Role of macrophages in outer membrane of Chronic Subdural Hematoma

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Research Article

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Abstract

Background

Chronic subdural hematoma (CSDH) is a common disease in the field of neurosurgery. Previous studies show that the outer membrane of CSDH is divided into four histological stages by HE staining and elasticavan Gieson staining. However, this method cannot show the proportion of specific inflammatory cell components at different stages. This study aims to explore the major inflammatory cells in the outer membrane of CSDH and discussion their function in CSDH.

Methods

A total of 18 patients were included in this study. A semi-quantitative analysis was performed on the results of Hematoxylin-eosin (HE), immunohistochemistry and Masson staining.

Results

Analysis results show, in stage II, that macrophages make up the highest number of inflammatory cells, followed by T cells, B cells and neutrophil. Eosinophil main express at stage II and stage III. Interestingly, Inflammatory cells in the outer membrane are arranged in this order at any stage. In stage III, the expression number of CD31 is more than in stage II. Masson staining shows that neomembrane fibrosis gradually increases from stage I to stage IV. The expression of Transforming growth factor β (TGF-β) in the outer membrane correlates with the expression of CD68 in neomembrane ($R^2 = 0.810, P = 0.015$).

Conclusion

The external membrane of CSDH may be a process of formation of scarring membrane according to the results of inflammatory elements in the outer membrane. The formation and development of the outer membrane of CSDH changes dynamically, macrophages play an important role in inflammation, neocapillaries and fibrosis, we considered macrophages play a critical role in the development of CSDH.

Background

CSDH is a common disease in the field of neurosurgery. The quantity of patients diagnosed with CSDH is increasing, attributable to the increasing average lifespan[1]. Its origin and development are still unknown, and were considered to be involved in multifactorial mechanisms. CSDH are encapsulated blood and blood products collected between dural and arachnoid with a characteristic outer “neomembrane“[2]. The neomembrane is considered important in onset and progression of CSDH[3].
Nagahori classified the histological characteristic by HE staining and elasticavan Gieson staining into four types: Type I, non-inflammatory membrane, this includes immature fibroblasts and collagen fiber, is associated with slight or sparse inflammatory cells, infiltration and neocapillaries. Type II, inflammatory membrane; containing one layer of immature connective tissue, this was associated with increasing inflammatory cell infiltration and neocapillaries throughout the membrane. Type III, hemorrhagic-inflammatory membrane; this type is multi-layered in structure and accompanied by capillaries with a large lumen, marker cell infiltration and many thin new vessels. Some patients showed only a layer consisting of collagen fibers and fibroblasts between two layers. Moreover, hemorrhaging was often seen in the membrane. Type IV, scar-inflammatory membrane; this type consists of connective tissue composed of mature fibroblasts and collagen, but associated with scarce neocapillaries and cell infiltration.[4]. Interestingly, Masahito and his colleagues researched the relationship between endoscopic findings and CSDH recurrence. This study inspected the association between Nagahori’s histopathological staging and external membrane color. The results of the study demonstrated that the outer membrane’s colors of white, red, yellow and white nearly correspond to the histopathological staging from type I to type IV in color, and suggest that a white membrane is a risk factor for CSDH recurrence[5]. Another study shows patients of CSDH with a GCS < 13 merely had type II outer membrane. Enhanced radiodensity and thickness of the hematoma on CT were in correlation with type IV neomembrane[6]. However, the histopathological staging of the outer membrane of CSDH are linked to recurrence and clinical feature was reported. The formation and development of the outer membrane are essential ingredients for the expansion of CSDH[7, 8]. Unfortunately, the proportion of specific inflammatory cells or expression of cytokines in different histopathological stages of outer membrane of CSDH still unclear. This study aims to explore subjects of outer membrane at various periods by HE, immunohistochemistry and Masson staining, and to discuss their effects on the development of CSDH.

Methods

Patients and sample collection

A total of 18 patients were included in this study. Patients were admitted to BeiJing TianTan hospital, and diagnosed with CSDH using computed tomography (CT) between April 2021 and October 2021. All patients were primary treatment and received burr hole surgery under local anesthesia and drainage at our hospital and received closed system drainage. None of the patients had recurrence. The Ethics Committee of BeiJing TianTan Hospital approved this experiment and each patient consented.

The outer membrane specimens obtained during the burr hole trepanation surgery were immediately placed in 4% paraformaldehyde solution for more than 24 hours. Next, the samples were taken out and prepared into paraffin sections.

Immunohistochemistry

The slices were roasted at 70°C for 20 minutes for dewaxing, soaked in different concentrations of ethanol for 5 minutes each and washed in distilled water 3 times for 5 minutes each. All sections for
immunostaining were processed for Citrate buffer high pressure antigen retrieval. After PBS washes for 15 min. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide incubated in a wet box for 15 min solution to the section. Fetal bovine serum was enclosed at room temperature for 20 minutes in order to block non-specific immunoreactivity. The samples were treated with primary antibodies against CD3(ab237721, Abcam, UK), CD20(ab64088, Abcam, UK), CD31(ab28364, Abcam, UK), CD68(ab213363, Abcam, UK), TGF-β (MAB1835, R D, USA), Galectin3(ab2785, Abcam, UK) at a 1:200 dilution overnight at 4°C. After washing, the samples were incubated with the enzyme-labelled anti-rabbit/mouse antibody (GK600705-B, GT, CHN) at room temperature for 1 h. DAB staining was performed for 5–10 minutes, and the staining was observed under microscope. Hematoxylin restained the nucleus for 1 min and differentiated with 1% alcohol hydrochloride, and then it returned to blue with water. Subsequently, samples were incubated in graded ethanol series and treated with xylene twice for 15 minutes. As a final step, neutral gum was used to seal the sections.

**Statistical analysis**

The results of CD3, CD20, CD68, Galectin 3 and TGF-β were scanned under an optical microscope with 200X magnification. 3 pictures were randomly selected containing six areas for counting, cell counting using Image J software (National Institutes of Health, USA). Masson staining count the ratio of staining area and total area using Image J. CD31 express in vascular endothelial cells and we counting the result of CD31 immunohistochemistry using Image Pro Plus (Media Cybernetics, Bethesda, USA) software, as described in reference[9]. We calculated the ratio of positive integral optical density (IOD) and tissue area, named average IOD, for each histological section. The average IOD of the corresponding fields was regarded as the protein expression.

Correlations between TGF-β and CD68 were evaluated by Spearman's correlation coefficient.

**Results**

**General data and HE**

General data were collected from 18 patients with CSDH, all patients are male, with an average age of 60.15 years. Histological stage was determined through HE(n = 18) reference by Nagahori[4], 3 cases(16.7%) of type I, 6 cases (33.3%) of type II, 7 cases (38.9%) of type III and 2 cases (11.1%) of type IV. The eosinophils count was (1.3 ± 1.9), (25.0 ± 10.9), (16.5 ± 23.6), (1.5 ± 1.5) from stage I to stage IV. However, eosinophil was found at stage II and stage III occasionally. The basic date of semiquantitative immunohistochemistry is summarized in Table 1. The HE staining are shown in Fig. 1.

**Immunohistochemistry**

The staining image of expression of CD 3(n = 12), CD 20(n = 12), CD 68(n = 12) and Galectin3(n = 11) at different histological periods are show in Figs. 2, 3, 4 and 5. As we can see, CD3 protein had low expressed at stage I and stage IV (2.00 ± 1.00 and 2.15 ± 0.21, respectively), had a high expressed at stage II and
stage III (20.40 ± 3.02 and 7.33 ± 3.86, respectively). CD20 protein also had a low expressed at stage I and stage IV (0.33 ± 0.58 and 0.15 ± 0.21, respectively), had highly expressed at stage II and stage III (14.47 ± 8.05 and 5.73 ± 4.9, respectively). CD68 protein higher expression in stage II and stage III (36.57 ± 14.01 and 14.45 ± 4.14, respectively) than stage I and stage IV (4.00 ± 1.73 and 3.95 ± 0.92, respectively). Galectin3 protein higher expression in stage II (6.50 ± 1.50), follow by stage III (2.39 ± 1.36), than stage I and stage IV (0.10 ± 0.09 and 0.23 ± 0.32, respectively). Macrophages are the dominant inflammatory cell in the outer membrane at any stages. The semi-quantitative result of HE, CD3, CD20, CD68 and Galectin3 are show in Fig. 6.

The CD31(n = 12) protein staining had shown a decrease at stage I and IV (0.24 ± 0.05 and 0.29 ± 0.04, respectively), and had expressed high at stage II and III (0.39 ± 0.08 and 0.49 ± 0.10, respectively). The mean of expression cells of TGF-β(n = 7) in the outer membrane was 23.8 ± 8.7. The result of CD31 and Masson staining are shown in Figs. 7 and 8. The result of Masson staining(n = 12) shows that neomembrane fibrosis gradually increases from stage I to stage IV shows that 0.21 ± 0.12, 0.46 ± 0.03, 0.59 ± 0.14 and 0.79 ± 0.02, respectively. The semi-quantitative result of CD31 and Masson are shown in Fig. 9. The expression of TGF-β in the outer membrane and the results of the relationship between TGF-β and CD68 ($R^2 = 0.810, P = 0.015$) are shown in Fig. 10 and Fig. 11.

**Discussion**

In this study, we performed HE, immunohistochemistry and Masson to examine elements of the outer membrane of CSDH in various histopathological stages. Nagahori and colleagues research the outer membrane with CSDH patients by HE staining and elasticavan Gieson staining and divide tissue histology into four stages. However, they did not observe changing of elements in the external membrane at different histological periods, especially the proportion of inflammatory cells in stage II and stage III. We carried out immunohistochemistry staining of CD3, CD20 and CD68 to explore the proportion of inflammatory cells in diverse histopathological stages. We suggested that macrophages comprise the largest number of inflammatory cells in any stage, followed by T cells and B cells. Moreover, the expression of eosinophils was mainly in stage II and stage III, especially in phase II.

The result of CD31 staining shows the most vascularization in stage III, followed by stage II. CD31 is expressed on cell-surface proteins of vascular cells, it showed homogeneous intense staining in all pulmonary endothelial cells and it is a symptom of angiogenesis[10]. Vascular endothelial growth factor (VEGF) plays a crucial role in vasculogenesis[11]. Tadahisa studies the vascular endothelial growth factor in CSDH. The results demonstrated that enhanced expression of VEGF was produced by macrophages and vascular endothelial cells in the outer membrane[7]. This suggests that macrophages promote the expression of VEGF and further regulates angiogenesis in the outer membrane.

TGF-β is a multifunctional protein and is involved in chronic inflammation and fibrosis. A lot of studies have demonstrated that activation of canonical or non-canonical TGF-β signaling promotes fibrosis in pathological condition[12]. Osuka has verified that the TGF-β/Smad signaling pathway might be activated
in the outer membrane and accelerate to formation and development of CSDH[13]. Importantly, previous studies have shown that TGF-β derived from wound healing-related macrophages and macrophage exhaustion effectively suppressed the activation of TGF-β signaling in various diseases[14, 15]. In our study, we found that the expression of TGF-β in the outer membrane is relevant to the expression of CD68 in the neomembrane by Spearman’s correlation coefficient. Thus, we speculate that macrophage is an important factor for fibrosis of the outer membrane in CSDH although it is not verified yet.

The results of Masson staining show fibrosis of the outer membrane of CSDH becomes increasingly severe from stage I to stage IV. In stage IV, collagen fibers account for and average 79% in the result of Masson staining. This is illustrated that the development of the outer membrane is a process of the formation of granulation tissue. There have many cytokines involved in tissue fibrosis such as interleukin-1 (IL-1), interleukin-6 (IL-6) and TGF-β[16]. Our results have shown evidence that TGF-β expresses in the outer membrane of CSDH. Undoubtedly, it is reasonable to conclude that the fibrosis of the outer membrane is regulated by TGF-β that origin from macrophages.

**Conclusion**

In summary, we suggested that macrophage is the largest quantity number of inflammatory cells in the outer membrane, followed by T cells and B cells. CD31 was maximum expressed in stage III in histological stages, followed by stage II. The expression of TGF-β is correlates with the level of CD68 in the outer membrane. Masson staining demonstrated that neomembrane fibrosis gradually increases from stage I to stage IV. Macrophages are involved in inflammatory, angiogenesis and fibrosis. In a word, macrophages may play a central role in the formation and growth of CSDH. It may be a target cell that prevents the progression of CSDH in a non-surgical manner. More fundamental research is needed to reveal the role of macrophages in the development of CSDH.

**Limitation and expectation**

There has much to be desired in this article. Firstly, the sample size is too small, meaning that the expression of inflammatory cells in various stages could not be analyzed by statistics. Secondly, based on our results, we could not explore the correlation the level of inflammatory cells or cytokines with clinical data such as CT and blood test results. Finally, we expectation to understand the pathophysiological mechanism of CSDH by researching the various elements changes in the outer membrane, and to find non-surgical therapeutic targets to provide personalized treatment for patients.

**Abbreviations**

CSDH: chronic subdural hematoma; HE: Hematoxylin-eosin; TGF-β: transforming growth factor β; CT: computed tomography; VEGF: Vascular endothelial growth factor; IL-1 Interleukin-1; IL-6 Interleukin-6.

**Declarations**
Ethics approval and consent to participate

The Ethics Committee of BeiJing TianTan Hospital approved this experiment. Informed consent was obtained from all the patients and/or their legal guardian(s) involved in the study. All investigations including human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Consent for publication

Not applicable.

Availability of data and materials

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

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

YL performed the histological examination of CSDH outer membrane and interpreted the pathological results. CY was a major contributor in writing the manuscript. All authors read and approved the final manuscript.

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References


Tables

Table 1 is available in the Supplementary Files section.

Figures

Figure 1

Histological hemotoxylin and eosin staining at four diverse stages. First stage I histology, there are sparse inflammatory cells and neocapillaries (A). In histological II stage, the number of inflammatory cells are abundant. Beside of inflammatory cells, we also can see fibrous cells of various shapes on the membrane. Neocapillies also found on the membrane. (B). Histological III stage, inflammatory cells are lower than at stage II. Fibrosis is gradually progression. (C). The last histological stage, a decreasing number of inflammatory cells could be found on the membrane. Most of fibrocytes remain. (D). Eosinophils are occasionally present in some patient with stage II and stage III, and the number of cells has varied widely. Black arrow: inflammatory cell; Blue arrow: eosinophil. All of photos were examined at magnification ×200.
Figure 2

CD3 immunohistochemical staining. Semi-quantitative shows rank of positive cells of staining is B C D A (result shows in Figure 6). ABCD corresponds to periods I through IV. Black arrow: positive staining cell. All images taken at a magnification of ×200.

Figure 3

CD20 immunohistochemical staining. Semi-quantitative shows rank of positive cells of staining is B C A D (result shows in Figure 6). ABCD corresponds to periods I through IV. Black arrow: positive staining cell. All images taken at a magnification of ×200.
Figure 4

CD68 immunohistochemical staining. Semi-quantitative shows rank of positive cells of staining is B C A D (result shows in Figure 6). ABCD corresponds to periods I through IV. Black arrow: positive staining cell. All images taken at a magnification of ×200.
Figure 5

Galectin3 immunohistochemical staining. Semi-quantitative shows rank of positive cells of staining is B C D A (result shows in Figure 6). ABCD corresponds to periods I through IV. Black arrow: positive staining cell. All images taken at a magnification of ×200.
Figure 6

Expression of inflammatory cells in outer membrane at different stages, picture shows result of semi-quantitative of HE and immunohistochemistry, including neutrophils, eosinophils, CD3, CD20 and CD68 (A, B, C, D, and E respectively). Data is displayed as Mean±SD.

Figure 7

CD31 immunohistochemical staining. Semi-quantitative shows rank of positive cells of staining is C B D A (result shows in Figure 8). ABCD corresponds to periods I through IV. Black arrow: positive staining. All images were taken at a magnification of ×100.
Figure 8

Masson immunohistochemical staining. Semi-quantitative shows rank of positive cells of staining is D C B A (result shows in Figure 8). ABCD corresponds to periods I through IV. The arrow points to the outer membrane. Picture A was taken at a magnification of ×100, and other images were taken at a magnification of ×200.

Figure 9

Result of Masson staining and CD31 staining for outer membrane. Masson staining showed progressive fibrosis of the outer membrane. CD31 staining showed intense staining for vascular endothelial cells at stage III, followed by stage II. Data is showed as Mean±SD.
Figure 10

The immunohistochemistry of TGF-β for the outer membrane. As we can see from the picture, the expression of TGF-β was found in vascular endothelial cells (red arrow), inflammatory cells (black arrow) and fibroblasts (blue arrow). The image was observed at a magnification of ×200.
Figure 11

Correlation between expression levels of TGF-β and CD68 in the outer membrane of CSDH. The TGF-β densitometries positively correlated with those of CD68 according to Spearman’s correlation coefficient ($R^2=0.810$, $P=0.015$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Table1.xlsx