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Research report

T lymphocytes infiltration promotes blood-brain barrier injury after experimental intracerebral hemorrhage

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ABSTRACT

T lymphocytes migrate into the brain after intracerebral hemorrhage (ICH) and promote cerebral inflammation, thus exacerbating neuronal injury. However, the relationship between of T lymphocytes infiltration and blood-brain barrier (BBB) injury after ICH has not been clarified. In this study, we investigated the spatial-temporal distribution of infiltrating T lymphocytes after ICH in C57BL/6 mice by immunofluorescence and flow cytometry, and the accompanying change rules of BBB permeability were detected by Evans blue dye leakage and tight junction protein expression. Furthermore, T lymphocyte-deficient nude mice and T lymphocyte-decreased C57BL/6 mice treated with fingolimod were used to verify the relationship between T lymphocytes infiltration and BBB leakage after ICH. Here, we reported that brain-infiltrating T lymphocytes in the hemorrhagic hemisphere began to accumulate on the first day and peaked on the fifth day after ICH; BBB leakage also at peaked on the fifth day. Moreover, T lymphocyte-deficient nude mice showed minor BBB leakage after ICH compared with C57BL/6 control mice. Similarly, fingolimod treatment can significantly decrease T lymphocyte infiltration and promote BBB integrity compared with a vehicle control. Overall, our results suggested that suppression of T lymphocyte infiltration may be a novel way to improve BBB integrity after ICH.

1. Introduction

Intracerebral hemorrhage (ICH) is a very challenging clinical problem that accounts for 10–15% of all hospitalizations for acute stroke. Compared with ischemic stroke, it is associated with substantially higher morbidity and mortality (Flower and Smith, 2011; Hwang et al., 2011; Zhou et al., 2014). Unfortunately, there are no effective prevention methods or specific therapies for ICH. Thus, better understanding of the pathogenesis of ICH-induced brain injury is urgently needed to improve the clinical outcomes of cerebral hemorrhage patients.

The BBB separates the central nervous system (CNS) from the peripheral blood circulation with highly specialized capillary endothelial cells, which use continuous intercellular tight junctions and extremely low pinocytotic activity to block circulating cells and molecules from crossing the barrier. However, T lymphocytes infiltrate into the brain parenchyma after BBB impairment; previous studies have demonstrated that an abrupt and massive influx of lymphocytes from the periphery to the hematoma region orchestrates focal inflammatory responses, facilitates tissue death, and worsens neurological outcomes after ICH, and inhibited T lymphocytes infiltration can ameliorate cerebral inflammation and improve neurological functions (Becher et al., 2006; Engelhardt, 2006). Nevertheless, the relationship between brain-infiltrating T lymphocytes and BBB injury is still unknown.

Several recent studies have evaluated cerebral ischemia-reperfusion injury in male SCID (severe combined immunodeficiency) and recombinase activating gene-deficient ($Rag1^{-/-}$) mice (both deficient in both T and B cells) and have consistently reported a smaller infarct volume and improved functional outcome in those lines than in wild-type controls (Wysoczynski et al., 2017). However, previous studies did not distinguish the beneficial effects due to T cells or B cells. Congenitally athymic nude mice (nu/nu) are used as a model for the study of cell-mediated immunologic deficiencies (Pelleitier and Montplaisir, 1975). These nude mice possess a vestigial thymus that is inca-

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pable of producing mature T lymphocytes. It is very meaningful to study BBB leakage in the absence of T lymphocytes in nude mice after ICH. Fingolimod (FTY720, Gilenya®) is a sphingosine 1-phosphate (S1P) analog that interacts with the S1P receptor (S1PR) and regulates various cellular biological process, including proliferation, apoptosis, and inflammation (Rolland et al., 2011). Previous study revealed that fingolimod prevents the egress of T lymphocytes from lymphoid organs by reducing the expression of S1PR-1 on T lymphocytes (Rolland et al., 2013). Therefore, fingolimod treatment can also be used to study BBB leakage after ICH in the partial absence of T lymphocytes.

In the present study, we hypothesized that T lymphocyte infiltration aggravated BBB leakage after ICH. Hence, the spatial-temporal distribution of infiltrating T lymphocytes in the brain and the patterns of change in BBB leakage were investigated using a C57BL/6 mouse ICH model. In addition, T lymphocyte-deficient nude mice and T lymphocyte-decreased C57BL/6 mice treated with fingolimod were used to verify the relationship between T lymphocyte infiltration and BBB leakage after ICH. The aim of this study is to further understand the relationship between T lymphocyte infiltration and BBB leakage and provide a feasible candidate for ICH treatment.

2. Results

2.1. Temporal changes in the distribution of brain-infiltrating T lymphocytes after ICH

T lymphocytes were isolated from the brain tissues of the hemorrhagic hemisphere at 0, 1, 3, 5, 7, and 14 days by flow cytometry after ICH. According to previous reports, $CD45^{+}$low/$CD11b^{+}$high cells were defined as microglia and were excluded from the calculation of infiltrating T lymphocyte numbers ($CD11b^{-}/CD45^{+}$) (Fig. 1B). At each time point, $CD4^{+}$ T lymphocytes were the predominant brain-infiltrating T lymphocyte subpopulation and peaked 5 days after surgery (Fig. 1C, $p < 0.01$). In addition, $CD8^{+}$ T cell counts constantly increased during the observation period (Fig. 1C). To characterize the spatial pattern of brain T lymphocyte infiltration in ICH, we performed CD3 immunohistochemistry at each time point (Fig. 1A). The number of CD3-positive cells reached a peak at 5 days after ICH, consistent with the flow cytometry results, and most of the positive cells were located around the hematoma. Concurrently, we also examined the changes in neurological function at each time point after ICH (Fig. 1D). Neurological function was significantly impaired at 1, 3, and 5 days and returned to normal on the 7th day after injury.

2.2. Temporal changes in BBB permeability and the expression of tight junction (TJ) proteins

The extravasation of Evans blue dye from blood vessels, a sensitive estimate of BBB permeability, was measured at 0, 1, 3, 5, 7, and 14 days post-ICH. The BBB permeability increased on the first day after ICH (Fig. 2F, $p < 0.05$), reaching its peak value at 5 days ($p < 0.01$) followed by reduction at 1 week ($p < 0.05$).

Tight junctions (TJ) are a type of connection between two endothelial cells and can limit the transfer of water and other substances through the BBB. To reveal the molecular mechanisms determining the degree of increase in BBB permeability, the expression changes of TJ proteins including claudin-5, occludin and ZO-1 were measured after ICH. Immunohistochemical analyses showed the relationship between claudin-5 and vascular endothelial cells (CD31) from 0 day to 14 days after ICH (Fig. 2A). Western blotting was used to examine the expression of claudin-5, occludin and ZO-1 (Fig. 2B); the results showed that the expression of those TJ proteins significantly decreased after ICH and reached their minimum values at 5 days (Fig. 2C, D-E, $p < 0.01$).

2.3. Decreasing the infiltration of T lymphocytes can protect the BBB after ICH

Brain sections from three groups were collected at 5 days after ICH, triple-labeling immunofluorescence was used to detect the relationship among blood vessels, claudin-5 and the infiltrating T lymphocytes by confocal analysis. ICH induced an increase in the number of T lymphocytes gathered around the blood vessels and a deficiency of claudin-5 compared with the sham group (Fig. 3A); however, the number of $CD3^{+}$ T lymphocytes decreased and the quantity of claudin-5 increased after daily fingolimod administration for 3 days (Fig. 3A). Meanwhile, the expression levels of claudin-5, occludin, and ZO-1 proteins were downregulated after ICH induction, according to Western blots (Fig. 3B), but fingolimod treatment can significantly upregulate the expression levels of the proteins compared with the levels in the Vehicle group (Fig. 3C, $P < 0.05$). In addition, we also analyzed the brain-infiltrating T lymphocytes into subsets by flow cytometry (Fig. 3D). ICH induced an increase in the numbers of $CD4^{+}$ and $CD8^{+}$ T lymphocytes (Fig. 3E, $P < 0.01$), and these effects were reduced by drug fingolimod treatment (Fig. 3E, $P < 0.05$). These results further demonstrated that T lymphocytes may be involved in the BBB after ICH.

In addition, the integrity of the BBB was also evaluated using Evans blue extravasation. Evans blue immunofluorescence demonstrated increased Evans blue dye leakage from vessels around the hematoma boundary 5 day after surgery, and fingolimod significantly reduced Evans blue leakage (Fig. 4A). Evans blue content was also examined, and the same results were obtained (Fig. 4B, $P < 0.05$). Finally, we also examined the effect of fingolimod on brain water content. Our results showed that the fingolimod intervention could markedly reduce brain water content (Fig. 4C) and improve neurological function (Fig. 4D).

2.4. Minor BBB damage in T lymphocytes deficient nude mice after ICH

In order to increase the reliability of our findings, T lymphocytes deficient nude mice were involved in our experiments. There is no significant differences between Sham-WT and Sham-nude mice groups in Evans blue permeability, TJ proteins' expression and brain water content (Fig. 5A-C, $P > 0.05$), indicating that nude mice had an normal BBB compared with C57BL/6 WT mice. However, after ICH, T lymphocytes deficient nude mice showed better BBB integrality compared with ICH-WT group, verified by testing Evans blue permeability, TJ proteins' expression and brain water content (Fig. 5D-F, $P < 0.05$). What's more, three-labelled immunofluorescence result reveled that there was no infiltrating T lymphocytes around hematoma in nude mice group after ICH, but more claudin5 expression on vessels peri-hematoma compared with ICH-WT group (Fig. G).

3. Discussion

In the present research, we found that the degree of T lymphocyte infiltration in the parenchyma was positively correlated with BBB leakage; both peaked on the fifth day after ICH in C57BL/6 mice. The BBB leakage was significantly alleviated in a nude mouse ICH model, which lacked T lymphocytes; similar results were also found in a C57BL/6 mouse ICH model after T lymphocytes were inhibited by fingolimod treatment. Taken together, these observations demonstrated that T lymphocyte infiltration aggravated BBB leakage after ICH and indicated that T lymphocyte inhibition may be a feasible candidate for ICH treatment.