Expression of Angiopoietin-2 is Correlated with Vascularization and Tumor Size in Human Colorectal Adenocarcinoma

HONG-LING WANG,1 CHANG-SHENG DENG,1 JUN LIN,1 DING-YU PAN,2 ZU-YU ZOU3 and XIAO-YANG ZHOU4

1Department of Gastroenterology, Zhongnan Hospital of Wuhan University, Wuhan, China
2Department of General Surgery, Zhongnan Hospital of Wuhan University, Wuhan, China
3Department of Pathology, Zhongnan Hospital of Wuhan University, Wuhan, China
4Department of Cardiology, Renmin Hospital of Wuhan University, Wuhan, China

WANG, H.-L., DENG, C.-S., LIN, J., PAN, D.-Y., ZOU, Z.-Y. and ZHOU, X.-Y. Expression of Angiopoietin-2 is Correlated with Vascularization and Tumor Size in Human Colorectal Adenocarcinoma. Tohoku J. Exp. Med., 2007, 213 (1), 33-40 — Angiopoietins are endothelial growth factors, which play crucial roles in normal vascular development and tumor angiogenesis. We examined the expression profiles of angiopoietin-1 (Ang-1), angiopoietin-2 (Ang-2), vascular endothelial growth factor (VEGF), and Tie-2, a receptor for Ang-1 and Ang-2, in both colorectal adenocarcinoma and adjacent normal tissues, as judged by histology, in order to elucidate their relationships with microvascular density (MVD) and clinicopathologic properties. Higher MVD was associated with a lower degree of differentiation of colorectal adenocarcinoma. Immunohistochemical analysis revealed that the expression of Ang-2 and VEGF was significantly increased in colorectal adenocarcinoma compared to adjacent normal tissues (p < 0.01), and the expression of Ang-2 positively correlated with that of VEGF (r = 0.997, p < 0.01). In contrast, the expression of Ang-1 was lower in adenocarcinoma tissues than in adjacent normal tissues (p < 0.01), while there was no significant difference in Tie-2 expression in both tissues. Moreover, MVD was increased in Ang-2- and VEGF-expressing adenocarcinoma tissues compared to the Ang-2- and VEGF-negative tissues, respectively (p < 0.01). Importantly, MVD was lower in Ang-1-expressing adenocarcinoma tissues relative to Ang-1-negative tissues (p < 0.01). Furthermore, expression of Ang-2 as well as VEGF was significantly up-regulated in colorectal adenocarcinoma with diameters ≥ 5 cm or with lymph-node metastases (p < 0.01). In conclusion, the increased expression of Ang-2 and the decreased expression of Ang-1 may be responsible for blood vessel formation and rapid growth of the colorectal adenocarcinoma. ——— angiopoietins; colorectal adenocarcinoma; microvascular density; clinicopathology

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Correspondence: Chang-Sheng Deng, Department of Gastroenterology, Zhongnan Hospital, Wuhan University, Wuhan 430071, Hubei Province, China.
e-mail: zhnwhl@yahoo.com.cn
Colorectal cancer is one of the most common malignancies worldwide, second only to lung cancer as a cause of cancer death. Angiogenesis, the formation of new blood vessels, has been well recognized to be essential for tumor growth and expansion by supplying malignant cells with sufficient oxygen and nutrition. Moreover, it has been shown that angiogenesis is an independent prognostic factor for colorectal cancer (Saclatides and Speziale 1994).

Angiogenesis involves the activation, migration, and proliferation of endothelial cells and is regulated by several peptides and nonpeptide molecules. Of these regulators, vascular endothelial growth factor (VEGF) is a hypoxia-inducible angiogenic factor, and its up-regulation is thought to mediate many of the angiogenic effects of growth factors that are not direct endothelial cell mitogens. Therefore, VEGF is crucial for blood vessel development (Leung et al. 1989; Katoh and Katoh 2006; Carvalho et al. 2007).

Angiopoietins are members of another novel family of angiogenic factors and participate in the formation of blood vessels. Of the four currently known family members, the best characterized ones are angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), both of which function as ligands for the endothelium-specific tyrosine kinase receptor Tie-2 (Zadeh and Guha 2003). Ang-1 reduces endothelial permeability of noncerebral vessels and has a major role in vascular stabilization and maturation; thus Ang-1 plays an important role in maintaining vessel integrity. Ang-2, which is thought to be an endogenous antagonist of the action of Ang-1, competes for binding to the Tie-2 receptor and blocks the Ang-1-induced Tie-2 autophosphorylation during vasculogenesis, subsequently leading to loosening of cell-matrix and cell-cell contacts and allowing access to angiogenic inducers. In the absence of angiogenic growth or survival signals, blockade of Ang-1 or activation of Ang-2 may destabilize capillaries and shift the balance to endothelial cell apoptosis and a regression of vessel structures (Hanahan 1997; Yancopoulos et al. 2000). In mice and humans, Ang-2 is selectively expressed in ovary, uterus and placenta (Maisonpierre et al. 1997), while Ang-1 is widely expressed in both the embryo and the adult (Wong et al. 1997). Furthermore, recent studies provide evidence that Ang-1 and Ang-2, via competing for their receptor Tie-2, are probably involved in the regulation of vasculogenesis in both normal and tumor tissues (Zhu and Chen 2004).

Here we investigated the expression profiles of Ang-1, Ang-2, Tie-2 and VEGF in human colorectal adenocarcinoma and their relationships with microvascular density (MVD) and clinicopathologic characteristics. The results obtained suggest that these proteins may be useful to determine clinical diagnosis and prognosis of colorectal carcinoma, and may represent novel targets for cancer therapy.

**MATERIALS AND METHODS**

**Clinical samples**

Surgical specimens, including cancer tissues and their adjacent normal tissues, were obtained from 45 patients with pathologically confirmed colorectal adenocarcinoma between January 2002 and December 2004 in Zhongnan Hospital of Wuhan University, China. None of the patients had undergone radiotherapy or chemotherapy prior to operation. All 45 subjects (29 males and 16 females) were included in this study, with a median age of 52.6 years (range 20-78 years). All resected surgical specimens were fixed with 10% buffered formalin, embedded in paraffin, and stained with hematoxylin-eosin for histopathologic classification. Depending on glandular architecture, cellular pleomorphism and mucosecretion of the predominant pattern, adenocarcinoma may present three degrees of differentiation: well, moderately and poorly differentiate. Furthermore, the tumors were staged according to Dukes’ classification system: A, carcinoma in situ limited to mucosa or submucosa; B, cancer that extends into the muscularis, into or through the serosa; C, cancer that extends to regional lymph nodes; and D, cancer that has metastasized to distant sites. Our study of human materials was approved by the ethical committee of Zhongnan Hospital of Wuhan University.

**Reagents**

The rabbit-derived polyclonal antibodies against human Ang-1, Ang-2, Tie-2 and VEGF were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).
The mouse anti-human CD34 monoclonal antibody was commercially acquired from Zhongshan Bio-company (Beijing, China). The streptavidin-peroxidase (S-P) based immunohistochemical detection kit was obtained from DAKO (Glostrup, Denmark). All other chemicals were supplied by Maixin Bio-company (Fujian, China).

**Immunohistochemical staining**

Paraffin sections of 4-μm thickness were deparaffinized in xylene and rehydrated, and then incubated with 3% hydrogen peroxide in methanol to block endogenous peroxidase. Each slide was incubated with normal horse serum for 20 min at room temperature. The primary antibody directed against Ang-1 (1:100 dilution), Ang-2 (1:100 dilution), Tie-2 (1:200 dilution) or VEGF (1:100 dilution) was applied to the sections and incubated overnight at 4°C. Sections were processed with a standard immunoperoxidase method using a streptavidin-peroxidase complex kit. The peroxidase reaction was then developed with diaminobenzidine and counterstained with hematoxylin. Human placenta was used as a positive control. For the negative control, phosphate-buffered saline (PBS) was used as a substitute for the primary antibody. Immunohistochemical staining was assessed from average luminosity and positive ratio by Automatic Magic Analysis System (Olympus BX50, Tokyo). Cells with brown-yellow or deep brown staining of the cytoplasm and plasma membrane were considered as positive cells. Because of intratumoral heterogeneity, the expression levels of Ang-1, Ang-2, Tie-2 and VEGF were graded based on corresponding positive cell ratio: negative (−) when positive cells were present in less than 20% of the area; positive (+) when positive cells were present in 20% to 50% of the area; and strongly positive (++) when positive cells were present in 50% or more of the area.

**MVD counting**

The MVD was evaluated according to the methods described by Weidner (1995). After immuno-staining with anti-CD34 monoclonal antibody (1:50 dilution), colorectal adenocarcinoma and adjacent normal tissues were first screened at a low-power magnification (×40) to identify areas of the highest MVD. Counts were taken in 5 highest MVD areas at a high power magnification (×400). A single blood vessel endothelial cell or cell colony stained with brown-yellow color was judged as a microvessel. The mean value of the fields counted was considered as the MVD.

**Statistical analysis**

All continuous variables are presented as mean ± s.d., and comparisons between two groups or multigroup were by unpaired student’s t-tests and ANOVA method, respectively. Categorical variables are presented as proportions, and comparisons between groups were by χ² tests. The q-test was used for identification and rejection of outliers. In addition, the correlations between the expression of Ang-1, Ang-2, or VEGF, and MVD in colorectal adenocarcinoma were determined by analysis of linear correlation. \( P < 0.05 \) was considered statistically significant.

**RESULTS**

**Distribution of Ang-1, Ang-2, Tie-2 and VEGF in colorectal adenocarcinoma and adjacent normal tissues**

Ang-1, Ang-2, and VEGF were expressed within the cytoplasm (Figs. 1-3). Ang-2 was highly expressed in colorectal adenocarcinoma, while Ang-1 was highly expressed in normal tissues adjacent to colorectal adenocarcinoma. VEGF was highly expressed in colorectal adenocarcinoma tissues compared to adjacent normal tissues (Figs. 1-3).

**Expression levels of Ang-1, Ang-2, Tie-2 and VEGF in colorectal adenocarcinoma tissues and adjacent normal tissues**

In terms of average luminosity and positive ratio, the expression levels of Ang-2 and VEGF were significantly higher in colorectal adenocarcinoma than in adjacent normal tissues \( (p < 0.01) \). In contrast, Ang-1 was significantly down-regulated in cancer tissues compared to adjacent normal tissues \( (p < 0.01) \). Moreover, there was no significant difference in Tie-2 expression in both normal and cancer tissues \( (p > 0.05) \). It is also noteworthy that the expression of these proteins may depend upon the degree of cancer differentiation. The lower the degree of differentiation of the adenocarcinoma, the higher the expression levels of Ang-2 and VEGF; highly differentiated adenocarcinoma demonstrated higher expression of Ang-1 (Table 1).
The correlation between expression of Ang-2 and expression of VEGF in colorectal adenocarcinoma and adjacent normal tissues

As shown in Fig. 4, there was a significantly positive correlation between Ang-2 and VEGF expression in various degrees of differentiation of adenocarcinoma, as well as in their adjacent normal tissues ($r = 0.997, p < 0.01$).

Comparison of MVD between colorectal adenocarcinoma and adjacent normal tissues

The MVD was significantly increased in any
of the poorly-, moderately- and well-differentiated colorectal adenocarcinoma, compared to adjacent normal tissues. Moreover, there was an increase in MVD as the degree of differentiation of the adenocarcinoma decreased (Table 2).

The expression levels of Ang-1, Ang-2 and VEGF correlate with MVD in colorectal adenocarcinoma

The MVD in Ang-1-expressing adenocarcinoma tissues was significantly lower compared to Ang-1 negative tissues ($p < 0.01$). In contrast, it was significantly increased in Ang-2-expressing tissues than in negative tissues ($p < 0.01$). Likewise, the MVD in VEGF-expressing tissues was significantly increased compared to negative tissues ($p < 0.01$) (Table 3).

The expression levels of Ang-2 and VEGF in colorectal adenocarcinoma correlate with clinicopathologic features

As shown in Table 4, the expression of Ang-2 was associated with tumor size and lymph-node metastases, but not with the age, gender, tumor location, invasion depth or Dukes’ stage. The expression of Ang-2 was significantly increased in colorectal adenocarcinoma with lymph-node metastases or in tumor $\geq 5$ cm in size ($p < 0.01$). Similar changes were observed for VEGF.

Discussion

Angiopoietins are regulatory factors involved in vasculogenesis. It has been recognized that angiopoietins play important roles in tumor blood vessel development (Loughna and Sato 2001). The present study was undertaken to analyze the expression profiles of Ang-1 and Ang-2 in human colorectal adenocarcinoma and adjacent normal tissues, in an effort to determine their importance in vasculogenesis and metastasis of colorectal cancer.

Our results indicate that Ang-1 and Ang-2 are expressed in both colorectal adenocarcinoma and adjacent normal tissues, but their expression levels varied between the two tissue types. In adenocarcinoma, the expression level of Ang-2

| Table 1. Expression of Ang-1, Ang-2, Tie-2 and VEGF in both colorectal adenocarcinoma and adjacent normal tissues (mean ± s.d.). |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Group           | Case number     | Average luminosity \(b\) | Positive ratio \(\%\) | \begin{align*}
| Colorectal cancer tissues & 45 & 0.158 ± 0.065* & 0.258 ± 0.062* & 37.4 ± 14.77* \\
| Poorly-differentiated & 18 & 0.108 ± 0.061* & 0.236 ± 0.042* & 67.97 ± 9.46* \\
| Moderately-differentiated & 16 & 0.134 ± 0.027 & 0.152 ± 0.035 & 39.53 ± 5.63 \\
| Well-differentiated & 11 & 0.204 ± 0.047 & 0.263 ± 0.042 & 69.84 ± 9.32 \\
| Adjacent normal tissues & 12 & 0.296 ± 0.068 & 0.382 ± 0.039 & 59.98 ± 12.26 \\

Compared with adjacent normal tissues, \(p < 0.01\); compared with the well-differentiated group, \(p < 0.01\); compared with the moderately-differentiated group, \(p < 0.05\); \(a\) average staining intensity; \(b\) the area of positive cells.
was significantly higher compared to the normal tissues, with an increase in Ang-2 expression as the degree of differentiation decreased. In normal tissues, however, Ang-1 was predominantly expressed rather than Ang-2. Furthermore, the expression level of Ang-1 was significantly higher
in the well-differentiated cancer tissues than in the poorly-differentiated cancer tissues. Tumor growth and metastasis are closely associated with tumor neoangiogenesis (Han et al. 2001). Tumor MVD is an effective index that reflects tumor angiogenesis, while CD34 is one of the best available markers to indicate the presence of blood vessel endothelial cells (Matsuyama et al. 1998; Mitsuhashi et al. 2003). The immunohistochemical examinations with a CD34 monoclonal antibody have confirmed that the level of MVD was closely correlated with the degree of cancer differentiation. In the present study, the MVD was significantly increased in the poorly-differentiated adenocarcinoma than in the well-differentiated adenocarcinoma. Therefore, we conclude that high MVD may indicate a poorer prognosis in colorectal adenocarcinoma.

Our study also demonstrated that the expression of Ang-1 and Ang-2 differentially correlated with the MVD. The MVD was significantly lower in Ang-1-expressing adenocarcinoma tissues, compared to Ang-1 negative tissues. In contrast, the MVD was significantly higher in Ang-2-expressing tissues than in Ang-2 negative tissues. These findings indicate that the differential expression of Ang-1 and Ang-2 may play a crucial role in angiogenesis of the colorectal adenocarcinoma.

It has been suggested that Ang-1 and Ang-2 may mediate the stability of endothelial cells via binding to the tyrosine kinase Tie-2 receptor. Through this binding, Ang-1 may enhance the adhesion of circumference blood vessels and stabilize neoangiogenesis, thereby limiting angiogenesis and tumor growth. In contrast, Ang-2 may enhance the sensitivity of endothelial cells to mitotic signals, and may promote angiogenesis, resulting in vessel instability and continuous tumor angiogenesis (Ellis et al. 2002).

Asahara et al. (1998) reported that Ang-2 may cooperate with VEGF in enhancing the formation of blood vessel net, thereby improving the recovery of damaged vessels and maintaining them in a plastic condition. Under such conditions, the blood vessels are likely to be more sensitive to VEGF signaling. The present study showed that the expression pattern of Ang-2 was.

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**Table 4.** The relationships between Ang-2 or VEGF expression in colorectal adenocarcinoma and clinicopathologic characteristics.

<table>
<thead>
<tr>
<th>Classification</th>
<th>Case number</th>
<th>Ang-2-expression rate (%)</th>
<th>VEGF-expression rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age: ≥60 years</td>
<td>24</td>
<td>62.5</td>
<td>70.8</td>
</tr>
<tr>
<td>&lt; 60 years</td>
<td>21</td>
<td>57.1</td>
<td>66.7</td>
</tr>
<tr>
<td>Gender: Male</td>
<td>29</td>
<td>58.6</td>
<td>72.4</td>
</tr>
<tr>
<td>Female</td>
<td>16</td>
<td>62.5</td>
<td>62.5</td>
</tr>
<tr>
<td>Tumor location: Colon</td>
<td>32</td>
<td>59.4</td>
<td>71.9</td>
</tr>
<tr>
<td>Rectum</td>
<td>13</td>
<td>61.5</td>
<td>61.5</td>
</tr>
<tr>
<td>Tumor size: ≥5 cm</td>
<td>27</td>
<td>77.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>81.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>&lt; 5 cm</td>
<td>18</td>
<td>33.3</td>
<td>50.0</td>
</tr>
<tr>
<td>Invasion depth: Early phase</td>
<td>12</td>
<td>58.3</td>
<td>66.7</td>
</tr>
<tr>
<td>Developed phase</td>
<td>33</td>
<td>60.6</td>
<td>69.7</td>
</tr>
<tr>
<td>Lymph node metastases: Yes</td>
<td>23</td>
<td>82.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>86.9&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>No</td>
<td>22</td>
<td>36.4</td>
<td>50.0</td>
</tr>
<tr>
<td>Dukes’ Stage: A, B</td>
<td>25</td>
<td>60.0</td>
<td>68.0</td>
</tr>
<tr>
<td>C, D</td>
<td>20</td>
<td>60.0</td>
<td>70.0</td>
</tr>
</tbody>
</table>

Compared with tumor < 5 cm in size, \( \chi^2 = 8.889, p < 0.01; \chi^2 = 4.994, p < 0.05 \); compared with no lymph node metastasis, \( \chi^2 = 10.020, p < 0.01; \chi^2 = 7.166, p < 0.01 \).
similar to that of VEGF, and there was a significant correlation between Ang-2 and VEGF. Furthermore, our findings revealed that high expression of Ang-2 and VEGF may be closely related to tumor size and lymph-node metastases. A previous study from Etoh et al. (2001) suggested that in the presence of VEGF, Ang-2 could strongly up-regulate the production of endothelial cell matrix metalloproteinases (MMP-1, MMP-2) and urokinase fibrinolysin activator, subsequently leading to the continuous production of highly instable and immature tumor blood vessels with high endothelial cell proliferation capacity. This might be a reason for causing lymph-node metastases, revealing the critical role of Ang-2 in the process of tumor metastatic spread (Lobov et al. 2002; Yoshiji et al. 2005).

Based upon these findings, we hypothesize that in colorectal cancer tissues, the differential expression of Ang-1 and Ang-2 leads to enhance the activity of Ang-2. Namely, Ang-2, via Tie-2 and with the help of VEGF, is able to promote continuous neoangiogenesis, thereby leading to increased MVD levels. Our study provides further evidence that the Ang-Tie system might play a critical role in regulating angiogenesis in human colorectal carcinoma, and suggests that this system, especially Ang-2, may represent a novel target for cancer therapy. In fact, a novel anti-angiogenesis strategy may decrease tumor development and metastasis (Pan and Tan et al. 2005).

References