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Original Research

Aspirin Inhibits Brain Metastasis of Lung Cancer via Upregulation of Tight Junction Protein Expression in Microvascular Endothelial Cells

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Abstract

Background: The brain is one of the most vulnerable metastasis sites in lung cancer; approximately 40–50% of lung cancer patients develop brain metastasis during the disease course, contributing to the poor prognosis and high mortality of lung cancer patients. Therefore, it is important to clarify the molecular mechanism underlying brain metastasis of lung cancer for improving the overall survival of lung cancer patients. The present study aimed to investigate the potential role of blood-brain barrier (BBB) permeability in the development of brain metastasis of lung cancer and explore the effect of aspirin in an *in-vitro* BBB model. **Methods**: An *in-vitro* BBB model was established. The expression of heat shock protein 70 (HSP 70), zonula occludens-1 (ZO-1), and occludin in rat brain microvascular endothelial cells was detected using Western blot at different time points following the administration of aspirin. **Results**: HSP70, ZO-1, and occludin expressions did not show significant changes before aspirin administration, but increased noticeably after aspirin administration. Tumor necrosis factor-α (TNF-α) could significantly attenuate the increased expression of these proteins induced by aspirin. Additionally, TNF-α also significantly reversed the aspirin-induced decrease of BBB permeability. **Conclusions**: Aspirin may inhibit brain metastasis of lung cancer in a time-dependent manner via upregulating tight junction proteins to reduce BBB permeability, and this effect can be reversed by TNF-α.

Keywords: aspirin; lung cancer; tight junction protein; blood-brain barrier; brain metastasis

1. Introduction

Brain metastasis is accepted as the most common fatal complication of lung cancer. Approximately 10–25% of lung cancer cases develop brain metastasis at the initial diagnosis, and 40–50% of lung cancer cases may experience brain metastasis during the disease course [1]; this leads to the dismal prognosis and high mortality of lung cancer patients. Therefore, it is important to determine the molecular mechanism underlying brain metastasis of lung cancer, thus contributing to improving the overall survival of lung cancer patients [2–4].

Aspirin is a nonsteroidal anti-inflammatory drug, which is commonly used to relieve pain, fever, and peripheral inflammation in clinics [5]. Emerging evidence has also indicated the potent role of aspirin in tumor prevention and treatment [6]. The combination of aspirin and antineoplastic agents has been reported to exhibit a potent anti-tumor activity [7], suggesting an anti-tumor effect of aspirin at the cell level.

The association between aspirin and lung cancer has been documented since 1989 [8]. Accumulating studies have demonstrated the anti-tumor effects of aspirin. For example, aspirin inhibits the proliferation of lung cancer cells by inhibiting cyclooxygenase activity [9]. Specific doses of aspirin can inhibit the proliferation and induce the apoptosis of human lung adenocarcinoma cells and small cell lung cancer cells; aspirin, in combination with radiotherapy, can exhibit a synergistic action. In addition to the anti-tumor effects, aspirin also enhances the therapeutic effect of chemotherapeutic drugs in lung cancer [10]. A previous study has shown that low-dose aspirin is associated with small tumor extents and few metastatic complications in lung cancer patients [11]. Therefore, aspirin is a promising agent for lung cancer. However, the underlying action mechanism of aspirin on lung cancer has not been thoroughly elucidated.

2. Materials and Methods

2.1 Lung Cancer Cell Culture

NCI-H209 lung cancer cells (from FuHeng Cell Center, Shanghai, China) were incubated in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS; 10270-106, HyClone; Thermo Fisher), 100 U/mL penicillin, and 100 µg/mL streptomycin under standard culture conditions. When the cells reached confluence, the medium was aspirated and fresh serum-free medium was added for further incubation (12 h). Subsequently, the cells were washed once with sterile phosphate-buffered saline (PBS) and then cultured with a fresh serum-free medium. Next, the cells were treated with aspirin (Sigma-Aldrich, St. Louis, MO, USA;

Merck KGaA) for 10, 30, 60, 120, 180, and 240 min. All cell lines were validated by STR profiling and tested negative for mycoplasma. Cells were all cultured in a humidified incubator at 37 °C and 5% CO_2.

2.2 Analysis of Tumor Necrosis Factor-α (TNF-α)

TNF-α expression was detected using the ELISA assay kit (cat. no. HK307-01; HyCult Biotechnology and R&D Systems, Inc., Minneapolis, MN, USA) according to the manufacturer's protocol. All experiments were performed in triplicate.

2.3 Establishment of the In-Vitro Blood-Brain-Barrier (BBB) Model

Primary rat brain microvascular endothelial cells (rBMECs) were isolated and cultured as previously described [12]. The *in-vitro* BBB model was established as described by Hurst and Fritz [13]. Initially, astrocytes (Cell Resource Center, IBMS, CAMS/PUMC) were seeded (1×10^6 cells) into the basolateral compartment of collagen-coated polytetrafluoroethylene Transwell chambers (Corning CoStar 3421; Cambridge, MA, USA) containing suitable culture medium. When the astrocytes reached 80% confluence, rBMECs were seeded into the apical compartment of the Transwell chambers. The cells were then cultured to 80% confluence.

2.4 Measurement of Horseradish Peroxidase (HRP) Flux

The status of BBB permeability was quantitatively evaluated by determining HRP extravasation. Lung cancer cell suspensions (5×10^5/mL) were added into the apical compartment of the Transwell chambers of the BBB model. Subsequently, the chamber liquid was removed, and the cells were treated with aspirin (8 mmol/L) at different time points (10, 30, 60, 120, 180, and 240 min). HRP (0.5 µM, Sigma-Aldrich) in serum-free DMEM was added into the apical compartment of Transwell chambers. The culture medium from the basolateral chamber was collected and HRP content was analyzed using a colorimetric assay (450 nm). HRP flux was expressed as pmol/cm^2 of the surface area [14].

2.5 In-Vitro Migration Assay

NCI-H209 cells were resuspended in DMEM containing 15% FBS to obtain a concentration of 1×10^7 cells/mL. Next, cells were seeded (1×10^6 cells/well) into the apical compartment of the *in-vitro* BBB model. The cells were treated with aspirin for 10, 30, 60, 120, 180, and 240 min. NCI-H209 cells that migrated to the basolateral compartment were counted under a light microscope. The experiments were performed in triplicate. The migration percentage of NCI-H209 cells was determined by calculating the number of migrated cells relative to the number of seeded cells.

2.6 Western Blot Analysis of Heat Shock Protein 70 (HSP70) and Tight Junction Proteins

The effects of aspirin on the level of HSP70, zonula occludens-1 (ZO-1), and occludin proteins in rBMECs were analyzed using the Western blot assay. The confluent rBMECs were collected at different time points and washed with Dulbecco's PBS containing 0.1 mM ethylenediamine tetraacetic acid (EDTA) in the absence of calcium and magnesium.

Next, rBMECs were homogenized (4 °C) in 1 mL lysis buffer (2 mM EDTA; 10 mM EGTA; 0.4% NaF; 20 mM Tris-HCl) containing protease inhibitor (pH = 7.5) to obtain the cell membrane fraction. The samples were then centrifuged (17,000 $\times$g, 1 h) at 4 °C. The protein concentration of soluble materials was determined using the Coomassie brilliant blue G-250 dye method. For each sample, 12 µg of protein was loaded per lane and then separated by 12% sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Next, the proteins were transferred onto nitrocellulose membranes (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). The membranes were blocked with 5% skim milk and then dissolved (overnight, 4 °C) in 0.1% Tween-Tris-buffered saline. The membranes were incubated (2 h) with the antibodies anti-HSP70 (1:500, cat. no. sc-24, Santa Cruz), anti-ZO-1 (1.0 µg/mL, cat. no. 61-7300; Invitrogen; Thermo Fisher), anti-occludin (0.5 µg/mL, cat. no. 33-1500; Invitrogen; Thermo Fisher), and anti-β-actin (1:5000, cat. no. ac026; Santa Cruz). The protein bands were visualized using an enhanced chemiluminescencsse (ECL) kit (Santa Cruz). The HSP70, ZO-1, occludin, and β-actin protein bands were scanned using the Chemi Imager 5500 V2.03 software (Bio-Rad, Hercules, CA, USA). The integrated density value (IDV) of proteins was calculated using the Fluor Chen 2.0 software (version 2.0; Bio-Rad) and normalized to the IDV of β-actin.

2.7 Statistical Analysis

The measurement data in normal distribution were described as mean $\pm$ standard deviation. The *t*-test was used for comparisons between two groups; A two-sided *p*-value of less than 0.05 indicated a statistically significant difference.

3. Results

3.1 Aspirin Reduced the Release of TNF-α from Lung Cancer Cells

To examine the effect of aspirin on TNF- α in NCI-H209 cells,We examined the concentration of TNF- α in each group. The concentration of TNF-α in the experimental groups was decreased by 1.21–5.03 folds when compared to that of the control group. TNF-α expression was noticeably decreased 30 min after aspirin administration (Fig. 1). Briefly, aspirin exerted an inhibitory effect on TNF-α concentration in NCI-H209 cells.

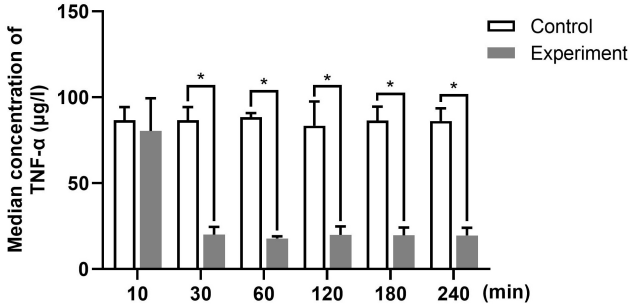

Fig. 1. The average concentrations of TNF-α in NCI-H209 lung cancer cells at different time points were detected. NCI-H209 lung cancer cells in a logarithmic growth phase were collected. Enzyme-linked immunosorbent assay (ELISA) was used to determine the concentration of TNF-α in lung cancer cells after aspirin administration at different time points (10, 30, 60, 120, 180, and 240 min). n = 12. Data are shown as mean ± SD, *t*-test, $^{*}p < 0.05$ vs. the control group. TNF, tumor necrosis factor.

3.2 Aspirin Decreased the Permeability of the In-Vitro BBB Model

In the present study, the permeability of the *in-vitro* BBB model was analyzed by measuring the HRP flux. The average concentrations of HRP flux at different time points are shown in Fig. 2. We found that aspirin administration to the rBMEC monolayer on Transwell filters could decrease HRP leakage. The HRP flux was significantly lower at 60 min after aspirin administration than that at 10 min ($p < 0.05$). The minimum HRP flux was observed 120 min after aspirin administration. These results indicated that aspirin could decrease the permeability of the *in-vitro* BBB model.

3.3 Aspirin Inhibited the Migration of Lung Cancer Cells

To examine the effect of aspirin on migration of Lung Cancer Cells,We examined the migration in each group. The migration of NCI-H209 lung cancer cells treated with aspirin at different time points was detected. The percentage of migrated cells with aspirin treatment varied at different time points (Fig. 3). Relative to the 10-min group, other groups showed a 1.8–4.4 times lower percentage of migrated cells. Further quantitative analysis indicated that the mean value of migrated cells in the experimental groups was significantly reduced at each time point (from 10 min to 240 min) compared with the control group (all $p < 0.05$) (Fig. 4). Altogether, these results suggest that aspirin could inhibit the migration of lung cancer cells.

3.4 Aspirin Induced the Expression of HSP70 in rBMECs

The IDV of HSP70 in rBMECs of different groups was detected, and was found to vary in different lanes (Fig. 5). IDV is the whole optical density value obtained after computer software measurement. According to Western blot analysis results, a higher IDV value was associated with a

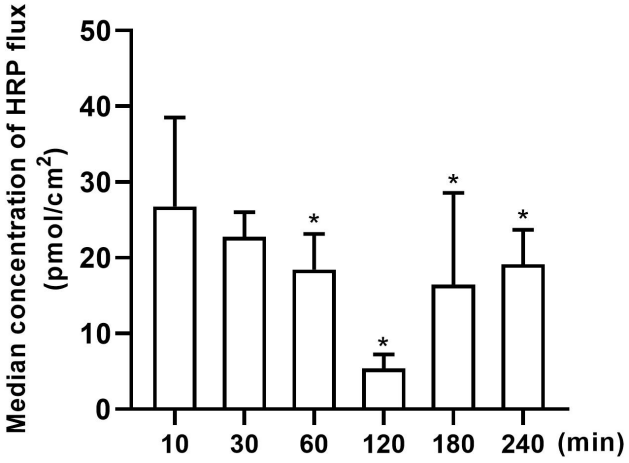

Fig. 2. The average concentrations of HRP flux at different time points were detected. Time course of aspirin-induced changes in HRP flux of the *in-vitro* BBB model. At different time points, rat brain microvascular endothelial cells were treated with aspirin (8 mM/L) for 10, 30, 60, 120, 180, and 240 min. n = 12. Data are shown as mean ± SD, *t*-test, $^{*}p < 0.05$ vs. the aspirin, 10 min group. HRP, horseradish peroxidase; BBB, blood-brain barrier.

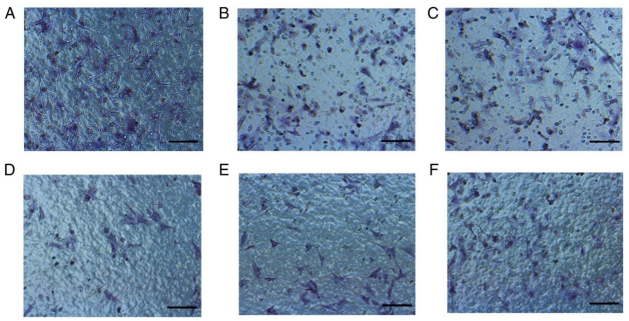

Fig. 3. The migration of NCI-H209 lung cancer cells treated with aspirin at different time points was detected. Migration of cells in the (A) 10, (B) 30, (C) 60, (D) 120, (E) 180, and (F) 240 min-groups. n = 12. Scale bars = 100 μm.

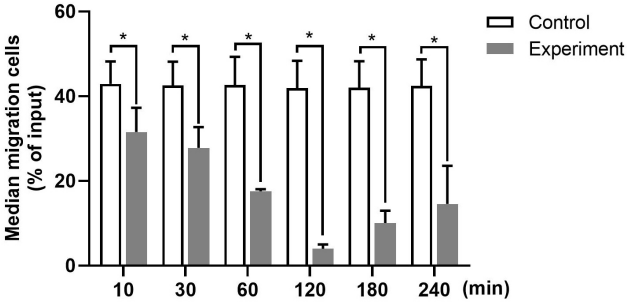

Fig. 4. The number of migrated NCI-H209 cells at different time points was detected. n = 12. Data are shown as mean ± SD, *t*-test, $^{*}p < 0.05$ vs. the control group.

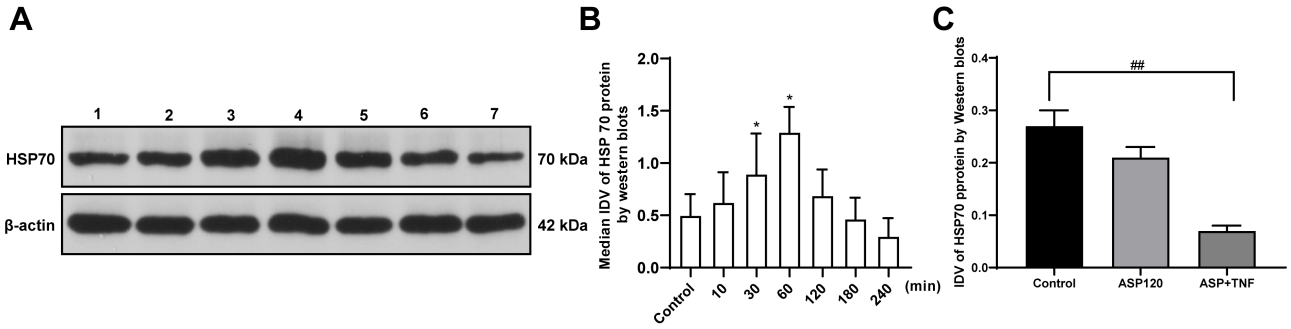

Fig. 5. The effects of aspirin on HSP70 protein level in rat brain microvascular endothelial cells at different time points after treatment were detected. (A) Lane 1: Control group; lane 2: ASP, 10 min group; lane 3: ASP, 30 min group; lane 4: ASP, 60 min group; lane 5: ASP, 120 min group; lane 6: ASP, 180 min group; lane 7: ASP, 240 min group. Representative Western blots showed differences in the bands of HSP70. Changes in the relative IDV of HSP70 (B) over 240 min and (C) with ASP and TNF-α. n = 12. Data are shown as mean $\pm$ SD, *t*-test, $^{*}p < 0.05$ vs. the control group and $^{\#\#}p < 0.01$ vs. the ASP, 120 min group. IDV, integrated density value; HSP70, heat shock protein 70; ASP, aspirin.

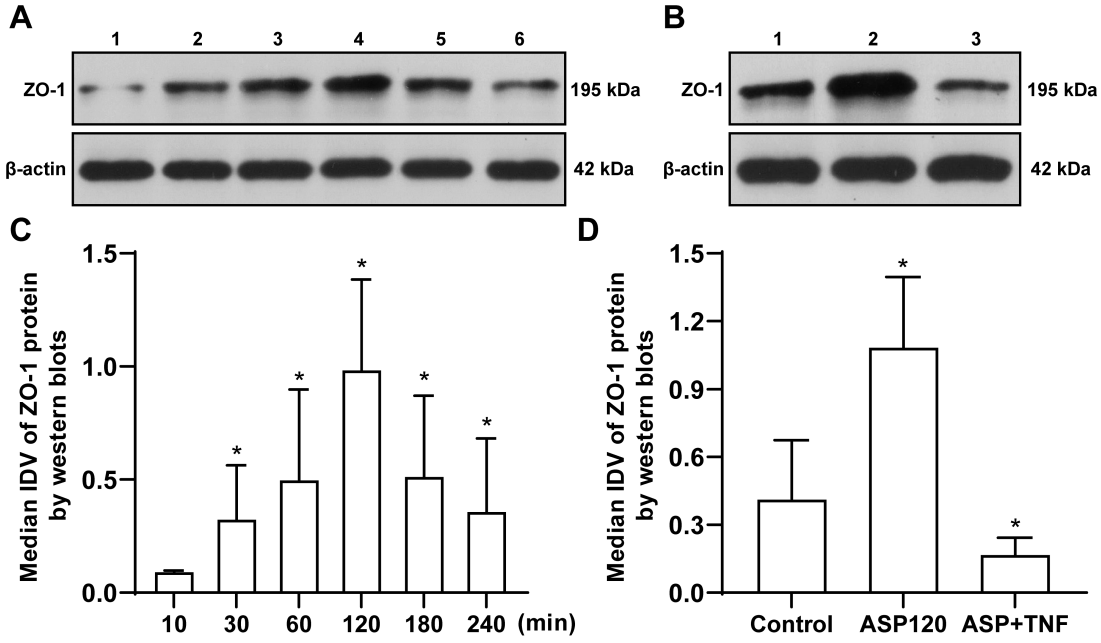

Fig. 6. The effects of ASP on ZO-1 protein level in rat brain microvascular endothelial cells at different time points after treatment were detected. (A) Lane 1: ASP, 10 min group; lane 2: ASP, 30 min group; lane 3: ASP, 60 min group; lane 4: ASP, 120 min group; lane 5: ASP, 180 min group; and lane 6: ASP, 240 min group. (B) Lane 1: control group; lane 2: ASP, 120 min group; and lane 3: ASP + TNF-α group. TNF-α inhibited the increase in the ZO-1 protein level induced by ASP (8 mM/L for 120 min). Representative Western blots showed differences in the bands of ZO-1. (C) Changes in the relative IDV of ZO-1 compared to 10 min treatment P ((C,D), n = 12 for each group). (D) Changes in the relative IDV of ZO-1 at 120 min and following ASP and TNF-α. Data are shown as mean $\pm$ SD, *t*-test, $^{*}p < 0.01$ vs. the control group (ASP, 10 min group). IDV, integrated density value; ZO-1, zonula occludens-1; TNF, tumor necrosis factor; ASP, aspirin.

higher protein expression. Compared with that in the control group, the mean IDV of HSP70 began to increase significantly 30 min after aspirin administration and reached its peak at 60 min after aspirin administration ($p < 0.05$). Moreover, 240 min after aspirin and TNF-α treatments, the IDV of HSP70 was significantly reduced in comparison to that in the control group ($p < 0.01$).

3.5 Aspirin Induced the Upregulation of Tight Junction Proteins ZO-1 and Occludin

The IDV ratios of tight junction proteins ZO-1 and occludin were analyzed using Western blot (Figs. 6,7). According to the results, the ZO-1 protein level in rBMECs was significantly upregulated 30 min after aspirin administration, reaching the highest at 120 min after aspirin admin-

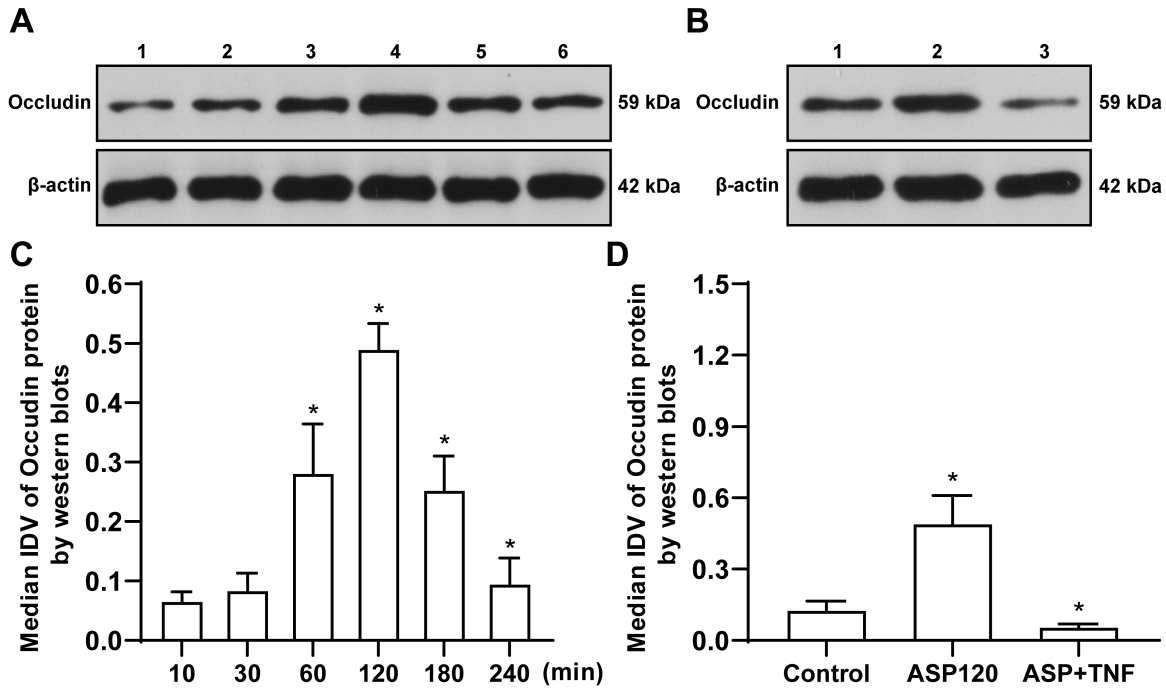

Fig. 7. The effects of aspirin on occludin protein level in rat brain microvascular endothelial cells at different time points after treatment were detected. (A) Lane 1: ASP, 10 min group; lane 2: ASP, 30 min group; lane 3: ASP, 60 min group; lane 4: ASP, 120 min group; lane 5: ASP, 180 min group; and lane 6: ASP, 240 min group. (B) Lane 1: control group; lane 2: ASP, 120 min group; and lane 3: ASP+ TNF-α group. TNF-α inhibited the increase in the occludin protein level induced by ASP (8 mM/L for 120 min). Representative Western blots showed differences in the bands of occludin. (C) Changes in the relative IDV of occludin compared to 10 min group ((C,D), n = 12 for each group). (D) Changes in the relative IDV of occludin at 120 min and following ASP and TNF-α ((C,D), n = 12 for each group). Data are shown as mean $\pm$ SD, *t*-test, $^{*}p < 0.01$ vs. the 10 min group and $^{*}p < 0.01$ vs. the ASP, 120 min group. TNF, tumor necrosis factor; IDV, integrated density value; ASP, aspirin.

istration. The upregulation trend of ZO-1 in rBMECs continued until 240 min after aspirin treatment (Fig. 6). The protein level of occludin was noticeably upregulated at 60 min after aspirin administration, and its highest level was observed at 120 min and lasted until 240 min after the treatment (Fig. 7). Furthermore, 240 min treatment with aspirin and TNF-α remarkably reduced the IDV of ZO-1 and occludin compared with the control group (Fig. 7C and D $p <$ 0.01).

4. Discussion

The BBB is a complex multicellular structure that separates the central nervous system (CNS) from the peripheral blood circulation and protects the CNS from toxins and pathogens, which maintains internal environmental homeostasis and enables the function of neurons [15–19]. The vast majority of patients with small cell lung cancer are prone to brain metastasis, which can be largely attributed to the increased permeability of the BBB. The tight junctions between BBB endothelial cells construct a robust physical barrier that can control the paracellular passage of molecules [20]. The tight junction complex is composed of different junctional molecules, including occludin, claudins, ZOs, and junctional adhesion molecules [21]. Accumulating studies have provided evidence that dysregulated tight junction proteins can contribute to altered BBB permeability [22,23]. ZO-1 is a member of the membrane-associated guanylate kinase-like protein family, and its presence at the plasma membrane is closely associated with the integrity of the tight junction [24,25]. Occludin, a class of transmembrane proteins, is specifically associated with tight junctions, contributing to the function of the intercellular seal [26].

Despite the remarkable progress in the development of *in-vitro* models of BBB, most studies are limited to simulating the BBB of the human body *in-vitro* [27–29]. Moreover, little is known about the factors that maintain the integrity of the BBB and the molecular mechanisms of various structures involved in the BBB formation. In the present study, the average values of multiple samples were obtained to compensate for the deficiencies of the modeling method, which can reduce the possible differences between the *in-vitro* model of BBB and the *in-vivo* BBB. Specifically, the *in-vitro* BBB model was established by the co-culture of astrocytes and rBMECs as previously reported [13]. Astrocytes reside in the abluminal surface of the microvascula-

ture of the BBB, where the cells constitute the major cell population in the neurovascular unit and play an important role in BBB maintenance. As previously evidenced, astrocytes can mediate metastatic cancer growth into the brain parenchyma by regulating matrix metalloproteinases [30]. Accumulating studies have also indicated the critical involvement of pericytes in BBB formation and BB permeability maintenance. However, the in-vitro BBB model in the present study did not contain pericytes and thus the effect of pericytes is not factored in in the present study.

In the present study, the effect of TNF-α and its possible molecular mechanism in the opening of the BBB and the blood-tumor barrier were investigated in the early stages of the disease. Studies have reported that glioma cells secrete TNF-α in an autocrine manner, thereby increasing the BBB permeability and the blood-tumor barrier [31,32]. Many other types of cancer cells have been shown to possess autocrine properties and can secrete certain cytokines or biologically active substances [33]. TNF-α, a common proinflammatory factor, acts in an autocrine manner. In the present study, we investigated whether lung cancer cells can secrete TNF-α in an autocrine manner and whether aspirin can inhibit brain metastases by inhibiting TNF-α secretion from lung cancer cells.

In prior works, aspirin displays notable inhibitory effects on the proliferation of lung cancer cells in a time- and concentration-dependent manner [34,35]. In the present study, after a series of preliminary experiments and dynamic index monitoring, the optimal dose of aspirin was determined to be 8 mmol/L. The results of this study revealed that lung cancer cells released TNF-α, while aspirin inhibited the release of TNF-α; these suggested that aspirin may restrain brain metastases by inhibiting lung cancer cells from releasing TNF-α. Moreover, the protein level changes of HSP70, ZO-1, and occludin in rBMECs were also investigated following aspirin treatment. Aspirin exerted slight effects on the protein level of HSP70, ZO-1, and occludin at 10 min after administration, which suggested that the BBB permeability reduced by aspirin at this time point was less associated with the opening of tight junctions. However, aspirin increased the level of HSP70 and tight junction proteins at 30 or 60 min after administration, respectively. HSP70, a type of molecular chaperone protein, is a master regulator in protein homeostasis. HSP70 expression was upregulated at 60 min after aspirin administration and tight junction protein expression peaked at 120 min after aspirin administration in the *in-vitro* BBB model. Meanwhile, the BBB permeability reached the lowest level. The aforementioned effect of aspirin on the level of HSP70 and tight junction proteins was reversed by TNF-α. These results demonstrated that aspirin may induce HSP70 expression and promote the synthesis of tight junction proteins. As the structural foundation of the BBB, the integrity of tight junctions directly affects the BBB permeability. Accumulating studies have shown that aspirin decreases BBB permeability by inhibiting the release of PGE2 from lung cancer cells in a time-dependent manner [36–38]. Therefore, we speculated that the decrease in the BBB permeability induced by aspirin was time-dependent, and aspirin enhanced the integrity of tight junctions via upregulating HSP70 and tight junction proteins. In this process, aspirin functioned as the dynamic factor, which inhibited lung cancer cells from releasing TNF-α. The present study demonstrated that aspirin decreased the number of invasive NCI-H209 lung cancer cells 120 min after administration, and this effect can be reversed by TNF-α, consistent with the changes in tight junction protein expression and BBB permeability over time. Taken together, aspirin could reduce the BBB permeability in a time-dependent manner and decrease the number of invasive NCI-H209 lung cancer cells via upregulating tight junction proteins; TNF-α acted as a key signaling molecule in this process.

5. Conclusions

In conclusion, the underlying molecular mechanism of aspirin reducing BBB permeability and further inhibiting the brain metastases of lung cancer is complicated. The results of the present study reveal that aspirin upregulates the expression of tight junction proteins via the TNF-α/HSP70 signaling pathway in a time-dependent manner. The results of this study may provide new evidence for understanding the mechanism of aspirin in suppressing brain metastasis of lung cancer, its involvement in the regulation of BBB permeability, and the delivery of molecules across brain microvessels following biochemical modulation.

Availability of Data and Materials

Raw data can be obtained by contacting corresponding author.

Author Contributions

LQ accounted for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work. DW, MT and WG performed the experiments and collected data and analyzed the data. DW, MT, WG and JL designed the study and wrote the manuscript. All authors read and approved the final manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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