Proapoptotic Role of Human Growth and Transformation-Dependent Protein in the Developing Rat Brain After Hypoxia-Ischemia

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Background and Purpose—Human growth and transformation-dependent protein (HGTD-P) is a new proapoptotic protein and an effector of cell death induced by hypoxia-ischemia (HI). The function of HGTD-P has been investigated in human prostate cancer cells and mouse neurons cultured in vitro. However, whether HGTD-P is involved in regulating the apoptosis of rat neurons is not clear, and the relevance of HGTD-P in HI animal models is still unknown. Therefore, in the present study, we tried to elucidate the role that HGTD-P plays in apoptosis of rat neurons subjected to HI, both in culture and in the developing rat brain in vivo.

Methods—Samples from primary cultured neurons and postnatal day 10 rat brains with HI were collected. RT-PCR, Western blotting, and immunocytochemistry were used to detect the expression and distribution of rat HGTD-P, cleaved caspase 3, and apoptosis-inducing factor (AIF). MTT assay, DAPI, TUNEL, and flowcytometry were used to detect cell viability and apoptosis.

Results—We found that HI upregulated the mRNA and protein levels of HGTD-P in rat neurons in vitro and in vivo. Antisense oligonucleotides (AS) targeted to HGTD-P inhibited the expression of HGTD-P, thus rescuing neuronal viability and attenuating neuronal apoptosis. In addition, we found that HGTD-P played its proapoptotic role by activating caspase 3 and inducing the translocation of AIF to nuclear.

Conclusions—Our findings show that HGTD-P plays a proapoptotic role in the developing rat brain after HI and that it may be a potential target in treating HI-induced brain damage. (Stroke. 2009;40:2843-2848.)

Key Words: HGTD-P □ brain □ hypoxia-ischemia □ apoptosis

HIF-1α, a key component of the cellular response to hypoxia, contributes to both cell survival and cell death induced by hypoxia-ischemia (HI) through regulating different target genes. It can induce the expression of neuroprotective genes such as VEGF and erythropoietin. It can also promote cell death by inducing the expression of prodeath molecules. Several prodeath molecules are known to be downstream effectors of HIF-1α such as BNIP-3, Noxa, and Puma, all of which play important roles in regulating brain damage after HI. Inhibiting the activity of these prodeath molecules will reduce the neurotoxicity of HIF-1α.

Recent studies showed that human growth and transformation-dependent protein (HGTD-P) is another downstream gene of HIF-1α. HGTD-P binds to the hypoxia responsive element of the HGTD-P gene promoter and induces its transcription. Studies on prostate cancer cell line PC3 cells have shown that HGTD-P induces the release of cytochrome C from mitochondria, thus activating the caspase-dependent apoptotic pathway. In contrast, studies on mouse neuronal cultures indicate that HGTD-P induces cell apoptosis by promoting the translocation of apoptosis-inducing factor (AIF) from mitochondria to the nucleus, independent of cytochrome C release and caspase activation.

Collectively, these findings suggest that HGTD-P may function differently in regulating apoptosis in different types of cells or animals.

No studies to date have examined whether HGTD-P is involved in regulating the apoptosis of rat neurons subjected to HI. Moreover, previous studies have been limited to cell culture, so little is known about the relevance of HGTD-P in HI animal models. In the present study, we explored the roles that HGTD-P plays in apoptosis of HI rat neurons, both in culture and in developing brain in vivo.

Materials and Methods

Primary Culture of Rat Cortical Neurons
All animal protocols were approved by the Sichuan University Committee on Animal Research. Cortical neurons were prepared from brains of 18-day-old Sprague-Dawley rat embryos (supplied by Experimental Animal Center of Sichuan University, Chengdu,
were cultured at 37°C in a humidified 5% CO2 atmosphere and used after 10 days in vitro.

To initiate oxygen-glucose deprivation/reperfusion (OGD/R), cortical neurons were exposed to DMEM without serum and glucose in a humidified atmosphere containing 95% nitrogen and 5% CO2 for 3 hours of OGD, neurons were fed with serum and glucose-supplemented original medium, and returned to the incubator under normoxic conditions. The viability of neurons was examined using 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on a Varioskan Flash microplate reader (Thermo Scientific).

**RT-PCR**

RT-PCR was used to determine the expression of the HGTD-P gene. Primer sequences of HGTD-P were designed using Premier 5.0 software as follows: upstream, 5′-ATGTTTCCTGCCCCACTAA-3′; downstream, 5′-CAGGGTGCAAGCATCTCAA-3′. The integrated density value (IDV) of each band was determined using Gel-pro image analysis software (Media Cybernetics). IDV values for HGTD-P were divided by the corresponding values for β-actin to give the relative expression levels of HGTD-P mRNA.

**Cell Extraction and Western Blotting**

Cells were extracted at 4°C in RIPA lysis buffer. Cytosolic and nuclear extracts were prepared using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce) according to the manufacturer’s protocol. Western blotting was carried out to detect HGTD-P, cleaved caspase 3 (CC3), and apoptosis-inducing factor (AIF) as previously described. Anti–HGTD-P polyclonal antibody was produced in rabbits as follows. New Zealand white rabbits were supplied by Experimental Animal Center of Sichuan University, Chengdu, China. Rabbis were inoculated with a keyhole limpet hemocyanin-conjugated polypeptide (KLH-PTDFEKILLWSGRF) corresponding to amino acids 63 to 78 of HGTD-P protein. After eight weeks, blood serum of the rabbit was collected and then purified by affinity chromatography to produce anti-HGTD-P antibody.

**Treatment of Neurons Using Antisense Oligonucleotides**

Two phosphorothioated oligonucleotides corresponding to nucleotides 36 to 56 of HGTD-P were synthetized as follows: sense (SE), 5′-ggcggctgg caattgtttta; and antisense (AS), 5′-taaacaat tcagcgcg. These oligonucleotides were dissolved in Hanks buffer. Cells were grown to 70% confluence in 100-mm dishes, and then transfected with different concentrations of oligonucleotides (1, 3, 5, 7, 10 µmol/L) using Lipofectamine (Roche) according to the manufacturer’s instructions. Control transfections were carried out by adding Hanks buffer to the medium instead of AS or SE. At 12 hours after transfection, cells were exposed to OGD/R. Inhibition of HGTD-P expression was confirmed by Western blotting. Neuronal viability was estimated using MTT as described above. To detect neuronal apoptosis, cells were stained with DAPI and propidium iodide, and the fraction in the sub-G0/G1 phases was determined using flow cytometry.

**Animal Protocols**

Postnatal day 10 Sprague-Dawley rats (half male and half female; supplied by Experimental Animal Center of Sichuan University, Chengdu, China) were anesthetized and then subjected to hypoxia and ischemia using previously described methods. Briefly, the right common carotid artery (CCA) was exposed and ligated with a 7-0 silk suture through a midline cervical incision. After ligation of the CCA, the pups were returned to the cage for 1 hour to recover from anesthesia. Pups were then exposed to hypoxia (8% O2/92% N2) for 2.5 hours and returned to their cage. Rats were killed at the indicated time points after HI, and their brains were removed. Cerebral cortex was dissected and the expression of HGTD-P was detected through RT-PCR, Western blotting, and immunohistochemistry.

**Antisense Oligonucleotide Treatment in the Animal Model**

HGTD-P AS and SE oligonucleotides in Lipofectamine (10 µg of oligonucleotide in a total volume of 5 µL) were injected into the right lateral ventricle 6 hours before HI. As a vehicle control, Lipofectamine mixed with Hanks buffer instead of AS or SE was injected into the right lateral ventricle. In a preliminary study, we tested different doses of AS (5, 10, 15, 20 µg), and found that AS doses above 10 µg did not increase the inhibition of HGTD-P expression. Therefore, we chose 10 µg for our study. The stereotactic intraventricular injection site was chosen to be 2 mm rostral to the bregma, 2 mm lateral to the sagittal suture, and 3 mm below the skull surface. At the indicated time points after HI, rats were killed and their brains were removed for proteins detection and injury evaluation.

**Figure 1.** Expression of HGTD-P in cultured rat neurons subjected to OGD. Neuronal cultures were subjected to OGD for 3 hours and then exposed to normal glucose and oxygen for the indicated periods. A, Total RNA was extracted from neurons and used to synthesize cDNA, which was analyzed by RT-PCR to determine the level of HGTD-P mRNA expression. Representative results of RT-PCR to amplify HGTD-P and β-actin are shown. B, The relative expression levels of HGTD-P mRNA were calculated as integrated density values (IDVs). C, Cell lysates (40 µg) were subjected to Western blotting, and representative results are shown. D, The relative expression levels of HGTD-P protein was calculated as IDVs. E, Analysis of cell viability by MTT measurement. Cell viability was expressed as percentage of the OGD-un-treated controls. It showed significant neuronal death after OGD treatment. From 0 to 72 hours after OGD, the cell viability decreased quickly. From 72 to 120 hours, the cell viability remained similar at a low level.
Evaluation of Neuropathological Injury
Brain injury was evaluated by calculating infarct volume as well as neuropathological scoring and neuronal apoptosis measuring.

To measure the infarct volume, brains were collected, flash frozen, and cut into 10-μm sections. Then 11 sections (500-μm apart) were stained with cresyl violet. The loss of cresyl violet staining was measured using Micro Image (Leica). Infarct volume was calculated by multiplying infarct areas with the distance between sections according to the Mallard method.11

Brain injury in 4 brain regions (cerebral cortex, hippocampus, striatum, and thalamus) was evaluated through neuropathological scoring described by Bona et al.12 Briefly, sections were stained with HE and scored by an observer blinded to the experimental protocol. The cortical injury was graded from 0 to 4; the damage in hippocampus, striatum, and thalamus was assessed both with respect to hypotrophy (shrinkage) (0–3) and observable cell injury/infarction (0–3).

Apoptosis of cerebral cortex was profoundly detected by TUNEL staining with the In Situ Cell Death Detection Kit (Roche) according to the manufacturer’s protocol.

Statistical Analysis
Data are presented as mean±SEM from at least 3 independent experiments. Student t test was used when comparing between 2 groups. ANOVA with Fisher post hoc test was used when comparing more than 2 groups. The value of P less than 0.05 was defined as the threshold for significance.

Results
OGD Upregulated HGTD-P Expression in Cultured Rat Neurons
We detected HGTD-P expression at the mRNA level using RT-PCR and at the protein level using Western blotting. After exposure to OGD/R, the neurons upregulated HGTD-P mRNA notably and the level reached a maximum at 18 hours after OGD/R, corresponding to a 5-fold increase (Figure 1A and 1B). Consistent with this result, Western blotting showed that HGTD-P protein was also upregulated and reached a maximum at 24 hours after OGD/R, corresponding to a 6-fold increase (Figure 1C and 1D). Analysis of cell viability by MTT measurement revealed a significant neuronal death after OGD treatment. From 0 to 72 hours after OGD, the cell viability decreased quickly. From 72 to 120 hours, the cell viability remained similar at a low level (Figure 1E).

Inhibition of HGTD-P Attenuated Apoptosis in Rat Neurons
Transfection with 5 μmol/L AS inhibited most of the endogenous HGTD-P expression (Figure 2A), whereas SE did not affect HGTD-P expression (Figure 2A). Similarly, transfection with the vehicle control did not affect HGTD-P expression (data not shown). Cell viability was higher and the proportion of apoptotic cells was lower in the AS group than in the groups transfected with SE or vehicle only (Figure 2B through 2D). In addition, we found that AS treatment downregulated CC3 in neurons subjected to OGD/R, and it inhibited the translocation of AIF into nucleus after OGD/R injury (Figure 2A).

HI Induced HGTD-P Expression in Rat Model
We found that both HGTD-P mRNA and protein were upregulated in rat brain, with the levels peaking at 36 to 48 hours after HI.
hours after HI. Activation of caspase-3 protein increased and prolonged for 96 hours after HI; and the nuclear AIF was upregulated at 8 hours, fluctuated between 24 to 72 hours, but still remained at a moderate level until 96 hours after HI. The expression patterns of CC3 and nuclear AIF differed between male and female rats from 0 to 24 hours after HI, but almost remained the same from 36 hours after HI. A representative immunohistochemical image of cerebral cortex section cut from a rat euthanized at 48 hours after HI. Arrows indicate the staining for HGTD-P. The results showed that HGTD-P localized predominantly to the neuronal cytoplasm, with extremely low levels in nonischemic regions and higher levels in ischemic regions. Magnification, 400×. E, TUNEL staining was conducted in paraffin-embedded sections of cerebral cortex using the In Situ Cell Death Detection Kit. The HI group contained more apoptotic cells than the sham control. Arrows indicate the positive staining. Magnification, 400×.

**Figure 3.** Expression of HGTD-P in HI rat brain. A, HGTD-P mRNA levels detected by RT-PCR. The results showed that HGTD-P mRNA expression was induced by HI, with levels peaking at 24 to 36 hours after HI. B, Protein levels detected by Western blotting and representative results were shown. C, The relative expression levels of proteins were calculated as IDVs. The results showed that HGTD-P protein was upregulated at 8 hours and peaked at 36 to 48 hours after HI; activation of caspase-3 protein increased and prolonged for 96 hours after HI, and the nuclear AIF was upregulated at 8 hours, fluctuated between 24 to 72 hours, but still remained at a moderate level until 96 hours after HI. The expression patterns of CC3 and nuclear AIF differed between male and female rats from 0 to 24 hours after HI, but almost remained the same from 36 hours after HI. D, A representative immunohistochemical image of cerebral cortex section cut from a rat euthanized at 48 hours after HI. Arrows indicate the staining for HGTD-P. The results showed that HGTD-P localized predominantly to the neuronal cytoplasm, with extremely low levels in nonischemic regions and higher levels in ischemic regions. Magnification, 400×. E, TUNEL staining was conducted in paraffin-embedded sections of cerebral cortex using the In Situ Cell Death Detection Kit. The HI group contained more apoptotic cells than the sham control. Arrows indicate the positive staining. Magnification, 400×.

**AS Targeted to HGTD-P Reduced Brain Damage in a Rat HI Model**

Western blotting showed that AS notably inhibited HGTD-P expression in rat brain subjected to HI, as well as inhibited the activation of caspase 3 and translocation of AIF to nuclear, to the same extent in male and female rats at 48 hours after HI, whereas vehicle alone or SE did not show these effects (Figure 4A). Cresyl violet staining showed that HI caused severe infarcts in rat brains at the same degree both in males and females, and AS decreased the infarct volume significantly (Figure 4B). HE staining and pathological scoring showed remarkable injuries after HI in cerebral cortex, striatum, thalamus, and hippocampus. The pathological scores in AS group were much lower than those in SE group (Figure 4B and 4C). TUNEL staining showed that the proportion of apoptotic neurons was much lower in the AS group than in the SE or vehicle group (Figure 4D and 4E).

**Discussion**

HGTD-P is a newly-discovered proapoptotic protein and an effector of HI-induced cell death. Previous studies showed that HGTD-P exerted proapoptotic effects in a cell type-specific manner. In the present study, we reported for the first time on the role and mechanism of HGTD-P in regulating neuronal apoptosis in developing rat brain subjected to HI. We found that HGTD-P played a proapoptotic role in developing rat brain subjected to HI, thus rescuing neuronal viability and attenuating neuronal apoptosis. These results showed that HGTD-P exerted its proapoptotic effect by activating caspase-3 and promoting AIF translocation into the nucleus. These findings differ from those obtained in mouse neurons, in which the proapoptotic effect of HGTD-P was found to depend only on AIF translocation and not on activation of caspases. This discrepancy may result from the difference in cell types in the 2 studies, or from the difference of the extent and duration of HI. How HGTD-P regulates neuronal apoptosis remains unclear. Previous investigations in PC3 cells showed that HGTD-P promotes cell death by inducing the mitochondrial permeability transition and facilitating the release of cytochrome c,
thus activating caspase 3. Whether this mechanism also functions in rat neurons and how HGTD-P modulates AIF translocation requires further investigation.

The kinetics of the expression of proteins was investigated in our study. In the neonatal HI model, HGTD-P protein was upregulated at 8 hours and peaked at 36 to 48 hours after HI; activation of caspase-3 increased and prolonged for 96 hours after HI; and the nuclear AIF was upregulated at 8 hours, fluctuated between 24 to 72 hours, but still remained at a moderate level until 96 hours after HI. Our results are consistent with those showing extended period of caspase-3 activation and apoptosis in neonatal HI. Since HGTD-P peaks at 36 to 48 hours after HI, it may contribute to the protracted period of caspase activation, AIF translocation, and brain damage. Another molecule related to prolonged neuronal death after HI is BNIP3, which peaks at 48 to 72 hours after HI. The late activation of these proapoptotic molecules suggests an extended therapeutic window for intervention of HI.

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**Disclosures**

None.
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