR amplification, specific oligonucleotide primer pairs (10 pmol each) were incubated with 1 μl of cDNA template in a 20-μl PCR reaction mixture containing 1.5 mM MgCl_2, 25 mM KCl, 10 mM Tris, pH 9.2, mixed deoxyribonucleotides (1 μM each), and 1 unit of Taq polymerase. The sequences of primers used in this study were as follows: rat Peg3/Pw1 sense primer, 5'-GAGAATTCCTTCAATTTATC-3' and rat Peg3/Pw1 antisense primer, 5'-TCTAGAATCTTGCGTTC-3'; human Peg3/Pw1 sense primer, 5'-TACGAGTATGCTGTTGCG-3' and human Peg3/Pw1 antisense primer, 5'-TTGCGGAGAATACACTCTG-3'; human Bax sense primer, 5'-ATGGACGGTTCGCCAGGAGGA-3' and human Bax antisense primer, 5'-CAGACGCTAGGGAAACCGGC-3'; human Grp78 sense primer, 5'-TTATTGGGAAAGAGTTATAC-3' and human Grp78 antisense primer, 5'-ATTGAGAAGCTTCAACGAC-3'; human Peg3/Pw1 sense primer, 5'-TACGAGTATGCTGTTGCG-3' and rat Grp78 antisense primer, 5'-CAATTTGCTTCTGAGTTC-3'; and as an internal control, rat or human β-actin sense primer, 5'-TGGCCCACTTTATGAGGGTCAGC-3' and rat or human β-actin antisense primer, 5'-TAGAAGGATTGCGGGTGACG-3'. Mus musculus Peg3, accession number AF038939; human Bax, accession number NM_004224; human Peg3, accession number XM_042345; human Peg3/Pw1, accession number NM_031445; rat Grp78, accession number S63521. Dilutions of cDNA were amplified for 23–32 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s. The amplified PCR products at each cycle number were analyzed by 1% agarose gel electrophoresis and ethidium bromide staining. The product of constitutively expressed β-actin mRNA served as the internal standard. All the products were assayed in the linear response range of the RT-PCR amplification process; the cycle number used was determined by finding the midpoint of linear amplification on a sigmoid curve for amplification products with cycle numbers of 24–40 plotted against band density. The identity of each PCR product was confirmed by subcloning the amplified cDNAs into the pGem-T vector (Promega) and sequencing. In Northern blot analysis, amplified cDNAs were used in probes to detect human and rat Peg3/Pw1 or human GRP78 transcripts.

Animals with Focal Ischemia—Unilateral permanent MCA-O was performed in 9-week-old male Sprague-Dawley rats (300–325 g) that were purchased from Japan SLC (Shizuoka, Japan) as described previously with minor modification (17, 18). Briefly, rats were anesthetized with halothane (4% for induction, 1.5% for maintenance) in a mixture of 70% oxygen and 30% nitrous oxide delivered through a clamping facemask during surgery. A vertical incision was made between the left orbit and the external auditory canal. The temporalis muscle was cut, and subtemporal craniotomy was performed without removing the zygomatic arch under an operation microscope. The main trunk of the MCA and olfactory tract were observed through the dura mater. The left MCA was exposed by a microsurgical approach, and the MCA was
occluded by bipolar electrocoagulation. The MCA from proximal to the 
ofactory tract to the inferior cerebral vein and the lenticulostriate 
arteries were permanently occluded and transected to avoid recanaliz-
tion. After occlusion of the MCA, the temporaJalis muscle and skin 
were closed in layers, and anesthesia was discontinued. Rectal tem-
perature of rats was maintained at 38.0±0.5°C with a heating pad (TR-100, 
PS-100; Fine Science Tools, Belmont, CA) during the surgery. Brains 
were removed, and serial brain sections (10–15 μm) were obtained from 
the frozen brains with a cryostat and stored in a tightly closed case at 
–80°C.

In Situ Hybridization and Immunohistochemistry—Frozen 
sections (10–15 μm-thick) of adult mouse brain were cut in various planes and 
thick-mounted onto poly-l-lysine-coated slides. [35S]-Labeled or digoxi-
genin-labeled cRNA probes (sense or antisense) were prepared by in 
vitro transcription using T7 or SP6 polymerase from the coding of rat 
Peg3/Pw1 cDNA subcloned into pGEM-T vector (Promega). All steps of 
in situ hybridization reactions were carried out essentially according to 
Simmons et al. (19). Briefly, the sections were dried, fixed in 4% formal-
hyde, treated with proteinase K (10 μg/ml, room temperature, 30 
min), acetylated, dehydrated, and air-dried prior to hybridization over-
night at 55°C in a humidified chamber with 150–200 μl of hybridiza-
tion buffer (10% sodium dextran sulfate, 20 mM Tris-HCl, pH 8.0, 0.3 M 
NaCl, 0.2% sarkosyl, 0.02% heat-denatured salmon sperm DNA, 1× 
Denhardt’s solution, 50% formamide) with a cRNA probe. After being 
rinse in 5× SSC at 60°C for 20 min and washed in 50% formamide/2× 
SSC at 60°C for 30 min, sections were subjected to RNase digestion for 
20 min at 37°C (1 μg/ml RNase A) and washed in 50% formamide/2× 
SSC at 60°C for 30 min. For detection of hybridized digoxigenin-labeled 
cRNA probes, anti-digoxigenin antibody conjugated to alkaline phos-
phatase (Roche Molecular Biochemicals) was reacted with 1:500 dilu-
tion, and color was developed by incubation with 4-nitro blue tetrazo-
lium chloride. For detection of hybridized [35S]-labeled cRNA probes, 
sections were exposed to x-ray film (Fuji Photo Film) and subjected to 
autorigraphy. For immunohistochemistry, frozen sections were 
dried, fixed in 4% paraformaldehyde solution, incubated in blocking 
buffer (1× PBS, 5% non-fat dried milk) for 1 h at room temperature, and 
reacted with anti-p53 monoclonal antibody (PharMingen) in blocking 
buffer for 1 h at room temperature, followed by incubation with Alexa 
Fluor 488 anti-mouse IgG conjugate (Molecular Probes, Eugene, OR) or 
biotinylated anti-mouse IgG secondary antibody (Vector Laboratories 
Inc., Burlingame, CA). For detection of fluorescent secondary antibody 
binding sites, sections were photographed under laser confocal micros-
copy (Carl Zeiss LSM510). Sites of biotinylated secondary antibody 
binding were visualized using a Vectastain Elite ABC kit according to 
manufacturer’s manual (Vector Laboratories Inc., Burlingame, CA). For 
in situ hybridization of p53 protein by immunohistochemistry and rat 
Peg/Pw1 mRNA by in situ hybridization, frozen sections were reacted 
with anti-p53 monoclonal antibody (PharMingen) followed by incubu-
tion with Alexa Fluor 488 anti-mouse IgG conjugate (Molecular Probes, Eugene, OR). The sections were photographed under laser confocal microscopy (Carl Zeiss LSM510) subsequent to in situ hybridization analysis using digoxigenin-labeled cRNA probe for rat Peg/Pw1.

RESULTS

Peg3/Pw1 Expression Is Enhanced under Ischemic/Hypoxic Conditions—Using primary cultured astroglial cells exposed to hypoxia, we have identified the genes whose expressions are enhanced in hypoxic insult by differential display methods as described previously (20). As shown in Fig. 1A, Peg3/Pw1 mRNA expression was induced 16–24 h after exposure to hy-
poxia and returned to basal levels upon re-oxygenation in rat primary cultured astroglial cells. Next, to investigate what kind of insults induce Peg3/Pw1 mRNA expression, we exposed primary cultured astroglial cells to various stresses and per-
fomed Northern blotting analysis using total RNAs extracted from 
those astroglial cells. Enhanced Peg3/Pw1 mRNA expressions were 
observed in hypoxia or heat shock stress (Fig. 1B). We then examined whether Peg3/Pw1 mRNA expression was up-regulated in primary cultured cortex neurons under hypoxic conditions by RT-PCR analysis. We exposed rat primary cultured cortex neurons to hypoxia or re-oxygenation for the indicated times, and performed RT-PCR analysis using first strand cDNAs synthesized from total RNAs extracted from those neu-
rons as templates. Similar to the results in astroglial cells, 

Peg3/Pw1 mRNA expression was enhanced 12–24 h post-hy-
poxia and returned to basal levels upon re-oxygenation in pri-
mary cultured neurons (Fig. 1C). Previous reports have shown that in human tissues Peg3/Pw1 expression is abundant in ovary, placenta, testis, and brain and that in adult mouse the highest expression levels are observed in brain and muscle (7–10). We then examined tissue distributions of Peg3/Pw1 in adult rat tissues by Northern blotting analysis. We found that Peg3/Pw1 mRNA expression was remarkably abundant in brain, and we could not detect Peg3/Pw1 mRNA expression in other organs including heart, lung, liver, muscle, and kidney in our experiment (Fig. 1D).

Peg3/Pw1 mRNA Expression Is Induced in Brain Ischemia/ 
Hypoxia—To address whether Peg3/Pw1 mRNA expression is enhanced in brain ischemia/hypoxia, we performed in situ hy-
bridization using [35S]-labeled Peg3/Pw1 cRNA probe in serial
brain coronal sections derived from the rat permanent MCA-O model. Fig. 2, A–D shows that in the rat permanent 6-h MCA-O model, Peg3/Pw1 mRNA expression was enhanced in peri-ischemic lesions including caudate putamen, globus pallidus, cingulate cortex, and thalamus. The signals in the contralateral side to ischemia were also observed at high levels in septum, thalamus, hypothalamus, and hippocampus, at moderate levels in neocortex, and at low levels in basal ganglia (globus pallidus, caudate putamen), which is consistent with previous reports that Peg3/Pw1 expression is abundant especially in hypothalamus (7–10). Signals were only observed in sections hybridized with the antisense probe (data not shown). Microautoradiographies show that marked induction of Peg3/Pw1 mRNA signals was observed in the deep peri-ischemic cortex layer (Fig. 2E) and mainly in medium to large-sized cells (about 30–40 μm in diameter) with large nuclei (Fig. 2F), suggesting that this Peg3/Pw1 mRNA expression was enhanced morphologically in typical neuronal cells. These findings indicate that Peg3/Pw1 mRNA is abundant in adult rat brain and that its expression is enhanced in neuronal cells located in peri-ischemic lesions including basal ganglia, thalamus, hypothalamus, and cortex in the rat permanent MCA-O model.

**Peg3/Pw1 mRNA Expression Is Enhanced Downstream of p53 under Ischemic/Hypoxic Conditions**—Previous studies have shown that p53 protein is enhanced under hypoxic conditions in various types of cells including neuronal cells (21) and that Bax gene contains the p53 response element in its promoter region (22), and Bax functions in the cell death pathway through activating caspase-3-like activity downstream of p53 in neurons (23). We then examined whether p53 and Bax expressions were enhanced under hypoxic conditions in human neuroblastoma-derived SK-N-SH cells by Western blotting analysis. As shown in Fig. 3A, both p53 and Bax protein expressions were induced 4 h after exposure to hypoxia, and its enhanced expression was sustained until 24 h after exposure to hypoxia in SK-N-SH cells. We performed Western blotting analysis using anti-p53, Bax, and β-tubulin antibodies. D, RT-PCR first strand cDNAs were synthesized from the total RNAs extracted from SK-N-SH cells being infected with Ad5CMVp53 or AdLacZ at the indicated m.o.i. and were subjected to RT-PCR analysis using the indicated primers. The cycle numbers used were 32 times for Peg3/Pw1, 32 times for Bax, and 25 times for β-actin.
with Ad5CMVP53 or AdLacZ (as control) at the indicated m.o.i. The mRNA expression of Peg3/Pw1 was up-regulated at the m.o.i. of 100, whereas Bax mRNA was up-regulated at the m.o.i. of 50–100, and β-actin mRNA expression levels were not enhanced (Fig. 3D). Similar to the previous reports that high dose adenovirus-mediated p53 overexpression is sufficient to induce cell death in some experimental systems (26), high dose (50–100 m.o.i.) adenovirus-mediated p53 overexpression was sufficient to induce cell death in SK-N-SH cells in our study (data not shown), suggesting that Peg3/Pw1 mRNA expression is up-regulated in the p53-mediated cell death pathway downstream of p53. However, the precise mechanism remains unclear in which Peg3/Pw1 mRNA expression is induced in the p53-mediated cell death pathway in this study.

**Peg3/Pw1 Is Co-localized with p53 in Peri-ischemic Lesions in the Rat Stroke Model—**As shown in Fig. 3, A and B, we can find that hypoxic insult causes the induction of p53 protein and Peg3/Pw1 mRNA in the neuronal SK-N-SH cell line. Moreover, we can show that Peg3/Pw1 mRNA expression is up-regulated in peri-ischemic neurons in the rat focal stroke model by *in situ* hybridization analysis (Fig. 2). Previous reports have shown that under normal conditions p53 protein is rapidly targeted for ubiquitination and degradation, whereas in diverse cellular stresses including ischemia/hypoxia, p53 protein is stabilized and accumulated by unknown mechanisms (27). Furthermore, it is already reported that immunoreactivity of p53 is remarkably up-regulated in severe neuronal damage using animal stroke models (3, 4). We then examined *in vivo* whether p53 and Peg3/Pw1 are co-localized in peri-ischemic neurons using the rat focal stroke model. First, we tested whether p53 immunoreactivity is up-regulated in peri-ischemic neurons of our rat stroke model by immunohistochemical analysis. Similar to previous reports (3, 4), p53 immunoreactivity was enhanced in peri-ischemic areas after 6 h of ischemia (Fig. 4, A–C), and almost no signal of p53 was observed on the contralateral side to ischemia (Fig. 4D). p53 immunoreactivity was induced in the deep layer of cortex on the ischemic side, and high magnification microscopy analysis indicated that p53 immunoreactivity was primarily up-regulated in peri-ischemic neurons (Fig. 4, B and C). Next, to investigate whether p53 and Peg3/Pw1 are co-localized, we performed double staining analysis for p53 with immunohistochemistry and for Peg3/Pw1 mRNA with in situ hybridization analysis in the rat stroke model. As the result, we found that p53 and Peg3/Pw1 mRNA were co-localized in peri-ischemic cortex (Fig. 4, E and F).

**Effect of Peg3/Pw1 on Cell Viability under Hypoxic Conditions and Its Subcellular Localization—**As mentioned above, recent studies have shown that p53 plays an important role in the hypoxia-mediated cell death pathway (5, 6), and Peg3/Pw1 is involved in p53-mediated apoptosis in some experimental systems (11, 12). We then examined whether Peg3/Pw1 is involved in p53-mediated neuronal cell death under hypoxic conditions. Previous studies have reported that truncated Peg3/Pw1 protein (amino acids 1–592; C-terminal-deleted mutant, designated as dnPeg3/Pw1) functions as dominant negative mutant in mediating NF-κB activity and p53/c-Myc-mediated apoptosis in mouse fibroblast cells (8, 12). First, we established stable SK-N-SH cell lines expressing N-terminal GFP-tagged full-length Peg3/Pw1 protein (amino acids 1–1380) or truncated Peg3/Pw1 protein (amino acids 1–592; dnPeg3/Pw1) and investigated the cell viability under hypoxic conditions. To generate stable transfected cell lines, SK-N-SH cells were transfected with empty pEGFP plasmid as the control, pEGFP-full-length Peg3/Pw1 plasmid, or pEGFP-dnPeg3/Pw1 plasmid, and were selected in neomycin. We confirmed that those stable cell lines actually express GFP-tagged proteins by Western blot analysis using anti-GFP monoclonal antibody. As shown in Fig. 5A, N-terminal GFP-tagged full-length Peg3/Pw1 (amino acids 1–1380) and dnPeg3/Pw1 (amino acids 1–592) protein were detected at the molecular mass of about 200 and 97 kDa, respectively. After stably transfected SK-N-SH cells were exposed to hypoxia for the indicated times, we estimated the cell viability by lactate dehydrogenase assay as described under “Experimental Procedures.” Overexpression of full-length Peg3/Pw1 significantly reduced cell viability at the late stage of hypoxia (at 36–48 h post-hypoxia), whereas dnPeg3/Pw1 conferred the protection against hypoxia-induced cell death (Fig. 5B). Furthermore, to confirm the involvement of Peg3/Pw1 in neuronal death, we generated SK-N-SH stable transfectants overexpressing antisense Peg3/Pw1 transcripts as described under “Experimental Procedures” (designated as antisense transfectants). We used SK-N-SH cells stably transfected with empty pCDNA3(-) vector as the control (designated as control transfectants). To test that the induction of Peg3/Pw1 mRNA by hypoxic insult is actually inhibited in antisense transfectants, we performed Northern blot analysis using total RNAs extracted from antisense Peg3/Pw1 transfectants or control transfectants being exposed to hypoxia for the indicated times. As in Fig. 5C, we found that Peg3/Pw1 mRNA induction was inhibited in antisense Peg3/Pw1 transfectants compared with control transfectants. In contrast, the enhanced expression of GRP78 mRNA by hypoxic insult is actually inhibited in antisense transfectants compared with control transfectants. We then assessed cell viability by lactate dehydrogenase assay after exposure of antisense transfectants or control transfectants to hypoxic stress for the indicated times. As in Fig. 5D, viability was significantly protected in antisense transfectants at the late stage of hypoxia (at 36–48 h post-hypoxia), compared with control transfectants, indicating that enhanced expression of Peg3/Pw1 by hypoxic stress promoted hypoxia-mediated neuronal death. For the experiments shown above, studies were performed in parallel with each of cell lines, and representative results are shown.

Previous reports have shown that Peg3/Pw1 is localized in the nucleus (10), so we examined the subcellular localization of Peg3/Pw1 in the stable SK-N-SH cell line. Both N-terminal GFP-tagged full-length Peg3/Pw1 and dnPeg3/Pw1 were detected in the nucleus in SK-N-SH cells (Fig. 5E) and human
cross-talk between Peg3/Pw1 and p53 in brain ischemia/hypoxia.

A, Western blot analysis. 30 µg of cell lysates were extracted from the stable SK-N-SH cell lines transfected with pEGFP plasmid (lane 1; control (Cont)), pEGFP-dnPeg3/Pw1 plasmid (lane 2, or pEGFP-dnPeg3/Pw1 plasmid (lane 3) and subjected to Western blot analysis with anti-GFP antibody. B, viability. The stable SK-N-SH cell lines transfected with pEGFP plasmid, pEGFP-dnPeg3/Pw1 plasmid, or pEGFP-Peg3/Pw1 plasmid were exposed to hypoxia for 0–48 h and then cell viability was assessed by lactate dehydrogenase assay at the indicated times as described under "Experimental Procedures." The viability was plotted as a mean ± S.E. of eight separate experiments. •, control (stable cell line transfected with empty pEGFP vector); ◆, Peg3/Pw1 (stable cell line transfected with pEGFP-Peg3/Pw1 vector); ▲, dnPeg3/Pw1 (stable cell line transfected with pEGFP-dnPeg3/Pw1 vector). * p < 0.05 comparing control against Peg3/Pw1 or dnPeg3/Pw1 using Student’s t test. C, Northern blot analysis. Total RNAs (20 µg/ lane) extracted from SK-N-SH cells stably transfected with empty pCDNA3(−) vector (Control transfectants; upper panels) or antisense P3/Pw1, C-terminal-deleted Peg3/Pw1, or antisense Peg3/Pw1 expressing pCDNA3(−) vector (Anti-sense transfectants; lower panels) after exposure to hypoxia for the indicated times (lane 1, 0 h; lane 2, 6 h; lane 3, 12 h; lane 4, 24 h) were subjected to Northern blot analysis using [32P]dCTP-labeled human GRP78 cDNA probe (left panels) and human Peg3/Pw1 cDNA probe (right panels). The RNA loading was measured by gel staining with ethidium bromide (attached lower panels). D, viability. The stable SK-N-SH cell lines transfected with empty pCDNA3(−) vector (◆, control) or antisense Peg3/Pw1 (carrier) were some differences in the localization between full-length Peg3/Pw1 and dnPeg3/Pw1 in the nucleus. That is, N-terminal peg3/pw1 was observed as a speckled pattern in the nucleus, whereas N-terminal GFP-tagged full-length Peg3/ Pw1 was observed as a rather homogenous pattern in the nucleus, suggesting that the C terminus of Peg3/Pw1 is involved in the determination of intranuclear localization. Fig. 5F shows that the localizations of N-terminal GFP-tagged full-length Peg3/Pw1 and dnPeg3/Pw1 do not change under hypoxic conditions in SK-N-SH cells. Taken together, these results show that Peg3/Pw1, localized in the nucleus, is involved in p53-mediated cell death pathway under hypoxic conditions in SK-N-SH cells.

**DISCUSSION**

In this study, we investigated whether Peg3/Pw1 is involved in the p53-mediated cell death pathway under ischemic/hypoxic conditions. Peg3/Pw1 mRNA expression was up-regulated after exposure of primary cultured astroglial cells, neuronal cells, and human neuroblastoma-derived SK-N-SH cells to hypoxic stress. In SK-N-SH cells, Peg3/Pw1 mRNA and p53 protein expression were induced at the late stage of hypoxic insulin (24 h post-hypoxia), and high dose (100 m.o.i.) adenovirus-mediated p53 transduction was capable of enhancing Peg3/Pw1 mRNA levels. These results, combined with the fact that only high dose adenovirus-mediated p53 overexpression is sufficient to induce neuronal death (26), suggest that Peg3/Pw1 mRNA expression is up-regulated in the p53-mediated cell death pathway by ischemic/hypoxic insult in vitro. In adult rat, Peg3/Pw1 mRNA was detected predominantly in brain, and in rat MCA-O model Peg3/Pw1 mRNA expression was induced abundantly in neurons located in peri-ischemic lesions, especially basal ganglia and thalamus by in situ hybridization analysis. Furthermore, double staining analysis for p53 by immunostaining and for Peg3/Pw1 by in situ hybridization showed that p53 and Peg3/Pw1 are co-localized in peri-ischemic lesions.

In stable SK-N-SH cell lines expressing full-length Peg3/ Pw1, C-terminal-deleted Peg3/Pw1, or antisense Peg3/Pw1, overexpression of full-length Peg3/Pw1 reduced cell viability at the late stage of hypoxia (36–48 h), whereas that of C-termi- nal-deleted Peg3/Pw1 and antisense Peg3/Pw1 inhibited hypoxia-mediated cell death. The localization of full-length or C-terminal-deleted Peg3/Pw1 was observed in the nucleus in SK-N-SH cells, and their localizations were not changed under hypoxic conditions. Taken together, these results suggest that Peg3/Pw1, induced in the nucleus downstream of p53, is involved in the p53-mediated neuronal death pathway in brain ischemia/hypoxia.

How does Peg3/Pw1 work during the p53-mediated cell death pathway in brain ischemia/hypoxia? According to previous reports, Peg3/Pw1 is thought to be involved in neuronal differentiation during development and in modulating offspring growth and maternal behavior in normal adult mouse (10, 28). Peg3/ Pw1 protein is a large molecule containing 12 zinc finger-like domains and 2 proline-rich periodic repeat domains (7), and Peg3/Pw1 protein is a large molecule containing 12 zinc finger-like domains and 2 proline-rich periodic repeat domains (7), and also interacts with Siah1A and Siah2 and is involved in p53-mediated apoptosis in mouse fibroblast cells (12). Both TRAF2 and Siah family contain the RING finger domain in their N terminus, which suggests that they are components of multiprotein complexes, and both are abundant in adult mouse brain (29, 30). Moreover, TRAF2 may reside in the nucleus and directly regulate transcription, independent of its role in cyto-
plasmic signal transduction (31). Siah1A and Siah2 are located in both the nucleus and cytoplasm and are involved in apoptosis, cell cycle arrest, or the protein degradation system (32, 33), indicating that Peg3/Pw1 probably forms multiprotein complexes in the nucleus, and plays a pivotal role in apoptosis, cell proliferation, or differentiation at the level of transcription complexes. Consistent with this speculation, it was reported that Siah1A is observed in the nucleus as punctuate or a speckled pattern in human embryonic kidney 293 cells transiently co-transfected with BAG-1 and Siah1A cDNAs (33). Taken together, these results indicate that downstream of p53, Peg3/Pw1 probably forms multiprotein complexes in the nucleus, and plays a pivotal role in apoptosis, cell proliferation, or differentiation signaling pathways at the transcriptional levels through interacting with some transcriptional co-factors including TRAF2 or Siah family members in the nucleus. However, the precise mechanism how Peg3/Pw1 is induced in p53-mediated neuronal death remains unclear, and it is possible that as in mouse fibroblast cell line (11), Peg3/Pw1 accelerates ischemia/hypoxia-induced neuronal death through translocating Bax proteins from cytosol to mitochondria.

In summary, we found that Peg3/Pw1 is involved in the p53-mediated neuronal cell death pathway in brain ischemia/hypoxia and that Peg3/Pw1 probably forms multiprotein complexes with other proteins in the nucleus and is involved in cell death signaling pathways at the transcriptional levels under stress conditions.

Acknowledgments—We are grateful to Dr. Sassoon (The Mount Sinai Medical Center, New York, NY) for providing mouse Peg3/Pw1 cDNA and C-terminal-deleted Peg3/Pw1 cDNA, Dr. Ross (University of Texas, Houston, TX) for Ad5CMVp53, and Dr. Saito (University of Tokyo, Tokyo, Japan) for AdLacZ.

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Cross-talk between Peg3/Pw1 and p53 in Brain Ischemia/Hypoxia

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Peg3/Pw1 Is Involved in p53-mediated Cell Death Pathway in Brain Ischemia/Hypoxia

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J. Biol. Chem. 2002, 277:623-629.
doi: 10.1074/jbc.M107435200 originally published online October 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M107435200

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