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Original Article

Expression of thioredoxin-1 and hypoxia inducible factor-1α in cerebral arteriovenous malformations: Possible role of redox regulatory factor in neoangiogenic property

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Abstract

Background: Recently it is reported that proliferative activity remains in vascular walls of cerebral arteriovenous malformations (AVMs). These reports indicate that endothelial cells in AVMs have the neoangiogenic property. In this study, we assess the role of thioredoxin-1 (Trx-1) and hypoxia-inducible factor 1a (HIF-1α) in AVMs. These factors are reported to play a role in neoangiogenesis.

Methods: We analyzed the expressions of Trx1 in the specimens of human cerebral AVMs. In addition, we also analyzed the expression of HIF-1α in these specimens by immunohistochemical method and RT-PCR. Furthermore, we assessed the effect of redox state and expression of Trx-1 during neoangiogenesis using *in vitro* angiogenesis assay.

Findings: Trx-1 and HIF-1α immunoreactivity was detected in almost all 17 specimens of AVMs. Trx-1 and HIF-1α immunoreactive cells were distributed mainly endothelium of intranidal arteries and enlarged veins with thickened vascular walls. Double staining shows that Trx-1 and VEGF (vascular endothelial growth factor) immunoreactivity were colocalized in the same cells. These cells were considered to be endothelial cells. HIF-1α immunoreactivity was also colocalized with VEGF immunoreactivity in endothelium. As for influencing factors, the presence of deep drainers and convulsion significantly associated with HIF-1α expression. Trx-1 assessed by western blotting decreased at 6 hours and 12 hours after plating on Matrigel, which is a model of angiogenesis.

Conclusions: We have shown that the endothelial induction of Trx-1 and HIF-1α in cerebral AVMs. Based on all findings obtained in this study, Trx-1 may affect the neoangiogenic property of cerebral AVMs.

Key Words: Angiogenesis, cerebral arteriovenous malformations, HIF-1α, Trx-1

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INTRODUCTION

Cerebral arterievenous malformations (AVMs) are tangled masses of abnormal arteries and veins that are directly connected without the interposition of capillaries.[3] Although they are a well-known vascular malformation, abnormalities in their molecular expression have not been fully clarified.[5] Some authors have indicated that AVMs have poorly differentiated and immature vessels.[5,14] Recently, it is reported that proliferative activity remains in vascular walls of AVMs in several reports.[6,15,17,18] In addition, the cases about recurrence of AVMs were shown.[8] These reports indicate that endothelial cells in AVMs have the neoangiogenic property.

Thioredoxin-1 (-) plays an important role in cellular processes via reduction/oxidation (redox regulation). The two-cysteine residues in its active sites undergo reversible redox reactions catalyzed by an NADPH-dependent enzyme, Trx-1 reductase.[1] Trx1 modulates the activities of various transcription factors by reducing families of proteins through modulation of their redox status. Cellular proliferation and differentiation require tightly coordinated control of gene expression. Cellular redox status plays an important role on the control of gene expression. Alteration of the cellular redox status can lead to change in DNA biding and transactivating activities of many transcription factors, and thus lead to alterations in gene expression which, in turn, may affect cellular behavior and adaptive responses.[4] Redox changes have been shown to regulate several eukaryotic transcription factors, both *in vitro* and *in vivo*. For example, oncogenes c-Jun and c-Fos, signal transducer NF-κB, c-Myb, and Egr-1 have all been shown to be regulated by redox mechanisms.[7,11,12]

In this study, we analyzed the expressions of Trx-1 in the specimens of human cerebral arteriovenous malformations. In addition, we also analyzed the expression of hypoxia inducible factor 1α (HIF-1α) in these specimens. The transcriptional activator complex HIF-1 is a key regulator of oxygen homeostasis and mediates many cellular and physiological responses necessary to adapt to changes in oxygen tension. HIF-1 has been implicated in many important aspects of tumor behavior, including increased angiogenesis, metastasis, and inhibited apoptosis.[23] Furthermore, we assessed the effect of redox state and expression of Trx-1 during neoangiogenesis using *in vitro* angiogenesis assay.

MATERIAL AND METHODS

Patients

Seventeen patients who underwent surgical procedures for treatment of standard indications of cerebral AVM at Kyoto University Hospital (Kyoto, Japan) were included in this study. Specimens were obtained from the patients during the surgical procedures. Clinical data on the patients are summarized in Table 1.

Immunohistochemistry

All specimens were fixed in 10% formalin overnight and

Table 1: Clinical summary of cases and results of immunohistochemical study

Case	Age/sex	Location	Onset	S-M grade	Neurological deficit	Preoperative embolization	Convulsion	Deep drainer	Trx-1	HIF-1α
1	27/F	Parietal	Headache	II	(-)	(+)	(-)	(-)	1	1
2	54/M	Occipital	Hemorrhage	II	Hemianopsia	(-)	(-)	(-)	1	1
3	23/M	Frontal	Hemorrhage	II	(-)	(-)	(-)	(-)	1	0
4	24/F	Parietal	Convulsion	III	(-)	(-)	(+)	(-)	1	1
5	47/M	Hippocampus	Convulsion	II	(-)	(-)	(+)	(+)	2	2
6	29/M	Temporal	Hemorrhage	I	(-)	(-)	(-)	(+)	3	2
7	40/M	Frontal	Headache	III	(-)	(-)	(-)	(+)	2	3
8	39/M	Frontal	Hemorrhage	I	(-)	(-)	(-)	(-)	2	3
9	52/M	Frontal	Steal	II	Sensory Disturbance	(-)	(-)	(+)	1	2
10	21/F	Temporal	Hemorrhage	I	Hemianopsia, Hemiparesis	(-)	(-)	(-)	3	1
11	23/M	Frontal	Hemorrhage	I	(-)	(-)	(-)	(-)	2	2
12	23/F	Parietal	Hemorrhage	I	Hemiparesis	(-)	(-)	(-)	1	1
13	31/M	Parietal	Hemorrhage	II	(-)	(-)	(-)	(-)	2	2
14	38/F	Occipital	Steal	I	Hemianopsia	(-)	(-)	(-)	2	2
15	45/M	Frontal	Convulsion	II	(-)	(-)	(+)	(-)	2	1
16	28/F	Parietal	Hemorrhage	II	(-)	(-)	(-)	(-)	2	1
17	28/F	Parietal	Hemorrhage	I	Hemianopsia	(-)	(-)	(-)	2	1

M: Male, F: Female, S-M grade: Spetzler and Martin grade,.Trx-1: Thioredoxin-1, HIF-1α: Hypoxia inducing factor 1α

then embedded in paraffin the next day. The specimens were stored at room temperature. In each case, multiple, sequential, 6-μm-thick tissue sections cut from paraffin blocks were deparaffinized in xylene, rehydrated, and prepared for immunohistochemical studies. Specimens with no perinidal tissue, those with destroyed, shredded brain parenchyma, and contaminated specimens were excluded. On that basis, we selected 17 specimens in which there was enough circulated brain parenchyma in good condition for light microscopy studies. The intranidal brain parenchyma was totally avoided when examining the slides.

The sections were washed for 5 minutes with 0.01 mol/L phosphate-buffered saline (PBS) (pH 7.2), followed by a 15-minute incubation with 10 mg/ml proteinase K. After having been blocked with 3% H2O2, the sections were preincubated with normal goat serum (diluted 1:50) and then incubated overnight at 4 degree C with the primary antibody. After three rinses with PBS, 15 minutes for each, the sections were incubated for 30 minutes with anti-rabbit or anti-mouse Envision/horseradish peroxidase (Dako, Glostrup, Denmark). After three more rinses with PBS, the sections were developed for color for 5 minutes at room temperature in a substrate medium containing 0.05% 3,3-diaminobenzidine and 0.02% H2O2 in Tris-HCl buffer (pH 7.6). The specificity of the staining was confirmed by the absence of specific staining when nonimmune rabbit IgG substituted for the primary antibody.

Antibodies

Anti-human Trx-1 (Redox Bio, Kyoto, Japan), anti-HIF-1α (NeoMarkers, Fremont, CA), and anti-VEGF, (NeoMarkers, Fremont, CA) antibodies were used in this study. As secondary antibodies, FITC-conjugated anti-rabbit IgG and Cy3-conjugated anti-mouse IgG (1:100; Jackson Immunoresearch, West Grove, PA) were used.

Double staining

Sections were washed for 5 minutes with 0.01 mol/L PBS (pH 7.2) and then incubated for a 15 minutes with 10 mg/ml proteinase K. They were next preincubated with 4% skim milk and were then incubated overnight at 4 0C with the primary antibodies. After three rinses with PBS, 15 minutes for each, the sections were incubated for 30 minutes with secondary antibodies. Following three additional rinses with PBS, the sections were incubated with DAPI (1:10000; Jackson Immunoresearch, West Grove, PA) for nuclear staining and were analyzed under a BX51 microscope (Olympus Optical Co., Tokyo, Japan)

RT-PCR

From frozen AVM specimens, total RNA was isolated using the RNeasy Fibrous Tissue Mini Kit (Qiagen). Extraction was performed according to manufacturer directions. A total of 50 ng of total RNA was converted to cDNA using Sensiscript reverse transcriptase (Qiagen). The conditions for the cDNA synthesis were: 60 minutes at 37°C followed by heating at 93°C for 5 minutes. A total of 2 μL of the samples was used in polymerase chain reaction (PCR) using HotStarTaq polymerase (Qiagen). PCR was started at 94°C for 15 minutes, followed by 45 cycles (94°C for 30 s, 55°C for 30 s, and 72°C for 60 s), ended with a 10-minute incubation at 72°C for Trx-1. In case of HIF-1a, cycles were 45 (95°C for 20 s, 62°C for 60 s, and 72°C for 60 s), VEGF were 40 cycles (94°C for 60 s, 60°C for 60 s, and 72°C for 60 s), ß-actin were 40 cycles (94°C for 30 s, 53°C for 30 s, and 72°C for 60 s). Primers were as follows: Trxe-1, forward 5'-GGATCCATTTCCATCGGTCCTTAC-3' and reverse 5'-GTGGTAATTACTTAATCAGATT-3', HIF-1a, forward 5'-TTACCATGCCCCAGATTCAG-3' and reverse 5'-CTTGCGGAACTGCTTTCTAATG-3', VEGF, forward 5'-TCGGGCCTCCGAAACCATG-3' and reverse 5'-GGTTCCCGAAACCCTGAGG-3', ß-actin, forward 5'-AAGATGACCCAGATCATGTTTGAG-3' and reverse 5'-AGGAGGAGCAATGATCTTGATCTT-3'. PCR products were separated by electrophoresis in 2% agarose gels.

In vitro angiogenesis assay

A 24-well tissue culture plate was prechilled at –20°C and carefully coated with Matrigel (100 μL/well; Becton Dickinson, Bedford, MA) avoiding bubbles. The plate was incubated at 37°C for 30 minutes to allow the Matrigel to solidify. F2 endothelial cells (30,000 per well) were suspended in 500 μL DMEM containing 5% FBS and gently added to the Matrigel-coated wells. Cells were untreated or treated with 2-mercaptoethanol (2-ME) or dithiothreitol (DTT). After indicated hours, the medium was removed and cells were fixed with cold 4% paraformaldehyde (PFA). Images were captured under phase contrast microscopy at 20× magnification and tube length was assessed by drawing a line along each tubule and measuring the length of the line in pixels then calibrating with a micrometer present in the image. Tube length was measured in five nonoverlapping fields under 20× magnification.

Western blotting

The cells were homogenized and then lysed for 30 min with 1 ml of a solubilizing solution (0.5% NP-40/10 mM Tris·HCl, pH 7.2/150 mM NaCl/1 mM PMSF/0.111 units/ml aprotinin) on ice. The extracts were cleared by centrifugation. Equal amounts of protein (10 μg protein/lane), estimated by the Bradford method using a protein assay (Bio-Rad), were electrophoresed on a 15% or 10% SDS-polyacrylamide gel, and then electrophoretically transferred to a poly(vinylidene difluoride) membrane (Millipore). After blocking with 5% BSA in PBS containing 0.05% Tween 20 at 4°C overnight, the membrane was incubated with the first antibody, and then with the peroxidase-linked second antibody (Amersham Pharmacia). Chemiluminescence was detected with an

ECL Western blot detection kit (Amersham Pharmacia) according to the manufacturer's recommendation.

Semiquantitative immunohistochemical analysis

The immunoreactivity was semiquantitatively scored by two observers (Y.T., K.K.) in a blind manner as follows: 0, no staining; 1, faint staining; 2, moderate staining; 3, intense staining. The mean score from both observers was recorded.

Statistical analysis

Mann–Whitney and Fisher's exact tests were used for statistical analysis (StatView; SAS Institute, Cary, NC). $P < 0.05$ was considered statistically significant.

RESULTS

Expression of Trx-1 in cerebral AVMs

Trx-1 immunoreactivity was detected all 17 specimens of AVMs [Table 1]. Trx-1 immunoreactive cells were distributed mainly endothelium of intranidal arteries and enlarged veins with thickened vascular walls. A small number of neurons also immunoreactive for Trx-1 [Figure 1a-c]. Normal pial arteries and veins far from the nidus were negative for Trx-1 [Figure 1d, e].

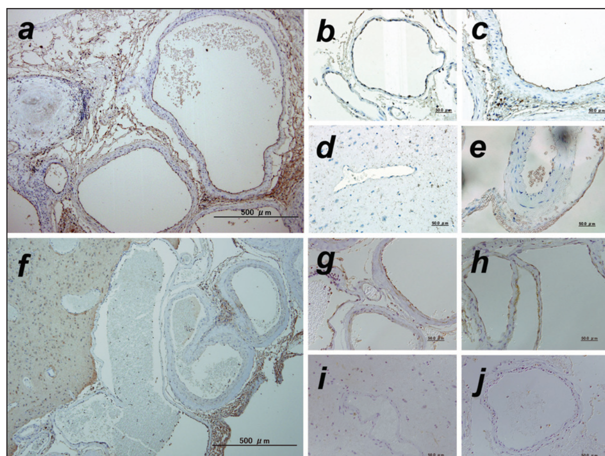

Figure 1: Expression of thioredoxin-1 (Trx-1) and hypoxia-inducible factor-1α (HIF-1α) in cerebral arteriovenous malformations. (a) Trx-1 immunoreactivity was elevated in endothelial cells and perivascular tissue in arteriovenous malformations. (b) Venous component of arteriovenous malformations. Trx-1 immunoreactivity was elevated in endothelial cells (c) Arterial component of arteriovenous malformations. Trx-1 immunoreactivity was also elevated in endothelial cells. (d) Normal small vein in perinidal area. Trx-1 immunoreactivity was hardly detected. (e) Normal small artery in perinidal area. Trx-1 immunoreactivity was also hardly detected. (f) HIF-1α immunoreactivity was elevated in endothelial cells and perivascular tissue in arteriovenous malformations. (g) Arterial component of arteriovenous malformations. HIF-1α immunoreactivity was elevated in endothelial cells (h) Venous component of arteriovenous malformations. HIF-1α immunoreactivity was also elevated in endothelial cells. (i) Normal small vein in perinidal area. HIF-1α immunoreactivity was hardly detected. (j) Normal small artery in perinidal area. HIF-1α immunoreactivity was also hardly detected

Expression of HIF-1α in cerebral AVMs

HIF-1α immunoreactivity was also detected 16 specimens of AVMs [Table 1]. HIF-1α immunoreactive cells were mainly detected in the endothelium of intranidal arteries and veins. The distribution of HIF-1α immunoreactivity in vascular walls was similar with that of Trx-1. Degenerating and a small number of perinidal neurons also immunoreactive for HIF-1α [Figure 1f-h]. HIF-1α immunoreactivities were not shown in normal pial arteries and veins far from the nidus [Figure 1i, j].

Double staining for Trx-1, HIF-1α and vascular endothelial growth factor

As shown by immunohistochmical method, Trx-1 and HIF-1α are expressed in the similar region of vessels in cerebral AVMs. Next, we assessed Trx-1 and HIF-1α expression in the relationship with vascular endothelial growth factor (VEGF), which is a one of the main anigiogenic factors expressed in AVMs. Double staining shows that Trx-1 and VEGF immunoreactivity were colocalized in the same cells [Figure 2a-d]. These cells were considered to be endothelial cells. HIF-1α immunoreactivity was also colocalized with VEGF immunoreactivity in endothelium [Figure 2e-h].

The factors influencing Trx-1 and HIF-1α expression

We assessed the factors which affected the expressions of Trx-1 and HIF-1α expression. As shown in Table 2, the presence of deep drainers and age significantly associated with HIF-1α expression. All other factors did not significantly affect the expressions of Trx-1 and HIF-1α.

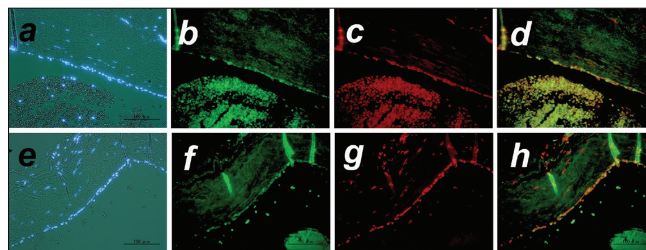

Figure 2: Double staining analysis for Trx-1, HIF-1α and vascular endothelial growth factor in cerebral arteriovenous malformations (a) nuclear staining. (b) Trx-1 immunoreactivity (green) is shown in vascular walls of arteriovenous malformations. It is mainly detected in the endothelial layer and media. (c) Vascular endothelial growth factor (red) immunoreactive cells are shown in the endothelial lyer of arteriovenous malformations. (d) Double staining is shown. Trx-1-positive cells in the endothelial cells are also positive for vascular endothelial growth factor (yellow, arrows indicated double-labeled cells). (e) nuclear staining (f) HIF-1α immunoreactivity (green) is shown in vascular walls of arteriovenous malformations. It is mainly detected in the endothelial layer and media. (g) Vascular endothelial growth factor (red) immunoreactive cells are shown in the endothelial layer of arteriovenous malformations. (h) Double staining is shown. Trx-1-positive cells in the endothelial cells are also positive for vascular endothelial growth factor (yellow, arrows indicated double-labeled cells)

RT-PCR analysis for Trx-1, HIF-1α and VEGF

To confirm the quantitative expression of Trx-1, HIF-1α, and VEGF, we performed RT-PCR for these mRNAs. As shown by Figure 3, these mRNAs were detected from AVM samples [Figure 3a]. Densitmetoric analysis indicated the levels of expression [Figure 3b].

Trx-1 expression and the effect of cellular redox state in *in vitro* angiogenesis assay

Matrigel is a basement membrane complex and promote endothelial differentiation. Endothelial cells cultured on Matrigel forms cord-like structure which is a model of angiogenesis. We assessed the expression of Trx-1 during angiogenesis by western blotting. Trx-1 decreased at 6 hours and 12 hours after plating on Matrigel [Figure 4a]. Next, we analyzed the effect of reducing agents on *in vitro* angiogenesis. DTT and 2-ME, which are strong reducing agents, promoted *in vitro* angiogenesis in a dose-dependent manner [Figure 4b, c].

Table 2: Clinical characteristics affecting Trx-1 and HIF-1a expression

		Trx-1	HIF-1α
Hemorrhage	Yes	2.0 ± 0.8	1.4 ± 0.8
	No	1.6 ± 0.5	1.7 ± 0.8
Deep drainer	Yes	2.0 ± 0.8	2.3 ± 0.5*
	No	1.7 ± 0.6	1.3 ± 0.8
>30 y.o.	Yes	1.8 ± 0.8	1.1 ± 0.6*
	No	1.6 ± 0.5	2.0 ± 0.8
Convulsion	Yes	1.7 ± 0.6	1.3 ± 0.6
	No	1.8 ± 0.7	1.6 ± 0.9
S-M grade (>II)	Yes	1.5 ± 0.5	1.4 ± 0.8
	No	2.1 ± 0.7	1.7 ± 0.8

Trx-1: Thioredoxin-1, HIF-1α: Hypoxia inducing factor-1α, y.o.: Years old, S-M grade: Spetzler and Martin grade, *$P<0.05$

DISCUSSION

In this study, we showed the expression of Trx-1 and HIF-1α was elevated in endothelial cells of cerebral AVMs. HIF-1α expression was significantly associated with the presence of deep drainers and age. These expressions were confirmed by RT-PCR. The expression of Trx-1 and HIF-1α were co-localized with VEGF. In addition, we assessed the effect of redox state in angiogenesis using *in vitro* angiogenesis assay. Cell redox state modified by Trx may affect *in vitro* angiogenesis.

Recently, the recurrence of AVMs was reported.[8] In addition, several reports indicate that the vascular walls of AVMs have neoangiogenic property.[6,15,18,20] Among the factors promoting angiogenesis, VEGF was reported to play a major role in the vascular walls of AVMs.[13] Sonstein *et al.*, demonstrated a high degree of astrocytic VEGF expression in four of four specimens with recurrent AVMs.[13] Uranishi *et al.*,[21] showed that the proportion of immunopositive vessels to VEGF receptors Flk-1 and Flt-1 was significantly greater in AVMs than in the control brain tissue samples. We also showed the expression of VEGF in AVMs. In addition, we analyzed the upstream molecules of angiogenesis.[18]

In this study, we focused on the expression of HIF-1α in AVMs. The increase in secreted biologically active VEGF from cells exposed to hypoxia is partly due to increased transcription of HIF-1 to a hypoxia responsive element (HRE) in the 5′-flanking region of the VEGF gene. HIF-1 is a dimeric transcription factor composed of HIF-1α

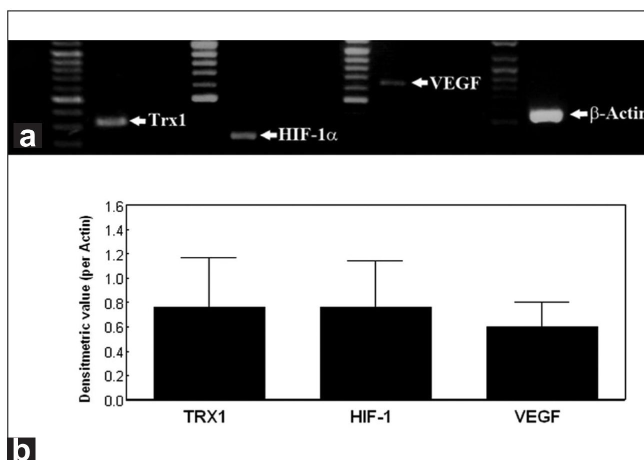

Figure 3: RT-PCR analysis for expression of Trx-1, HIF-1α, and VEGF in cerebral arteriovenous malformations (a) The expression of Trx-1, HIF-1α, vascular endothelial growth factor, and β-actin were shown as single band assessed by RT-PCR. (b) Densitmetric values of Trx-1, HIF-1α and vascular endothelial growth factor are shown. All values are the mean of three separate experiments and bars are SD

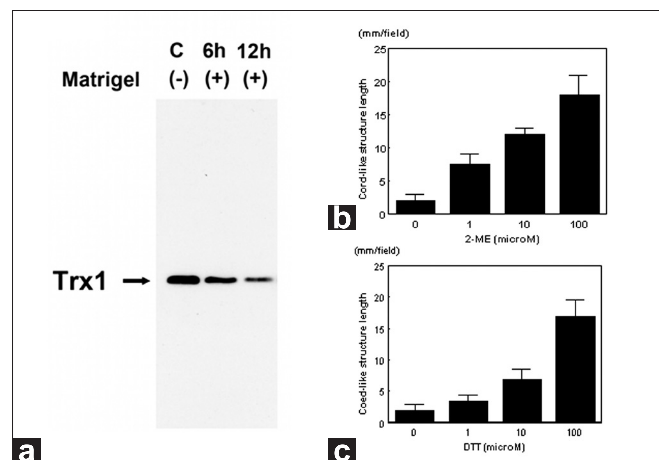

Figure 4: Western blot analysis for Trx-1 during in vitro angiogenesis and the effect of reducing agents on *in vitro* angiogenesis. (a) The level of Trx-1 assessed by western blot is decreased during differentiation on Matrigel. (b) The effect of 2-mercaptoethanol (2-ME) on cord-like structure length assessed on Matrigel. (c) The effect of dithiothreitol on cord-like structure length assessed on Matrigel

and HIF-1β subunits.[23] Under normoxia, the HIF-1α subunit is subjected to ubiquitination and proteosomal degradation. In response to hypoxia, ubiquitination and degradation is inhibited resulting in accumulation of the HIF-1α protein. In addition, the activity of the HIF-1α transactivation domains is induced by hypoxia.[23] HIF-1α dimerizes with HIF-1β which is constitutively expressed, resulting in the formation of active HIF-1 protein that binds to the core sequence 5′-RCGTG-3′ within HREs that are present in target genes, including VEGF and its receptor Flt-1.[23] Two reports indicated that HIF-1α was overexpressed in cerebral AVMs.[13,16] Sure *et al*.,[16] showed that HIF-1α expression was significantly higher in AVMs treated with embolization before surgery than in patients who did not have previous endovascular treatment. In our study, only one patient received preoperative embolization. Thus, we think that preoperative embolization was not major cause of HIF-1α expression. Ng *et al*.,[13] suggested that hematoma or steal phenomenon induced HIF-1α upregulation. They did not find the significant association between the presence of hematoma and HIF-1α expression. In our study, we also did not indicate significant association.

The major finding obtained in this study was the Trx-1 elevation in the endothelial cells of AVMs. Trx-1 is a small redox protein that undergoes reversible NADPH-dependent reduction by selenocysteine containing flavoprotein Trx-1 reductases.[1] Trx-1, thorough its redox activity, regulates the activity of various enzymes. Trx-1 is a potent cell growth factor. Trx-1 expression is increased in several human primary cancers, including lung, colon, cervix, liver, pancreatic, colorectal, and squamous cell cancer and has been linked to aggressive tumor growth and inhibited apoptosis.[1,2,19] Trx-1 was also reported to regulate HIF-1α expression via its redox property. Stable transfection of Trx-1 caused a significant increase in HIF-1α protein levels.[22] Trx-1 increased hypoxia-induced HIF-1 transactivation activity measured using luciferase reporter under the control of the HRE. Trx-1 also caused a significant increase in the products of hypoxia-responsive genes, including VEGF. Trx-1 was induced under hypoxic condition.[9,22] Trx-1 was also elevated after ischemia and ischemia-reperfusion.[17] Our results may indicate that trx-1 overexpression is one of the major origins of HIF-1α elevation in endothelial cells of AVMs.

Finally, we assess the role of redox factor in neoangiogenesis using *in vitro* angiogenesis assay. Trx-1 protein level was decreased during angiogenesis assessed by Matrigel. This result indicates that undifferentiated stage of endothelial cell overexpressed Trx-1. Farina *et al*., also showed that endothelial cells cultured on Matrigel did not differentiate and make tube-like formation on the presence of Trx-1.[2] Other redox chemical agents such as 2-ME and DTT did not show this effect. On the contrary, they promoted *in vitro* angiogenesis in a dose-dependent manner. This finding may show that the effect of Trx-1 did not depend on its only reducing activity.

In this study, we used subjective scoring method in this study. To overcome this weak point, we analyzed mRNA expressions by RT-PCR. As described above, previous biochemical studies indicated that HIF-1α is regulated by redox state. Trx-1 is a major molecule ruling cellular redox state. Thus, we studied the expression of Trx-1 and HIF-α. In addition, we could not use normal brain tissue obtained from epilepsy surgery of brain tumor surgery as alternative negative control. Perinidal tissue may be affected by nidal circumstances. This point is one of the limitations of our study. We showed that age significantly was associated with HIF-1α expression. According to previous studies, HIF-1α plays an modulator of aging.[10,24] The result obtained in this study may be influenced by aging effect.

In summary, we have shown that endothelial induction of Trx-1 and HIF-1α of cerebral AVMs. Considering all findings obtained in this study indicate that trx-1 may affect the neoangiogenic property of cerebral AVMs.

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