Angiopoietin-2 Is Related to Tumor Angiogenesis in Gastric Carcinoma: Possible in Vivo Regulation via Induction of Proteases

Tsuyoshi Etoh, Hiroshi Inoue, Shinji Tanaka, Graham F. Barnard, Seigo Kitano, and Masaki Mori

Department of Surgery, Medical Institute of Bioregulation, Kyushu University, Beppu 874-0838, Japan [T. E., H. I., M. M.]; Department of Surgery II, Kyushu University, Fukuoka, Japan [S. T.]; Division of Digestive Disease and Nutrition, University of Massachusetts Medical School, Worcester, MA [G. F. B.]; and Department of Surgery I, Faculty of Medicine, Oita Medical University, Oita, Japan [T. E., S. K.]

ABSTRACT

Tumor angiogenesis progresses by a dynamic balance between tumor vascular regression and growth. Angiopoietin (Ang)-2 (the natural antagonist for the angiogenic Tie-2 receptor) and vascular endothelial growth factor (VEGF) are thought to be critical regulators in this process; therefore, these may play a critical role in cancer aggressiveness. The aim of this study was to clarify the clinical and biological significance of the expression of Ang-2 in human gastric cancers and to investigate the relationship between Ang-2 together with VEGF and the induction of proteases such as matrix metalloproteinases (MMPs) in the process of tumor development. Eighty-five individuals with gastric cancer, who had undergone surgery without preparative treatment, were studied. A stable transfectant of the human MKN-7 gastric cancer cell lines with an Ang-2 expression vector was used for the experimental study. First, we examined the relationship between the mRNA expression of Angs by Northern blot analysis and clinicopathological features. High Ang-2-expression cases showed more frequent vascular involvement and more advanced stages of disease compared with low Ang-2-expression cases (P < 0.05). With regard to prognosis, the survival time for patients in the high-Ang-2 mRNA group was significantly shorter (P < 0.05). When we examined the localization of Ang-2 in human gastric cancers, immunohistochemical analysis revealed that this protein was expressed predominantly in cancer tissues when compared with normal tissues. Interestingly it was expressed not only in endothelia cells (ECs) but also in cancer cells. Second, Ang-2-transfected cells were implanted in vivo into the gastric walls of nude mice. Ang-2-transfectant mice developed highly metastatic tumors with hypervascularity as compared with MKN-7 or control vector-transfectant tumors. There was a significant correlation between Ang-2 mRNA expression and lower grade of vessel maturation. Third, on the basis of the in vivo data, we focused on production of proteases such as MMPs to investigate possible mechanisms in these processes. MMP-1, MMP-9, and urokinase-type plasminogen activator in ECs were strongly up-regulated by Ang-2 in the presence of VEGF in vitro. These data suggest that production of Ang-2 is implicated in tumor development in human gastric cancers. Its production may contribute to tumor angiogenesis by induction of proteases in ECs, which may be enhanced in the presence of VEGF.

INTRODUCTION

Several studies suggest that solid tumor growth to a clinically relevant size depends on an adequate blood supply (1–3). Solid tumors recruit blood vessels from neighboring tissue by angiogenesis with the sprouting of capillaries from preexisting vessels that migrate into the tumor and form a new vascular network. To stimulate angiogenesis, tumor and form a new vascular network. To stimulate angiogenesis, tumors secrete growth factors that act on ECs.3 It is thought that the resulting neovascularization supports tumor expansion and metastasis.

MATERIALS AND METHODS

Animals and Cell Cultures. These experiments used male athymic BALB/c nude mice (4 weeks of age). The human gastric cancer cell lines AZS21, NUGC3, SOH, NS, MKN-1, and MKN-7 were obtained from Tohoku Institute and maintained in RPMI 1640 supplemented with 10% FBS at 37°C in a 5% humidified CO2 atmosphere. HUVECs were obtained from Dainippon Pharmaceutical Co., Ltd. (Osaka, Japan) and maintained in gelatin-coated dishes (Falcon Laboratories, McLean, VA) in MCDB 131 medium (Life Technologies, Inc., Rockville, MD) containing 10% FBS.

Clinical Samples. Fresh surgical specimens were obtained from 85 patients with primary gastric cancer and their paired adjacent normal gastric reverse transcription-PCR; vWF, von Willebrand factor; uPA, urokinase-type plasminogen activator; SMA, smooth muscle actin; PAI, plasminogen activator inhibitor; CM, conditioned medium.
They had undergone surgery at the Department of Surgery, Medical Institute of Bioregulation, Kyushu University (Beppu, Japan) from 1988 to 1997. None of these patients received preoperative treatment such as radiation and chemotherapy, although, 28 patients received postoperative adjuvant chemotherapy. Data concerning patient outcome, including overall survival and development of metastases, were available for all 85 patients, and the observation periods ranged from 6 months to 105 months (the median follow-up period was 41.2 months). Of 85 patients, 15 had died as a result of recurrence.

**RT-PCR and Northern Blot Analysis.** Total RNA was extracted using the acid guanidine phenol chloroform method, then DNase treatment and the reverse transcriptase reaction was as described previously (21). Each full coding sequence of Ang-1 or Ang-2 was obtained by RT-PCR and confirmed, with no mutation documented using sequence analysis. As shown in Table 1, all primers for PCR amplification, including Ang-1, Ang-2, VEGF, and Tie-2 proteases and their inhibitors, are listed. PCR amplification was performed for 25 cycles under the following conditions: (a) denaturing at 95°C for 1 min; and (b) polymerization at 72°C for 1 min. Each annealing condition for amplification of these cDNAs is included.

RNA extraction and Northern blot analysis were performed as described (22). In brief, total cellular RNA was isolated from cell lines and surgical specimens, electrophoresed in formaldehyde-agarose gels, transferred to Hybond N nylon filters (Amersham International), and then hybridized with $^{32}$P-labeled by random priming cDNA probes for Ang-1, Ang-2, VEGF, Tie-2, proteases and their inhibitors, and e-En, which were generated by RT-PCR. Filters were exposed to autoradiography for 2 h, and the mRNA levels were quantitated using a Bio-Image analyzer BAS 1000 and corrected by the levels of GAPDH as a control.

Transfection Assays and Production of Stable Cell Lines. Endogenous mRNA expression of both Ang-1 and Ang-2 were not detected in the human MKN-7 gastric cancer cell line. Each Ang-1 and Ang-2 cDNA were subcloned into a pC DNA3 vector (Invitrogen) and transfected into the cell line by the lipofection method (Life Technologies, Inc.) as described (21). Subsequently, stable transfectants were selected with 800 μg/ml of G418 treatment. Two clones of MKN-7 cells, expressing abundant Ang-2 mRNA, and 2 clones of MKN-7 cells, expressing abundant Ang-1 mRNA, were used for the subsequent experiments. A mock-transfected clone of each cell line was used for the subsequent experiments.

**Invasion and Proliferation Assays.** The invasive potential of the Ang-transfected MKN-7 cells was determined using a modified two-chamber invasion assay as described (23). Briefly, six-well transflects plates with an 8-μm pore size were coated with gelatin. Cells on the lower side of the membrane were stained and counted. Furthermore, we performed proliferation assays using titrated-thymidine (1 μCi; NEN, Boston, MA) as described previously (24). Cells (3 x 10^5) were seeded on 24-well plates and cultured in RPMI 1640 in the absence or presence of FBS. The medium was changed every 48 h. All experiments were performed in triplicate.

**Implantation of Tumor Tissues into Subcutis and Gastric Wall of Mice.** To investigate the tumorigenicity and biological effect of Ang-2 expression in MKN-7 cells, we injected Ang-2 transfectant cells (1 x 10^6) in 50 μl of PBS into the subcutis of nude mice. Tumors in the exponential phase were resected and necrotic tissue was removed. The viable tumor tissue was cut into pieces 2 mm in diameter. Under anesthesia with diethylether, the tumor piece was fixed on the anterior wall of the stomach by a superficial serosal suture with 6-0 propylene (Ethicon, Somerville, NJ). Parent MKN-7 tumors and control vector-transfected MKN-1 tumors were used as controls. Nude mice (n = 12) were analyzed for histological examination or detection of mRNA of tumor at 4 weeks after implantation. The macroscopic number and size of metastases to lung, liver, mesentery, other abdominal organs, and lymph nodes were evaluated.

**In Situ Hybridization.** Paraffin sections from 30 samples of human gastric cancer were studied. Antisense RNA probes were generated using SP6 RNA polymerase and labeled with immunofluorescence using an RNA color kit (Amersham International) as described (25). Sense probes were used as the negative control.

**Immunohistochemistry.** To evaluate the protein, Western blot analysis was performed using a polyclonal antibody against Ang-1 or Ang-2 (Santa Cruz Biochemicals) as described previously (24). Next, to determine the localization of Ang-1, Ang-2, Tie-2, and proteases including MMP-1, MMP-9, and uPA in cancer tissue specimens, an immunohistochemical analysis was performed as described previously (24). ECs were detected by vWF antibody (DAKO, Kyoto, Japan), and vascular smooth muscle cells or pericytes were detected by α-SMA antibody (Sigma). Antibodies against MMP-1, MMP-9, and uPA were purchased from Fuji Chemical Industries (Takaoka, Japan). These proteins were detected using the avidin-biotin-peroxidase method (LSAB Kit; DAKO, Kyoto, Japan). Furthermore, these enzymes were also detected using immunofluorescence microscopy.

**Microvessel Count and Vessel Maturation Index.** The microvessel count was quantitatively examined as described previously (26). The fraction of blood vessels found to be associated with both α-SMA-positive periendothelial cells and vWF-positive ECs was defined as the vessel maturation. This measurement was determined by scoring blood vessels larger than capillaries (i.e., vessels containing a lumen large enough for several erythrocytes), because capillaries are sparsely coated by pericytes and, therefore, may falsely appear as uncovered in these sections. Quantification of the type of vessel in several high-power fields of each tumor specimen allowed the assignment of a vessel maturation index to each tumor.

**Coculture Assay of HUVECs and Ang-1 or Ang-2-Transfected MKN-7 Cells with or without Exogenous VEGF.** In this study, 1 x 10^4 or 1 x 10^5 HUVECs were cultured in two-chamber, six-well plates (Falcon Laboratories, McLean, VA). After HUVECs attached to the lower chamber, the medium was changed to RPMI 1640 without FBS used as a CM. Ang-1- or Ang-2-transfectant MKN-7 cells (1 x 10^5) were incubated in the upper chamber with an 8-μm pore size for 12, 24, and 48 h. Furthermore, these conditions were divided into two subgroups: with or without recombinant human VEGF 165 (R&D). Concentrations of recombinant VEGF added into the supernatant of cocultured medium ranged from 10 to 100 ng/ml. After coculture with Ang-1- or Ang-2-transfectant, HUVECs were harvested and used for additional examination.

**Statistical Analysis.** The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the main frame computer (4381; IBM, Armonk, NY) was used for all analyses. Associations between the variables were tested by Student’s t test or by Fisher’s exact probability test. The BMDP PIL program was used for survival analysis (Kaplan-Meier method) and for testing the equality of the survival curves (Mantel-Cox method). The BMDP P2L program was used for multivariate adjustments for some covariates, simultaneously.

### Table 1. Primers and PCR products of genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upper primer (5’ to 3’)</th>
<th>Lower primer (5’ to 3’)</th>
<th>Product size (bp)</th>
<th>AT* (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang-1</td>
<td>ATGACAGTTTCTTCTTTCTTTGCG</td>
<td>CAGCCTTCTGGGATTTCTTTGT</td>
<td>550</td>
<td>55</td>
</tr>
<tr>
<td>Ang-2</td>
<td>GGAAGAGCATGAGCAAGCATAGGA</td>
<td>GCCATTTGCGTTGCTCTGATT</td>
<td>821</td>
<td>56</td>
</tr>
<tr>
<td>Tie-2</td>
<td>CACCCATCAGGGCAGCATCACA</td>
<td>TCTCCGCAGGATCTCTTCAA</td>
<td>554</td>
<td>62</td>
</tr>
<tr>
<td>VEGF</td>
<td>ATGACAGTTTCTTCTTTCTCTG</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>520</td>
<td>60</td>
</tr>
<tr>
<td>MMP-1</td>
<td>GATCCCTCTCAGTTCTCTCTG</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>560</td>
<td>60</td>
</tr>
<tr>
<td>MMP-2</td>
<td>GACGCTACCTCGACTTCTCTG</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>561</td>
<td>56</td>
</tr>
<tr>
<td>MMP-7</td>
<td>GGCTCAGTCTCTTCTCTCCTCTCTTCTOTGA</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>620</td>
<td>56</td>
</tr>
<tr>
<td>MMP-9</td>
<td>CCGGCTTCCTCTCTCTCTCTCTCTCTCTOTGA</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>350</td>
<td>56</td>
</tr>
<tr>
<td>TIMP-1</td>
<td>TTCTTGCTGCTCTCTGCTCTCT</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>361</td>
<td>58</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>CCTCTCAGTCTCTCTCTCTCTCTCTCTOTGA</td>
<td>CAGCCTTCTGGCATTCTTTGT</td>
<td>602</td>
<td>60</td>
</tr>
</tbody>
</table>

* AT, annealing temperature.
with Cox’s proportional hazards model. Model selection was performed using the forward stepwise method. All statistical differences were deemed significant at the level of \( P < 0.05 \).

The histopathological type and staging of gastric carcinomas were classified on the basis of the criteria set up by the Japanese Society for Cancer of the Stomach (27).

**RESULTS**

**Clinical Significance of Expression of Ang-2 in Human Gastric Cancer.** To assess a critical role of Angs as modulators of tumor angiogenesis in human gastric cancer, the mRNA expression of Angs and their receptor, Tie-2, in tumor tissues and adjacent normal mucosa was examined by Northern blot analysis. In addition, the distribution and cellular localization of these proteins were examined by immunohistochemistry. mRNA expression levels of Ang-1, Ang-2, and Tie-2 were increased in human gastric cancers. Of these, the mRNA expression level of each Ang-2 and Tie-2 in tumor tissues was significantly higher compared with that in normal tissues (Table 2). Fig. 1 shows representative cases. In particular, the expression of Ang-2 mRNA in 75% of the 85 cases was greater in tumor tissue than normal tissue (Table 2). The average levels of Ang-2 mRNA expression in groups showing increased vascular involvement and advanced stage were significantly higher than those of the other groups (Table 3). However, there was no significant correlation between each level of Ang-1 mRNA, Tie-2 mRNA, and clinicopathological features (data not shown).

In practical evaluations, we set several cutoff values for these mRNA expression levels in tumor tissues. We selected the average as the most appropriate cutoff value, as shown in Table 2. Using these averages, we classified expression into two groups: a high group \( (n = 43) \) and a low group \( (n = 42) \). With regard to prognosis, a difference in survival time was significant between the high-Ang-2 mRNA-expression group and the low-Ang-2 mRNA-expression group \( (P < 0.05; \text{ Mantel-Cox method; Fig. 2}) \). On the other hand, there was no significant correlation between the levels of Ang-1 mRNA or Tie-2 mRNA expression and survival time (data not shown). In a multivariate analysis for prognosis, parameters included vascular involvement, lymph node metastasis, clinical stage, postoperative adjuvant chemotherapy, and Ang-2 mRNA expression. This analysis demonstrated that high Ang-2 mRNA expression was not an independent prognostic factor (Hazard ratio, 1.54; 95%CI, 1.01–2.15).

On the basis of the above results, it was noted that Ang-2 might be an important factor in tumor aggressiveness in gastric cancer. Thus we focused on the possible role of Ang-2 in tumor tissue. First, we performed immunohistochemistry to evaluate the localization of Ang-2 in 65 gastric cancer tissues. We used a cultured EC line (HUVECs) as a positive control and the cultured gastric cancer cell line MKN-7 as a negative control for the use of Ang-2 antibodies. As a result, Ang-2 was found to be expressed not only in ECs but also in cancer cells (Fig. 3, A–E). Ang-2 was detected in cancer cells of both intestinal and diffuse types of gastric cancer not forming a solid mass. Using in situ hybridization, transcripts of Ang-2 were found in cancer epithelial cells (Fig. 3, F and G). In addition, there was a significant association between Ang-2 mRNA and VEGF mRNA expressions in cancer tissues by Northern blot analysis using Student’s \( t \) test (data not shown).

**Table 2** Expression of Ang-1, Ang-2, and Tie-2 mRNA in 85 paired cases of gastric cancer and adjacent noncancerous tissues by Northern blot analysis compared with GAPDH mRNA

<table>
<thead>
<tr>
<th>Variables</th>
<th>Ang-1 mRNA expression</th>
<th>Ang-2 mRNA expression</th>
<th>Tie-2 mRNA expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor tissue</td>
<td>2.5 ± 1.2</td>
<td>5.6 ± 1.8*</td>
<td>4.1 ± 1.2*</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>1.1 ± 0.9</td>
<td>1.4 ± 0.5</td>
<td>1.5 ± 1.1</td>
</tr>
</tbody>
</table>

* \( P < 0.05 \) versus normal tissue. Mean ± SD.

**Table 3** Association between clinicopathological features and Ang-2 mRNA expression of tumor tissues in human gastric carcinoma

<table>
<thead>
<tr>
<th>Variables</th>
<th>( n )</th>
<th>Ang-2 mRNA expression ( a )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>47</td>
<td>5.5 ± 3.2</td>
<td>NS*</td>
</tr>
<tr>
<td>Female</td>
<td>38</td>
<td>7.5 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Histology</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intestinal type</td>
<td>40</td>
<td>4.2 ± 5.3</td>
<td>NS</td>
</tr>
<tr>
<td>Diffuse type</td>
<td>45</td>
<td>5.1 ± 1.2</td>
<td></td>
</tr>
<tr>
<td>Serosal invasion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>37</td>
<td>5.2 ± 3.1</td>
<td></td>
</tr>
<tr>
<td>Absent</td>
<td>48</td>
<td>6.2 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Vascular involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>32</td>
<td>7.8 ± 3.2</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>Absent</td>
<td>53</td>
<td>3.5 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>Lymphatic involvement</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>45</td>
<td>6.1 ± 2.6</td>
<td>NS</td>
</tr>
<tr>
<td>Absent</td>
<td>40</td>
<td>3.5 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>51</td>
<td>7.5 ± 1.2</td>
<td>NS</td>
</tr>
<tr>
<td>Absent</td>
<td>34</td>
<td>5.5 ± 3.2</td>
<td></td>
</tr>
<tr>
<td>Stage</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1, 2</td>
<td>42</td>
<td>4.5 ± 2.2</td>
<td>( P &lt; 0.05 )</td>
</tr>
<tr>
<td>3, 4</td>
<td>43</td>
<td>7.1 ± 3.4</td>
<td></td>
</tr>
</tbody>
</table>

* Mean ± SD.

* NS, not significant.
Biological Significance of Ang-2 Expression in Gastric Cancer Cells. We then investigated whether transfection with Ang-2 into gastric cancer cells affects their biological behavior. In brief, we examined the expression of Ang-1, Ang-2, and their receptor, Tie-2, in several gastric cancer cell lines and HUVECs. Among these cells, MKN-1 and MKN-7 gastric cancer cells expressed neither Ang-1 mRNA nor Ang-2 mRNA. Tie-2 mRNA was only expressed in HUVECs. Shown in Table 4, the mRNA levels of six gastric cancer cell lines and the EC line used in this study are listed. We used the MKN-7 cell line for additional experiments. First, we transfected Ang-2 expression vectors into MKN-7 gastric cancer cells and selected stable clones. Ang-2 mRNA expression and protein expression were confirmed by Northern and Western blot analyses, respectively. Western blot analysis showed that Ang-2 protein was found in cell lysates of transfected MKN-7 cells and in the supernatant of cultured medium (Fig. 4A, right). With regard to Ang-1, transfection assays and the evaluation of Ang-1 protein were performed similarly (Fig. 4A, left).

Using proliferation assays, we next analyzed whether Ang-2 transfection stimulates the in vitro growth of gastric cancer cells. Under both serum-free and serum-containing conditions, growth of the cells was not affected by transfection with Ang-2 (Fig. 4B). These findings thus demonstrated that the addition of exogenous Ang-2 did not alter cell proliferation of the gastric cancer cell lines. Furthermore, invasion assays revealed that addition of exogenous Ang-2 did not alter cell invasiveness of the gastric cancer cell lines (data not shown). After transfected cells were injected into the subcutis of nude mice, tumors arising from Ang-2-transfected MKN-7 cells developed large and marked hemorrhages compared with tumors from mock-transfected clones (Fig. 4C, a and c). There was a significant association between Ang-2 expression and macroscopic tumor growth in vivo (Table 5). To investigate the organ-microenvironment influence on tumor growth
and metastasis, tumors were then transplanted into an orthotopic site (gastric wall) of nude mice. Ang-2-transfected cells formed tumors in 10 of 12 nude mice, whereas mock-transfected clones formed tumors in 2 of 12 nude mice. We also found peritoneal dissemination and liver metastasis of tumors only in the Ang-2-transfectant group (Fig. 4C, d and e). Immunohistochemical examination of the tumors derived from Ang-2 transfecants revealed that a huge mass was formed in the subserosal space of the gastric wall (Fig. 4D, a and b). In addition, Ang-2 was strongly expressed in cancer cells and also partly expressed in ECs at sites surrounding the cancer cells (Fig. 4D, c). On the other hand, Ang-2 was not detected in cancer cells of the mock-transfectant tumors (Fig. 4D, d). Its receptor, Tie-2, was only expressed in ECs at sites surrounding the cancer cells (Fig. 4D, e).

Finally, we investigated whether the increased tumorigenesis and metastasis of the tumors were associated with increased angiogenesis and vessel maturation. For examination of microvessel counts, tumor vessel density in the tumors derived from Ang-2 transfectants was significantly higher than that of the mock-transfected tumors (Table 5). Next, to evaluate the relationship between expression of Ang-2 and vessel maturation in these tumors, we determined the vessel maturation index, defined as the fraction of vessels that are associated with α-SMA-positive cells around ECs (Fig. 5, A and B). The maturation index in the primary and metastatic liver tumors resulting from the Ang-2 transfecants was significantly lower than that of parent and/or control vector-transfected cell tumors (Fig. 5C).

Ang-2 Together with VEGF Induces Expression of MMPs and uPA in ECs. To characterize the morphological and biological differences between the tumors arising from Ang-2-transfected cells and mock-transfected cells or parent cells, we focused on the role of proteases, such as MMPs, uPA, and tPA and their inhibitors, such as tissue inhibitor of metalloproteinase-1 and -2 and PAI-1 and PAI-2, in these tumors. The principal role of MMP activity is thought to be to remove the extracellular matrix constituents of mature vessel walls and to allow EC migration. Furthermore, because it is considered that VEGF acts as a key mediator for proliferation, migration, and survival of ECs, we investigated how collaboration with Angs (in particular,
Ang-2 and VEGF) during tumor angiogenesis affects the induction of MMPs and their inhibitors in the tumors. In coculture assays, Ang-1 and Ang-2 proteins were detected in culture medium from Ang-1 or Ang-2 transfectants, respectively (Fig. 4A). VEGF protein was also detected in culture medium and subsequently in CM with added recombinant VEGF (10, 50, and 100 ng/ml) from parental MKN-7 cells (Fig. 6A). Fifty ng/ml of recombinant VEGF was added into the coculture medium.

First, we examined the induction of mRNA expression of MMPs and their inhibitors in ECs in the presence or absence of each of Ang-1, Ang-2, or VEGF and in various combinations in CM by Northern blot analysis. Among these MMPs and their inhibitors, MMP-1, MMP-9, and uPA were significantly up-regulated by Ang-2 in the presence of VEGF compared with other conditions, but they were not significantly up-regulated by Ang-1 or Ang-2 alone compared with control (Fig. 6, B and C). Next, we examined the cellular expression and distribution of MMP-1, MMP-9, and uPA in ECs by immunofluorescence microscopy. Similar to the mRNA expression of these enzymes, they were strongly induced only by Ang-2 in the presence of VEGF (Fig. 6D). Finally, mRNA expression of c-Ets1, which is a promoter of MMP-1, MMP-9, or uPA, was examined by Northern blot analysis (Fig. 7A). The mRNA expression of c-Ets1 in ECs was up-regulated by Ang-2 in the presence of VEGF (Fig. 7B). In addition, different cell numbers of HUVECs did not influence the fold induction of the proteases and c-Ets1 mRNA expression.

**DISCUSSION**

The role of Angs in tumor angiogenesis has not been clarified, although many critical roles for VEGF in gastric carcinoma have been reported (8, 28, 29). Similar to VEGF, the specificity of the Angs for the vascular endothelium is ascribed to the distribution of its receptor, Tie-2, which is present on these cells. It has been reported that Tie-2 expression is associated with advanced grade in breast carcinoma (30). However, the relationship between the levels of expression of Angs and tumor aggressiveness in clinical samples has not been studied, although its expression pattern in tumor tissues by in situ hybridization was examined (18). In this study, mRNA expression levels of both Ang-2 and Tie-2 in tumor tissues were significantly higher compared with normal tissues. These results suggest that the Ang/Tie-2 system may play an important role in gastric carcinoma. Our study of the relationship between clinicopathological features and the expression of Angs and Tie-2 disclosed that high Ang-2 mRNA expression was significantly associated with increased vascular involvement and advanced stage, but neither Ang-1 or Tie-2 were significantly associated with clinicopathological features. With regard to prognosis, the high Ang-2-expression group showed a significantly worse prognosis.

Recently, it has been reported that many tumors rapidly co-opt existing host vessels to form an initially well-vascularized tumor mass, and Ang-2 plays an important role in the process (17, 31). In tumors forming a solid mass, such as glioblastoma or astrocytoma, Ang-1 is expressed in tumor cells and Ang-2 is strikingly induced in co-opted vessels adjacent to the tumor cells (16, 18). It is considered that the intense autocrine expression of Ang-2 by ECs in tumor-associated vessels contributes to tumor expansion. In this study, we investigated the patterns of Ang-2 expression in clinical samples of gastric carcinoma by immunohistochemistry. We determined that Ang-2 was expressed not only in ECs in tumor-associated vessels, but also in cancer cells in both the intestinal type forming a solid mass and in the diffuse type of gastric cancer not forming a solid mass. This result suggests that Ang-2 derived from cancer cells may undergo the regression of tumor-associated vessels and robust angiogenesis.

To assess the biological role of Ang-2 in the tumor angiogenesis of gastric cancer, we performed transfection experiments and analyzed the tumorigenicity and metastatic potential of Ang-2 transfected into MKN-7 cells in nude mice. As a result, Ang-2-expressing MKN-7 cells developed tumors with a highly metastatic potential with hyper-vascularity. The initiation of new-vascular formation needs destabilization of the mature structure of vessels and, subsequently, degradation of the extracellular matrix surrounding ECs. Vessels that are formed in the marked hypervascular tumors producing Ang-2 tend to be immature. In fact, the immaturity of the vessels and the microvascular counts in these tumors were significantly associated with Ang-2 production. However, it has not been clarified how the process from destabilization of vessels to degradation of the extracellular matrix is regulated. In this study, we focused on proteinases that are required to degrade the extracellular matrix (32–36). We investigated whether the expression of proteases of ECs in tumor angiogenesis was regulated by Ang-2. We also evaluated the influence of VEGF on the role of Ang-2, because there was a significant correlation between mRNA expression levels of Ang-2 and VEGF in tumor tissues of human gastric cancer. In ECs constitutively expressing the Tie-2 receptor, mRNA and the protein expression of MMP-1, MMP-9, and uPA were

**Table 5: Analysis of Ang-2 transfected tumors in MKN-7 cells**

<table>
<thead>
<tr>
<th>Cell line</th>
<th>n</th>
<th>Tumor size (mm³)</th>
<th>Vessel count</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental</td>
<td>12</td>
<td>125 ± 8</td>
<td>6.6 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Mock</td>
<td>12</td>
<td>166 ± 7</td>
<td>7.3 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Ang-2 transfectant</td>
<td>12</td>
<td>546 ± 10</td>
<td>20.1 ± 7.9*</td>
<td></td>
</tr>
</tbody>
</table>

* P < 0.05 versus parental or mock.
significantly up-regulated by Ang-2 in the presence of VEGF; in contrast, these were not significantly up-regulated by Ang-1 or Ang-2 alone. On the other hand, the expression of these enzymes was not significantly altered in cell lines lacking Tie-2 expression, such as the Ang-1 or Ang-2 transfectants. The different protease expression pattern between ECs and Tie-2-nonexpressing cells is ascribed to the Ang/Tie-2 pathway. These properties suggest that induction of proteases in ECs by Ang-2 together with VEGF during tumor angiogenesis could be partly regulated not only in an autocrine manner but also in a paracrine manner via the Ang/Tie-2 pathway.

Why does Ang-2 require the presence of VEGF to induce these proteases compared with Ang-2 or VEGF or Ang-1 alone? Because Ang-2, unlike Ang-1, cannot induce autophosphorylation of the Tie-2 receptor in human ECs, Ang-2 is considered to inhibit Ang-1/Tie-2 activity. Induction of MMP-1, MMP-9, and uPA is regulated by common promoter regions such as c-Ets1 (37, 38). It has recently been reported that Tie promoter activity is also controlled by EC Ets factors (39). We therefore consider that Ets-1 may play a key role in tumor angiogenesis via the Ang/Tie system. We hypothesized that the signaling by Ang-1 via the Tie-2 receptor may act as a negative regulator of c-Ets1 activity and result in the suppression of the production of these proteases; once Ang-2 blocks the activation of Tie-2 by Ang-1, subsequent c-Ets1 activity may be increased. In fact, c-Ets1 mRNA expression in ECs was significantly up-regulated by Ang-2 in the presence of VEGF. VEGF alone was added in cultured medium of MKN-7 cells used as a control (a, c, and e); VEGF was added in cocultured medium containing Ang-2 (b, d, and f). *, P < 0.05 compared with medium alone; and #, P < 0.05 compared with other conditions.

Fig. 6. Enhancement of expression of MMPs, MMP-inhibitors, uPA, and PAI-1 in ECs by Angs derived from transfectants and exogenous VEGF. A, expression of VEGF protein in CM with MKN-7 cells with or without exogenous VEGF. VEGF protein was weakly detected in the supernatant of MKN-7 cells alone, whereas, it was strongly detected in CM from MKN-7 cells in the presence of exogenous VEGF (10–100 ng/ml). In particular, VEGF was markedly detected using concentrations of VEGF above 50 ng/ml. B and C, expression of mRNAs for MMPs, MMP-inhibitors, uPA, and PAI-1 in ECs by Ang-1, Ang-2, and VEGF (50 ng/ml) alone or in combination, by Northern blot analysis. Among these MMPs and their inhibitors, MMP-1, MMP-9, and uPA were significantly up-regulated by Ang-2 in the presence of VEGF compared with the other conditions. They were not significantly up-regulated by Ang-1 or Ang-2 alone compared with control. In addition, up-regulation of MMP-1, MMP-9, and uPA mRNA expression was inhibited by the presence of both Ang-1 and Ang-2. The mean of three experiments is shown. D, immunohistochemical staining for MMP-1 (a and b), MMP-9 (c and d), or uPA (e and f) in ECs. Similar to the mRNA expression, these enzyme proteins were enhanced by Ang-2 in the presence of VEGF. VEGF alone was added in cultured medium of MKN-7 cells used as a control (a, c, and e); VEGF was added in cocultured medium containing Ang-2 (b, d, and f). *, P < 0.05 compared with medium alone; and #, P < 0.05 compared with other conditions.
41). On the other hand, Ang-2 could induce metalloproteinases to degrade vessel basement membranes via activation of c-Ets1. Furthermore, this induction might be enhanced in the presence of VEGF. The implication is that VEGF acts as a powerful promoter of tumor angiogenesis and results in tumor progression, and Ang-2 acts as an initiator to begin neovascular formation. In malignant diseases such as gastric carcinoma, the dynamic balance between Ang-2 and VEGF might be impaired. Thus, overexpressed Ang-2 together with VEGF derived mainly from cancer cells might promote tumor angiogenesis in gastric cancer development. Recent studies have demonstrated that Ang-2 mRNA levels were increased by VEGF or hypoxia in bovine microvascular ECs (42), although the detailed regulation of Ang-2 mRNA levels in gastric cancer cells remains unclear and will require additional investigation for clarification.

In conclusion, our findings demonstrate that Ang-2 is produced not only in ECs, but also in cancer cells in human gastric carcinoma. On the basis of the experiments in this study, Ang-2 produced from gastric cancer cells may play an important role in tumor angiogenesis in the presence of VEGF.

ACKNOWLEDGMENTS

We thank to Dr. Yasuji Yoshikawa, Department of Pathology, for advice on histopathological analysis. We also thank J. Miyake, T. Shimooka, and D. Mori for excellent technical assistance.

REFERENCES


Fig. 7. Expression of c-Ets1 in ECs induced by Ang-1, Ang-2, and VEGF (50ng/ml) alone or in combination with CM by Northern blot analysis.

Expression of c-ets1 mRNA was significantly up-regulated by Ang-2 in the presence of VEGF. In addition, up-regulation of c-Ets1 mRNA expression was inhibited by both Ang-1 and Ang-2. The mean of three experiments is shown. * P < 0.05 compared with other conditions.

2152

Downloaded from cancerres.aacjrournals.org on October 18, 2017. © 2001 American Association for Cancer Research.
Angiopoietin-2 Is Related to Tumor Angiogenesis in Gastric Carcinoma: Possible in Vivo Regulation via Induction of Proteases

Tsuyoshi Etoh, Hiroshi Inoue, Shinji Tanaka, et al.


Updated version  Access the most recent version of this article at: http://cancerres.aacrjournals.org/content/61/5/2145

Cited articles  This article cites 40 articles, 14 of which you can access for free at: http://cancerres.aacrjournals.org/content/61/5/2145.full#ref-list-1

Citing articles  This article has been cited by 42 HighWire-hosted articles. Access the articles at: http://cancerres.aacrjournals.org/content/61/5/2145.full#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.