Senescent Phenotype of Astrocytes Leads to Microglia Activation and Neuronal Death

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Abstract

Astrocyte, the most abundant cell type in the central nervous system, is increasingly recognized and is thought to depend on curial and diverse roles in maintaining brain homeostasis, the blood-brain barrier, ion homeostasis, secrete neurotrophic factors and regulate synaptic transmission which is essential to tune individual-to-network neuronal activity. Senescence in astrocytes has been discovered to be an important contributor to several age-related neurological diseases like Alzheimer's and Parkinson's disease. However, the latest research about astrocytes from aged subjects or aged astrocytes in vitro is not yet adequate to be well elucidated on their curial process in the regulation of brain function. In this study, an in vitro cell model of aged astrocytes was constructed by serial passaging until passage 20-25, and those passages within 1-5 were used as young astrocytes. Meanwhile, oxidative induced astrocyte senescence model was also constructed by H_2O_2 induction. Our results indicate that after serial passaging or oxidative stress-induced astrocytes, all showed manifest changes in several established markers of cellular senescence like P53, P21, the release of inflammatory cytokine IL-6 and SA- β -gal positive cells. Results also showed mitochondrial dysfunction in the oxidative stress-induced astrocyte senescence model and treatment of berberine could reverse these alterations. What's more interests us is that those two types of senescent astrocytes' conditioned medium co-cultured with neuronal cells could do impact on neuron apoptosis no matter in direct or indirect ways. This study may help us better understand the fundamental role of astrocyte senescence on the regulation of normal and pathological brain aging.

Introduction

Aging is a major risk of many diseases. As the elderly population has been expanding rapidly, the growing prevalence of age-related diseases and disability is a major public concern. Arguably, one of the most devastating is the changes that occur in the central nervous system, leading to the loss of cognitive, motor, and emotional function that in essence make us who we are. When aging comes, all those brain cells could be undergoing the process of senescence. Recent studies have demonstrated that clearance of senescent cells results in prolonged life span in mice [1]. Researches [2–3] published in Nature simultaneously reported that organ aging is an "asynchronous" process, and various types of cells as the basic unit of organ composition also follow this "asynchronous" feature. As is well known, the mammalian brain is composed of a multitude of cell types like astrocytes, microglia, and neurons, so here leaves the question that is there a precedence among this different type of cells and how they interact and affect each other.

Astrocytes are the predominant glial cells in the brain and serve multiple functions including maintain the formation of brain, secrete various extracellular matrix proteins and neurotrophic factors, also regulate the transmission efficiency at pre- and post-synaptic sites, and modulate synapse formation and turnover [2, 4]. Thus, astrocytes play crucial roles in maintaining normal brain function and homeostasis [5–6]. Meanwhile, microglia, the resident macrophages of the central nervous system, have been widely considered as a homogeneous population of cells involved in stable brain patterns. Researchers have

found that the activation of microglia will do large effect on cells in brain, the most common behavior of these types of cells is to release a large number of inflammatory factors such as IL-1 β , IL-6, TNF- α and finally do impact on other cells in brain. However, whatever the mechanism of astrocytes, microglia, other glia cells or endothelial cells in brain, will eventually lead to the functional loss or death of neuron, and ultimately manifest through a variety of physical behaviors. Questions arise as to how astrocytes, microglia, and neurons actual cross talk with others.

In the present study, we first investigated whether neuronal damage occurs at the same time as aging happens at the whole animal level, then we explored primary cultures of rat astrocytes for natural senescence and oxidative stress-induced senescence to detect their interaction with microglia and neurons, and, we found that berberine treatment maybe an effectively therapeutic approaches to protect astrocyte from senescence.

Materials And Methods

Animals

C57bl6/J mice (8-week-old) were purchased from Vital River Laboratory Animals Technology Co, Ltd, housed in a temperature and climate condition-controlled barrier system ($23 \pm 2^{\circ}\text{C}$ and 45–60% relative humidity, 12 h light-dark cycle) and fed regular rodent chow (Laboratory Animal Center of Jilin University). Then, the mice were divided into 2 groups for analysis: 4-month-old mice (age group; young; $n=10$), 15-month-old mice (aged group; initial old; $n=10$).

Tissue processing

Fresh brain tissues were dissected and soaked overnight in 4% paraformaldehyde, dehydrated in an ascending ethanol series, and equilibrated with xylene, followed by embedding in paraffin and sectioning into 3 to 5 μm slices. Then, the samples were dewaxed with xylene and a descending ethanol series.

Materials

Berberine dilutions were obtained from a 10mM berberine chloride stock solution prepared in Dimethyl sulfoxide.

Cell culture and primary astrocyte cultures

Mouse BV2 and Neuro-2a(N2a) cell lines were purchased from National Collection of Authenticated Cell Cultures(Shanghai, China) and were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, 100 IU $\cdot\text{ml}^{-1}$ penicillin, 100 $\mu\text{g}\cdot\text{ml}^{-1}$ streptomycin and reseeded at a 1:7 dilution every 3 days. Primary rat astrocytes were isolated from 1- to 2-day-old Wistar rat pups as described previously [7]. In brief, cortices were removed from the rat pups; the meninges were stripped and homogenized. After incubation with trypsin(0.05%) for 30 min in a 37 $^{\circ}\text{C}$ thermostatic shaker, the homogenate was resuspended in a trypsin inhibitor/DNase solution, triturated, and dissociated cortical cells were suspended in DMEM/F12 (Life Technology,11965-084) containing 25 mM glucose, 4 mM

glutamine, 1 mM sodium pyruvate, 100 IU·ml⁻¹ penicillin, 100 µg·ml⁻¹ streptomycin, and 10% FBS and plated on poly-L-lysine coated 10cm dishes at a density of 1×10⁵ cells cm² at 37°C with 5% CO₂ in air. 24 hours later, the culture medium was changed to fresh medium. Monolayers of type 1 astrocytes were obtained 7 days after plating. Non-astrocytic cells such as microglia and neurons were detached from the flasks by shaking and removed by changing the medium. Cells were trypsinised and reseeded at a 1:3 dilution every 3 days on poly-L-lysine coated 10cm dishes.

Senescence induction.

Induction of astrocyte senescence by oxidative stress was performed as previously described [8]. In brief, early-passage (no more than five passages) astrocytes were incubated for 2 h in complete astrocyte medium containing 80 µM H₂O₂, then washed with PBS and cultured in medium without serum for 24 hours. Induction of astrocyte senescence by natural senescence was performed by trypsinised astrocytes when reaching 80-90% confluency, cells within passages 20-25 were used as aged-astrocytes and within passages 1-5 were used as young-astrocytes.

Senescence-associated-β-galactosidase (SA-β-gal) staining

β-galactosidase activity was measured using the Senescence β-Galactosidase Staining Kit (Beyotime, Shanghai, China). Briefly, cells were washed with PBS and fixed with 1×fixative solution for 15 min. Then, β-galactosidase staining solution with a final pH between 5.9 and 6.1 was prepared and added to the fixed cells. Samples were sealed with parafilm to prevent evaporation and placed in 37°C incubator without CO₂ overnight. Imaging was performed using an inverted microscope.

Preparation of Astrocyte-conditioned medium and microglia-conditioned medium

Astrocytes were grown in 96-well plates, when reaching 80% confluency, cells were treated with 80µM H₂O₂ or normal culture medium for 2 h, then the medium was removed and washed with PBS for 1 time and DMEM/F12 without serum was added for 24 h, finally the supernatant was collected for downstream experiments. Conditioned medium from microglia was collected after treating with astrocyte conditioned medium for 24 h.

MTT assay

Cell viability was determined using a modified MTT assay [9]. Briefly, 5000 cells well⁻¹ were plated in 96-well plates and incubated overnight. Cells were then treated with astrocyte, microglia or astrocyte-microglia conditioned medium for 24h. At the end of the follow-up period, MTT in PBS was added and the cultures were incubated for 2 h at 37°C incubator. After discarding the supernatant, the formazan was dissolved in DMSO; then, the optical density (OD) values were determined at 492 nm. The cell viability was calculated by taking the cell viability in the non-treatment group as 100%.

ATP measurement

Intracellular ATP level was determined by ATP assay kit (Beyotime, Shanghai, China), which can perform cell lysis and generate a luminescent signal proportional to the amount of ATP present. The preparation of samples was conducted according to the manual of the product. The supernatants of each sample (20 μ l) were added to the ATP detection solution (100 μ L) attached to the kit. Then, Infinite 200 Pro (Tecan) was utilized to record the RLU values. The protraction of the standard curve was conducted on the basis of the RLU values of ATP with the concentration of 0, 0.01, 0.05, 0.1, 0.5, 1, 5, and 10 nmol/L. Finally, the protein concentration was used to standardize the results, which were presented as ATP/protein (nmol/mg)

Intracellular ROS detection

Intracellular ROS level was detected by the membrane-permeable ROS-sensitive fluorescent indicator, 2',7'-dichlorodihydro-fluorescein diacetate (DCFH-DA), using the ROS Assay Kit (Beyotime). Briefly, cells were seeded into 6-well plates and treated with different interventions, then were incubated with 10 μ mol/L DCFH-DA for 20–30 min at 37°C in the dark, and were washed with cold wash buffer 3 times to wash off excess DCFH-DA. Finally, cells were harvested for flow cytometry (BD, USA) analysis.

Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi_m$) loss was assessed by a Mitochondrial Membrane Potential Assay Kit with JC-1. After treatment for 24 h, cells were incubated with JC-1 for 20 min, washed and visualized under BX53 Fluorescence microscope (Olympus). Red fluorescence indicates normal $\Delta\Psi_m$ with JC-1 aggregates in mitochondria, and green reflects cytosolic JC-1 monomer indicative of $\Delta\Psi_m$ loss.

Western blot analysis

The harvested cells were digested by RIPA buffer, following sonication, the samples were centrifuged for 15 min at 12,000 g at 4°C. For the determination of total protein density, a BCA Protein Assay Kit (Thermo fisher) was applied. Then, after separating the proteins on SDS-PAGE, the proteins were transferred onto the PVDF membrane (Millipore). In all, 5% non-fat milk was utilized for the sealing of membranes in Tris-buffered saline (pH 7.5). The membrane went through overnight hybridization with the primary antibodies and second antibodies. The protein bands were revealed by an ECL kit (Thermo fisher). The expression levels of proteins were evaluated by Image J (National Institutes of Health, USA). The primary antibodies were: anti-P53(proteintech), anti-P21(proteintech), anti-P16(abcam), anti-OPA-1 (proteintech), anti-Mfn2 (abcam), anti-DRP-1 (proteintech), anti- β -actin (proteintech).

HE staining

Fixed, paraffin-embedded brain tissue was sectioned and underwent hematoxylin eosin (HE) staining according to the following procedure. Sections were deparaffinized, washed in distilled water, and incubated in hematoxylin solution for 5 min; excess hematoxylin solution was washed off with running tap water. To remove background staining, sections underwent a differentiation step in hydrochloric acid alcohol, after which they were fully washed in running tap water. Sections were then counterstained in

eosin solution for 2-3 min, washed in running tap water, dehydrated through graded alcohol, and mounted with neutral resin. Pathological changes in neurons were observed under CX31(Olympus).

Nissl staining

Paraffin-embedded, fixed brain tissue was deparaffinized, washed 1-2 min in distilled water, dipped in 1% thionin lyosol at 37°C for 30 min, and washed again for 1-2 min in distilled water. To moderately differentiate the nucleus, sections were incubated in 0.5% hydrochloric acid alcohol, washed back to blue, and differentiated using 95% alcohol until the Nissl substance was visualized. Sections were then dehydrated (twice for 5 min each) in 100% anhydrous alcohol, permeabilized with xylene twice (5 min each), and mounted with neutral gum. Changes observed in neurons and Nissl bodies of the hippocampal CA1, CA2, CA3 and DG regions were detected under a light microscope CX31(Olympus).

Immunofluorescence and Immunohistochemistry

Total cells on the slides went through permeabilization in 0.3% Triton X-100 subsequent to the fixation in 4% paraformaldehyde. After that, goat serum was utilized for blocking. Cells or tissues were later cultivated overnight with anti-GFAP (Abcam), Then, the slide was subjected to 1-h incubation with the Fluorescein (PE)–conjugated Affinipure Goat Anti-Mouse IgG(H+L) (proteintech) under the room temperature, again, cells or tissues were cultivated overnight with anti- beta-Galactosidase (proteintech) at 4°C and then subjected to 1h incubation with the FITC–conjugated Goat Anti-Rabbit IgG(H+L) (proteintech). The counterstaining of the nucleus was accomplished by using DAPI. For immunohistochemistry (IHC), slides were incubated in 0.9% H₂O₂ for 30 min. Afterward slides were placed in blocking buffer (nomal goat serum 1:100 in PBS/BSA) for 30 min at room temperature. And then anti-iba-1 (Abcam) antibody was used, tissues were further blocked with Biotin for 15min each. Antibodies were detected using a rabbit peroxidase ABC Kit (MXB biotechnologies). Each sample was viewed with the use of BX53 Fluorescence microscope (Olympus).

Iba1⁺ cell density and soma size quantifications

Scans of the hippocampus were imaged using BX53 Fluorescence microscope (Olympus) in brightfield on IHC sections stained for Iba1⁺. The hippocampus was then subdivided into CA3 and CA1 regions in ImageJ. The number of Iba1⁺ cells in region, and the area of each region were recorded. Cell numbers were expressed as number of Iba1⁺ cells per mm [10] in a 5 µM thick section. The area of the soma of Iba1⁺ cells were manually traced and measured in ImageJ.

Elisa assays

Rat IL-6 Elisa kit (Dakewe Biotech), Mouse IL-1 beta Elisa kit (Dakewe Biotech), Rat IL-1 beta Elisa kit (proteintech), Mouse IL-6 Elisa kit (proteintech). Young/aged astrocytes were plated on 12-well plates, after reaching confluency, the medium was removed and DMEM/F12 without serum was added for 24h, Rat IL-6 and IL-1 beta were detected by collecting the supernatant. Similarly, after culturing with H₂O₂ treated astrocyte conditioned medium for 24h, the supernatant of BV2 was collected for detecting Mouse IL-6 and Mouse IL-1 beta.

Statistical analysis

Data computation was accomplished by SPSS software 16.0 (SPSS Inc., Chicago, IL, USA). For determining the significance of differences between two groups or among multiple groups, Student's t-test or one-way ANOVA was applied. Each experiment was conducted for three times at minimum. The statistical significance in differences were confirmed when $p < 0.05$.

Results

Neuron loss in aged mice and the emergence of senescent astrocytes

HE staining has been widely used as a neuronal degeneration marker after a variety of brain damage [11]. HE staining of the hippocampal neurons revealed normal neurons in the group of young mice with rich cytoplasm and slightly and round stained nucleus and clear formation, on the contrary, in the group of aged mice, degenerated neurons showed shrinking cytoplasm and bodies, and most of the degenerated neurons revealed deep stained nucleus and irregular morphology. The neuronal loss was evaluated by Nissl staining. As shown in Fig. 1b, young mice showed highly dense pyramidal layer neurons with intact structure, in contrast, the neurons appeared atrophied and pyknotic in aged mice, meanwhile, the numbers were also lower than those in young mice, which can be seen intuitively from Fig. 1b. β -gal staining can be used as a senescence marker [12], with increasing age of mice, the β -gal positive cells were significantly increased (Fig. 1c) and most of the positive expression of β -gal was co-stained with GFAP, this phenomenon suggests that the senescence of astrocyte may play a role in neuronal degeneration of aging. Fig. 1d indicated that a progressive enlargement of microglial size as the activation state and phagocytic capacity of the cells enhances, usually, amoeboid microglia was considered in higher activation state [13].

Serial passaged astrocytes show phenotypes of aged cells and affected neuronal viability

To deeply investigate the changes in a range of physiological functions of astrocytes during senescence in vitro, astrocytes were isolated from Wistar rat pups and made identification by the marker of GFAP (Fig. 2a), the data showed highly purity of astrocytes. To simulate natural senescence, serial passaging under standard culture conditions (passages between 20-25, named as aged-astrocytes) was made and found that SA- β -gal positive astrocytes were increased compared with those from passages 1-5, which were named as young-astrocyte (Fig. 2a). To systematically explore the altered phenotype of young and aged astrocytes, senescence markers of P53, P16, P21 and IL-6, which were collectively known as the senescence-associated secretory phenotype (SASP) [14] were performed. As is shown in Fig. 2b, after serial passaging of astrocytes, the expression of P53, P21 and P16 were gradually increased, meanwhile, the concentration of IL-6 of aged-astrocytes in supernatant were elevated either (Fig. 2c). Mitochondrial membrane potential was selected to detect whether the mitochondrial function remained normally,

notably, the MMP that showed significantly decreased in those aged astrocytes(Fig. 2d). To confirm whether these changes of astrocyte would do deleterious effects on microglia or neurons(Fig. 2e), we examined the neurotoxicity to Neuro-2a cells by using different conditioned medium isolated from young-astrocytes or aged-astrocytes supernatant treated BV2 cells(Fig. 2f). As might be expected, aged-astrocytes-derived CM or aged-astrocytes-derived CM treated BV2 supernatant significantly decreased Neuro-2a cell's viability(Fig. 2g, 2i). Meanwhile, aged-astrocytes-derived CM promoted negative contribution for the survival of BV2 cells(Fig. 2h), suggest that substances released from aged-astrocytes may did activate the BV2 cells and may prompt cells to release inflammatory mediators to damage neurons.

Astrocytes activate a senescence program in response to oxidative stress and showed mitochondrial dysfunction

To establish another model of cellular senescence instead of cellular death in response to oxidative stress in human astrocytes, rodent astrocytes were treated with 80uM H_2O_2 for 2 h and then removed and added new culture medium without serum for 24 h. The cell viability showed no significance when the concentration reached 80 μ M(Fig. 3a). Next, senescence markers were also detected to find out if the cells already reached the SASP phenotype. Data shown in Fig. 3b, the proteins expression of P53, P21 and P16 were significantly elevated after treating with H_2O_2 . Meanwhile, administration of berberine showed a certain degree of decrease in these indicators, although the expression of P16 decreased but showed no significance. And, the concentration of IL-6 in supernatant of astrocytes treated with H_2O_2 were elevated compared with those didn't(Fig. 3e). Mitochondrial membrane potential also showed that exogenous H_2O_2 treatment caused MMP decrease and administration of berberine would do recovery of the damaged MMP(Fig. 3d). ROS production is the most common product upon cells facing to oxidative stress, compared with control cells without H_2O_2 addition, H_2O_2 treatment increased intracellular ROS accumulation, and, markedly lowered cellular ATP levels (Fig. 3f, Fig. 3g). What's more, H_2O_2 induces mitochondrial fission by elevating the expression of mitochondrial fission protein drp1, and decreasing the expression of mitochondrial fusion proteins Mfn2 and opa-1, although the mitochondrial fusion protein showed no significant decrease. Concurrently, this adverse effect on mitochondrial function caused by oxidative stress was reversed by the treatment of berberine(Fig. 3h, Fig. 3i).

Direct interactions between senescent astrocytes and neurons

To confirm that H_2O_2 -treated astrocytes' mitochondrial dysfunction is responsible for neuron viability, we examined the neurotoxicity to Neuro-2a cells by culturing with conditioned medium isolated from H_2O_2 -stimulated astrocytes(Fig. 4a). As expected, H_2O_2 -treated astrocyte-derived CM significantly decreased Neuro-2a cells' viability, However, CM from AS treated without H_2O_2 didn't affect the viability of Neuro-2a cells. To confirm if apoptosis happens after treating with conditioned medium for 24 h, we also detected caspase-3 activity of Neuro-2a cells and found that Neuro-2a cells treated with H_2O_2 -treated astrocyte

supernatant presented a significant increase of caspase-3 activity at 24 h, what's more, this effect was reversed by administration of berberine.

Indirect effects of senescent astrocytes to neurons

Before, we found that the conditioned medium of astrocytes treated with H_2O_2 could have direct toxic effects on Neuro-2a cells. However, what is known to us is that there're so many types of cells in brain, will astrocytes do some indirect effects like firstly activate the microglia cells, which act as immune cells in brain, make them secrete neuronal inflammation cytokines and do harm to neuron cells. To confirm this hypothesis, we first examined the viability of BV2 cells treated with H_2O_2 -stimulated astrocytes or supernatant and found that after culturing with condition medium for 24 h, the viability of BV2 cells were significantly decreased(Fig. 5b), what's more interesting thing is that the IL-6, also seemed as a neuronal toxic cytokine, was significantly elevated compared to those treated without H_2O_2 (Fig. 5c). After treating with H_2O_2 -treated astrocyte-derived CM for 24 h, the supernatant of BV2 cells was collected and cultured with Neuro-2a cells. Data showed that after incubation with the conditioned medium of BV2 cells, the viability of neuro-2a cells decreased significantly compared to other groups(Fig. 5d), moreover, the activity of caspase-3 of these neuro-2a cells showed that astrocytes treated with H_2O_2 supernatant treated BV2 supernatant was also capable of increasing caspase-3 activity of neuron-2a cells, and administration of berberine would have ameliorating effects(Fig. 5e).

Discussion

Astrocytes play curial physical and molecular roles in the mammalian brain, any interference to their normal physiological function may lead to the pathology of central nervous system diseases, thence, the aging of astrocytes may have immense impact on the function and micro-environment of the brain. As a potential candidate of aging, cellular senescence was thought to be the inducing factor of aged-related neurodegenerative diseases. However, knowledge of the impact of senescent astrocytes in the brain is fragmented, and, what's more troubling us is that it remains unclear how these senescent astrocytes may interact with microglia or neurons or any other cell types in the mammalian brain. In our in vivo aging model of mice, it has been found that the hippocampus of old mice showed thinning and loosening of pyramidal cells, as well as pyknotic and atrophied neurons, while the relative neuron loss of young mice was less than that of old mice, while the activation of microglia is more severe than those in young mice, which consistent with findings in other studies [15–16]. However, finding interests us most is that through the co-immunostain of β -gal and astrocyte marker GFAP, it could be found that this cell senescence marker appears in the same position of astrocytes, which suggests that astrocyte senescence has occurred first in the process of brain aging, and may bring adverse factors to other type of cells in brain. Therefore, we made attempts to establish astrocytes models of aging in vitro to assist in the study of brain aging.

Several reports have shown that late passage of primary or mesenchymal stem cells can appear sign of senescence. The cultured primary MEF cells aged through later passage, which causes the cells to swell

and eventually stop proliferating [17–18]. The replicative senescence of astrocytes has also been confirmed in primary cultures derived from normal or post-AD brain tissue [19–20]. In the course of our experiments about astrocytes and adipocyte-derived mesenchymal stem cells, it was found that higher passage number cells have indeed reduced proliferative and differentiation capabilities (data not shown). Therefore, we here constructed a serial passage model and oxidative stress model of astrocytes to imitate senescent cells during aging. Actually, previous articles have reported astrocytes that underwent serial passage cultivation or oxidative stress showed aged phenotypes like nuclear enlargement of primary astrocytes [20–21], elevated expression of P53 and P21 and P16 [22]. Senescence-associated beta-galactosidase, a widely used cellular senescence marker, which is overexpressed and accumulated in the lysosomal specifically in senescent cells [23]. Senescent astrocytes in our study also found these similar changes of indicators. What's more is that the mitochondrial function of senescent astrocytes may decline with increased passaging [24], especially the level of proton leak and ATP, but we found that increased passaging did not affect the level of ATP, which is different from the research before, speculating that this discrepancy may be caused by the difference of length of culturing time and self-regulation of astrocyte itself, but ATP level of the model of oxidative stress of astrocyte indeed changed. We also examined the mitochondrial membrane potential (MMP) and molecular changes in senescent astrocytes. Found that no matter the serial passing model or the oxidative model of senescent astrocytes model all appeared MMP decline, which suggests that mitochondrial function is negatively affected. However, indeed, a drop in mitochondrial membrane potential does not necessarily lead to the loss of mitochondrial functions, research has reported that in Huntington's disease occurred imbalanced mitochondrial fusion-fission [25], our results showed that oxidative stress induced astrocyte senescence occurred with elevated expression of DRP1, suggests that fission in mitochondria of this type of senescent astrocyte was enhanced. Administration of berberine could reverse these mitochondrial dysfunctions caused by H_2O_2 . Thus, here leaves the question that how the mitochondrial dysfunction of senescent astrocytes does impact on the process of brain aging.

It has been proposed that astrocytes can establish communications with microglia, neurons and other astrocytes [26], and may involve in synapse formation and elimination to different degrees in a wide range of disease conditions [27]. Astrocyte-microglia are considered to have immune-related functions in the mammalian brain. Initially, all the inflammatory responses or regulatory process are activated to against with the disruption of steady-state brain homeostasis. This rapid response induces the process of brain repair mostly by those activated glia cells. However, sustained secretion of inflammatory mediators of astrocyte or microglia may induce chronic inflammation, which usually happens in brain aging and finally make contribute to neurodegeneration and cognitive decline [28–29]. Recently, research has found that a subset of microglia named as disease-associated microglia, which are associated with the genes of many of which were found in human genome that are linked to Alzheimer's disease (AD) and other neurodegenerative conditions [30–32]. This type of microglia will sustain secrete IL-6 and do impact on neurons and finally lead to neuroinflammation. However, how the microglia be activated, numerous explanations are aroused. Some research suggests that microglial maybe activated by a repertoire of pattern recognition receptors (PRRs) which allow microglia to detect “harmful signals” such as

substances containing pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) including ATP or DNA or different types of interleukin released from astrocyte or other cell type in brain [33–34]. Once upon detecting these messages, microglia migrate to those damaged positions and engulf those materials with an amoeboid-like “reactive” morphology [35–36]. Our study found that in the brain of aged mice, the microglial morphology was significantly altered, and in vitro data also found that serial passaging or oxidative stress induced senescent astrocytes secreted higher IL-6 compared to those young-astrocytes, so these mitochondrial dysfunction or interleukin release by astrocyte maybe a cause of microglia activation and finally lead to neurons death due to the sustained secrete of neuroinflammatory cytokines. Our cell viability assay of astrocyte-microglia-neuron also proved this cell-to-cell interaction would eventually lead to neuronal death.

Except for the astrocyte-microglia-neuron interaction referred above, our data also suggests that different types of senescent astrocytes could do damage to neurons directly. Although astrocytes were initially considered as nonfunctional fillers of the neuronal network. However, with time and technology advances, the significance of these types of cells for many complicated biological processes has been elucidated. In vivo, they interact closer with neurons and participate in the “tri-partite synapse”, which couples neurotransmission between pre- and postsynaptic materials [37]. Further contributions like against trauma, infection, and neurodegeneration to maintain neuronal health. However, many researches have reported that astrocyte senescence may directly influence neuronal health through multiple process. Like SASP, P16, P21 [38–39], which were referred above, and DNA damage induced by ionizing radiation [40]. What’s more, research also reported that ROS-induced senescence in human astrocytes showed downregulated genes of neuronal development and differentiation and upregulated genes of proinflammation [41], these may be partially responsible for the neuronal damage or death. In our study, we found that after neurons incubated with the supernatant of senescent astrocytes, the viability was dramatically decreased, combined with elevated caspase-3 expression compared to those astrocytes treated without H₂O₂. Interestingly, these adverse effects were reversed upon inclusion of berberine when inducing oxidative stress with H₂O₂. These results suggest that senescent astrocytes could do impact on neuronal survive in a direct way.

Meanwhile, we used berberine as a positive control to deal with the dysfunction of mitochondria induced by oxidative stress, since it had long been reported to have the function of mitochondria targeting and previous studies also proved that it could possess several pharmacological properties [42–45], including anti-inflammatory, antifibrotic, and correct the fission of mitochondria. Our data shows that berberine may have the favorable potency of astrocytes from escaping the process of senescence.

Conclusions

In summary, we have shown that in vivo, astrocytes maybe the earliest sign of aging in numerous types of brain cells, and senescent astrocytes could trigger neurons death both direct and indirect ways. However, future investigation on cell-to-cell interactions and the decent mechanism of them is needed. Astrocyte senescence is a brand-new field of study and more research needs to be designed and

implemented so that the autonomous and non-autonomous mechanisms of senescent astrocytes and its bandage between age-related neurodegenerative diseases would eventually be uncovered.

Declarations

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Author Contributions WZ and XY designed the overall research experiments. MZ and LC performed the experiments. WZ and JL analyzed the data. WZ, XY wrote and revised the manuscript. All authors read and approved the final manuscript.

Data Availability All the data used to support the findings of this study are included within the article. Additional data related to this paper may be requested from the authors.

Compliance with Ethical Standards

Conflict of Interest All authors declare that they have no conflicts of interest.

Statement on the Welfare of Animals All animal experimental procedures were approved by the Ethics Committee for the Use of Experimental Animals of College of Basic medical Sciences, Jilin University [SCXK(Jing)2014-0004].

Consent to participate No applicable.

Consent to publish No applicable.

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Figures

Figure 1

The neuron loss of Young and Aged mice were examined and senescent astrocytes were found accompanied by microglia activation. **(a)** HE staining of mouse brain. $\times 200$ magnification. **(b)** Nissl staining in the hippocampus of each group. $\times 200$ magnification. **(c)** Representative brain sections co-immunostained for Gfap (red) (markers of astrocytes) and β -gal (green) in young and aged mice (hippocampus). $\times 200$ magnification. **(d)** brain sections immunostained for iba-1 (activated markers of

microglia). ×200 magnification. (e) Quantification of the average soma size of microglia in the CA3 hippocampal region.

Figure 2

Astrocytes acquired senescent phenotype through serial passaging and SASP secretion may cause direct or indirect neuronal damage. (a) Immunofluorescent staining using GFAP antibody as astrocytes' marker, and cell senescence staining of β -gal. (b) Representative immunoblot and (c) quantitation of P53, P21, P16 and β -Actin, in Young and Aged astrocytes. β -Actin was a loading control and data are expressed relative to Young, n=3. (d) Cytokine ELISA in culture medium of young and aged astrocyte expressed as pg cytokine per mL of culture medium without serum. (e) JC-1 staining. The red and green fluorescence reflects changes in the mitochondrial membrane potential of young and aged astrocytes, n=3. (f) Scheme of conditioned media (CM) and cell viability assay by using young and aged astrocytes or astrocyte-CM

treated BV2 cells. (g) cell viability assay by using young and aged astrocytes supernatant treated N2a cells. (h) cell viability assay by using young and aged astrocytes supernatant treated BV2 cells. (i) cell viability assay of N2a cells by using supernatant of young and aged astrocytes supernatant treated BV2 cells. All experiments were expressed as the mean $\pm$ S.D, analyzed by ANOVA followed by Tukey's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Figure 3

Mitochondrial functions in aged astrocytes were found declined and berberine may have reversal effect. **(a)** cell viability assay of astrocytes by using different concentration of H₂O₂. **(b)** Representative immunoblots and **(c)** quantitation of P53, P21, P16 and β -actin, in astrocytes treated with H₂O₂ and berberine. β -Actin was a loading control and data are expressed relative to control, n=3. **(d)** JC-1 staining. The red and green fluorescence reflects changes in the mitochondrial membrane potential of astrocytes treated with or without H₂O₂ and berberine, the group of CCCP is used as a positive control. n=3. $\times 200$ magnification. **(e)** Cytokine ELISA in culture medium of astrocytes treated with or without H₂O₂ expressed as pg cytokine per mL of culture medium without serum. **(f)** Intracellular ROS levels were measured using flow cytometry. **(g)** ATP content was detected by the ATP Assess Kit. **(h)** Representative immunoblots and **(i)** quantitation of drp1, mfn2, opa-1 and β -actin, in astrocytes treated with H₂O₂ and berberine. β -Actin was a loading control and data are expressed relative to control, n=3. All experiments were expressed as the mean $\pm$ S.D, analyzed by ANOVA followed by Tukey's test, *P<0.05, **P<0.01, ***P<0.001.

Figure 4

The direct effect of aged astrocytes in communicating with neurons. **(a)** A schematic diagram of the interaction of cell supernatants. **(b)** cell viability assay of Neuro-2a cells by using astrocytes treated with or without H₂O₂ supernatant. **(c)** Caspase-3 activity in Neuro-2a cells (treated with astrocyte-CM for 24 h). Green fluorescence indicates the activity of Caspase-3. Blue indicates nuclear stained by Hoechst33342. Merge is the overlapped green and blue. ×200 magnification. All experiments were expressed as the mean ±S.D, analyzed by ANOVA followed by Tukey's test, *P<0.05, **P<0.01, ***P<0.001.

Figure 5

The indirect effect of aged astrocytes in communicating with microglia and neurons. **(a)** A schematic diagram of the interaction of cell supernatants. **(b)** cell viability assay of BV2 cells by using astrocytes treated with or without H₂O₂ supernatant. **(c)** Cytokine ELISA in culture medium of BV2 treated with astrocyte supernatant (treated with or without H₂O₂) expressed as pg cytokine per mL of culture medium without serum. **(d)** cell viability assay of N2a cells by using BV2 treated with astrocyte supernatant. **(e)** The caspase-3 activity was measured with a caspase-3 assay kit. All experiments were expressed as the mean $\pm$ S.D, analyzed by ANOVA followed by Tukey's test, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.