Lipocalin-2 Mediated Polarization of Neutrophil in Cerebral Ischemia-Reperfusion Injury

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Research Article

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Abstract

Cerebral ischemia-reperfusion (I/R) injury is a difficult point in the treatment of ischemic stroke. Lipocalin-2 (LCN2) and neutrophils play an important role in I/R injury. We explored the effect of anti-LCN2 antibody (LCN2mAb) and to further clarify the relationship between LCN2mAb and neutrophil polarization (N1/N2 neutrophils) in I/R injury.

A mouse middle cerebral artery occlusion (MCAO) model was used to induce transient cerebral ischemia. LCN2mAb was administered 1h before MCAO; Anti-Ly6G was administered for 3d before MCAO. The expression of LCN2 and Ly6G was Measured by western blot. Infarct size, behavior and the blood-brain barrier (BBB) damage were assessed. The polarized N2 neutrophils were measured by western blot and Flow Cytometry. Using HL-60 as a cell model explores the role of LCN2mAb in the polarity transition of neutrophils.

The expression of LCN2 and Ly6G in the brain at different time points reached a peak after I/R 24h and then gradually decreased. As demonstrated previously, LCN2mAb-treated mice had neuroprotective effects in cerebral infarction volume, behavior, and blood-brain barrier damage. The expression of Ly6G was not significantly different, but the expression of N2 neutrophils was increased. The expression of anti-inflammatory factor CD206 was significantly increased, and pro-inflammatory factor TNF- α was significantly reduced. Compared with mice treated with Anti-Ly6G, Anti-Ly6G-LCN2mAb combined treatment of I/R, there was no further improvement in behavior and pathology. Based on HL-60 as a cell model, N1-HL-60 cells were pretreated with LCN2mAb, and N2-HL-60 cells were significantly increased.

LCN2 may affect the prognosis of ischemic stroke by mediating neutrophils polarization.

Introduction

Ischemic stroke is a major disease that seriously endangers human health and quality of life. The main pathophysiological process is the stenosis or occlusion of the cerebral artery lumen in a short period, which causes the interruption of blood supply and leads to ischemic injury[1]. The main early treatment is vascular recanalization (intravenous thrombolysis and mechanical thrombectomy) to restore blood perfusion in the ischemic area[2,3]. However, blood flow reperfusion is a double-edged sword. It may cause secondary damage to tissues by increasing the release of free radicals and inflammatory factors and further aggravate nerve cell damage[4]. This damage is called I/R injury.

After ischemic stroke, neurons die and release a large number of inflammatory factors and stimulate the activation of Intrinsic cells such as astrocytes. The release of ROS, chemokines, and cytokines in ischemic brain tissue will recruit neutrophils that are the first to be recruited outside the brain, which lead to oxidative stress, destruction of BBB, brain edema, and brain damage[5-7]. In addition to inflammatory damage, neutrophils have also been found to have neuroprotective effects-N2 neutrophils[8,9]. Neutrophils show an atypical phenotype, N1 neutrophils aggravate neuroinflammation which is pro-

inflammatory, and N2 neutrophils are conducive to the resolution of inflammation which is anti-inflammatory after stroke[10].

LCN2 is a member of the lipocalin family and is a multifunctional protein in the acute phase of diseases. LCN2 is a regulator of various cell phenotypes in the central nervous system(CNS), such as cell death, survival, morphology, migration, invasion, differentiation, and functional polarization[11,12]. After the central nervous system is injured, LCN2 will induce many chemokines to participate in the inflammatory response[13]. However, the effect of LCN2 on neutrophils in ischemic stroke has not been explored. The polarization of neutrophils to N2 type is a new perspective to reduce inflammatory damage after acute ischemic stroke, but its mechanism is still not fully elucidated. The purpose of this study is to explore whether LCN2 can induce neutrophil phenotypic changes, which may affect the outcome of stroke in a mouse MCAO model.

Materials And Methods

Animals

C57BL/6J male mice (9-10 weeks, weight 22-25g) were purchased from Shanghai Jihui Experimental Animal Co., Ltd. The mice were housed in the Experimental Animal Center of Soochow University. The feeding standard was rodent feed and water. The temperature was controlled at 22-26°C and illumination was controlled at the light-dark cycle of 12h of darkness/12h. Animals should adapt to the breeding environment for at least one week before performing relevant experimental operations. All experimental operations comply with the requirements of the Animal Care and Use Committee of Soochow University.

Construction of mouse MCAO model

Mice were anesthetized with 4% Chloral hydrate. Keep mice at a temperature of 37°C. The mice were fixed after anesthesia and performed a median neck incision, then separated bluntly the skin, muscles, and fascia to avoid damaging the vagus nerve until the right common carotid artery was separated. Then the internal carotid artery outside the carotid was separated in turn artery. The external carotid artery was cut off and made an incision. The polylysine-coated thread was plug approximately 10mm from the incision, through the external carotid artery to the internal carotid artery, and occluded the start of the middle cerebral artery along the internal carotid artery. After 75min, the thread was pulled out gently to restore blood flow. The blood vessel was ligated, and the incision was sutured. After modeling, keep the mice warm.

Medicine administration

Before MCAO or sham surgery, LCN2mAb and isotype control IgG was administered through the lateral ventricle of the brain. According to the literature, we used LCN2mAb to neutralize the effect of LCN2. After

LCN2mAb binds to LCN2, it destroyed the formation of disulfide bonds in the LCN2 structure, thereby destroying the cup-shaped structure which affected the function of LCN2[14]. 30min before MCAO, LCN2mAb (0.6ug/ul 3ul, RD system) or isotype control IgG 3ul was administered to the lateral ventricle [14,15]. Before MCAO, anti-Ly6G antibody[16,17] (200ug/3d, bioxcell) was intraperitoneally administered[18]. The mice were randomly divided into the following experimental groups: (1) sham, (2) MCAO+I/R 12h, (3) MCAO+I/R 24h, (4) MCAO+I/R 48h, (5) MCAO+ I/R 96h, (6) sham+LCN2mAb, (7) MCAO+IgG, (8) MCAO+LCN2mAb, (9) MCAO+Anti-Ly6G, (10) MCAO+LCN2mAb+Anti-Ly6G. All behavioral results were evaluated by the double-blind method.

Western blot

After I/R 24h, the mice were killed by rapid cervical dislocation. The brain tissues were decapitated and were lysed by cell lysate. After standing for 1h, the tissues were centrifuged at 13200r/min for 20min. the collected supernatant was the cerebral ischemic tissue protein. BCA protein quantification kit was used to detect protein concentration. we performed SDS-PAGE gel electrophoresis with the same amount of protein mass (30mg), then transferred membrane(PVDF membrane). PVDF membranes were dissolved 5% skim milk in TBST and seal at room temperature for 2h, washed with TBST three times for 10min each time. Immunolabeling included primary antibody goat anti-LCN2 (1:500; R&D Systems), rat anti-Ly6G (1:500; Invitrogen), rabbit anti-occludin (1:1000; proteintech), rabbit anti-ZO-1 (1:1000; proteintech), rabbit anti-CD206 (1:500; Abcam). PVDF membranes were incubated overnight at 4°C, washed three times with TBST at room temperature for 10min each time, then was applied secondary antibody, goat IgG secondary antibodies (1:1000; Millipore), rat IgG secondary antibodies (1:1000; Absin), rabbit IgG secondary antibodies (1:1000; Jackson).

Reverse transcriptase polymer chain reaction

After 24h of I/R, the mice were killed by rapid cervical dislocation. The brain was decapitated and the total RNA (2 mg) of the sample was extracted. Then, the reverse kit (#K1622, Thermo Fisher Scientific) was used to reverse transcription RNA into cDNA. The samples were subjected to RT-PCR to determine the expression of inflammatory factors at the transcription level. 18s was used as an internal reference. The result of RT-PCR was calculated by the $2^{-\Delta\Delta CT}$ method to calculate the Ct value.

Cerebral infarction volume analysis

After 24h of I/R in mice, the brain was removed by rapid cervical dislocation and decapitation, and the coronal section was cut into 2mm thick sections, stained with 2,3,5-triphenyltetrazolium chloride (TTC, Sigma), and then 4 % Paraformaldehyde(PFA, Sigma) was used to fix cerebral infarction volume. the AlphaEaseFC program and the following formula were used to measure the infarct volume = infarct

volume / (whole brain volume-contralateral brain volume) * 100%. unstained area (light color) is defined as ischemic lesions.

Neurological score and behavioral testing

After 24h of I/R in mice, neurological function scores and behavioral tests were performed. Zea-longa score is 0-5 scale: 0 = no obvious defect, 1 = weakness of the ipsilateral forelimb (right), 2 = circling to the ipsilateral side (right), 3 = body imbalance, the trunk tilted to the ipsilateral side, 4 = Cannot go spontaneously; 5 = No spontaneous movement or death. The purpose of the open field is to explore the autonomous behavior, behavior, and tension of experimental animals in a new environment. In our experiment, we mainly explored the active movement ability, including movement distance and movement speed within a fixed time of mice after MCAO. The passive gait test of mice explores the passive movement ability of mice, mainly detecting their movement speed and movement time.

Measurement of BBB permeability

Evans blue (EB) was used to detect leakage after BBB injury. After reperfusion 24h, EB (2% diluted in saline) (Sigma-Aldrich) was injected into the tail vein, and the injection dose was 3ml/kg. After 1h of reperfusion, normal saline was perfused through the heart until a colorless perfusion solution was obtained from the right atrium. The brain was decapitated and weighed. It was sonicated into a homogenate in 100mmol/L PBS 0.75ml, and then 0.25ml 100% trichloroacetic acid was added. Samples were incubated overnight at 4°C and centrifuged at 15000r.p.m for 15min the next day. We measured the absorbance of the supernatant at 620nm with a spectrophotometer and quantified the content m of EB from the linear standard curve. The EB result is expressed as m/M.

Flow cytometry

After 24h of I/R in mice, the brain was quickly decapitated and removed. The whole-brain tissues were treated with the Adult Brain Dissociation Kit (Miltenyi Biotec 130-107-677), and the cells were washed by centrifugation to obtain a cell suspension. Add flow cytometry antibody and rupture fixative. After incubating in the dark at 37°C for 60min, samples were washed twice and then resuspended in 400ul PBS. Flow cytometry was used to detect cell phenotype. The following antibodies were used: CD11b FITC M1/70 (BD), Ms Ly-6G PE 1A8 (BD), Ms CD206 Alexa 647 (BD), CD66b (BD).

Cell culture

Human promyelocytic leukemia cells (HL-60) were purchased from Wuhan Procell company and cultured in IMDM medium with 10% (v/v) fetal bovine serum (FBS) and 1% double-antibody. HL-60 was grown in an incubator containing 5% CO₂ and 95% N₂ at 37°C. Untreated cells were denoted as N0-HL-60 cells.

When HL-60 cells were cultured to a concentration of 5×10^5 cells/mL, they were first cultured with 1 μ M all-trans retinoic acid (ATRA) medium, and the medium was exchanged with 1 μ M ATRA medium on the third day, and the cells obtained from culture to the fifth day which were recorded as N1-HL-60. On the 5th day, the cells were obtained by culturing for 24 hours with 50ng/mL TGF- β which were designated as N2-HL-60[19,20]. The cells were collected on the 6th day, and the N1-HL-60 and N2-HL-60 were separated by CD66b antibody flow cytometry analysis.

data analysis

The data are expressed by mean $\pm$ standard error (mean $\pm$ SEM). GraphPad Prism7 (GraphPad Software Inc., La Jolla, CA, USA) was used for analysis. The T-test is used to compare the differences between two groups of data; ANOVA analysis of variance is used to compare multiple groups of data. $P < 0.05$ considered the difference to be statistically significant.

Results

The expression of LCN2 and neutrophil after I/R injury

After ischemic stroke, neutrophils are driven by inflammatory factors at the injured site to enter the brain through the damaged blood-brain barrier. LCN2 is an acute-phase protein related to stroke, and can also be secreted and produced by neutrophils. To study whether the two are related in ischemic stroke at different time points, we detected the changes of neutrophils Ly6G and LCN2 using Western blot. from 12h to 96h after I/R (Figure 1a-b). The LCN2 was an obvious upward trend at 12 hours ($P < 0.05$), and the highest at 24 hours ($P < 0.05$), then the expression level gradually decreased to the normal level. Western blot was also used to explore the changes in neutrophils after I/R. The results showed (Figure 1c-d) that neutrophils began to rise 12h and to be the highest at 24h ($P < 0.05$), then began to show a downward trend and gradually recovered. These results suggested that both Ly6G and LCN2 changed after I/R, maintained at a high level in the 24h acute phase, and had a similar trend of change. After I/R, the Zea-longa score of neurological injury also showed a similar trend (Figure 1e). The behavioral function of I/R injury gradually improved over time. Both Neutrophils Ly6G and LCN2 are involved in the pathophysiological process of I/R injury, and the changes of the protein have a similar trend in time. We speculate that the two are involved in the acute phase of cerebral I/R injury.

The protective effect of LCN2mAb treatment on I/R

In the above result, the expression level of LCN2 had changed over time, so would LCN2 affect the outcome of acute ischemic stroke? 30min before I/R, the mice were pretreated with LCN2mAb and control IgG. We selected the time point of high expression of LCN2 24h after I/R to observe the behavioral and pathological results of the mice.

Zea-longa score (Figure 2a), the neurobehavioral open field test, and the gait analysis results showed that there was no significant difference in neurological damage between the sham group and the LCN2mAb treatment group. Compared with mice without surgery, mice after an I/R injury had obvious neurological impairment; but compared with control IgG-treated mice, the neurological impairment of mice treated with LCN2mAb was significantly reduced after I/R ($P<0.05$). LCN2mAb alone would not cause obvious damage to nerve function, but the nerve function of mice with I/R was more preserved after using LCN2mAb. The results of the open field test reflected the differences in the autonomous activity and movement speed of the mice (Figure 2b-c). The gait experiment included standing time (Stance time) and gait time (Stride time) (Figure 2d-f). The above behavioral results all proved that the neurological function of mice after I/R would be significantly damaged, but compared with the control, LCN2mAb could partially improve the neurological damage caused by I/R.

TTC could intuitively and efficiently assess cerebral ischemic volume. Compared with control IgG-treated mice, TTC after LCN2mAb treatment showed a reduction in total infarct volume (Figure 2g-h) ($P<0.05$). The integrity of the BBB is an effective indicator for detecting the degree of ischemic brain damage. We evaluated the effectiveness of LCN2mAb treatment in reducing the damage of the BBB (Figure 2i). The EB test showed that compared with mice treated with control IgG, LCN2mAb can reduce the leakage of the BBB after MCAO ($P<0.05$). Western blot evaluated the expression of tight junction proteins Occludin and ZO-1 after MCAO (Figure j-l) and found that compared with the control IgG-treated mice, in the LCN2mAb-treated mice the expression of Occludin was significantly increased ($P<0.05$). LCN2mAb can reduce BBB damage after I/R injury.

The effect of LCN2mAb treatment on the level of neutrophils

In the above experiments, we had explored that the expression level of neutrophils would also change after I/R injury. LCN2mAb treatment had improved the I/R injury. So did LCN2mAb treatment cause changes in the expression of neutrophils in the brain tissue? We measured the expression of neutrophils in the brain tissue of mice after I/R for 24h. Because there was no significant difference in neurological damage between the sham group and the sham+LCN2mAb group, this group of experiments was divided into the sham group, the I/R+IgG group, and the I/R+LCN2mAb group. We analyzed the whole brain cells by flow cytometry (Figure 3a-b) and explored the percentage of neutrophils ($CD11b^+ Ly6G^+$) in whole brain cells. Compared with the sham group, flow cytometry showed that the expression of $CD11b^+ Ly6G^+$ was significantly increased with I/R ($P<0.05$), but there was no significant difference between the mice treated with control IgG and LCN2mAb before surgery ($P>0.05$). This was inconsistent with our guess, so we used Western blot for analysis, and the results were consistent with flow cytometry (Figure 3 c-d). The above experiments showed that the protective effect of LCN2mAb after treatment had no obvious correlation with changes in the total number of neutrophils in the brain.

LCN2mAb affects neutrophils to N2-type polarization

In the above results, there was no significant difference in the total number of neutrophils between I/R+control IgG and I/R+LCN2mAb mice. However, except for inflammatory damage, neutrophils had also been found to have neurological Protection effects which were named N2 neutrophils. Therefore, we explored whether there is a difference in the polarity of neutrophils. Referring to previous articles[21,19], we used CD206 to label polarized N2 neutrophils in mice. Mice were divided into sham, I/R+control IgG, I/R+LCN2mAb groups. After I/R 24h, compared with the control IgG groups, flow cytometry analysis showed that the I/R+LCN2mAb group N2 neutrophils (CD11b⁺Ly6G⁺CD206⁺) accounted for the percentage of neutrophils (CD11b⁺Ly6G⁺) increased significantly(Figure 4a-b) ($P<0.05$). Similarly, Western blot results showed (Figure 4c-d) that the expression of N2 neutrophils in the I/R+control IgG group was also significantly lower than that in the I/R+LCN2mAb group ($P<0.05$). This was consistent with our expected, the number of N2 neutrophils increased after treatment with LCN2mAb. These results explained that LCN2mAb treatment did not affect the overall number of neutrophils in the mouse brain, but it would increase the polarization of N2 neutrophils.

This was consistent with the known results that changes in neutrophil polarity.

LCN2mAb has no further protective effect after neutrophil knockout

To further confirm that neutrophils play a role in the protection of LCN2mAb, we used Anti-Ly6G (1A8) to knock out neutrophils. Referring to the experimental groups of published literature[17], the mice had divided into I/R+control IgG, I/R+Anti-Ly6G, and I/R+Anti-Ly6G+LCN2mAb groups. The Zea-longa score was used to evaluate the behavior of mice after I/R 24h (Figure 5a). Compared with the I/R+control IgG group, I/R+Anti-Ly6G and I/R+Anti-Ly6G+LCN2mAb group behavior was significantly improved. TTC and BBB leakage experiments showed the same results ($P<0.05$). However, compared with the I/R+Anti-Ly6G group, anti-Ly6G followed by LCN2mAb treatment did not significantly improve behavior, and the cerebral infarction volume and BBB damage were not significantly reduced ($P>0.05$) (Figure 5b-d). This confirmed our hypothesis that neutrophils played a role in the protective effect of LCN2mAb.

Verification of Polarity Transition of Neutrophils by LCN2mAb in HL-60 Cell Model

To further verify that LCN2 can mediate the polarity transition of neutrophils, we conducted cell experiments. The short lifespan of primary neutrophils limited their experimental use, so we used human neutrophils HL-60 cells to explore whether HL-60 cells also have transformative properties. HL-60 cells (N0-HL-60) were induced to differentiate into N1 neutrophils (N1-HL-60). And N2 neutrophils (N2-HL-60). Flow cytometry was used to filter HL-60 cells of different polarities. N2-HL-60 cells had a higher surface

CD66b expression level (Figure 6a-b). The results reported in the literature were consistent, indicating that the cell polarity was successfully induced ($P<0.05$). Whether the LCN2mAb used in the mouse experiment can also exert the same inhibitory effect in human cell lines, Western blot results showed (Figure 6c-d) that the expression of LCN2 was significantly reduced after LCN2mAb treatment ($P<0.05$). To prove whether the LCN2mAb treatment could induce the conversion of N1-HL-60 to N2-HL-60 as in vivo, we induced N1-HL-60 cells with 100ng/mL LCN2mAb or control IgG culture medium. Flow cytometry analysis (Figure 6e-f) showed that, compared with the control IgG, the expression of CD66b increased after LCN2mAb treatment, indicating that LCN2mAb indeed induced the formation of N2-HL-60 ($P<0.05$). To verify whether LCN2mAb induced the polarity of neutrophils and whether it is accompanied by changes in the inflammatory response, we used RT-PCR to detect the expression of inflammatory factors at the cell transcription level (Figure 6g-j). We found that, compared with the control IgG group, the cells treated with LCN2mAb can significantly reduce the expression of the pro-inflammatory factor TNF- α . Consistent with the above results, the process of neutrophil polarity changes was also accompanied by inflammatory change. This proved that LCN2mAb can induce neutrophils to polarize to N2 neutrophils in vitro.

Discussion

Our study illustrated that LCN2 affects the outcome of I/R injury by regulating the polarization of neutrophils. We found that LCN2 plays a crucial role in the polarization function of neutrophils. In the I/R injury, LCN2mAb inhibits the function of LCN2 and induces neutrophils to polarize to the N2 phenotype, showing an anti-inflammatory effect, which is related to neuroprotection. Therefore, we propose that LCN2 mediates neutrophil polarization to regulate the outcome of I/R injury.

In the central nervous system, LCN2 is mainly expressed on the surface of endothelial cells, neurons, and astrocytes[22]. LCN2 has been related to a variety of central nervous system diseases, such as stroke and multiple sclerosis[12]. LCN2 plays a variety of roles after ischemic strokes, such as promoting the release of neurotoxicity and inflammatory factors after I/R injury, aggravating the damage of the BBB, and increasing the volume of cerebral infarction[23]. Now, the mechanism of the above-mentioned damage is mainly mediated by LCN2 through the activation of the Astrocyte pathway[24-26].

LCN2 is a small molecule protein secreted in neutrophils, also known as neutrophil gelatinase-associated lipocalin (NGAL). The current research direction of LCN2 and neutrophils is mainly through LCN2 regulating neutrophils in anti-infection such as hepatitis and dermatitis[27-29]. For example, some scholars have found that in psoriasis-like mouse models, the neutralization of LCN2 by LCN2mAb can reduce epidermal hyperplasia, inflammation, and the infiltration of neutrophils[30]. In vitro experiments, LCN2 passes 24p3R on neutrophils to stimulate neutrophils to produce important pro-inflammatory mediators, such as IL-6, IL-8, tumor necrosis factor- α , and IL-1 α [27,13].

After ischemic stroke, there are many studies on immune cells. Neutrophils are the first immune cells that cross the damaged BBB to reach the damaged brain tissue. Despite the high degree of heterogeneity in different studies, the time of immune response after stroke is similar between patients and rodents. In

rodents, immune cell infiltration depends on the time, location, and type of blood vessel blockage. The density of infiltrating immune cells is positively correlated with the infarct volume[31].there are few studies on LCN2 and neutrophils in the brain, so we hypothesized whether LCN2 will also affect the outcome of ischemic stroke by regulating neutrophils.

In this study, we first explored the correlation between LCN2 and neutrophils after I/R injury. Our results showed that the expression of LCN2 and neutrophils is time-dependent. After I/R, the protein expression level tended to increase first and then decreased and reached a peak at 24h, which was similar to the neurological symptoms of MCAO. Due to the limited experimental conditions, we used LCN2mAb to neutralize the function of LCN2. Consistent with the experimental results in the reference[13,32,33], we evaluated the pathology and behavior of the mice and found that the MCAO mice treated with LCN2mAb were significantly improved. Therefore, we speculated that the protective effect of LCN2mAb may be related to neutrophils. We used Western blot and flow cytometry to evaluate the expression of neutrophils in the injured brain, but there was no significant difference in neutrophils, which was also different from the previously reported results. It was described in the previous literature that after using LCN2-KO mouse MCAO[25] or intraperitoneal injection of LCN2mAb[14], that the accumulation of neutrophils in the injured brain tissue was reduced. In our experiment, we used lateral ventricle injection. There may be differences in the method of medication. After I/R, neutrophils mainly enter the injured site from the peripheral circulation through the damaged BBB. Stereotactic injection of LCN2mAb directly into the lateral ventricle makes the effect more limited to neutrophils entering the brain tissue and weakens the migration effect of peripheral neutrophils. Studies have found that in the MCAO mouse model of TLR4-KO[34], the expression of neutrophils was increased, but the neurological damage was also improved. This is similar to the results of our research, and there may be similar mechanisms that prove the reliability of our experimental results.

Interestingly, although there was no significant difference in the overall number of neutrophils in the brain, the neuroprotection induced by LCN2mAb was associated with the expression of N2 neutrophils. The percentage of N2 neutrophils in brain tissues was detected by flow cytometry and Western blot. It was found that the expression of N2 neutrophils increased significantly after treatment with LCN2mAb compared with the control IgG group. Our previous experiments were also verified that the infarct volume and the behavior after LCN2mAb treatment were significantly improved. So we speculated that there were neutrophil subpopulations in the LCN2mAb treatment group which had different pathophysiological effects. The change in polarity of neutrophils was first verified in tumors. Recent studies on tumors have shown that neutrophils maintain phenotypic plasticity in different environments. Neutrophils with different functions are adapting to their environment and being differentially polarized instead of being a specific subtype from the beginning[9,35]. Numerous neutrophils with different functional characteristics have been researched in the tumor-related study, including pro-inflammatory and anti-tumor type, anti-inflammatory and anti-tumor type, pro-inflammatory and anti-tumor type, anti-inflammatory, and tumor-promoting neutrophils. Pro-inflammatory neutrophils are N1 type in tumors, and N2-neutrophils are anti-inflammatory. In ischemic stroke, we consider whether the above-mentioned anti-inflammatory and pro-inflammatory neutrophils are also present in the brain tissue of mice treated with LCN2mAb, similar to

macrophages of different polarities[36]. Previous articles have also found the existence of neutrophils of different polarities in ischemic stroke. We also found that after I/R, LCN2mAb polarized neutrophils to N2 type[37]. Through flow cytometry, the proportion of N2 neutrophils increased and the ratio of N1 neutrophils decreased in the infiltrating neutrophils, which are consistent with the observed neuroprotective effects. We have also tested the expression of several inflammatory factors at the transcription level. According to the published literature, we have also explored and found that the anti-inflammatory factor CD206 was increased, the pro-inflammatory factor TNF- α was significantly reduced [38], while IL-10 and IL-1 β had no obvious signs, which proved that LCN2 affects the transition of neutrophils and is accompanied by changes in inflammatory factors. But it may be is selective for the expression of inflammatory factors.

Previous studies found that the role of LCN2 is mainly played by astrocytes in acute cerebral ischemia[26,25]. To verify that the protective effect of LCN2mAb can be mediated through the neutrophil pathway, we refer to the previous literature using anti-neutrophil antibodies to knock out neutrophils. We found that compared with Anti-Ly6G treatment, the combination of Anti-Ly6G and LCN2mAb has no obvious neuroprotective function, which indicates that the presence or absence of neutrophils may affect the LCN2mAb neuroprotective effect.

To clarify the specificity and reliability of LCN2 to neutrophil N1/N2 polarization, we used HL-60 cells for analysis. HL-60 cells are an effective laboratory research model system for human neutrophils. HL-60 cells may respond to different cytokines, damage-related molecular patterns, growth factors released, and other different molecules after stroke, which are reported to be related to their polarization. We successfully induced HL-60 cells in different polarities in vitro and found that LCN2mAb can induce HL-60 cells into N2-HL-60. Besides, the expression of pro-inflammatory and anti-inflammatory factors in N1-HL-60 and N2-HL-60 cells was detected from the transcription level. There is a slight discrepancy between the experiment and the animal experiment. There is no significant change in the expression level of IL-1 β in the animal experiment. It may be that the expression level of IL-1 β in vivo is affected by many factors. In vivo and vitro experiences have highlighted the important role of LCN2 on neutrophil polarity transition after I/R injury. N1 neutrophils promote neuroinflammation, and N2 neutrophils have the effect of inhibiting neuroinflammation. Both neutrophils and inflammatory factors are due to changes in the inflammatory response. So LCN2mAb may mediate neutrophil polarization to improve the outcome of I/R injury.

In our experiment, we still have some shortcomings. For example, we have not discussed the pathway mechanism of LCN2mAb promoting the polarity transition of N1 and N2 neutrophils; we explored the role of LCN2 using the monoclonal antibody LCN2mAb. It has not been verified in LCN2-KO mice, and we will further improve it in the next study; we have initially verified that LCN2 may function through neutrophils, but the literature also mentions that LCN2 will play a role through Astrocytes. The contribution of neutrophils and astrocytes to the outcome of ischemic stroke is unknown, and we will further verify it. In summary, our data support that LCN2mAb has a protective effect on I/R injury and proves that it is achieved by affecting the polarity of N1 and N2 neutrophils. The heterogeneity of the neutrophil and

promoting the transformation of LCN2mAb in ischemic brain tissue is a new process, which is likely to help restore tissue homeostasis and improve ischemia. As a result of stroke, there are few studies on N1 and N2 neutrophils, and our research has opened up a new perspective for them.

Conclusion

In the animal model of ischemic stroke, the expression of LCN2 and neutrophil Ly6G is significantly correlated with time, and the expression level increases significantly in the acute phase. Both are involved in I/R injury. The use of LCN2mAb can reduce cerebral infarction volume and BBB damage and improve neurological symptoms after I/R injury. Using LCN2mAb can increase the proportion of N2 neutrophils and reduce inflammation. The above results suggest that LCN2 may affect the prognosis of ischemic stroke by mediating the polarization of neutrophils, which provides a new idea for clinical stroke treatment.

Declarations

Availability of data and material:All data generated or analysed during this study are included in this published article.

Code availability:Not applicable

Authors' contributions:Conceptualization:Zhiliang Guo; Methodology:Zhiliang Guo,Yongjun Cao ; Formal analysis and investigation:Guoli Xu and Yaqian Huang and Jiaping Xu;Writing-original draft preparation:Zhiliang Guo,Guoli Xu;Writing-review and editing:Yongjun Cao and Chunfeng Liu; Funding acquisition:Zhiliang Guo; Resources:Chunfeng Liu; Supervision: Yongjun Cao .

Compliance with Ethical Standards

Disclosure of potential conflicts of interest: The authors have no relevant financial or non-financial interests to disclose.

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Research involving Human Participants and/or Animals:Cell Line: HL-60 cell line; Procell;CL-0110;This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Second Affiliated Hospital of Soochow University(Date:2019.3.1;No.JD-LK-2019-004-02).

Informed consent:Not applicable

Aknowledge

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Figures

Figure 1

Changes in the expression of LCN2 and neutrophils after I/R. a-b western blot showed the changes of LCN2 in the ipsilateral brain tissue and related statistical results at different time points (12h, 24h, 48h, 96h) after cerebral ischemia and reperfusion. c-d western blot showed the changes of Ly6G in the ipsilateral brain tissue and related statistical results at different time points (12h, 24h, 48h, 96h) after cerebral ischemia and reperfusion. e Longa score evaluates the acute neurological damage in mice after surgery. N=6, #P<0.05, ##P<0.01, ###P<0.001.

Figure 2

LCN2mAb treatment has a protective effect on I/R. a Zea-longa score evaluated the changes in neurological function of mice under different treatments of sham, sham+LCN2mAb, I/R+IgG, I/R+LCN2mAb. b-c Distance and Mean speed of the open field evaluated the spontaneous movement and movement speed. d-f Gait detection experiment evaluated the standing time, walking time, and instantaneous speed of mice. g-h TTC evaluates the infarct volume of mice under different treatments with sham, I/R+IgG, and I/R+LCN2mAb. As shown in the figure, the red part is live cells and the white part is cell death. i Assess the damage of the BBB in mice. Evans blue BBB damage leak detection. j-l Western blot showed the expression of ZO-1 and Occludin in the BBB of mice under the above treatment. N=4-6, # $P < 0.05$, ## $P < 0.01$, ns, no significant difference.

Figure 3

The effect of LCN2mAb treatment on the level of neutrophils a-b Whole-brain tissues were collected after I/R 24h and analyzed the percentage of Ly6G (CD11b+Ly6G+) by flow cytometry. c-d Brain tissues were collected in the ischemic penumbra after I/R 24h and analyzed the expression level of Ly6G by Western blot. N=6, #P<0.05, ns, no significant difference.

Figure 4

LCN2mAb affects neutrophils to N2-type polarization a-b All brain tissues were collected after I/R 24h and analyzed the percentage of CD206 (CD11b+ Ly6G+CD206+) in neutrophils (CD11b+Ly6G+) by flow cytometry. c-d The brain tissues were collected from the ischemic penumbra after I/R 24h and analyzed the expression of CD206 protein by Western blot. N=4-6, #P<0.05, ##P<0.01.

Figure 5

LCN2mAb has no further protective effect after neutrophil knockout a The Zea-longa score evaluated the changes in neurological function under different treatments of I/R+IgG, I/R+Anti-Ly6G, I/R+Anti-Ly6G+LCN2mAb. b To assess the damage of the BBB in mice under the above-mentioned different treatments, we used EB to detect BBB damage. c-d TTC staining, the red part was live cells, and the white part was cell death. N=4-6, # $P \leq 0.05$, ## $P \leq 0.01$, ns, no significant difference.

Figure 6

LCN2mAb induced the formation of N2-HL-60 a-b Under different treatment conditions, N0 $\rightarrow$ N0-HL-60 $\rightarrow$, N0+ATRA $\rightarrow$ N1-HL-60 $\rightarrow$, N0+ATRA+TGF- β 1 $\rightarrow$ N2-HL-60 $\rightarrow$, Flow cytometry was used to detect the percentage of

CD66b+ in different polarization states of HL-60. c-d HL-60 cells were collected which were treated with ATRA+IgG or ATRA+LCN2mAb and analyzed the level of CD66b protein expression by Western blot. e-f Under different treatment conditions, N0+ATRA+IgG, N0+ATRA+LCN2mAb in different polarization states, the percentage of CD66b+ was evaluated by flow cytometry analysis. g-j The expression of various inflammatory factors was detected at the cell transcription level, including IL-1 β , TNF- α , CD206, and IL-10. N=4, #P $\leq$ 0.05, ##P $\leq$ 0.01, ns, no significant difference.