Activation of Signal Transducer and Activator of Transcription 3 in Endothelial Cells of Chronic Subdural Hematoma Outer Membranes

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■ OBJECTIVE: **Chronic subdural hematoma (CSDH) is considered an angiogenic and inflammatory disease. Interleukin-6, a well-known inflammatory cytokine, activates the Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway. We previously reported that the JAK/STAT pathway is activated in fibroblasts in the outer membrane of CSDH. More recently, signal transducer and activator of transcription 3 (STAT3) has been shown to have a role in angiogenesis. We examined the expression of STAT3 in endothelial cells in the outer membrane of CSDH.**

■ METHODS: **This study included 7 patients whose outer membranes were successfully obtained during trephination surgery. The expression of STAT3 and phosphorylated STAT3 was examined by Western blot analysis and immunohistochemistry. We also examined whether CSDH fluid could activate STAT3 in cultured endothelial cells in vitro.**

■ RESULTS: **STAT3 and phosphorylated STAT3 were detected in all cases. Immunostaining showed that STAT3 and phosphorylated STAT3 were expressed not only in fibroblasts but also in vascular endothelium. Expression of phosphorylated STAT3 in endothelial cells was significantly induced immediately after treatment of CSDH fluid in vitro. The activation of STAT3 was significantly inhibited by treatment with antibodies that were directed against interleukin-6; however, this was suppressed by antibodies that were directed against vascular endothelial growth factor, but not significantly.**

■ CONCLUSIONS: **Interleukin-6 might dominantly activate STAT3 in endothelial cells, which might have a central role in endothelial cell proliferation and angiogenesis of CSDH outer membranes.**

INTRODUCTION

Chronic subdural hematoma (CSDH) occurs following mild head trauma in elderly adults. Inflammation and angiogenesis play some role in growth of the disease. However, the development of CSDH is considered to involve multifactorial mechanisms, many of which are not fully understood. The inflammatory cytokine interleukin-6 (IL-6) is increased in hematoma fluid compared with serum.[1,2] The Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling pathway is well known to play essential roles in cytokine function, especially in IL-6 families.[3] We have previously reported that the JAK/STAT3 signaling pathway is activated by IL-6 in fibroblasts.[4] We have also shown that transforming growth factor β in CSDH fluid activates the Smad signaling pathway in fibroblasts.[5] These data suggest that these molecules are involved in the growth of fibroblasts in the CSDH outer membrane.

We previously revealed that the Ras/mitogen-activated protein kinase/extracellular signal-regulated kinase kinase/extracellular signal-regulated kinase signaling pathway, phosphatidylinositol 3-kinase/protein kinase B signaling pathway, p38 mitogen-activated protein kinase, and c-jun N-terminal kinase are activated by vascular endothelial growth factor in the endothelial cells within the CSDH outer membrane.[6-8] Recently, we have also shown the activation of the nuclear factor κB signaling pathway by double-stranded

Key words
- Angiogenesis
- Chronic subdural hematoma
- STAT3

Abbreviations and Acronyms
CSDH: Chronic subdural hematoma
IL-6: Interleukin-6
JAK: Janus kinase
STAT: Signal transducer and activator of transcription
STAT3: Signal transducer and activator of transcription 3
VEGF: Vascular endothelial growth factor

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RNA—activated protein kinase in endothelial cells of vessels (K.O., et al., unpublished data, 2016). These data suggest that these molecules are involved in the angiogenesis of CSDH. Nuclear factor κB and STAT3 pathways are central signaling pathways in inflammation and angiogenesis.[9] IL-6 induces basic fibroblast growth factor—dependent angiogenesis through the activation of the JAK/STAT3 and phosphatidylinositol 3-kinase/protein kinase B pathway in basal cell carcinoma.[10] Epidermal growth factor—like domain 7 induces endothelial cell migration and angiogenesis via the phosphorylation of extracellular signal-regulated kinase and STAT3 in bone.[11] STAT3 has a central role in modulating angiogenesis under both physiologic and pathologic conditions as a regulator of gene transcription.[12] These data suggest that crosstalk between STAT3 and these molecules might be involved with the angiogenesis of CSDH; however, the expression of STAT3 in endothelial cells has not been determined in CSDH outer membranes. Therefore, in this study, we sought to determine whether STAT3 is activated in endothelial cells in the outer membrane of CSDH. We performed immunoblot and immunohistochemical analyses using outer membranes of CSDH and cultured vascular endothelial cells.

MATERIALS AND METHODS

Patients

The Ethics Committee of Aichi Medical University approved this clinical experiment. Informed consent was obtained from each patient or relative. This study included 7 patients (4 men, 3 women; 59—79 years old; mean age, 67 years) with CSDH, which was confirmed by computed tomography or magnetic resonance imaging. All patients underwent burr hole drainage surgery under local anesthesia at the Aichi Medical University Hospital. All patients had a history of mild head injury, no patient had a hemostatic disorder, and no patients were receiving antiplatelet or anticoagulation therapy. Clinical data are shown in Table 1.

Materials

All chemicals, unless specified, were from Sigma-Aldrich (St. Louis, Missouri, USA).

Western Blot Analysis

Samples of outer membranes and CSDH fluid from CSDH were obtained during trephination surgery. The outer membranes were homogenized in 80 μL of homogenization buffer, which contained 50 mmol/L tris base/tris-HCl (pH 7.5), 0.1 mmol/L dithiothreitol, 0.2 mmol/L ethylenediaminetetraacetate, 0.2 mmol/L ethyleneglycol-bis-(β-aminoethylether)tetraacetate, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1.25 μg/mL Pepstatin A, 0.2 μg/mL aprotinin, 1 mmol/L sodium orthovanadate, 50 mmol/L sodium fluoride, 2 mmol/L sodium pyrophosphate, and 1% Nonidet P-40. The homogenates were later centrifuged at 12,000*g* at 4°C for 10 minutes. The protein concentrations of the supernatants were determined by Bradford assay, using bovine serum albumin as the standard. The crude samples (25 μg of protein each) were subjected to 7.5% sodium dodecyl sulfate—polyacrylamide gel electrophoresis. The proteins were then transferred to polyvinylidene difluoride membranes and incubated with primary polyclonal antibodies against actin (Sigma-Aldrich) and phosphorylated STAT3 at Tyr^{705} (Cell Signaling Technology, Inc., Danvers, Massachusetts, USA) at a dilution of 1:750 overnight at 4°C. After washing, the membranes were incubated with secondary antibodies that were conjugated to horseradish peroxidase (Sigma-Aldrich) at a dilution of 1:3000 for 30 minutes at room temperature. Reactions were developed with ECL or ECL Plus (GE Healthcare UK Limited, Buckinghamshire, United Kingdom). Phosphorylated STAT3 at Tyr^{705} immunoblots were stripped from polyvinylidene difluoride membranes and reblotted with primary polyclonal STAT3 (Cell Signaling Technology, Inc.) at a dilution of 1:750 overnight at 4°C. Finally, the membranes were developed with ECL. Band intensities were quantitated by densitometry using ImageQuant software (GE Healthcare UK Limited).

Histologic Examinations

To study the cellular localization of STAT3, immunohistochemical staining was performed according to the avidin-biotinylated peroxidase complex technique at room temperature ($n = 3$). Outer membranes from CSDH were preserved in 10 mL of ice-cold 4% paraformaldehyde in 0.1 mol/L phosphate buffer (pH 7.4) for 3 hours. Serial axial cryostat sections (10 μm) were placed on slides

Table 1. Clinical Data of 7 Patients with Chronic Subdural Hematoma

Case	Age (years)/Sex	Side	CT Appearance	MRI Appearance (T1, T2, FLAIR)	Size (cm)*	Shift (cm)†	Symptoms	Trauma (weeks ago)
1	66/F	Left	Isodense	iso, high, iso	1.5	1.3	Headache, aphasia	8
2	59/M	Right	Isodense	iso, iso, iso	2.0	0.7	Gait disturbance, hemiparesis	12
3	68/F	Right	Isodense	iso, iso, high	2.5	1.0	Gait disturbance, hemiparesis	8
4	74/M	Right	Isodense	iso, iso, iso	2.0	1.0	Headache	6
5	79/F	Left	Isodense	iso, high, iso	2.0	0.7	Gait disturbance, hemiparesis	8
6	63/M	Right	Mixed, layering	high, low, iso	3.0	0.8	Gait disturbance, hemiparesis	12
7	60/M	Left	Mixed, layering	high, low, iso	2.5	1.0	Gait disturbance, hemiparesis	16

CT, computed tomography; MRI, magnetic resonance imaging; FLAIR, fluid attenuated inversion recovery; F, female; M, male; iso, isointensity; high, high intensity; low, low intensity.
*Size refers to the largest extent measured in preoperative CT slices.
†Shift refers to the midline shift.