厚生労働科学研究費補助金
第３次対がん総合戦略研究事業

血管新生とリンパ管新生の同時制御による制癌法の確立に関する研究

平成19年度　総括研究報告書

主任研究者　佐　藤　靖　史

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総括研究報告書

血管新生とリンパ管新生の同時制御による制癌法の確立に関する研究

主任研究者　佐藤靖史　東北大学加齢医学研究所教授

研究要旨
主任研究者の発見した新規血管新生抑制因子Vasohibin-1と、そのホモログのVasohibin-2の血管新生調節における役割分担を明らかにした。特にVasohibin-1は、血管新生のみならずリンパ管新生をも抑制し、マウスへの高リンパ節転移性肺がんの移植モデルにおいてリンパ節転移を抑制する活性のあることを示した。

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A．研究目的
癌の治療標的として血管新生とリンパ管新生が注目されている。理想としては血管新生とリンパ管新生の双方を同時に制御する治療法が望ましいが、そのような治療法は未だ確立していない。主任研究者は、血管新生刺激に反応して血管内皮細胞が産生して血管新生を抑制する新規血管新生抑制因子Vasohibin-1とそのホモログのvasohibin-2を発見した。本研究は、以上のオリジナルな研究成績を背景として、血管新生とリンパ管新生の同時制御による制癌法の確立を目指している。本研究を推進することにより、癌の血管新生とリンパ管新生の双方に対し安全で効果的な制御法を確立し、本邦発の新しい治療法として国民のがん医療に大きく貢献することを目的としている。

B．研究方法
（１）マウスの皮下血管新生モデルを用いてVasohibin-1とVasobihin-2の時間・空間的発現様式を明らかにする。作製したVasohibin-1とVasobihin-2の遺伝子改変マウスについて、血管新生を惹起させてその過程を野生型マウスと比較する。ヒトVasohibin-1またはVasobihin-2遺伝子搭載非増殖型アデノウイルスを尾静注して肝臓で発現させ、血管新生に与える効果を観察する。
（２）マウス角膜法を用いて、VEGF-A、VEGF-C、FGF-2、PDGF-BBなど種々の増殖因子によって惹起される血管新生、リンパ管新生に対するVasohibin-1の効果を検討する。
（３）高転移性ヒト非小細胞肺がん細胞をSCIDマウスに皮下移植、ヒトVasohibin-1遺伝子搭載非増殖型アデノウイルスを尾静注して肝臓で発現させ、移植４週間後に屠殺して癌の発育、血管新生、リンパ管新生、リンパ節転移に対する効果を観察する。この治療後の気管粘膜の血管とリンパ管の形態をコントロールのLacZ遺伝子搭載非増殖型アデノウイルス注射群と比較する。
（４）培養血管内皮細胞にVasohibin-1遺伝子を安定導入し、血清飢餓あるいはH_2O_2処理後の細胞死を解析する。
（倫理面への配慮）
全ての動物実験は所属施設での審査を受けた後に行う。

C．研究結果
（１）マウス皮下の血管新生モデルを用いてVasohibin-1，2の時間的・空間的発現を詳細に検討したところ、野生型マウスでは、Vasohibin-1は、血管新生先端の発芽部位でなく、後方の血管新生終止部位の血管内皮に発現するのに対し、Vasohibin-2は主に発芽部位に浸潤する骨髄由来の単核球に発現していた。これに対し、Vasohibin-1遺伝子改変マウスでは血管新生が終息しなかった。一方、Vasohibin-2遺伝子改変マウスでは発芽部位での血管新生が障害されていた。アデノウイルスベクターを用いてVasohibin-1やVasohibin-2を補充すると、それらの異常は正常化した。
（２）Vasohibin-1は種々の増殖因子によって惹起される血管新生、リンパ管新生を広い作用スペクトルムで抑制した。
（３）高転移性ヒト非小細胞肺がん細胞の移植実験では、LacZ遺伝子搭載非増殖型アデノウイルス注射群と比較して、ヒトVasohibin-1遺伝子搭載非増殖型アデノウイルス注射群では、腫瘍の発育、腫瘍内血管密度、腫瘍周囲リンパ管密度、所属リンパ節の転移はいずれも有意に抑制された。また、気管粘膜の血管とリンパ管の形態はヒ

トVasohibin-1遺伝子搭載非増殖型アデノウイルス注射群で変化を認めなかった。
（4）培養内皮細胞にVasohibin-1を発現させると、血管内皮細胞の増殖は抑制され、血清飢餓やH_2O_2など各種の細胞死を惹起する処理に曝されても血管内皮細胞の細胞死は顕著に抑制された。

D．考察
Vasohibin-1は、発芽部位より後方の血管新生終止部位の血管内皮に発現して、血管新生を止める働きがあるのに対して、Vasohibin-2は主に発芽部位に局在し、Vasohibin-1と拮抗して血管新生を促進すると考えられた。Vasohibin-1は、血管新生のみならずリンパ管新生をも広い作用スペクトルムで抑制するが、正常内皮を障害せず、癌のリンパ節転移制御に対しても有用と考えられた。

E．結論
Vasohibin-1は、血管新生のみならずリンパ管新生に対しても広いスペクトルムで抑制作用を有しており、血管新生とリンパ管新生の同時制御による制癌法への応用が期待される。

F．健康危険情報
健康に対する危険性を認めない。

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瓶子隆弘，宮下浩輝，高橋隆，尾池雄一，佐藤靖史．Vasohibin suppresses lymph node metastasis via the inhibition of lymphangiogenesis in mice．The U.S.-Japan Cooperative Cancer Research Program，京都，2008.

H．知的財産権の出願・登録状況
1．特許
特許出願「バソヒビンからなるリンパ管新生抑制剤」
基礎出願2006-324773
WO PCT/JP2007/072838
2．実用新案登録
なし
3.その他
まし

-03-

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

骨髄細胞・内皮前駆細胞に関する研究

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研究要旨
マウス骨髄細胞中の種々の血液細胞分画におけるvasohibin-1およびvasohibin-2の発現解析から、骨髄細胞中では特に造血幹細胞分画の細胞がこれらを高く発現することが解明された。

A．研究目的

近年、腫瘍環境においては、骨髄から動員された血液細胞が、サイトカインなどの液性因子を分泌して血管新生を正や負に制御することが明らかとなってきた。そこで、本研究では骨髄細胞の中で、いかなる細胞分画の細胞が、vasohibin-1,2を分泌し、腫瘍環境においてどのような役割を果たすのかを解析し、血液細胞による血管新生の制御機構を明確にすることを目的として研究を遂行する。

B．研究方法

マウス大腿骨より骨髄細胞を採取し血液細胞を以下の抗体により染色し、フローサイトメトリーによる解析にて各種細胞を分画採取した。これらの細胞からRNAを抽出して、vasohibin-1, 2の発現をリアルタイムPCRにて半定量的に解析した。

CD11b弱陽性細胞：造血前駆細胞
CD11b強陽性細胞：単球系細胞
CD11b陰性細胞：上記２種と異なる細胞
Lin（CD8、CD4、B220、CD11b、Gr-1、Ter119）陰性、c-Kit陽性、Sca-1陽性細胞：造血幹細胞

（倫理面への配慮）

本研究はマウスを用いた解析であり、実験動物の使用に関しては、大阪大学微生物研究所の定める倫理規定に則って研究を行った。

C．研究結果

骨髄では、造血前駆細胞、単球細胞やその他の成熟した血液細胞ではvasohibin-1, 2ともに発現が認められなかったが、造血幹細胞分画において、vasohibin-1,2のいずれも発現が高いことが判明した。

D．考察

分担研究者は骨髄細胞中では造血幹細胞分画の細胞はAngiopoietin-1を産生して血管形成を促進し、また血管壁細胞に分化して血管安定化あるいは成熟化を誘導することを従来より解明してきた。さらに腫瘍内には多くの造血幹細胞が侵入して血管形成に関与することを明らかにしている。今回血管の成熟化に関するvasohibin-1および血管形成の促進に機能するvasohibin-2の両者を造血幹細胞が発現することから、造血幹細胞の腫瘍環境における血管形成の正の促進作用と負の制御の両者があり得ることが考えられた。

E．結論

造血幹細胞は血管およびリンパ管形成に関与するvasohibin-1およびvasohibin-2の両者を発現し、血管形成に関与することが示唆された。

F．健康危険情報

G．研究発表

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H. 知的財産権の出願・登録状況
（予定を含む。）

なし

厚生労働科学研究費補助金（第３次対がん総合戦略研究事業）
分担研究報告書

リンパ管内皮細胞の遺伝子発現に関する研究

分担研究者　渡部徹郎　東京大学大学院医学系研究科・助教

研究要旨　癌の悪性化を抑制するためには血管新生とリンパ管新生の両方を同時に制御できる治療法を開発することが急務であり、内因性の血管・リンパ管新生抑制因子であるVasohibin-1に注目が集まっている。本研究ではES細胞由来の血管・リンパ管内皮細胞などの培養細胞を用いてVasohibinによる抑制作用の分子機構の解明を試みる。本年度は主にリンパ管発生を調節するProx1転写因子の機能についての研究を行い、リンパ管内皮細胞の分化においてProx1がVEGF受容体３などのさまざまなリンパ管マーカー分子の発現を調節することを見出した。

A．研究目的

リンパ管は末梢組織で血管から漏出した間質液、タンパク質、細胞などを血管系へと環流することにより血液の量や組成を一定に保ち、成体の恒常性を維持するとともに、癌組織から細胞を運んでリンパ節転移を引き起こしたりもする。このためリンパ管形成機構の解明は医学的に必要性が高いが、その研究の歴史は比較的新しく、未解明な点が多く残されている。本研究においてはVasohibinによるリンパ管新生抑制作用の分子機構の解明を試みるために、まずはリンパ管新生において重要な役割を果たすProx1に注目した。

B．研究方法

我々はマウス胚性幹(ES)細胞由来血管細胞およびヒト臍帯静脈内皮細胞(HUVEC)ならびにヒト皮膚由来リンパ管内皮細胞(HDLEC)を用いてProx1の作用を検討した。ES細胞由来血管内皮細胞においてはtetracycline遺伝子発現誘導システムを用いて、HUVECにおいてはアデノウィルスを用いてProx1を発現させた。またHDLECにおいてはsiRNAを用いてProx1の発現を低下させた。

(倫理面への配慮)

ヒトの遺伝子解析ならびに相手方の同意を得る研究は本研究計画には含まれていない。また本研究ではマウス胚性幹(ES)細胞を用いるが、ヒトES細胞を用いる予定はない。

C．研究結果

血管内皮細胞においてProx1を発現させることで、VEGFR3の発現が亢進し、VEGF-C (VEGFR3に対するリガンド)に対する走化性が上昇した。また、Prox1を発現した血管内皮細胞ではシート形成が阻害され、運動性が亢進することが示された。この作用はProx1により発現が亢進するintegrin α9に対する中和抗体により抑制された。さらに成熟したリンパ管内皮細胞におけるProx1の発現を低下させることでVEGFR3やintegrin α9の発現が減少し、VEGF-Cへの走化性が低下することが示された(Mishima, Watabe et al., 2007)。

D．考察

以上の結果からProx1は血管内皮細胞において、さまざまなシグナル伝達因子の発現を調節することで、内皮細胞の増殖や走化性などを調節し、リンパ管発生を推し進めていることが示唆された。

E．結論

Prox1はリンパ管内皮細胞の性質を決定・維持するマスター因子であり、リンパ管新生抑制の新たな標的となりうる。現在VasohibinがProx1の発現や機能に与える影響を検討している。

F．健康危険情報

該当なし

G．研究発表

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2. Oka M, Iwata C, Suzuki H, Kiyono K, Morishita Y, Watabe T, Komuro A, Kano MR, Miyazono K. (2008) Inhibition of endogenous TGF-β signaling enhances lymphangiogenesis *Blood*, in press
3. Suzuki Y, Montagne K, Nishihara A, *Watabe T, Miyazono K. (2008) BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and Angiopoietin-1/Tie2 signals. *Journal of Biochemistry. In press.* (*: corresponding author)
4. Moore ML, Teitell MA, Kim Y, Watabe T, Reiter RE, Witte ON, Dubey P. (2008) Deletion of PSCA increases metastasis of TRAMP-Induced prostate tumors without altering primary tumor formation. *Prostate.* 68:139-151.
5. Mishima K, Watabe T, Saito A, Yoshimatsu Y, Imaizumi N, Masui S, Hirashima M, Morisada T, Oike Y, Araie M, Niwa H, Kubo H, Suda T, Miyazono K. (2007) Prox1 induces lymphatic endothelial differentiation via integrin α9 and other signaling cascades. *Molecular Biology of the Cell.* 18:1421-1429.
6. Ogawa K, Saito A, Matsui H, Suzuki H, Ohtsuka S, Shimosato D, Morishita Y, Watabe T, Niwa H, Miyazono K. (2007) Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells. *Journal of Cell Science.* 120:55-65.

2. 学会発表

国際学会および集会

European Society of Hemamtology (ESH) Euroconference: Angiogenesis (Albufeira, ポルトガル) 2007年5月11日～14日
Tetsuro Watabe, Koichi Mishima, Natsuko Imaizumi, Shinji Masui, Hajime Kubo, Yuichi Oike, Toshio Suda, Kohei Miyazono: ROLES OF PROX1 DURING LYMPHATIC DIFFERENTIATION OF EMBRYONIC STEM (ES) CELL-DERIVED ENDOTHELIAL CELLS (口頭発表)

第4回 Korea-Japan Joint Symposium on Vascular Biology, 2007 (Chuncheon, 韓国) 2007年8月16日～17日
Tetsuro Watabe, Koichi Mishima, Masanori Hirashima, Tohru Morisada, Yuichi Oike, Toshio Suda, and Kohei Miyazono: Prox1 induces lymphatic endothelial differentiation via integrin α9 and other signaling cascades (口頭発表)

Gordon Research Conferences (Molecular Mechanisms In Lymphatic Function & Disease) (Ventura, アメリカ合衆国) 2008年3月2日～7日
Tetsuro Watabe, Yasuhiro Yoshimatsu, Tomoko Yamazaki, Masato Motikawa, Kohei Miyazono: Regulation of Prox1-mediated lymphatic differentiation of endothelial cells by transcriptional regulators (口頭発表)

The U.S.-Japan Cooperative Cancer Research Program, Workshop 2008 (京都) 2008年3月19日～21日
Tetsuro Watabe, Yasuhiro Yoshimatsu, Tomoko Yamazaki, Masato Morikawa, and Kohei Miyazono Regulation of Prox1-mediated lymphatic differentiation of endothelial cells by transcriptional regulators (ポスター)

● 国内学会および集会

第15回 日本血管生物医学会学術大会 (福岡) 2007年11月29日～30日
渡部 徹郎：シンポジウム リンパ管発生における転写・シグナルネットワークの役割

第39回 第30回日本分子生物学会年・第80回日本生化学会大会合同大会 (BMB2007)ワークショップ「血管・リンパ管発生の新展開」(横浜) 2007年12月11日～15日
渡部 徹郎、三嶋 弘一、今泉 奈津子、升井 伸治、丹羽 仁史、久保肇、平島正則、尾池雄一、須田年生、宮園 浩平：血管・リンパ管発生における転写・シグナルネットワークの役割

H. 知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

書籍

著者氏名	論文タイトル名	書籍全体の編集者名	書　籍　名	出版社名	出版地	出版年	ページ
Sato, Y	Endogenous inhibitors of angiogenesis	ME Maragoudakis, E Papadimittiou	Angiogenesis: Basic Science and Clinical Applications	Transworld Research Network	India	2007	1-14
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Suzuki Y, Montagne K, Nishihara A, Watabe T, Miyazono K.	BMPs promote proliferation and migration of endothelial cells via stimulation of VEGF-A/VEGFR2 and Angiopoietin-1/Tie2 signals.	Journal of Biochemistry	in press		2008
Moore ML, Teitell MA, Kim Y, Watabe T, Reiter RE, Witte ON, Dubey P.	Deletion of PSCA increases metastasis of TRAMP-Induced prostate tumors without altering primary tumor formation.	Prostate	68	139-151	2008
Mishima K, Watabe T, Saito A, Yoshimatsu Y, Imaizumi N, Masui S, Hirashima M, Morisada T, Oike Y, Araie M, Niwa H, Kubo H, Suda T, Miyazono K.	Prox1 induces lymphatic endothelial differentiation via integrin ・9 and other signaling cascades.	Molecular Biology of the Cell	18	1421-1429	2007
Ogawa K, Saito A, Matsui H, Suzuki H, Ohtsuka S, Shimosato D, Morishita Y, Watabe T, Niwa H, Miyazono K.	Activin-Nodal signaling is involved in propagation of mouse embryonic stem cells.	Journal of Cell Science	120	55-65	2007

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

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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)

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

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with second primary carcinoma were excluded from this series, because the endometrioid type and the others are referred to as type 1 and type 2 endometrial cancer are considered to originate from different mechanisms and exhibit different clinical behaviors.[17]

We also examined 12 sections of normal cyclic endometria derived from surgically resected specimens for benign uterine diseases, six of which were in the proliferative phase and six of which were in the secretory phase. The average age of these patients was 38.7 ± 4.9 and 40.3 ± 6.1 years, respectively. All specimens were routinely processed (i.e. 10% formalin fixed for 24–48 h), paraffin embedded, and thin sectioned (3 μm).

Immunohistochemical staining and scoring of immunoreactivity. We performed immunohistochemical staining for vasohibin, VEGFR-2, CD34 as a marker for vascular endothelial cells, and D2-40 as a lymphatic vessel marker. Ki-67 and VEGF-A were investigated in endometrial carcinoma cells. Paraffin-embedded tissue sections from human endometrial cancers were deparaffinized, rehydrated, and incubated with 3% H_2O_2 for 10 min to block endogenous peroxidase activity. Sections were incubated for 30 min at room temperature (RT) in a blocking solution of 10% goat serum (Nichirei Biosciences, Tokyo, Japan), and then stained for 12 h at 4°C with primary antibodies, followed by staining for 30 min at RT with secondary antibodies. The primary antibodies were all mouse monoclonal antibodies and were used as follows: 2 μg/mL antihuman vasohibin monoclonal antibody, anti-VEGFR-2 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted 1:100, anti-CD34 (Dako, Copenhagen, Denmark) diluted at 1:200, Ki-67 (Dako) diluted 1:100, anti-D2-40 (Dako) diluted 1:100, and anti-VEGF-A (Lab Vision, Fremont, CA, USA) diluted 1:100. We have previously described a mouse monoclonal antibody against a synthetic peptide corresponding to the 286–299 amino acid sequence of vasohibin.[13] The positive control slide for CD34 antigen was prepared from paraffin-fixed breast cancer tissue that was known to contain a high microvessel density. Nuclei were counterstained with hematoxylin.

Three investigators (A.T., H.T., M.K.) independently evaluated the immunohistochemical staining of the tissue sections. They were blinded to the clinical course of the patients and the average of the numbers counted by the three investigators was adopted for subsequent analysis. We carefully selected for investigation areas where cancer cells came into contact with or invaded into the stroma. First, microvessels were counted by searching for CD34-positive signals after scanning the immunostained section at low magnification. The areas with the greatest number of distinctly highlighted microvessels were selected. Any cell clusters with CD34-positive signals were regarded as a single countable microvessel, regardless of whether a lumen was visible or not. Unstained lumina were considered artifacts even if they contained blood or tumor cells. Microvessel density (MVD) was assessed by light microscopy in areas of invasive tumor containing the highest numbers of capillaries and small venules per area (neovascular hot spots) according to the original method.[19] In endometrioid adenocarcinoma, the ratio of stroma per total area (as neovascular hot spots) decreased significantly with poorer histological differentiation. Thus, we measured the ratio of stroma per total area using National Institute of Health imaging (200 × magnification hot spot picture captured with Nikon imaging) and revised the true vessel counts per 1 mm^2 of stroma (microvessel density) for each case.

Investigation of lymphatic vessel density (LVD) was performed using the same procedure described as above, by searching for D2-40-positive signals.

Next, immunostaining for vasohibin and VEGFR-2 was evaluated in serial thin sections. Positive immunoreactive signals for vasohibin and VEGFR-2 in the CD34-positive microvessels were counted and calculated as positive ratios of vasohibin and VEGFR-2 in microvessels. Evaluation of Ki-67 immunoreactivity was performed at high-power field (400X) and used as a marker of cell proliferation. More than 500 tumor cells from each of three different representative fields were counted and the percentage of the number of positively stained nuclei relative to the total numbers of cells were determined as a labeling index (LI). The protein expression of selected angiogenic factor (VEGF-A) was examined by immunohistochemistry using an established antibody. For this marker, cytoplasmic staining intensity and the proportion of positive tumor cells were recorded and a staining index (values of 0–9) was calculated as the product of staining intensity (0–3) and the area of positive staining (1, <10%; 2, 10–50%; 3, > 50%).[20]

Statistical analysis. Statistical analysis, such as the Student's *t*-test and Peason's correlation coefficient test, were performed using StatView (version 4.5; SAS Institute Inc., Cary, NC, US). The results were considered significant when the *P*-values were <0.05.

Results

Microvessel density of endometrioid adenocarcinoma. CD34-positive microvessel density (counts per mm^2) were 41.1 ± 3.1, 36.9 ± 2.3, and 29.7 ± 1.7 in G1, G2, and G3, respectively; 35 ± 2.70 in the proliferative phase; and 36.3 ± 1.83 in the secretory phase (Figs 1 and 2). We measured the ratio of the stromal area per total hot spot area in the location with the greatest number of distinctly highlighted microvessels. In G1, the ratio was 32.7% ± 0.24; in G2, 27.5% ± 0.19; in G3, 5.8% ± 0.05; 73.2% ± 2.70 in the proliferative phase; and 70.0% ± 1.83 in the secretory phase. The ratio of the stromal area per total area of G3 was significantly lower than that of G1 and G2. CD34-positive microvessel density (counts per mm^2), as revised by the ratio of the stroma per total area, was 141.65 ± 13.2 in G1, 168.62 ± 19.38 in G2, and 788.94 ± 105.8 in G3, respectively; 48.71 ± 2.70 in the proliferative phase; and 52.45 ± 1.83 in the secretory phase. The MVD of G3 was significantly higher than the MVD of G1 and G2 (Fig. 3a).

Lymphatic vessel density of endometrioid adenocarcinoma. D2-40 positive lymphatic vessel density (counts per mm^2) was 4.73 ± 0.70 in G1, 8.83 ± 2.24 in G2, and 2.88 ± 0.54 in G3, respectively; 1.80 ± 0.20 in the proliferative phase; and 6.10 ± 0.50 in the secretory phase (Figs 1 and 2). D2-40 positive lymphatic vessel density (counts per mm^2), as revised by the ratio of stroma per total area, was 15.90 ± 2.30 in G1, 9.47 ± 1.95 in G2, and 91.01 ± 23.06 in G3, respectively; 2.53 ± 0.36 in the proliferative phase; and 8.83 ± 0.82 in the secretory phase. The LVD of G3 was significantly higher than the LVD of G1 and G2 (Fig. 3b).

Vasohibin expression in microvessels in endometrial tissues. The positive ratios of vasohibin in microvessels were 31.4% and 43.1% in the proliferative and secretory phases, respectively (Fig. 2). The positive ratios were significantly different between the two phases (Fig. 4a). In addition, the endothelium of the spiral arteries characteristically exhibited positivity in the secretory phase; the positive ratio of vasohibin was 95.4% and the positive ratio of VEGFR-2 was 87.4%. (Fig. 2).

The positive ratio of vasohibin in microvessels was 56.6% ± 12.8 in the endometrioid adenocarcinoma cases examined. Interestingly, the positive ratios differed according to grade: 52.5 ± 13.9 in G1 (well differentiated), 57.8% ± 13.5 in G2 (moderately differentiated), and 59.9% ± 10.0 in G3 (poorly differentiated) (Fig. 1). The positive ratio of vasohibin in microvessels in G3 was significantly higher than that in G1 (Fig. 4a). Positive ratios of vasohibin in microvessels in each histlogical grade were significantly higher than that of the proliferative phase ($P < 0.05$). Positive ratios were compared in clinical stages: 56.2% ± 11.2 in stage 1, 61.9% ± 15.5 in stage 2,

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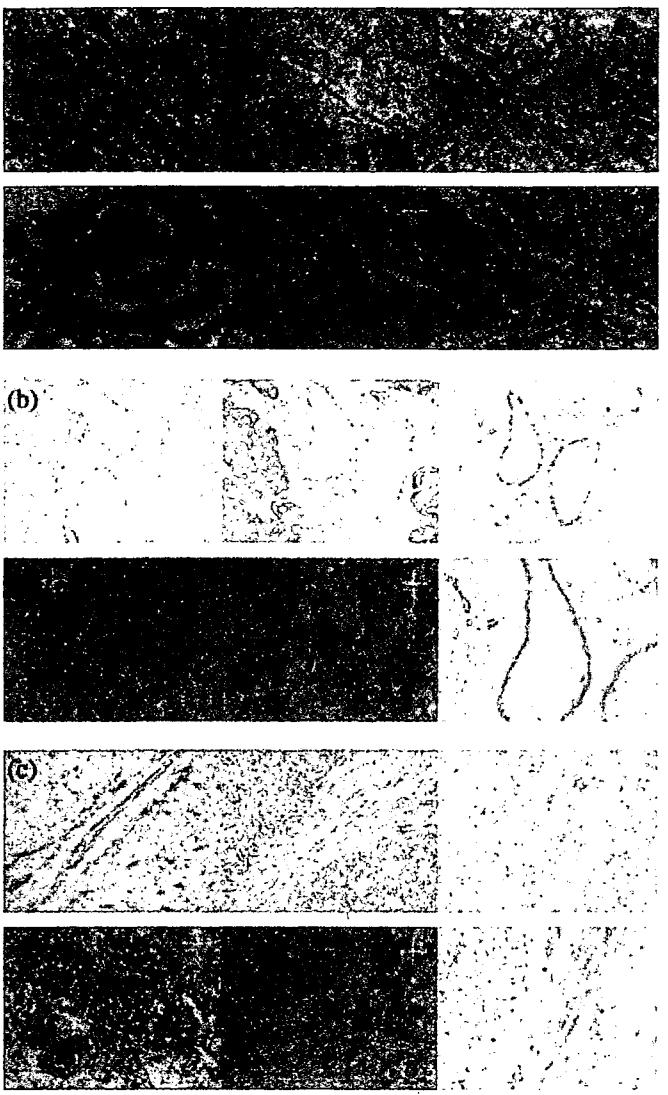

Fig. 1. (a) Immunohistochemistry in well-differentiated endometrioid adenocarcinomas. All sections stained positively for CD34 (upper left, original magnification 200×), vascular endothelial growth factor receptor-2 (VEGFR-2) (upper middle, original magnification 200×), vasohibin (upper right, original magnification 200× and lower right, original magnification 400×), and vascular endothelial growth factor-alpha (VEGF-A) (lower left, original magnification 200×). D2-40 (lower middle, original magnification 200×). (b) Immunohistochemistry in moderately differentiated adenocarcinoma. All sections stained positively as for (a). (c) Immunohistochemistry in poorly differentiated adenocarcinoma. All sections stained positively as for (a).

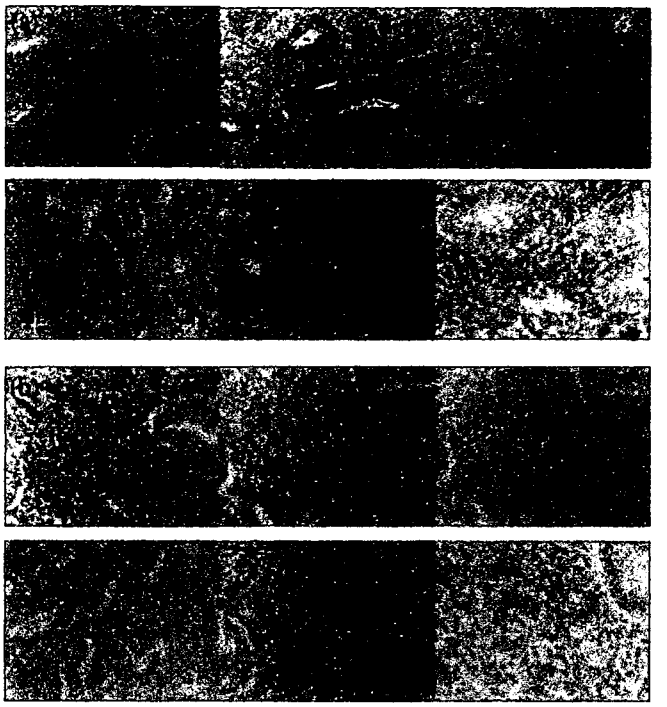

Fig. 2. (a) Immunohistochemistry in proliferative phase of cyclic endometria. All sections stained positively for CD34 (upper left, original magnification 200×), vascular endothelial growth factor receptor-2 (VEGFR-2) (upper middle, original magnification 200×), vasohibin (upper right, original magnification 200× and lower right, original magnification 400×), and vascular endothelial growth factor-alpha (VEGF-A) (lower left, original magnification 200×). D2-40 (lower middle, original magnification 200×). (b) Immunohistochemistry in secretory phase of cyclic endometria. All sections stained positively as for (a). Immunopositivity was significantly different between the two phases for vasohibin and VEGFR-2. The endothelium of the spiral arteries exhibited characteristic positivities in the secretory phase.

56.7% ± 12.9 in stage 3, and 57.7% ± 21.3 in stage 4. There was no significant difference between each group.

VEGFR-2 expression in microvessels in endometrial tissues. The VEGFR-2 positive ratio of the microvessels was 8.6% ± 1.0 and 22.5% ± 3.0 in the proliferative and secretory phases, respectively (Fig. 2). VEGFR-2 positive vessel ratio in the proliferative phase was significantly higher than in the secretory phase (Fig. 4b).

VEGFR-2 positive ratios in G1, 2, and G3 were 22.3% ± 13.0, 39.8% ± 12.8, and 47.6% ± 11.5, respectively (Fig. 1). The VEGFR-2 positive ratio in G2 was significantly higher than in G1 and cyclic endometria. The VEGFR-2 positive ratio in G3 was significantly higher than those of G1 and G2 (Fig. 4b). Positive ratios were compared between clinical stages: 32.8% ± 15.4% in stage 1, 33.9% ± 19.0 in stage 2, 37.5% ± 15.1 in stage 3, and 46.4% ± 16.8 in stage 4. There was no significant difference between each group.

VEGF-A expression in endometrial tissues. VEGF-A expression was detected in the cytoplasm of epithelial cells. The staining index of VEGF-A is shown in Figure 5. The VEGF-A positive ratio of cytoplasmic staining intensity was 0.67 ± 0.49 and 2.33 ± 0.76 in the proliferative and secretory phases, respectively (Fig. 5). In endometrioid adenocarcinomas VEGF-A positive ratios of cytoplasmic staining in grades 1, 2, and 3 were 3.85 ± 0.39, 4.2 ± 0.37, and 5.88 ± 0.37, respectively (Fig. 5). The VEGF-A positive ratio in G3 was significantly higher than in G1 and 2. The VEGF-A positive ratio in the proliferative phase was significantly lower than G1, G2, and G3, respectively, and the VEGF-A positive ratio in secretory phase was significantly lower than G2 and G3, respectively.

Correlation between vasohibin and VEGFR-2 positive ratios in microvessels, and cell proliferation and expression of angiogenic factor. A strongly positive correlation was found between vasohibin and VEGFR-2 positive ratios in microvessels in endometrioid adenocarcinomas ($P < 0.0001$, $r^2 = 0.591$) (Fig. 6).

We then analyzed vasohibin and VEGFR-2 positive ratios in comparison to cell proliferation and expression of angiogenic factor. The ratios of both vasohibin- and VEGFR-2-positivity did not correlate significantly to the LI of Ki-67 (data not

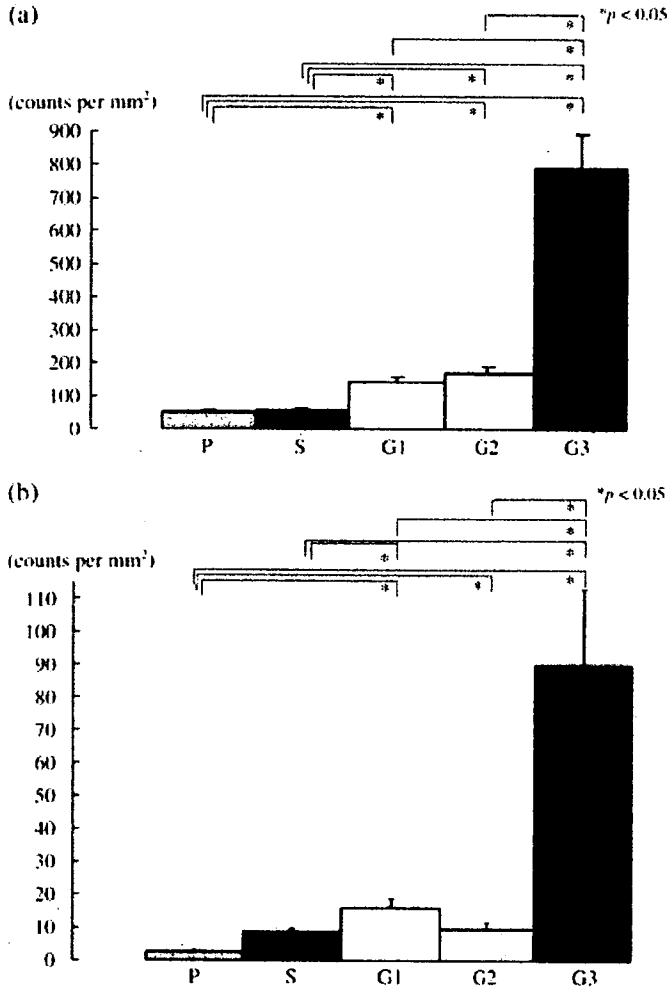

Fig. 3. (a) Microvessel density of cyclic endometria and endometrioid adenocarcinoma. Microvessel density of G3 was significantly higher than that of G1 and G2. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma, $*P < 0.05$. (b) Lymphatic vessel density of cyclic endometria and endometrioid adenocarcinoma. Lymphatic vessel density of G3 was significantly higher than that of G1 and G2. G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma, $*P < 0.05$.

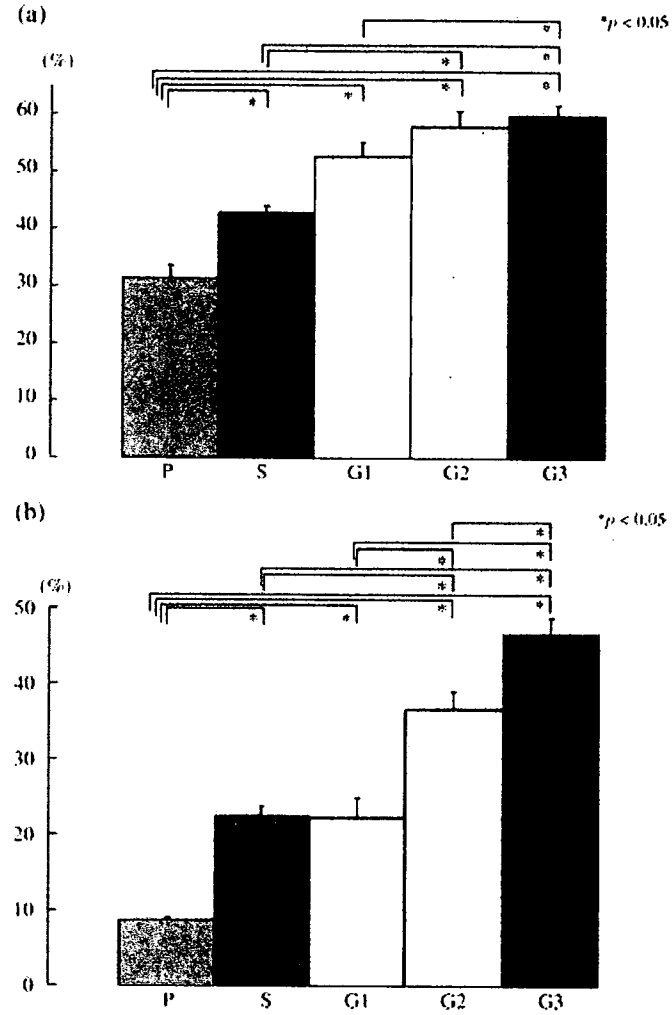

Fig. 4. (a) Proportion of vasohibin/CD34-positive vessels in cyclic endometria and endometrioid adenocarcinoma. Vasohibin-immunopositivity in microvessels in G3 was significantly higher than that in G1. S, secretory phase; P, proliferative phase; G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma, $*P < 0.05$. (b) Proportion of vascular endothelial growth factor receptor-2 (VEGFR-2)/CD34-positive vessels in cyclic endometria and endometrioid adenocarcinoma. VEGFR-2-immunopositivity of vessels in the proliferative phase was significantly higher than in the secretory phase. S, secretory phase; P, proliferative phase; G1, well-differentiated adenocarcinoma; G2, moderately differentiated adenocarcinoma; G3, poorly differentiated adenocarcinoma, $*P < 0.05$.

shown). No significant correlation was observed between the vasohibin- and VEGFR-2-positive ratios and VEGF-A expression in endometrioid adenocarcinoma (data not shown).

Discussion

Here we examined the vascular density of endometrial cancer and compared it with that of normal endometrium. Some reports have previously indicated that MVD of endometrial cancer increases from well differentiated to poorly differentiated adenocarcinomas[4,21,22] but this relationship is not universally accepted.[23] Moreover, most of them have failed to consider the vessel numbers in normal endometrium and to compare it with those of adenocarcinoma. In the present study, we confirmed that the vessel number increased from normal endometrium to endometrial cancer, and that this increase was significantly augmented in poorly differentiated adenocarcinoma.

Recently, focus has been given to the importance of lymphangiogenesis for tumor metastasis.[24,25] Here, we investigated LVD in normal endometrium and endometrioid adenocarcinoamas. Our analysis revealed that LVD increased significantly in poorly differentiated adenocarcinoma, similar to MVD. Some studies have reported the presence of peritumoral lymphatic vessels in 40% of the cases in endometrial cancer and demonstrated that high LVD was strongly associated with the features of aggressive endometrial carcinomas, including high histological grade, presence of necrosis, and vascular invasion by tumor cells.[26–28] Although it was expected that frequent LVD correlated with lymph node metastasis, there was no significant correlation between LVD and lymph node metastasis. In the present study, only two cases out of 78 cases exhibited lymph node metastasis. Therefore, investigation of a greater number of cases with lymph node metastasis in endometrioid adenocarcinoma will be necessary to further elucidate this correlation. The

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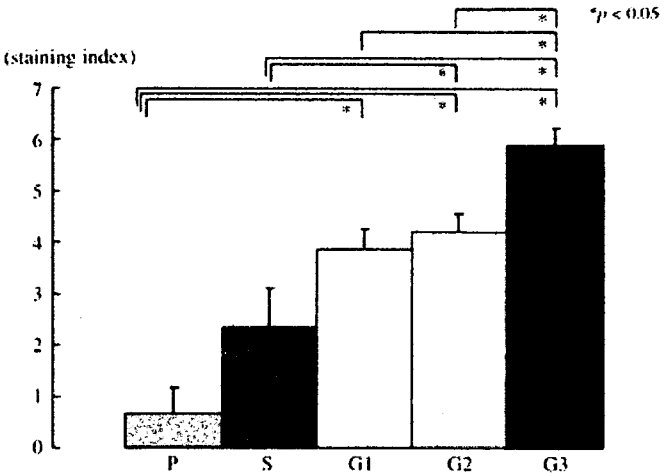

Fig. 5. Vascular endothelial growth factor-alpha (VEGF-A) staining indexes of the cytoplasm of the tumor cell and cyclic endometrial glands. $*P < 0.05$.

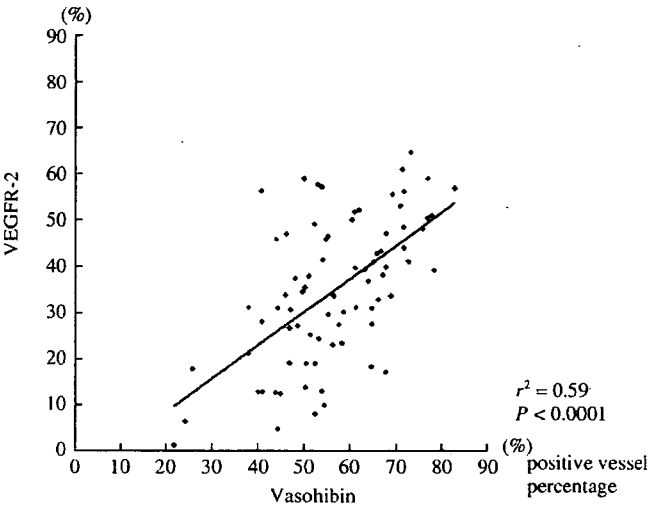

Fig. 6. Correlation between vasohibin and vascular endothelial growth factor receptor-2 (VEGFR-2). A strongly positive correlation was found between vasohibin- and VEGFR2-positive ratios in microvessels in endometrioid adenocarcinomas ($P < 0.0001$, $r^2 = 0.591$).

mechanism of the alteration of lymphangiogenesis from cyclic endometria to endometrioid adenocarcinoma remains unclear. Our analysis of MVD and LVD demonstrated that the secretory phase is similar to well differentiated and moderately differentiated endometrioid adenocarcinomas, which suggests that the function of cyclic endometria may be retained until adenocarcinomas become moderately differentiated.

We then examined the expression of vasohibin. Vasohibin is an endogenous endothelium-derived angiogenesis inhibitor that we have previously isolated.[13] Here we confirmed that the expression of vasohibin was restricted to vascular endothelium, and further observed that the ratio of vasohibin-positive vessels increased from normal endometrium to poorly differentiated adenocarcinomas. This is the first study to profile the expression of vasohibin in human gynecologic malignancy.

Several clinicopathologic studies have demonstrated a direct association between VEGF expression and increased MVD in human solid tumors, including breast[29] lung,[30] and gastric[31] malignancies. A similar association has been reported for normal[32] and malignant endometrium.[33–35] Between the two VEGF signal transducing receptors, VEGFR-2 transduces most of the angiogenesis-related signals in ECs. The VEGF/VEGFR-2 signaling pathway is also important for the induction of vasohibin in ECs.[16] We previously revealed that the VEGF-A-mediated induction of vasohibin was preferentially mediated via the VEGFR-2 signaling pathway.[16]

Our present analysis revealed that the ratio of VEGFR-2-positive vessels, as well as the ratio of vasohibin-positive vessels, also increased from normal endometrium to poorly differentiated endometrial adenocarcinomas. In addition, a significantly positive correlation existed between the positive ratios of vasohibin and VEGFR-2 expression in endometrioid endometrial carcinomas. This is the first study to elucidate this correlation between expression of these factors in human cancer. This result suggested the value of vasohibin as a biomarker of angiogenesis at least in endometrial cancer.

Angiogenesis is determined by the local balance between angiogenic stimulators and inhibitors. Therefore, one may anticipate the application of angiogenesis inhibitors towards antiangiogenic therapy for the treatment of human malignancies including endometrial cancer. A number of angiogenesis inhibitors have been investigated and identified, including pigment epithelium-derived factor (PEDF), angiostatin, endostatin, and thrombospondin-1 (TSP-1). Vasohibin is a newly identified negative feedback regulator for angiogenesis. We previously reported that transfection of Lewis lung carcinoma (LCC) cells with the vasohibin gene did not affect the proliferation of cancer cell *in vitro*, but did inhibit tumor growth and tumor angiogenesis *in vivo*.[13] The growth of vasohibin-producing LLC cells in mice was significantly attenuated. In addition, tumors of mock-transfectants contained large luminal vessels, whereas those of vasohibin-producing LLC cells contained very small vessels, even when the size of tumors did not differ extremely.[14] These results suggest that vasohibin may play a very important role in regulating tumor angiogenesis.

Among the various angiogenesis inhibitors, thrombospondin-1 (TSP-1) has been extensively studied in cancers, although the role of TSP-1 in endometrial tumor angiogenesis and progression still remains controversial.[36] The expression of TSP-1 in epithelial cells and/or caner cells is up-regulated by the tumor suppressor gene *p53*, and down-regulated by oncogenes such as Myc and Ras. Thus, the mutation of *p53* or activation of *myc* and *ras* results in the down-regulation of TSP-1, which may alter tumor growth by modulating angiogenesis in a variety of tumor types. Herein, we demonstrated that the expression of vasohibin increased in ECs of endometrial cancer. As the expression of vasohibin was restricted to normal ECs, the alteration of tumor suppressor gene and/or oncogenes in tumor cells would not influence the expression of vasohibin. However, angiogenesis inhibitors may function in a concerted manner. Therefore, vasohibin alone may not be sufficient to control tumor angiogenesis, if other inhibitors become deregulated.

Nevertheless, since the expression of vasohibin increased in correlation with that of VEGFR-2, vasohibin could be an important biomarker of angiogenesis in both normal endometrium and endometrial cancer. However, further investigations are required to clarify the precise roles of vasohibin in regulating antiangiogenic activity in normal endometrium and its disorders.

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Editorial

VEGFR1 for Lymphangiogenesis
An Alternative Signaling Pathway?

Yasufumi Sato

The lymphatic vascular system is a conduit for interstitial fluid extravasated from blood vessels and also plays important roles in maintaining immune responses, lipid uptake, and tissue homeostasis. In recent years, much attention has been given to lymphangiogenesis, a formation of new lymphatic vessels, because lymphangiogenesis has been shown to be involved in lymph node metastasis of tumors.[1]

See accompanying article on page 658

The development of blood and lymphatic vascular systems is primarily regulated by vascular endothelial growth factor (VEGF) family members. This family consists of 5 members: VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). There are 3 members of VEGF receptor (VEGFR) tyrosine kinases: VEGFR1, VEGFR2, and VEGFR3. Members of the VEGF family show different affinities for these receptors. VEGFR1 is able to bind VEGF-A, VEGF-B, and PlGF. VEGFR2 is activated primarily by VEGF-A, but cleaved forms of VEGF-C and VEGF-D may also activate this receptor. VEGFR3 is activated by VEGF-C and VEGF-D. Vascular endothelial cells (ECs) express VEGFR1 and VEGFR2, whereas lymphatic ECs express VEGFR2 and VEGFR3 in the adult. The most important molecule in the VEGF family that controls angiogenesis is VEGF-A, and VEGFR2 is the major mediator of VEGF-A driven responses in vascular ECs. VEGFR1, on the other hand, has a higher affinity for VEGF-A but weaker tyrosine kinase activity. Thus, VEGFR1 on vascular ECs may act as a counter-regulator of VEGFR2. VEGFR1 is also expressed by monocytes/macrophages and hematopoietic stem cells, and in those cases, VEGFR1 transduces signal for the migration of those cells. Therefore, VEGFR-1 has a dual function, acting in a positive or negative manner in different cell types or circumstances. The most important molecule in the VEGF family that controls lymphangiogenesis are VEGF-C and VEGF-D, and VEGFR3 is the major mediator of VEGF-C and VEGF-D driven responses in lymphatic ECs. However, VEGF-A may stimulate VEGFR2 on lymphatic ECs and induce lymphangiogenesis directly.[2]

The induction of VEGF-A is important for the initiation of angiogenesis. The principal trigger of angiogenesis is hypoxia, which induces the expression VEGF-A in various cell types. This induction in hypoxia is mediated by a transcription factor known as hypoxia-inducible factor 1 (HIF-1), a heterodimeric complex of HIF-1α and HIF-1β subunits, which binds to hypoxia responsive element (HRE) in the promoter of VEGF-A gene. Under normal oxygen tension, the expression of VEGF-A is suppressed by the product of a tumor suppressor gene known as the von Hippel Lindau (VHL) gene, which is involved in the degradation of HIF-1α through the ubiquitin proteasome system.[2] Angiogenesis is normally associated with, or followed by, lymphangiogenesis, because defective lymphangiogenesis should cause tissue edema. However, little is known about the trigger of lymphangiogenesis. The aberrant expression of VEGF-C or VEGF-D and its correlation with lymph node metastasis was described in various tumors.[1] Nevertheless, only a few studies have focused on the regulation of the expression of VEGF-C or VEGF-D. A transcription factor known as nuclear factor-kappa B (NF-κB) or an enzyme known as cycloxygenase-2 (COX-2) is involved in the upregulation of VEGF-C in certain cancer cells.[3–5]

In this issue of *Arteriosclerosis, Thrombosis, and Vascular Biology*, Murakami et al propose that the signal via VEGFR1 promotes angiogenesis in parallel with lymphangiogenesis through the recruitment of macrophages. To test the function of VEGFR1, they use K14 *Vefg-A* Tg mice, *Vegfr1* $tk^{-/-}$ mice, and a double mutant of K14 *Vefg-A* Tg *Vegfr1* $tk^{-/-}$ mice. K14 *Vefg-A* Tg *Vegfr1* $tk^{-/-}$ mice show a significant decrease in angiogenesis and lymphangiogenesis in subcutaneous tissue where VEGF-A is overexpressed. To address the mechanism underlying this decrease of angiogenesis and lymphangiogenesis in K14 *Vefg-A* Tg *Vegfr1* $tk^{-/-}$ mice, they focus on the recruitment of macrophages into subcutaneous tissue. VEGF-A augments the recruitment of macrophages through the activation of VEGFR1 on macrophages. This recruitment of macrophages is reduced in K14 *Vefg-A* Tg *Vegfr1* $tk^{-/-}$ mice. Moreover, K14 *Vefg-A* Tg mice that receive bone marrow transplantation from *Vegfr1* $tk^{-/-}$ mice show the reduction of macrophage recruitment as well as the decrease of angiogenesis and lymphangiogenesis. Thus, they conclude that VEGF-A stimulates mobilization and recruitment of macrophages from bone marrow through VEGFR1, and that is required for angiogenesis and lymphangiogenesis.

The role of macrophage recruitment in angiogenesis has been well documented.[6] In addition, several recent articles described the involvement of macrophage recruitment in lymphangiogenesis as well. Schoppmann et al showed that, in certain human cancers, the density of lymphatic microvessels was significantly increased in peritumoral stroma, and that a subset of cells in peritumoral stroma, namely tumor-

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associated macrophages (TAMs), expressed VEGF-C and VEGF-D. Their data indicated that the density of TAMs producing VEGF-C and VEGF-D correlated with peritumoral inflammatory reaction, peritumoral lymphangiogenesis, and frequency of lymph node metastasis.[7,8] Cursiefen et al reported the relationship between VEGF-A stimulated lymphangiogenesis and macrophage recruitment in the suture-induced inflammatory corneal model of mice. Administration of VEGF Trap, a receptor-based fusion protein that neutralized VEGF-A but not VEGF-C or VEGF-D, completely inhibited both angiogenesis and lymphangiogenesis after corneal injury. Moreover, either systemic depletion of bone marrow–derived cells by irradiation or local depletion of macrophages in the cornea by clodronate liposome significantly inhibited angiogenesis and lymphangiogenesis in the cornea.[9] Maruyama et al revealed that the decreased number of macrophages correlated with the reduced lymphangiogenesis in the diabetic skin wound healing model.[10] These reports together with the present Murakami's work point out the involvement of macrophage recruitment in lymphangiogenesis in certain conditions.

The scenario of angiogenesis and associating (or following) lymphangiogenesis may be as follows. The trigger of these phenomena should be the induction of VEGF-A. VEGF-A stimulates vascular ECs via VEGFR2 and that initiates angiogenesis. Simultaneously, VEGF-A induces the mobilization of bone marrow cells including monocytes via VEGFR1. Monocytes recruit to the area of angiogenesis, differentiate to macrophages, and produce both angiogenesis and lymphangiogenesis stimulators. Angiogenesis stimulators include VEGF-A, whereas lymphangiogenesis stimulators include VEGF-C and VEGF-D. Accordingly, VEGF-A from macrophages potentiates angiogenesis via VEGFR2 on vascular ECs, whereas VEGF-C and VEGF-D from macrophages induce lymphangiogenesis via VEGFR3 on lymphatic ECs. Of course, this scenario is drawn from data obtained by the models of pathological conditions. It is still unclear how physiological lymphangiogenesis is regulated. What cell type is the main source of VEGF-C and VEGF-D in the physiological condition? How is the expression of VEGF-C and VEGF-D regulated? Further studies are expected to resolve these questions.

Disclosures

None.

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