Telomerase Reverse Transcriptase Upregulation Attenuates Astrocyte Proliferation and Promotes Neuronal Survival in the Hypoxic–Ischemic Rat Brain

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Background and Purpose—Telomerase reverse transcriptase (TERT) is tightly related to the resistance of cells to stress and injury. However, little is known about the roles of TERT in the nervous system. We try to investigate the effects of TERT on the function of astrocytes in developing rat brains subjected to hypoxia–ischemia.

Methods—TERT expression was detected in rat brains with hypoxia–ischemia. In in vitro study, the function of astrocytes with TERT overexpression was measured, and the effects of astrocyte on neuronal apoptosis were examined. Moreover, overexpression or inhibition of TERT was conducted in vivo by gene transduction. Astrocyte proliferation was examined through Ki67 staining. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining and brain infarct volume calculation were used to detect neuronal injury.

Results—Both TERT mRNA and protein were upregulated in neurons within 2 days but shifted to astrocytes at Day 3 after hypoxia–ischemia. Astrocyte proliferation was inhibited with TERT overexpression due to the upregulation of cell-cycle regulatory protein p15. Meanwhile, the apoptosis of neurons increased, whereas neurons were cocultured with conditioned media from astrocytes with TERT inhibition compared with TERT overexpression due to the decrease of neurotrophin-3 expression in astrocytes. Furthermore, Ki67-positive astrocytes and neuronal injury were found to be inhibited in TERT-overexpressing rat brains with hypoxia–ischemia.

Conclusions—TERT attenuates astrocyte proliferation and promotes neuronal survival in the developing rat brain after hypoxia–ischemia, partly through its enhancement of p15 and neurotrophin-3 expression in astrocytes. (Stroke. 2011; 42:3542-3550.)

Key Words: astrocyte • hypoxia–ischemia • neonatal • rat brain • TERT
established both in vivo and in vitro ischemia models using postnatal Day 10 rats and cultured astrocytes and investigated the function of TERT in neonatal HI injury.

Materials and Methods

Detection of TERT Expression in the HI Rat
Sprague-Dawley rats were obtained from Medical Animal Center of Sichuan Province (Chengdu, China). All animal research was approved by the Sichuan University Committee on Animal Research. Postnatal Day 10 rats without gender selection were subjected to right common carotid occlusion plus 8% hypoxia for 2.5 hours for ischemia and hypoxia treatment13 (Supplement I; http://stroke.ahajournals.org). The expression of TERT was detected through reverse transcriptase–polymerase chain reaction, Western blot, and immunohistochemistry (Supplement I).

Cell Culture and Gene Transduction
Astrocytes and neurons were prepared from primary cell cultures of cortical tissues from postnatal Day 1 and embryonic Day 16 pups, respectively (Supplement II).

Plasmids, pcDNA-rTERT bearing the full-length cDNA of rat telomerase reverse transcriptase (rTERT), pcDNA-AS bearing the antisense sequence against rTERT, and pcDNA-SE bearing the sense sequence against rTERT were synthesized and constructed by Jinsite Biotechnology (Supplement III).

Plasmid pcDNA-rTERT (pT) was transduced into astrocytes at passage 3 using the Lipofectamine 2000 reagent (Invitrogen) and the control astrocytes were made by transducing mock plasmid (Mo). To inhibit TERT expression, pcDNA-AS (pAs) was transduced into astrocytes, and the control astrocytes were made by transducing with pcDNA-SE (pSe). Gene transduction was performed using the method as described previously.14 The positive clone cells were selected by 400 μg/mL G418 and maintained in Dulbecco’s modified Eagle’s medium supplemented with 100 μg/mL G418 and 10% fetal bovine serum. Astrocytes at passage 3 after G418 selection were used to perform subsequent experiments.

Detection of Astrocytes Proliferation In Vitro
Astrocytes were plated into a 96-well plate and cultured in serum-free Dulbecco’s modified Eagle’s medium for 24 hours, then serum (10% fetal bovine serum) and 600 ng/mL 5-bromodeoxyuridine (Sigma) were added into the media for incubation for 24 hours and cells were collected for 5-bromodeoxyuridine assay (Supplement IV). In parallel, cells with only serum induction were collected for cell-cycle distribution analysis using flow cytometry (Supplement V) and for cell-cycle regulator detection through Western blot (Supplement VI).

Determination of the Effect of p15 and p21 on Astrocyte Proliferation
Phosphorothioate antisense and sense oligodeoxynucleotides against cell-cycle regulatory proteins p15 or p21 were synthesized by Jinsite Biotechnology. These oligonucleotides were transduced in pT group astrocytes mediated by Lipofectin TM (Invitrogen; Supplement VII). Cell-cycle distribution analysis was conducted using flow cytometry as described previously. Inhibition of p21 or p15 expression was confirmed by Western blot.

Evaluation of the Functional State of Astrocytes
The expression of glutamate aspartate transporter GLAST, glutamate transporter-1, glutamine synthetase, and calcium regulatory protein S-100B were assessed using Western blot. Neural growth factors produced by astrocytes such as transforming growth factor β1, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and neurotrophin-3 (NT-3) were detected using Western blot and enzyme-linked immunosorbent assays (Supplement VIII).

In Vitro Combined Hypoxia and Glucose Deprivation Model of Astrocytes
All cell culture dishes were cultured in glucose-free Dulbecco’s modified Eagle’s medium placed in a humidified incubation chamber at 37°C and flushed with a gas mixture of 93% N2/5% CO2/2% O2 for combined hypoxia and glucose deprivation (CHGD). For 5-bromodeoxyuridine analysis, astrocytes were incubated with 600 ng/mL 5-bromodeoxyuridine (Sigma) for 6 hours before CHGD, then at 6, 12, and 24 hours of treatment, the cells were fixed for 5-bromodeoxyuridine analysis (Supplement IV). NT-3 expression in CHGD astrocytes was also detected through Western blot and enzyme-linked immunosorbent assays as described previously.

Determination of the Effect of Astrocyte-Conditioned Media on Neuronal Apoptosis After Hypoxia
Astrocytes were cultured in glucose-free Dulbecco’s modified Eagle’s medium placed in a humidified incubation chamber at 37°C and flushed with a gas mixture of 93% N2/5% CO2/2% O2 for CHGD treatment. The conditioned media (10 mL) from the astrocytes (a density of 6.4×10⁵ cells/ml) were collected at 0, 12, and 24 hours of the CHGD and centrifuged at 500 g to remove cellular debris. The supernatants were used for the subsequent tests using the method previously described.15

Astrocyte-conditioned media were transferred in equal volume into 3 wells (1 mL/well) with each well containing 4×10⁵ neurons and incubated for 6 hours in a humidified incubation chamber at 37°C and flushed with a gas mixture of 93% N2/5% CO2/2% O2 for hypoxia treatment.

At 6 hours of hypoxia, neuronal apoptosis was detected using the In Situ Cell Death Detection Kit (Roche) for terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and apoptotic index calculation (Supplement IV).

Overexpression or Inhibition of TERT In Vivo
Plasmids of pcDNA-GFAP-EGFP (Mock), pcDNA-GFAP-TERT-EGFP (T), pcDNA-GFAP-SE-EGFP (SE), and pcDNA-GFAP-AS-EGFP (AS) were constructed by Jinsite Biotechnology (Supplement X) and solved in lipofectamine (7 μg of plasmid in 5 μL of lipofectamine). For TERT overexpression analysis, T and Mock were injected into the right lateral ventricle at 12 hours before HI. For TERT inhibition analysis, AS and SE were injected into the right lateral ventricle at 12 hours before HI. As a vehicle control, lipofectamine mixed with Hanks buffer was injected into the right lateral ventricle (Supplement XX). At 1, 3, 5, and 7 days after HI, rats were euthanized and the brains were removed for detection of the plasmid distribution (Supplement XI) and subsequent tests.

Evaluation of Astrocyte Proliferation and Neuropathological Injury
To evaluate astrocyte proliferation induced by HI injury, rat brains were immunolabeled by glial fibrillary acidic protein and Ki67. The numbers of Ki67(+) astrocytes were counted (Supplement XII).

Brain injury was evaluated by measuring infarct volume using cresyl violet staining (Supplement XIII). Apoptosis of cerebral cortex neuron was detected by terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling and neuronal nuclei double-labeling staining (Supplement XIV).

Statistical Analysis
Data are presented as mean±SEM from at least 3 independent experiments. Student t test was used when comparison was made between 2 groups. Analysis of variance with Fisher post hoc test was used when comparing 2 groups. The probability value <0.05 was defined as the threshold for significance.

Results
TERT Expression Upregulated in the Developing Rat Brain With HI
TERT expression was detected by reverse transcriptase–polymerase chain reaction, Western blot, and immunohisto-
chemistry in the developing rat brain subjected to HI treatment. We found that both TERT mRNA and protein were upregulated in rat brain with the mRNA level peaking at 24 hours and protein level peaking at 72 hours after HI (Figure 1A–B). TERT/NeuN or TERT/glial fibrillary acidic protein double-labeling assays showed that TERT was expressed mainly in neurons within 2 days but shifted mainly to astrocytes from 3 to 4 days after HI. In sham controls, TERT was not detectable (Figure 1C).

TERT Inhibits Astrocyte Proliferation and Reduces Astrocytes in the S Phase
To determine the role of TERT in regulating astrocyte proliferation, plasmid containing TERT, Mock, sense, or antisense fragment were transduced into astrocytes. The positive clones were, respectively, termed as pT, Mo, pAs, and pSe. Western blot analyses demonstrated TERT overexpression in pT-transduced astrocytes but not in other groups (Figure 2A). To test cellular proliferation, 5-bromodeoxyuridine assays and flow cytometry detection were performed using pT and control transduced astrocytes. We found that TERT inhibits astrocyte proliferation and reduces the number of astrocytes in the S phase (Figure 2B).

TERT Upregulates p15 and p21 Expression in Astrocytes
Because TERT was found to affect cell-cycle distribution, we studied whether cell-cycle regulators such as p15, p21, p27, and p53 were involved in this distribution. Western blot showed that p15 and p21 proteins were upregulated in pT but not in control transduced astrocytes (Figure 2C). To further determine the effects of p15 and p21 on astrocyte proliferation, antisense oligonucleotides were used to inhibit p15 and p21 expression. We found that p15 but not p21 inhibition enhanced the number of astrocytes in the S phase (Figure 2D).

TERT Upregulates NT-3 Expression in Astrocytes
To investigate the functional state of astrocytes with TERT overexpression, we analyzed the expression of glutamate aspartate transporter (GLAST), glutamate transporter-1, glutamine synthetase, and calcium regulatory protein S-100B using Western blots. Moreover, we detected neural growth

Figure 1. Expression and localization of TERT in the HI rat brain. Reverse transcriptase–polymerase chain reaction (RT-PCR) was used to detect TERT mRNA expression, Western blot was used to detect TERT protein expression, and double-staining confocal immunocytochemistry was used to identify TERT localization in the HI rat brain. A, Total RNA was isolated from the injured hemisphere (n=6 for each time point) and RT-PCR analysis was performed. The integrated density values (IDVs) for TERT were divided by the corresponding values for glyceraldehyde-3-phosphate dehydrogenase to give the relative expression levels of TERT mRNA. TERT mRNA appeared at 12 hours, peaked at 18 to 24 hours after HI, and then decreased gradually. B, Western blot was used to determine TERT protein expression. Equal amount of protein (40 μg) from tissues of injured hemisphere (n=6 for each time point) was separated on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and the expression signals were detected by enhanced chemiluminescence. IDV values for TERT were divided by the corresponding values for β-actin to give the relative expression levels of TERT protein. It showed that TERT protein was upregulated at 1 day and remained at a high level until the fifth day after HI. C, Coexpression of TERT/GFAP (green/red) or TERT/NeuN (green/red) from slides of injured hemisphere (n=6 for each time point) was identified by double-staining confocal immunocytochemistry. It showed that TERT expressed mainly in neurons within 2 days after HI. However, TERT expressed mainly in astrocytes from 3 to 4 days after HI. In sham control, TERT was not expressed. Scale bar=50 μm, and arrows indicated TERT-positive cells. TERT indicates telomerase reverse transcriptase; HI, hypoxia–ischemia; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclei.
factors produced by astrocytes such as transforming growth factor β1, glial cell line-derived neurotrophic factor, brain-derived neurotrophic factor, and NT-3 using Western blot and enzyme-linked immunosorbent assays. We found that NT-3, but not GLAST, glutamate transporter-1, glutamine synthetase, S-100B, transforming growth factor β1, brain-derived neurotrophic factor, or glial cell line-derived neurotrophic factor, was significantly increased in pT transduced astrocytes (Figure 3A–B). Our findings suggest that the important physiological functions of astrocytes such as the capacity to clear glutamate, synthesize glutamine, and buffer calcium were not changed by TERT overexpression. However, NT-3 upregulation is an important functional change by TERT overexpression in astrocytes.

**Proliferation of Astrocytes Subjected to CHGD In Vitro**

Because TERT overexpression inhibits astrocyte proliferation, we further determined the proliferation of astrocytes with or without TERT inhibition after CHGD. Astrocytes were subjected to glucose-free media with 93% N₂/5% CO₂/2% O₂ treatment for 6 hours, 12 hours, or 24 hours to mimic HI in vitro, and 5-bromodeoxyuridine analysis was used to detect cell proliferation. We found that cell proliferation in the pT group was much less than that in Mock, pAs, and pSe groups. Moreover, Western blot showed that TERT overexpression inhibited cell proliferation and number in the S phase compared with other groups without TERT expression (P < 0.05). Western blot was used to detect the expression of cell-cycle regulators such as p15, p21, p27, and p53. It showed that astrocytes overexpressing TERT upregulated p15 and p21 but not p27 and p53 proteins. D, pT astrocytes were treated with antisense oligonucleotides against p21 or p15. Western blot showed that antisense oligonucleotides inhibited p21 or p15 expression. Flow cytometry detection showed that astrocytes with p15 inhibition increased the number of astrocytes in the S phase compared with other groups without p15 inhibition (P < 0.05), whereas astrocytes with p21 inhibition did not change the number of astrocytes in the S phase compared with other groups without p21 inhibition. TERT indicates telomerase reverse transcriptase; BrdU, 5-bromodeoxyuridine.
TERT Prolongs NT-3 Expression in CHGD Astrocytes

Because TERT overexpression enhanced NT-3 expression in astrocytes, we further determined the roles of TERT in regulating NT-3 expression in astrocytes with CHGD. We found that TERT and NT-3 were induced by CHGD treatment in vitro. TERT expression appeared at 6 hours and gradually increased until 24 hours after CHGD. NT-3 expression appeared earlier at 2 hours and also gradually increased until 24 hours after CHGD. However, when TERT was inhibited using antisense oligonucleotides (pAs), the increased NT-3 expression was inhibited. Enzyme-linked immunosorbent assays showed the NT-3 level in pAs group was only approximately 40% of pSe group at 24 hours of HI, which is similar to the findings detected by Western blots (Figure 4C).

Astrocyte-Conditioned Media Protects Neurons From Hypoxia Damage In Vitro

Because TERT can regulate the expression of neural growth factors of astrocytes such as NT-3, the supernatant from astrocytes with different TERT expression levels might have different neuronal protection potential. To detect the role of astrocytic TERT in neuronal apoptosis after hypoxia, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining was performed to test neuronal apoptosis with TERT sense (pSe) or antisense (pAs) treated astrocyte-conditioned media. We found that neurons cultured with astrocyte-conditioned media without CHGD treatment had significant apoptosis after hypoxia for 6 hours. However, when neurons were cultured in astrocyte-conditioned media with 12 hours or 24 hours of CHGD treatment, apoptosis decreased. Moreover, there were more apoptotic neurons cultured in media from the pAs group compared with the pSe group (Figure 5A). After measuring the apoptotic index, we found that the apoptosis of neurons cultured in media from the pAs group was much higher than that in the pSe group ($P<0.05$; Figure 5B). To further determine the involvement of NT-3 in neuronal protection, we detected the NT-3 amount in astrocytes supernatant using enzyme-linked immunosorbent assays. We found that the NT-3 amount was lower in pAs than that in the pSe group with CHGD treatment for 12 hours and 24 hours ($P<0.05$; Figure 5C).

Effect of TERT on Astrocyte Proliferation and Brain Injury Induced by HI In Vivo

Plasmids producing TERT protein (T) or TERT antisense oligonucleotides (AS) were used to overexpress or inhibit TERT expression in vivo. Western blot analyses revealed that TERT was overexpressed in the T group and inhibited in the As group, whereas neither Mock (M) nor sense oligonucleotides (SE) affected TERT expression (Figure 6B). Accordingly, the expression of p15 and NT-3 was enhanced or attenuated with TERT overexpression or inhibition (Figure 6A). Double immunolabeling showed that plasmids were expressed mainly in astrocytes but not in neurons after transduction (Figure 6B).

To further study the TERT effect on astrocyte proliferation induced by HI, we studied Ki67 (cellular proliferation marker) expression in astrocytes after HI treatment using immunohistochemistry. In sham controls, Ki67(+) astrocytes were rarely detected. However, Ki67(+) astrocytes were increased at Days 3, 5, and 7 after HI. Moreover, compared with Mock and SE groups, the Ki67(+) astrocytes were significantly decreased in the T group and increased in the AS group at the previously mentioned time points (Figure 6C).

To determine the effect of TERT on brain injury, we measured infarct volume of brains at Days 3, 5, and 7 after HI using cresyl violet staining. We found a significant decrease of brain infarct volume in the T group and an increase of brain...
infarct volume in the AS group compared with that in Mock or SE groups (Figure 6D). To analyze the effects of TERT on neuronal apoptosis after HI, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling staining with neuronal marker, neuronal nuclei expression was detected. Terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling-positive neurons were increased at Days 3, 5 and 7 after HI compared with that in sham rats. Moreover, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling-positive neurons were significantly decreased in the T group and increased in the AS group compared with those in Mock and SE groups (Figure 6D).

**Discussion**

In this study, we first demonstrated that TERT upregulation attenuates astrocyte proliferation and promotes neuronal survival in developing rat brains after HI. This newly found function of TERT may be related to the enhancement of p15 and NT-3 expression in astrocytes.
In vivo experiments, we found that TERT expressed mainly in neurons within 2 days but then shifted to astrocytes the third day after HI. The early regulation of TERT in neurons may suggest an instant reaction of TERT on neurons with HI. This is in agreement with previous reports that TERT upregulation in neurons ameliorates neuronal death induced by HI. The later upregulation of TERT in astrocytes suggests a chronic role of TERT, which is unknown at present. Therefore, in this study, we focused on investigating the functions of TERT in astrocytes and the possible mechanisms behind it.

TERT is induced in the developing rat brain and cultured astrocytes after HI. However, the telomerase RNA component and telomerase activity are undetectable. Because telomerase RNA serves as a template for telomere synthesis and is essential for telomerase activity, the absence of telomerase RNA may contribute to the lack of telomerase activity in the developing rat brain. Our findings are in line with a previous report that telomerase activity and telomerase RNA are undetectable in rat adult brains. Therefore, TERT might exert its functions through a telomerase activity-independent way in neonatal rat brains.

As we know, the original function of TERT is to construct telomerase activity and thus to maintain telomere length during cell division. However, recent advances have clearly demonstrated that TERT exerts many other functions beyond maintaining telomere length. These functions include the regulation of Ca²⁺ distribution, metabolism, growth factor secretion, mitochondria function, energy balance, and apoptosis. However, whether these functions of TERT exist in the central nervous system, especially in developing brains with HI, is not clear. In the present study, we found that TERT did not change the expression of GLAST, glutamate transporter-1, glutamine synthetase, and S-100B, suggesting that the important physiological function of astrocytes such as the capacity to clear glutamate, synthesize glutamine, and buffer calcium are not changed by TERT. However, TERT was found to enhance NT-3 level and prolong the time window of NT-3 expression after HI in developing rat brains. Because NT-3 exerts a neuroprotective function in cerebral ischemia, the prolongation of NT-3 expression might contribute to the neuroprotective effect of TERT against HI damage in developing rat brains.

Interestingly, we found that TERT upregulation in astrocytes attenuated cell proliferation rather than promotes cell propagation that has been found in other cell types such as vascular endothelium and hepatocyte cells. To investigate the possible mechanisms behind this, we detected cell-cycle regulators such as p15, p21, p27, and p53 in astrocytes with TERT overexpression. We found that TERT upregulated p15 and p21 proteins. Furthermore, attenuating p15 but not p21 inhibited the effect of TERT on astrocyte, suggesting that TERT inhibited astrocyte proliferation through upregulating p15 expression but not p21. The protein of p15 is an important cyclin-dependent kinase inhibitor, which can bind to either Cdk4 or Cdk6 and inhibit the action of cyclin D. During the G1 phase in cell cycle, retinoblastoma protein is phosphorylated by cyclin D/cdk4 and cyclin D/cdk6 complexes. Phosphorylation of Rb inactivates the complexes and allows the release of E2F, thereby allowing entry of cells into the S phase. Therefore, we speculated that p15 upregulation might block the cell-cycle progression of astrocytes through inhibiting the cyclin D-E2F pathway. Unlike p15, p21 upregulation in TERT overexpression astrocytes did not affect cell proliferation and cell-
Figure 6. Effects of TERT on astrocyte proliferation and brain injury induced by HI in vivo. Plasmids producing TERT protein or TERT antisense oligonucleotides were used to overexpress or inhibit TERT expression in vivo. Ki67(+) astrocytes were counted through immunohistochemical experiments to examine astrocyte proliferation induced by HI, and brain injury was evaluated by calculating infarct volume as well as neuronal apoptosis measurement. A, Western blot analysis revealed that pcDNA-GFAP-TERT-EGFP (T) overexpressed TERT and antisense (AS) oligonucleotides inhibited TERT expression induced by HI (*compare AS with T, \( P < 0.05 \)), whereas neither sense (SE) oligonucleotides nor Mock affected TERT expression. Accordingly, the expression of p15 and NT-3 was enhanced or attenuated with TERT overexpression or inhibition (\( n = 6 \) for each group and each time point). B, Double immunolabeling was used to detect plasmid distribution. Representative immunolabeling showed plasmids expressed in astrocytes rather than neurons of brain cortex 24 hours after HI (\( n = 6 \) for each group, scale bar=50 \( \mu m \), arrows indicated EGFP-positive cells). C, Astrocyte proliferation was evaluated through GFAP (green) and Ki67 (red) double staining. The panels showed representative GFAP/Ki67-stained images at Days 3, 5, and 7 after HI (scale bar=20 \( \mu m \)). Percentage of Ki67(+) astrocytes was calculated as described in “Methods” (\( n = 6 \) for each group and each time point). It showed that at Days 3, 5, and 7 after HI, the percentage of Ki67(+) astrocytes in the T group was significantly lower than that in Mock and SE groups (*\( P < 0.05 \)), and the percentage of Ki67(+) astrocytes in the AS group was significantly higher than that in Mock and SE groups (**\( P < 0.05 \)). D, The infarct volume was calculated as described in “Methods.” The average ratio of infarct volume of rats in the T group was smaller than that in Mock and SE groups (\( *P < 0.05 \)), and the average ratio of infarct volume of rats in the AS group was larger than that in Mock and SE groups (\( *P < 0.05 \)), and the average ratio of infarct volume of rats in the AS group was larger than that in Mock and SE groups at 3, 5, and 7 days after HI (\( **P < 0.05, n = 6 \) for each group and each time point). Furthermore, TUNEL-positive neurons were calculated as described in “Methods.” The amount of TUNEL-positive neurons of rats in the T group was less than that in Mock and SE groups (\( *P < 0.05 \)), and the amount of TUNEL-positive neurons of rats in the AS group was more than that in Mock and SE groups at Day 5 after HI (\( **P < 0.05, n = 6 \) for each group). Representative TUNEL and NeuN double staining showed a decreased neuronal apoptosis in the T group and an increased neuronal apoptosis in the AS group at Day 5 after HI compared with Mock and SE groups. Scale bar=50 \( \mu m \), and arrows indicate TUNEL-positive neurons. SH indicates sham group; M, Mock group; T, TERT overexpression group; SE, sense group; AS, antisense group; VE, vehicle control; TERT, telomerase reverse transcriptase; HI, hypoxia-ischemia; NT-3, neurotrophin-3; EGFP, enhanced green fluorescent protein; GFAP, glial fibrillary acidic protein; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end labeling; NeuN, neuronal nuclei.
cycle distribution, suggesting that cell-cycle inhibitors might be cell-specific or tissue-specific-dependent.

In vivo study, we found that TERT attenuated astrocyte proliferation in the developing rat brain subjected to HI. As we know, excessive astrocyte proliferation might be detrimental and contribute to neuronal damage. Reactive astrocytes can form a local biochemical and physical barrier, which will attenuate neuronal survival, axonal regeneration, and circuitry re-establishment after brain damage. Therefore, early inhibition of proliferating reactive astrocytes would inhibit the accumulation of molecules involved in neuronal damage and thus achieve an environment more suitable for neural repair. In this study, we found that with the attenuation of astrocyte proliferation induced by HI, the neuronal apoptosis was also inhibited, suggesting that TERT might exert its neuroprotective function through deleting the detrimental effect of reactive astrocytes. However, whether TERT would inhibit the accumulation of molecules involved in neuronal apoptosis was also inhibited, suggesting that TERT would attenuate neuronal survival, axonal regeneration, and circuitry re-establishment after brain damage. Therefore, early inhibition of proliferating reactive astrocytes would inhibit the accumulation of molecules involved in neuronal damage and thus achieve an environment more suitable for neural repair.

Conclusions

In conclusion, TERT upregulation attenuates astrocyte proliferation and neuronal apoptosis partly by enhancing p15 and NT-3 expression in astrocytes. The later activation of TERT in astrocytes at 3 days after HI suggests an extended therapeutic window for HI-induced neuronal brain damage.

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Disclosures

None.

References

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1. Hypoxia and ischemia treatment for animal

For hypoxia and ischemia (HI) treatment, each pup was anesthetized with ethyl ether, the right common carotid artery (CCA) of each pup 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 h to recover from anesthesia. Pups were then exposed to hypoxia (8% O\textsubscript{2}/92% N\textsubscript{2}) for 2.5 h, and returned to their cage. Sham pups underwent anesthesia and the right CCA was separated without ligation or subsequent hypoxic inhalation. Rats were killed at the indicated time points after HI and their brains were removed for further detection.

To investigate TERT mRNA expression, we performed RT-PCR. Total RNA was isolated from injured hemisphere using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. cDNA was produced using reverse transcriptase (Omniscript, Qiagen). Briefly, each 20 μl reaction contained 500 μM dNTP, 1 μM random decamers, 1 unit reverse transcriptase, and 2.5 μg of total RNA. The reaction was conducted at 37 °C for 60 min and then 92 °C for 10 min to end the reaction. The PCR reactions were performed using a PCR Kit (Ampli-Taq Gold; Perkin-Elmer) with conditions at 95°C for 1 min, 55 °C for 1 min, 68 °C for 90s for 30 cycles. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping gene, was used as an internal control. The primers used in this study were as follows:

TERT forward primer, 5’ -CAGGGAAGCTGGTGA-3’
TERT reverse primer, 5’ -GCAGGAAGGTGCAAGGAAG-3’
GAPDH forward primer, 5’ -GTGA TGGGTGTGAACCACGAG-3’
GAPDH reverse primer, 5’ -CAGTGAGCTTCCCGTTCAGCT-3’

The integrated density value (IDV) of each band was determined using Gel-pro image analysis software (Media Cybernetics). IDV values for TERT (359bp) were divided by the corresponding values for GAPDH (250bp) to give the relative expression levels of TERT mRNA.
We also performed Western blot and immunochemistry to determine TERT protein level. For Western blot, tissues of injured hemisphere were collected in lysis buffer (50mM Tris-HCl, pH8.0, 150mM NaCl, 1% NP40, 0.1% SDS), which includes proteinase inhibitors (1μg/ml aprotinin and leupeptin, 1mM PMSF). Protein concentrations were measured using DeProtein Assay Kit (Bio-Rad). Equal amounts of proteins (40μg) were separated in 10% SDS-polyacrylamide gel, and then transferred on a PVDF membrane (Amersham). The membranes were blocked in 5% milk for 2 h, and incubated with a 1:600 TERT antibody (sc-7212, Santa Cruz) for 2 h at room temperature. After washing with TBS-Tween, the membranes were incubated with 1:2000 horseradish peroxide-conjugated anti-rabbit IgG (NA934, Amersham). Signals were visualized by enhanced chemiluminescence (Amersham). Expression of β-actin was also measured as an internal loading control using an anti β-actin antibody (sc-10731, Santa Cruz, 1:400). IDV values for TERT were divided by the corresponding values for β-actin to give the relative expression levels of TERT protein.

For immunohistochemistry, brain sections (8μM) were cut on a cryostat at the level of 1 mm relative to bregma and mounted on poly-L-lysine-coated slides, then the slides were fixed, dehydrated, and rehydrated. Endogenous peroxidase was blocked, and antigen retrieval was performed. Slides were then treated with primary antibodies against GFAP (TA500337, mouse anti rat GFAP, OriGene, 1:150) or NeuN (MAB377, mouse anti rat NeuN, Millipore, 1:200), and rTERT (sc-7212, rabbit anti rat TERT, Santa Cruz, 1:100) overnight at 4°C. Primary antibodies were visualized with Cy3 or FITC-conjugated secondary antibodies.

2. Cell culture

Brains were cleaned of meninges and blood vessels. Cortical tissues were dissected and then incubated with 0.25% trypsin at 37°C for 10 min. After addition of fetal bovine serum (FBS), the dissociated cells were forced through 70 μM mesh. Cell pellets were collected and plated in poly-L-lysine coated flasks in DMEM with 10% FBS (Hyclone) for astrocytes, in Neurobasal (Gibco) with 2% B27 (Gibco) for neurons, with the final cell densities adjusted to 1x10⁶/ml. The purity of astrocytes
and neurons were assessed separately by staining with GFAP (sc-71141, Santa Cruz, 1:200) and NeuN (MAB377, Millipore, 1:200) using immunocytochemistry.

3. Plasmids used in vitro.

The plasmid pcDNA-rTERT was constructed by insertion of a 3360 bp rat full length TERT cDNA (accession number AY53971) into the BamH I and Not I-digested plasmid pcDNA3 (Invitrogen), pcDNA-AS was constructed by insertion of a 60bp antisense sequence against rTERT into the BamH I and Not I-digested plasmid pcDNA3, and pcDNA-SE was constructed by insertion of a 60bp sense sequence against rTERT into the BamH I and Not I-digested plasmid pcDNA3.

The TERT antisense sequence is
5' -gggatggcatcataggcccctgtcacatctgcttaacaaagtacatcctgggtgtctgg-3';
The TERT sense sequence is:
5' -ccagacacccaggatgtaccttgaagcagatgtgacaggggcctatg atgccatcc-3’.

4. BrdU analysis

Cells were collected and fixed in 100% methanol for 10 min at room temperature. After rehydration in PBS, the cells were treated with 2 M Hcl for 30 min to denature the DNA and allow the antibody access to incorporated BrdU. The membranes of fixed cells were permeabilized with Triton X-100 (0.1% v/v in PBS) for 5 min. Nonspecific antibody binding was blocked by incubation with 5% BSA in PBS at room temperature for 1 h. The cells were then simultaneously incubated with both monoclonal mouse anti-BrdU (MS-1058-P, Neomarkers, 1:100) and polyclonal rabbit anti-GFAP (RB-087-A, Neomarkers, 1:100) overnight at 4 ºC. The incorporated BrdU and GFAP were visualized by successive incubation in goat anti-mouse IgG conjugated to FITC (PA1-84390, Pierce) and donkey anti-rabbit IgG conjugated to Cy3 (771-165-152, Jackson ImmunoResearch) both diluted 1:100. To determine the proportion of proliferating cells, the nuclei were labeled with DAPI. Ten fields were chosen randomly at 400x magnification to count BrdU+ cells and total cells. BrdU+ % = (number of BrdU+ cells/total number counted) ×100%.

5. Cell-cycle distribution analysis
Cells were collected and washed with PBS, fixed in ethanol, then suspended in Guava cell cycle reagent (Guava Technologies), and cell cycle was analyzed using a Guava EasyCyte flow cytometer running Guava ExpressPlus software (Guava Technologies).

6. Analysis of cell cycle regulators by Western blot

Astrocytes were collected in lysis buffer (50mM Tris-HCl, pH8.0, 150mM NaCl, 1% NP40, 0.1% SDS), which includes proteinase inhibitors (1μg/ml aprotinin and leupeptin, 1mM PMSF). Protein concentrations were measured using DCProtein Assay Kit (Bio-Rad). Equal amounts of proteins (30μg) were separated on 12% SDS PAGE gels and transferred onto polyvinylidene difluoride (Millipore). Primary antibodies raised against p53 (3036R-100, BioVision, 1:500), p27 (AB3003, Millipore, 1:400), p21 (ABIN105216, Antibodies-online.com, 1:500), and p15 (RB-028-P0, Thermo Scientific, 1:500) were applied. Antibody binding was detected by peroxidase conjugate secondary antibodies and visualized by the enhanced chemiluminescence detection system (Amersham Biosciences). Expression of β-actin was also measured as an internal loading control using an anti β-actin antibody (sc-10731, Santa Cruz, 1:400).

7. Determination of the effect of p15 and p21 on astrocyte proliferation

The p21 antisense oligonucleotide is 5’-GACATCACCAGGATCGGACAT-3’. The p21 sense sequence is 5’-ATGTCCGATCCTGGTGATGTC-3’. The p15 antisense oligonucleotide is 5’-TTCCAGGAGCTGCCGCACCGT-3’. The p15 sense sequence is 5’-ACGGTGCGGC AGCTCCTGGAA-3’. These oligonucleotides were dissolved in Hanks buffer. For transduction, astrocytes of pT group with TERT expression were grown to 80% confluence, and then 5 μM of oligodeoxynucleotide which we have determined as a optical amount in our preliminary study was mixed with 7μl of Lipofectin™ (Invitrogen) per ml of Opti-MEM medium and was added to the cells for 4 h at 37°C. The cells were washed and serum-free medium (without oligodeoxynucleotide) was added overnight, then proliferation was induced with 24 h of serum stimulation (10% FBS). Cell-cycle distribution analysis was conducted using
flow cytometry as described above. Inhibition of p21 or p15 expression was confirmed by Western blot.

8. Evaluation of functional state of astrocytes

For Western blot, cytosolic proteins of astrocyte were extracted and the content of proteins was determined by following antibodies: anti-GLAST (ABIN343469, Antibodies online, 1:500), anti GLT-1/EAAAT2 (GLT11-S, Alpha Diagnostic Intl.Inc, 1:800), anti-GS (MAB302, Millipore, 1:500), anti- S-100β (GTX11178, Abcam, 1:400), anti-TGF-β1 (sc-31609, Santa Cruz, 1:400), anti-GDNF (sc-13147, Santa Cruz, 1:500), anti-BDNF (sc-20981, Santa Cruz, 1:600), anti-NT-3 (sc-545, Santa Cruz, 1:500), and anti-β-actin (sc-10731, Santa Cruz, 1:400).

For Elisa assay, astrocyte supernatants were collected and centrifuged at 500 ×g to remove cellular debris. The supernatants were used to determine the amounts of TGF-β1, GDNF, BDNF and NT-3 by specific ELISA kits (KAC1688, Biosource; CHC2423, Biosource; E0011r, Usclife; CYT 302, Chemicon). The procedures used were based on the protocols provided by the company. Sample values were within the ranges of the standard curves. The measuring range for TGF-β1 was between 0 and 250 pg/ml, for GDNF was between 0 and 62.5 pg/ml, for BDNF was between 0 and 500pg/ml and for NT-3 was between 0 and 250 pg/ml.

9. Apoptotic index calculation

Ten fields were chosen randomly at 400x magnification to count apoptotic cells and total cells. The apoptotic index (AI) was calculated as follows: AI= (number of apoptotic cells/total number counted) ×100%.

10. Plasmids used in vivo

The plasmid pcDNA-GFAP-EGFP was constructed by insertion of a 1765 bp GFAP promoter (accession number 279974) into the Blg II and HindIII-digested plasmid pcDNA3-EGFP (Addgene). The plasmid pcDNA-GFAP-TERT-EGFP was
constructed by inserting TERT fragment cleaved using BamH I and Not I from pcDNA-rTERT into the same sites of pcDNA-GFAP-EGFP. Meanwhile, internal ribosome entry site (IRES) was cleaved using Not I and Sma I from pIRE-S-neo (Clontech) and inserted into Not I and end-filling Xho I sites of pcDNA-GFAP-EGFP. Therefore, the plasmid pcDNA-GFAP-TERT-EGFP contains IRES between TERT and EGFP coding region, which permits both TERT and EGFP genes to be translated from a single bicistronic mRNA but not in a fusion protein form. The plasmid pcDNA-GFAP-SE-EGFP was constructed by inserting sense sequence against rTERT cleaved from pcDNA-SE between BamHI and Not I cleavage sites of pcDNA-GFAP-EGFP. The plasmid pcDNA-GFAP-AS-EGFP was constructed by inserting antisense sequence against rTERT cleaved from pcDNA-AS between BamHI and Not I cleavage sites of pcDNA-GFAP-EGFP.

In a preliminary study, we tested different doses of plasmids (3, 5, 7, 10, and 15 µg), and found that plasmids doses above 7 µg did not increase the inhibition or overexpression of TERT expression. Therefore, we chose 7 µg for our study. The intraventricular injection site was chosen under stereotaxic apparatus (ASI) to be 1 mm rostral to the bregma, 1.5 mm lateral to the sagittal suture, and 3 mm below the skull surface. At the indicated time points after HI, rats were sacrificed and the brains were removed for subsequent experiments.

11. Detection of the plasmid distribution through double immunolabelling

Brain sections (8µm) were cut on a cryostat at the level of 1 mm relative to bregma and mounted on poly-L-lysine-coated slides, then the slides were fixed, dehydrated, and rehydrated. Endogenous peroxidase was blocked, and antigen retrieval was performed. Slides were then treated with primary antibodies against GFAP (TA500337, OriGene, 1:150) or NeuN (MAB377, Millipore, 1:200) overnight at 4ºC. Primary antibodies were visualized with Cy3-conjugated secondary antibodies. Slides were viewed through a fluorescence microscope.

12. Evaluation of astrocyte proliferation through Ki67 immunolabelling

The brain sections (8µm) were cut on a cryostat from the level of 1 mm relative to bregma and mounted on poly-L-lysine-coated slides, then they were treated with
primary antibodies against mouse anti-GFAP (TA500337, OriGene, 1:100) and rabbit anti-Ki67 (RM-9106, Neomarkers, 1:100) overnight at 4ºC. Primary antibodies were visualized with FITC or Cy3-conjugated secondary antibodies. Numbers of Ki67 (+) astrocytes was counted in sections which selected six unreduplicative visual fields with amplification of 400 times. The mean of the positive cell number from the six fields in each section was defined as positive cell number. Three continuous sections were chosen in each animal. The mean of the positive cell number in these three slices was the number of Ki67 (+) cell.

13. Calculating infarct volume

To measure the infarct volume, brains were collected, flash frozen, and cut into 10 µm sections. Then eleven sections from anterior striatum to posterior hippocampus were selected and taken at equally spaced 0.5-mm intervals. 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 Mallard’s method [1].

14. TUNEL staining

Brain sections (8µm) were cut on a cryostat from the level of 1 mm relative to bregma and mounted on poly-L-lysine-coated slides. Apoptosis of neuron was profoundly detected by TUNEL and NeuN double-labeling staining with the In Situ Cell Death Detection Kit (Roche) and anti-NeuN (MAB377, Chemicon, 1:100) according to the manufacturer's protocol. Numbers of TUNEL positive neurons was counted in sections which selected six unreduplicative visual fields and amplified 400 times. The mean of six number in each section was positive cell number. Three contiguous slices were selective in each sample. The mean of their positive cell number was the number of TUNEL positive neurons.

References: