Angiopoietin-1, 2 and Tie2 expressions in endometrial adenocarcinoma – the Ang2 dominant balance up-regulates tumor angiogenesis in the presence of VEGF

M. Saito[1], *M.S.*; **J. Watanabe**[1,2], *M.D.*; **T. Fujisawa**[1], *M.D.*; **Y. Kamata**[1], *M.D.*; **Y. Nishimura**[1], *M.S.*; **T. Arai**[1], *M.D.*; **T. Miyamoto**[1], *M.S.*; **A. Obokata**[1], *M.S.*; **H. Kuramoto**[1,3], *M.D.*

[1]Department of Clinical Cytology, Graduate School of Medical Sciences, [2]Departments of Pathology and [3]Obstetrics and Gynecology, Kitasato University, Kanagawa (Japan)

Summary

We investigated Ang1, Ang2 and Tie2 expressions including balance and intratumoral vessels in the role of angiogenesis of endometrial adenocarcinoma. Immunohistochemical staining was performed on 133 patients with endometrial (endometrioid) adenocarcinoma, including 73 with G1, 34 with G2, and 26 with G3. The levels of Ang1, Ang2 and Tie2 expressions were expressed as staining score. Total vessel count (TVC), microvessel count (MVC) and mean vessel diameter (VD) in the CD34-stained tissues were measured in five hot spot areas at x 200 magnification by image cytometry. These results were compared with high and low vascular endothelial growth factor (VEGF) expressions. Ang1, Ang2, Tie2 and CD34 were expressed in the cytoplasm of tumor cells. A significant correlation was found among Ang1, Ang2 and Tie2 expressions. In high VEGF cases, Ang1 expression was correlated negatively with TVC and MVC, but positively with VD, and the Ang1 < Ang2 group was significantly higher in TVC and MVC and tended to be smaller in VD than the Ang1 > Ang2 group. VD was significantly larger in G3 than in G1. The Ang1 < Ang2 balance may be one of the key factors for angiogenesis of endometrial carcinoma in the presence of high VEGF expression.

Key words: Angiopoietins; Tie2; Endometrial adenocarcinoma.

Introduction

Angiopoietins (Angs) are a second family of growth factors, specific to the vascular endothelium, which consists of four known members: Ang1, 2, 3 and 4 [1]. Ang1 was originally identified as a specific ligand of Tie2 providing 70 kDa ligands for Tie2 [2], and induces its tyrosine phosphorylation [3], stabilizes and tightens preexisting vessels by mediating the interaction between endothelial cells and pericytes [4]. Tie2, specifically expressed in the vascular endothelial cells, is a member of the tyrosine kinase receptor family. A study has been reported that overexpression of Ang1 in vivo resulted in a dramatic increase in the number, size and branching of blood vessels in transgenic mice [5]. Ang2 was first characterized as a structural homolog of Ang1 that binds Tie2 and antagonizes Ang1 [6]. Ang2 does not induce phosphorylation of Tie2, but blocks Ang1-mediated tyrosine phosphorylation of Tie2 [6], and thus induces vascular destabilization. Angs do not induce endothelial cell proliferation in vitro [2], unlike other angiogenic growth factors such as vascular endothelial growth factor (VEGF), but they are involved in sprouting endothelial cells [1]. Recently, various studies have reported that the Ang/Tie2 system seems to act in a complementary and coordinating role for the VEGF/VEGF receptor (VEGFR) system [7].

A number of studies on angiogenesis in endometrial carcinoma have been reported [8]; however, there is only one study investigating Angs. Holland *et al.* [9] revealed using a small number of samples that Ang2 level was higher in endometrial carcinoma than in benign endometrium, although it was not significantly correlated. Therefore, the present study with a large number of cases investigated Angs and Tie2 expressions, and their relation to VEGF expression, intratumoral vessels and histological grade. As intratumoral vessel indicators, we analyzed not only microvessel count (MVC), a known marker of angiogenic grade, but also total vessel count (TVC), and mean vessel diameter (VD).

Materials and Methods

Tissue samples

Tissue samples of endometrial adenocarcinoma (endometrioid type) were obtained, with informed consent, from 133 patients who underwent surgical treatment at Kitasato University Hospital between 1983 and 2000. Of these patients, 73 had well-differentiated (G1), 34 had moderately differentiated (G2), and 26 had poorly differentiated (G3) adenocarcinomas. No patients received either adjuvant chemotherapy or radiotherapy before surgery. Tumor classification was performed according to the International Federation of Gynecology and Obstetrics (FIGO) [10]. The mean age of these patients was 57 years (range, 30-83 years).

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Immunohistochemical staining

Immunohistochemical staining for Ang1, Ang2, Tie2 and CD34 was performed using the Universal Immuno-enzyme polymer method (UIP method, Histofine Simple stain MAX PO, NICHIREI, Tokyo, Japan) on formalin-fixed and parafin-embedded tumor tissues of 4 μm thick sections. For the UIP method, sections deparaffinized in xylene and then rehydrated were treated with 0.3% hydrogen peroxide for 20 min to block endogenous peroxidase activity, and the antigen retrieval slides were autoclaved in a 10 mM citrate buffer (pH 6.0) at 121°C for 15 min. The sections were incubated with either goat polyclonal anti-human Ang1 antibody (N-18, Santa Cruz Biotechnology, California, USA, 1:50 dilution), goat polyclonal anti-human Ang2 antibody (N-18, Santa Cruz Biotechnology, California, USA, 1:50 dilution), rabbit polyclonal anti-human Tie2 antibody (C-20, Santa Cruz Biotechnology, California, USA, 1:50 dilution) or mouse monoclonal anti-human CD34 antibody (QBEnd 10, DAKO, Glostrup, Denmark, 1:200 dilution) at 4°C overnight. Next, the slides were incubated with peroxidase-labelled amino acid polymer with Ig for 30 min, and then reacted with 0.05% diaminobenzidine containing 0.6% hydrogen peroxide. Finally, the nuclei were counterstained with Mayer's hematoxylin, and the sections were dehydrated, cleared and mounted.

Evaluation of immunohistochemical staining

Immunohistochemical staining for Ang1, Ang2, Tie2 and CD34 was performed using serial sections. Therefore, similar areas were evaluable. The five fields with the largest number of blood vessels within the tumor (hot spot) were searched by screening the sections with CD34 staining at low magnification (x 40 or x 100). The intensity of Ang1, Ang2 and Tie2 expressions in tumor cells was classified into negative (score 0), positive (score 1) and strongly positive (score 2) groups at x 200 magnification, and the total score of these five hot spot areas was calculated (full score 10). The Ang1 and Ang2 scores in each case were compared and classified into three groups: Ang1 > Ang2, Ang1 = Ang2 and Ang1 < Ang2, to evaluate the intensity balance of Ang expression. For evaluating blood vessels, image cytometry (Image-Pro Plus ver.4.5, MEDIA CYBERNETICS) was performed in the counting of all CD34 positive blood vessels on the five hot spot areas at x 200 magnification. We defined three vessel indicators as follows: the total number of blood vessels on the five fields as total vessel count (TVC), the number of micro blood vessels with diameters of 8 μm or less as MVC, and the average diameter of total blood vessels (34 to 253 vessels per case) as mean vessel diameter (VD).

Comparison with VEGF expression

One hundred cases of 104 previously examined for VEGF expression [11] were included in the present study, and compared with Ang1, Ang2, Tie2 and vessel indicators. In brief, immunohistochemical staining for VEGF was performed using the labeled streptavidin-biotin method (LSAB method, LSAB-kit, DAKO, California, USA). The LSAB method has been described in detail elsewhere [11]. The level of VEGF expression was classified as the high group when it was more intense than that of myometrial cells and the low group when it was similar to, or less than, that of myometrial cells [11].

Statistical analysis

The results of Ang1, Ang2 and Tie2 expressions and the three intratumoral vessel indicators were expressed as mean ± standard deviation (SD). Statistical analysis on the correlations among Ang1, Ang2 and Tie2 expressions, and the three intratumoral vessel indicators, was performed using Spearman's rank correlation test. The correlations of Ang1, Ang2 and Tie2 expressions and the three intratumoral vessel indicators with Ang balance and histological grade, were conducted using the Mann-Whitney U-test; p values less than 0.05 were considered statistically significant.

Results

Ang1, Ang2 and Tie2 were expressed in the cytoplasm of tumor cells as well as endothelial and stromal cells (Figures 1A-C and 2A-C). The intensity of Ang1, Ang2 and Tie2 expressions in endometrial carcinoma cells was stronger than that of the adjacent endothelial or stromal cells. An immunoreaction against CD34 was expressed in the cytoplasm of endothelial cells (Figures 1D and 2D).

Ang2 expressions were correlated significantly with Ang1 ($p < 0.001$, $r_s = 0.62$; Spearman's rank correlation test) and Tie2 ($p < 0.001$, $r_s = 0.465$; Spearman's rank correlation test; Figure 3) expressions. Ang1 expressions were also correlated significantly with Tie2 expressions ($p < 0.001$, $r_s = 0.345$; Spearman's rank correlation test).

The average scores of Ang1 expression in G1, G2 and G3 endometrial carcinomas were 3.5 ± 2.0, 3.1 ± 2.0 and 3.5 ± 1.9, respectively. Those of Ang2 expression were 3.6 ± 2.1, 3.0 ± 1.8 and 3.3 ± 2.2, respectively. Those of Tie2 expression were 5.0 ± 2.0, 4.9 ± 1.5 and 4.8 ± 2.5, respectively. Ang1, Ang2 and Tie2 expressions were not correlated with histological grade.

The high VEGF group consisted of 64 cases (64.0%) and the low VEGF group consisted of 38 cases (36.0%). Ang1, Ang2 and Tie2 expressions were not correlated with either the high or low VEGF expression group (Table 1).

Table 1. — *Correlation of Ang1, Ang2 and Tie2 expressions with VEGF expression.*

		Expression Score (Mean ± SD)			
VEGF	n	Ang1	Ang2	Tie2	
Low	36	3.5 ± 2.1	3.2 ± 2.2	5.0 ± 1.9	
High	64	3.3 ± 2.0	3.2 ± 2.2	4.9 ± 2.0	N.S.
Total	100	3.4 ± 2.0	3.2 ± 2.1	5.0 ± 1.9	

(N.S.: not significant; Mann-Whitney U test); Ang1: Angiopoietin 1, Ang2: Angiopoietin 2, VEGF: Vascular endothelial growth factor.

Figure 1. — Immunohistochemical staining of angiopoietin (Ang) 1 (A), Ang2 (B), Tie2 (C) and CD34 (D) in an Ang1 > Ang2 case being performed in the serial sections (x 200).
The staining scores of Ang1, Ang2 and Tie2 are 2, 1 and 2, respectively. Figure 1D shows low TVC, low MVC and large VD in an Ang1 > Ang2 case. TVC: total vessel count, MVC: microvessel count, VD: mean vessel diameter

Figure 2. — Immunohistochemical staining of Ang1 (A), Ang2 (B), Tie2 (C) and CD34 (D) in an Ang1 < Ang2 case being performed in the serial sections (x 200).
The staining scores of Ang1, Ang2 and Tie2 are 1, 2 and 2, respectively. Figure 2D shows high TVC, high MVC and small VD in an Ang1 < Ang2 case.

Fig. 1a

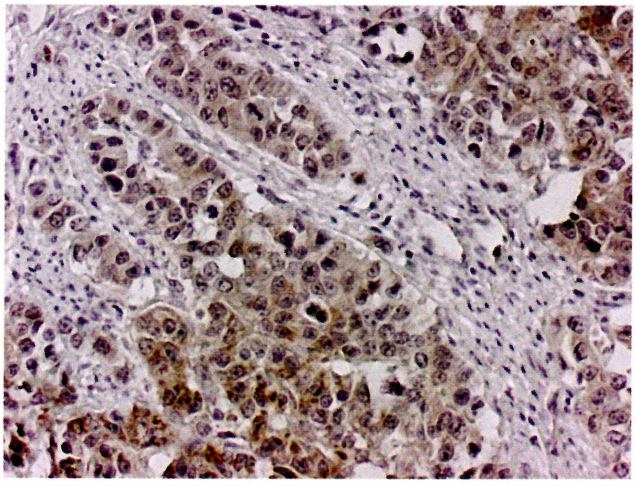


Fig. 1b

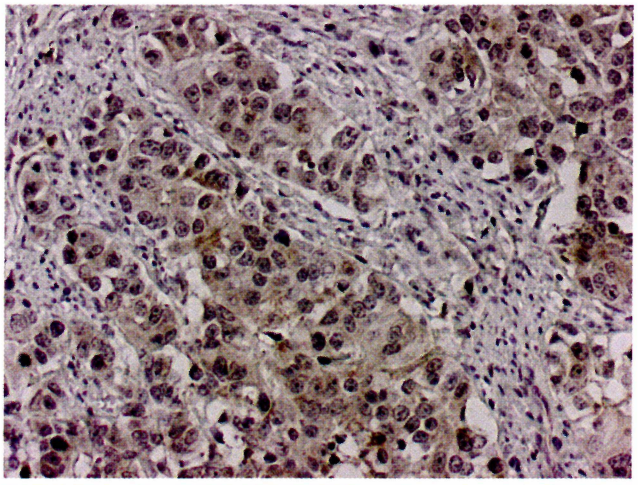


Fig. 1c

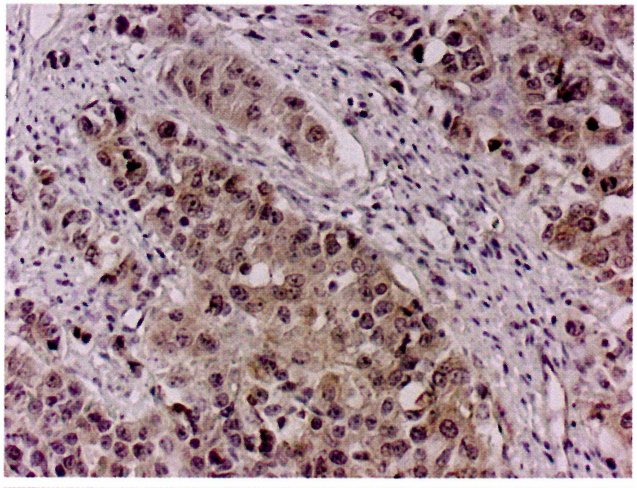


Fig. 1d

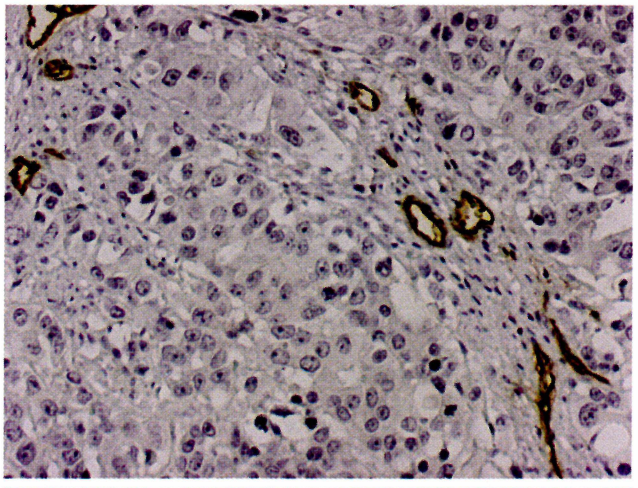


Fig. 2a

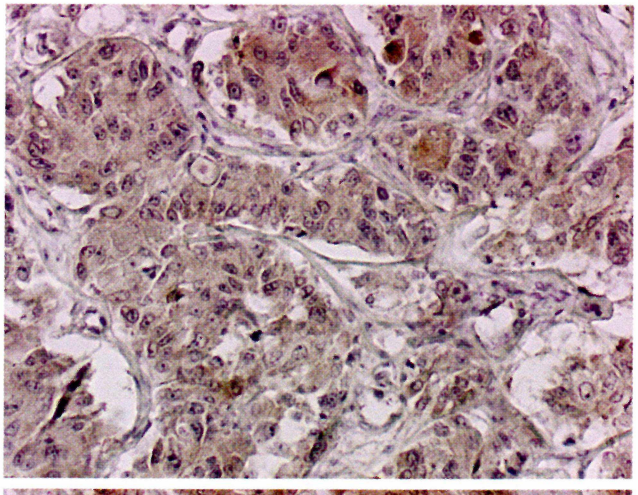


Fig. 2b

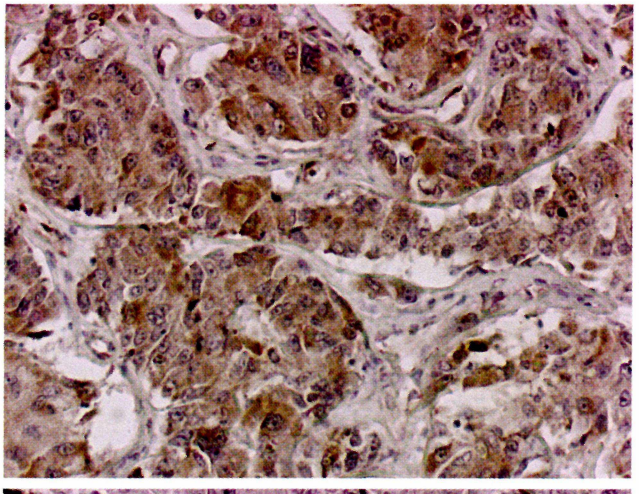


Fig. 2c

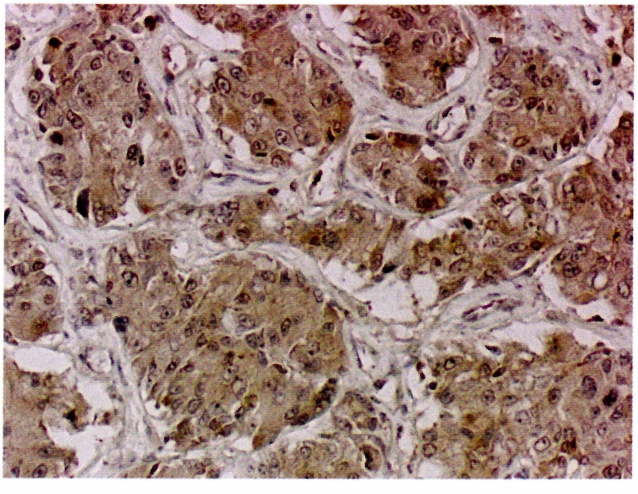
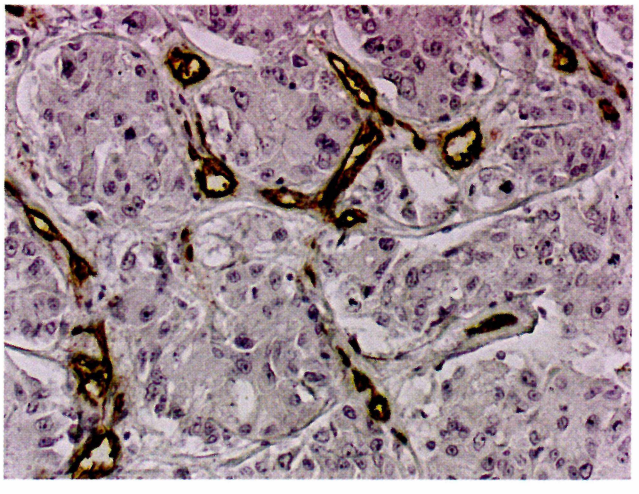


Fig. 2d



TVCs calculated with CD34 staining specimens in the whole G1, G2 and G3 endometrial carcinomas were 104 ± 32, 104 ± 28 and 102 ± 43, respectively. MVCs were 70 ± 29, 65 ± 22 and 62 ± 38, respectively. VDs were 9.2 ± 2.1, 9.6 ± 2.3 and 10.3 ± 2.7 μm, respectively. The VD was significantly larger in G3 than in G1 ($p = 0.0459$; Mann-Whitney U-test; Figure 4), although both TVC and MVC were not correlated with histological grade. The three vessel indicators were not correlated with Ang1, Ang2 or Tie2, except for a significant correlation between VD and Tie2 expressions ($p = 0.0233$, $r_s = -0.197$; Spearman's rank correlation test; Figure 5). In high VEGF cases, Ang1 was correlated significantly with a lower TVC and MVC ($p = 0.0403$ and $r_s = -0.258$, $p = 0.0315$ and $r_s = -0.271$, respectively; Spearman's rank correlation test; Table 2), whereas Ang1 was correlated significantly with a larger VD ($p = 0.0285$ and $r_s = 0.276$; Spearman's rank correlation test; Table 2). In contrast, there was no correlation between vessel indicators and Ang2 or Tie2. These three vessel indicators were also not correlated with VEGF expression (Table 3).

Table 2. — *Correlation of Ang1, Ang2 and Tie2 expressions with intratumoral vessels in high VEGF cases.*

	TVC	MVC	VD
Ang1	$p = 0.0403$	$p = 0.0315$	$p = 0.0285$
	$r_s = -0.258$	$r_s = -0.271$	$r_s = 0.276$
Ang2	$p = 0.9907$	$p = 0.5138$	$p = 0.5766$
	$r_s = -0.001$	$r_s = 0.082$	$r_s = -0.070$
Tie2	$p = 0.7895$	$p = 0.3495$	$p = 0.2869$
	$r_s = 0.034$	$r_s = 0.118$	$r_s = -0.134$

(Spearman's rank correlation test); TVC: total vessel count; MVC: microvessel count; VD: mean vessel diameter.

Table 3. — *Correlation of intratumoral vessels with VEGF expression.*

	n	TVC (Mean ± SD)	MVC (Mean ± SD)	VD (Mean ± SD)	p value
Low	36	111 ± 34	76 ± 31	9.0 ± 2.2	
High	64	105 ± 36	68 ± 29	9.4 ± 2.3	N.S.
Total	100	107 ± 35	71 ± 30	9.2 ± 2.2	

(N.S.: not significant; Mann-Whitney U test).

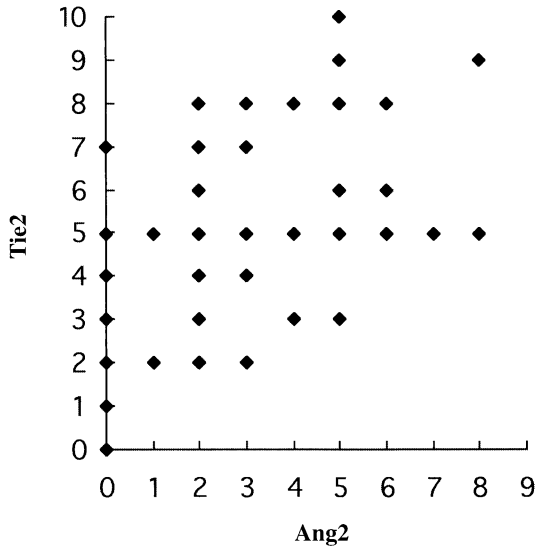


Figure 3. — Correlation between Ang2 and Tie2 expressions Ang2 expression was significantly correlated with those of Tie2 ($p < 0.001$ and $r_s = 0.465$; Spearman's rank correlation test).

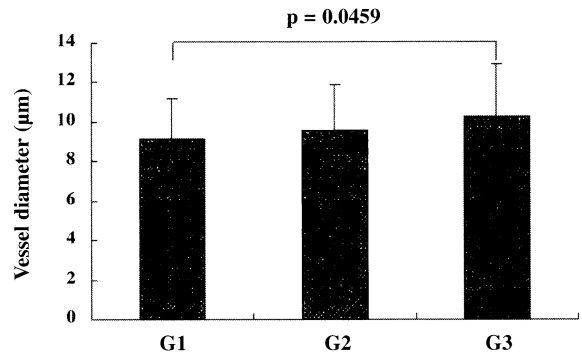


Figure 4. — Correlation between VD and histopathological grade VD was significantly larger in G3 than in G1, well, G2, moderately and G3, poorly differentiated adenocarcinoma (Mann-Whitney U test).

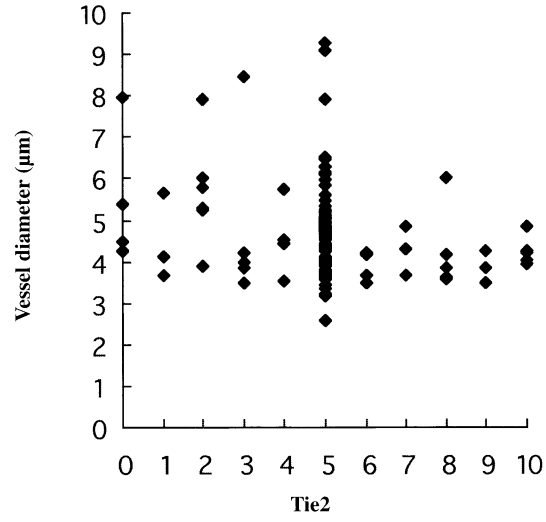


Figure 5. — Correlation between VD and Tie2 expression VDs were significantly correlated with Tie2 expressions ($p = 0.0233$ and $r_s = -0.197$; Spearman's rank correlation test).

The Ang1 > Ang2, Ang1 = Ang2 and Ang1 < Ang2 groups among the total endometrial carcinomas consisted of a total of 29 cases (21.8%), 75 cases (56.4%) and 29 cases (21.8%), respectively. In 64 high VEGF cases, these groups consisted of 17 cases (26.6%), 35 cases (54.7%) and 12 cases (18.7%), respectively, and the TVCs in Ang1 > Ang2, Ang1 = Ang2 and Ang1 < Ang2 groups were 98 ± 38, 102 ± 30 and 123 ± 44, respectively (Figure 6). The MVCs were 61 ± 31, 65 ± 21 and 88 ± 37, respectively (Figure 7). VDs were 10.3 ± 3.3, 9.3 ± 1.8 and 8.4 ± 1.5 mm, respectively (Figure 8). The Ang1 < Ang2 group in high VEGF cases was significantly higher in TVC than the Ang1 > Ang2 group ($p = 0.0189$; Mann-Whitney U-test; Figure 6). The Ang1 < Ang2 group was significantly

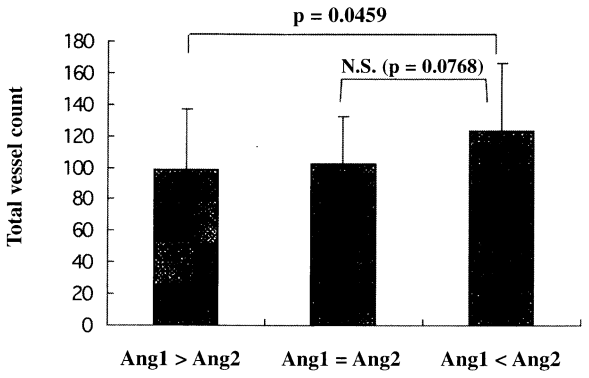


Figure 6. — Correlation between TVC and Ang expression balance in high VEGF cases TVC in the Ang1 < Ang2 group was significantly higher than that in the Ang1 > Ang2 group and tended to be higher than that in the Ang1 = Ang2 group (*p = 0.0189, **p = 0.0768; Mann-Whitney U test).

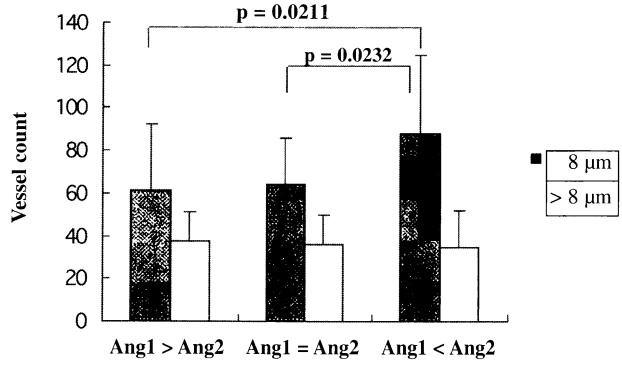


Figure 7. — Correlation between MCV and Ang expression balance in high VEGF cases MVC was significantly higher in the Ang1 < Ang2 group than in the Ang1 > Ang2 and Ang1 = Ang2 groups, whereas large vessels were not correlated with the Ang expression balance (*p = 0.0211, **p = 0.0232; Mann-Whitney U test).

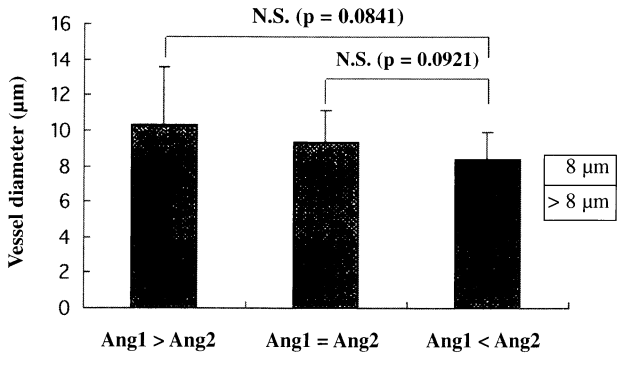


Figure 8. — Correlation between VD and Ang expression balance in high VEGF cases D tended to be smaller in the Ang1 < Ang2 group than in the Ang1 > Ang2 and Ang1 = Ang2 groups, although not significant (*p = 0.0841, **p = 0.0923: Mann-Whiney U test).

higher in MVC (vessel count < 8 μm in diameter) than the Ang1 > Ang2 group (p = 0.0211; Mann-Whitney U-test; Figure 7) and the Ang1 = Ang2 group (p = 0.0232; Figure 7), although large vessels larger than 8 μm in diameter were not correlated with Ang expression balance. The Ang1 < Ang2 group tended to be smaller in VD than other groups, although it was not significant (Figure 8). Additionally, the Ang balance was not correlated with intratumoral vessels in low VEGF cases.

Discussion

It has been reported that Ang1 and Ang2 are expressed in the stromal, glandular and endothelial cells of human endometrium in both the proliferative and secretary phases, and that Tie2 is expressed in the glandular and endothelial cells [12, 13]. In our study, positive expressions of Ang1, Ang2 and Tie2 were also expressed in the cytoplasm of endometrial carcinoma cells. This confirmed that endometrial carcinoma cells produced those factors. These expressions were stronger in tumor cells than in endothelial and stromal cells. To the best of our knowledge, Ang1, Ang2 and Tie2 expressions have not been reported in endometrial carcinoma cells.

Ang1, Ang2 and Tie2 expressions were positively correlated with each other in our study. A few studies have reported on the correlation among Angs and Tie2 expressions. Ang2 allows angiogenic sprouts of vessels by promoting the destabilization of blood vessels, and Ang1 promotes the stabilization and tightening of preexisting vessels [4]. Therefore, our study indicates that efficient angiogenesis in endometrial carcinoma is mediated by both Ang1 and Ang2, by performing both steps at the same time.

No comparative study on the relationship between Angs and Tie2 expressions, and histological tumor grade in malignant tumors has been reported yet. In our results, Angs, Tie2 and VEGF expressions were not correlated with histological grade; however, the VD in G3 cases was larger than that in G1 cases, although TVC and MVC were not correlated. It has been suggested that high grade endometrial carcinomas require larger vessels due to high proliferative activity [14].

It has been shown that both Ang2 [6, 15-19] and VEGF [19] were up-regulated and that both Ang1 and Tie2 were down-regulated [8] by hypoxia. A significant correlation between VEGF and Ang2 expressions [20, 21] and also between VEGF and Tie2 expressions [21] has been demonstrated in papillary thyroid and gastric carcinomas. In ovarian carcinoma [16], a significant correlation between VEGF and Ang2, but not between VEGF and Ang1 or Tie2, has been reported. In this study, VEGF expression in endometrial carcinoma was not correlated with individual Ang1, Ang2 or Tie2, although it was reported that Ang2 expression in bovine microvascular endothelium was up-regulated by VEGF [15].

The Ang expression balance [22, 23] and the relationship between Ang and VEGF expressions [6, 7, 23] may play a role in the angiogenesis. Holash *et al.* [22] reported that the

quantitative balance between Ang1 (agonist) and Ang2 (antagonist) activities defined the Tie2 signaling pathway. In hepatocellular carcinoma, the Ang2/1 relationship was associated with portal vein invasion, tumor diameter and microvessel density (MVD), and the prognosis for patients with a high Ang2/1 ratio was significantly poor [24]. In non-small cell lung cancer, the MVD of Ang2 positive cases was significantly higher than that of Ang2 negative ones [25]. Etoh *et al.* [20] demonstrated that Ang2 required the presence of VEGF to play a role in the angiogenesis of gastric cancer [20]; however, no study on endometrial carcinoma has been reported. Therefore, we classified cases into three groups depending on the intensity of Ang1 and Ang2 expressions. In high VEGF cases, the TVC and MVC in the Ang2 dominant group were significantly higher than those in the Ang1 dominant group. It is said that Ang2 is involved in the sprouting of endothelial cells by destabilizing vessel walls, and contributes to tumor angiogenesis by cooperating with VEGF to induce blood vessel growth [6]. In endometrial carcinoma, our study demonstrates that in the Ang2 dominant group, rich angiogenesis of endometrial carcinoma has been induced in the presence of VEGF, i.e., Ang2 dominant expression under high VEGF conditions is correlated significantly with higher TVC and MVC, and with smaller VD. These results suggest that the Ang expression balance is quite important for the angiogenesis of endometrial carcinoma. The Ang2 dominant backgrounds up-regulate tumor angiogenesis in the presence of VEGF.

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Address reprint requests to:
J. WATANABE, M.D.
Department of Pathology
School of Medicine
Kitasato University
1-15-1 Kitasato, Sagamihara
Kanagawa, 228-8555 (Japan)