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Preclinical study

Angiopoietin 2 expression in invasive ductal carcinoma of the breast: its relationship to the VEGF expression and microvessel density

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Summary

Angiopoietin (Ang) is a ligand for the endothelium-specific tyrosine kinase receptor Tie-2, while a shift in the Ang-1:Ang-2 expression ratio in favor of Ang-2 was found to be associated with tumor angiogenesis. In the present study, we analyzed the immunohistochemical expression of Ang-2 in a series of 198 breast cancers, in which VEGF expression and microvessel density (MVD) were previously determined. Ang-2 expression was negative in 24 (12%), positive in 50 (25%) and strongly positive in 124 (63%) of 198 cases. A significant correlation was found between Ang-2 and VEGF expressions ($p = 0.0004$) and between Ang-2 expression and MVD ($p = 0.0006$), while a high MVD was found in 10 (77%) of 13 tumors with a strongly positive VEGF and positive Ang-2 expression and in 40 (71%) of 56 tumors with a strongly positive VEGF and strongly positive Ang-2 expression. Although there was no difference in the disease free survival (DFS) stratified according to Ang-2 expression alone, the 69 patients with a strongly positive VEGF and a strongly positive or positive Ang-2 expression had a significantly ($p = 0.0316$) worse DFS than those with other combinations of VEGF and Ang-2 expressions. A multivariate analysis indicated lymph node metastasis and MVD to be independently significant prognostic factors for DFS, while the combination of VEGF and Ang-2 expressions was not a significant factor for DFS. In conclusion, the Ang-2 expression was found to be closely correlated with VEGF expression and MVD in breast cancer, while a high MVD was frequently found in tumors with a high expression of both VEGF and Ang-2. The survival analysis demonstrated a high MVD, which was induced by a high expression of both VEGF and Ang-2, to therefore have a strong prognostic significance in breast cancer.

Introduction

Angiogenesis is an important and essential step in the growth and metastasis of the tumor [1]. The microvessel density (MVD) has been evaluated as a quantitative marker of new vessel formation in tumor tissue and the prognostic significance of MVD has also been demonstrated in various tumors [2]. We previously demonstrated the prognostic significance of MVD in breast cancer [3, 4]. On the other hand, pro- and anti-angiogenic factors are known to play various roles in tumor angiogenesis. Among these factors contributing angiogenesis, VEGF has so far been the best characterized and it is also thought to be the most potent pro-angiogenic factor in a tumor angiogenesis [5]. A high VEGF expression has been demonstrated in various tumors, while we previously demonstrated a close correlation between the VEGF expression and MVD and a prognostic significance of VEGF expression in breast cancer [4].

Angiopoietin (Ang), a novel endothelial growth factor, was found to be a ligand for the endothelium-specific tyrosine kinase receptor Tie-2 [6]. Ang-1 plays a role in maintaining and stabilizing mature vessels by promoting the interaction between endothelial cells and surrounding support cells, whereas Ang-2 is expressed at sites of vascular remodeling and is thought to antagonize the stabilizing action of Ang-1 [7,8]. Tait et al. reviewed 56 published series regarding the Ang-1 and Ang-2 expression in various tumors and described that Ang-1 and Ang-2 expressions were both elevated in the tumor tissues, while the Ang-2 expression was more frequently up-regulated than Ang-1 or Tie2 expressions [9]. A shift in the Ang-1:Ang-2 ratio in favor of Ang-2 clearly appears to be associated with angiogenesis in the tumor tissues [9]. Experimental studies [10–13] have suggested a close relationship between VEGF and Ang-2 functions in angiogenesis and a correlation between Ang-2 and VEGF expressions have been found in

various tumors [14–17], however, no correlation between Ang-2 and VEGF expressions was found in breast cancer tissues [18,19]. On the other hand, a correlation between Ang-2 expression and MVD has been found in various tumors [15, 20–25], while conflicting results have been published regarding the relationship between Ang-2 expression and MVD in breast cancer tissue specimens [18,19]. In the present study, the Ang-2 expression was therefore evaluated in 198 breast cancer tissue specimens in which the VEGF expression and MVD had been determined previously [3,4]. The aim of the present study was to evaluate the relationship between Ang-2 and VEGF expressions and between Ang-2 expression and MVD in breast cancer, and the prognostic value of Ang-2 expression in relation to the VEGF expression and MVD in breast cancer was also evaluated.

Patients and methods

Patients

This study comprised 198 women with breast cancer who underwent surgery for breast cancer, without any evidence of distant metastasis at the time of surgery, between 1985 and 1998 at the Beppu Medical Center. The histological type of breast cancer in all patients was invasive ductal carcinoma, while all types other than invasive ductal carcinoma were excluded in this study. No cases of non-invasive carcinoma were included in this study. The patients' ages ranged from 27 to 86 years, with a mean age of 58.4 years. The patients were either treated by a mastectomy (180 patients) or by breast conservation treatment (18 patients). A lymph node dissection was performed in 196 patients. Adjuvant postoperative hormone therapy was administered to 170 patients and 168 patients received adjuvant chemotherapy, while 33 patients received postoperative radiotherapy. The median follow-up duration was 6.54 years.

Immunohistochemistry

For an immunohistochemical analysis of the Ang-2 protein, paraffin-embedded sections (3-μm) were deparaffinized and rehydrated, and were heated at 95 °C for 5 min in 10 mM sodium citrate buffer (pH 6.0) with a microwave for antigen retrieval. Endogenous peroxidase in the sections was inactivated in 0.1% pepsin in 0.01 N HCL buffer, pH 2.5, followed by the incubation in 0.05% saponin for 30 min at room temperature. The sections were blocked in 1.5% normal horse serum in PBS and then were incubated at 4 °C overnight with goat polyclonal anti-Ang-2 antibody (C-19, Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:250 in PBS. The sections were subsequently stained according to the labelled streptavidin biotin (LSAB) method using DAKO LSAB + kit (DAKO, Kyoto, Japan) and were visualized using 3,3′-diaminobenzidine, followed by counterstaining with hematoxylin.

In the present study, we evaluated the correlation of Ang-2 expression to the VEGF expression, MVD and the cell proliferation (MIB-1 counts) that have been previously studied by immunohistochemistry for the tumors in the present series [3,4,26]. The immunohistochemical procedures and results regarding these parameters have all been described previously [3,4,26].

The immunohistochemical expression of Ang-2 was assessed in the tumor cells but not in the stromal cells. The Ang-2 expression was determined by a method previously described by Volm et al. [27], while the determination method used in the present study was the same as that used previously to determine the VEGF expression [4]. Briefly, the sections were graded, respectively, according to the percentage of positively stained tumor cells (0: 0% immunopositive cells, 1: $<25\%$ positive cells, 2: 26–50% positive cells and 3: $>50\%$ positive cells) and the staining intensity (0: negative, 1: weak, 2: moderate and 3: high). The score of the percentage of positively stained cells and the staining intensity between 0 and 2 was regarded as negative, the score between 3 and 4 was regarded as positive, and the score between 5 and 6 was regarded as strongly positive. The Ang-2 protein expression was determined independently by two authors (S.T. and H.I.) while a final determination was made by the author (H.I.) did not know any clinicopathological information about each patient.

Statistical analysis

The χ^2-test was used to investigate any significance of the relationships between the Ang-2 expression and the individual variables. The disease free survival (DFS) was estimated using the Kaplan and Meier method, and any differences in the survival curves were compared by the Log-rank test. A multivariate analysis was performed by Cox's proportional hazards model. The p-value of <0.05 was regarded as statistically significant. All statistical analyses were performed using the StatView 5.0 software package (SAS Institute Inc. Cary, NC).

Results

Ang-2 and VEGF expressions and MVD in 198 breast cancers

Ang-2 expression was found to be negative in 24 (12%) cases, positive in 50 (25%) cases and strongly positive in 124 (63%) cases, while VEGF expression was found to be negative in 45 (23%) cases, positive in 82 (41%) cases and strongly positive in 71 (36%) cases. A low MVD was found in 127 (64%) cases and a high MVD was found in 71 (36%) cases. Table 1 shows the relationship of Ang-2 expression to clinicopathological factors in breast cancer. There was a significant correlation between Ang-2 and VEGF expressions ($p=0.0004$) and between Ang-2 expression and MVD ($p=0.0006$) in 198

Table 1. Ang-2 expression and clinicopathological factors in breast cancer

	No. of patients	Ang-2			*p*-value
		(−) ($n=24$)	(+) ($n=50$)	(++) ($n=124$)	
Tumor size (cm)					0.5750
–2.0	43	3 (7)	13 (30)	27 (63)	
2.1–5.0	128	19 (15)	30 (23)	79 (62)	
5.1–	27	2 (7)	7 (26)	18 (67)	
Lymph node metastasis[a]					0.6729
Absent	106	11 (10)	27 (25)	68 (64)	
Present	90	13 (14)	23 (26)	54 (60)	
Nuclear grade					0.9431
I or II	131	16 (12)	34 (26)	81 (62)	
III	67	8 (12)	16 (24)	43 (64)	
Estrogen receptor					0.5339
Positive	84	12 (14)	23 (27)	49 (58)	
Negative	114	12 (11)	27 (24)	75 (66)	
MIB-1 counts					0.0031
Negative (<10%)	115	21 (18)	31 (27)	63 (55)	
Positive (10% ≤)	83	3 (4)	19 (23)	61 (73)	
VEGF					0.0004
(−)	45	12 (27)	12 (27)	21 (47)	
(+)	82	10 (12)	25 (30)	47 (57)	
(++)	71	2 (3)	13 (18)	56 (79)	
MVD					0.0006
Low	127	22 (17)	37 (29)	68 (54)	
High	71	2 (3)	13 (18)	56 (79)	

[a]Lymph node dissection was performed in 196 patients.

breast cancers. There was also a significant correlation between the Ang-2 expression and the MIB-1 counts while no correlation was found between the Ang-2 expression and the tumor size, lymph node metastasis, nuclear grade and estrogen receptor.

MVD in relation to VEGF and Ang-2 expression

Table 2 shows the MVD according to the combination of VEGF and Ang-2 expressions. A high MVD was found in 10 (77%) of 13 cases with a strongly positive VEGF and positive Ang-2 expression and in 40 (71%) of 56 cases with a strongly positive VEGF and strongly positive Ang-2 expression. A significant ($p<0.0001$) correlation was thus observed between the MVD and the combination of VEGF and Ang-2 expressions.

Survival analysis based by univariate and multivariate analyses

Figure 1(a) shows the survival (DFS) curves stratified according to Ang-2 expression. There was no difference in DFS according to Ang-2 expression alone. Next, 69 patients with a strongly positive VEGF and strongly

Table 2. MVD according to the combination of VEGF and Ang-2 expressions

Combination of VEGF/Ang-2 expressions	No. of patients	MVD		*p*-value
		Low ($n=127$)	High ($n=71$)	
(−)/(−)	12	11 (92)	1 (8)	<0.0001
(−)/(+)	12	12 (100)	0 (0)	
(−)/(++)	21	17 (81)	4 (19)	
(+)/(−)	10	10 (100)	0 (0)	
(+)/(+)	25	22 (88)	3 (12)	
(+)/(++)	47	35 (74)	12 (26)	
(++)/(−)	2	1 (50)	1 (50)	
(++)/(+)	13	3 (23)	10 (77)	
(++)/(++)	56	16 (29)	40 (71)	

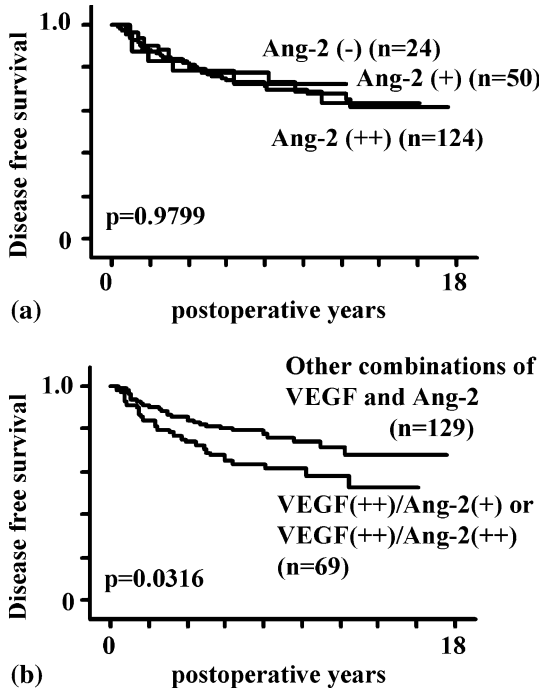

Figure 1. The disease free survival (DFS) curve stratified according to the Ang-2 expression (a) and the combination of VEGF and Ang-2 expressions (b).

positive or positive Ang-2 expression, which was found to be related to a high MVD, was compared with the 129 patients with other combinations of VEGF and Ang-2 expressions. The 69 patients with a strongly positive VEGF and strongly positive or positive Ang-2 expression had a significantly ($p = 0.0316$) worse DFS than those with other combinations of VEGF and Ang-2 expressions (Figure 1(b)). A multivariate analysis indicated lymph node metastasis and MVD to be independently significant prognostic factors for DFS, while the combination of VEGF and Ang-2 expressions was not a significant factor for DFS (Table 3).

Discussion

There have been several studies regarding to the relationships between Ang-2 and VEGF expressions and between Ang-2 expression and MVD in various tumor tissues [14–25], while many studies have been conducted on Ang-1 and Ang-2 expressions in various tumors [9]. Table 4 shows the published series regarding to the relationship of Ang-2 expression to VEGF expression and MVD. A significant correlation was found between the Ang-2 and VEGF expressions in brain (glioma) tumor [14] and liver [15], stomach [16] and ovary [17] cancers. No significant correlation, however, was found between the Ang-2 and VEGF expressions in breast cancer [18,19]. On the other hand, a significant correlation was found between the Ang-2 expression and MVD in liver [15,20], lung [21,22], colon [23], ovary [24] and prostate [25] cancers. Regarding to the relationship between the Ang-2 expression and MVD in breast cancer, Curiie et al. [18] found no significant correlation while Sfiligoi et al. [19] demonstrated a significant ($p = 0.04$) correlation between the Ang-2 expression and MVD. The difference in the correlation between breast cancer and other tumor tissues seems to be due that the sample size of the two studies [18,19] on breast cancer was relatively small, compared with the studies on other tumor tissues (Table 4). The present study on 198 breast cancers demonstrated a statistically significant correlation between Ang-2 and VEGF expressions and between Ang-2 expression and MVD. More noteworthy in the present study is the fact that a high MVD was frequently found in the cases with a strongly positive VEGF and positive Ang-2 expression or with a strongly positive VEGF and strongly positive Ang-2 expression. Similar findings have also been demonstrated in lung [21] and colon [23] cancers. The average MVD for Ang-2 positive tumor was found to be significantly higher than that for Ang-2 negative tumor in non-small cell lung cancers,

Table 3. Multivariate analyses for DFS

Variables	*p*-value	Relative risk	95% CI
Tumor size (cm)			
2.1–5.0 (vs. –2.0)	0.3969	0.70	0.31–1.59
5.1– (vs. –2.0)	0.8308	1.11	0.42–2.98
Lymph node metastasis			
Present (vs. absent)	< 0.0001	6.12	2.96–12.7
Nuclear grade			
III (vs. I or II)	0.2518	1.41	0.78–2.55
Estrogen receptor			
Negative (vs. positive)	0.9627	1.01	0.57–1.82
MIB-1 counts			
10% ≤ (vs. < 10%)	0.0953	1.73	0.91–3.31
MVD			
High (vs. low)	0.0035	2.94	1.43–6.06
VEGF/Ang-2 expressions:			
(++)/(+) or (++)/(++) (vs. others)	0.5482	0.82	0.42–1.58

Table 4. Published series regarding the relationship of Ang-2 expression to VEGF expression and microvessel density

Author, year [Ref. No.]	Site of tumor	Method	No. of patients	Correlation with VEGF	Correlation with MVD
Currie, 2001 [18]	Breast	mRNA	38, 42	N. S. ($n=38$)	N. S. ($n=42$)
Sfiligoi, 2003 [19]	Breast	mRNA	14	N. S.	$p=0.04$
Osada, 2001 [14]	Brain (glioma)	mRNA	39	$p=0.039$	
Moon, 2003 [15]	Liver	mRNA	49	$p<0.001$	$p=0.001$
Mitsuhashi, 2003 [20]	Liver	mRNA	46		$p=0.010$[a]
Tanaka, 2002 [21]	Lung	IHC	236		$p=0.032$
Takanami, 2004 [22]	Lung	mRNA	77		$p<0.0001$
Sun, 2004 [16]	Stomach	IHC	72	$p=0.0055$	
Ochiumi, 2004 [23]	Colon	IHC	152		$p<0.01$
Hata, 2004 [24]	Ovary	mRNA	85		$p<0.0001$[a]
Zhang, 2003 [17]	Ovary	mRNA	52	$p<0.01$	
Lind, 2005 [25]	Prostate	IHC	64		$p=0.002$

[a]The Ang-1/Ang-2 expression ratio showed a correlation with MVD.

and such an angiogenic effect of Ang-2 was only seen when the VEGF expression was high [21]. The MVD was not significantly correlated with the Ang-2 expression alone but it was significantly up-regulated by the VEGF expression under positive Ang-2 conditions in colon cancers [23]. These findings suggested a high MVD to be frequently found under a high expression of both VEGF and Ang-2, while a high Ang-2 expression in the absence of high VEGF expression did not contribute to a high MVD.

Although the role and mechanism of Ang-2 in tumor angiogenesis has not yet been fully clarified, experimental studies have demonstrated a close relationship of VEGF and Ang-2 functions in angiogenesis. Ang-2 was found to promote a vessel sprouting in the presence of abundant VEGF, whereas Ang-2 contributed to vessel regression in the absence of VEGF [7,8,10]. VEGF induced both a time- and concentration-dependent increase of Ang-2 expression in bovine microvascular endothelial cells [11]. On the other hand, ectopically administered Ang-2 was shown to promote endothelial cell proliferation and sprouting of new blood vessels in the presence of endogenous VEGF [12]. Futhermore, a study using transgenic mice ectopically expressing VEGF, Ang-1 and Ang-2 demonstrated Ang-2 to stimulate VEGF-mediated angiogenesis, thus indicating the synergistic effect of Ang-2 and VEGF on the induction of angiogenesis [13]. These experimental studies all indicated the complementary and coordinated role of VEGF and Ang-2 in angiogenesis.

The prognostic significance of Ang-2 expression has been demonstrated in the breast [19], stomach [28], bladder [29], lung [21,22] and prostate [25] cancers, while the prognostic significance of the Ang-1/Ang-2 expression ratio was also demonstrated in liver [20] and ovary [24] cancers. Sfiligoi et al. [19] demonstrated a significant and independent association between the Ang-2 mRNA level and both disease free ($p<0.0001$) and overall survival ($p<0.0003$) in 38 breast cancer. In the present study, however, the prognostic significance of the Ang-2 expression alone was not found in 198 breast cancers, while the Ang-2 expression was correlated with MIB-1 counts which had been found to be a strong prognostic factor for breast cancer [26]. On the other hand, the patients with a strongly positive VEGF and strongly positive or positive Ang-2 expression, which was closely related to a high MVD, had a significantly worse prognosis than those with other combinations of Ang-2 and VEGF expressions. The prognostic value of the combination of Ang-2 and VEGF expressions has also been demonstrated in lung [21], colon [23] and ovary [24] cancers. The patients with the tumors showing both positive Ang-2 and positive VEGF expression had a significantly worse prognosis than those with the other combinations in lung [21] and colon [23] cancers, while the combination of Ang-1/Ang-2 gene expression ratio and VEGF gene expression was significantly associated with a poor prognosis in ovarian cancers [24]. Our previous study [3] demonstrated that patients with a strongly positive VEGF expression had a significantly worse prognosis than those with a positive or negative VEGF expression. On the other hand, a multivariate analysis in the present study demonstrated MVD to be an independently significant prognostic factor for breast cancer while the combination of Ang-2 and VEGF expressions was not a significant factor. This finding indicated that a high MVD, which was induced by a high expression of both Ang-2 and VEGF, demonstrated prognostic significance and the MVD to be a more powerful prognostic factor than VEGF and Ang-2 expressions in breast cancer.

In conclusion, the present study demonstrated a close correlation of the Ang-2 expression to both VEGF expression and MVD in breast cancer. The finding that a high MVD was frequently found in tumors with a strongly positive VEGF and strongly positive or positive Ang-2 expression suggested the complementary and coordinated role of VEGF and Ang-2 in tumor angiogenesis. The survival analysis demonstrated a high MVD, which was induced by a high expression of both VEGF and Ang-2, to therefore have a strong prognostic significance in breast cancer.

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