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Cancer Res 2001;61:2145-2153.

[CANCER RESEARCH 61, 2145–2153, March 1, 2001]

Angiopoietin-2 Is Related to Tumor Angiogenesis in Gastric Carcinoma: Possible *in Vivo* Regulation via Induction of Proteases[1]

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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 preoperative 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, tumors secrete growth factors that act on ECs.[3] It is thought that the resulting neovasculature supports tumor expansion and metastasis (4, 5). Indeed, recent studies have shown that the incidence of metastasis can be correlated with the number and density of blood vessels in breast, lung, prostate, esophageal, colon, and gastric carcinoma and melanomas (3–6).

There have been many studies attempting to isolate the molecular mediators of tumor angiogenesis. Until recently, most of the work in the field has focused on polypeptide growth factors such as fibroblast growth factor and VEGF, which are mitogenic for ECs *in vitro*, and which produce an angiogenic response *in vivo* (7–10). Ang-1 and its naturally occurring antagonist, Ang-2, are novel ligands that regulate tyrosine phosphorylation of the Tie2/Tek receptor on ECs (11, 12). Proper regulation of Tie2/Tek is absolutely required for normal vascular development, seemingly by regulating vascular remodeling and EC interactions with supporting pericytes/smooth muscle cells (12–15). Recently, it has been reported that the expression pattern of Ang-2 is strongly associated with the expression of VEGF in the process of tumor angiogenesis and, subsequently, in tumor expansion (16–19).

Angs are mainly produced by ECs and pericytes, and their receptor Tie2/Tek is also expressed in ECs and partly in hematopoietic cells (13). In particular, Ang-2 is expressed in ECs of tumor-associated vessels (18, 20). Therefore, Angs, especially in the Ang-2 and Tie system, may act via an autocrine manner in ECs in tumor angiogenesis. However, we have recently found that hypervascular hepatomas with aberrant vasculature show high levels of Ang-2 expression in their epithelium, and Ang-2-transfected human hepatocellular carcinoma cells may contribute to tumor development and extensive hemorrhage in nude mice (21). With respect to gastrointestinal tumors, there have not been any studies that establish a causal role for Angs. In this study, to define a putative role for the Ang/Tie2 system in gastric cancer angiogenesis, we investigated the expression pattern of Angs and its clinical significance. On the basis of the clinical results, we examined further the biological behavior of Ang-2 stably transfected human gastric cancer cells in culture and after orthotopic implantation into nude mice. We focused especially on the role of proteases such as MMPs with regard to the possible mechanism resulting in hypervascular conditions induced by Angs in tumor angiogenesis.

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 AZ521, 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 CO_2 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

Received 6/12/00; accepted 1/2/01.

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[1] This study was supported in part by the Ministry of Education, Culture, and Science of Japan (Grants 11139252, 10470260, and 10671198).

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[3] EC, endothelial cell; VEGF, vascular endothelial growth factor; Ang, angiopoietin; MMP, matrix metalloproteinase; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HUVEC, human umbilical venous endothelial cell; RT-PCR, reverse transcription-PCR; vWF, von Willebrand factor; uPA, urokinase-type plasminogen activator; SMA, smooth muscle actin; PAI, plasminogen activator inhibitor; CM, conditioned medium.

mucosa. 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 42.1 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 α-^{32}P labeled by random priming cDNA probes for *Ang-1, Ang-2, VEGF, Tie-2*, proteases and their inhibitors, and *c-Ets1*, 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. Endogeneous 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 pcDNA3 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 transwell 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 tritiated-thymidine (1 μCi; NEN, Boston, MA) as described previously (24). Cells (3×10^3) 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 (1×10^4) 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.

Immunoblot Analysis and 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 measure 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-Transfectant MKN-7 Cells with or without Exogenous VEGF. In this study, 1×10^3 or 1×10^5 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 (1×10^5) 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*

Gene	Upper primer (5′ to 3′)	Lower primer (5′ to 3′)	Product size (bp)	AT[a] (°C)
Ang-1	ATGACAGTTTTCCTTTCCTTTGC	CAGCTTCTCCGGATTTCTTTGT	550	55
Ang-2	GGAAGAGCATGGACAGCATAGGA	GCCATTTGTGGTGTGTCCTGATT	821	56
Tie-2	CCACCATCGAGCGGCATCTACA	TTGATCCGGGGCATCGTCTCTAA	554	62
VEGF	ATGACAGTTTTCCTTTCCTTTG	CAGCTTCTCCCGGATTTCTTTGT	520	60
MMP-1	GGACTCTCCCATTCTACTGAT	CCTTCTTTGGACTCACACCAT	561	56
MMP-2	CGGAAAAGATTGATGCGGTAT	CAGTGAAAAGCCAGGGGTCTG	325	56
MMP-7	CGACTCACCGTGCTGTGTGCT	TCAGAGGAATGTCCCATACCC	455	56
MMP-9	CCCTTCACTTTCCTGGGTAAG	CATCTTCCCCCTGCCACTCC	620	56
MT1-MMP	GGGTCATCTGCTCCTTTTCCA	TCCTTTCTCTGGCTGCCCTAC	350	56
uPA	TTCTCTGCGTCCTGGTCGTGA	CTCCGGTTGTCTGGGTTCCTG	361	58
PAI-1	TTGGTGAAGGGTCTGCTGTGC	GCTGCCGTCTGATTTGTGGAA	602	60
TIMP-1	GGCTGGGATAGACACCAATG	CCCTGGAAGGAACCTCACAA	360	56
TIMP-2	CTGAAGAGCATTTGGGGTCC	ATGGCCTTAGCCTTAGACTGAA	480	56

[a] AT, annealing temperature.

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*

	Average mRNA expression		
	Ang-1	Ang-2	Tie-2
Tumor tissue	2.5 ± 1.2	5.6 ± 1.8[a]	4.1 ± 1.2[a]
Normal tissue	1.1 ± 0.9	1.4 ± 0.5	1.5 ± 1.1

[a] $P < 0.05$ *versus* normal tissue. Mean ± SD.

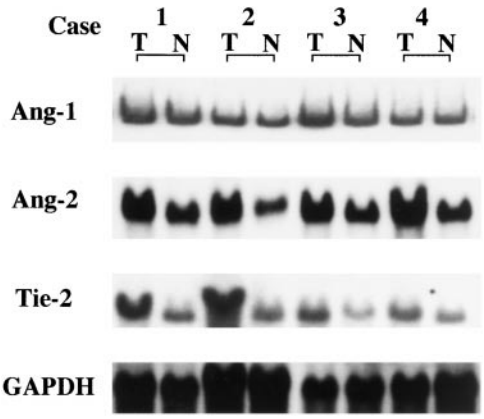

Fig. 1. Four representative cases of Northern blot analysis for Angs and Tie-2. The expression of Ang-2 and Tie-2 in tumor tissue (*T*) was greater than in normal tissue (*N*). GAPDH mRNA levels served as the internal control.

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 the 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 (*T*) than normal tissue (*N*). 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 ($P < 0.05$; 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 3 *Association between clinicopathological features and Ang-2 mRNA expression of tumor tissues in human gastric carcinoma*

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

[a] Mean ± SD.
[b] NS, not significant.

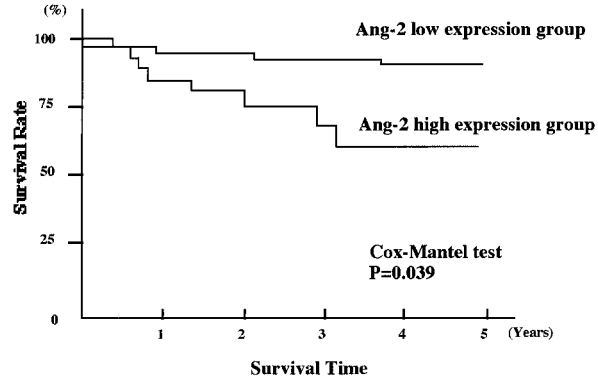

Fig. 2. Overall survival of patients with human gastric carcinoma with regard to Ang-2 mRNA expression in tumor tissues. A difference in survival time was significant between the high-Ang-2 mRNA expression group ($n = 43$) and the low-Ang-2 mRNA expression group ($n = 42$; $P < 0.05$; Mantel-Cox method).

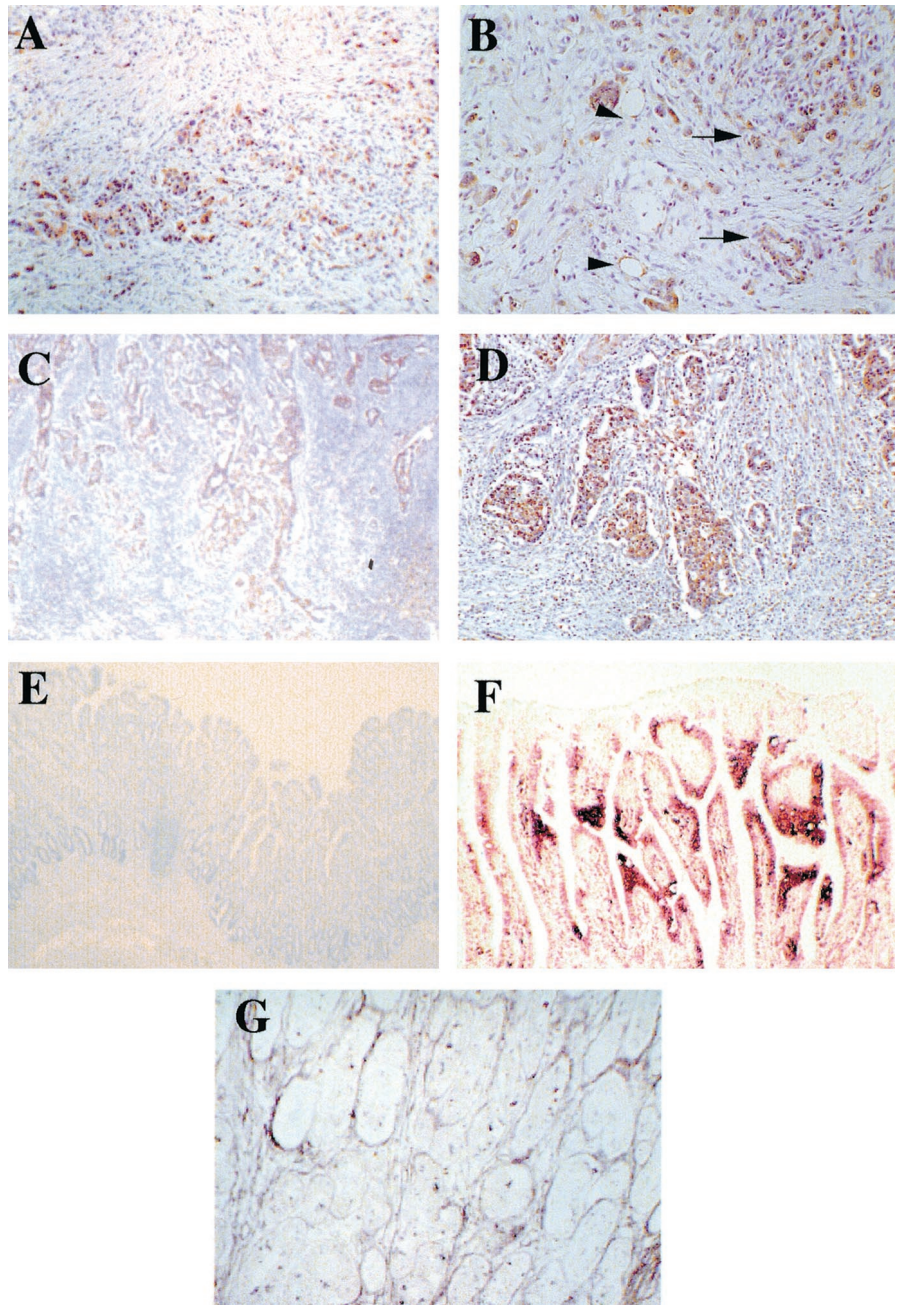

Fig. 3. Immunohistochemical staining and *in situ* hybridization for Ang-2 in human gastric cancers. *A* and *B*, Ang-2 was expressed not only in ECs but also in cancer cells in the diffuse type of gastric cancer. *C* and *D*, in another case, Ang-2 was expressed mainly in the cancer cells in the intestinal type of gastric cancer, especially at invasive areas, and it was also detected in the stromal cells. *E*, Ang-2 was not detected in normal gastric mucosa. *F*, similarly, Ang-2 mRNA expression was detected in gastric cancer cells by *in situ* hybridization. *G*, Ang-2 was not detected in gastric cancer cells using a sense probe. Original magnification: ×20 (*C* and *E*); ×40 (*A*, *D*, and *E*); and ×80 (*B*, *F*, and *G*).

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. 4*A*, *right*). With regard to Ang-1, transfection assays and the evaluation of Ang-1 protein were performed similarly (Fig. 4*A*, *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. 4*B*). 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. 4*C*, *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

Table 4 *mRNA expression of Angs and Tie-2 in gastric cancer cell lines and HUVECs*

The levels of mRNA were examined by Northern blot analysis and standardized by GAPDH mRNA.

Cell line	Average mRNA expression[a]		
	Ang-1	Ang-2	Tie-2
MKN-1	ND[b]	ND	ND
MKN-7	ND	ND	ND
AZ521	0.8 ± 0.4	ND	ND
NUGC3	ND	1.3 ± 0.8	ND
SOH	1.5 ± 1.1	ND	ND
NS	ND	0.9 ± 0.5	ND
HUVECs	1.2 ± 0.5	2.1 ± 1.2	2.8 ± 1.1

[a] Mean ± SD.
[b] ND, not detected.

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. 4*C*, *d* and *e*). Immunohistochemical examination of the tumors derived from Ang-2 transfectants revealed that a huge mass was formed in the subserosal space of the gastric wall (Fig. 4*D*, *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. 4*D*, *c*). On the other hand, Ang-2 was not detected in cancer cells of the mock-transfectant tumors (Fig. 4*D*, *d*). Its receptor, Tie-2, was only expressed in ECs at sites surrounding the cancer cells (Fig. 4*D*, *e*).

Finally, we investigated whether the increased tumorigenesis and metastasis of the tumors were associated with increased angiogenesis and vessel maturation. For examination for 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 transfectants was significantly lower than that of parent and/or control vector-transfected cell tumors (Fig. 5*C*).

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,

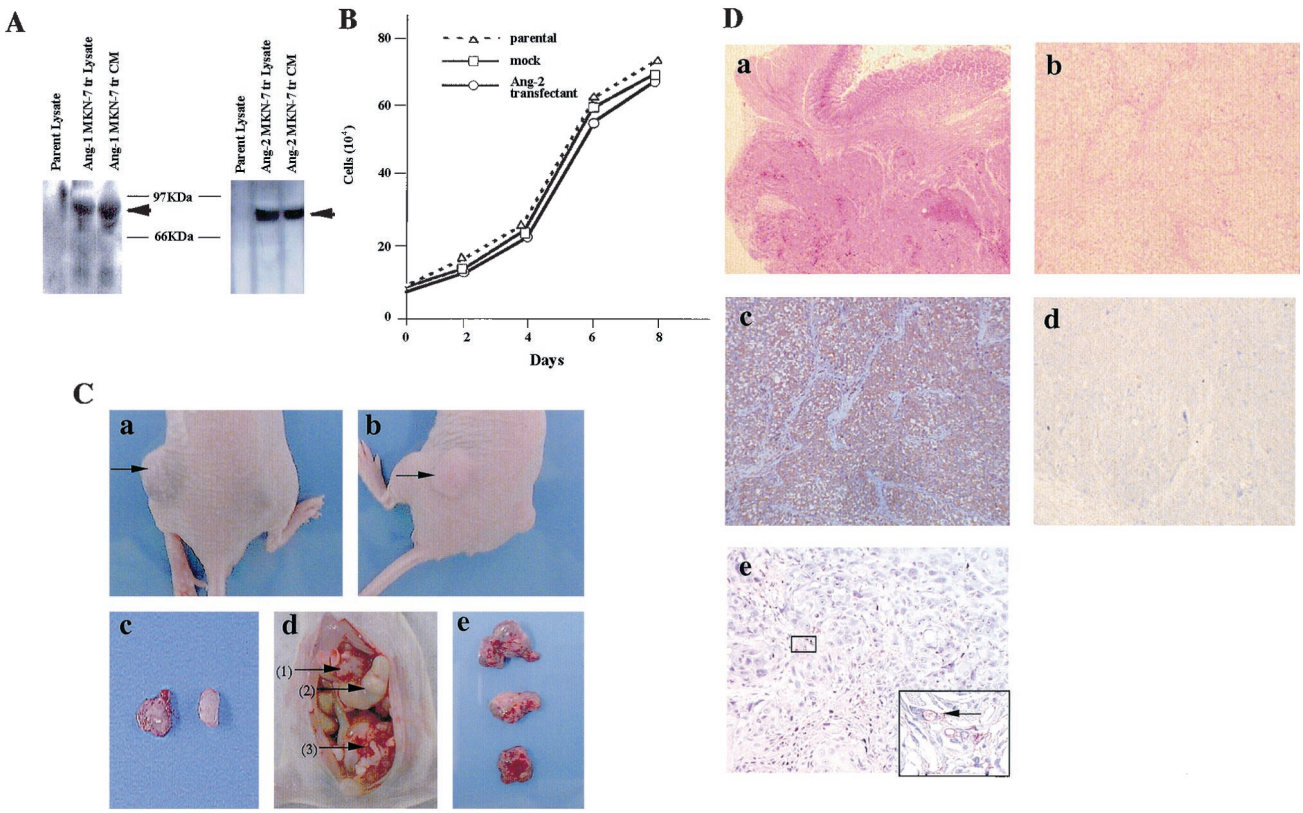

Fig. 4. Biological characteristics of Ang-2-transfected tumors. *A*, Ang-1 or Ang-2 protein expression in cell lysates or CM with Ang-transfected MKN-7 cells using Western blot analysis; *tr*, transfectant. *B*, *in vitro* growth of MKN-7 cells, transfected with the *Ang-2* gene, in the presence of FBS. Cell number was counted in triplicate cultures. This is one representative experiment of three. *C*, implantation of Ang-2-transfected MKN-7 cells or mock-transfected MKN-7 cells into s.c. tissues of BALB/c nude mice. A representative example of tumor development derived from Ang-2-transfected MKN-7 cells (*a*); mock-transfectant (*b*); and transitional slices of resected specimens (*c*, Ang-2 transfectant, *left*; mock, *right*). Ang-2-expressing MKN-7 cells developed markedly hypervascular tumors (*d*); Ang-2-expressing MKN-7 cells developed tumors of metastatic potential. Liver metastasis and peritoneal dissemination in nude mice inoculated into the gastric wall with Ang-2-transfected MKN-7 cells, liver metastasis (*1*); implanted primary gastric cancer (*2*); and peritoneal dissemination (*3*). Severe hemorrhage was found in these liver metastatic tumors (*e*). *D*, immunohistochemical staining for Ang-2 and Tie-2 in Ang-2-transfected tumors. Sections of implanted primary tumor of the stomach by H&E staining show a huge mass formation in the subserosal space of the gastric wall (*a* and *b*); Ang-2 was strongly detected in cancer cells and also detected in stromal cells (*c*); Ang-2 was not detected in cancer tissues of the mock-transfectant tumors (*d*); Tie-2 was detected only in ECs of Ang-2-transfected tumors (*e*). *Inset* shows a higher magnification of Tie-2-positive cells. Original magnification: ×10 (*D*, *a*); ×40 (*D*, *b*, *c*, *d*, and *e*); and ×80 (*D*, *c*; *inset*).

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

The latency of tumor appearance is 4 wk after implantation. Tumor size (mean ± SD) resected from mice is expressed in cubic volume. Vascularization of tumors is expressed as the number of capillaries/field as described in "Materials and Methods."

Cell line	*n*	Tumor size (mm^3)	Vessel count
Parental	12	125 ± 8	6.6 ± 2.1
Mock	12	166 ± 7	7.3 ± 2.5
Ang-2 transfectant	12	546 ± 10[a]	20.1 ± 7.9[a]

[a] $P < 0.05$ *versus* parental or mock.

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. 4*A*). 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. 6*A*). 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. 6*D*). Finally, mRNA expression of c-Ets1, which is a promoter of MMP-1, MMP-9, or uPA, was examined by Northern blot analysis (Fig. 7*A*). The mRNA expression of c-Ets1 in ECs was up-regulated by Ang-2 in the presence of VEGF (Fig. 7*B*). 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 hypervascularity. The initiation of new-vessel 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

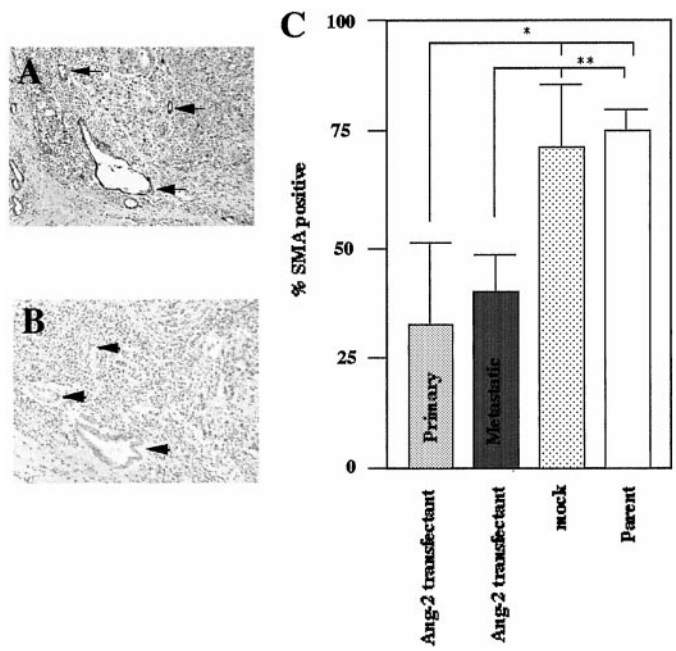

Fig. 5. Most blood vessels in the Ang-2-transfected tumors are immature. Serial sections of Ang-2-transfected tumors stained with anti-vWF (*A*) and anti-α-SMA (*B*) showed only few α-SMA-positive vessels. Original magnification: ×40. *C*, comparison of maturation index between Ang-2 transfectant and control. Vessels larger than capillaries were scored (between 45 and 185 for each specimen), and the percentage of α-SMA-positive vessels is presented. For comparison, the maturation index of Ang-2-expressing primary and metastatic liver tumors was significantly lower than that of non-Ang-2-expressing tumors. Averages of five high power fields in Ang-2-transfected (primary and metastatic), mock-transfected, and MKN-7 (parent) tumors were 35, 45, 78, and 85% respectively. * and **, $P < 0.05$.

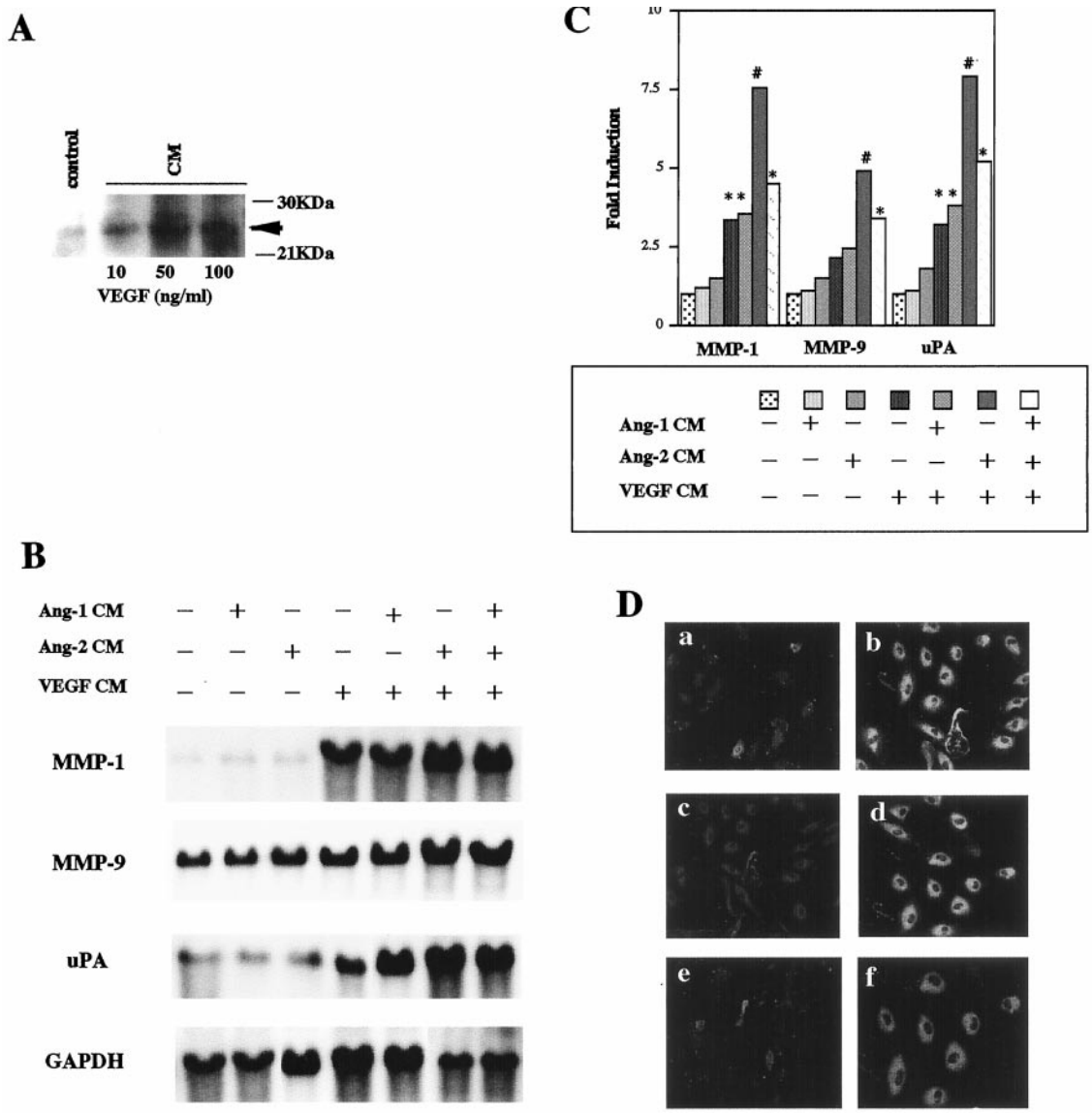

Fig. 6. Enhancement of expression of MMPs, MMP-inhibitors, uPA, and PAI-1 in ECs by Angs derived from transfectants and exogenous VEGF. *A*, expression of VEGF protein in CM with MKN-7 cells with or without exogenous VEGF. VEGF protein was weakly detected in the supernatant of MKN-7 cells alone, whereas, 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.

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 by Northern blot analysis. Interestingly, up-regulation of MMP-1 and MMP-9, uPA, or c-Ets1 mRNA expression by Ang-2 was inhibited by Ang-1. This result suggests that Ang-1 competes with Ang-2 for up-regulation of these enzymes via c-Ets1. The role of VEGF in tumor angiogenesis is to activate c-Ets1 and, in addition, to promote the proliferation and cell growth of ECs (37, 40,

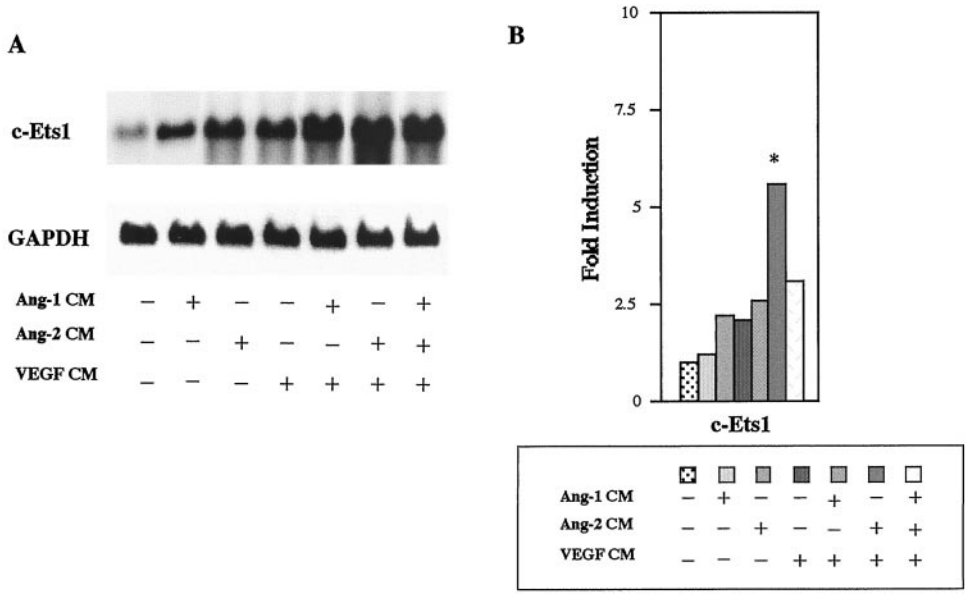

Fig. 7. Expression of c-Ets1 in ECs induced by Ang-1, Ang-2, and VEGF (50ng/ml) alone or in combination with CM by Northern blot analysis. Expression of c-ets1 mRNA was significantly up-regulated by Ang-2 in the presence of VEGF. In addition, up-regulation of c-Ets1 mRNA expression was inhibited by both Ang-1 and Ang-2. The mean of three experiments is shown. *, $P < 0.05$ compared with other conditions.

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.

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