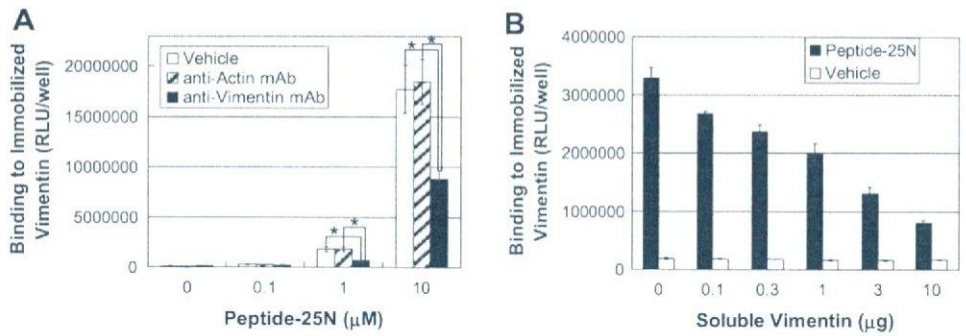


Fig. 3. Specific binding of Peptide-25N to immobilized vimentin protein. (A) Binding of Peptide-25N to immobilized vimentin protein was verified by adding anti-vimentin mAb clone VIM3B, unrelated anti-α-actin mAb clone ASM-1 or vehicle. The data are shown as the mean and S.D. from four samples. $^{*}P < 0.005$. (B) Binding of Peptide-25N to immobilized vimentin protein was investigated by adding soluble vimentin. Background of the system is also shown. The data are shown as the mean and S.D. from four samples.

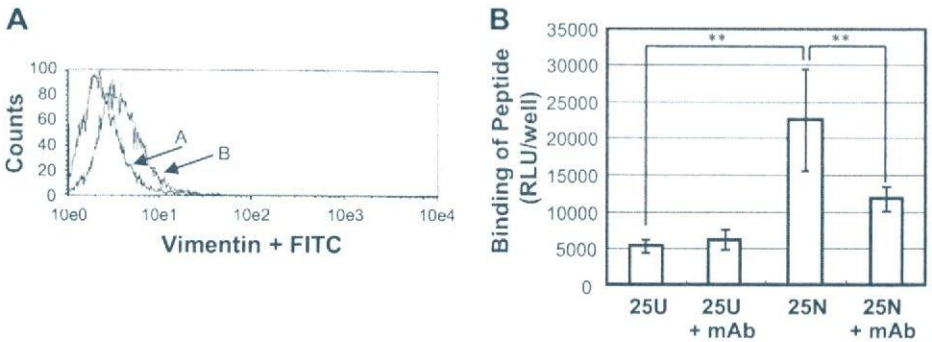


Fig. 4. Contribution of vimentin in binding of Peptide-25 to cell surface of BAECs. (A) The expression of vimentin on cell surface of BAECs was analyzed by FACS. A, Mouse IgG2a as a negative control; B, anti-vimentin mAb (VIM3B4). The intensity of VIM3B4 (B) was significantly higher than that of control (A) ($P < 0.001$). (B) Binding of biotin-labeled (25N) or unlabeled (25U) peptide to BAECs was measured as described in Section 2. In some experiments, anti-vimentin mAb (VIM3B4) was added. The data are shown as the mean and S.D. of eight samples. $^{**}P < 0.01$.

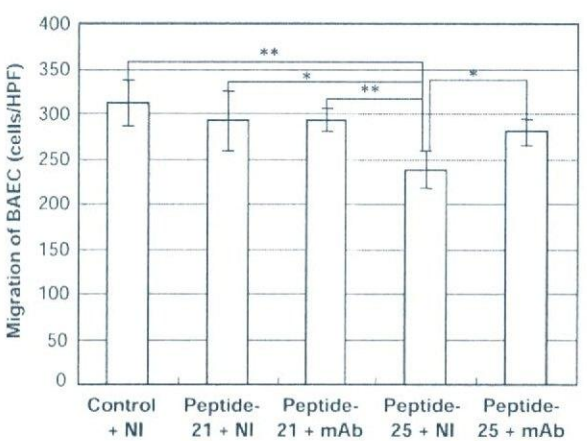


Fig. 5. Contribution of vimentin in LTGF-β activation by Peptide-25. To detect activity of TGF-β, migration assay was performed in the presence of Peptide-25 or Peptide-21 (negative control) as described in Section 2. In some experiments anti-vimentin mAb or mouse IgG1 as a negative control was added. The data are shown as the mean and SD of four samples. $P < 0.05$ and $^{**}P < 0.01$.

Glu113 to Arg131 in the LAP molecule) could not augment LTGF-β binding to cells and activation [9]. Therefore, we concluded that vimentin is involved in the latent TGF-β activation by Peptide-25.

4. Discussion

Vimentin is one of the cytoskeletal proteins classified as an intermediate filament. However, vimentin is secreted by activated macrophages in response to inflammatory signals for efficient killing of microorganisms [13], suggesting the involvement of vimentin in immune function. Macrophages are thought to be the principle source of TGF-β during inflammatory response [14] and active TGF-β is thought to be involved in various inflamed diseases such as rheumatoid arthritis [15]. One of the mechanisms of LTGF-β activation is partial enzymatic cleavage or degradation of LTGF-β by such as plasmin, matrix metalloprotease, and calpain [5,6,8] and the inflamed diseases show the elevated activity of those cell-associated proteases [5,16]. We speculate the mechanism of LTGF-β activation by the LAP fragments as following: (1) degradation of LAP by elevated proteolysis on cell surface generates LAP fragments, (2) active TGF-β is released from latent complex and exerts its activities, (3) the LAP fragments bind to vimentin on cell surface, (4) binding of LTGF-β to the cell surface is augmented by the LAP fragment, and (5) the additional activation occurs. To our knowledge, this is the first report indicating that vimentin is involved in the LTGF-β activation mechanism. The precise mechanisms remain to be elucidated how the LAP fragments and vimentin can work together to draw the LTGF-β complex to the cell surface. It is critical to control the excessive activation of LTGF-β for the treatment. We expect to develop a new type of TGF-β inhibitors through the study of activation of LTGF-β.

References

[1] Annes, J.P., Munger, J.S. and Rifkin, D.B. (2003) Making sense of latent TGFβ activation. J. Cell Sci. 116, 217–224.

[2] Ahamed, J., Burg, N., Yoshinaga, K., Janczak, C.A., Rifkin, D.B. and Coller, B.S. (2008) In vitro and in vivo evidence for shear-induced activation of latent transforming growth factor-β1. Blood 112, 3650–3660.

[3] Young, G.D. and Murphy-Ullrich, J.E. (2004) The tryptophan-rich motifs of the thrombospondin type 1 repeats bind VLAL motifs in the latent transforming growth factor-β complex. J. Biol. Chem. 279, 47633–47642.

[4] Annes, J.P., Chen, Y., Munger, J.S. and Rifkin, D.B. (2004) Integrin $\alpha_v\beta_6$-mediated activation of latent TGF-β binding protein-1. J. Cell Biol. 165, 723–734.

[5] Jakins, G. (2008) The role of proteases in transforming growth factor-β activation. Int. J. Biochem. Cell. Biol. 40, 1068–1078.

[6] Sato, Y. and Rifkin, D.B. (1989) Inhibition of endothelial cell movement by pericytes and smooth muscle cells: Activation of a latent transforming growth factor-β1 molecule by plasmin during co-culture J. Cell Biol. 109, 309–315.

[7] Sato, Y., Okada, F., Abe, M., Seguchi, T., Kuwano, M., Sato, S., Furuya, A., Hanai, N. and Tamaoki, T. (1993) The mechanism for the activation of latent TGF-β during co-culture of endothelial cells and smooth muscle cells: Cell-type specific targeting of latent TGF-β to smooth muscle cells. J. Cell Biol. 123, 1249–1254.

[8] Abe, M., Oda, N. and Sato, Y. (1998) Cell-associated activation of latent transforming growth factor-β by calpain. J. Cell. Physiol. 174, 186–193.

[9] Abe, M., Oda, N., Sato, Y., Shibata, K. and Yamasaki, M. (2002) Augmented binding and activation of latent transforming growth factor-β by a tryptic fragment of latency associated peptide. Endothelium 9, 25–36.

[10] Clement, S., Velasco, P.T., Murthy, S.N.P., Wilson, J.H., Lukas, T.J., Goldman, R.D. and Lorand, L. (1998) The intermediate filament protein, vimentin, in the lens is a target for cross-linking by transglutaminase. J. Biol. Chem. 273, 7604–7609.

[11] Kojima, S., Vernooy, R., Moscatelli, D., Amanuma, H. and Rifkin, D.B. (1995) Lipopolysaccharide inhibits activation of latent transforming growth factor-β in bovine endothelial cells. J. Cell. Physiol. 163, 210–219.

[12] Kalo, E., Buganim, Y., Shapira, K.E., Besserglick, H., Goldfinger, N., Weisz, L., Stambolsky, P., Heins, Y.I. and Rotter, V. (2007) Mutant p53 attenuates the SMAD-dependent transforming growth factor β1 (TGF-β1) signaling pathway by repressing the expression of TGF-β receptor type II. Mol. Cell. Biol. 27, 8228–8242.

[13] Mor-Vankin, N., Punturieri, A., Sitwala, K. and Markovitz, D.M. (2003) Vimentin is secreted by activated macrophages. Nat. Cell Biol. 5, 59–63.

[14] Wahl, S., McCartney-Francis, N., Allen, J.B., Dougherty, E.B. and Dougherty, S.F. (1990) Macrophage production of TGF-β and regulation of TGF-β. Ann. NY Acad. Sci. 593, 188–196.

[15] Lettesjo, H., Nordstrom, E., Strom, H., Nilsson, B., Glinghammar, B., Dahlstedt, L. and Moller, E. (1998) Synovial fluid cytokines in patients with rheumatoid arthritis or other arthritic lesions. Scand. J. Immunol. 48, 286–292.

[16] Fukui, I., Tanaka, K. and Murachi, T. (1989) Extracellular appearance of calpain and calpastatin in the synovial fluid of the knee joint. Biochem. Biophys. Res. Commun. 162, 559–566.

Vasohibin-1 in human breast carcinoma: A potential negative feedback regulator of angiogenesis

Kentaro Tamaki,[1,4,6] Takuya Moriya,[2] Yasufumi Sato,[3] Takanori Ishida,[1] Yohei Maruo,[4] Kousuke Yoshinaga,[5] Noriaki Ohuchi[1] and Hironobu Sasano[4]

[1]Department of Surgical Oncology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [2]Department of Pathology 2, Kawasaki Medical School, 577 Matsushima, Kurashiki, Okayama 701-0192; [3]Department of Vascular Biology, Institute of Department, Aging, and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [4]Department of Pathology, Tohoku University Hospital, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574; [5]Department of Gynecology, Tohoku University School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai, Miyagi 980-8574

(Received August 11, 2008/Revised September 21, 2008/Accepted September 22, 2008/Online publication November 25, 2008)

Vasohibin-1 is a recently identified negative feedback inhibitor or suppressor of angiogenesis induced by vascular endothelial growth factor (VEGF)-A. The status of vasohibin-1 in human breast carcinoma has not been examined. We examined 151 breast specimens including 98 cases of invasive ductal carcinoma (IDC), 12 of ductal carcinoma *in situ* (DCIS), 16 of fibroadenoma (FA), six of inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue. We immunolocalized vasohibin-1 and compared its immunoreactivity to that of VEGF-A, basic fibroblastic growth factor (bFGF), VEGF receptor 2 (Flk-1), CD31, CD34 and Ki-67/MIB-1. The correlation of vasohibin-1 immunoreactivity with overall survival (OS), and disease-free survival (DFS) of the patients with breast carcinoma was also evaluated. In addition, we evaluated Ki-67 and CD31, and Ki-67 and vasohibin-1 double-immunostaining for further characterization of neovascularization. Vasohibin-1 was detected in endothelial cells of human breast and its immunodensity was significantly higher in IDC and inflammatory lesions than the other types ($P < 0.001$). In addition, a significant positive correlation was detected between vasohibin-1 and VEGF-A, bFGF or Flk-1 ($P < 0.001$). There was also positive associations between vasohibin-1 and OS ($P = 0.004$) and between vasohibin-1 and DFS ($P \leq 0.001$) in carcinoma cases. Results of double-immunostaining demonstrated the ratio of Ki-67-positive cells among vasohibin-1-positive endothelial cells (46.5%) was significantly higher than those among CD31-positive cells (23.5%). This is the first study demonstrating the status of vasohibin-1 in human breast lesions, which indicates that vasohibin-1 is associated with neovascularization and may especially play important roles in the regulation of intratumoral angiogenesis in human breast cancer. (*Cancer Sci* 2009; 100: 88–94)

Angiogenesis or the formation of new blood vessel networks, not only plays a pivotal role in human normal development, but also in pathophysiological conditions such as inflammatory diseases and neoplasms. Angiogenesis is generally regulated by an *in situ* balance between stimulatory and inhibitory factors of angiogenesis.[1,2] However, this "angiogenic homeostasis" may be disrupted in pathological conditions such as cancer and dysregulated or excessive production and/or secretion of angiogenesis inducers result in excessive formation of abnormal blood vessels. In general, various biological phenomena in physiological conditions are under stringent control by numerous negative feedback systems as seen in endocrine mechanisms the including hypothalamic–pituitary–adrenal system to maintain their homeostasis. However, little has been known about such negative feedback mechanisms of angiogenesis in both physiological and pathological conditions.

Vasohibin-1 has been very recently identified as one of the first established negative feedback regulators of angiogenesis.[2–5] This interesting factor was identified as one of vascular endothelial growth factor (VEGF)-induced genes with anti-angiogenic properties in endothelial cells (EC) using cDNA microarray analysis.[3,4,6] Vasohibin-1 was subsequently demonstrated to be specifically expressed in EC in response to angiogenic stimulators such as VEGF and basic fibroblastic growth factor (bFGF).[3,6] Vasohibin-1 is also abundantly present in human placenta and fetus[2,3,5] in which angiogenic events markedly occur *in vivo*. VEGF-A is the most potent factor for angiogenesis among known VEGF family members, stimulating protease synthesis, migration and proliferation of EC.[7] In addition, the great majority of VEGF-A-mediated signals are transduced via VEGF receptor 2 (Flk-1)[8] and protein kinase Cδ (PKCδ), one of the signals located in important downstream intrasignaling pathway of Flk-1, and they also induced vasohibin-1 expression markedly.[4] Yoshinaga *et al.* demonstrated that the VEGF-A-mediated induction of vasohibin-1 was preferentially mediated via the Flk-1 signaling pathway in human endometrial carcinoma.[9] However, the status of vasohibin-1 in other human malignancies has not been examined in detail.

Therefore, in this study, we first immunolocalized vasohibin-1 in human breast disorders including breast cancer in order to examine whether this factor is expressed in endothelial cells or not in human breast tissues. We then correlated the findings with various clinicopathological factors of the cases including microvessel density (MVD)[10,11] in order to correlate the status of vasohibin-1 with vascularity of the lesions. We also correlated vasohibin-1 immunoreactivity with neovascularization or proliferating endothelial cells using double immunostaining of Ki-67 in order to further characterize vasohibin-1 expression and its clinical and/or biological significance in human breast disorders.

Materials and Methods

Breast tissue specimens. We retrieved 151 Japanese female cases of breast tissues from surgical pathology files of Tohoku University Hospital (Sendai, Japan). These subjects were operated on between 1995 and 1998 at the Department of Surgery, Tohoku University Hospital. The median age of the patients was 48 years (range, 15–81). The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). The relevant clinicopathological information including age, histological type, stage classification, histological grade for invasive ductal carcinoma (IDC), grading scheme for ductal carcinoma *in situ* (DCIS) (van Nuys classifications[12] for DCIS and T1mic) are summarized in Table 1. Histological findings were 98 cases of IDC including eight cases of T1mic, 12 of DCIS, 16 fibroadenoma (FA), six of

[6]To whom correspondence should be addressed.
E-mail: nahanisikenta@yahoo.co.jp

doi: 10.1111/j.1349-7006.2008.01015.x

Table 1. Clinicopathological characters of examined cases

Histological type	
IDC	98
(T1mic)	8
DCIS	12
FA	16
Inflammatory lesion	6
Fibrocystic change	9
Non-pathological breast tissue	7
Age, years (range)	
All cases	48 years (15–81)
IDC	53 years (28–81)
DCIS	47 years (40–81)
FA	39 years (15–52)
Inflammatory lesion	49 years (35–70)
Fibrocystic change	47 years (39–48)
Non-pathological breast tissue	40 years (34–48)
UICC stage grouping	
Stage 0	12
Stage I	38
Stage II	36
II A	23
II B	13
Stage III	18
III A	8
III B	6
III C	4
Stage IV	6
Histological grade (for IDC)	
G 1	34
G 2	42
G 3	14
Van Nuys scheme (for DCIS and T1mic)	
Group 1	8
Group 2	12

DCIS, ductal carcinoma *in situ*; FA, fibroadenoma; IDC, invasive ductal carcinoma; UICC, International Union Against Cancer staging.

inflammatory lesion, nine of fibrocystic change and seven of non-pathological breast tissue taken from the lumpectomy specimen for breast cancer operation. In the nine cases of fibrocystic change, we evaluated vessels in the areas adjacent to adenosis or ductal hyperplasia. Stage grouping was based on *TNM Classification of Malignant Tumors Sixth Edition* by the International Union Against Cancer (UICC).[13] The tumor grade was determined according to the criteria of Elston and Ellis.[14]

Immunohistochemistry. We performed immunohistochemical staining for vasohibin-1, Flk-1, CD31, Ki-67, VEGF-A and FGF-2. The specimens had been fixed in 10% formalin, embedded in paraffin, cut into 4-μm thick sections and placed on glue-coated glass slides. Sections were deparaffinized in xylene, and hydrated with graded alcohols and distilled water. Endogenous peroxidase activity was blocked by 3% hydrogen peroxidase for 10 min at room temperature. Antigen retrieval was performed using Autoclave (TOMY SX-500 HIGH PRESSURE STEAM STERILIZER, TOMY SEIKO CO., LTD, Tokyo, Japan) in 10 nmol ethylene diamine tetra acetate (EDTA; pH 8) for vasohibin-1 and in citrate buffer for Flk-1, CD31, Ki-67 and FGF-2, heated at 121°C for 5 min, and for VEGF-A using microwave in citrate buffer for 15 min. Sections were subsequently incubated for 30 min at room temperature (RT) in a blocking solution of 10% rabbit serum (Nichirei Biosciences, Tokyo, Japan) for vasohibin-1, Flk-1, CD31, CD34 and Ki-67, and a blocking solution of 10% goat serum (Nichirei Bioscience) for VEGF-A and FGF-2, and then immunostained for 16 h at 4°C with primary antibodies. The primary antibodies of vasohibin-1, Flk-1, CD31, Ki-67, VEGF-1 were mouse monoclonal antibodies, whereas the primary antibody against FGF-2 was a rabbit polyclonal antibody, and were used as follows: antihuman vasohibin-1 monoclonal antibody[9,15] diluted at 1:3200; anti-VEGFR-2 (Flk-1; Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted at 1:3200; anti-CD31 (Dako, Copenhagen, Denmark) diluted at 1:40; anti-CD34 (Nichirei Bioscience) diluted at 1:100, Ki-67 (Dako) diluted 1:300; anti-VEGF-A (Laboratory Vision, Fremont, CA, USA) diluted at 1:50; and anti-FGF-2 (Santa Cruz Biotechnology) diluted at 1:100. Antihuman vasohibin-1 monoclonal antibody (mAb) was raised against the synthetic fragment (Gly286-Arg299) of human vasohibin-1 as described by Watanabe *et al.*[3] The specificity and sensitivity of this mAb was confirmed by both western blotting and immunohistochemical analysis.[3] For vasohibin-1, Flk-1, CD31, CD34 and Ki-67 immunohistochemistry, secondary antibody reactions were performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a dilution of 1:100 for 30 min at RT and peroxidase-conjugated avidin (Nichirei Bioscience) was used according to the manufacturer's instructions. Envision (Dako) was used for immunostaining of VEGF-A and FGF-2. Reacted sections were visualized using 3,3′-diaminobenzidine-tetrachloride (DAB)/30% H_2O_2 in 0.05 mol/L Tris buffer (pH 7.6) and counterstained with hematoxylin–eosin (HE) for nuclear staining.

Double staining procedure. For the quantification of proliferating endothelial cells, Ki-67/CD31 and Ki-67/vasohibin-1 double-labeling immunohistochemical staining was performed. A mAb directed against Ki-67 (Dako) was diluted at 1:300 following antigen retrieval using Autoclave in a citrate buffer, and incubated for 30 min at RT in a blocking solution of 10% rabbit serum (Nichirei Bioscience). A secondary antibody reaction was performed using biotinylated rabbit antimouse antibody (Nichirei Bioscience) at a distribution of 1:100 for 30 min at RT. Peroxidase-conjugated avidin (Nichirei Bioscience) was subsequently used in this study. DAB was used to visualize the binding of the first antibody. Antigen retrieval was then performed using a microwave for 15 min in 10 nmol EDTA (pH 8) for vasohibin-1 and in a citrate buffer for CD31. The reacted sections were then incubated for 30 min with antibodies against vasohibin-1 diluted at 1:3200 and CD31 (Dako) diluted at 1:40. Following the reaction with biotinylated rabbit antimouse antibody (Nichirei Bioscience) diluted at 1:100 as a secondary antibody and alkaline phosphatase-conjugated avidin (Nichirei Bioscience), an alkaline phosphatase substrate kit III (Vector Laboratories, Burlingame, CA, USA) was employed.[16,17]

Immunohistochemical analysis. Two of the authors (K. T. and Y. M.) independently evaluated the immunohistochemical staining of the tissue sections. They were blinded to the clinical course of the patients and the average of numbers counted by the two investigators was used for subsequent analysis. We used Olympus (Tokyo, Japan) BX50 and 20X objectives for the analysis.

The number of microvessels was counted within the tumor of IDC and FA, whereas in DCIS, the number of vessels in the stroma among intraductal components was evaluated. In inflammatory lesions, fibrocystic change and non-pathological breast tissues, the greatest number of vessels in the tissue sections was determined as MVD.[10,11,18–20] Microvessels were identified based on the architecture, lumen lined by endothelial cells, complemented by positivity of the endothelial cells with anti-CD31 after scanning the immunostained section at low magnification (×40 and ×100).[10,11] The areas with the greatest number of distinctly highlighted microvessels were selected, and counted at one higher power (×200).[10,11] Any immunostained endothelial cells or clusters separated from adjacent vessels were counted as a single microvessel, even in the absence of vessel lumen. Each single count was defined as the highest number of microvessels identified at the "hot spot". Vasohibin-1- and Flk-1-positive signals

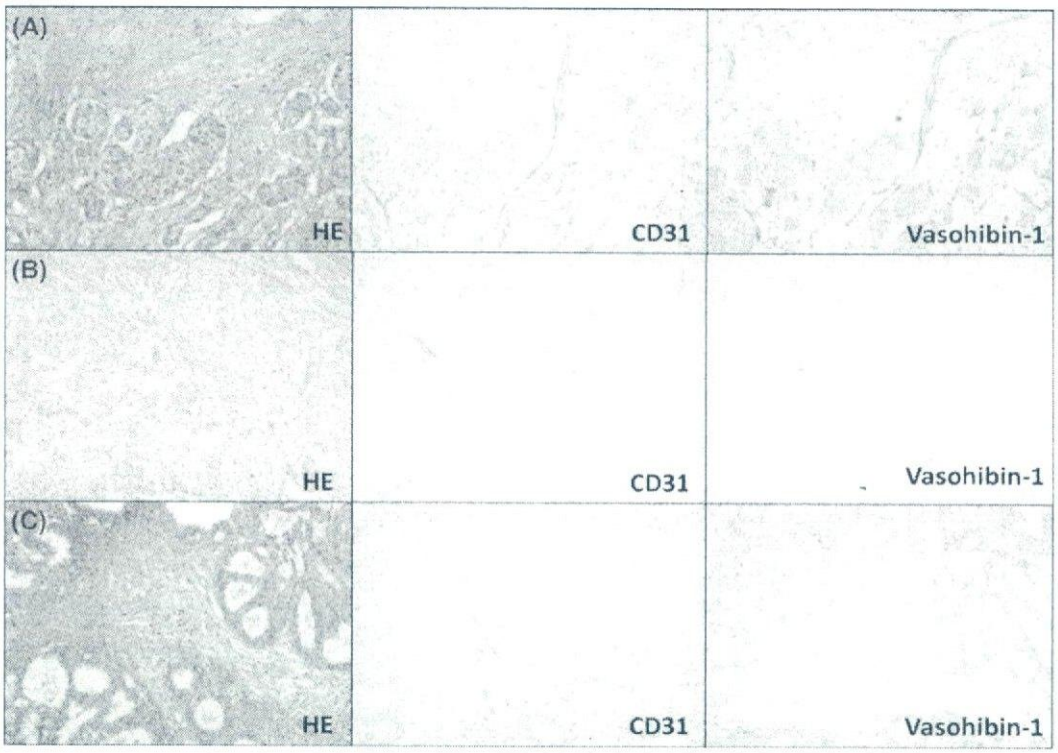


Fig. 1. Representative illustrations of histological and immunohistochemical findings of breast carcinoma cases examined. (A,B) Two invasive ductal carcinoma (IDC) cases stained positively for CD31 and vasohibin-1, whereas (C) a ductal carcinoma *in situ* (DCIS) case stained positive only for CD31 and not for vasohibin-1. (Original magnification, ×200.)

were counted in the hot spot in which the highest number of anti-CD31-positive vessels was identified. We also counted the average of vasohibin-1-positive vessels in 10 representative fields per case (×200). We defined vasohibin-1-positive ratio as the number divided by the number of vasohibin-1-positive vessels by that of CD31-positive vessels in the hot spot. An evaluation of Ki-67 immunoreactivity was performed at high power field (×400) and used as a marker of cell proliferation. More than 500 tumor cells from each of three different representative fields were evaluated and the labeling index was subsequently obtained. VEGF-A immunoreactivity was evaluated using grading, interpreting both relative immunointensity and the proportion of tumor cells associated with an unequivocal positive reaction.[21,22] Relative immunointensity was graded 0 (no staining) to 3 (strong staining), percentage of cells staining positive as 0 (no tumor cells positive), 1 (positive staining in <10% of the tumor cells), 2 (positive staining in 10–50% of the tumor cells) and 3 (positive staining in >50% of the tumor cells).[20,21] A semiquantitative method was used to evaluate the degrees of FGF-2 immunostaining ranging from 0 (no expression), 1 (weak), 2 (moderate) to 3 (highest level of expression).[23] The proportion of proliferating endothelial cells (CD31 and vasohibin-1-positive vessels) was defined as the number of endothelial cells with Ki-67-stained nuclei divided by the total number of endothelial cells.

Analyzes of OS and disease-free survival (DFS) curves were performed by employing the Kaplan–Meyer method. The segregation point of the parameter at 21 for vasohibin-1-positive vessels was determined by the Cox proportional hazards regression model. The values of survival rates represented estimated survival rates. Factors independently associated with OS and DFS – vasohibin-1, MVD, VEGF-A and Ki-67 – were identified by multivariate analyses using multiple regression analysis.

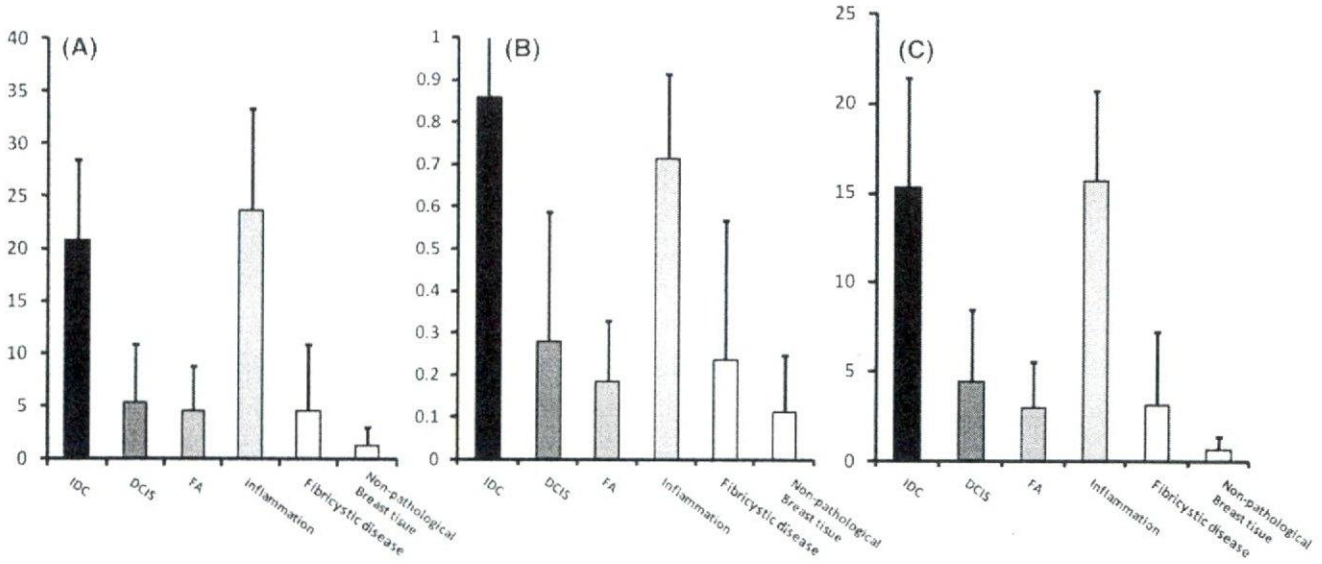
Statistical analysis, such as the one-factor ANOVA and simple regression analysis, were performed using StatMate III for Windows ver. 3.18 (ATMS, Tokyo, Japan). The results were considered significant at $P < 0.05$.

Results

MVD. The representative findings of immunostaining for HE, CD31 and vasohibin-1 are illustrated in Fig. 1. The average number of microvessels detected by CD31 was 24.6 ± 8.3 in IDC, 21.7 ± 11.7 in DCIS, 26.3 ± 15.7 in FA, 34.2 ± 15.4 in inflammatory lesions, 20.6 ± 14.4 in fibrocystic change and 13.6 ± 10.3 in non-pathological breast tissue, respectively. Statistically significant differences of MVD among the lesions were detected only between IDC and non-pathological breast tissue ($P = 0.001$).

Vasohibin-1 immunohistochemistry. Vasohibin-1 immunoreactivity was detected only in endothelial cells (Fig. 1). Vasohibin-1-positive microvessels in the hot spot were 20.9 ± 7.7 in IDC, 5.3 ± 5.5 in DCIS, 4.6 ± 4.1 in FA, 23.7 ± 9.7 in inflammatory lesions, 4.6 ± 6.3 in fibrocystic change and 1.3 ± 1.8 in non-pathological breast tissue. There were statistically significant differences between IDC and four other histological types of breast tissues examined (DCIS, FA, fibrocystic change and non-pathological breast tissue; $P < 0.001$) (Fig. 2A). The ratio of vasohibin-1/CD31[4] was 0.857 ± 0.193 in IDC, 0.279 ± 0.308 in DCIS, 0.183 ± 0.146 in FA, 0.713 ± 0.200 in inflammatory lesions, 0.237 ± 0.332 in fibrocystic change and 0.112 ± 0.136 in non-pathological breast tissue. There were significant differences between IDC and all other histological types ($P < 0.001$) (Fig. 2B). The average number of vasohibin-1-positive vessels per 10 fields (×200) were 15.3 ± 6.1 in IDC, 4.4 ± 4.1 in

doi: 10.1111/j.1349-7006.2008.01015.x



IDC	20.9 ± 7.7
DCIS	5.3 ± 5.5
FA	4.6 ± 4.1
Inflammation	23.7 ± 9.7
Fibrocystic disease	4.6 ± 4.1
Non-pathological breast tissue	1.3 ± 1.8

IDC	0.857 ± 0.193
DCIS	0.279 ± 0.308
FA	0.183 ± 0.146
Inflammation	0.713 ± 0.200
Fibrocystic disease	0.237 ± 0.332
Non-pathological breast tissue	0.112 ± 0.136

IDC	15.3 ± 6.1
DCIS	4.4 ± 4.1
FA	2.9 ± 2.6
Inflammation	15.7 ± 5.0
Fibrocystic disease	3.1 ± 4.1
Non-pathological breast tissue	0.7 ± 0.7

Fig. 2. Analysis of vasohibin-1 immunohistochemistry according to histological subtypes. (A) Number of vasohibin-1-positive vessels in the 'hotspot'. (B) Vasohibin-1-positive ratio defined as the vasohibin-1-positive vessels/CD31-positive vessels. (C) Average of vasohibin-1-positive vessels in 10 different fields. The lower boxes are the statistical analysis compared with invasive ductal carcinoma (IDC) cases.

DCIS, 2.9 ± 2.6 in FA, 15.7 ± 5.0 in inflammatory lesions, 3.1 ± 4.1 in fibrocystic change and 0.7 ± 0.7 in non-pathological breast tissue. There were also statistically significant differences between IDC and four histological types (DCIS, FA, fibrocystic change and non-pathological breast tissue, $P < 0.001$). No significant differences were detected between IDC and inflammatory lesions ($P = 0.781$) (Fig. 2C).

Correlation between vasohibin-1-positive vessels and Ki-67 labeling index in carcinoma cells. A significant positive correlation was detected between the number of vasohibin-1-positive vessels and Ki-67 labeling index in breast tumor cells ($P < 0.001$).

Correlation between vasohibin-1-positive vessels and VEGF-A status in carcinoma cells. The number of vasohibin-1-positive vessels was 5.8 ± 5.5 in VEGF-A of score 0, 11.0 ± 9.4 of score 2, 15.1 ± 10.0 of score 3, 17.5 ± 10.1 of score 4, 22.1 ± 8.9 of score 5 and 22.7 ± 5.7 of score 6. There was a statistically significant association between vasohibin-1 in the vessels and VEGF-A scores in carcinoma cells ($P < 0.001$) (Fig. 3A).

Correlation between vasohibin-1-positive vessels and FGF-2 in carcinoma cells. The number of vasohibin-1-positive vessels was 6.3 ± 6.1 in FGF-2 of score 0, 19.1 ± 6.5 of score 1, 21.9 ± 7.2 of score 2 and 26.8 ± 8.4 of score 3. A statistically significant association was detected between vasohibin-1 immunoreactivity in the vessels and FGF-2 scores in carcinoma cells ($P < 0.001$) (Fig. 3B).

Correlation between vasohibin-1 and Flk-1 in microvessels in breast carcinoma. A significantly positive correlation was detected between vasohibin-1 and Flk-1 positive ratios in microvessels ($P < 0.001$) (Fig. 3C).

Correlation between vasohibin-1 and clinical stage of breast carcinoma cases. The number of vasohibin-1-positive vessels was 5.3 ± 5.5 in TNM Stage 0, 19.6 ± 6.7 in Stage I, 18.7 ± 8.6 in Stage II A, 22.1 ± 8.3 in Stage II B, 23.8 ± 5.8 in Stage III A, 28.7 ± 7.5 in Stage III B, 23.0 ± 7.5 in Stage III C and 21.2 ± 5.6 in Stage IV. Statistically significant differences were detected only between IDC and DCIS ($P < 0.001$) with no significant differences among the different stages of IDC.

Correlation between vasohibin-1 and histological grades of breast carcinoma cells. The number of vasohibin-1-positive vessels among different groups of carcinoma cases and histological grade was 18.4 ± 7.5 in grade I, 20.8 ± 7.0 in grade II and 28.0 ± 8.0 in grade III. There were statistically significant differences of vasohibin-1 density between grade I and III, and grade II and III cases ($P < 0.001$) with no significant difference between grade I and II cases ($P = 0.14684$).

Correlation between vasohibin-1 and overall survival or DFS in breast carcinoma patients. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more. The 10-year overall survival rates were 0.932203 and 0.72549 among these two groups, respectively. (The total 10-year overall survival rate in this cohort of patients was 0.838836.) Statistically significant differences in the 0–20 and 21 or more groups was $P = 0.004$ (Fig. 4A). The 10-year DFS were 0.92736 and 0.708333, respectively, in these two groups. Statistically significant differences were also detected in the 0–20 and 21 or more groups was at $P \leq 0.001$. (The total 10-year DFS rate was 0.81777; Fig. 4B.) The following variables were included in the multivariate analysis of OS: vasohibin-1, MVD, VEGF-A and Ki-67. This multivariate analysis demonstrated that vasohibin-1 was associated with VEGF-A ($P = 0.038$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.083$). The multivariate analysis of

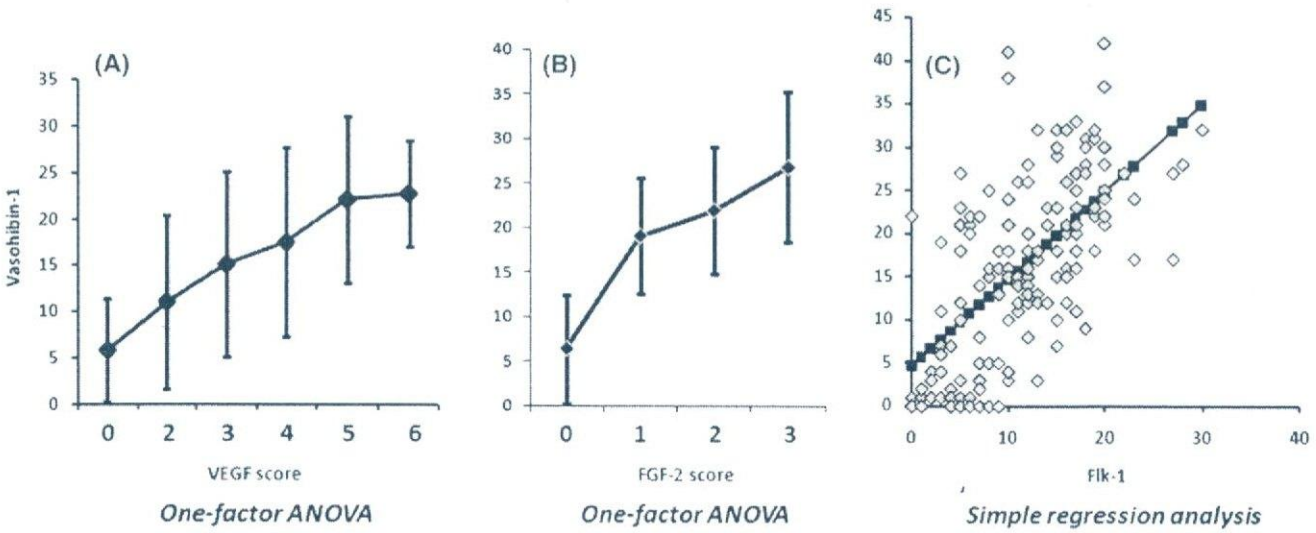
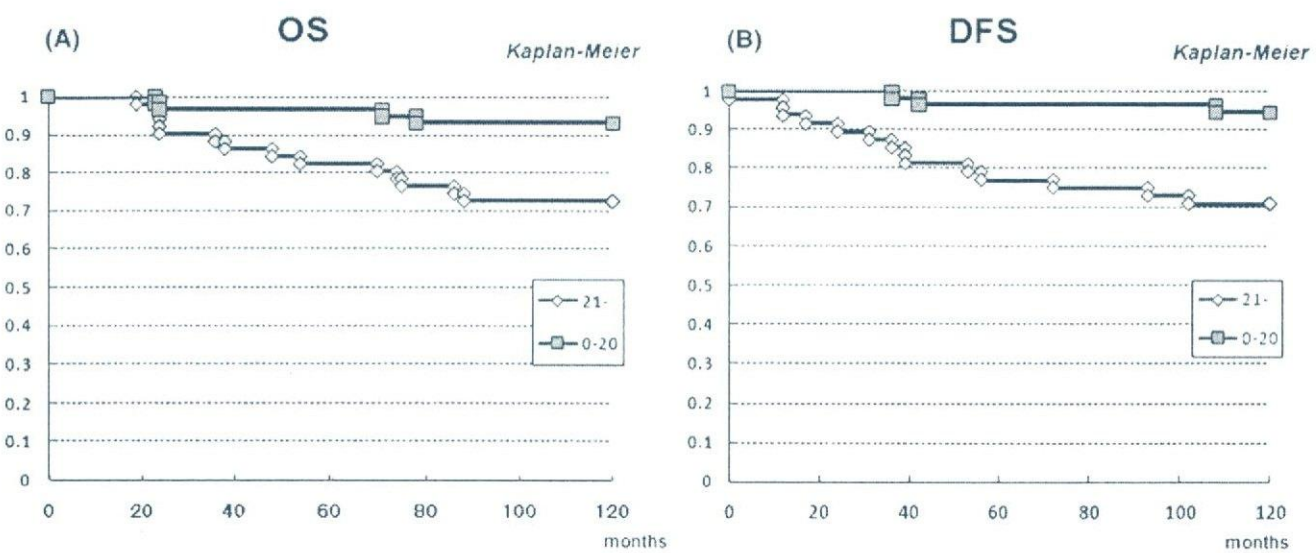


Fig. 3. (A) Result of the correlation between vasohibin-1-positive vessels and vascular endothelial growth factor (VEGF)-A expression in the tumor cells. (B) Result of the correlation between vasohibin-1-positive vessels and fibroblastic growth factor (FGF)-2 expression in the tumor cells. (C) Correlation between vasohibin-1 and Flk-1 in the 'hot spot'.



Vasohibin-1 positive vessels	10 year OS
0-20	0.932203
21-	0.72549

Vasohibin-1 positive vessels	10 year DFS
0-20	0.927356
21-	0.708333

Fig. 4. Summary of analysis of (A) overall survival and (B) disease free survival in relation to the status of vasohibin-1 expression. Patients were tentatively classified into two different groups according to the number of vasohibin-1-positive vessels: 0–20 and 21 or more.

DFS also revealed that vasohibin-1 was associated with VEGF-A ($P = 0.004$) and Ki-67 ($P < 0.001$), but was not associated with MVD ($P = 0.081$).

Double immunostaining with Ki-67 in microvessels. Ki-67/vasohibin-1 double immunostaining analysis demonstrated that Ki-67 labeling index of vasohibin-1-positive vessels was 46.5% (33.3–62.5%), whereas that of CD31-positive vessels was 23.5% (12.7–37.5%) (Fig. 5A,B).

Discussion

One of the most important functions of vasculature in general is to supply nutrients the distal organs. Three major types of regulation occur in the maintenance of vasculature: (i) vasodilation; (ii) changes in capillary permeability; and (iii) growth and development of new vessels, also known as angiogenesis.[24–26] Angiogenesis is a pivotal event in various biological processes

doi: 10.1111/j.1349-7006.2008.01015.x

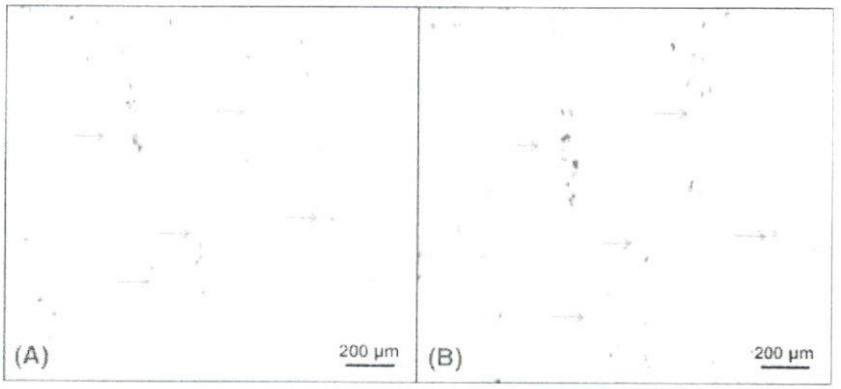


Fig. 5. Representative illustrations of double immunostaining for determining proliferating endothelial cells. (A) CD31/Ki-67 double staining; (B) vasohibin-1/Ki-67 double staining (arrow). (A) CD31 and (B) vasohibin-1 were colored blue, and Ki-67 was colored brown.

under both physiological and pathological conditions. Physiological conditions include embryonic development, reproduction and wound healing, and pathological conditions include cancers and inflammatory conditions.[2] *In situ* balance between angiogenesis stimulators such as VEGF and bFGF and inhibitors such as thrombospondin-1 (TSP-1) and pigment epithelium derived factor (PEDF) is generally considered to regulate the process of angiogenesis.[1] Negative feedback regulation is considered one of the most important physiological mechanisms with which bodies are endowed, and has been demonstrated to be involved in a wide range of biological phenomena.[27] This regulation is most effectively performed through the factors produced in endothelial cells but the endothelium-derived negative feedback regulators of angiogenesis have not been elucidated. Vasohibin-1 is therefore the first secretory anti-angiogenic factor from endothelial cells themselves induced by VEGF in EC.[2–4,28] The other anti-angiogenic regulator has been very recently identified and termed vasohibin-2 but this factor lacks the property of VEGF-A or bFGF inducibility in contrast to vasohibin-1.[28] Vasohibin-1 immunoreactivity was exclusively detected in endothelial cells in the present study, which is also consistent with results of previous studies of endometrial carcinoma[9] in lung carcinoma[3] and ischemic retina.[29] This is the first study to examine the status of vasohibin-1 in human breast disease in which angiogenesis also plays important roles in both physiological and pathological conditions.

Breast cancer has also been considered an angiogenic-dependent disease as in other human malignancies and angiogenesis has been demonstrated to play an essential role in breast cancer development, invasion and metastasis.[30–32] MVD assessed by CD31, CD34 and Factor VIII is generally considered as a gold-standard surrogate marker of tumor angiogenesis and has been also proposed by some investigators to identify patients at high risk of recurrence more precisely than classical indicators.[10,11]

In this study, we first examined how the vasohibin-1 expression was correlated to the MVD status. Vasohibin-1 immunodensity tended to be concordant with MVD in human breast tissues but they were not always parallel. The vasohibin-1 immunodensity was significantly higher in IDC than in DCIS but there was no difference of MVD between these two lesions. In addition, results of double immunostaining analysis which could simultaneously demonstrate two different proteins in the same cells, demonstrated the significant positive correlation between Ki-67-positive proliferating vascular endothelial cells, which may represent neovascular formation[16,17] and vasohibin-1-positive endothelial cells. Indeed, the Ki-67 labeling index among vasohibin-1-positive endothelial cells was significantly higher than Ki-67 in all CD31-positive endothelial cells. These results will clearly indicate that vasohibin-1 is considered a more appropriate biomarker for intratumoral neovascularization compared to CD31, which may detect all the vasculature including both resting and proliferating endothelial cells.

Results of our study also demonstrated the positive correlation between vasohibin-1 and VEGF-A or bFGF in carcinoma cells or Flk-1 in intratumoral endothelial cells, which also suggest that the vasohibin-1 in vasculature in human breast carcinoma is induced by VEGF-A, bFGF/Flk-1 signaling pathway. PKCδ was reported to play an important role in an induction of vasohibin-1 in endothelial cells.[4] Therefore, vasohibin-1 is supposed to be induced in the downstream of VEGF-A, bFGF/Flk-1 signaling pathway. Further investigations are necessary to reach the final conclusion.

The expression of vasohibin-1 in EC was proposed to be regulated either positively or negatively by certain factors at the transcriptional level, and this may influence the process of angiogenesis.[4] Another *in vivo* study also demonstrated the significantly positive correlation between vasohibin-1 and Flk-1 expression in vasculature of human endometrial carcinoma.[9] Significantly higher vasohibin-1 immunodensity in IDC than DCIS in our present study of human breast also indicate that the anti-angiogenic compensatory mechanism may be operational in invasive breast carcinoma, possibly in response to induction of angiogenesis by various factors related to carcinoma invasion into the surrounding stroma.

Results of several recent studies demonstrated the possible correlation between VEGF status in carcinoma cells and clinical outcome in breast cancer patients. VEGF was proposed to be correlated with worse DFS and overall survival rates especially in the patients with early-stage breast cancer.[33] VEGF expression in carcinoma cells was also reported as an independent prognostic marker in both node-positive and node-negative breast cancers.[34] Many previous immunohistochemical studies of MVD assessed by CD31, CD34 or Factor VIII antigen in human breast cancer demonstrated that high MVD in invasive ductal carcinoma is usually correlated with a greater likelihood of metastatic disease,[10] shorter relapse-free intervals and reduced overall survival in patients with node-negative breast cancer.[11] We therefore examined whether vasohibin-1 immunoreactivity is correlated with OS and DFS of the patients. Results of our study demonstrated that the cases with a higher number of vasohibin-1-positive vessels tended to be associated with better and statistically significant OS. In addition, a statistically negative or inverse correlation was detected between vasohibin-1 immunodensity and DFS. These results all suggest that an evaluation of the number of vasohibin-1-positive vessels may become one of the prognostic markers for metastasis and prognosis but it awaits further investigations to establish this approach as a surrogate marker such as MVD.

Recently, newer targeted therapies toward the control of tumor neovascularization such as anti-VEGF therapy have been developed in phase II and III clinical trials and demonstrated the clinical effects such as reduction of tumor angiogenesis and inhibition of solid tumors proliferation, either alone or in combination with chemotherapy.[35–38] In our present study, vasohibin-1 immunohistochemical staining was demonstrated to reasonably reflect the status of angiogenesis, and vasohibin-1 itself may be considered for anti-VEGF and anti-angiogenesis drugs to control tumor angiogenesis in future.

Acknowledgments

We thank Yayoi Takahashi, MT, for her excellent technical assistance. This work was partly supported by the grants from the Japanese Ministry of Health, Labor and Welfare for Researches on Intractable Diseases, Risk Analysis Research on Food and Pharmaceuticals, and Development of Multidisciplinary Treatment Algorithm with Biomarkers and Modeling of the Decision-making Process with Artificial Intelligence for Primary Breast Cancer. This work was also partly supported by a Grant-in-Aid for Scientific Research (no. 18390109) from the Japanese Ministry of Education, Culture, Sports, Science and Technology, and the Yasuda Medical Foundation.

References

1 Folkman J. Angiogenesis in cancers, vascular, rheumatoid and other disease. *Nat Med* 1995; **1**: 27–31.
2 Sato Y, Sonoda H. The Vasohibin Family: a negative regulatory system of angiogenesis genetically programmed in endothelial cells. *Arterioscl Thromb Vasc Biol* 2007; **27**: 37–41.
3 Watanabe K, Hasegawa Y, Yamashita H *et al.* Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. *J Clin Invest* 2004; **114**: 898–907.
4 Shimizu K, Watanabe K, Yamashita H *et al.* Gene regulation of novel angiogenesis inhibitor, vasohibin, in endothelial cells. *Biochem Biophys Res Commun* 2005; **327**: 700–6.
5 Kerbel RS. Vasohibin. The feedback on a new inhibitor of angiogenesis. *J Clin Invest* 2004; **114**: 884–6.
6 Sonoda H, Ohta H, Watanabe K *et al.* Multiple processing forms and their biological activities of a novel angiogenesis inhibitor vasohibin. *Biochem Biophys Res Commun* 2006; **342**: 640–6.
7 Abe M, Sato Y. cDNA microarray analysis of the gene expression profile of VEGF-activated human umbilical vein endothelial cells. *Angiogenesis* 2001; **4**: 289–98.
8 Ferrara N. Vascular endothelial growth factor: basic science and clinical progress. *Endocr Rev* 2004; **25**: 581–611.
9 Yoshinaga K, Ito K, Moriya T *et al.* Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer. *Cancer Sci* 2008; **99**: 914–9.
10 Weidner N, Semple JP, Welch WR *et al.* Tumor angiogenesis and metastasis-correlation in invasive breast carcinoma. *N Engl J Med* 1991; **324**: 1–8.
11 Weidner N, Folkman J, Pozza F *et al.* Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J Natl Cancer Inst* 1992; **84**: 1875–87.
12 Silverstein MJ. Prognostic classification of breast ductal carcinoma *in situ*. *Lancet* 1995; **345**: 1154–7.
13 Sobin LH, Wittekind C. *TNM Classification of Malignant Tumours* 131–41.
14 Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a larger study with long-term follow-up. *Histopathology* 1991; **19**: 403–10.
15 Yamashita H, Abe M, Watanabe K *et al.* Vasohibin prevents arterial neointimal formation through angiogenesis inhibition. *Biochem Biophys Res Commun* 2006; **345**: 919–25.
16 Nijsten T, Copaert CG, Vermeulen PB *et al.* Cyclooxygenase-2 expression and angiogenesis in squamous cell carcinoma of the skin and its precursors: a paired immunohistochemical study of 36 cases. *Br J Dermatol* 2004; **151**: 837–45.
17 Hoskin PJ, Sibtain A, Daley FM *et al.* The immunohistochemical assessment of hypoxia, vascularity and proliferation in bladder carcinoma. *Radiother Oncol* 2004; **72**: 159–68.
18 Uzzan B, Nicolas P, Cucherat M *et al.* Microvessel density as a prognostic factor in women with breast cancer: a systematic review of the literature and meta-analysis. *Cancer Res* 2004; **64**: 2941–55.
19 Guidi AJ, Schnitt SJ, Fischer L *et al.* Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in patients with ductal carcinoma *in situ* of the breast. *Cancer* 1997; **80**: 1945–53.
20 Cao Y, Paner GP, Kahn LB *et al.* Non-invasive carcinoma of the breast: angiogenesis and cell proliferation. *Arch Pathol Lab Med* 2004; **128**: 893–6.
21 Aas T, Borresen AL, Geisler S *et al.* Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. *Nat Med* 1996; **2**: 811–14.
22 Toi M, Hoshina S, Takayanagi T *et al.* Association of vascular endothelial growth factor expression with angiogenesis and with early relapse in primary breast cancer. *Jpn J Cancer Res* 1994; **85**: 1045–9.
23 Begum S, Zhang Y, Shintani T *et al.* Immunohistochemical expression of heparin-binding protein 17/fibroblast growth factor-binding protein-1 (HBp17/FGFBP-1) as an angiogenic factor in head and neck tumorigenesis. *Oncol Report* 2007 Mar; **17** (3): 591–6.
24 Folkman J. Fundamental concepts of the angiogenic process. *Curr Mol Med* 2003; **3**: 643–51.
25 Risau W. Mechanism of angiogenesis. *Nature* 1997; **386**: 671–4.
26 Carmeliet P. Angiogenesis in health and disease. *Nat Med* 2003; **9**: 653–60.
27 Lord BI. Feedback regulators in normal and tumor tissues. *J Cell Sci Suppl* 1988; **10**: 231–42.
28 Shibuya T, Watanabe K, Yamashita H *et al.* Isolation of vasohibin-2 as a sole homologue of VEGF-inducible endothelium-derived angiogenesis inhibitor vasohibin: a comparative study on their expressions. *Arterioscl Thromb Vasc Biol* 2006; **26**: 1051–7.
29 Shen JK, Yang XR, Sato Y *et al.* Vasohibin is up-regulated by VEGF in the retina and suppress VEGF receptor 2 and retinal neovascularization. *FASEB J* 2006; **20**: 723–5.
30 Lichtenbeld HC, Barendsz-Janson AF, ven Essen H *et al.* Angiogenic potential of malignant and non-malignant human breast tissue in an *in vivo* angiogenesis model. *Int J Cancer* 1998; **77**: 455–9.
31 Dabrosin C, Palmer K, Muller WJ *et al.* Estradiol promotes growth and angiogenesis in polyoma middle T transgenic mouse mammary tumor explants. *Breast Cancer Res Treat* 2003; **78**: 1–6.
32 Scneider BP, Miller KD. Angiogenesis of breast cancer. *J Clin Oncol* 2005; **22**: 609–14.
33 Sledge GW Jr. Vascular endothelial growth factor in breast cancer: biologic and therapeutic aspects. *Semin Oncol* 2002; **29** (Suppl 11): 104–10.
34 Linderholm B, Lindh B, Beckman L *et al.* The prognostic value of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) and associations to first metastasis site in 1307 patients with primary breast cancer. *Proc Am Soc Clin Oncol* 2001; **20**: 4a.
35 Rugo HS. Bevacizumab in the treatment of breast cancer: rationale and current data. *The Oncologist* 2004; **9** (Suppl 1): 43–9.
36 Fox SB, Generail DG, Harris AL. Breast tumour angiogenesis. *Breast Cancer Res* 2007; **9**: 216.
37 Miller KD, Chap LI, Holmes FA *et al.* Randomized phase III trial of capecitabine compared with bevacizumab plus capecitabine in patients with previously treated metastatic breast cancer. *J Clin Oncol* 2005; **23**: 792–9.
38 Wedam SB, Low JA, Yang SX *et al.* Antiangiogenic and antitumor effects of bevacizumab in patients with inflammatory and locally advanced breast cancer. *J Clin Oncol* 2006; **24**: 769–777.

doi: 10.1111/j.1349-7006.2008.01015.x

Expression of Vasohibin, an Antiangiogenic Factor, in Human Choroidal Neovascular Membranes

RYOSUKE WAKUSAWA, TOSHIAKI ABE, HAJIME SATO, MADOKA YOSHIDA, HIROSHI KUNIKATA, YASUFUMI SATO, AND KOHJI NISHIDA

• PURPOSE: To determine whether vasohibin, an antiangiogenic factor produced by vascular endothelial cells, is expressed in the choroidal neovascular (CNV) membranes obtained from human eyes with age-related macular degeneration (AMD) or polypoidal choroidal vasculopathy (PCV).

• DESIGN: Retrospective, interventional case series.

• METHODS: The medical charts of 21 eyes of 21 patients with AMD or PCV who underwent surgical removal of the CNV membrane were reviewed. The removed tissues were immunostained for von Willebrand Factor (vWF), vascular endothelial growth factor (VEGF), and vasohibin. The levels of the messenger ribonucleic acid of VEGF, VEGFR2, and vasohibin were determined by real-time reverse-transcriptase polymerase chain reaction (RT-PCR) from the CNV membranes excised from nine AMD and nine PCV patients.

• RESULTS: The patients were divided into three groups; four patients were placed in the most active group (Group H), 13 in the less active group (Group E), and four in the nonactive group (Group S). Immunohistochemistry showed that vasohibin, vWF, and VEGF were expressed in the vascular endothelial cells in the CNV membranes and in the polypoidal vessels. RT-PCR showed that there was a strong correlation between the level of expression of VEGFR2 and vasohibin ($P = .0002$). Eyes with a lower vasohibin-to-VEGF ratio tended to have larger subretinal hemorrhages or vitreous hemorrhages, whereas eyes with higher vasohibin-to-VEGF ratio had subretinal fibrosislike lesions. Statistical analysis of the vasohibin-to-VEGF ratio among the three groups was significant ($P = .0209$).

• CONCLUSIONS: Vasohibin is expressed in human CNV membranes. Our results indicate that the vasohibin-to-VEGF ratio may be related with the activity of the CNV.

AJO.com Supplemental Material available at AJO.com.
Accepted for publication Mar 13, 2008.

From the Department of Ophthalmology and Visual Science (R.W., H.S., Y.M., H.K., K.N.); the Division of Clinical Cell Therapy, Center for Translational and Advanced Animal Research (T.A.); and the Department of Vascular Biology, Institute of Development, Aging, and Cancer (Y.S.), Tohoku University Graduate School of Medicine, Miyagi, Japan.

Inquires to Ryosuke Wakusawa, Department of Ophthalmology and Visual Science, Tohoku University Graduate School of Medicine, 1-1, Seiryo-cho, Aoba-ku, Sendai 980–8574, Miyagi, Japan; e-mail: ckd19390@rio.odn.ne.jp

(Am J Ophthalmol 2008;xx:xxx. © 2008 by Elsevier Inc. All rights reserved.)

THREE FACTORS HAVE BEEN SUGGESTED TO CONTROL vasculogenesis and angiogenesis. These factors are: vascular endothelial growth factors (VEGFs) and VEGF receptors, angiopoietins (Ang) and Tie receptors, and the ephrins and Eph receptors.[1,2] The Ang–Tie system stabilizes the vascular wall, and the ephrin-B2–EphD4 system is required for the differentiation of vascular endothelial cells (ECs) during the earliest stages of arterial and venous development. The VEGFs and VEGF receptors play important roles in vasculogenesis, angiogenesis, and stabilization of mature vessels. VEGF receptor 2 (VEGFR2) is well accepted as the receptor that mediates functional VEGF signaling in ECs.

Neovascularization is the major cause of blindness in many ocular diseases. Age-related macular degeneration (AMD) is the most common cause of central vision loss in the elderly population of developed countries,[3] and the development of choroidal neovascularizations (CNVs) is characteristic of the exudative form of AMD. A CNV leads to subretinal hemorrhages, exudative lesions, serous retinal detachment (RD), and disciform scars.[4]

Endothelial cells, retinal pigment epithelial (RPE) cells, and macrophage-like mononuclear cells have been reported to be the major cellular components of CNV membranes, and they produce many different kinds of proangiogenic and antiangiogenic factors.[5–12] VEGF is one of the proangiogenic factors that plays major roles in the development of CNVs.[13]

Polypoidal choroidal vasculopathy (PCV), first described by Yannuzzi and associates, is characterized by an abnormal network of choroidal vessels with polyp-like dilations at the terminals of the branches.[14] Whether PCV represents a kind of CNV is still being debated, but PCV resembles AMD clinically and is relatively more common in the Japanese[15] and Chinese persons than in White persons.[16] Recent studies have demonstrated that VEGF was expressed in the ECs and RPE cells of a PCV lesion.[11]

Vasohibin recently was reported to be a VEGF-inducible gene in human cultured ECs,[17] and it is a secretory protein made up of several processed forms, for example, 27, 32, 36, 42, and 44 kDa.[18] It has been reported that recombinant vasohibin inhibited the network formation of ECs in

0002-9394/08/$34.00
doi:10.1016/j.ajo.2008.03.019

TABLE 1. Clinical Characteristics of 11 Patients with Age-Related Macular Degeneration and 10 with Polypoidal Choroidal Vasculopathy

Case No.	Age (yrs)	Gender	Diagnosis	TAP Classification	Lesion Size*	Subgroup	Analysis
1	56	M	AMD	Predominantly classic	0.9	E	IHC
2	72	M	AMD	Minimally classic	2.8	E	IHC
3	73	F	PCV	Occult	3.4	E	IHC
4	76	M	AMD	Minimally classic	4.0	H	RT-PCR
5	65	M	AMD	Occult	4.8	H	RT-PCR
6	49	F	AMD	Predominantly classic	1.3	E	RT-PCR
7	85	M	AMD	Predominantly classic	3.1	E	RT-PCR
8	68	F	AMD	Minimally classic	2.7	E	RT-PCR
9	64	M	AMD	Minimally classic	3.3	E	RT-PCR
10	78	M	AMD	Minimally classic	4.1	E	RT-PCR
11	73	M	AMD	Staining†	1.4	S	RT-PCR
12	65	M	AMD	Staining†	1.9	S	RT-PCR
13	76	M	PCV	Occult	6.2	H	RT-PCR
14	56	M	PCV	Vitreous hemorrhage‡		H	RT-PCR
15	69	M	PCV	Minimally classic	2.1	E	RT-PCR
16	77	M	PCV	Minimally classic	3.0	E	RT-PCR
17	73	F	PCV	Occult	1.1	E	RT-PCR
18	65	M	PCV	Occult	2.0	E	RT-PCR
19	66	F	PCV	Occult	2.2	E	RT-PCR
20	66	M	PCV	Staining†	1.8	S	RT-PCR
21	72	M	PCV	Staining†	3.2	S	RT-PCR

AMD = age-related macular degeneration; F = female patients; Group E = exudative lesion excluded in subgroup H; Group H = massive subretinal hemorrhage or vitreous hemorrhage; Group S = quiescent lesion including disciform scar; IHC = immunohistochemistry; M = male; PCV = polypoidal choroidal vasculopathy; TAP = Treatment of Age-Related Macular Degeneration with Photodynamic Therapy Study; RT-PCR = reverse-transcriptase polymerase chain reaction.

*In disk diameters.

†Not applicable to TAP classification because of quiescent lesion.

‡Not applicable to TAP classification because of dense vitreous hemorrhage. Diagnosed with angiography before vitreous hemorrhage.

vitro[17] and also inhibited the retinal neovascularizaion in the mouse model of oxygen-induced ischemic retinopathy.[19] Vasohibin is different from other angiogenic inhibitors because it is induced selectively in ECs by proangiogenic factors such as VEGF and basic fibroblast growth factor (bFGF).[17,20] Vasohibin is considered to be an intrinsic and highly specific negative feedback regulator of activated ECs engaged in the process of angiogenesis.

The purpose of this study is to determine whether vasohibin is expressed in human CNV membranes obtained from eyes with AMD or PCV. We also examined the relationship between the degree of expression of vasohibin, VEGF, and VEGFR2 and considered whether the level of expression of these genes was related to the clinical manifestations among the patients.

METHODS

• SUBJECTS AND TREATMENTS: We reviewed the medical records of 11 eyes of 11 AMD patients and 10 eyes of 10 PCV patients who had undergone vitrectomy and surgical removal of a CNV with or without autologous iris pigment epithelial cell transplantation.[21] These were the initial treatment and were performed between May 10, 1996 and April 15, 2003. Photodynamic therapy was not available in Japan at that time.

In addition to the routine ophthalmologic examination, fluorescein angiography and indocyanine green angiography (ICGA) were performed on all patients, and optical coherence tomography (OCT) was performed on 12 patients before and after the surgery. The criteria used to determine whether surgery was required were: best-corrected visual acuity (BCVA) less than 0.2, progressive deterioration of vision during the three months before surgery, and other factors as described.[21]

The eyes were classified retrospectively into three subgroups according to the clinical findings: eyes in Group H had massive subretinal hemorrhage that extended beyond the temporal retinal vessel arcade or a vitreous hemorrhage; eyes in Group E had active exudative lesions such as subretinal hemorrhage, hard exudates, and serous RD within the vascular arcade; and eyes in Group S had resolved and no exudative lesions at the time of surgery,

but these patients had undergone surgery for the transplantation of autologous iris pigment epithelial cells. The clinical characteristics of the patients are summarized in Table 1.

• TISSUE PREPARATION AND IMMUNOHISTOCHEMISTRY: The CNV membranes from two patients with AMD and one patient with PCV were fixed in 4% formalin and embedded in paraffin. Serial 5-μm sections were cut, and adjacent sections were stained with hematoxylin and eosin.

Immunohistochemical staining for the von Willebrand Factor (vWF), VEGF, and vasohibin was performed using the horseradish peroxidase (HRP) method. All steps were performed at room temperature unless otherwise stated. Briefly, sections were deparaffinized, rehydrated, and then treated with 3% hydrogen peroxide to block endogenous peroxidase activity. After blocking with 1% bovine serum albumin in phosphate buffered saline (PBS) for 30 minutes, rabbit polyclonal antibodies against vWF (1:500; DAKO, Glostrup, Denmark), rabbit polyclonal antibodies against VEGF (1:200; Santa Cruz Biotechnology, Santa Cruz, California, USA), or mouse monoclonal antibodies against vasohibin (1:1000; made by Watanabe and associates) were applied to the sections overnight at 4 C.[17] The following morning, the sections were incubated in biotin-conjugated antirabbit immunoglobulin (Ig) G or antimouse IgG antibodies (Vector Laboratories, Burlingame, California, USA) for 30 minutes. The slides then were incubated with HRP-conjugated streptavidin (Vector Laboratories) for 30 minutes. Brown chromogen diaminobenzidine (Sigma-Aldrich, St Louis, Missouri, USA) was used for all sections. The sections were washed three times with PBS between each step. For control, preimmune rabbit IgG or mouse IgG was used instead of the primary antibody. Sections were examined under a standard light microscopy.

• PREPARATION OF TOTAL RIBONUCLEIC ACID AND REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION: The CNV membranes from nine eyes with AMD and from nine eyes with PCV were separately mixed with denaturing solution, and messenger ribonucleic acid (mRNA) was prepared using QuickPrep micromRNA Purification Kit (Amersham Biosciences, Buckinghamshire, United Kingdom) according to the manufacturer's instructions. The purified mRNA was reverse-transcribed into complementary deoxyribonucleic acid (cDNA) using First-Strand cDNA Synthesis Kit (Amersham Biosciences). One microliter of cDNA was used for real-time reverse-transcriptase polymerase chain reaction (RT-PCR) amplification using a Light Cycler (Roche, Meylan, France) and the Light Cycler FastStart DNA Master SYBR Green I reagent Kit (Roche).

The primers for VEGF[22] and VEGFR2[12] were synthesized from the reported sequences. Primers for vasohibin and glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) were designed to be intron spanning to preclude amplification of genomic DNA using GeneWorks version 2.45N (IntelliGenetics; Mountain View, California, USA) computer software.

TABLE 2. Semiquantitative Reverse-Transcriptase Polymerase Chain Reaction Conditions Used for the Amplification of Vasohibin and Other mRNAs

Gene	Sequence of Primers	Annealing Temperature
Vasohibin	5′-CTTACCTGCTTGCTGTCTGC-3′	59 C
	5′-CATGGATGGTGACTAAGGCC-3′	
VEGF	5′-GCAGAATCATCACGAAGTGG-3′	58 C
	5′-AAGGACTGTTCTGTCGATGG-3′	
VEGFR2	5′-CCAGATGAACTCCCATTGGATG-3′	56 C
	5′-CTCTTGCTCCTCAGGTAAGTGGAC-3′	
GAPDH	5′-AAGGTGAAGGTCGGAGTCAA-3′	55 C
	5′-TTGAGGTCAATGAAGGGGTC-3′	

GAPDH = glyceraldehyde-3-phosphate-dehydrogenase; mRNA = messenger ribonucleic acid; VEGF = vascular endothelial growth factor; VEGFR2 = vascular endothelial growth factor receptor 2.

The sequence of the primers and the experimental conditions for optimized amplification are summarized in Table 2. PCR conditions were: step 1, 95 C for 10 minutes; step 2, 95 C for 10 seconds; hybridization temperature as indicated in Table 2 for 10 seconds; and 72 C for 10 seconds. Step 2 was repeated for 45 cycles. All data were normalized to the GAPDH transcript level, thus giving the relative transcript level.

The relative transcript level of VEGF, VEGFR2, and vasohibin obtained from the CNV membranes of eyes with AMD or PCV were compared using the Mann–Whitney *U* test. The correlations between the transcription level of VEGF, VEGFR2, and vasohibin were calculated by the Pearson simple correlation coefficient. The relative transcript level of vasohibin and the transcription ratio of vasohibin to VEGF also were compared among Groups H, E, and S using Mann–Whitney *U* test.

RESULTS

• CLINICAL CLASSIFICATION AND CHARACTERIZATION: The 21 eyes (11 with AMD and 10 with PCV) were divided into three groups according to the clinical findings: Group H included four eyes that had the most active lesions, Group E had 13 eyes with less active lesions, and Group S had four eyes whose lesion were not active (Table 1). The median visual acuity was 0.02 in Group H, 0.04 in Group E, and 0.05 in Group S. The median size of the CNV estimated from the ICGA images[23] was 2.4 disk diameters (DD) in Group H, 1.3 DD in Group E, and 1.7

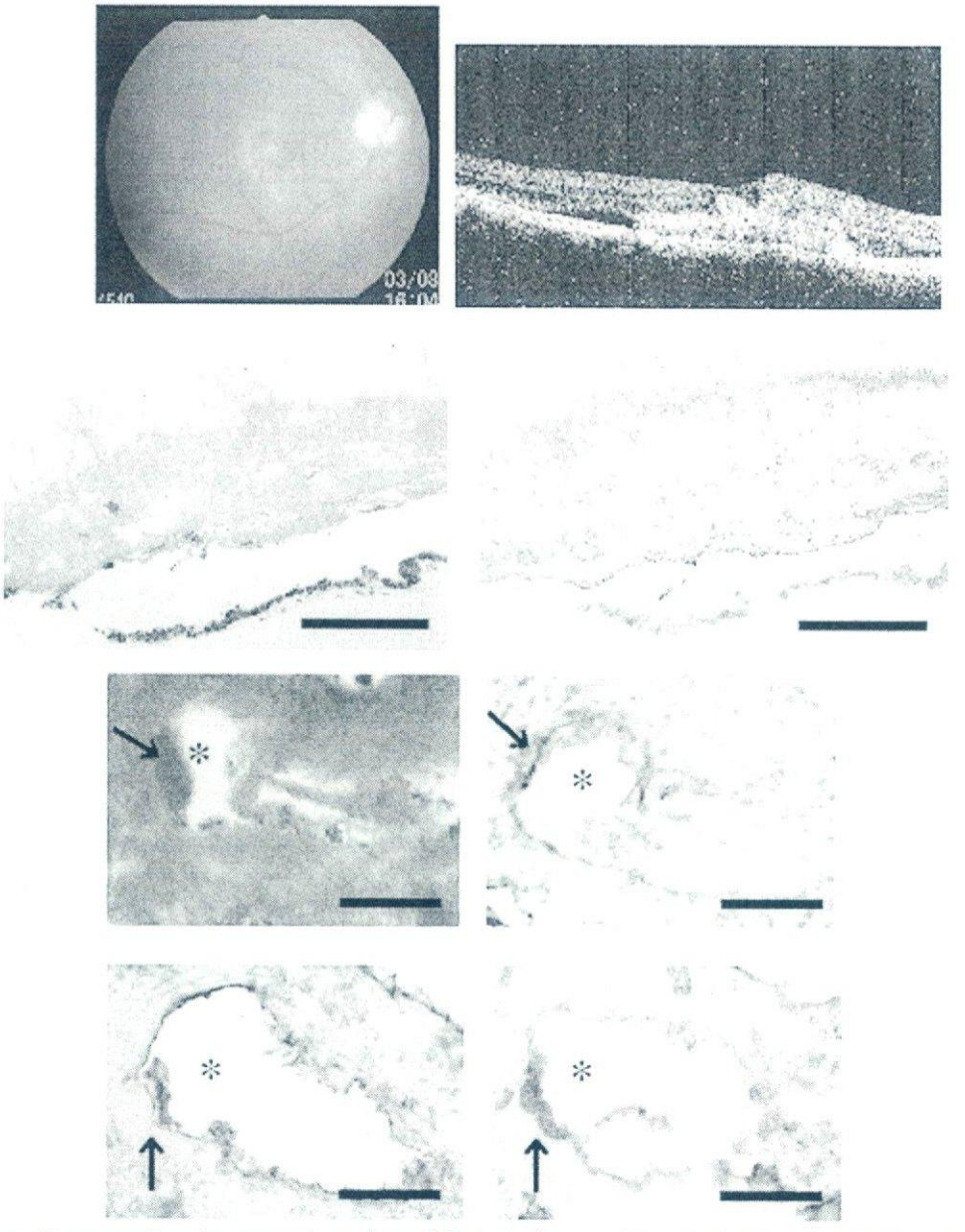


FIGURE 1. Images demonstrating the expression of vasohibin on the vascular endothelial cells (ECs) within the choroidal neovascularization (CNV) membrane obtained from an eye with age-related macular degeneration [AMD] (Cases 1 and 2 in Table 1). (Top left) Case 1: fundus photograph showing exudative lesion with subretinal hemorrhage. (Top right) Case 1: optical coherence tomogram showing CNV above the retinal pigment epithelium (RPE) layer. (Second row left) Photomicrograph of CNV membrane from Case 1 showing fibrovascular tissue beneath the outer segments of the photoreceptors. The brown linear structure under the CNV membrane is a sheet of RPE cells (stain, hematoxylin and eosin; original magnification, ×200). (Second row right) Photomicrograph showing vasohibin of CNV membrane from Case 1. Vasohibin is expressed on vascular ECs and some of the photoreceptor outer segments (stain, immunohistochemical staining for vasohibin; original magnification, ×200). (Third row left) Photomicrograph showing CNV membrane from Case 2 (stain, hematoxylin and eosin; original magnification, ×400). (Third row right) Photomicrograph showing vasohibin in CNV membrane from Case 2 (stain, immunohistochemical staining for vasohibin, original magnification, ×400). (Bottom left) Photomicrograph showing von Willebrand Factor (vWF) original magnification, ×400 in Case 2. (Bottom right) Photomicrograph showing vascular endothelial growth factor (VEGF) in Case 2 (stain, immunohistochemical staining for VEGF; original magnification, ×400). Arrows point to vascular ECs that are positive for each staining. Asterisks indicate the vessel lumens. Bar = 200 μm (second row); 20 μm (third row and bottom).

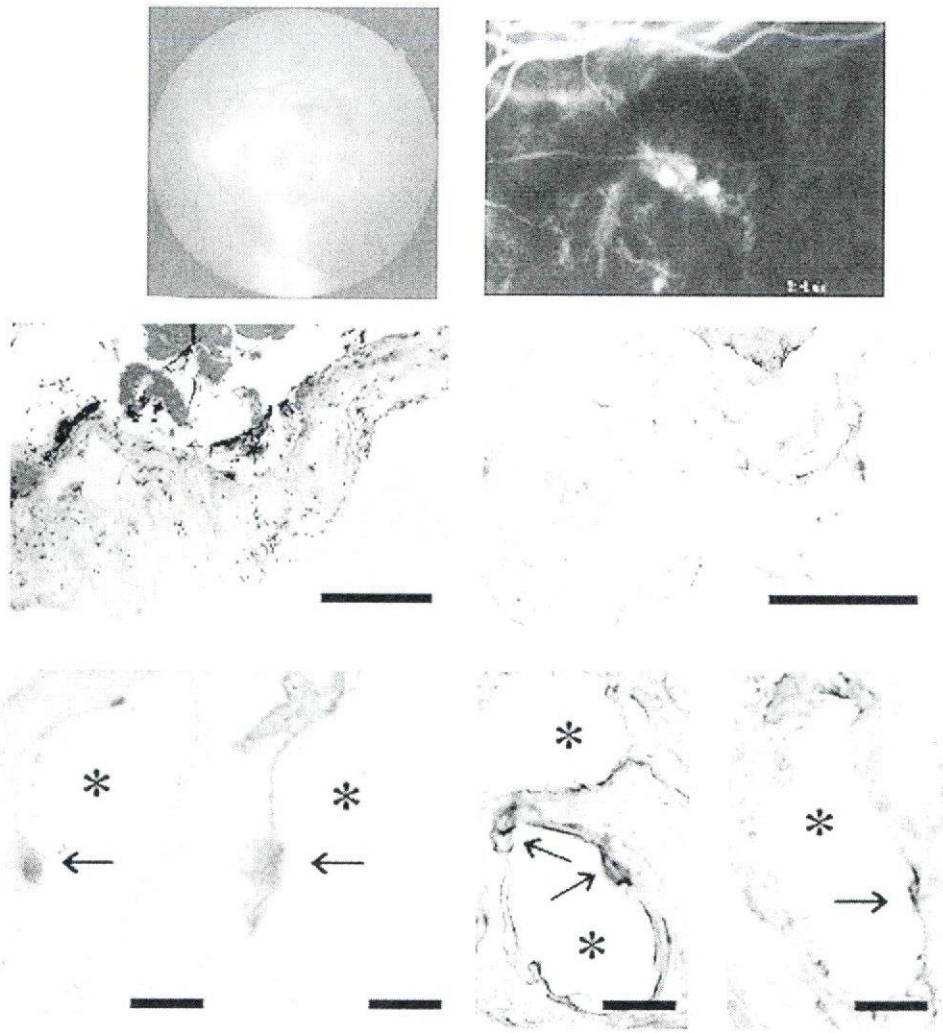


FIGURE 2. Images demonstrating that vasohibin is expressed on vascular ECs in dilated abnormal vessels obtained from an eye with polypoidal choroidal vasculopathy [PCV] (Case 3 in Table 1). (Top left) Fundus photograph showing orange-colored lesion with exudation. (Top right) Indocyanine green angiogram (ICGA) showing abnormal polypoidal choroidal vessels (PCV). (Middle left) Photomicrograph of CNV membrane showing dilated abnormal choroidal vessels under the disrupted RPE layer. The vessels are surrounded by loose connective tissue (stain, hematoxylin and eosin; original magnification, ×200). (Middle right) Photomicrograph showing vasohibin in CNV membrane (original magnification, ×200). (Bottom far left) Photomicrograph showing hematoxylin and eosin staining results (original magnification, ×400). (Bottom near left) Photomicrograph showing immunohistochemistry results for vasohibin (original magnification, ×400). (Bottom near right) Photomicrograph showing results for vWF (original magnification, ×400). (Bottom far right) Photomicrograph showing results for VEGF (original magnification, ×400). Arrows point to vascular ECs in polyp-like dilated vessels, positive for each staining. Asterisks indicate the vessel lumens. Bar = 200 μm (Middle); 20 μm (Bottom).

DD in Group S. The differences in these values in the three groups were not statistically significant.

• IMMUNOHISTOCHEMISTRY: Vasohibin-positive cells were detected in all specimens. The results from Cases 1 and 2 with AMD (Table 1) are shown in Figure 1. The type II CNV membrane (Figure 1, Top row) was removed and histologic examination revealed that the CNV membrane included not only fibrous tissue and vessels but also possible photoreceptor outer segment and RPE cells (Figure 1, Second row left). The photoreceptor-like cells were stained by mouse monoclonal antibody against opsin (1:1000; Sigma-Aldrich; data not shown). Immunohistochemistry demonstrated that vWF, a marker of ECs, stained the inside of the vascular lumens. Vasohibin, VEGF, or both were present in some of the ECs (Figure 1, Third row and Bottom). Interestingly, the outer segments of the photoreceptors also were positive for vasohibin. The results from Patient 3 with PCV are shown in Figure 2 and Table 1. Vasohibin and VEGF were present in some of the

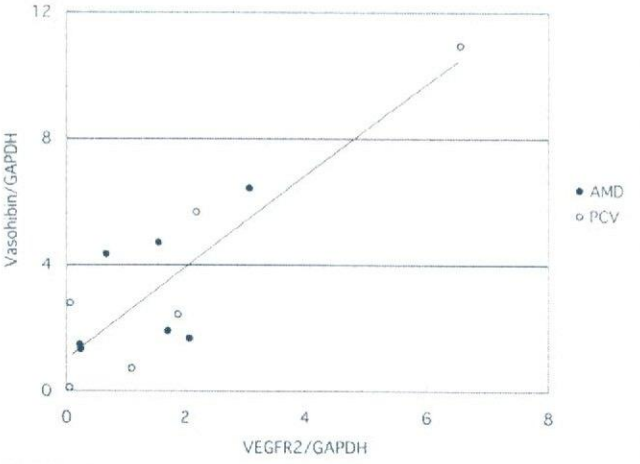


FIGURE 3. Scatterplot showing quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) results demonstrating a strong positive correlation between the expression level of vascular endothelial growth factor receptor 2 (VEGFR2) and vasohibin (Pearson simple correlation coefficient, $r = 0.858$; $P < .01$). GAPDH = glyceraldehyde-3-phosphate-dehydrogenase.

ECs of the dilated abnormal choroidal vessels (Figure 2, Second row and Bottom).

• REAL-TIME REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION: The tissues from the nine eyes with AMD (two from Group H, five from Group E, and two from Group S) and nine specimens from nine eyes with PCV (two from Group H, five from Group E, and two from Group S) were used for the RT-PCR analysis. The average degree of gene expression of VEGF, VEGFR2, and vasohibin was not significantly different between patients with AMD and PCV ($P = .508$, .500, and .627, respectively). However, there was a strong positive correlation between the level of expression of VEGFR2 and vasohibin ($r = 0.858$; $P = .000173$; Figure 3). The correlations between VEGF and VEGFR2 ($r = 0.322$; $P = .283$) and between VEGF and vasohibin ($r = 0.145$; $P = .565$) were not significant.

The expression of vasohibin ranged from 0.1 to 1.36 (median, 0.945) in Group H, from 0.71 to 6.41 (median, 2.61) in Group E, and from 1.46 to 10.93 (median, 6.985) in Group S. The level of expression in Group H was significantly lower than that in Group E ($P = .0109$) and Group S ($P = .0209$). The difference between Groups E and S was not significant ($P = .2578$).

The ratio of vasohibin to VEGF ranged from 0.04 to 0.54 (median, 0.135) in Group H, from 0.19 to 0.68 (median, 0.40) in Group E, and from 0.65 to 4.50 (median, 1.32) in Group S. The difference between Groups H and E ($P = .0477$), between Groups E and S ($P = .0109$) and that between Groups H and S ($P = .0209$) were significant (Figure 4).

DISCUSSION

EARLIER STUDIES HAVE SHOWN THAT VEGF,[4–12] BFGF,[5,6] transforming growth factor-β,[5] tumor necrosis factor-α (TNF-α),[10] pigment epithelium-derived factor (PEDF),[11] and other cytokines were expressed in surgically excised CNV membranes in patients with AMD and PCV. VEGF was expressed in the ECs, RPE cells, and macrophage-like mononuclear cells in the CNV membranes. Our results also showed that VEGF is expressed in the ECs in the lesions of eyes with AMD and PCV. Secreted VEGF stimulates the migration of ECs, proliferation of ECs, and canalization of the vessels through VEGFR2,[1,2] and thus must play an important part in the development of a CNV.[13] These findings have led to the develop anti-VEGF treatments for AMD.[24,25]

A large number of endogenous antiangiogenic factors have been identified.[26] PEDF, a 50-kDa noninhibitory member of the serine protease inhibitor gene family, is a well-described antiangiogenic factor.[27,28] It is expressed in the RPE cells and also in CNV membranes,[11] cornea, and ciliary epithelium. It is secreted into the interphotoreceptor matrix, aqueous humor, and vitreous. The concentration of PEDF in the vitreous and aqueous decreases with increasing age and is very low in AMD patients.[29] It has been suggested that the level of PEDF in CNV membranes is enhanced by stimulation of VEGF receptor 1. This may constitute a negative feedback loop against the VEGF-induced neovascularization.[28,30]

Vasohibin, however, was reported to be an antiangiogenic factor produced by ECs.[17] We have also found that vasohibin is expressed on a part of the ECs of choroidal vessels, retinal vessels, and RPE cells of normal mouse eyes (see Supplemental Figure available at AJO.com). However, the physiologic function of the vasohibin secreted by ECs and RPE cells has not been determined. Our immunohistochemical results demonstrated the expression of vasohibin on ECs in human CNV membranes and on the abnormally dilated vessels of PCV. In addition, the RT-PCR findings showed a strong positive correlation between the expression of VEGFR2 and vasohibin but not other combinations, such as VEGF and VEGFR2. Shimizu and associates reported that vasohibin was expressed by human cultured ECs, and the level increased after stimulation by VEGFR2.[20] Our findings also showed the possibility that vasohibin is secreted by the ECs in CNV membranes after the stimulation of VEGFR2. Shen and associates showed that a knockdown of the mRNA of vasohibin in ischemic retinas caused a significant elevation of the mRNA of VEGFR2 in mice.[19] However, they reported a significant negative correlation between vasohibin and VEGFR2. The discrepancy between our results and those of Shen and associates may be because the excised CNV membrane was only from a limited area, and also because the stage of the disease may have been different. We examined only the CNV membrane, and not all of the retina and choroid.

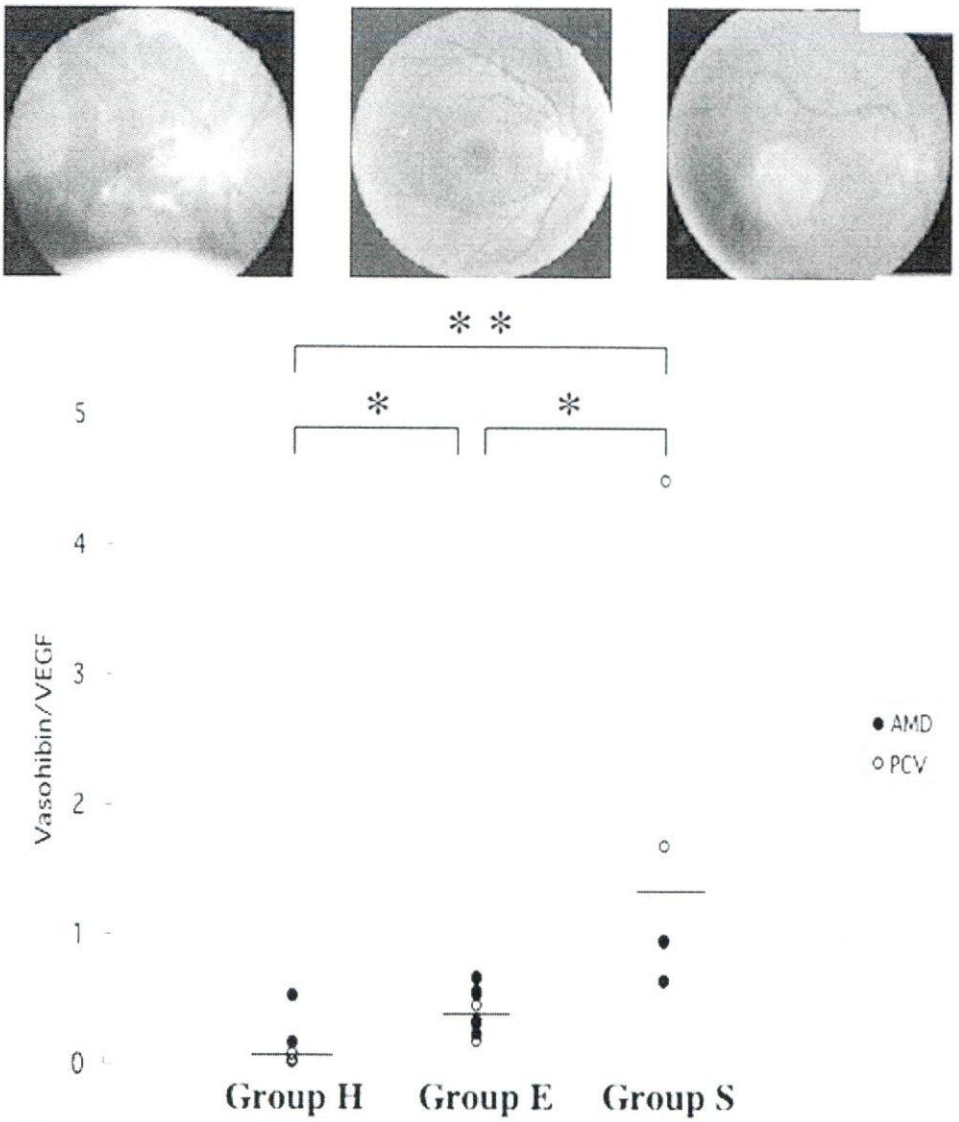


FIGURE 4. Images demonstrating the relationship between the transcription ratio of vasohibin/VEGF and the activity of the lesion. Higher transcription ratio of vasohibin/VEGF is associated with less active lesions. (Top left) Fundus photograph from a representative case in Group H showing massive subretinal hemorrhage (Case 13, vasohibin/VEGF = 0.09). (Top middle) Fundus photograph from a representative case in Group E showing exudative lesion (Case 6, vasohibin/VEGF = 0.67). (Top right) Fundus photograph from a representative case in Group S showing disciform scar (Case 20, vasohibin/VEGF = 4.50). (Bottom) Graph showing a significant difference between Groups H and E, between Groups E and S, and between Groups H and S (*$P < .05$, **$P < .01$, Mann–Whitney U test). Bar indicates median.

Furthermore, most of the excised CNVs were considered to be very active for neovascularization. The level of expression of vasohibin in the CNV membranes in our study may not have been sufficient to suppress VEGFR2. VEGF, TNF-α, and many other factors have been reported to control VEGFR2 expression.[31,32] We suggest that high levels of vasohibin may downregulate VEGFR2 in CNV membranes.

It is difficult to draw any conclusion on the relationship between vasohibin and clinical manifestations from our experimental results because of the small sample number. However, we have tried to estimate the participation of vasohibin using semiquantitative methods of RT-PCR and immunohistochemistry. We observed that the cases with massive hemorrhage showed markedly lower levels of vasohibin expression, although statistical analysis was not as significant as that of comparison of the vasohibin-to-VEGF ratio. The cases with low a vasohibin-to-VEGF expression ratio had larger subretinal hemorrhages or vitreous hemorrhages, and inversely, cases with a higher vasohibin-to-VEGF ratio had low exudative activity such as fibrosis. Although our sample number was small,

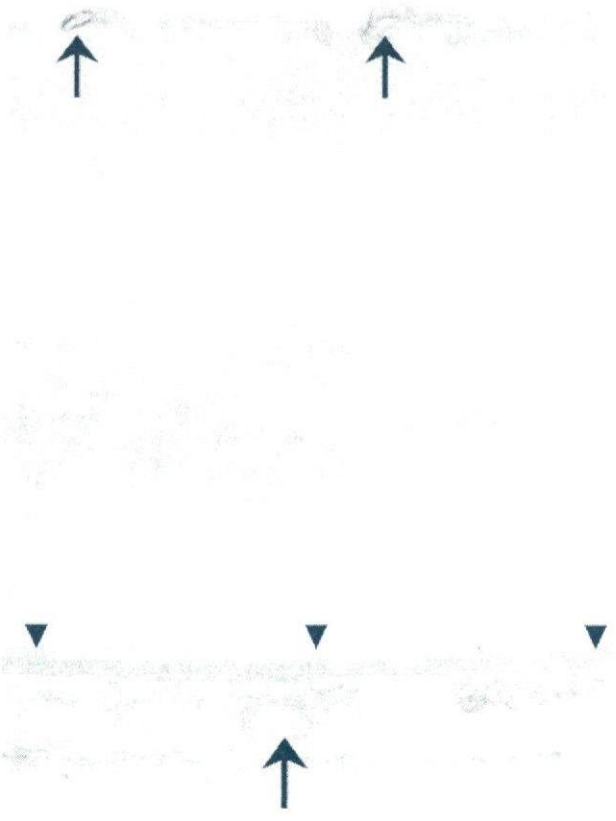
we suggest that lower vasohibin expression together with higher VEGF expression represent an active, that is, hemorrhagic or exudative, CNV, and conversely, high vasohibin expression together with lower VEGF expression represent decreased exudative activity in the CNV. Watanabe and associates reported that VEGF and bFGF increased the expression of vasohibin, and hypoxia and TNF-α inhibited VEGF-stimulated vasohibin expression in vitro.[17] However, a correlation between VEGF and vasohibin expression in vivo still has not been reported. Further examinations on the balance between VEGF and vasohibin would be important for determining their relationship with the activity of CNV membranes. Animal models and clinically available samples may help in answering this question. If vasohibin, other than PEDF, is a negative feedback regulator, it may provide us an alternative target for antineovascularization therapy. Experiments on the suppression of neovascularization are ongoing in our laboratory.

THIS STUDY WAS SUPPORTED IN PART BY GRANTS FROM GRANTS-IN-AID FOR SCIENTIFIC RESEARCH 18659508 AND 18591907 (Dr Abe) from the Japan Society for the Promotion of Science, Chiyoda-ku, Tokyo, Japan. The authors indicate no financial conflict of interest. Involved in design of study (R.W., T.A.); conduct of study (R.W., T.A., K.N.); data collection (R.W., M.Y., H.K.); analysis and interpretation of data (R.W., T.A., Y.S.); writing and preparation of the manuscript (R.W., T.A.); and review and approval of the manuscript (T.A., Y.S., K.N.). This study was not a clinical trial. Institutional review board approval was obtained from the Tohoku University Hospital. The procedures used in this study conformed to the guidelines of the Declaration of Helsinki. Written informed consent for the treatment and the research analysis of these pathologic specimens was obtained from each patient. All treatments of the animals conformed to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

REFERENCES

1. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J. Vascular-specific growth factors and blood vessel formation. Nature 2000;407:242–248.
2. Hofer E, Schweighofer B. Signal transduction induced in endothelial cells by growth factor receptors involved in angiogenesis. Thromb Haemost 2007;97:355–363.
3. Klein R, Peto T, Bird A, Vannewkirk MR. The epidemiology of age-related macular degeneration. Am J Ophthalmol 2004;137:486–495.
4. Bressler NM, Bressler SB, Fine SL. Age-related macular degeneration. Surv Ophthalmol 1998;32:375–413.
5. Amin R, Puklin JE, Frank RN. Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. Invest Ophthalmol Vis Sci 1994;35:3178–3188.
6. Frank RN, Amin RH, Eliott D, Puklin JE, Abrams GW. Basic fibroblast growth factor and vascular endothelial growth factor are present in epiretinal and choroidal neovascular membranes. Am J Ophthalmol 1996;122:393–403.
7. Lopez PF, Sippy BD, Lambert M, Thach AB, Hinton DR. Transdifferentiated retinal pigment epithelial cells are immunoreactive for vascular endothelial growth factor in surgically excised age-related macular degeneration-related membranes. Invest Ophthalmol Vis Sci 1996;37:855–868.
8. Kvanta A, Algvere PV, Berglin L, Seregard S. Subfoveal fibrovascular membranes in age-related macular degeneration express vascular endothelial growth factor. Invest Ophthalmol Vis Sci 1996;37:1929–1934.
9. Otani A, Takagi H, Oh H, et al. Vascular endothelial growth factor family and receptor expression in human choroidal neovascular membranes. Microvas Res 2002;64:162–169.
10. Oh H, Takagi H, Takagi C, et al. The potential angiogenic role of macrophages in the formation of choroidal neovascular membranes. Invest Ophthalmol Vis Sci 1999;40:1891–1898.
11. Matsuoka M, Ogata N, Otsuji T, Nishimura T, Takahashi K, Matsumura M. Expression of pigment epithelium derived factor and vascular endothelial growth factor in choroidal neovascular membranes and polypoidal choroidal vasculopathy. Br J Ophthalmol 2004;88:809–815.
12. Hera R, Keramidas M, Peoc'h M, Mouillon M, Romanet JP, Feige JJ. Expression of VEGF and angiopoietins in subfoveal membranes from patients with age-related macular degeneration. Am J Ophthalmol 2005;139:589–596.
13. Spilsbury K, Garrett KL, Shen WY, Constable IJ, Rakoczy PE. Overexpression of vascular endothelial growth factor (VEGF) in the retinal pigment epithelium leads to the development of choroidal neovascularization. Am J Pathol 2000;157:135–144.
14. Yannuzzi LA, Sorenson J, Spaide RF, Lipson B. Idiopathic polypoidal choroidal vasculopathy (IPCV). Retina 1990;10:1–8.
15. Uyama M, Matsubara T, Fukushima I, et al. Idiopathic polypoidal choroidal vasculopathy in Japanese patients. Arch Ophthalmol 1999;117:1035–1042.
16. Chan WM, Lai TY, Tano Y, Liu DT, Li KK, Lam DS. Photodynamic therapy in macular diseases of Asian populations: when East meets West. Jpn J Ophthalmol 2006;50:161–169.
17. Watanabe K, Hasegawa Y, Yamashita H, et al. Vasohibin as an endothelium-derived negative feedback regulator of angiogenesis. J Clin Invest 2004;114:898–907.
18. Sonoda H, Ohta H, Watanabe K, Yamashita H, Kimura H, Sato Y. Multiple processing forms and their biological activities of a novel angiogenesis inhibitor vasohibin. Biochem Biophys Res Commun 2006;342:640–646.
19. Shen J, Yang X, Xiao WH, Hackett SF, Sato Y, Campochiaro PA. Vasohibin is up-regulated by VEGF in the retina and suppresses VEGF receptor 2 and retinal neovascularization. FASEB J 2006;20:723–725.
20. Shimizu K, Watanabe K, Yamashita H, et al. Gene regulation of a novel angiogenesis inhibitor, vasohibin, in endothelial cells. Biochem Biophys Res Commun 2005;327:700–706.
21. Abe T, Yoshida M, Yoshioka Y, et al. Iris pigment epithelial cell transplantation for degenerative retinal diseases. Prog Retin Eye Res 2007;26:302–321.

22. Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 1989;246:1306–1309.
23. Abe T, Yoshida M, Kano T, Tamai M. Visual function after removal of subretinal neovascular membranes in patients with age-related macular degeneration. Graefes Arch Clin Exp Ophthalmol 2001;239:927–936.
24. Krzystolik MG, Afshari MA, Adamis AP, et al. Prevention of experimental choroidal neovascularization with intravitreal anti-vascular endothelial growth factor antibody fragment. Arch Ophthalmol 2002;120:338–346.
25. Rosenfeld PJ, Brown DM, Heier JS, et al, MARINA Study Group. Ranibizumab for neovascular age-related macular degeneration. N Engl J Med 2006;355:1419–1431.
26. Dorrel M, Uusitalo-Jarvinen H, Aguilar E, Freidlander M. Ocular neovascularization: basic mechanisms and therapeutic advances. Surv Ophthalmol 2007;52:S3–S19.
27. Dawson DW, Volpert OV, Gillis P, et al. Pigment epithelium-derived factor: a potent inhibitor of angiogenesis. Science 1999;285:245–248.
28. Tong JP, Yao YF. Contribution of VEGF and PEDF to choroidal angiogenesis: a need for balanced expressions. Clin Biochem 2006;39:267–276.
29. Holekamp NM, Bouck N, Volpert O. Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. Am J Ophthalmol 2002;134:220–227.
30. Ohno-Matsui K, Yoshida T, Uetama T, Mochizuki M, Morita I. Vascular endothelial growth factor upregulates pigment epithelium-derived factor expression via VEGFR-1 in human retinal pigment epithelial cells. Biochem Biophys Res Commun 2003;303:962–967.
31. Singh AJ, Meyer RD, Band H, Rahimi N. The carboxyl terminus of VEGFR-2 is required for PKC-mediated down-regulation. Mol Biol Cell 2005;16:2106–2118.
32. Giraudo E, Primo L, Audero E, et al. Tumor necrosis factor-alpha regulates expression of vascular endothelial growth factor receptor-2 and of its co-receptor neuropilin-1 in human vascular endothelial cells. J Biol Chem 1998;273:22128–22135.



SUPPLEMENTAL FIGURE. Immunohistochemistry demonstrated vasohibin was expressed on a part of endothelial cells of choroidal vessels, retinal vessels, and retinal pigment epithelial (RPE) cells. Arrows indicate retinal or choroidal vessels and arrowheads for RPE layer. These regions are positive for vasohibin. A 8-week-old, Male Institute of Cancer Research (ICR) mice was used (original magnification, ×200). After anesthesia with ketamine hydrochloride (100 mg/kg body weight) the mice were killed. The eyes were enucleated and fixed in 4% formalin overnight at 4 C. They were cryoprotected in 10% to 20% sucrose-phosphate buffered saline (PBS) and then sectioned (10 μm). Immunohistochemical staining for vashohibin was performed using the horseradish peroxidase (HRP) method. All steps were performed at room temperature unless otherwise stated. Briefly, sections were treated with 3% hydrogen peroxide to block endogenous peroxidase activity. After blocking with 1% bovine serum albumin in PBS for 30 minutes, rabbit polyclonal antibodies against vasohibin (1:400; made by Watanabe and associates) were applied to the sections overnight at 4 C. The following morning, the sections were incubated in HRP-conjugated anti-rabbit IgG antibody overnight at 4 C. Brown chromogen diaminobenzidine was used for all sections. Nuclei were stained with methylgreen. The sections were washed three times with PBS between each step. For control, preimmune rabbit IgG was used instead of the primary antibody. Sections were examined under a standard light microscope. All treatments of the animals conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Expression of vasohibin as a novel endothelium-derived angiogenesis inhibitor in endometrial cancer

Kousuke Yoshinaga,[1] Kiyoshi Ito,[1,4] Takuya Moriya,[2] Satoru Nagase,[1] Tadao Takano,[1] Hitoshi Niikura,[1] Nobuo Yaegashi[1] and Yasufumi Sato[3]

[1]Department of Gynecology and [2]Department of Pathology, Tohoku University Graduate School of Medicine, [3]Department of Vascular Biology, Institute of Development, Aging, and Cancer, Tohoku University, Seiryo-cho 1-1 Aoba ward, Sendai City, Japan

(Received September 13, 2007/Revised January 8, 2008/Accepted January 14, 2008/Online publication March 5, 2008)

We have previously reported on vasohibin as a novel endothelium-derived vascular endothelial growth factor (VEGF)–inducible inhibitor of angiogenesis. The aim of our present study was to define the role of vasohibin in endometrioid endometrial adenocarcinoma. We collected 78 sections of endometrial carcinoma for assessment using immunohistochemistry. Twenty-seven were well differentiated (G1), 25 were moderately differentiated (G2), and 26 were poorly differentiated endometrioid adenocarcinomas (G3). We also included 12 sections of normal cyclic endometria, six of which were in the proliferative phase and six were in the secretory phase. We investigated the expression of vasohibin, and compared it to VEGF receptor-2 (VEGFR-2: KDR/flk-1), CD34, Ki-67, VEGF-A, and D2-40 (as a lymphatic vessel marker). We assessed the ratio of vasohibin- and VEGFR-2-positive vessels in the stroma of endometrial carcinoma. Immunohistochemical assessment was classified as negative or positive based on staining intensity. Vasohibin was selectively expressed on vascular endothelial cells in both cyclic endometria and endometrial carcinomas. Vasohibin was highly expressed in the normal functional endmetrium of the secretory phase, especially in the spiral artery, and was highly expressed in all grades of endometrioid adenocarcinomas. The stromal endothelial cells in G3 expressed vasohibin and VEGFR-2 more frequently than these in G1. In endometrioid adenocarcinomas, there was a significant correlation between the expression percentage of vasohibin and that of VEGFR-2 ($P < 0.0001$, $r^2 = 0.591$). This is the first study to elucidate the correlation between expression of vasohibin in the stromal endothelial cells and that of VEGFR-2 in human carcinomas. (*Cancer Sci* 2008; 99: 914–919)

Endometrial carcinoma is one of the most common gynecologic malignancies in women worldwide, and its incidence, especially that of endometrioid endometrial carcinoma, has recently increased.[1] The morbidity of endometrial cancer is rapidly increasing in Japan. In order to predict the behavior of aggressive tumors, various factors and/or phenomena associated with endometrial cancer have been studied extensively. It is well recognized that angiogenesis, the process of formation of new vessels, is requisite for tumor growth and enables hematogenous spread of tumor cells throughout the body. Several studies have documented the association between the microvessel density (MVD) and/or the extent of endothelial proliferation and tumor stage, as well as recurrence of endometrial cancer.[2–7] Angiogenesis is determined by the local balance between angiogenic stimulators and inhibitors. The expression of various angiogenesis stimulators, such as vascular endothelial growth factors (VEGFs), angiopoietins, and thymidine phosphorylase, has been described in endometrial cancer.[8–12] However, the significance of endogenous angiogenesis inhibitors in endometrial cancer is poorly documented.

We recently isolated a novel angiogenesis inhibitor, vasohibin, which is specifically expressed in endothelial cells (ECs). Its basal expression in quiescent ECs is low, but it is induced in response to angiogenic stimuli, such as VEGF-A and fibroblast growth factor (FGF)-2, and inhibits angiogenesis in an autocrine manner.[13,14] We therefore propose that vasohibin inhibits angiogenesis as a negative feedback regulator. Among the VEGF family members, VEGF-A is the most important factor for angiogenesis, and most of the VEGF-A-mediated signals for angiogenesis are transduced via VEGF receptor-2 (VEGFR-2).[15] We observed that the VEGF-A-mediated induction of vasohibin was preferentially mediated via the VEGFR-2 signaling pathway.[16]

In the present study, we aimed to elucidate the significance of vasohibin in human endometrium and its disorder(s). We also studied MVD and lymphatic vessel density (LVD). Physiological periodic angiogenesis is observed in functional endometria. We therefore enrolled functional endometria and endometrioid adenocarcinoma, as endometriotic-type endometrial adenocarcinoma, and compared the expression of vasohibin and VEGFR-2. Our analysis revealed a significantly positive correlation between vasohibin and VEGFR-2 in endometrial cancer. This is the first study to profile the expression of vasohibin, a negative feedback regulator of angiogenesis, in gynecologic malignancy.

Materials and Methods

Tissue specimens and clinical data. Seventy-eight endometrioid endometrial carcinomas (27 well differentiated, 25 moderately differentiated, 26 poorly differentiated; 50 stage I, 3 stage II, 20 stage III, 5 stage IV) were retrieved from the surgical pathology files of Tohoku University Hospital, Sendai, Japan. The average age of the patients was 55.6 ± 10.7 years. The protocol for this study was approved by the Ethics Committee at Tohoku University School of Medicine (Sendai, Japan). Each patient provided written informed consent before her surgery. None of the patients examined had received irradiation, hormonal therapy, or chemotherapy prior to surgery. The clinicopathological findings of the patients, including age, histology, stage, grade, and preoperative therapy was retrieved by extensive review of the charts. A standard primary treatment for endometrial carcinoma at Tohoku University Hospital was surgery consisting of total abdominal hysterectomy, salpingo-oopholectomy, pelvic and/or para-aortic lymphadenectomy, and peritoneal washing cytology. The lesions were classified according to the Histological Typing of Female Genital Tract Tumors by the World Health Organization, and staged according to the International Federation of Gynecology and Obstetrics system.[17,18] Patients with subtypes other than endometrioid or

[4]To whom correspondence should be addressed.
E-mail: kito@mail.tains.tohoku.ac.jp

doi: 10.1111/j.1349-7006.2008.00777.x