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Expression of Vascular Endothelial Growth Factor and E-Cadherin in Human Ovarian Cancer: Association with Ascites Fluid Accumulation and Peritoneal Dissemination in Mouse Ascites Model

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Ascites formation and peritoneal dissemination are critical problems in patients with advanced ovarian cancer. Vascular endothelial growth factor (VEGF), also known as angiogenic growth factor, is a potent mediator of peritoneal fluid accumulation and angiogenesis of tumors. E-Cadherin is an adhesion molecule that is important for cell-to-cell interaction. To elucidate the molecular mechanism of ascites formation and peritoneal dissemination of ovarian cancer, we examined the expression of VEGF and E-cadherin in different ovarian cancer cell lines and utilized nude mice to compare the biological characteristics of ovarian cancer cells. Three human ovarian cancer cell lines (AMOC-2, HNOA and HTBOA) were used in this study. Expression of genes was analyzed by northern blotting and RT-PCR methods. AMOC-2 expressed E-cadherin, but not VEGF. HNOA expressed VEGF without E-cadherin expression. HTBOA expressed both VEGF and E-cadherin. Each human ovarian cancer model revealed a specific feature. The AMOC-2 mouse had a single large peritoneal tumor without ascites or remarkable peritoneal dissemination. HTBOA and HNOA mice had bloody ascites and marked peritoneal dissemination. Introduction of VEGF antisense into HTBOA cells could inhibit the ascites formation. It is suggested that VEGF is important for the ascites formation via the increased vascular permeability effect. The deregulation of E-cadherin expression might be involved in the peritoneal dissemination. These molecules are important for the formation of specific features of advanced ovarian cancer. Ovarian cancer cell lines that had different gene expression patterns produced nude mouse human ovarian cancer models with different characteristics.

Key words: Vascular endothelial growth factor — E-Cadherin — Ovarian cancer — Ascites — Dissemination

Ovarian cancer is the second most common gynecologic malignancy, with an estimated 6000 new cases in 1999 in Japan, leading to 4000 deaths.[1)] Advanced ovarian cancer is characterized by peritoneal dissemination and ascites formation. The major route of spread is direct extension. Patients with advanced ovarian cancer may suffer from this specific spread to any peritoneal surface, including the omentum. Many investigators have shown that angiogenesis plays an important role in the growth, progression, and metastasis of solid tumors.[2–4)] Several molecules have been identified as angiogenic factors. Among them, vascular endothelial growth factor (VEGF) plays an important role in tumor angiogenesis. VEGF, also called vascular permeability factor (VPF), is believed to be a potent mediator of peritoneal fluid accumulation and a primary stimulant of the vascularization in ascites tumors. The expression of VEGF has been widely demonstrated in various normal tissues[5)] and many human and animal tumors,[5, 6)] including ascites tumors.[7, 8)] The accumulation of tumor ascites fluid might result from increased permeability of the blood vessels lining the peritoneal cavity and the hyperpermeability of vessels might be mediated by VEGF secreted from ascites tumor cells.[7, 9)] VEGF is easily detected in the peritoneal fluid of two well-characterized transplantable mouse tumors, as early as 2 to 5 days after the injection of 106 tumor cells, by both a functional bioassay and an immunofluorometric assay.[10)] Therefore, it has been proposed that VEGF secretion by tumor cells is responsible for initiating and maintaining the ascites pattern of tumor growth.[10)]

Adhesion molecules such as connexin, cadherin, and catenin correlate with cell migration and dissemination.[11–14)] Cadherins are structurally and functionally related transmembrane glycoproteins that mediate the Ca^{2+}-dependent homophilic adhesion, development, and morphogenesis.[11, 15, 16)] Several types of cadherins have been identified: E-cadherin is expressed on most epithelia and some car-

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cinoma cells of epithelial origin, N-cadherin in nervous tissues, and P-cadherin in the placenta. Their decreased expression and dysfunction might be related to invasion and metastasis,[15, 17–19] as well as peritoneal dissemination,[20] by disruption of the tumor cell-to-cell junctions. Epithelial cells acquire invasive properties after the loss of E-cadherin function due to specific antibody treatment and antisense RNA treatment.[17, 21] It is believed that well-differentiated carcinoma, which has cell-cell adhesion, highly expresses E-cadherin, whereas poorly differentiated carcinoma, which has weak cell-cell adhesion, shows loss of expression and abnormal location of E-cadherin.[22–24]

To elucidate the molecular mechanisms of ascites formation and peritoneal dissemination of human ovarian cancer, we focused on the expression of VEGF and E-cadherin in different ovarian cancer cell lines and produced human ovarian cancer ascites models using nude mice to compare the tumor biology.

MATERIALS AND METHODS

Cells and cell culture Three established ovarian carcinoma cell lines were used for this study. AMOC-2 was established from serous cyst adenocarcinoma,[25] and HTBOA and HNOA were established from poorly differentiated adenocarcinoma,[26] and endometrioid adenocarcinoma,[27] respectively. Cells were cultured in RPMI 1640 (Gibco BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) in a CO_2 incubator (5% CO_2) at 37°C.

RNA extraction and northern blot hybridization Total RNA was isolated from cells with an Ultraspec RNA isolation system (Biotex, Houston, TX). Fifteen micrograms of each cellular RNA sample was denatured and subjected to electrophoresis in 1.0% agarose gels containing 0.22 *M* formaldehyde as described by Tsang *et al.*[28] The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. Northern blotting was performed as described previously.[29] For northern blot analysis, probes were labeled with ^{32}P-dCTP, using a Prime-it II kit (Stratagene, La Jolla, CA). The specific cDNA probe for VEGF was a 0.6-kb *Eco*RI-*Hin*dIII fragment.[30] A 531 bp human glyceraldehyde-3-phosphate dehydrogenase (GAPDH: EC 1.2.1.12) cDNA was used[31] as an internal control for the amount of RNA in each RNA blot. Labeled probes were purified through a Nick column (Amersham Pharmacia Biotech UK, Ltd., Buckinghamshire, UK) before hybridization.

RT-PCR Five micrograms of total RNA was treated with 1 unit of Dnase I (Gibco BRL) at room temperature for 15 min. After inactivation of Dnase I, the total RNA was reverse-transcribed by using a SuperScript Preamplification System for First Strand cDNA Synthesis Kit (Gibco BRL) as specified in the manufacturer's protocol. We used oligo dT as a primer for reverse transcription. PCR was carried out with the following primers: forward primer 5′-TGGAATCCAAGCAGAATTGC-3′ and reverse primer 5′-GGAGGATTATCGTTGGTGTC-3′ for E-cadherin; forward primer 5′-TCGGGCCTCCGAAACCATGA-3′ and reverse primer 5′-CCTGGTGAGATCTGGTTC-3′ for VEGF[32]; forward primer 5′-CGCAGAAGGAGATCACTGCC-3′ and reverse primer 5′-CTCGTAGGGGGTTTCAAGTG-3′ for human β-actin. The reaction mixture of 50 μl contained 1/20 volume of cDNA, 200 m*M* of each dNTP, 0.125 unit of TaKaRa EX *Taq* (TaKaRa Shuzo Co., Ltd., Shiga), 1× buffer (supplied with enzyme), and 100 pmol each of the forward and reverse primers. The mixture was subjected to amplification using a TaKaRa MP Thermal cycler (TaKaRa Shuzo Co., Ltd.). The reaction with E-cadherin primers was performed with 30 cycles at 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min. A final 10-min extension step was added after the last cycle. The reactions with VEGF and β-actin primers were performed with 30 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Then 10 μl of the PCR product was electrophoresed on a 1.0% agarose gel and photographed under UV light after staining with ethidium bromide.

Human ovarian cancer ascites model To evaluate the tumor biology of ovarian cancer cells, 5- to 6-week-old BALB/cAnNCrj-*nu*/*nu* nude mice (Charles River Japan, Inc., Chiba) were used. Three ovarian cancer cell lines that were exponentially growing were trypsinized and counted. For each, 1×10^7 cells suspended in 1 ml of phosphate-buffered saline were inoculated intraperitoneally into nude mice. Twenty-one days after the inoculation, the mice were killed by deep anesthesia using diethyl ether. The ascites was collected and intraperitoneal findings were carefully studied. We observed the macroscopic peritoneal dissemination, and the size and the number of tumors in the abdomen. The ascites fluid was stored at −80°C after centrifugation to remove cancer cells. A part of the tumor was fixed in 10% formaldehyde for further analysis.

Quantitative assay of VEGF in ascites fluid The VEGF concentration of cell-free ascites fluid accumulated in HTBOA and HNOA mice was assayed using a human VEGF measuring kit (Immuno-Biological Laboratories Co., Ltd., Gunma). Sandwich enzyme immunoassay was done with 20-fold diluted ascites fluid.

Vascular permeability assay Miles' assay[33] was conducted to analyze the vascular permeability. A guinea pig was anesthetized and 0.5 ml of 0.5% Evans blue (Sigma, St. Louis, MO) was injected into the intracardiac space. Ten minutes later, 200 μl of samples and control physiological saline were intracutaneously injected into the shaved back, and leakage of dye was observed as the presence of blue spots surrounding the injected site after another 30 min.

Microvessel density Tissues of nude mouse tumors were

fixed with 10% formalin solution for one week before being dehydrated by alcohol replacement and embedded in paraffin. The tumors generated in the nude mice were fixed with 10% formalin, and embedded in paraffin.

For immunohistochemistry, we used an anti-CD31 antibody (DAKO, Glostrup, Denmark), which was specific to lymphocyte protein and endothelial cells of mouse blood vessels.[34] For the detection of the primary antibody, we used a biotinylated anti-mouse serum peroxidase-conjugated streptavidin HISTFINE SAB-PO kit (Nichirei Co., Tokyo). For secondary and tertiary antibodies, we also used a SAB-PO kit which utilized streptavidin as an agent. 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was used to detect the protein. Negative control was performed in all cases by omitting the primary antibody. We counted the number of individual microvessels within an area of 0.6 mm^2 under ×200 power magnification with an Olympus BX50 microscope (Olympus, Tokyo) on anti-CD31 antibody-stained sections.[34]

Plasmid construction and transfection The mammalian expression vector pcDNA3.1(−) (Invitrogen, Carlsbad, CA) was used. A 0.6-kb *Eco*RI-*Hin*dIII fragment of VEGF cDNA was cloned into pcDNA3.1(−) in the antisense direction. Lipofectamine 2000 (Invitrogen) was used to transfect the HTBOA cell line with either the control vector (pcDNA3.1(−) with no insert) or the pcDNA3.1(−) vector containing VEGF cDNA fragment in the antisense direction. Selection and maintenance of neo-resistant transfectants were performed in the presence of G418 (Sigma). After 14 days, single colonies were picked up.

Statistics The VEGF concentration and microvessel density quantification were compared by means of Student's *t* test. A P value of <0.05 was considered to be statistically significant.

RESULTS

Expression of VEGF and E-cadherin mRNA in human ovarian cancer cell lines The expression of VEGF mRNA was examined in 3 human ovarian cancer cell lines. The results of northern blot analysis are shown in Fig. 1a. Two major bands were observed in HNOA and HTBOA. The expression of VEGF was very weak in AMOC-2. We performed RT-PCR to investigate the form of the VEGF mRNA. Oligonucleotide primers corresponding to the 5′ noncoding region and 3′ noncoding region of the gene were used to amplify the whole coding region of all known splicing forms of VEGF mRNA.[32] By RT-PCR in three human ovarian cancer cell lines, two major products (530 and 662 base pairs) corresponding to VEGF121 and VEGF165, which are known to be secreted efficiently, were detected in HNOA and HTBOA (Fig. 1b).

The expression of E-cadherin was also examined. As shown in Fig. 1b, a 721bp E-cadherin-specific product was detected in HTBOA and AMOC-2. However, no expression of E-cadherin was found in HNOA.

Tumor biology of ovarian cancer cell lines To determine the characteristics of the tumor biology of each cell line, we utilized the nude mouse models. Cells of each line were inoculated into groups of 10 nude mice. Each mouse was killed by deep anesthesia with diethyl ether 21 days after inoculation and the tumor biological characteristics were compared. Table I summarizes the results. The photograph in Fig. 2 shows a representative of each mouse. The AMOC-2 mouse frequently generated a single large tumor in the peritoneal cavity (7 of 10 mice) without ascites formation or remarkable peritoneal dissemination. Seven of the 10 HTBOA mice and 5 of the 10 HNOA mice had

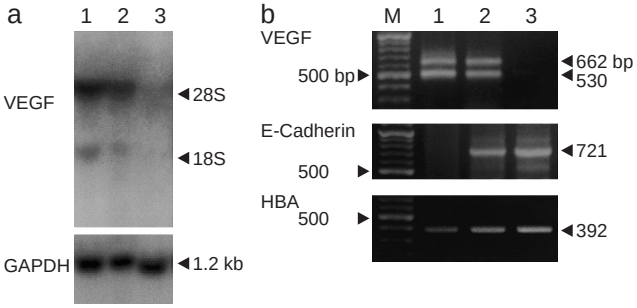

Fig. 1. Expression of VEGF and E-cadherin mRNA in human ovarian cancer cells. (a) Northern blot analysis of VEGF mRNA in 3 human ovarian cancer cell lines. Fifteen micrograms of total RNA from each cell line was used for the analysis. Filter membranes were hybridized with ^{32}P-labeled VEGF cDNA probes. Arrowheads indicate the positions of 18S and 28S rRNA. VEGF mRNA were detected in HNOA and HTBOA. A GAPDH cDNA probe was used for the internal control of RNA. (b) RT-PCR analysis of VEGF and E-cadherin mRNA in three cell lines. Five micrograms of total RNA was reverse-transcribed to 1st strand cDNA. 1/20 volumes of 1st strand cDNAs were amplified using VEGF- and E-cadherin-specific primers as described in "Materials and Methods." The human β-actin primer was used for the internal control. M, 100-bp ladder; lane 1, HNOA; lane 2, HTBOA; lane 3, AMOC-2.

Table I. Biological Property of Each Human Ovarian Cancer Model

	Cell line		
	AMOC-2	HTBOA	HNOA
Tumorigenicity	7/10	8/10	10/10
Ascites formation	1/10	7/10	5/10
Peritoneal dissemination	1/10	7/10	9/10

The numbers represent the numbers of mice that exhibited each biological property.

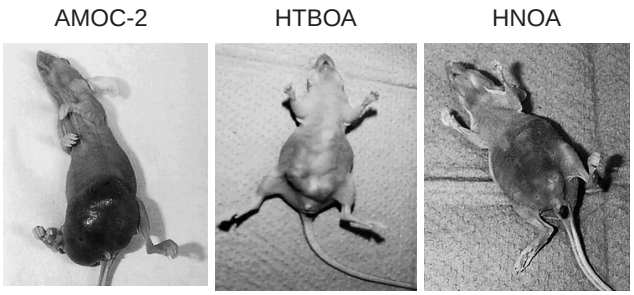

Fig. 2. Tumor biology of ovarian cancer cells. Cells (1×10^7) of each ovarian cancer cell line were inoculated intraperitoneally into groups of 10 BALB/cAnNCrj-*nu*/*nu* nude mice. The mice were killed at 21 days, and the tumorigenicity, ascites formation and peritoneal dissemination were observed. The photographs show a representative of each type of mouse.

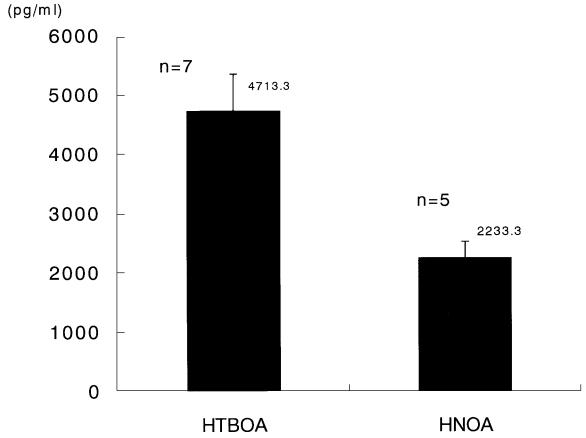

Fig. 3. ELISA of human VEGF in ascites fluid.

bloody ascites fluid. Seven of the 10 HTBOA mice and 9 of the 10 HNOA mice had extensive macroscopic peritoneal dissemination. HNOA mice tended to have diffuse peritoneal dissemination, as well as marked weight loss, and they weakened rapidly. HTBOA mice produced bloody ascites most frequently.

Measurement of VEGF in the ascites fluid The concentrations of VEGF in the ascites of HTBOA and HNOA mice were measured using a human VEGF ELISA kit. Although one of the 10 AMOC-2 mice produced ascites fluid, the volume was too small to measure the concentration. Fig. 3 shows the average levels of human VEGF in 7 HTBOA mice and 5 HNOA mice in which ascites accumulated. The average VEGF concentration was higher in the ascites fluids of HTBOA mice (4713.3±1083.0 ng/ml) than in those of HNOA mice (2233.3±481.8 ng/ml) ($P<0.05$).

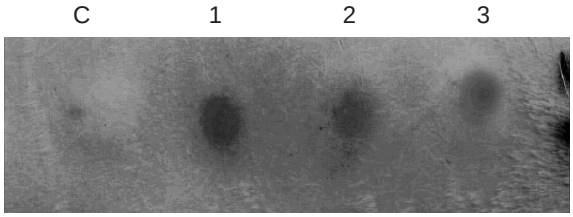

Fig. 4. Vascular permeability activity of the ascites from HTBOA mice. Aliquots of 200 μg of the control and 3 samples of the ascites from HTBOA mice were injected into the back of a guinea pig for Miles' assay. Although the control did not show any blue spot, the 3 samples of the ascites from HTBOA mice showed blue spots due to increased vascular permeability. C, control (200 μg of physiological saline); 1–3, 3 representative samples of ascites from HTBOA mice.

Vascular permeability of the ascites fluid We performed Miles' assay using a guinea pig to confirm the vascular permeability-enhancing activity. Fig. 4 shows the result. Three representative HTBOA mouse ascites showed blue spots due to the leakage of Evans blue.

Microvascular density of the tumor To evaluate the angiogenic activity of VEGF in ovarian cancer cells, we examined the microvascular densities of HTBOA and AMOC-2 tumors by immunohistochemical analysis using an anti-CD31 antibody specific for endothelial cells of mouse blood vessels. Microvascular density was determined by counting the number of stained cells. Fig. 5 shows the results of the microvascular density assay. The HTBOA tumor that produced VEGF had higher microvascular density than the AMOC-2 tumor that did not express VEGF.

Suppression of ascites by VEGF antisense plasmid In order to confirm the role of VEGF in ascites form accumulation, we constructed an antisense expression vector. Several clones were picked up after the transfection and we confirmed the expression of antisense VEGF by RT-PCR using primers located at the cloning sites (data not shown). Two clones designated HTBOA-VEGFas and HTBOA-pcDNA3.1 were used for further experiments. Expression of VEGF protein was analyzed by western blotting. The expression of VEGF was decreased in HTBOA-VEGFas (data not shown).

We inoculated aliquots of 1×10^7 cells into nude mice. Twenty-one days after the inoculation, the mice were killed. One of 10 HTBOA-VEGFas mice revealed obvious bloody ascites, while seven of 10 HTBOA-pcDNA3.1 mice revealed bloody ascites (Table II). Although antisense VEGF reduced the ascites accumulation, the frequencies of tumor formation and peritoneal dissemination were not altered between the antisense and control mice.

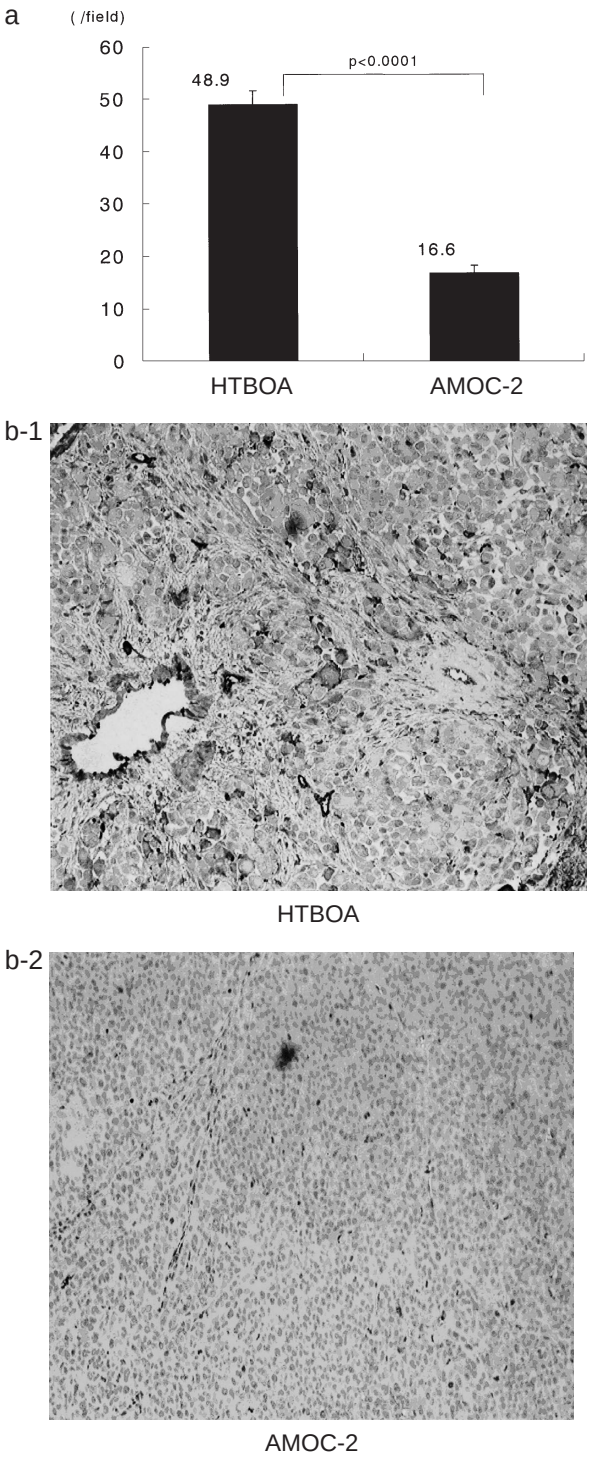

Fig. 5. Microvascular densities of the tumors of HTBOA and AMOC-2 mice. (a) Immunohistochemistry of HTBOA and AMOC-2 tumors. Endothelial cells were stained by an anti-CD31 antibody. (b) CD31-positive microvessels were counted at ×200 magnification. Five fields of 3 tumors each from HTBOA (b-1) and AMOC-2 (b-2) mouse were used for the analysis.

Table II. Tumor Biology of HTBOA-VEGFas and HTBOA-pcDNA3.1

	HTBOA-VEGFas	HTBOA-pcDNA3.1
Tumorigenicity	7/10	8/10
Ascites formation	1/10	7/10
Peritoneal dissemination	7/10	8/10

Cells (1×10^7) of each cell line were inoculated intraperitoneally into groups of 10 BALB/cAnNCrj-*nu*/*nu* nude mice. The mice were killed at 21 days, and the tumorigenicity, ascites formation and peritoneal dissemination were observed. The numbers represent the numbers of mice that exhibited each biological property.

DISCUSSION

The aim of our study was to analyze the molecular mechanism of the peritoneal dissemination and ascites formation by human ovarian cancer cells. We analyzed the expression of VEGF and E-cadherin mRNA in 3 human ovarian cancer cell lines. Then human ovarian cancer models using nude mice were analyzed to assess the tumor biology of each cell line. Interestingly, the 3 cell lines showed different characteristics of gene expression. AMOC-2 expressed E-cadherin, but not VEGF, as shown by northern blot hybridization and RT-PCR. HNOA expressed VEGF without E-cadherin expression. HTBOA expressed both VEGF and E-cadherin. Furthermore, each human ovarian cancer model revealed a specific feature. These findings suggested that the lack of E-cadherin expression was related to rapid dissemination, and VEGF expression played an important role in ascites formation.

VEGF is a multifunctional molecule that has been implicated in vasculogenesis, endothelial cell proliferation and migration, vascular permeability, lymph node metastasis, and stromal degradation through the activation of proteolytic enzymes involved in tumor invasiveness and angiogenesis.[35–40] Our data strongly indicated a relationship between VEGF expression and ascites formation. In addition, VEGF produced by ovarian cancer cells in this study showed vascular permeability-enhancing activity and angiogenic activity. Luo *et al.* reported that VEGF neutralizing antibody can inhibit fluid accumulation and tumor growth in two mouse ascites tumors.[41] We also demonstrated the inhibitory effect of VEGF antisense on ascites accumulation of human ovarian cancer cells. The inhibition of VEGF is suggested to be an important strategy to prevent ascites accumulation.

Cell dissociation and acquisition of cell motility are believed to affect the initial steps in the metastatic process.[42] Suppressed expression or dysfunction of the cadherin-catenin complex might reduce cell-cell adhesion and trigger the escape of cancer cells from the primary tumor.[43] Fixen *et al.* reported that E-cadherin-mediated

cell-to-cell adhesion prevents invasiveness of human carcinoma cells.[22] From these studies, the lack of E-cadherin expression is related to the spread of cancer cells. The lack of E-cadherin expression in HNOA cells might be associated with the most rapid progress of peritoneal dissemination. However, the difference of molecular characteristics between AMOC-2 and HTBOA as regards peritoneal dissemination is not clear. It is suggested that other factors are related to the dissemination of HTBOA.

It has been reported that the expression of matrix metalloproteinases (MMPs) and VEGF, and reduction of E-cadherin correlate with lymph node metastasis of esophageal cancer[40, 44] and the metastatic potential of pancreatic cancer.[45] The formation of ascites is directly associated with expression of VEGF, and survival is inversely associated with expression of IL-8 by human ovarian carcinomas in nude mice.[46] However, there has been little information about the relationships between VEGF and E-cadherin, and human ovarian cancer.

In summary, we have demonstrated the importance of VEGF expression and the lack of E-cadherin expression for the progression of human ovarian cancer cells, suggesting that these factors closely correlate with the malignant potential of ovarian cancer cells. The correlation of the clinical outcomes of patients with VEGF and E-cadherin expression should be examined. These molecules could be molecular targets for ovarian cancer therapy. Furthermore, our human ovarian cancer ascites model should be useful to analyze the mechanism of progression of ovarian cancer, and to evaluate the efficacy of therapeutic agents.

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REFERENCES

1) Cancer Statistics in Japan databases (http://www.ncc.go.jp/jp/statistics/2001/tables).
2) Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.*, **82**, 4–6 (1990).
3) Liotta, L. A., Steeg, P. S. and Stetler, S. W. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell*, **64**, 327–336 (1991).
4) Weidner, N., Semple, J. P., Welch, W. R. and Folkman, J. Tumor angiogenesis and metastasis—correlation in invasive breast carcinoma. *N. Engl. J. Med.*, **324**, 1–8 (1991).
5) Dvorak, H. F., Brown, L. F., Detmar, M. and Dvorak, A. M. Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am. J. Pathol.*, **146**, 1029–1039 (1995).
6) Berse, B., Brown, L. F., Van de Water, L., Dvorak, H. F. and Senger, D. R. Vascular permeability factor (vascular endothelial growth factor) gene is expressed differentially in normal tissues, macrophages, and tumors. *Mol. Biol. Cell*, **3**, 211–220 (1992).
7) Yeo, K. T., Wang, H. H., Nagy, J. A., Sioussat, T. M., Ledbetter, S. R., Hoogewerf, A. J., Zhou, Y., Masse, E. M., Senger, D. R., Dvorak, H. F. and Yeo, T. K. Vascular permeability factor (vascular endothelial growth factor) in guinea pig and human tumor and inflammatory effusions. *Cancer Res.*, **53**, 2912–2918 (1993).
8) Luo, J. C., Yamaguchi, S., Shinkai, A., Shitara, K. and Shibuya, M. Significant expression of vascular endothelial growth factor/vascular permeability factor in mouse ascites tumors. *Cancer Res.*, **58**, 2652–2660 (1998).
9) Senger, D. R., Galli, S. J., Dvorak, A. M., Perruzzi, C. A., Harvey, V. S. and Dvorak, H. F. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. *Science*, **219**, 983–985 (1983).
10) Nagy, J. A., Masse, E. M., Herzberg, K. T., Meyers, M. S., Yeo, K. T., Yeo, T. K., Sioussat, T. M. and Dvorak, H. F. Pathogenesis of ascites tumor growth: vascular permeability factor, vascular hyperpermeability, and ascites fluid accumulation. *Cancer Res.*, **55**, 360–368 (1995).
11) Sommers, C. L., Walker, J. D., Heckford, S. E., Worland, P., Valverius, E., Clark, R., McCormick, F., Stampfer, M., Abularach, S. and Gelmann, E. P. Vimentin rather than keratin expression in some hormone-independent breast cancer cell lines and in oncogene-transformed mammary epithelial cells. *Cancer Res.*, **49**, 4258–4263 (1989).
12) Musil, L. S., Cunningham, B. A., Edelman, G. M. and Goodenough, D. A. Differential phosphorylation of the gap junction protein connexin 43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.*, **111**, 2077–2088 (1990).
13) Ozawa, M., Baribault, H. and Kemler, R. The cytoplasmic domain of the cell adhesion molecule uvomorulin associates with three independent proteins structurally related in different species. *EMBO J.*, **8**, 1711–1717 (1989).
14) Tran, N. L., Nagle, R. B., Cress, A. E. and Heimark, R. L. N-Cadherin expression in human prostate carcinoma cell lines. An epithelial-mesenchymal transformation mediating adhesion with stromal cells. *Am. J. Pathol.*, **155**, 787–798 (1999).
15) Sommers, C. L., Thompson, E. W., Torri, J. A., Kemler, R., Gelmann, E. P. and Byers, S. W. Cell adhesion molecule uvomorulin expression in human breast cancer cell lines: relationship to morphology and invasive capacities. *Cell*

Growth Differ., **2**, 365–372 (1991).
16) Takeichi, M. Cadherin cell adhesion receptors as a morphogenetic regulator. *Science*, **251**, 1451–1455 (1991).
17) Vleminckx, K., Vakaet, L., Mareel, M., Fiers, W. and Van Roy, F. Genetic manipulation of E-cadherin expression by epithelial tumor cells reveals an invasion suppressor role. *Cell*, **66**, 107–119 (1991).
18) Matsuura, K., Kawanishi, J., Fujii, S., Imamura, M., Hirano, S., Takeichi, M. and Niitsu, Y. Altered expression of E-cadherin in gastric cancer tissues and carcinomatous fluid. *Br. J. Cancer*, **66**, 1122–1130 (1992).
19) Takeichi, M. Cadherins in cancer: implications for invasion and metastasis. *Curr. Opin. Cell Biol.*, **5**, 806–811 (1993).
20) Furuyama, H., Arii, S., Mori, A. and Imamura, M. Role of E-cadherin in peritoneal dissemination of the pancreatic cancer cell line, panc-1, through regulation of cell to cell contact. *Cancer Lett.*, **157**, 201–209 (2000).
21) Behrens, J., Mareel, M. M., Van Roy, F. and Birchmeier, W. Dissecting tumor cell invasion: epithelial cells acquire invasive properties after the loss of uvomorulin-mediated cell-cell adhesion. *J. Cell Biol.*, **108**, 2435–2447 (1989).
22) Frixen, U. H., Behrens, J., Sachs, M., Eberle, G., Voss, B., Warda, A., Lochner, D. and Birchmeier, W. E-Cadherin-mediated cell-cell adhesion prevents invasiveness of human carcinoma cells. *J. Cell Biol.*, **113**, 173–185 (1991).
23) Schipper, J. H., Frixen, U. H., Behrens, J., Unger, A., Jahnke, K. and Birchmeier, W. E-Cadherin expression in squamous cell carcinomas of head and neck: inverse correlation with tumor dedifferentiation and lymph node metastasis. *Cancer Res.*, **51**, 6328–6337 (1991).
24) Shiozaki, H., Tahara, H., Oka, H., Miyata, M., Kobayashi, K., Tamura, S., Iihara, K., Doki, Y., Hirano, S. and Takeichi, M. Expression of immunoreactive E-cadherin adhesion molecules in human cancers. *Am. J. Pathol.*, **139**, 17–23 (1991).
25) Yabushita, H., Ueno, N., Sawaguchi, K., Higuchi, K., Noguchi, M. and Ishihara, M. Establishment and characterization of a new human cell-line (AMOC-2) derived from a serous adenocarcinoma of ovary. *Acta Obstet. Gynaecol. Jpn.*, **41**, 888–894 (1989).
26) Ishiwata, I., Ishiwata, C., Soma, M., Ono, I., Nakaguchi, T. and Ishikawa, H. Establishment and characterization of a human ovarian anaplastic carcinoma cell line. *Gynecol. Oncol.*, **30**, 35–43 (1988).
27) Ishiwata, I., Ishiwata, C., Soma, M. and Ishikawa, H. Establishment and characterization of two human ovarian endometrioid carcinoma cell lines (with or without squamous cell component). *Gynecol. Oncol.*, **25**, 95–107 (1986).
28) Tsang, S. S., Yin, X., Guzzo, A. C., Jones, V. S. and Davison, A. J. Loss of resolution in gel electrophoresis of RNA: a problem associated with the presence of formaldehyde gradients. *Biotechniques*, **14**, 380–381 (1993).
29) Nishikawa, A., Iwasaki, M., Akutagawa, N., Manase, K., Yamashita, S., Endo, T. and Kudo, R. Expression of various matrix proteases and Ets family transcriptional factors in ovarian cancer cell lines: correlation to invasive potential. *Gynecol. Oncol.*, **79**, 256–263 (2000).
30) Sawano, A., Takahashi, T., Yamaguchi, S., Aonuma, M. and Shibuya, M. Flt-1 but not KDR/Flk-1 tyrosine kinase is a receptor for placenta growth factor, which is related to vascular endothelial growth factor. *Cell Growth Differ.*, **7**, 213–221 (1996).
31) Tso, J. Y., Sun, X. H., Kao, T. H., Reece, K. S. and Wu, R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: genomic complexity and molecular evolution of the gene. *Nucleic Acids Res.*, **13**, 2485–2502 (1985).
32) Weindel, K., Marme, D. and Weich, H. A. AIDS-associated Kaposi's sarcoma cells in culture express vascular endothelial growth factor. *Biochem. Biophys. Res. Commun.*, **183**, 1167–1174 (1992).
33) Miles, A. A. and Miles, E. M. Vascular reactions to histamine, histamine liberators or leukotoxins in the skin of the guinea pigs. *J. Physiol.*, **118**, 228–257 (1952).
34) Vecchi, A., Garlanda, C., Lampugnani, M. G., Resnati, M., Matteucci, C., Stoppacciaro, A., Schnurch, H., Risau, W., Ruco, L. and Mantovani, A. Monoclonal antibodies specific for endothelial cells of mouse blood vessels. Their application in the identification of adult and embryonic endothelium. *Eur. J. Cell Biol.*, **63**, 247–254 (1994).
35) Senger, D. R., Van de Water, L., Brown, L. F., Nagy, J. A., Yeo, K. T., Yeo, T. K., Berse, B., Jackman, R. W., Dvorak, A. M. and Dvorak, H. F. Vascular permeability factor (VPF, VEGF) in tumor biology. *Cancer Metastasis Rev.*, **12**, 303–324 (1993).
36) Roberts, W. G. and Palade, G. E. Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor. *J. Cell Sci.*, **108**, 2369–2379 (1995).
37) Carmeliet, P., Ferreira, V., Breier, G., Pollefeyt, S., Kieckens, L., Gertsenstein, M., Fahrig, M., Vandenhoeck, A., Harpal, K., Eberhardt, C., Declercq, C., Pawling, J., Moons, L., Collen, D., Risau, W. and Nagy, A. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature*, **380**, 435–439 (1996).
38) Ferrara, N. Vascular endothelial growth factor. *Eur. J. Cancer*, **32A**, 2413–2422 (1996).
39) Ferrara, N., Carver, M. K., Chen, H., Dowd, M., Lu, L., O'Shea, K. S., Powell, B. L., Hillan, K. J. and Moore, M. W. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature*, **380**, 439–442 (1996).
40) Sato, F., Shimada, Y., Watanabe, G., Uchida, S., Makino, T. and Imamura, M. Expression of vascular endothelial growth factor, matrix metalloproteinase-9 and E-cadherin in the process of lymph node metastasis in oesophageal cancer. *Br. J. Cancer*, **80**, 1366–1372 (1999).
41) Luo, J. C., Toyoda, M. and Shibuya, M. Differential inhibition of fluid accumulation and tumor growth in two mouse ascites tumors by an antivascular endothelial growth factor/permeability factor neutralizing antibody. *Cancer Res.*, **58**,

2594–2600 (1998).
42) Liotta, L. A. Tumor invasion and metastases—role of the extracellular matrix: Rhoads Memorial Award lecture. *Cancer Res.*, **46**, 1–7 (1986).
43) Shimoyama, Y., Nagafuchi, A., Fujita, S., Gotoh, M., Takeichi, M., Tsukita, S. and Hirohashi, S. Cadherin dysfunction in a human cancer cell line: possible involvement of loss of alpha-catenin expression in reduced cell-cell adhesiveness. *Cancer Res.*, **52**, 5770–5774 (1992).
44) Uchida, S., Shimada, Y., Watanabe, G., Tanaka, H., Shibagaki, I., Miyahara, T., Ishigami, S. and Imamura, M. In oesophageal squamous cell carcinoma vascular endothelial growth factor is associated with p53 mutation, advanced stage and poor prognosis. *Br. J. Cancer*, **77**, 1704–1709 (1998).
45) Bruns, C. J., Harbison, M. T., Kuniyasu, H., Eue, I. and Fidler, I. J. *In vivo* selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia*, **1**, 50–62 (1999).
46) Yoneda, J., Kuniyasu, H., Crispens, M. A., Price, J. E., Bucana, C. D. and Fidler, I. J. Expression of angiogenesis-related genes and progression of human ovarian carcinomas in nude mice. *J. Natl. Cancer Inst.*, **90**, 447–454 (1998).