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Expression of the TGF-β–ALK-1 Pathway in Dura and the Outer Membrane of Chronic Subdural Hematomas

Atsushi SAITO,[1] Ayumi NARISAWA,[1] Hiroki TAKASAWA,[1] Takahiro MORITA,[1] Seiya SANNOHE,[2] Tatsuya SASAKI,[1] Hidekachi KUROTAKI,[2] and Michiharu NISHIJIMA[1]

Departments of [1]Neurosurgery and [2]Pathology, Aomori Prefectural Central Hospital, Aomori, Aomori

Abstract

Neovascularization of the outer membrane plays a critical role in the development and enlargement of chronic subdural hematomas (CSHs) and vascular endothelial growth factor (VEGF) may promote their progression. However, the precise mechanisms remain to be determined. We focused on the signaling pathway upstream of VEGF, transforming growth factor β (TGF-β), and activin receptor-like kinase 1 (ALK-1) to identify the mechanisms underlying the neovascularization of the outer membrane of CSH. Retrospective comparative study was conducted on 15 consecutive patients diagnosed as CSH with burr-hole drainage. Dura and the outer membrane were collected. We immunohistochemically examined the expression of VEGF, integrin-α, TGF-β, and ALK-1 on the outer membrane and dura of CSH and compared our findings with control samples and the signal intensity of hematomas on computed tomography (CT) scans. VEGF and integrin-α expression was markedly up-regulated in both the dura and outer membrane of CSH, the expression of TGF-β and ALK-1 in the dura was slightly increased in the dura and markedly up-regulated in the outer membrane. There was no significant correlation between their expression and CT density. Here we first report the expression of TGF-β and ALK-1 in the outer membrane and dura mater of CSH. We suggest that the TGF-β–ALK-1 pathway and VEGF affect neovascularization and the progression of CSH.

Key words: chronic subdural hematoma, transforming growth factor, activin kinase

Introduction

The detailed pathologic mechanisms underlying the initiation and progression chronic subdural hematoma (CSH), one of the most common traumatic entities seen in routine neurosurgical care,[1)] remain to be elucidated. It has been suggested that CSH is associated with an imbalance in factors involved in the regulation of coagulation and fibrinolysis and that the combination of fragile, permeable microvessels, and coagulopathy results in the progression of CSH.[1–3)]

The pathogenesis of CSH may be related to the signaling pathway of vascular endothelial growth factor (VEGF).[2,4–7)] VEGF can induce angiogenesis and increase the permeability of immature vessels in the outer membrane because its expression is correlated with angiogenesis and vascular leakage in diverse physiological and pathological conditions.[1,8)] While VEGF is known to be involved in the pathological mechanisms of CSH, its source and the upstream mediation of the VEGF signaling pathway remains unclear.[1)]

Transforming growth factor-β (TGF-β) is a multifunction protein involved in diverse biological processes including growth, differentiation, and inflammation.[9)] TGF-β1 plays an important role in vascular remodeling and exerts a biphasic effect on angiogenesis.[10)] At low concentrations TGF-β1 synergistically enhances, and at high concentrations it decreases VEGF-induced angiogenesis.[10–12)] To date, 7 type I receptors have been identified and designated activin receptor-like kinase (ALK) 1–7.[13)] ALK-1 mediates signals important for the formation or remodeling of blood vessels; it mediates TGF-β1 signaling in endothelial cells and the ALK-1 signaling pathway can inhibit TGF-β1-dependent transcriptional activation mediated by the TGF-β1

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type I receptor ALK-5.[10] The balance between the ALK-1 and ALK-5 signaling pathway downstream of TGF-β1 plays a crucial role in determining vascular endothelial properties during angiogenesis.[10] However, it is unknown whether the TGF-β1–ALK-1 signaling pathway is involved in the development of CSH and whether it affects the VEGF signaling pathway in CSH neovascularization. We investigated the existence of the TGF-β1–ALK-1 signaling pathway on the CSH outer membrane and its correlation with clinical and radiological findings on CSH.

Materials and Methods

This study includes 15 consecutive patients (12 men and 3 women) with a total of 16 CSHs. Their age ranged from 43 to 86 years (mean 64.6 years). All had undergone surgery at our department during the past 3 years and their pathological specimens were available. The surgical procedure in all 15 patients was burr-hole craniotomy with closed-system drainage. Prior written informed consent to sample and analyze the dura mater and outer membrane was obtained from all patients or their legal representatives. We selected the outer membrane of CSH as samples for the reason that the outer membrane contains rich vascular layer of dural border cells. Control dura mater (n = 8) was obtained from patients who underwent the clipping of unruptured intracranial aneurysms. Our study was approved by our institutional ethic committee and all enrolled patients provided informed consent.

Small pieces of the dura mater and outer membrane were obtained simultaneously and fixed in 4% buffered formalin. For immunohistochemical analysis samples were embedded in paraffin and 3-μm thick sections were stained with hematoxylin and eosin. Immunohistochemical staining was with the streptavidin-biotin-immunoperoxidase method. The primary antibodies (all from Santa Cruz Biotechnology, Santa Cruz, California, USA) were anti-VEGF antibody (mouse monoclonal), anti-TGF-β1 antibody (mouse monoclonal), anti-ALK-1 antibody (goat polyclonal), and integrin-α antibody (mouse monoclonal). The sections were deparaffinized, endogenous peroxidase activity was blocked, and the primary antibodies were applied and reacted. Peroxidase activity was visualized with diaminobenzidine; this was followed by counterstaining with hematoxylin. Four investigators (A.N., H.T., T.M., and T.S.) independently evaluated immunohistochemical staining. The results for VEGF, TGF-β1, ALK-1, and integrin-α were scored semi-quantitatively as 0 (no positive staining), 1 (focal or diffuse weak staining), 2 (focal moderate staining), and 3 (diffuse moderate or focal strong staining) according to the method of Nanko et al.[1] All hematomas were classified as homogenous, laminar, separated, or trabecular according to their density and internal architecture on computed tomography (CT) images.[14] The degree of immunostaining was classified as ± (0–0.5), + (0.5–1.0), and ++ (1.0–1.5) by using the average scores of immunopositivities. CT findings and the semi-quantitative staining scores of the four types of hematoma were compared.

Data were analyzed using Statview for Windows Abacus Concepts, Inc., Piscataway, New Jersey, USA). For intergroup comparison, data were compared using Wilcoxon rank sum test. Probability values less than 0.05 were considered significant.

Results

The average interval between the head injury and admission was 2.68 ± 1.05 months. Control cases were 6 men and 2 women and their age ranged from 51 to 76 years (mean 67.2 years). There was no significant difference in sex and age between CSH group and control cases. Neurological deficits were consciousness disturbance and hemiparesis in 16 and 15 patients, respectively. None of our patients suffered recurrence. VEGF, TGF-β1, ALK-1, and integrin-α were immunopositive in the duramater of most patients (Fig. 1). Both the dura mater and outer membrane expressed VEGF and integrin-α. VEGF was strongly expressed in endothelial cells of the dura mater. On the other hand, the expression of TGF-β1 and ALK-1 was stronger in the outer membrane than the dura mater. ALK-1 was strongly present in the outer membrane adjacent to the dura mater. We performed sub-quantitative comparisons of the dural expressions of the 4 angiogenic factors in CSH and control samples (Table 1). The average scores of those factors immunopositivities were as follows: VEGF (SCH: 1.44 ± 0.74, Normal: 0.24 ± 0.09, P = 0.03), integrin-α (SCH: 1.26 ± 0.52, Normal: 0.03 ± 0.02, P = 0.03), and TGF-β1 (SCH: 1.21 ± 0.33, Normal: 0.74 ± 0.25, P = 0.04) was significantly higher in the dura mater of CSH samples. ALK-1 (SCH: 1.10 ± 0.35, Normal: 0.83 ± 0.31, P = 0.08) was strongly expressed in CSH- and control samples.

Preoperative CT scans were available for all 16 patients. The density of the hematomas on CT images was scored as low in 6 (37.5%), iso in 9 (56.3%), and high in 1 (6.3%) of the 16 patients. As shown in Table 2, 4 hematomas (25%) were homogeneous; of the 12 (75%) heterogeneous hematomas, 1 each (8.3%) was laminar or segmental and the other 10 were trabecular (n = 10, 83.3%). There was no significant correlation between the

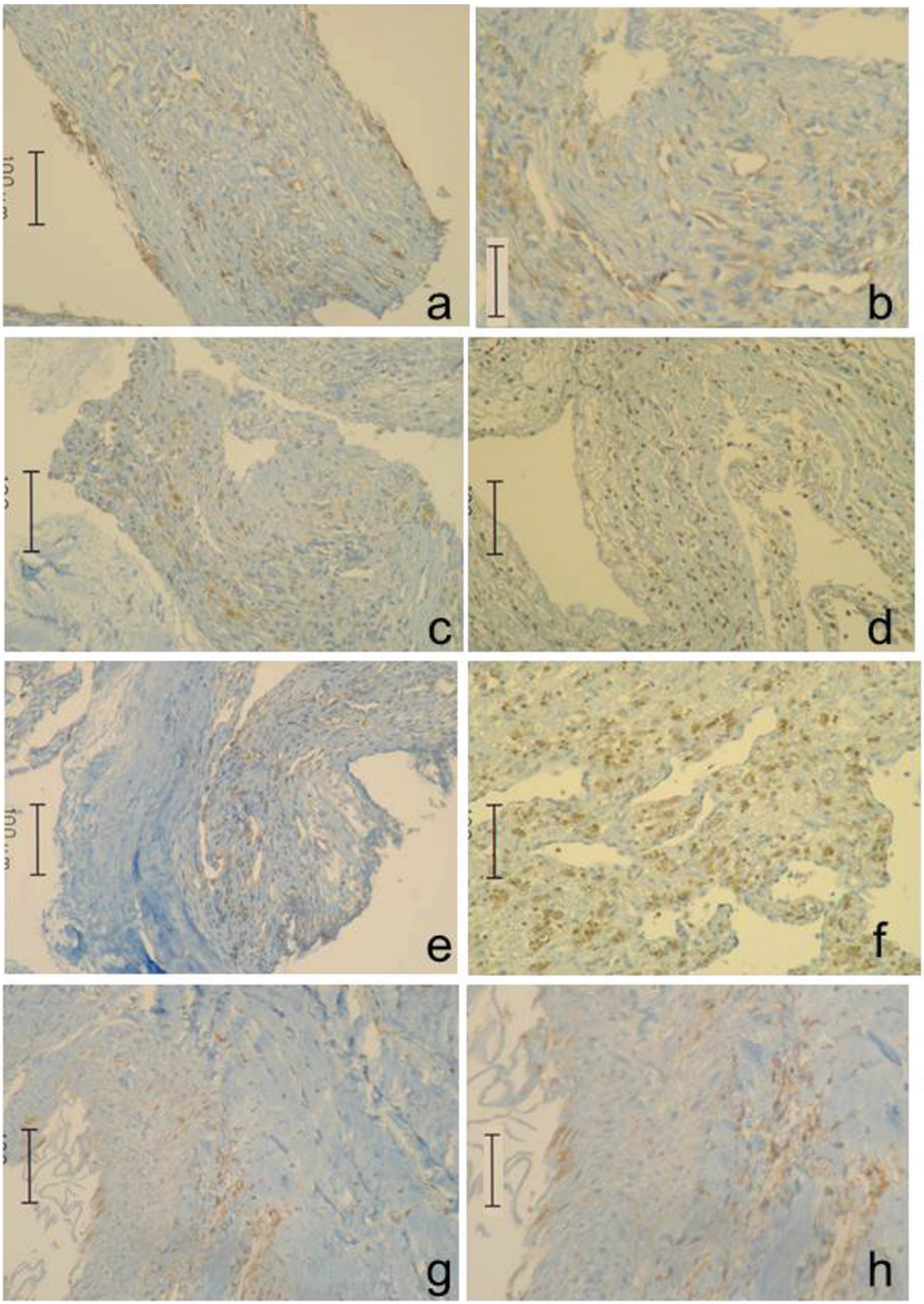

Fig. 1 Representative photographs showing the immunoreactive expression of vascular endothelial growth factor (VEGF) in the dura mater (a) and outer membrane of chronic subdural hematoma (CSH) (b). Integrin-α was expressed in the dura mater (c) and outer membrane (d). The expression of VEGF was particularly strong in endothelial cells in the dura mater. Transforming growth factor β (TGF-β1) and activin receptor-like kinase-1 (ALK-1) expression was observed in the dura mater (e and g, respectively) and the outer membrane (f and h, respectively). TGF-β1 and ALK-1 expression was stronger in the outer membrane than the dura mater. The expression of ALK-1 was remarkable in the outer membrane adjacent to the dura mater. Bar, 100 μm.

Table 1 Average scores of dural expressions of angiogenic factors

	Normal	CSH	P value
VEGF	0.24 ± 0.09	1.44 ± 0.74	0.03
Integrin α	0.03 ± 0.02	1.26 ± 0.52	0.03
TGF-β	0.74 ± 0.25	1.21 ± 0.33	0.04
ALK-1	0.83 ± 0.31	1.10 ± 0.35	0.08

ALK-1: activin receptor-like kinase 1, CSH: chronic subdural hematoma, TGF-β: transforming growth factor β, VEGF: vascular endothelial growth factor.

Table 2 Immunohistological findings of neovascularization factors and computed tomography findings

Density		VEGF	Integrin α	TGF-β	ALK-1
Low	6	+	+	±	+
Iso	9	+	+	+	+
High	1	++	+	+	±
Type					
Homogenous	4	++	+	+	±
Heterogenous	12	+	+	+	+
Laminar	1	+	+	+	+
Segmental	1	+	+	±	+
Mix	10	+	+	+	+

±: 0–0.5, +: 0.5–1.0, ++: 1.0–1.5, ALK-1: activin receptor-like kinase 1, TGF-β: transforming growth factor β, VEGF: vascular endothelial growth factor.

degree of staining and the density/architecture of the hematomas on CT.

Discussion

CSH, considered as a circumscribed chronic inflammatory disorder, involves the dura mater.[14,15] Pathological delamination in restricted areas of the dura-arachnoid junction due to the circumscribed accumulation of extravasated blood or cerebrospinal fluid evokes local aseptic inflammatory and angiogenic reactions accompanied by the proliferation of dura mater border cells.[15] The precise mechanism of development of CSH is unclear. There are two different theories: angiogenesis on the outer membrane and osmotic theory. Osmotic theory is based on high osmotic pressure inducing fluid collection inside the hematoma membrane via semipermeable membrane.[16] Weir reported negative findings of osmotic theory for hematoma expansion and the precise machinery of hematoma growth is controversial.[17] We focused on only angiogenetic factors on CSH in this present study. Being a well-vascularized structure, the dura mater reacts by actively producing granulation tissue comprised of numerous newly-formed permeable blood vessels, inflammatory cells, and proliferating fibroblasts. This results in the formation of a capsule, also called a neomembrane, with a thicker outer and a thinner inner membrane.[14,18] While the importance of angiogenesis in vascular-rich networks with respect to the membrane and content fluid is known, the precise underlying mechanisms remain to be elucidated.

VEGF, a key regulator of vasculogenesis and angiogenesis, can be induced in response to environmental states and by the stimulation of cells including endothelial cells, fibroblasts, smooth muscle cells, macrophages, neutrophils, neurons, and glial cells.[8,19] Shono et al. reported expression of VEGF in the infiltrated cells into outer membrane of CSH for the first time to our knowledge.[4] Weigel et al. evaluated expressions of VEGF, fibroblast growth factor, and platelet derived growth factor in both hematoma and serum.[7] Nanko et al. focused on membraneous neovascularization and upstream of VEGF.[1] They observed a strong immunohistochemical expression of VEGF in various cells in the outer membrane and its high concentration in the fluid of CSH.[1] The upstream mediation of VEGF hyper-induction has been suggested to involve interleukin-6, tumor necrosis factor, and hypoxia-inducible factor-1α, however, the direct connection with VEGF and other angiogenic cascade reactions remains to be documented.[1,20]

We first document the immunohistochemical expression of TGF-β and of ALK-1 in the dura mater and outer membrane of CSH. We also show that the expression of TGF-β and ALK-1 was markedly stronger in the outer membrane than the dura mater. Our findings suggest that the TGF-β–ALK-1 pathway participates in angiogenic processes in CSH as do VEGF and integrins. Furthermore, the TGF-β–ALK-1 pathway may regulate VEGF upstream as was observed in the earlier *in vitro* trials.[10] There is no clear evidence about relationship between radiological findings of hematoma and natural history of CSH, such as tendency of recurrence and growth. We hypothesized that angiogenic factors might control vascularization or permeability of outer membrane and affect how hematoma accumulates. According to CT classification of CSH reported by Moskala M et al., immunoreactive aspects of angiogenic factors were compared to hematoma formation.[14] However, there was no significant relation between them. Biochemical findings of hematoma itself might be better target for relationship with membraneous expression of angiogenic factors rather than radiological characteristics. Further studies should be required to elucidate the association of angiogenic factors and natural history of CSH.

Our study has some limitations. We did not evaluate a direct relationship between TGF-β and ALK-1, and we did not analyze our data quantitatively. We also cannot confirm direct cross-talk between VEGF and the TGF-β–ALK-1 pathway. There is a limitation in the evaluation of co-relation of immunological data and clinical findings of CSH in this study. Especially, promotion of TGFβ-ALK signaling factors might affect recurrence of CSH. Further studies will be required to evaluate characteristic findings and clinical meanings of TGFβ-ALK expressions in CSH. Semi-quantitative method of scoring was performed focusing on the number of immune-positive cells, but could not exclude the effect of sensitivity of background completely. Another limitation was the number of objects. The number of 15 patients might be small to be evaluated for pathological study and more objects might be better for classification by CT imaging. Further study and accumulation of data will be required for evaluation of pathological and radiological findings. Consequently, our data do not provide direct evidence for the role of the TGF-β–ALK-1 pathway in VEGF-related angiogenesis involved in the progression of CSH. Our study focused on the role of the TGF-β–ALK-1 signaling pathway in angiogenesis of the outer membrane of CSH and we first document the significant expressions of these factors. Our findings may make it possible to identify new angiogenic mechanisms and may facilitate the development of new therapeutic strategies to control molecular mechanism involved in CSH progression. Additional studies are underway to elucidate the TGF-β–ALK-1 signaling pathway involved in CSH angiogenesis.

Conflicts of Interest Disclosure

The authors report no conflict of interest concerning the case report used in this study or the finding specified in this article.

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Address reprint requests to: Atsushi Saito, MD, PhD, Department of Neurosurgery, Aomori Prefectural Central Hospital, 2-1-1 Higashitsukurimichi, Aomori, Aomori 030-8553, Japan.
e-mail: satsushi2002@yahoo.co.jp