Abstract. Cerebral ischemia results in severe brain damage, and is a leading cause of death and long-term disability. Previous studies have investigated methods to activate astrocytes in order to promote repair in injured brain tissue and inhibit cell death. It has previously been shown that n-myc downstream-regulated gene 2 (NDRG2) was highly expressed in astrocytes and associated with cell activity, but the underlying mechanism is largely unknown. The present study generated NDRG2 conditional knockout (Ndrg2-/-) mice to investigate whether NDRG2 can block ischemia-induced astrocyte necroptosis by suppressing receptor interacting protein kinase 1 (RIPK1) expression. This study investigated astrocyte activity in cerebral ischemia, and identified that ischemic brain injuries could trigger RIP-dependent astrocyte necroptosis. The depletion of NDRG2 was found to accelerate permanent middle cerebral artery occlusion-induced necroptosis in the brain tissue of Ndrg2-/- mice, indicating that NDRG2 may act as a neuroprotector during cerebral ischemic injury. The present study suggested that NDRG2 attenuated astrocytic cell death via the suppression of RIPK1. The pharmacological inhibition of astrocyte necroptosis by necrostatin-1 provided neuroprotection against ischemic brain injuries after NDRG2 knockdown. Therefore, NDRG2 could be considered as a potential target for the treatment of cerebral ischemia.

Introduction

Severe craniocerebral trauma is characterized by high mortality and disability, and has been a focus of neuroscientific research (1). Cerebral ischemia is an important pathophysiological feature of secondary brain injury after trauma (2). Secondary injury related mechanisms include primary mechanical injury of blood vessels caused by cerebral tissue ischemia, hematoma and other placeholder lesions caused by local cerebral ischemia (3). In addition, secondary brain injury also includes diffuse brain swelling after trauma, increased intracranial pressure leading to low perfusion in the brain, cerebral vasospasm, microvascular lesions and alterations in local cerebral blood flow, which can influence systemic circulations (3).

Astrocytes are the most widely distributed glial cell type in the central nervous system and play a crucial role in protecting neurons, regulating synaptic function, and promoting nerve regeneration and repair (4,5). Astrocytes also play an important role in the process of ischemic brain injury (6). It was previously shown that astrocytes are regularly distributed in the brain, and establish positional relationships with neurons via close connections to exchange information and material between cells (7). After cerebral ischemia, the extent of the damaged area of brain tissue is determined by the degree of damage to neurons and glial cells (8). The activation of astrocytes can promote neuron damage repair, axon regeneration and neuron migration (9). Previous studies have shown that astrocytes maintain the homeostasis of the extracellular microenvironment of brain tissue, and can promote neuron survival after cerebral ischemia by interacting with neurons and glial cells, and participating in the process of endogenous nerve protection (10,11). Therefore, understanding how to activate the neuroprotective mechanisms of astrocytes and inhibit cell death could facilitate the treatment of ischemia-induced brain tissue injury.

Necroptosis is a programmed type of necrosis, which can be regulated and reversed under certain conditions (12,13). Necroptosis is mediated by the interaction between receptor interacting protein (RIP) kinase (RIPK) members, such as RIPK1 and RIPK3 (12,13). After external signal stimulation, a
series of death receptors are activated, such as tumor necrosis factor receptor (TNFR1, TNFR2 and Fas ligand (14). RIPK1 is then activated and interacts with death receptors to form complex-I, which can recruit RIPK3 to form a necrosome, which is critical for the process of necroptosis (14). A previous study showed that the cerebral ischemic environment could lead to necroptosis of neurons and astrocytes, and increase the area of brain injury (15). Based on the importance of astrocytes in the repair of injured brain tissue, inhibiting astrocytic death and promoting its normal activation is key to the functional recovery of the nervous system, as well as the treatment and prognosis of traumatic cerebral ischemia.

N-myc downstream-regulated gene 2 (NDRG2) belongs to the NDRG family that consists of four identified members (NDRG1-4), and has been implicated in the regulation of cell differentiation and proliferation (16,17). NDRG2 is closely related to neurological diseases (18). In the brains of patients with Alzheimer’s disease, NDRG2 expression is upregulated (18,19). Overexpression of NDRG2 in nerve growth factor induced-differentiated PC12 cells promotes axon regeneration (19). In astrocytes, NDRG2 is specifically and more widely expressed than glial fibrillary acidic protein (GFAP), which is a commonly used astrocyte marker; thus, NDRG2 is considered as a new astrocyte marker (20). It was previously shown that NDRG2 could regulate astrocyte activity by promoting cell differentiation and stabilizing cell morphology (21). The present study generated NDRG2 conditional knockout mice (Ndrg2-/-) to investigate the association of NDRG2 and astrocytic activity during cerebral ischemia.

Materials and methods

Animals. NDRG2 conditional knockout (Ndrg2-/-) mice were generated via cooperation with Shanghai Biomodel Organism Science & Technology Development Co., Ltd. and maintained on C57BL6/J background as previously described (22). Briefly, Ndrg2lox/lox mice were crossed with B6.c-Tg(cMV-cre)1cgn/J mice (Jackson Laboratory) to generate Ndrg2-/- mice. The line was backcrossed with c57Bl/6J a minimum of 8 times before use in any experiments in this study. Heterozygous mice carrying the knockout mutation were interbred to obtain the homozygous Ndrg2-deficient mice and their wild-type (WT) littermates. All animals were raised under specific pathogen-free conditions. The incubator temperature was maintained at 21±2˚C with 45-60% humidity, on a 12/12 h day/night cycle free conditions. The incubator temperature was maintained at 37.0±0.5˚C using a temperature-regulated heating pad during the procedure. Animals were randomly assigned to different treatment groups (n=8/group). Infarct volume was assessed with a direct method using ImageJ v2.1 (National Institutes of Health). The grip forces of the mice were measured as previously described (24).

Primary cortical astrocyte culture and NDRG2 lentivirus infection. The 1- or 2-day-old neonates (n=12) were used for primary cortical astrocyte culture as previously described (25). The cerebral cortices were digested with 0.25% trypsin for 10 min at 37˚C and filtered with a sterile nylon cell strainer. The cells were grown at 37˚C in conditions of 5% CO2 and 95% O2 in DMEM/F12 (1:1) supplemented with 10% FBS (both purchased from Gibco; Thermo Fisher Scientific, Inc.), streptomycin (100 µg/ml), and penicillin (100 U/ml). For NDRG2 overexpression in astrocytes, cells were infected with 1x10^5 TU/ml NDRG2-overexpressing lentivirus (pHBLV-CMVIE-NDRG2), which was packaged by Hanbio Biotechnology Co., Ltd., with 5 µg/ml polybrene. At 48 h after infection, subsequent experiments were performed.

Oxygen-glucose deprivation (OGD) treatment. OGD was performed as previously described (26). Cells in the OGD group were cultured in glucose-free DMEM (Gibco; Thermo Fisher Scientific, Inc.) and kept for 6 h or 12 h at 37˚C in a hypoxic incubator chamber (Billups-Rothenberg, Inc.) filled with 95% N2/5% CO2. Cells grown in conditions of 5% CO2 and 95% O2 in standard DMEM were used as the control group. Necrostatin-1 (Nec-1; 10 µM; Sigma-Aldrich; Merck KGaA) dissolved in DMSO was added to the cells 0.5 h before OGD treatment.

Western blotting. Cells were lysed in RIPA buffer (Thermo Fisher Scientific, Inc.) and the concentrations were measured by bicinechonic acid protein assay kit (Thermo Fisher Scientific, Inc.). In all samples, 40 µg proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membranes (Hybond-ECL; GE Healthcare Life Sciences). Then, nitrocellulose membranes were incubated with 5% skimmed milk for 1 h at room temperature. Primary antibodies were then introduced at dilutions of 1:1,000 for anti-RIPK1 (cat. no. ab106393; Abcam), anti-GFAP (cat. no. AB5541; EMD Millipore), anti-NDRG2 (cat. no. 5667; Cell Signaling Technology, Inc.) and anti-β-actin (cat. no. 4970; Cell Signaling Technology, Inc.), membranes were incubated at 4˚C overnight. The following secondary antibodies (1:5,000) were then added against the primary antibodies, horseradish peroxidase (HRP)-conjugated anti-mouse IgG (cat. no. 7076; Cell Signaling Technology, Inc.) or HRP-conjugated anti-rabbit IgG (cat. no. 7074; Cell Signaling Technology, Inc.), and membranes were incubated at 4˚C overnight. The blots were detected by chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.) or Odyssey Imaging System (LI-COR Biosciences).

Immunofluorescence assay. The brain tissue was fixed with 10% formalin overnight at 4˚C and embedded in paraffin. The sections (thickness, 6 µm) were then dewaxed in xylene through the internal carotid to the MCA. Rectal temperature was maintained at 37±0.5˚C using a temperature-regulated heating pad during the procedure. Animals were randomly assigned to different treatment groups (n=8/group). Infarct volume was assessed with a direct method using ImageJ v2.1 (National Institutes of Health). The grip forces of the mice were measured as previously described (24).
three times for 5 min each at room temperature and rehydrated in a descending series of ethanol (100 and 95%) at room temperature for 10 min each. For the cells, the primary cells were fixed in 4% paraformaldehyde for 10 min at room temperature. For immunofluorescent staining, the sections were blocked with 5% normal goat serum (cat. no. 5425; Cell Signaling Technology, Inc.) and 0.3% Triton X-100 for 1 h at room temperature to minimize non-specific staining. They were then incubated overnight at 4˚C with the following primary antibodies (1:200), anti-riPK1 (cat. no. ab106393; abcam), anti-GF aP (cat. no. A B5541; eMd Millipore) and anti-neun (cat. no. 24307; cell Signaling Technology, inc.). Then, the samples were incubated with H rP-conjugated secondary antibodies (1:500) for 1 h at room temperature (cat. nos. A32935, A32731 and A32759; Thermo Fisher Scientific, Inc.), and further incubated with 10 µg/ml da Pi for 5 min at room temperature for visualization of the cell nuclei. The cells were imaged by confocal laser scanning microscopy (magnification, x1,000; nikon corporation) and the fluorescence intensity of immunofluorescence images was measured by ImageJ v2.1 (National Institutes of Health) for quantification of five fields per sample.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was isolated from brain tissue or primary cells using TRIZol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and then quantified. Then, 2 µg of total RNA was reverse-transcribed using reverse transcriptase (Promega...
Corporation) according to the manufacturer's instructions. qPCR was performed with TB Green® Fast qPCR kit (Takara Bio, Inc.) for 35 cycles of: 10 sec at 98°C, 10 sec at 55°C and 20 sec at 72°C. The primer sequences used were as follows: GFalphaP, forward 5'-cGGaGacGcaTcaccTcTG-3' and reverse 5'-TGGaGGaGTcaTTcGaGacaa-3'; riPK, forward 5'-GacaGaccTaGacaGcGGaG-3' and reverse 5'-ccaGTaGcTTcaccacTcGac-3'; GaPDH, forward 5'-aGGTcGGTGTGaacGGaTTTG-3' and reverse 5'-GGGGTcGTTGaTGGcaaca-3'. The mean cQ values for the target genes were normalized to the mean cQ value for the endogenous control GaPDH as previously described (27). The ratio of mRNA expression of target gene to GaPDH was defined as 2−ΔΔcQ.

Cell growth assay. For the crystal violet assays, 1x10^5 astrocytes were seeded into each well of a 12-well plate with three replicates for each group. Cells in conditions of 5% CO2 and 95% O2 in DMEM/F12 supplemented with 10% FBS were used as a negative control. After 12 h of OGD treatment, cell proliferation was assessed by crystal violet staining at 37°C for 4 h, as described previously (28). For the cell survival assays, 1x10^4 cells from each treatment condition were plated in triplicate in a 96-well plate at 37°C. The survival rate was measured using a Guava Nexin assay (EMD Millipore). Results are shown as the mean % of viable cells.

Lactate dehydrogenase (LDH) assay. A total of 1x10^5 astrocytes were seeded into each well of a 6-well plate. The release of LDH was measured using the LDH assay kit (cat. no. A020-2-2; Nanjing Jiancheng Bioengineering Institute Co., Ltd.) according to the manufacturer's protocols. Samples without coenzyme I treatment were used as a negative control. LDH leakage was calculated as follows: LDH leakage (%) = (A positive/A positive blank) / (A negative/A negative blank) x100%.

Statistical analysis. Data are presented as the mean ± SD. Statistical analysis was conducted using SPSS 10.0 software (SPSS, Inc.) to perform ANOVA followed by a Student-Newman-Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Ischemic brain injuries trigger RIP-dependent astrocyte necroptosis. To determine the association of cerebral ischemic injury and astrocytic features, a brain injury mouse pMCAO model was established. The present results showed decreased grip strength and an increased rate of right forelimb utilization in pMCAO mice (Fig. 1A and B). In addition, a significant infarct was found in the ipsilateral cortex of pMCAO brains compared with sham-operated brains (Fig. 1C and D),
indicating that the pMCAO mouse model was successfully established. The present results showed that protein and mRNA expression levels of GFAP were decreased at 12 and 24 h after pMCAO in the ischemic cortex (Fig. 1 e, F, H and i). The protein and mRNA expression levels of RIPK1, an initiator of necroptosis, were increased in the pMCAO group compared with the control (Fig. 1 e, G, H and J). Therefore, the present results suggested that ischemic brain injuries could be associated with RIPK-dependent necroptosis. Protein and mRNA expression levels of RIPK1 were also increased in astrocytes after OGD exposure, in addition to decreased cell survival rate and increased LDH leakage (Fig. 1K-o).

**NDRG2 knockdown accelerates cerebral ischemic injury-induced necroptosis.** Previous studies have demonstrated that the tumor suppressor NDRG2 has a neuroprotective function in various diseases (29,30). Therefore, the present study investigated whether NDRG2 was involved in the regulation of cerebral ischemic injury and necroptosis. A mouse line carrying the NDRG2 conditional knockout allele (Ndrg2-/-) with exons 2-6 flanked by locus of X-over P1 sites (Fig. 2A) was generated. Female homozygotes (Ndrg2-/-) were crossed with CMV-Cre transgenic male mice, which have ubiquitous Cre activity. After the pMCAO operation, an increased infarction volume was observed in Ndrg2-/- mice (Fig. 2B and C); thus, NDRG2 may provide neuroprotection against pMCAO insult. Moreover, NDRG2 knockout led to a significant decrease in GFAP expression levels, and a significant increase in the protein and mRNA expression levels of RIPK1 (Fig. 2D-I). Therefore, the depletion of NDRG2 could accelerate pMCAO-induced necroptosis in brain tissue. NDRG2 may function as a neuroprotectant during cerebral ischemic injury.

**NDRG2 attenuates astrocytic cell death via the suppression of RIPK1.** To further determine the role of NDRG2 in regulating astrocyte survival, astrocytes from the neonate mice were isolated and NDRG2 was overexpressed using a lentivirus (Fig. 3a). After OGD exposure, NDRG2 overexpression could significantly block OGD-dependent decreases in cell viability and survival, and induction of LDH leakage (Fig. 3B- D). Furthermore, NDRG2 overexpression increased GFAP expression levels and decreased RIPK1 expression levels after OGD exposure (Fig. 3A). After OGD exposure, NDRG2 overexpression could significantly block OGD-dependent decreases in cell viability and survival, and induction of LDH leakage (Fig. 3B-D). NDRG2 knockout increased OGD-induced cell death and LDH leakage (Fig. 3G-I). NDRG2 knockout also upregulated RIPK1 and downregulated under OGD conditions GFAP (Fig. 3J). Therefore, the present results suggested that NDRG2 could block OGD-induced astrocytic cell death by suppressing RIPK1.

**Necroptosis inhibitor necrostatin-1 blocks astrocytic cell death after NDRG2 knockdown.** In order to determine
whether the role of NDRG2 in neuroprotection is the result of blocking necroptosis, the present study used a necroptosis inhibitor Nec-1, which can inhibit necrosome formation via the suppression of riPK1 activity (31). Nec-1 treatment prevented the pMCAO-induced decrease in GFAP protein and mRNA expression levels in the ischemic cortex of Ndrg2-/- mice (Fig. 4A and B). In addition, after Nec-1 treatment, the reduced viability and survival rates of astrocytes from Ndrg2-/- mice following OGD exposure were partly reversed (Fig. 4C and D). OGD-induced LDH leakage from Ndrg2-/- astrocytes was also attenuated following Nec-1 treatment (Fig. 4E). The present results indicated that NDRG2 could function as a neuroprotector by blocking necroptosis, and that pharmacological inhibition of astrocyte necroptosis promotes neuroprotection against ischemic brain injuries after NDRG2 knockdown.

Discussion

Cerebral ischemia is an important component of secondary brain injury after trauma (2). Inhibiting ischemic injury has become one of the key therapeutic methods for improving the prognosis of brain injury (3). A highly regulated form of necrosis, termed necroptosis, elicited significant interest in the study of human diseases such as ischemic stroke in order to understand its implications in pathologies (32). The present study investigated astrocyte activity during cerebral ischemia, and identified that ischemic brain injuries may trigger RIP-dependent astrocyte necroptosis. In a NDRG2 conditional knockout mouse model, the present study found that knockdown of NDRG2 could accelerate pMCAO-induced necroptosis in brain tissue, therefore indicating that NDRG2 may function as a neuroprotector during cerebral ischemic injury. The present study suggested that NDRG2 may attenuate astrocytic cell death via the suppression of RIPK1. The present results suggested that pharmacological inhibition of astrocyte necroptosis by Nec-1 produced neuroprotection against ischemic brain injuries after NDRG2 knockdown. Therefore, NDRG2 could be considered as a target for the treatment of cerebral ischemia.

Astroglycer are the most widely distributed glial cells in the nervous system (4,5). Astrocytes can not only provide metabolic and nutritional support for neurons, but also play a crucial role in promoting neuronal survival, synaptic function, nerve regeneration and nerve repair (4,5). It is of clinical importance to explore the mechanism of astrocyte death to facilitate the treatment and prognosis of post-traumatic cerebral ischemia. To determine the association between cerebral ischemic injury and astrocytes, the present study established a brain injury mouse pMCAO model and identified that RIPK1, which is an initiator of necroptosis, was upregulated after injury. OGD exposure also led to increased RIPK1 expression levels and LDH leakage, and decreased cell survival rate. Therefore, the present results suggested that ischemic brain injuries may be associated with RIP-dependent astrocyte necroptosis.

NDRG2 has been shown to function as a tumor suppressor via the reduction of cell proliferation and metastasis, and induction of cell differentiation in numerous cancer types (33,34). In the central nervous system, NDRG2 is highly expressed in astrocytes, and regulates astrocytic activity by promoting cell differentiation and stabilizing cell morphology (21). To investigate the role of NDRG2 in astrocytes, the present study generated Ndrg2-/- mice. After ischemic injury, increased infarction volume, reduction of GFAP and induction of RIPK1 were observed; thus, NDRG2 may provide neuroprotection against cerebral ischemia. Moreover, astrocytes from Ndrg2-/- mice showed decreased cell viability and survival, and increased LDH leakage after OGD exposure. The present study suggested that NDRG2 could block OGD-induced astrocytic cell death by inhibiting RIPK1. A similar conclusion was reached following Ndrg2 overexpression in astrocytes. Therefore, the present study suggested that NDRG2 plays an important role in blocking cerebral ischemic injury-induced necroptosis, potentially via the suppression of RIPK1 expression.

Figure 4. Necroptosis inhibitor Nec-1 blocks astrocytic cell death after NDRG2 knockdown. Nec-1 (10 µM) was administrated to primary cultured astrocytes for 30 min prior to OGD. (A) GFAP protein and (B) mRNA expression levels were determined 6 h after OGD treatment. (C) Cell viability, (D) survival and (E) LDH leakage were determined 12 h after OGD treatment. *P<0.05, **P<0.01, ***P<0.001. Nec-1, necrostatin-1; NDRG2, N-myc downstream-regulated gene 2; WT, wild-type; RIPK1, receptor interacting protein kinase 1; GFAP, glial fibrillary acidic protein; LDH, lactate dehydrogenase; OGD, oxygen-glucose deprivation.
Necroptosis is different to traditional apoptosis. When cells are stimulated by tumor necrosis factor-α and other apoptotic inducers, necrotic morphological features can be induced, and treatment with caspase inhibitors can block apoptosis pathways (35). Nec-1 is one of the most commonly used RIPK1 inhibitors. The effect of Nec-1 on necroptosis is associated with allosteric inhibition of the activity of RIPK1 by interacting with the T-loop of the N-terminal kinase domain (36). A previous study showed that Nec-1 can decrease ischemic brain injury area dose-dependently, and improve nerve function score, prolong nerve protective effect and delay the onset of necroptosis by inhibiting the activation of RIPK1/RIPK3 pathways after ischemic brain injury (19). In addition, Nec-1 can protect neurovascularulature and improve the prognosis of animals following neurological injury (37,38). The present results indicated that Nec-1 treatment prevented pMCAO-induced reductions of GFAP, ODG-induced suppression of astrocyte viability and survival, and induction of LDH leakage in the ischemic cortex of Ndrg2-/- mice. Therefore, pharmacological inhibition of astrocyte necroptosis may provide neuroprotection against ischemic brain injuries after NDRG2 knockdown. NDRG2 could be considered as a target for the treatment of cerebral ischemia.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YHW and KX contributed to the analysis and interpretation of data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All animal experiments were in accordance with the relevant provisions of the Laboratory Animal Center and were approved by the Experimental Animal Ethics Committee of the 101 Hospital of PLA of Anhui Medical University.

Patient consent to participate

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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