Involvement of Hypoxia-Inducible Factor-1α and Vascular Endothelial Growth Factor in the Mechanism of Development of Chronic Subdural Hematoma

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Abstract

Vascular endothelial growth factor (VEGF), a potent inducer of angiogenesis and vascular permeability in diverse physiological and pathological conditions, may be involved in the pathophysiology of chronic subdural hematoma (CSDH). The present study investigated the source and mechanisms for the induction of VEGF in CSDH by measuring the concentration of VEGF in the hematoma of 102 patients (122 hematomas) using the enzyme-linked immunosorbent assay technique. The relationship between the VEGF concentration in hematoma and the intrahematoma membranous structure confirmed by preoperative T2*-weighted magnetic resonance image was examined in 46 of these patients. VEGF and hypoxia-inducible factor-1α (HIF-1α) expression was immunohistochemically studied and microvessel density (MVD) in the outer membrane was identified using anti-CD31 antibody in 30 patients. VEGF and HIF-1α were positive in the outer membranes of all 30 patients. VEGF expression was significantly correlated to HIF-1α expression ($r = 0.651$, $p = 0.0084$) and VEGF concentration in the hematoma ($r = 0.654$, $p = 0.0013$). VEGF concentration in layered hematomas, which have intrahematoma membranous structure, was significantly higher than in non-layered hematomas ($p < 0.01$). Although MVDs of the outer membranes were comparable to those described in tumors, there was no significant relationship with VEGF expression. The present study suggests that VEGF in CSDH, which may be induced in the neomembrane by HIF-1 release, may give rise to the excessive development of fragile microvessels and hyperpermeability, resulting in the enlargement of CSDH.

Key words: chronic subdural hematoma, vascular endothelial growth factor, hypoxia-inducible factor-1α

Introduction

Chronic subdural hematoma (CSDH) is one of the most common entities seen in routine neurosurgical care, but the pathogenic principles underlying the development are not yet fully understood. CSDH is most prevalent in elderly patients, and usually occurs after trauma. The hematoma cavity is characteristically covered by a neomembrane, which can be differentiated into inner (visceral) and outer (parietal) membranes. The inner membrane consists of connective tissue containing few blood vessels, whereas the outer membrane contains large numbers of blood vessels. Ultrastructural investigation has clarified that microvessels in the outer membrane have endothelial fenestrations and loose gap junctions, partial lack of basal membrane and pericytes, and are fragile and permeable. On the other hand, CSDH is also associated with an imbalance of factors involved in the regulation of coagulation and fibrinolysis. The combination of fragile and permeable microvessels with coagulopathy is believed to be one of the causes of CSDH.

The pathogenesis of CSDH may be related to expression of vascular endothelial growth factor (VEGF) based on the observation of high concentrations of this cytokine in the fluid of CSDH. VEGF can induce angiogenesis and increase the
permeability of immature vessels in the outer membrane, because expression of VEGF is correlated with angiogenesis and vascular leakage in diverse physiological and pathological conditions.\textsuperscript{1,3,5} The involvement of VEGF in the pathophysiological mechanisms of CSDH is clear, but the source and upstream mediation of VEGF expression remain controversial.

Hypoxia-inducible factor (HIF)-1 is a heterodimeric transcriptional factor integral to the response to hypoxia. One of the important functions of HIF-1 is to promote angiogenesis through the regulation of VEGF transcription. HIF-1 consists of $\alpha$ and $\beta$ subunits, which both contain basic-helix-loop-helix motifs and per-ARNT-Sim domains, and are necessary for deoxyribonucleic acid binding and dimerization. The HIF-1/$\beta$ subunit is constitutively expressed, but HIF-1/$\alpha$ subunit expression, which determines HIF-1 activity, is regulated by oxygen. The HIF-1/$\alpha$ subunit is degraded by proteasomes, including proline-hydroxylase-2 and von Hippel-Lindau-ubiquitin ligase complexes, under normoxic conditions.\textsuperscript{20}

The present study investigated the source and mechanisms of VEGF induction in CSDH by measuring VEGF levels in the hematoma fluid and serum of patients with CSDH by the enzyme-linked immunosorbent assay (ELISA) technique, examining the relationship between VEGF concentration in the hematoma fluid and the intrahematoma membranous structure confirmed by preoperative T\textsubscript{2}* weighted magnetic resonance (MR) imaging, and analyzing the correlation between VEGF and HIF-1 expression in the outer membrane. Furthermore, any correlation between VEGF and microvessel density (MVD) was evaluated by measuring the MVD in the outer membrane using JC 70 monoclonal antibody, which binds to CD31 panendothelial antigen.

**Material and Methods**

This study included 102 consecutive patients with a total of 122 CSDHs, 62 men and 40 women aged from 27 to 91 years (mean 70.2 years), who received surgical treatment at our department in the last 3 years. The surgical procedures were burr-hole craniostomy for 76 cases and small craniotomy ranging from 40–50 mm in diameter for 46 cases, both with closed-system drainage. The choice of operative technique depended on the preference of each neurosurgeon. Informed consent was obtained from all patients or legal representatives to sample and analyze hematoma fluid, serum, and the outer membrane of the hematoma.

Gradient-echo T\textsubscript{2}* -weighted MR imaging (repetition time 550 msec, echo time 34 msec, flip angle 40°) was preoperatively performed in 46 patients using a 1.5-tesla MR system (Gyroscan ACS-II; Philips, Eindhoven, the Netherlands). These cases were retrospectively divided into two groups according to the intrahematoma membrane structure on T\textsubscript{2}* -weighted MR images, as described previously.\textsuperscript{16} The first group included 22 patients with non-layered subdural hematoma which had no intrahematoma membrane or had monolayer multilobuli. The second group included 24 patients with layered hematoma which was divided into multiple layers by the intrahematoma membrane (Fig. 1).

Samples of hematoma fluid and venous blood (10 ml) were obtained simultaneously using a serum separator tube and allowed to clot for 30 minutes. All samples were kept on ice and transferred to a laboratory for centrifugation (1000 g, 15 min, 4 °C). Supernatants of the hematoma and serum were removed and stored at −80 °C until assayed. The concentration of VEGF was measured using a sandwich enzyme immunoassay (Sandwich ELISA kit; R&D Systems, Minneapolis, Minn., U.S.A.) with monoclonal antibody against human VEGF. Serum was used undiluted, whereas hematoma fluid was diluted up to 1:100 with assay diluent reagent. Standard, control, or sample fluids (100 $\mu$l) were incubated in an antibody-coated well for 2 hours, then the unbound VEGF was washed off and the secondary polyclonal antibody conjugated to horseradish peroxidase (HRP) added for 2 hours. A 1:1 mixture of hydrogen peroxide and tetramethylbenzidine was used to visualize the presence of HRP as blue coloration. This reaction was stopped at the appropriate time by addition of 2N sulfuric acid, and the optical density of the resulting yellow color was determined using a microtiter plate reader set to 450 nm. Each sample was assayed in duplicate. A standard curve

![Fig. 1 Representative T\textsubscript{2}* -weighted magnetic resonance images of non-layered (left) and layered (right) chronic subdural hematomas.](image-url)
was generated and used to determine the actual VEGF concentration in individual samples with the background intensity subtracted.

Immunohistochemical studies of surgically resected outer membrane were performed in 30 cases. Specimens were fixed in 4% buffered formalin and embedded in paraffin. Sections 3 µm thick were stained with hematoxylin and eosin. Immunohistochemical staining was performed by the streptavidin-biotin-immunoperoxidase method (HISTOFINE immunostaining kit; Nichirei, Tokyo) or EnVision™ Systems (ENVISION+/HRP; Dako Denmark A/S, Glostrup, Denmark). The primary antibodies were anti-VEGF antibody (rabbit; Santa Cruz Biotechnology, Santa Cruz, Calif., U.S.A.) diluted 1/50, rabbit polyclonal anti-HIF-1α antibody (cat.#100-449; Novus Biologicals, Littleton, Colo., U.S.A.) diluted 1/25, and mouse anti-human CD31 antibody (M0823; Dako Denmark A/S) diluted 1/50. Subsequently, sections were deparaffinized by treatment in xylene for 15 minutes and rehydrated in graded ethanol series. Endogenous peroxidase activity was blocked using 0.3% hydrogen peroxide in methanol for 30 minutes. Sections were rinsed with phosphate-buffered saline and incubated with normal blocking serum for 60 minutes to minimize background staining. Next, the primary antibodies were applied and reacted overnight in a cold room. Peroxidase activity was visualized with diaminobenzidine, followed by counterstaining with hematoxylin. Negative control staining substituted rabbit or mouse serum for the primary antibodies. For double immunostaining to detect the VEGF-expressing cells, deparaffinized and rehydrated sections were immersed into preheated Target Retrieval Solution (diluted Dako S1699; Dako Denmark A/S) and microwaved for 30 minutes. After cooling to room temperature, sections were washed in Tris-Buffered NaCl Solution with Tween 20 (TBST) pH 7.6 (diluted Dako S3306; Dako Denmark A/S), blocked in Protein Block Serum-Free (X0909; Dako Denmark A/S), and incubated in rabbit anti-VEGF antibody (Santa Cruz Biotechnology) diluted 1/50 and mouse anti-human CD31 immunoglobulin G (IgG) diluted 1/50, mouse anti-human CD68 IgG (M0876; Dako Denmark A/S) diluted 1/100, or mouse anti-human smooth muscle actin IgG (M0851; Dako Denmark A/S) diluted 1/100 in Antibody Diluent Background Reducing (S3022; Dako Denmark A/S) overnight at 4°C in a humidified chamber. After washing in TBST, slides were incubated in goat anti-rabbit Alexa Fluor®546 antibodies and goat anti-mouse Alexa Fluor®488 antibodies from Molecular Probes (A11035 and A11029; Invitrogen, Carlsbad, Calif., U.S.A.), diluted 1/1000 in Antibody Diluent Background Reducing, for 1 hour at room temperature. After washing in TBST and phosphate-buffered saline, sections were mounted in ProLong Gold Antifade Reagent with DAPI (P36935; Invitrogen) in a dark box overnight at room temperature. Slides were examined under a confocal fluorescent microscope (Zeiss Pascal LSM 5; Carl Zeiss, Jena, Germany) equipped with 63 × objective lens and images were analyzed using the Zeiss LSM Image Browser.

Two investigators (M.F., H.T.) independently evaluated the immunohistochemistry staining. The immunohistochemical results for HIF-1α and VEGF were scored semi-quantitatively in five categories as follows: −, no positive staining; +, focal or diffuse weak staining; ++, focal moderate staining; ++++, diffuse moderate or focal strong staining; and +++++, diffuse strong staining. To assess the degree of angiogenesis in each specimen, microvessels were counted stained by JC 70 monoclonal antibody, which binds to CD31 panendothelial antigen (platelet-endothelial cell adhesion molecule). The 3 areas with the highest degree of vascularization in each specimen were identified at low power (×100), then the number of vessels in the selected fields was counted at high power (×400) with a computer image analyzer, ImageJ (http://rsb.info.nih.gov/ij/). The highest of these 3 vessel counts for each specimen was recorded as the MVD. Only vessels with a clearly defined lumen or well-defined linear vessel shape were counted as microvessels. Newly forming vessels that consisted of only one layer of endothelial cells were ignored.

Data were analyzed using Statview for Windows, version 4.54 (Abacus Concepts, Inc., Piscataway, N.J., U.S.A.). For intergroup comparison, continuous data were compared using the Mann-Whitney U-test. The Spearman rank correlation coefficient (rs) was used to look for statistical correlations of positive staining for HIF-1α in the outer membrane, positive staining for VEGF in the outer membrane, concentration of VEGF in the hematoma fluid, and MVD in the outer membrane. All continuous data are presented as means ± standard error of the mean, and a probability value less than 0.05 was considered significant.

Results

The concentration of VEGF was 289 ± 18 pg/ml in serum, and 11,739 ± 914 pg/ml in hematoma fluid, approximately 41 times higher (p < 0.01). No significant difference was found in mean VEGF concentration in hematoma fluid in the 11 cases of recurrent hematomas (11,887 ± 1055 pg/ml) compared with the 65 nonrecurrent hematomas (11,724
Table 1 Results of immunohistochemical studies of the outer membrane of chronic subdural hematoma

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HIF-1α: hypoxia-inducible factor-1α, MVD: microvascular density, VEGF: vascular endothelial growth factor, -: no positive staining, +: focal or diffuse weak staining, ++: focal moderate staining, +++: diffuse moderate or focal strong staining, ++++: diffuse strong staining.

± 962 pg/ml), all treated with burr-hole craniotomy. The concentration of VEGF was 7454 ± 895 pg/ml in the 22 patients with non-layered hematoma, and 17,105 ± 2427 pg/ml in the 24 patients with layered hematoma (p < 0.01).

Histological examination of the surgically resected outer membrane of 30 hematomas found the characteristic findings of subdural neomembranes in all cases, such as the presence of fibrous tissue, capillaries, and inflammatory cells (Table 1). Immunohistochemical studies disclosed strong positive staining for VEGF in these tissues, including endothelial cells, fibroblasts, smooth muscle cells, macrophages, and other inflammatory cells. Positive staining for VEGF was located in the cytoplasm (Fig. 3).
VEGF staining was positive in the outer membrane in all 30 cases, including 4 focal or diffuse weakly positive, 13 focal moderately positive, 11 diffuse moderately or focal strongly positive, and 2 diffuse strongly positive (Table 1). Furthermore, double staining for VEGF and CD68 (Fig. 2D), CD31 (Fig. 2E), or smooth muscle actin (Fig. 2F) revealed that VEGF was expressed in the smooth muscle cells, epithelial cells, and macrophages. Positive staining for HIF-1α was found in the nucleus and/or the cytoplasm in all 30 cases, including 3 focal or diffuse weakly positive, 8 focal moderately positive, 13 diffuse moderately or focal strongly positive, and 6 diffuse strongly positive (Fig. 3, Table 1). MVD of the outer hematoma membrane, determined by counting the number of CD31-positive microvessels in selected fields, was 7 to 47.7 (mean 33.7 ± 1.7) (Fig. 2, Table 1). Spearman's correlation analysis showed that expression of HIF-1α in the outer membrane was correlated with the expression of VEGF ($r_s = 0.651$, $p = 0.0084$), and expression of VEGF in the outer membrane was correlated with VEGF concentration in the hematoma fluid ($r_s = 0.654$, $p = 0.0013$). MVD in the outer hematoma membrane was not correlated with either the expression of VEGF in the outer membrane ($r_s = 0.273$, $p = 0.48$) or the concentration of VEGF in the hematoma fluid ($r_s = 0.037$, $p = 0.84$).

**Discussion**

VEGF can be induced in response to various environmental stimuli in various cells, including endothelial cells, fibroblasts, smooth muscle cells, macrophages, neutrophils, neurons, and glial cells. The strong expression of VEGF in the outer membrane of CSDH has been confirmed using immunohistochemical studies, reverse transcription-polymerase chain reaction (RT-PCR) analysis, and in situ hybridization. On the other hand, RT-PCR analysis revealed strong expression of VEGF in cells obtained from the hematoma fluid in comparison to the outer membrane, in which the level of VEGF messenger ribonucleic acid was conspicuously low. In the present study, immunohistochemical study clarified the strong expression of VEGF in various cells in the outer membrane of all 30 CSDHs. The expression of VEGF in the outer membrane significantly correlated with the concentration of this cytokine in the fluid of hematoma. Therefore, the extremely high concentration of VEGF in the fluid of CSDH can be considered to originate from the hematoma membrane. This conclusion may also be supported by our finding of significantly higher concentration of VEGF in layered than in non-layered hematomas, due to the high degree of membranous structure in layered hematomas, although histological confirmation was not obtained.

The upstream mediation of this hyperinduction of VEGF in CSDH has not been established. Inflammatory mechanisms like those in wound healing are thought to be involved in the induction of VEGF in CSDH, because they are considered to be related to the pathogenesis of CSDH. Furthermore, high concentrations of cytokines that can induce VEGF, such as interleukin-6 and tumor necrosis factor-α, were revealed in the fluid of hematoma. The most potent inducer for VEGF expression is considered to be hypoxia, which is induced by hypoxia, growth factors, and oncogenes, and HIF-1α, which is a heterodimeric transcription factor consisting of HIF-1α and HIF-1β subunits, and regulates VEGF expression at the transcriptional level. The present study found strong positive staining for VEGF in various cells in the outer membrane, including endothelial cells and fibroblasts as well as inflammatory cells, and strong positive staining for HIF-1α, showing a significant correlation between expression of VEGF and HIF-1α in the outer membrane of CSDH. These results suggest that HIF-1α is one of the main inducers of VEGF in CSDH. On the other hand, the expression of HIF-1α in the outer membrane of CSDH was rather diffuse and quite different from tumor tissue, which showed negative staining for HIF-1α in perivascular regions. The mechanism for hyperinduction of HIF-1α in the outer membrane of CSDH may be different from that in tumor tissue, and requires further study.

VEGF is a potent inducer for angiogenesis. Therefore, we examined the MVD in the outer membrane using immunostaining with anti-CD31 antibody. However, no significant correlation was found between MVD in the outer membrane and either VEGF expression in the outer membrane or VEGF concentration in the hematoma fluid. On the other hand, MVD in the outer membrane of CSDHs was similar to those described in tumors. Therefore, the outer membrane of CSDH may be saturated with microvessels promoted by the extremely high levels of VEGF in the hematoma fluid and membrane. Such new vessels, with loose junctions between adjacent endothelial cells and the partial absence of basal membrane and pericytes, are abnormally permeable and fragile. VEGF can also induce excessive vascular permeability. Therefore, bleeding and leakage from those vessels can occur easily, and result in the enlargement of CSDH.

In conclusion, the extremely high levels of VEGF

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in the fluid of CSDH, thought to be related to the pathophysiology of this lesion,11,12,13,14,17,18 may originate in the neomembrane of hematoma, and VEGF in CSDH may be induced by release of HIF-1. Furthermore, excessive development of fragile microvessels in the outer membrane of hematoma, probably induced by VEGF in the hematoma fluid and membrane, can easily suffer bleeding and leakage, especially in the presence of VEGF, and result in the enlargement of CSDH.

References


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Commentary

Nanko and collaborators investigated 122 patients with chronic subdural hematoma. They found evidence for high expression of vascular endothelial growth factor (VEGF) in the outer membrane of chronic subdural hematoma. VEGF is known to be related to angiogenesis and increased permeability of vessels. Thus the authors have possibly identified a plausible explanation for the phenomenon of spontaneous enlargement of chronic subdural hematomas.

The authors are to be congratulated upon their findings. Obviously we all still do not know why this mysterious enlargement over weeks and months is reversed by a temporary drainage of a few days,
which leads to spontaneous resolution of the hematoma long after the drainage has been removed. Some years ago looking at histological findings of membranes we had found evidence for the production of red blood cells in the membranes of chronic subdural hematomas.1) Possibly a number of different unresolved mechanisms contribute to the phenomenon of chronic subdural hematomas and the working group of Nanko et al. are to be credited for their achievements.

Reference


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Chronic subdural hematoma (CSDH) is one of the common diseases in neurosurgical practice. However, some studies have investigated the formation of CSDH and why it become larger and larger in size, but till now the mechanism was not fully understood. Therefore, investigating and appreciating the fundamental mechanism is essential for neurosurgeons to treat patients with CSDH.

This study investigated the source and mechanisms for the induction of VEGF involved in CSDH. The relationship between the concentration of VEGF in hematoma fluid and venous blood and two kinds of CSDH classified by preoperative T2-weighted MR imaging was scrutinized. VEGF and HIF-1α were studied by immunohistochemical technique, and microvessel density was also examined using JC70 monoclonal antibody. The authors present this study to demonstrate that HIF-1α would induce the expression of VEGF in CSDH, and could attribute to the excessive formation of fragile microvessels and hyperpermeability finally leading to the progression of CSDH. The authors suggest that HIF-1α is one of the main inducers of VEGF in CSDH. But the authors do not explain why HIF-1α shows high expression on the outer membrane of CSDH. What is the mechanism? In order to further support the hypothesis “VEGF is related to the pathophysiology of CSDH, and may originate in the neomembrane of hematoma, and VEGF in CSDH may be induced by release of HIF-1,” a further study must investigate whether the expression of HIF-1 (up- or down-regulation) affects the progression of CSDH.

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