Inhibition of Infiltration and Angiogenesis by Thrombospondin-1 in Papillary Thyroid Carcinoma

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ABSTRACT

Purpose: Angiogenesis is essential for tumor growth and is controlled by the balance between angiogenic and antiangiogenic factors. We studied the expression of angiogenic factors and antiangiogenic factors in papillary thyroid carcinoma.

Experimental Design: We investigated immunohistochemically the expression patterns and levels of angiogenic factor and its receptor, thrombospondin-1 (TSP-1) and CD36, and four angiogenic factors, vascular endothelial growth factor (VEGF), VEGF-C, angiopoietin-2 (Ang-2), and Tie-2, in the primary tumors of 75 papillary thyroid carcinoma patients. We also examined the microvessel count (MVC), using CD31 staining.

Results: VEGF expression strongly correlated with other angiogenic factors. The cytoplasm of cancer cells stained positive for all factors. Tie-2 and TSP-1 receptor also appeared in endothelia of microvessels. TSP-1 inversely correlated with the degree of invasion of the primary tumor to other adjacent organs and with MVC. A higher MVC correlated with poorer survival. To clarify the balance between angiogenic and antiangiogenic factors in the same tumor, we calculated the ratio of each angiogenic factor against TSP-1 as the antiangiogenic factor. The ratios VEGF/TSP-1, VEGF-C/TSP-1, and Ang-2/TSP-1 significantly correlated with a higher MVC. Furthermore, the ratios VEGF/TSP-1 and Ang-2/TSP-1 significantly correlated with the degree of infiltration.

Conclusions: To the best of our knowledge, this is the first report demonstrating that the balance between angiogenic and antiangiogenic factors correlates with distinct invasion to other organs and neovascularization of papillary thyroid carcinoma.

INTRODUCTION

Angiogenesis is essential for tumor growth and progression (1). Among many angiogenic factors, VEGF3 is considered the most important (2). Ang-2 is an angiogenic factor that binds to the Tie-2 receptor with an antagonistic effect for binding of Ang-1 and Tie-2 (3). Recently, Ang-2 has been reported to play a key role in initiating neovascularization before activation of VEGF (4, 5). Holash et al. (5) have reported that induced Ang-2 destabilized endothelium, smooth muscle, and the extracellular matrix and that VEGF led these unstable vessels to angiogenesis.

Antiangiogenic factors have also been identified, including TSP-1 (6), TSP-2 (7), angiostatin (8), endostatin (9), and vasostatin (10). TSP-1 is a high molecular weight protein and is recognized as a mediator of the components of the extracellular matrix (11). TSP-1 binds to some receptors, such as D51/CD61 (α3β1 integrin; Ref. 12). The Val-Thr-Cys-Gly sequence binds to CD36, so-called platelet GPIIb (13), and the COOH-terminal domain binds to a Mv 8,000/105,000 receptor (14) to mediate each cell. TSP-1 is an antiangiogenic factor (15), but its biological role is still controversial. The antiangiogenic effects of TSP-1 have been reported in bladder cancer (16), squamous cell carcinoma (17, 18), breast cancer (19), and gastrointestinal tumors (19). However, TSP-1 has also been reported to be an angiogenic factor in cancers of the colon and rectum (20), pancreas (21), and breast (22) and a lymphangiogenic factor in gallbladder cancer (23). In addition, TSP-1 has been demonstrated to be an inhibitor of adhesion activity in breast cancer cells and a promoter of infiltration of cancer cells (24). Other studies have demonstrated that TSP-1 is an antitumor factor in melanoma (25) and small cell lung carcinoma (26), independent of angiogenesis. Thus, TSP-1 has been demonstrated to play diverse roles in each organ. CD36 and CD51 have also been investigated in some carcinoma tissues. CD36 has been demonstrated on breast cancer cells (22, 27, 28), cutaneous squamous cells (17), and colorectal cancer cells (29). Some authors observed no expression of CD36 on thyroid (30) or colon (31) cancer cells, but expression of CD51 has been demonstrated on breast (27) and thyroid (30) cancer cells. The patterns of expression vary in each organ, and the relationship between clinical data and the expression of CD36 was further investigated. We therefore investigated the effect of TSP-1 on cells through CD36 or CD51 and studied the expression levels of CD36.

In this study, we examined immunohistochemically the
expression patterns and levels of TSP-1 and CD36 in addition to four angiogenic factors, VEGF, VEGF-C, Ang-2, and Tie-2, in the primary tumors of papillary thyroid carcinoma patients. To clarify the roles of these factors in papillary thyroid carcinoma, we studied the correlation among the angiogenesis-related factors and the relationship between their expression and clinical data.

MATERIALS AND METHODS

The subjects of this study were 25 recurrent papillary thyroid carcinoma patients with distant metastases and 50 non-recurrent patients who were treated in our hospital. As disease-free patients, we selected at random two nonrecurrent patients for each recurrent patient matched according to age, sex, the TNM classification of the postoperative diagnosis, and the surgical method used. All patients were euthyroid before primary operations. All patients had received thyroxin to suppress serum thyroid-stimulating hormone during follow-up after the initial surgical treatment. The dose of thyroxin necessary to maintain the serum thyroid-stimulating hormone level under the lower limit for each patient was determined by measuring the level every 6 months during follow-up. Distant recurrence of disease was defined by echography, chest X-rays, computed tomography, and/or thallium or radioiodine scintigraphy during follow-up.

For this study, we used embedded paraffin sections of the primary tumor from each patient, which were resected at each initial surgical procedure.

Informed consent was obtained from all enrolled patients. We determined the clinical stage and TNM classification of the postoperative diagnosis, and the surgical method used. All patients were euthyroid before primary operations. All patients had received thyroxin to suppress serum thyroid-stimulating hormone during follow-up after the initial surgical treatment. The dose of thyroxin necessary to maintain the serum thyroid-stimulating hormone level under the lower limit for each patient was determined by measuring the level every 6 months during follow-up. Distant recurrence of disease was defined by echography, chest X-rays, computed tomography, and/or thallium or radioiodine scintigraphy during follow-up.

For this study, we used embedded paraffin sections of the primary tumor from each patient, which were resected at each initial surgical procedure.

We determined the clinical stage and TNM classification of the primary tumor according to the International Union Against Cancer classification (32).

Immunohistochemical Procedures. The paraffin-embedded sections were cut into sections 4 μm thick and fixed on aminopropyltriethoxysilane-coated glass slides (Matsunami, Osaka, Japan). After deparaffinization by standard methods, normal rabbit serum was applied, and the sections were incubated for 10 min at room temperature to block nonspecific binding of the antibody. The slides were then incubated for 2 h at room temperature with each of the following primary antibodies: VEGF antibody, a mouse monoclonal IgG2a antibody (sc-7269; Santa Cruz Biotechnology, Santa Cruz, CA), at a concentration of 10 μg/ml; VEGF-C antibody, a mouse polyclonal antibody (sc-9047; Santa Cruz Biotechnology), at a concentration of 20 μg/ml; Ang-2 antibody, a goat polyclonal antibody (sc-7016; Santa Cruz Biotechnology), at a concentration of 10 μg/ml; and Tie-2 antibody, a rabbit polyclonal antibody (sc-324; Santa Cruz Biotechnology), at a concentration of 4 μg/ml. CD31 antibody (Clone JC/70A, mouse monoclonal antibody; Neo Markers, Fremont, CA) was used to determine the intratumoral MVC. TSP-1 monoclonal antibody (MAB054; Chemicon International, Inc., CA) was used at 2 μg/ml, and CD36 monoclonal antibody (MAB1204; Chemicon, Temecula, CA) was used at a 200-fold dilution. After incubation, the slides were washed in PBS for 15 min, and each secondary antibody (mouse, rabbit, and goat) was applied, using the LSAB kit (DAKO Corp., Carpinteria, CA) according to the manufacturer’s protocol. After washing, the color was developed with 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazo-
patients, the percentages of cells positive for VEGF, VEGF-C, Ang-2, and Tie-2 were 96, 88, 98.7, and 100%, respectively.

The immunohistochemical scores for VEGF, VEGF-C, Ang-2, and Tie-2 were 5.31/11006, 2.9, 5.08/11006, 2.4, and 6.4/11006, 2.1, respectively. The mean MVC for all of the investigated tumors was 16.75/11006, 2.9. Fig. 1 shows examples of immunohistochemical staining for Ang-2, Tie-2, and CD31. Strong positive staining for TSP-1 and CD36 was also seen in the cytoplasm of the cancer cells. TSP-1 and CD36 also appeared in the endothelia of microvessels. The percentage of positive cells was 100% for both TSP-1 and CD36. The immunohistochemical scores for TSP-1 and CD36 in all patients were 6.41/11006, 1.8, and 5.89/11006, 2.0, respectively. Fig. 2 shows examples of staining for TSP-1 and CD36. Fig. 3 shows examples of staining for CD31, TSP-1, CD36, and Tie-2 in the endothelia of adjacent tissues.

Correlation among the Expression Levels of Angiogenesis-related Factors. VEGF expression showed a significant correlation with all other factors except TSP-1 (Table 2 and Fig. 4). VEGF-C expression showed a significant correlation with Ang-2 and Tie-2. Ang-2 expression was significantly correlated with Tie-2. The expression of TSP-1 correlated with that of CD36 and inversely with MVC. Fig. 4 shows examples of the correlations between VEGF and VEGF-C and between TSP-1 and MVC.

Relationship between Clinical Data and the Expression of Angiogenesis-related Factors. Table 3 shows the relationship between clinical data and each angiogenesis-related factor. In the presence of lymph node involvement, there was a significant correlation with the expression of VEGF-C. The degree of

<table>
<thead>
<tr>
<th>Characteristic of the enrolled patients</th>
<th>All patients (n = 75)</th>
<th>Recurrent group (n = 25)</th>
<th>Nonrecurrent group (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>(11–74)</td>
<td>(11–74)</td>
<td>(16–74)</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>57/18</td>
<td>8/17</td>
<td>6/34</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
<td>0.8 (0.3–8.5)</td>
<td>0.8 (0.6–8.7)</td>
<td>0.8 (0.6–8.5)</td>
</tr>
<tr>
<td>With poor differentiation of primary tumor</td>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Metastatic sites (n)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymph node</td>
<td>24/51</td>
<td>8/17</td>
<td>16/34</td>
</tr>
<tr>
<td>Bone</td>
<td>24/51</td>
<td>9/24</td>
<td>15/36</td>
</tr>
<tr>
<td>Brain</td>
<td>24/51</td>
<td>8/24</td>
<td>15/36</td>
</tr>
<tr>
<td>Liver</td>
<td>24/51</td>
<td>5/24</td>
<td>10/36</td>
</tr>
<tr>
<td>Brain</td>
<td>24/51</td>
<td>8/24</td>
<td>15/36</td>
</tr>
<tr>
<td>Outcome</td>
<td>Alive and well: 47</td>
<td>Alive and well: 14</td>
<td>Alive and well: 47</td>
</tr>
<tr>
<td>Alive with disease: 15</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Dead of other causes: 5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead of other causes: 10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dead of disease: 15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Disease-free survival (months)</td>
<td>70 (18–275)</td>
<td>24 (18–275)</td>
<td>24 (18–275)</td>
</tr>
<tr>
<td>Overall survival (months)</td>
<td>94 (18–275)</td>
<td>8.6 (18–275)</td>
<td>97 (25–275)</td>
</tr>
</tbody>
</table>

NS, not significant.

Fig. 1 Immunohistochemical detection of Ang-2 (A), Tie-2 (B) in the cytoplasm of thyroid cancer cells, and CD31 (C), dark staining indicates the presence of the factors. For these immunohistochemical analyses, except for that of CD31, we evaluated both the intensity and distribution of stained cells in each section. To evaluate the intensity of staining, we divided the stained cells in the largest population of the section into four categories based on degree of staining (from 0 to 3). To evaluate the distribution of stained cells, we divided the sections into four categories based on percentage of stained cells: 0 (no stained cells observed), 1 (<30% of the entire population of cells stained), 2 (<50% of the entire population of cells stained), and 3 (>60% of the entire population of cells stained). Multiplication of both scores produced a final score ranging from 0–9. The immunohistochemical scores of A and B were 6 and 9, respectively. In C, the microvessels in papillary thyroid carcinoma were revealed by CD31 staining.
EX of the primary tumor inversely correlated with the expression of TSP-1. The overall survival correlated with stronger expression of CD36 and with a lower MVC score. Other clinical factors, such as age, gender, and tumor size, did not correlate with the expression of the angiogenesis-related factors investigated. In addition, no relationship was found between the expression levels of any of the angiogenic factors and recurrence.

To clarify the balance of expression of angiogenic and antiangiogenic factors in the same tumor, we calculated the ratio of each angiogenic factor versus TSP-1 as the antiangiogenic factor. Table 4 shows the relationship between each ratio and the clinical data. The increased ratio of VEGF to TSP-1 significantly correlated with a higher degree of EX and a higher MVC. The increased ratio of VEGF-C to TSP-1 correlated with a higher MVC. The increased ratio of Ang-2 to TSP-1 correlated with a higher EX and a higher MVC.

**DISCUSSION**

Angiogenesis, which is essential for tumor growth and progression (1), does not involve a single pathway, but is a complex of many factors and signal transduction systems. Within this angiogenesis complex, the balance between angiogenic and antiangiogenic factors is important (33). In the present study, we investigated the expression and distribution of TSP and CD36 in papillary thyroid carcinomas to detect the expression of two antiangiogenic factors and four angiogenic factors: VEGF, VEGF-C, Tie-2, and Ang-2. All of these factors appeared in the cytoplasm of the tumor, demonstrating production by thyroid cancer cells. It is generally recognized that cancer cells produce VEGF and VEGF-C (34, 35). Ang-2 is expressed in the cytoplasm of cancer cells in hepatocellular carcinoma (36), Kaposi’s sarcoma (37), and gastric carcinoma (38). Its expression is localized in the endothelial cells of glioblastoma (39), astrocytoma (40), and non-small cell lung cancer (41), but not in the cancer cells. Tie-2 has been reported in the cytoplasm of cancer cells in glioma (5) and Kaposi’s sarcoma (37), but its expression is localized in intratumoral endothelial cells in gastric cancer (38), glioblastoma (39), and breast cancer (42). In our study, Tie-2 was present in the cytoplasm of both cancer and endothelial cells. Some investigators have reported TSP-1 expression in the cytoplasm of thyroid hyperplasia (43) and breast (27, 28), colorectal (44), and prostate cancer (45) cells. However, there have also been many reports of TSP-1 expression only in cells adjacent to the stromal tissue of the tumor and/or in the intratumoral stromal tissue in bladder (16), mouth (18), pancreas (21), and thyroid cancer (30). CD36 has been identified in the cytoplasm of cancer cells of cutaneous squamous cell carcinoma (17), breast cancer (27, 28), and colon cancer (29), and also in the endothelial cells of colon cancer (29, 31). Therefore, the reported expression sites of these angiogenesis-related factors vary. In our study, CD36 appeared in the cytoplasm of both cancer cells and endothelial cells.

Regarding the relationship among the factors studied, VEGF was closely related to the other angiogenic factors. Among the antiangiogenic factors, two factors were closely related to each other. MVC showed a significant positive correlation with the expression of VEGF and an inverse correlation with TSP-1. These results indicate that VEGF is a major angiogenic...
genic factor (2) and that TSP-1 should be recognized as an antiangiogenic factor (15).

In the present study, there was no correlation between the clinical data, such as age, gender, and tumor size, and the expression levels of angiogenesis-related factors. In papillary thyroid carcinoma, these factors have been considered independent prognostic factors (46), but they were not related to the expression levels of angiogenic factors. The presence of node involvement was closely related to higher expression of VEGF-C. In a recent study, it was clearly demonstrated that VEGF-C promoted lymph node metastasis and distant metastasis (35). TSP-1 has been reported as an antiangiogenic factor (16–19, 31). In thyroid cancer, Bunone et al. (47) reported that a decrease in TSP-1 enabled hematogenous spread. In our findings, TSP-1 showed a significant inhibitory effect on the MVC of tumors.

Some studies have shown that TSP-1 suppresses tumor growth through antiangiogenic effects in cutaneous squamous cell carcinoma (17), breast carcinoma (48), thyroid cancer (49), and melanoma (50). In other studies, TSP-1 has been demonstrated as an antitumor factor in melanoma (25) and small cell lung carcinoma (26), independent of angiogenesis. In other studies of the relationship between tumor invasion and TSP-1, however, TSP-1 has been reported to promote invasion through up-regulation of the plasminogen/plasmin system (24, 28, 50). In our study, higher expression of TSP-1 inversely correlated with EX. Whether this inverse result is derived from a property of each organ should be investigated. As for overall survival, a higher MVC significantly correlated with poorer survival, as in our and other previous studies (51, 52). In other studies, the expression of TSP-1 correlated with a good prognosis (18, 44), but there was no relationship in the results of the present study similar to the relationship reported by Gasparini et al. (53).

The balance between angiogenic and antiangiogenic factors has been recognized as important in this angiogenesis complex (33). The concept has been described by Prehn (54), and it has been shown that fibrosarcomas counterbalance their own secretion of TSP-1 by overproducing angiogenic factors (25). Recently, Filleur et al. (33) demonstrated that tumor resistance to the antiangiogenic effect of TSP-1 develops as a result of in vivo outgrowth of tumor cell variants that secrete increased amounts of angiogenic factors (VEGF), which counterbalance the inhibitory effect of TSP-1 on neovascularization. Morelli et al. (19) reported that the expression of VEGF was elevated in approximately half of the sera from gastric and breast cancer patients and that these sera stimulated endothelial cell growth, whereas sera that inhibited endothelial growth contained high levels of TSP. On the basis of these findings, we studied the effect of the balance between angiogenic and antiangiogenic factors on clinical factors. Two clinical factors, EX and MVC, were affected by the balance between angiogenic and antiangiogenic factors. Two clinical factors, EX and MVC, were affected by the balance between angiogenic and antiangiogenic factors. In an independent factor analysis, no relationship was found between VEGF and Ang-2 and higher EX or between VEGF-C and Ang-2 and a higher MVC. However, from the standpoint of the balance between angiogenic and antiangiogenic factors, it was clear that the superiority of production of angiogenic factors over that of antiangiogenic factors affects EX and MVC.

To the best of our knowledge, this is the first report

<table>
<thead>
<tr>
<th>VEGF-C</th>
<th>Ang-2</th>
<th>Tie-2</th>
<th>TSP-1</th>
<th>CD36</th>
<th>MVC</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>P &lt; 0.0001</td>
<td>P = 0.0004</td>
<td>P = 0.0026</td>
<td>NS</td>
<td>P = 0.0247</td>
</tr>
<tr>
<td>r = 0.603</td>
<td>r = 0.408</td>
<td>r = 0.35</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>P &lt; 0.0001</td>
<td>P = 0.0074</td>
<td>NS</td>
<td>NS</td>
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<tr>
<td>r = 0.572</td>
<td>r = 0.311</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Ang-2</td>
<td>P = 0.004</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>r = 0.335</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>Tie-2</td>
<td></td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>TSP-1</td>
<td>P = 0.026</td>
<td>P = 0.044</td>
<td>r = 0.258</td>
<td>r = -0.234</td>
<td></td>
</tr>
<tr>
<td>CD36</td>
<td></td>
<td></td>
<td></td>
<td>NS</td>
<td>NS</td>
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</table>

*NS, not significant.

**Table 2** Correlation among the expression levels of the factors

**Fig. 4** Correlation between VEGF and VEGF-C and between TSP-1 and MVC. Two examples from Table 2 are shown. A, correlation between VEGF and VEGF-C; B, correlation between TSP-1 and MVC. Diagonal lines are simple regression lines.
showing that the balance between angiogenic and antiangiogenic factors is closely related to distinct invasion to other adjacent organs and neovascularization of the primary tumor.

REFERENCES


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