

Aging-associated inflammation and fibrosis in arachnoid membrane

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Abstract

Background

The physiological and pathological significance of the arachnoid membrane (AM) is still unknown. In this study, we investigated various characteristics of the AM, focusing on the influence of inflammation and fibrosis.

Methods

Small pieces of AM sample were obtained during neurosurgical procedures from 74 cases. The clinical and pathological characteristics of the hyperplastic AM group ($\geq 50 \mu\text{m}$) and the non-hyperplastic AM group ($< 50 \mu\text{m}$) were compared. Then, potential correlations between AM thickness and clinical characteristics were analyzed. Moreover, VEGF α , TGF β , and TNF α levels were quantitated by real time PCR. Then, the potential correlations between AM thickness and these inflammatory or anti-inflammatory markers, and the influence of the original disease were calculated.

Results

The median age of the patients in hyperplastic AM group was significantly older than that of the non-hyperplastic AM group. Moreover, the number of fibroblasts, CD68⁺ cells, CD86⁺ cells, and CD206⁺ cells in the hyperplastic AM group was significantly higher than that in the non-hyperplastic AM group. The AM thickness was significantly correlated to age and number of fibroblasts, CD68⁺ cells, CD86⁺ cells, and CD206⁺ cells. The thickness of the AM was significantly correlated to the messenger RNA expression levels of VEGF α ($p = 0.337$), and the VEGF α expression levels were significantly correlated with TGF β , which in turn was significantly correlated with TNF α .

Conclusions

The AM hyperplasia was influenced by aging and could be a result of inflammation and fibrosis through cytokine secretion from the inflammatory cells and fibroblasts in the AM.

Background

The arachnoid membrane (AM) is a tissue named “choroid meninx” by Herophilus in 3 B.C.[1]. Presently, the AM is considered to exist between the dura matter and pia matter and forms a subarachnoid cavity that plays a role in cerebrospinal fluid circulation. From the viewpoint of pathology, the AM comprises two laminar structures: the outer layer and inner layer[1, 2]. The outer layer is the arachnoid barrier cell layer constructed with tight junctions, whereas the inner layer is an arachnoid reticular cell layer loosely

constructed with denser cytoplasm, intermediate filaments, and mitochondria[2, 3]. These delicate structures constitute the space of cerebrospinal fluid circulation to exclude unnecessary and harmful materials and to act as cushioning material to protect brain tissue[1, 4]. The AM is relatively hyperplastic in the posterior fossa or around the Sylvian fissure[1]. However, there are few reports on the pathological and physiological significance of AM characteristics, and the role of the AM is still not elucidated at present.

Recently, low-level and chronic systematic inflammation has been found to influence the onset and recurrence of various lifestyle diseases[5–7]. This chronic inflammation is mainly caused by macrophages and lymphocytes through various cytokines and their polymorphisms including TNF α , IL-6, TGF β , and VEGF[8–10], and affects the incidence of stroke or cardiovascular events through atherosclerosis[11, 12]. In addition, chronic inflammation induces fibrosis as shown in idiopathic interstitial pneumonias, constrictive pericarditis, and hepatic cirrhosis[13–17]. Such fibrosis can cause organ failure or organopathy in the final stage. The AM is an avascular tissue. Therefore, it is assumed that inflammation is hardly caused in the AM. However, from the neurosurgical perspective, during intracranial microscopic operation, varying opacity of the AM can be recognized after opening the dura, and we hypothesized that the degree of transparency of the AM might be associated with chronic inflammation and fibrosis. In this study, we investigated the various characteristics of the AM focusing on the influence of chronic inflammation and fibrosis.

Methods

Description of patient population

This study was approved by the Ethics Committee of Sapporo Medical University Hospital (no. 292 – 128) and was performed in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. This study was a prospective and observational study. Six institutes with sufficient surgical experience participated in the study. All patients provided informed consent before participating. From August 2017 to August 2019, the AM was obtained during neurosurgical procedure. In the patients with vascular disease, only the AM was obtained, whereas cerebral cortex samples were obtained along with AM in patients with brain tumor, cerebral infarction, and severe trauma. AM sampling was performed above the middle frontal gyrus, and was avoided around the Sylvian fissure, superior sagittal sinus, posterior fossa, and basal cistern because these parts are physiologically hyperplastic. The AM above the cortical artery (prefrontal or precentral artery) was dissected under a microscope, and small pieces (10–30 mm²) of the sample were obtained. Using the obtained samples, we assessed the following: (1) pathological characteristics of the AM, (2) messenger RNA expression of the cytokines.

Preparation of the AM and pathological assessment

For measuring the thickness of the AM in each sample, the specimens needed to be flattened and then embedded in 6.5% Sabouraud agar (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) as a 1.0 cm square cube sample. For flattening the sample, 4 points of the corners of the AM were sewn on a silicon sheet

(5MS01, Kono Seisakusho Co Ltd., Ichikawa, Japan) under a microscope using 10 – 0 nylon. After sewing onto the silicon sheet, the sample was infiltrated in formalin-fixation liquid for 5 days, and then embedded in 6.5% Sabouraud agar. Finally, the Sabouraud agar-embedded sample was set in paraffin. The paraffin-embedded specimens were sectioned to a thickness of 3 μm .

Infiltration of inflammatory cells and fibrosis was assessed by H-E and immunohistochemical staining. CD68s (macrophage markers), CD86s (type I [M1] macrophage markers), and CD206s (type II [M2] macrophage markers) were used to assess inflammation. Antibodies against the following antigens were used for assessment: CD68 (M0876 Dako clone PG-M1; Agilent Technologies, Santa Clara, CA, USA, 1:100); rabbit anti-CD86 (ab53004, Abcam plc, Cambridge, UK, 1:500); rabbit anti-mannose receptor antibody (anti-CD206; ab64693, Abcam plc, 1:200). In each sample with H-E stain, the thickness of AM was measured in 3 places and the mean value was calculated (HPF, $\times 400$). The number of fibroblasts was then counted in 3 areas of each glass slide (HPF, $\times 400$), and the mean value was calculated. In addition, the local existence of fibroblasts was assessed. Next, the number of CD68⁺ cells, CD86⁺, and CD206⁺ cells were counted in 3 areas using immunohistochemical staining, and the mean value was similarly calculated.

Stratification of clinical data

Patient clinical data were stratified based on the following 11 clinical variables: (1) age; (2) sex; (3) disease; (4) smoke; (5) systolic blood pressure; (6) total cholesterol; (7) high density lipoprotein (HDL) cholesterol; (8) low-density lipoprotein (LDL) cholesterol; (9) glucose metabolism disorders; (10) a family history of coronary artery disease; and (11) degree of atherosclerosis. The degree of atherosclerosis was determined using the ASCVD score and Suita score.[18, 19] The ASCVD score was advocated in the American College of cardiology and the American Heart Association guideline to evaluate prediction of the 10-year risk of development of ASCVD events. Suita score was advocated by the Japan Atherosclerosis Society guidelines for prevention of atherosclerotic cardiovascular diseases. The clinical and pathological factors were compared between the hyperplastic AM group ($\geq 50 \mu\text{m}$) and non-hyperplastic AM group ($< 50 \mu\text{m}$).

mRNA expression analysis using real-time qRT-PCR

Only AM was used to assess mRNA expression. Samples with adhered blood components or brain tissue were excluded in this analysis. First, RNA extraction was performed using the RNeasy Fibrous Tissue Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. The total RNA concentration and the A260/A280 ratio were measured using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). Samples in which the A260/A280 ratio was less than 1.30 were excluded. GoScript™ Reverse Transcription Kit (Promega Corporation, WI, USA) was used to reverse-transcribe 500 ng of total RNA. PCR was performed in a 10 μL volume with 2 μL of cDNA diluted 1:5. TaqMan Universal Master Mix II with UNG and TaqMan Gene Expression Assays for glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs02786624_g1), vascular endothelial growth factor A (VEGF α ; Hs00998133_m1), transforming growth factor beta 1 (TGF β ; Hs00998133_m1), and tumor necrosis

factor (TNF α ; HS00174128) were purchased from Thermo Fisher Scientific Inc. qRT-PCR was performed in duplicate on the Applied Biosystems StepOnePlus™ (Thermo Fisher Scientific Inc., Tokyo, Japan). The thermal cycling protocol consisted of 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The expression of each mRNA was calculated using a calibration curve with another reference sample such as the unknown level of mRNA expression. Each mRNA expression was revised to the ratio of a reference mRNA expression (target mRNA expression/GAPDH expression) based on relative quantification. Using the mRNA expression data, correlations between the thickness of the AM and mRNA expression levels of TNF α , TGF β and VEGF α were investigated.

Statistical analysis

Data are expressed as median (interquartile range). Mann-Whitney U test and Fisher's exact probability tests were used for comparison between the hyperplastic AM group and non- hyperplastic AM group. Spearman's correlation coefficient and regression analysis were used to compare the thickness of the AM and potential factors. Similarly, correlations between the thickness of the AM and mRNA expression levels were calculated using Spearman's correlation coefficient and regression analysis. A correlation coefficient (ρ) larger than 0.7 indicated a strong correlation, a ρ value between 0.5 and 0.7 indicated moderate correlation, and a $\rho < 0.5$ indicated weak correlation. All statistical analyses were conducted using the SPSS software package (version 24.0, IBM Corp., NY, USA), and $p < 0.05$ was considered indicative of statistical significance.

Results

Patient data

This study included 98 cases operated at our Hospital and an affiliated hospital from September 2017 to April 2019. Twenty-three cases could not be measured because the specimens were over-minute, or due to thermal denaturation during the preparation process. One case was excluded because the AM could not be separated from the meningioma. Consequently, 74 cases could be used to measure the thickness of the AM. Of these 74 cases, 17 cases had partially missing clinical data. The messenger RNA (mRNA) expression levels were measured completely in 57 patients. Of the 74 patients, 44 cases suffered from cerebrovascular disease, 21 cases had brain tumor, and 9 cases were of other diseases. Of the 44 cases of cerebrovascular disease, 14 cases were unruptured aneurysms, 13 cases were of moyamoya disease, 10 cases were of atherosclerotic steno-occlusive disease, 4 cases were of cerebral hemorrhage, and 3 cases were of cerebral arteriovenous malformation. Of the 21 cases of brain tumor, 7 cases were high grade glioma, 5 cases were meningioma, 3 cases were malignant lymphoma, 2 cases were low grade glioma, 1 case was ependymoma, 1 case was craniopharyngioma, 1 case was pituitary adenoma, and 1 case was metastatic carcinoma. The median (interquartile range) age of the patients at the time of surgery was 54.0 years (39.0–68.0). Thirty-one patients were male and 43 were female. The AM was collected from the frontal lobe in 29 cases, the temporal lobe in 35 cases, parietal lobe in 3 cases,

occipital lobe in 4 cases, and from the cerebellum in 3 cases. The median Atherosclerotic Cardiovascular Disease (ASCVD) score and Suita score was 6.0 (2.1–24.3) % and 41.0 (23.0–51.3), respectively.

Pathological characteristics of the AM

H-E staining showed fibroblasts, fibers, and the barrier cell layer in the AM. Fibroblasts tend to exist at the inward pial side, and the fibroblast decreased at the outward dural side being replaced by fibers (Fig. 1A, B). In the 16 specimens including brain tissue, hyperplasia of the AM was observed around the arteries in 6 cases. CD68⁺, CD86⁺, CD206⁺ staining showed the infiltration of macrophages, M1 macrophages and M2 macrophages respectively, in the AM (Fig. 1C, 1D, 1E.). CD68⁺ cells and CD206⁺ cells tended to be accumulated at inward pial side. The median thickness of AM was 49.62 (34.05–69.13) μm . The median number of fibroblasts counted by Hematoxylin-Eosin (H-E) stain was 88.48 (41.14–125.97) $\times 10^{-3}/\mu\text{m}^2$. A total of 46 cases were stained positively for CD68, and the median number of CD68⁺ cells was 2.85 (0.00–12.56) $\times 10^{-3}/\mu\text{m}^2$. Of these 46 cases stained positively for CD68, 13 cases were stained positively for CD86 as well, and the median number of CD86⁺ cells was 0.00 (0.00–0.00) $\times 10^{-3}/\mu\text{m}^2$. Additionally, 32 cases were stained positively for CD206, and the median number of CD206⁺ cell was 0.00 (0.00–10.24) $\times 10^{-3}/\mu\text{m}^2$.

Table 1 shows the clinical and pathological characteristics associated with the AM thickness. We divided patients into two groups; hyperplastic AM group ($\geq 50 \mu\text{m}$) and non-hyperplastic AM group ($< 50 \mu\text{m}$). The hyperplastic AM group and the non-hyperplastic AM group included 37 cases each. The median age of the patient was 59.0 years (45.0–70.0) in the hyperplastic AM group, and 47.0 years (17.0–65.0) in the non-hyperplastic AM group indicating that the older patients exhibited hyperplastic AM ($p = 0.020$). The AM thickness was significantly correlated to age ($\rho = 0.332$, $p = 0.004$) (Fig. 2A). Patients' sex did not affect the hyperplasia of the AM ($p = 0.814$). With regards to other clinical factors including disease, smoke, systolic blood pressure, total cholesterol, HDL cholesterol, LDL cholesterol, glucose metabolism disorders, and familial history of coronary artery disease, there was no significant difference between hyperplastic AM group and non-hyperplastic AM group. The median ASCVD score in the hyperplastic AM group [11.1 (2.2–27.2) %] was higher than that in the non-hyperplastic AM group [4.3 (1.2–16.4) %], though it was not significant ($p = 0.176$). The median Suita score in the hyperplastic AM group [45.0 (31.00–54.00)] was higher than that in the non-hyperplastic AM group [40.0 (-1.50–48.50)] and was also not significant ($p = 0.153$). The median number of fibroblasts in the hyperplastic AM group and non-hyperplastic AM group were 110.16 (69.83–138.35) $\times 10^{-3}/\mu\text{m}^2$ and 65.84 (19.28–97.01) $\times 10^{-3}/\mu\text{m}^2$, respectively, with a significant difference between the 2 groups ($p = 0.004$). The AM thickness was significantly correlated to the number of fibroblasts ($\rho = 0.336$, $p = 0.003$) (Fig. 2B). The median number of CD68⁺ cells in the hyperplastic AM group and non-hyperplastic AM group was 7.28 (1.12–13.46) $\times 10^{-3}/\mu\text{m}^2$ and 0.00 (0.00–5.75) $\times 10^{-3}/\mu\text{m}^2$, respectively, with a significant difference ($p = 0.023$). The AM thickness was significantly correlated to the number of CD68⁺ cells ($\rho = 0.274$, $p = 0.018$) (Fig. 2C). The median number of CD86⁺ cells in the hyperplastic AM group and non-hyperplastic AM group was 0.00 (0.00–9.83) $\times 10^{-3}/\mu\text{m}^2$ and 0.00 (0.00–0.00) $\times 10^{-3}/\mu\text{m}^2$, respectively, with a significant difference ($p =$

0.005). The AM thickness was significantly correlated to the number of CD86⁺ cells ($\rho = 0.271$, $p = 0.020$) (Fig. 2D). The median number of CD206⁺ cell in the hyperplastic AM group and non-hyperplastic AM group was $3.63 (0.00-12.11) \times 10^{-3}/\mu\text{m}^2$ and $0.00 (0.00-2.34) \times 10^{-3}/\mu\text{m}^2$, respectively, with a significant difference ($p = 0.020$). The AM thickness was significantly correlated to the number of CD206⁺ cells ($\rho = 0.312$, $p = 0.007$) (Fig. 2E). These findings suggested that fibrosis and chronic inflammation might be seen, especially in patients with hyperplastic AM.

mRNA expression of cytokines

The macrophages and lymphocytes liberate TNF α , IL-6, TGF β , and VEGF. To study whether the cytokines affect thickness of AM, we measured mRNA levels of TNF α , TGF β , and VEGF and compared their expression levels and thickness of AM. mRNA expression levels were measured in 58 cases including 28 cases in the hyperplastic AM group and 30 cases of the non-hyperplastic AM group. Firstly, VEGF α was assessed, because VEGF α is key factor for tissue repair released by fibroblasts and inflammatory cells. The median value of VEGF α mRNA expression was 0.68 (0.09–2.24). The median value of VEGF α mRNA expression levels was 1.30 (0.29–2.85) in the hyperplastic AM group, and 0.23 (0.07–1.60) in the non-hyperplastic AM group ($p = 0.043$). The value of VEGF α mRNA expression levels was significantly correlated to the thickness of the AM ($\rho = 0.337$, $p = 0.010$) (Fig. 3A). Next, TNF α and TGF β were assessed, because these are inflammatory mediators. The median values of TNF α and TGF β were 48.02 (0.00–1361.71) and 0.50 (0.03–1.76), respectively. The median value of TNF α mRNA expression levels was 114.89 (0.00–1008.17) in the hyperplastic AM group, and 32.12 (0.00–1428.72) in the non-hyperplastic AM group ($p = 0.936$). The median value of TGF β mRNA expression levels was 0.61 (0.00–2.13) in the hyperplastic AM group, and 0.48 (0.18–1.63) in the non-hyperplastic AM group ($p = 0.445$). The VEGF α mRNA expression was significantly correlated with the TGF β mRNA expression ($\rho = 0.703$, $p < 0.001$) (Fig. 3B). TNF α mRNA expression was significantly correlated with the TGF β mRNA expression ($\rho = 0.879$, $p < 0.001$) (Fig. 3C). These findings indicate that VEGF α and other inflammatory mediators are associated with AM hyperplasia.

Discussion

In the AM, hyperplasia due to fibroblast proliferation, and infiltration of CD68⁺ cells, CD86⁺ positive cells, and CD206⁺ cells were observed, suggesting the presence of chronic inflammation focusing on macrophages in the AM. Macrophages are a key regulator of chronic inflammation and fibrosis in various disorders such as pericarditis, and hepatic cirrhosis and arteriosclerotic plaque [20–22], and in this study, among all cases, 63.5% involved CD68⁺ cells. In the AM, inflammatory and anti-inflammatory cells should be derived from the cerebrospinal fluid of the subarachnoid space, and/or the cerebral arteries and brain tissue. The macrophages are divided into two phenotypes varying in their physiological roles: M1 and M2 macrophages [23, 24]. The M1 macrophage appears in inflammatory conditions and promotes damage of the tissue organization by producing pro-inflammatory cytokines (IL-1 β , TNF α , IL-6, and IL-12) [25]. On the contrary, the M2 type macrophages appear in anti-inflammatory conditions and produces anti-

inflammatory cytokines (IL-10, IGF-1, and TGF β) working in phagocytosis and restoration of tissue organization [25]. The distribution of various macrophage phenotypes is considered to allow prediction of the inflammatory stage. In atherosclerosis, some reports show that the dominance of M1 macrophages results in a vicious cycle of plaque formation [26]. In this study, 17.6% were CD86⁺ cells and 43.2% were CD206⁺ cells, and the M2 marker was superior. The M2 macrophages facilitate healing and repairing of the inflammatory process, show anti-inflammatory action, and promote fibrosis [23]; it is thought that these cells contribute to hyperplasia of the AM. Further, TGF β and TNF α in the AM were strongly correlated in this study. M1 and M2 macrophages co-exist in various inflammatory states, and dynamic changes in the M1/M2 phenotype of recruited mononuclear phagocytes have been observed in other disease model as well [27–29].

Fibroblast proliferation is observed in the regeneration process of chronic inflammation due to anti-inflammatory cytokines [30]. Our results indicate that the infiltration of chronic inflammatory cells and fibroblasts tends to distribute in the inner layer adjacent to the brain surface, and that extracellular matrix including collagen fibers mainly exists at the dura matter side, in the barrier cell layer. It was considered that the AM hyperplasia originates from the inner side of the AM, and that inflammation begins from the subarachnoid space near the arteries (Fig. 4). TGF β causes tissue fibrosis due to the proliferation of fibroblasts and production of extracellular matrix [31]. In this study, VEGF α levels correlated with the thickness of the AM. VEGF α is produced by several types of cells including fibroblasts and inflammatory cells [32, 33]. In moyamoya disease, high VEGF expression was correlated with TGF β [34], and might be associated with abnormal vascular hyperplasia. Although the pathological meaning of excessive AM hyperplasia is still obscure, in other organs including the lung, liver, and kidney, excessive fibrosis leads to defective repair and induces organ failure in the final stages [27, 35]. In the brain, fibrosis of the AM might affect the pulsative circulation of cerebrospinal fluid. Recently, the glymphatic system of the perivascular and subarachnoid space has gained attention and has been shown to be associated with Alzheimer's disease [36]. In future work, understanding the harmful effect of AM hyperplasia on the glymphatic system or cognitive function will be crucial. From the clinical viewpoint, only aging was associated with the AM thickness in this study. Progressive fibrosis is a hallmark of the aging process and has been implicated in the pathogenesis of diseases of the heart, lungs, liver, kidneys, and bone marrow [37]. Contrary to expectations, there were no correlations between the AM hyperplasia and clinical factors, including atherosclerosis scores. In stroke, chronic cerebral ischemic state, brain tumor, and brain abscess, these pathologies should be a state of microglia or macrophage activation [38, 39], though the influence of each pathology was minimal in the AM. Therefore, it will be necessary to compare the groups with equivalent ages to assess the influence of the disease.

There are some limitations to this study. First, this study included various kinds of diseases, and the degree of the disease was different in each case. For instance, local factors might depend on the stage of the original disease especially in extra-axial tumors. On the other hand, it was considered that the AM could be affected widely in chronic ischemic disease, though the degree of the ischemia was different in each case. This study did not exclude the influences of disease specificity. Secondly, the quantity of

specimens was not sufficient for analysis by real-time qRT-PCR. Samples in which the A260/A280 ratio was set less than 1.30 were excluded in this study, though their value was relatively low. Third, there were problems in terms of AM fixation. Because AM is a very thin and delicate tissue, thermal denaturation might have occurred in the Sabouraud agar fixation process. Although, an alternative technique is needed to assess the cerebral environment, this could not be achieved to find pathological significance.

In conclusion, AM hyperplasia was influenced by aging. Pathologically, fibroblasts and M2 macrophages proliferated in the inward pia side especially around the arteries. AM hyperplasia was correlated with VEGF α and TGF β and could be a result of anti-inflammatory changes and fibrosis. These alterations might be affected by cerebral ischemia, especially in moyamoya disease. Further analysis of the AM in various diseases And more number of cases will provide us with answers to some of these hypothesis.

Conclusion

In conclusion, AM hyperplasia was influenced by aging. Pathologically, fibroblasts and M2 macrophages proliferated in the inward pia side especially around the arteries. AM hyperplasia was correlated with VEGF α and TGF β and could be a result of anti-inflammatory changes and fibrosis. Further analysis of the AM in various diseases and more number of cases will provide us with answers to some of these hypothesis.

Abbreviations

AM: arachnoid membrane; mRNA: messenger RNA; ASCVD: Atherosclerotic Cardiovascular Disease; H-E: Hematoxylin-Eosin

Declarations

Ethical approval and consent participate:

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee (The ethics committee of Sapporo Medical University Hospital) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All participants provided written informed consent and were able and willing to participate.

Consent for publication:

Not applicable.

Availability of dataset and materials:

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request. The data are not publicly available due to privacy or ethical restrictions.

Competing interests:

The authors declare that they have no competing interests.

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Authors' contribution:

HS and TM analyzed and interpreted the patient data regarding the thickness of arachnoid membrane and the influence of inflammation and fibrosis. NI, RH, YH, and AK assessed mRNA expression analysis using real-time qRT-PCR. YA, MW, KK, RY, TH, and NM provided surgical specimens and data. All authors read and approved the final manuscript.

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Tables

Table 1 Comparison of the characteristics of patients in the hyperplastic AM group and the non-hyperplastic AM group

Median (interquartile range)	Total	AM thickness		<i>p</i> value
		Hyperplastic AM (50 μ m \geq)	Non-hyperplastic AM (<50 μ m)	
AM thickness (μ m)	49.62 (34.05-69.13)	71.42 (55.68-97.60)	33.99 (24.96-39.39)	<0.001
Age	54.00 (39.00-68.00)	59.00 (45.00-70.00)	47.00 (17.00-65.00)	0.020
Sex (male/female)	31/42	16/21	15/22	0.814
Disease				
Cerebrovascular disease	44	23	21	0.192
Brain tumor	21	12	9	
Others	9	2	7	
Smoke (yes/no)	34/40	20/17	14/23	0.162
Systolic BP (mmHg)	120.00 (110.00-132.50)	130.00 (110.00-140.00)	120 (110.00-130.00)	0.249
Total Cholesterol (mg/dL)	201.00 (168.50-233.00)	205.00 (176.00-234.50)	187.00 (164.00-228.00)	0.465
HDL (mg/dL)	54.00 (42.50-67.00)	52.50 (42.50-65.00)	58.00 (43.00-70.00)	0.322
LDL (mg/dL)	121.00 (85.00-136.80)	126.00 (85.00-146.00)	107.00 (88.20-130.00)	0.335
Glucose metabolism disorders (yes/no)	15/59	7/30	8/29	0.772
A family history of CAD (yes/no)	1/72	1/36	0/36	0.321
ASCVD score [%]	6.00 (2.10-24.30)	11.10 (2.20-27.20)	4.30 (1.15-16.35)	0.176
Suita score	41.00 (23.00-51.30)	45.00 (31.00-54.00)	40.00 (-1.50-48.50)	0.153
Fibroblasts ($\times 10^{-3}/\mu\text{m}^2$)	88.48 (41.14-125.97)	110.16 (69.83-138.35)	65.84 (19.28-97.01)	0.004
CD68 ⁺ cell ($\times 10^{-3}/\mu\text{m}^2$)	2.825 (0.00-12.57)	7.28 (1.12-13.46)	0.00 (0.00-5.75)	0.023
CD86 ⁺ cell ($\times 10^{-3}/\mu\text{m}^2$)	0.00 (0.00-0.00)	0.00 (0.00-9.83)	0.00 (0.00-0.00)	0.005
CD206 ⁺ cell ($\times 10^{-3}/\mu\text{m}^2$)	0.00 (0.00-10.25)	3.63 (0.00-12.11)	0.00 (0.00-2.34)	0.020

TGFβ [fold change]	0.50 (0.03-1.76)	0.61 (0.00-2.13)	0.48 (0.18-1.63)	0.445
TNFα [fold change]	48.02 (0.00-1361.71)	114.89 (0.00-1008.17)	32.12 (0.00-1428.72)	0.936
VEGFα [fold change]	0.68 (0.09-2.24)	1.30 (0.29-2.85)	0.23 (0.07-1.60)	0.043

AM: arachnoid membrane, CAD: coronary artery disease, ASCVD: Atherosclerotic Cardiovascular Disease

Figures

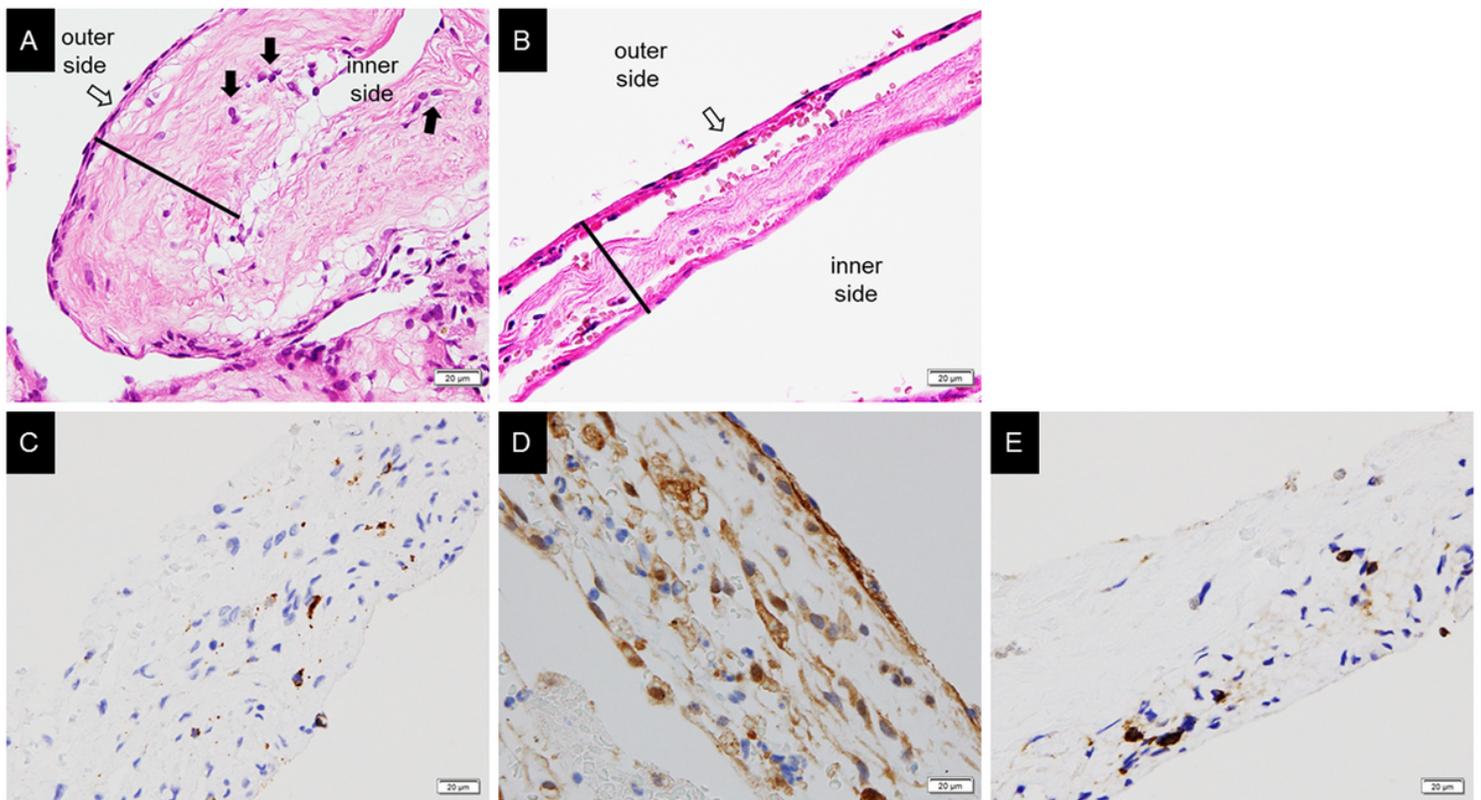


Figure 1

Pathological findings of the AM (×400) in a representative case of hyperplastic AM (A), and non-hyperplastic AM (B). The arachnoid membrane includes a barrier cell layer beside the dura matter (white arrow). The fibroblast cells increased and localized beside the inner layer (black arrow). The membrane thickness was measured as the black bar. Immunostaining of the AM (×400); Macrophages were stained with CD68 (C), CD86 (D), and/or CD206 (E). The number of positive cells for CD68, CD86, and CD206 staining were counted (black arrow).

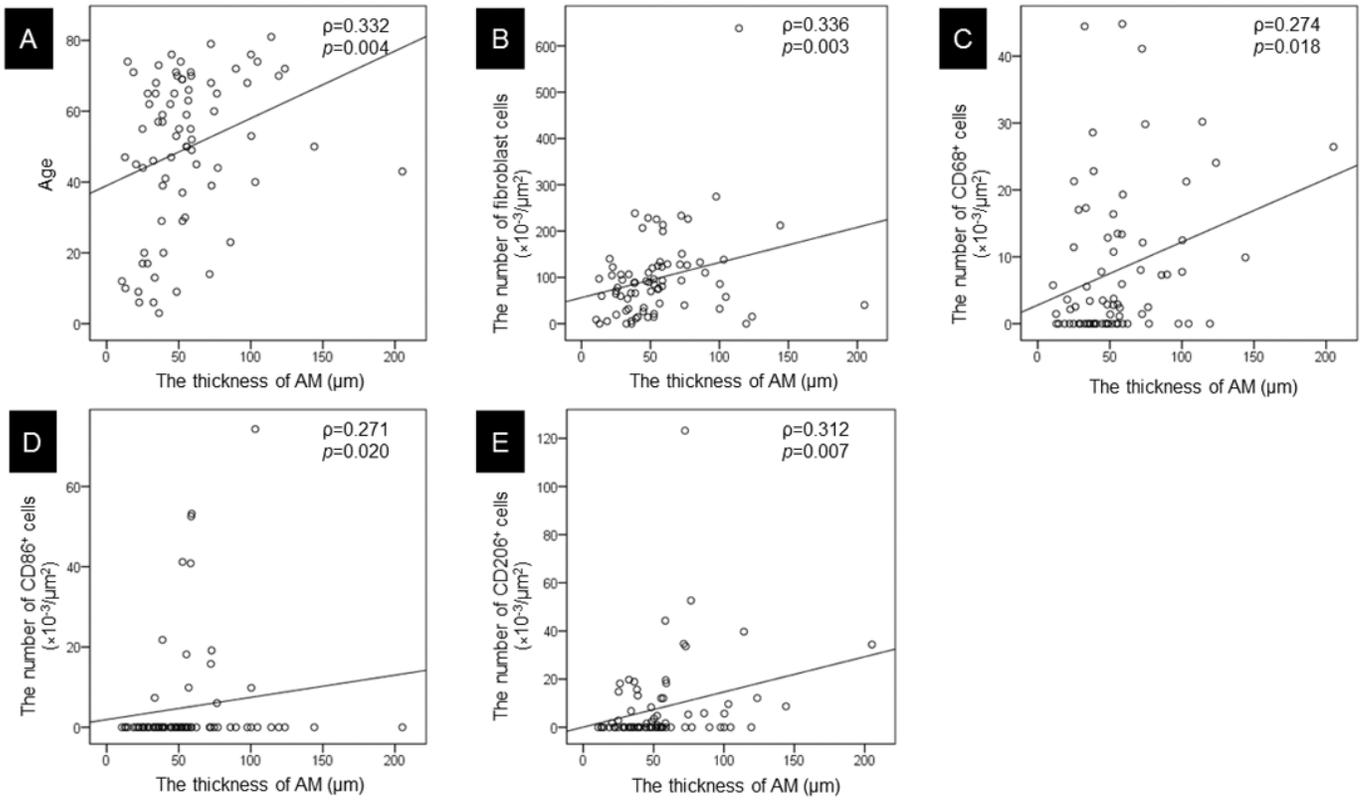


Figure 2

Correlation between the thickness of the AM and age (A), fibroblasts (B), CD68+ cells (C), CD86+ cells (D), and CD206+ cells (E) cells. The correlation between these were significant ($\rho = 0.332, p = 0.004; \rho = 0.336, p = 0.003; \rho = 0.274, p = 0.018; \rho = 0.271, p = 0.020; \rho = 0.312, p = 0.007$), respectively.

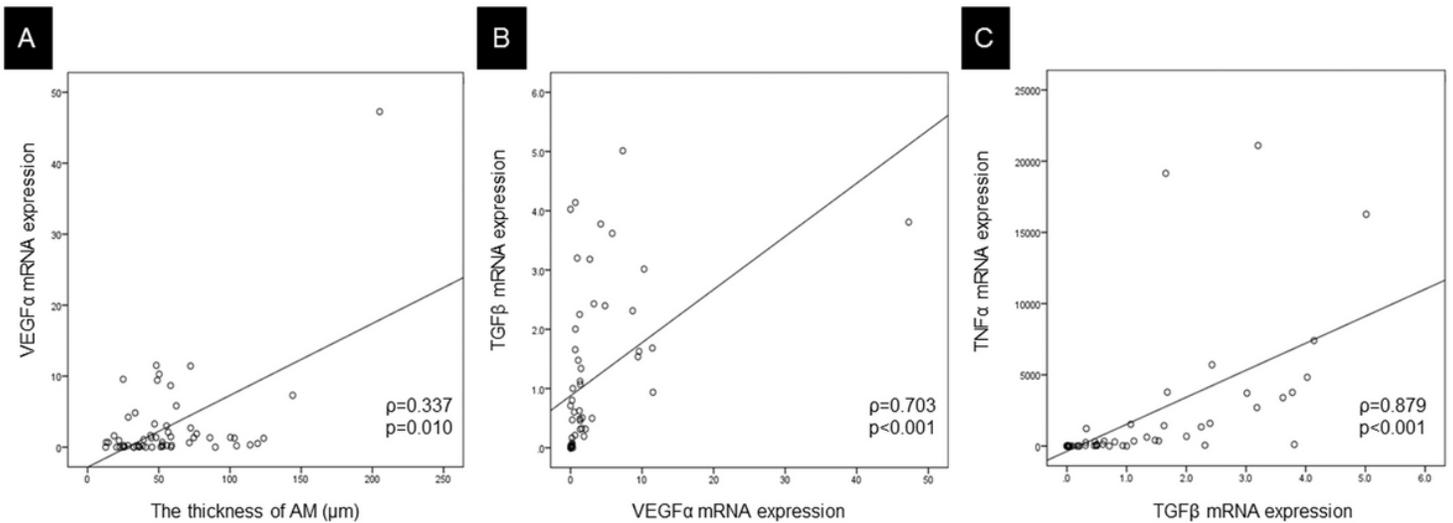


Figure 3

Correlation between the thickness of AM and the VEGF α , TGF β , and TNF α mRNA expression. The AM thickness was significantly correlated with the mRNA expression of VEGF α ($\rho = 0.337, p = 0.010$) (A).

VEGF α mRNA expression was significantly correlated to the TGF β mRNA expression ($\rho = 0.703, p < 0.001$) (B). The value of TGF β mRNA expression was significantly correlated to the value of TNF α mRNA expression ($\rho = 0.879, p < 0.001$) (C).

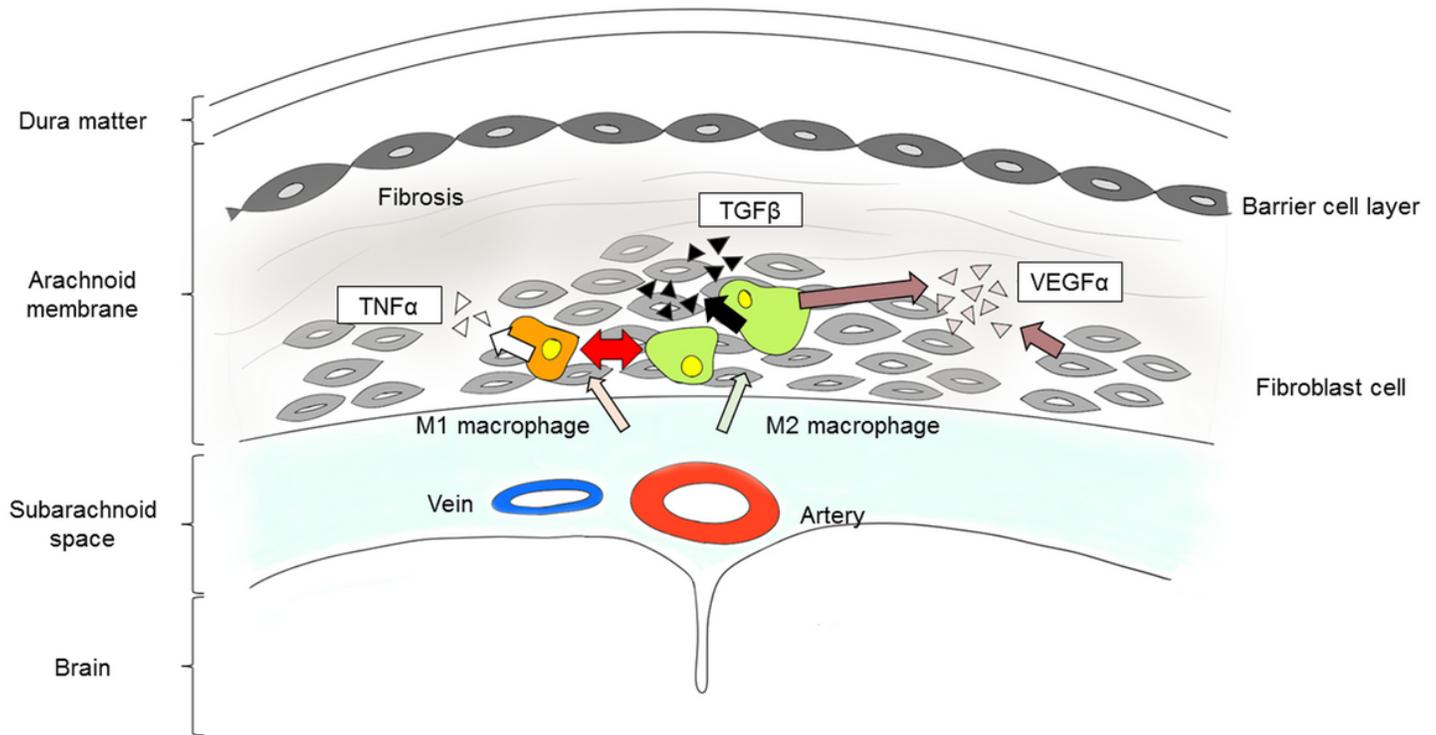


Figure 4

Illustration of the process of AM hyperplasia. Macrophages play an important role in chronic inflammation. Infiltration of macrophages and fibroblasts tends to distribute inward to the pial side adjacent to the brain surface, and the extracellular matrix including collagen fibers mainly existed at the outward dural side. Macrophage activation accelerates the elevation of TNF α and TGF β , and promotes of fibrosis. Consequently, VEGF levels increase in the AM.