Biochemical and Biophysical Research Communications 417 (2012) 468–472

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

SIRT2 activity is required for the survival of C6 glioma cells

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ARTICLE INFO

Article history:
Received 19 November 2011
Available online 7 December 2011

Keywords:
Glioma cells
SIRT2
Cell survival
Apoptosis
Caspase-3

ABSTRACT

SIRT2 is a tubulin deacetylase, which can play either detrimental or beneficial roles in cell survival under different conditions. While it has been suggested that reduced SIRT2 expression in human gliomas may contribute to development of gliomas, there has been no study that directly determines the effects of decreased SIRT2 activity on the survival of glioma cells. In this study we applied both pharmacological and molecular approaches to determine the roles of SIRT2 in the survival of glioma cells. Our studies, by conducting such assays as flow cytometry-based Annexin V assay and caspase-3 immunostaining, have indicated that decreased SIRT2 activity leads to apoptosis of C6 glioma cells by caspase-3-dependent pathway. Our experiments have further shown that reduced SIRT2 activity produces necrosis of C6 glioma cells. Moreover, our study applying SIRT2 siRNA has also shown that decreased SIRT2 leads to both necrosis and apoptotic changes of C6 glioma cells. Collectively, our study has provided novel evidence indicating that SIRT2 activity plays a key role in maintaining the survival of glioma cells, and that reduced SIRT2 activity can induce both necrosis and caspase-3-dependent apoptosis of C6 glioma cells. These results have also suggested that inhibition of SIRT2 might become a novel therapeutic strategy for gliomas.

1. Introduction

Sirtuins are the mammalian homolog of Sir2 – a NAD^+-dependent histone deacetylase that has been shown to mediate the aging process of yeast [1]. There are seven members in sirtuin family, including SIRT1–SIRT7 [1]. SIRT2 is a tubulin deacetylase [2], which can play either beneficial or detrimental roles in cell survival under different conditions: While SIRT2 inhibition can reduce α-synuclein-induced cytotoxicity in cellular and *Drosophila* models of Parkinson's disease [3], SIRT2 reductions can also induce apoptosis of HeLa cells by affecting the levels of p53 [4].

Human SIRT2 is most predominantly expressed in the brain [5]. It has been reported that SIRT2 mRNA expression is severely reduced in approximately 70% human gliomas [6]. Overexpression of SIRT2 has also been shown to decrease the survival of glioma cell lines [6]. Based on these two observations, it has been suggested that SIRT2 may play a role in growth suppression of glioma, and the reduced SIRT2 expression in human gliomas may contribute to development of cellular malignancy [6,7]. However, there has been no study that directly demonstrates that reduced SIRT2 activity can promote growth of glioma cells.

In this study we decreased SIRT2 activity of C6 glioma cells by applying both pharmacological and molecular approaches, so as to determine the effects of reduced SIRT2 activity on the survival of the glioma cells. Our study has suggested that SIRT2 activity is required for the survival of C6 glioma cells, and that caspase-3 mediates the decreased SIRT2 activity-induced apoptosis of the glioma cells.

2. Materials and methods

2.1. Cell cultures

C6 glioma cells were purchased from the Cell Resource Center of Shanghai Institute of Biological Sciences, Chinese Academy of Sciences. The cells were plated onto 24-well cell culture plates at the initial density of 2×10^5 cells/ml in Dulbecco's Modified Eagle Medium (containing 4500 mg/l D-glucose, 584 mg/l L-glutamine, 110 mg/l sodium pyruvate) (Thermo Scientific, Waltham, MA, USA) that contains 1% penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA) and 10% fetal bovine serum (Gibco, Grand Island, NY, USA).

2.2. Experimental procedures

Experiments were initiated by replacing the culture medium with cell culture medium containing various concentrations of drugs. The cells were left in an incubator with 5% CO_2 at 37 °C for various durations.

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doi:10.1016/j.bbrc.2011.11.141

2.3. Extracellular and intracellular lactate dehydrogenase (LDH) assay

Extracellular LDH assay was conducted to determine cell death, as described previously [8]. In brief, 100 μl of extracellular media was mixed with 150 μl potassium phosphate buffer (500 mM, pH 7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate. Subsequently changes of the A_{340nm} of the samples were monitored over 90 s.

Intracellular LDH assay was also conducted to determine cell survival, as described previously [9]. Briefly, cells were lysed for 20 min in lysing buffer containing 0.04% Triton X-100, 2 mM HEPES, 0.2 mM dithiothreitol, 0.01% bovine serum albumin, and 0.1% phenol red (pH 7.5). Fifty microliter cell lysates were mixed with 150 μl potassium phosphate buffer (500 mM, pH 7.5) containing 1.5 mM NADH and 7.5 mM sodium pyruvate. Subsequently changes of the A_{340nm} of the samples were monitored over 90 s. Percentage of cell survival was calculated by normalizing the LDH activity of the lysates of a sample to the LDH activity of the lysates of controls.

2.4. SIRT2 RNA silencing

C6 cells were approximately 50% confluent at the time of transfection. Three small interfering RNA (siRNA) duplexes against rat SIRT2 (NM_001008368) at nucleotides 754–772 (CCUUGCUAAGGAGCUCUAUTT), 843–862 (GCUGCUACACGCAGAAUAUTT), or 1393–1411 (GGAGCAUGCCAACAUAGAUTT), respectively, were commercially synthesized (Genepharma, Shanghai, China). For controls, scrambled RNA oligonucleotides were used. For each well, 33.3 nM of each of the three oligos were transfected using lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After incubation for 6 h, the media was replaced with DMEM containing 10% fetal bovine serum.

2.5. Western blot

C6 cells were harvested and lysed in RIPA buffer (Millipore, Temecula, CA, USA) containing Complete Protease Inhibitor Cocktail (Roche Diagnostics, Mannheim, Germany) plus 1 mM PMSF. Lysates were centrifuged at 12,000*g* for 20 min at 4 °C. After quantifications of the protein samples using the BCA Protein Assay Kit (Pierce Biotechonology, Rockford, IL, USA), 30 μg of total protein was electrophoresed through a 10% SDS–polyacrylamide gel, and then transferred to 0.45 μm nitrocellulose membranes (Millipore, CA, USA) on a semi-dry electro transferring unit (Bio-Rad Laboratories, CA, USA). Blots were incubated overnight at 4 °C with a rabbit polyclonal anti-SIRT2 antibody (1:500 dilution) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), then incubated with appropriate HRP-conjugated secondary antibody (EPITOMICS, Hangzhou, Zhejiang Province, China). Protein signals were detected using an ECL detection system (Pierce Biotechonology, Rockford, IL, USA). An anti-tubulin antibody (Sigma, St. Louis, MO, USA) was used to normalize sample loading and transfer. The intensities of the bands were quantified by densitometry using Gel-Pro Analyzer.

2.6. Nuclear condensation determinations

The nuclear size of cells was assessed by Hoechst staining [9]. In brief, cells were treated with 20 μg/ml Hoechst 33258 in phosphate buffered saline (PBS) for 20 min. The stained nuclei were photographed under a fluorescence microscope. To quantify the size of the nuclei, three randomly picked fields in each well were photographed.

2.7. Flow cytometry-based Annexin V/7-AAD staining

The flow cytometry assay was performed to measure the degrees of both apoptosis and necrosis using ApoScreen Annexin V kit (SouthernBiotech, Birmingham, AL, USA) according to the manufacturer's protocol. Briefly, C6 cells were digested by 0.1% trypsin and resuspended in cold binding buffer (10 mM HEPES, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$, 0.1% BSA) at concentrations between 1×10^6 and 1×10^7 cells/ml. Ten microliter of labeled Annexin V was added into 100 μl of the cell suspension. After 15-min incubation on ice, 380 μl binding buffer and 10 μl 7-AAD solution were added into the cell suspension. Subsequently the number of stained cells was assessed by a flow cytometer (BD FACSAriaII).

2.8. Statistical analyses

All data are presented as mean ± SE. Data were assessed by one-way ANOVA, followed by Student–Newman–Keuls *post hoc* test. *P* values less than 0.05 were considered statistically significant.

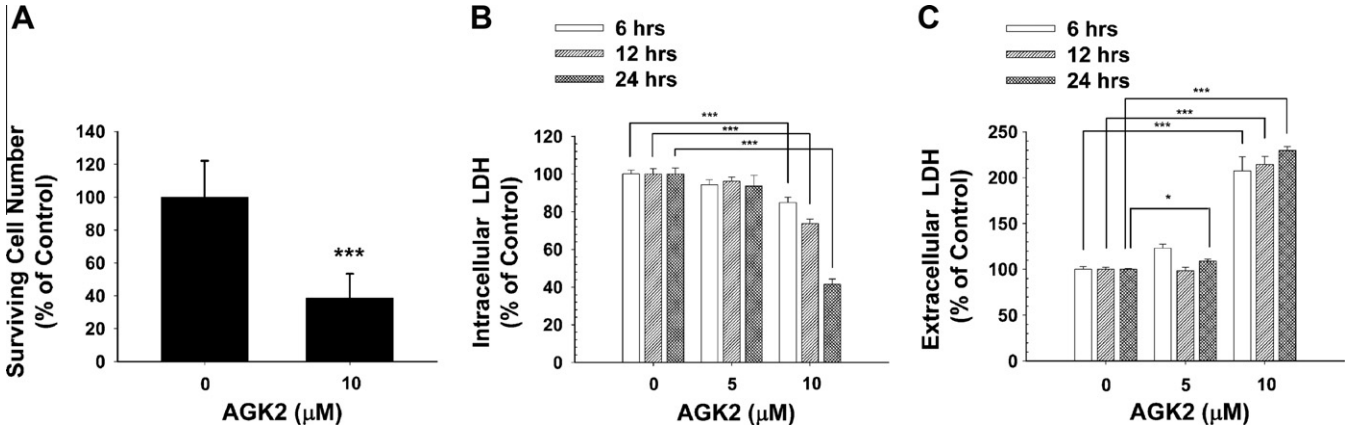

Fig. 1. Treatment of C6 glioma cells with the SIRT2 inhibitor AGK2 significantly decreased the survival of C6 glioma cells. (A) Flow-cytometry-based assay of surviving cells showed that treatment of the cells with AGK2 led to significant decreases in the number of surviving cells. The cells were treated with 10 μM AGK2 for 24 h, and subsequently the number of surviving cells was assessed by flow-cytometry-based assay of surviving cells. $N = 6$. Data were collected from four independent experiments. $^{***}p < 0.001$. (B) Determinations of cell survival by intracellular LDH assay showed that treatment of the cells with AGK2 led to significant decreases in cell survival. The cells were treated with 5 or 10 μM AGK2 for 6, 12 or 24 h, and subsequently cell survival was assessed by determining intracellular LDH levels. $N = 11–20$. Data were collected from three independent experiments. $^{***}p < 0.001$. (C) Determinations of cell death by extracellular LDH assay showed that treatment of the cells with AGK2 led to significant increases in cell death. The cells were treated with 5 or 10 μM AGK2 for 6, 12 or 24 h, subsequently cell death was assessed by determining extracellular LDH levels. $N = 11–20$. Data were collected from three independent experiments. $^{*}p < 0.05$; $^{***}p < 0.001$.

3. Results

3.1. The SIRT2 inhibitor AGK2 decreased survival of C6 glioma cells

We studied the effects of AGK2, a specific SIRT2 inhibitor [10], on the survival of C6 glioma cells by flow cytometry-based assay, and by both intracellular LDH assay and extracellular LDH assay. In our flow cytometry-based assay, surviving cells are defined as the cells that are neither Annexin V-positive (Annexin V-positive cells are cells undergoing early-stage apoptosis) nor 7-AAD-positive (7-AAD-positive cells are necrotic cells). Our study showed that treatment of the cells with 10 μM AGK2 for 24 h decreased the number of surviving C6 glioma cells by approximately 60% (Fig. 1A). Consistent with this observation, our intracellular LDH assays also showed that 10 μM AGK2 led to significant decreases in intracellular LDH levels (Fig. 1B). Assays of extracellular LDH levels showed that 10 μM AGK2 induced significant LDH release at 6, 12 or 24 h after AGK2 treatment (Fig. 1C), which indicates increased cell death.

3.2. The SIRT2 inhibitor AGK2 induced caspase-3-dependent apoptosis of C6 glioma cells

We further determined if AGK2 may induce apoptotic changes of the cells. Caspase-3-dependent pathway is a critical pathway of cell apoptosis. We determined if AGK2 treatment may induce caspase-3 activation by caspase-3 immunostaining, showing that AGK2 can induce marked increases in the immunostaining of activated caspase-3 (Fig. 2A). Because nuclear condensation is a hallmark of cell apoptosis, we also determined if AGK2 treatment may induce nuclear condensation of the cells by Hoechst staining. Ten micromole of AGK2, but not 5 μM AGK2, was shown to significantly decrease the nuclear size, which was dose-dependently prevented by the caspase-3 inhibitor Ac-DEVD-CHO (Beyotime, Jiangsu, China) (Fig. 2B).

We also used flow cytometry-based assay to determine cell apoptosis by assessing the levels of Annexin V-positive cells. We found that 10 μM AGK2 led to significant increases in Annexin V-positive cells and Annexin V-positive/7-AAD-positive cells

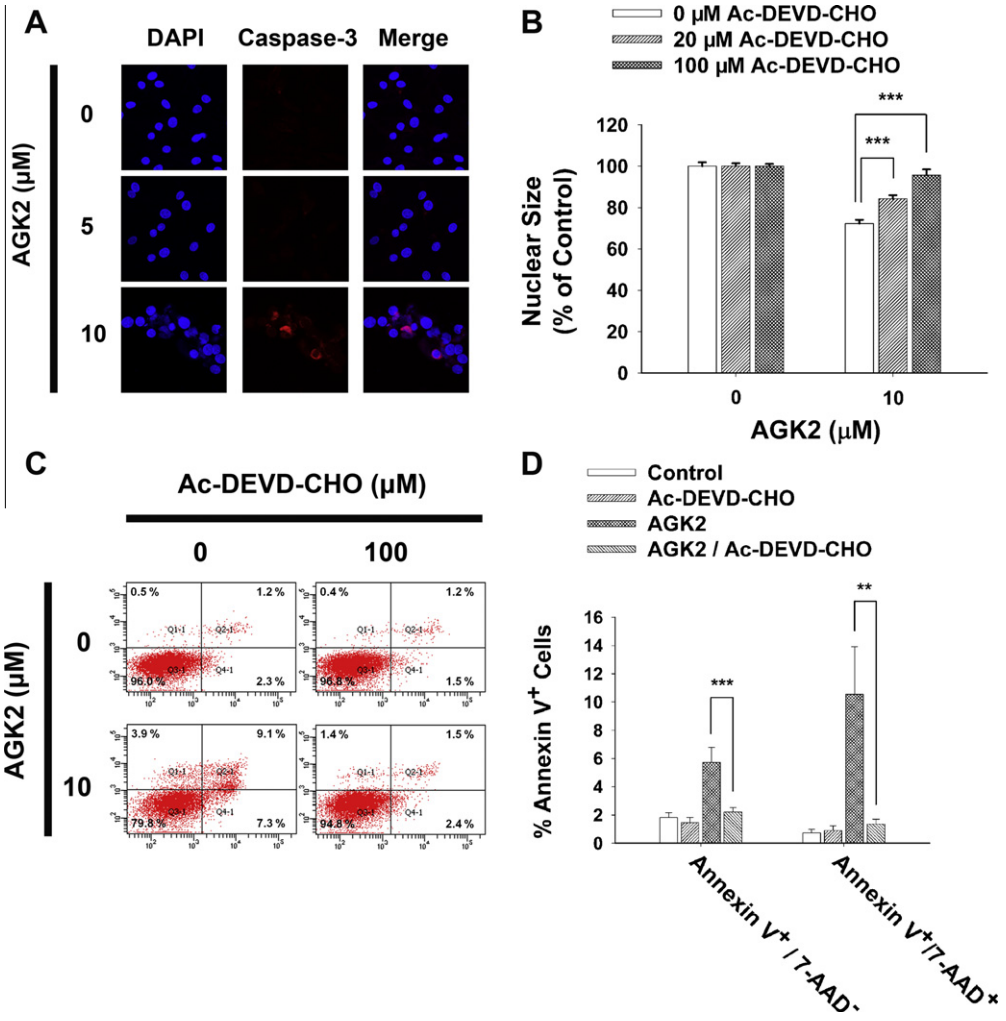

Fig. 2. Treatment of C6 glioma cells with the SIRT2 inhibitor AGK2 led to significant increases in apoptosis of C6 glioma cells by caspase-3-dependent pathway. (A) Immunostaining of activated caspase-3 showed a marked increase in the immunostaining of activated caspase-3. The cells were treated with 5 or 10 μM AGK2 for 24 h, and subsequently immunostaining of activated caspase-3 was conducted. $N = 9$. The photographs were representatives of the photographs taken in three independent experiments. (B) Hoechst staining assay showed that AGK2 led to significant decreases in the nuclear size of C6 glioma cells, which was prevented by the caspase-3 inhibitor Ac-DEVD-CHO. $N = 9$. Data were collected from three independent experiments. $^{***}p < 0.001$. (C) Flow-cytometry-based assay showed that AGK2 induced increases in the number of Annexin $V^+/7\text{-}AAD^-$ and Annexin $V^+/7\text{-}AAD^+$ cells. In the four fields of the original images from the flow cytometry-based study, the number of the dots indicates the number of Annexin $V^-/7\text{-}AAD^-$ (the bottom-left field), Annexin $V^+/7\text{-}AAD^-$ (the bottom-right field), Annexin $V^-/7\text{-}AAD^+$ (the top-left field), and Annexin $V^+/7\text{-}AAD^+$ cells (the top-right field), respectively. (D) Quantifications of the results from the flow-cytometry-based study showed that AGK2 induced significant increases in the number of Annexin $V^+/7\text{-}AAD^-$ and Annexin $V^+/7\text{-}AAD^+$ cells, which were prevented by caspase-3 inhibitor Ac-DEVD-CHO. $N = 6$. Data were collected from four independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

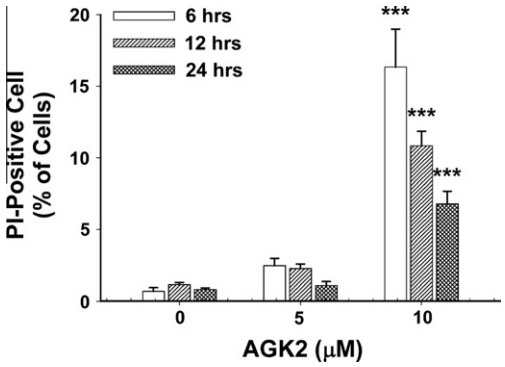

Fig. 3. Treatment of C6 glioma cells with the SIRT2 inhibitor AGK2 led to significant increases in cell necrosis. C6 glioma cells were treated with AGK2 for 6, 12 or 24 h, and subsequently necrosis of the cells was determined by PI staining. $N = 9$. Data were collected from three independent experiments. $^{***}p < 0.001$.

(Annexin V-positive/7-AAD-positive cells are cells undergoing late-stage apoptosis), suggesting that AGK2 can induce both early-stage and late-stage apoptosis (Fig. 2C and D). Our study further showed that the caspase-3 inhibitor Ac-DEVD-CHO significantly reduced the number of both early-stage and late-stage apoptosis of the cells (Fig. 2C and D).

3.3. The SIRT2 inhibitor AGK2 induced necrosis of C6 glioma cells

We further determined the effects of AGK2 treatment on the levels of necrosis of C6 glioma cells by both PI staining and flow-cytometry-based assay on 7-AAD-positive cells. We found that treatment of the cells with 10 μM AGK2 led to significant increases in PI-positive cells, suggesting that AGK2 can lead increased necrosis of C6 glioma cells (Fig. 3). Similar results were obtained by flow cytometry-based 7-AAD assay (data not shown).

3.4. SIRT2 silencing induced both necrosis and apoptotic changes of C6 glioma cells

We also applied SIRT2 siRNA to further determine the roles of SIRT2 in the survival of C6 glioma cells. C6 glioma cells were treated with SIRT2 siRNA for 24 or 48 h, and SIRT2 levels in the cells were assessed by Western blot, showing that SIRT2 siRNA significantly decreased SIRT2 levels (Supplemental Fig. 1A and B). Our study indicated that SIRT2 siRNA can induce apoptotic changes of the cells: SIRT2 siRNA produced significant decreases in nuclear size, as determined by Hoechst staining (Fig. 4A and B); and SIRT2 siRNA also led to increased immunostaining of activated caspase-3 (Fig. 4C). We also found that SIRT2 siRNA led to a significant increase in PI-positive cells (Fig. 4D), suggesting increases in cell necrosis.

4. Discussion

The major findings from this study include: first, reduced SIRT2 activity in C6 glioma cells can lead to significantly decreased survival of C6 glioma cells by inducing both apoptosis and necrosis of the cells; and second, caspase-3 mediates the reduced SIRT2 activity-induced apoptosis of C6 glioma cells. These results have

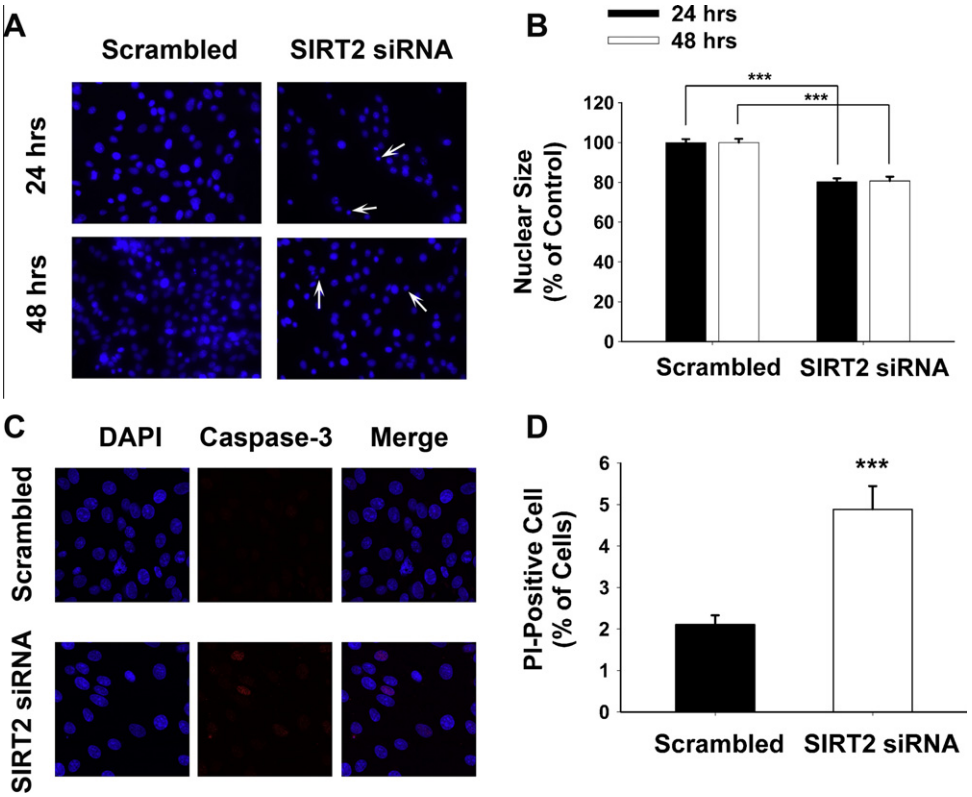

Fig. 4. Treatment of C6 glioma cells with the SIRT2 siRNA induced apoptotic changes and necrosis of the cells. (A) Hoechst staining assay showed that SIRT2 siRNA led to decreases in the nuclear size of C6 glioma cells. The arrows point to the cells with reduced nuclear size. (B) Quantifications of the Hoechst staining assay indicate that SIRT2 siRNA led to significant decreases in the nuclear size of C6 glioma cells. $N = 9$. Data were collected from three independent experiments. $^{***}p < 0.001$. (C) Immunostaining of activated caspase-3 showed that SIRT2 siRNA induced increases in the staining of activated caspase-3. The cells were treated with SIRT2 siRNA for 48 h, and subsequently immunostaining of activated caspase-3 was conducted. The photographs were representatives of the photographs taken in three independent experiments. (D) SIRT2 siRNA led to significant increases in PI-positive cells. C6 glioma cells were treated with SIRT2 siRNA for 48 h, and subsequently necrosis of the cells was determined by PI staining. $N = 9$. Data were collected from three independent experiments. $^{***}p < 0.001$.

provided evidence indicating that the decreased SIRT2 expression observed in a large portion of human gliomas [6] does not promote tumor growth. Instead, the decreased SIRT2 activity could be detrimental to glioma cell survival.

It has been found that Sir2 – a NAD^+-dependent histone deacetylase – mediates the aging process of yeast [1]. Sirtuins are the mammalian homolog of Sir2. In sirtuin family proteins, there are seven members, including SIRT1–SIRT7, in which SIRT1 has been the most intensively studied member [11]. SIRT2 is a tubulin deacetylase [2]. It has been found that SIRT2 activation can lead to cell apoptosis by inducing increased expression of Bid [12]. However, SIRT2 can also produce cytoprotective effects by inducing increased expression Mn–SOD [12].

The article of Hiratsuka et al. proposes that the decreased SIRT2 expression in a large portion of human gliomas may promote glioma growth [6]. The major experimental evidence used for supporting their claim is that overexpression of SIRT2 in glioma cell lines led to decreased survival of the cells [6]. However, it is not solid to conclude that decreased SIRT2 expression promotes growth of glioma cells, solely based on observation that overexpression of SIRT2 in glioma cell lines led to decreased survival of the cells: In many biological systems, both abnormally high and low levels of certain physiological parameters, such as glucose levels, can lead to decreased cell survival [13,14].

Our current study has provided evidence arguing against the proposal that decreased SIRT2 activity in human gliomas leads to increased glioma growth. Our study has shown that reduced SIRT2 activity by both pharmacological and molecular approaches can induce both apoptosis and necrosis of glioma cells. This finding is consistent with our previous observations that decreased SIRT2 activity can impair both energy metabolism and survival of PC12 cells [15].

Three lines of evidence in our study using AGK2 has strongly indicated that decreased SIRT2 activity can induce apoptosis of C6 glioma cells: Decreased SIRT2 activity can induce a decreased in nuclear size and increases in Annexin V-positive cells and caspase-3 immunostaining. By applying a specific caspase-3 inhibitor, our study has indicated that caspase-3-dependent pathway is the major apoptotic pathway induced by reduced SIRT2 activity. These results have further been supported by our study using SIRT2 siRNA.

Collectively, our study has indicated that the SIRT2 activity in C6 glioma cells is required for the cell survival. Decreased SIRT2 activity can induce both necrosis and capase-3-dependent apoptosis of C6 glioma cells. Gliomas are the most common type of primary malignant brain tumor. Our current study suggests that inhibition of SIRT2 may become a novel strategy to decrease the survival of glioma cells.

Acknowledgments

This study was supported by a Chinese National Science Foundation Grant # 81171098 (to W.Y.), a Pujiang Scholar Program Award 09PJ1405900 (to W.Y.), a National Key Basic Research '973 Program Grant' # 2010CB834306 (to W.Y. and W. Xia), and a Key Shanghai Jiao Tong University Grant for Interdisciplinary Research on Medicine and Physical Sciences (to W.Y).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.141.

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