Table 1. Peritoneal metastasis of *Reg IV* transfectants

Cells	Treatment	Metastasis[a]	Number	Size (mm)
MKN28				
R0	Vehicle	2/9[b]	1.5 ± 0.7[b]	1.0 ± 0.1[d]
R1	*Reg IV*	9/9[b]	5.3 ± 1.0[b]	4.2 ± 1.2[d]
R2	*Reg IV*	9/9[b]	5.6 ± 0.9[b]	5.2 ± 1.4[d]
TMK1				
R0	Vehicle	2/8[b]	0.7 ± 0.6[c]	0.6 ± 0.1[d]
R1	*Reg IV*	8/8[b]	9.8 ± 1.5[c]	4.6 ± 0.8[d]
MKN45				
Control	siRNA mix	8/8	14.4 ± 2.1[d]	3.5 ± 0.7[d]
siRNA	*Reg IV* siRNA	5/8	0.55 ± 0.7[d]	0.9 ± 0.5[d]

[a]Metastasis determined at 2 weeks post inoculation.
[b]$P < 0.01$, [c]$P < 0.001$, [d]$P < 0.0001$.

from those of MKN28-R0 cells. Anti-Reg IV antibody added to the culture media to neutralize secreted Reg IV did not affect invasion of three types of MKN28 cells (Fig. 2d). In TMK1 and MKN45 cells, transfection or knockdown of *Reg IV* did not affect the cell growth or invasion (Fig. 2b,c,e,f). We next treated these cells with nitric oxide (NO) using SNP as an NO donor. NO cytotoxicity depends on NO concentration and sensitivity of the cells (36). MKN28-R0 cells were decreased by SNP treatment in a dose-dependent manner (Fig. 2g). In contrast, MKN28-R2 and MKN28-R1 cells attenuated cell decrease in SNP concentration higher than 10^{-7} M. At 10^{-1} M, the relative cell numbers compared to untreated MKN28 parental cells were $28 \pm 3\%$ and $57 \pm 5\%$ in MKN28-R1 and MKN28-R2 cells, respectively, which were higher than that in MKN28-R0 cells ($13 \pm 2\%$) (both $P < 0.0001$). TMK1-R1 cells also showed lower sensitivities to SNP-induced cytotoxicity than that in TMK1-R0 cells. In contrast, MKN45-siRNA cells showed higher SNP sensitivities than that in MKN45-Cont cells (Fig. 2h,i).

Peritoneal tumours of Reg IV-transfectants

Reg IV-transfected MKN28 cells were inoculated into the peritoneal cavities of nude mice. Peritoneal tumours of *Reg IV*-transfected MKN28 cells were compared to MKN28-R0 tumours (Table 1, Fig. 3). Tumorigenicity of MKN28-R1 and MKN28-R2 cells was significantly higher (both 9/9) than that of MKN-R0 cells (2/9) ($P = 0.0023$). Numbers of peritoneal tumours in MKN28-R1 and MKN28-R2 cells were higher (5.3 ± 1.0 and 5.6 ± 0.9 foci, respectively) than of MKN28-R0 cells (1.5 ± 0.7 foci) ($P = 0.0364$). Sizes of tumours of MKN28-R1 and MKN28-R2 cells were significantly larger (4.2 ± 1.2 mm and 5.2 ± 1.4 mm, respectively) than of MKN28-R0 cells (1.0 ± 0.1 mm) ($P = 0.0005$). MKN28-R2 cells formed larger tumours in the peritoneum than MKN28-R0 cells (Fig. 1a). *Reg IV*-transfected TMK1-R1 cells also showed higher tumorigenicity (8/8) than TMK1-R0 cells (2/8). Numbers and sizes of the peritoneal tumours were larger (9.8 ± 1.5 foci and 4.6 ± 0.8 mm, respectively) in TMK1-R1 cells than those in TMK1-R0 cells (0.7 ± 0.6 foci and 0.6 ± 0.1 mm, respectively) ($P < 0.001$ and $P < 0.0001$). In contrast, knockdown of *Reg IV* decreased tumourigenicity, tumour number and tumour growth in MKN45 cells. *Reg IV* siRNA-treated MKN45 cells showed lower tumorigenicity (5/8) than siRNA mixture-treated MKN45 cells (8/8). The number and sizes of the peritoneal tumours in *Reg IV* siRNA-treated MKN45 cells were smaller (0.55 ± 0.7 foci and 0.9 ± 0.5 mm, respectively) than those in siRNA mixture-treated MKN45 cells (14.4 ± 2.1 foci and 3.5 ± 0.7 mm, respectively) ($P < 0.0001$ and $P < 0.0001$).

Histologically, MKN28-R0 tumours showed large areas of necrosis, whereas no necrosis was found in the MKN28-R2 tumours (Fig. 3b). Production of Reg IV in the tumours was confirmed by immunohistochemistry (Fig. 3b, Table 2). MKN28-R0 tumours contained few Reg IV-positive cells, whereas MKN28-R2 tumours showed marked Reg IV immunoreactivity in the cytoplasm in all tumour cells (Allred's grade 8). As shown in Table 2, MKN28-R1, TMK1-R1 and siRNA mixture-treated MKN45 cells showed marked Reg IV expression (grades 7, 8 and 8, respectively), whereas MKN28-R0, TMK1-R0 and *Reg IV* siRNA-treated MKN45 cells showed no Reg IV expression (grade 0).

Cell proliferation and apoptotic properties of MKN28-R1 and -R2 tumours were compared to those of MKN28-R0 tumours (Fig. 3c, Table 2). Proliferating cell nuclear antigen (PCNA) indices in MKN28-R1 and MKN28-R2 tumours were $87 \pm 5\%$ and $83 \pm 4\%$, respectively, which were similar to those in MKN28-R0 tumours ($84 \pm 5\%$). In contrast, TUNEL indices in MKN28-R1 and MKN28-R2 tumours were $0.8 \pm 0.8\%$ and $0.5 \pm 0.7\%$, respectively, which were significantly lower than in MKN28-R0 tumours ($6.2 \pm 1.6\%$, $P < 0.0001$). We examined labelling indices of PCNA and TUNEL in TMK1-R1 and *Reg IV* siRNA-treated MKN45 tumours, which were compared to those in TMK1-R0 and siRNA mixture-treated MKN45 tumours. PCNA indices in TMK1-R0 and TMK1-R1 tumours were 58 ± 7 and 65 ± 7, respectively, which were similar to each other. In contrast, the TUNEL index in *Reg IV*-transfected TMK1-R1 tumours was significantly lower (2.3 ± 1.8) than that in TMK1-R0 tumours (8.6 ± 2.4) ($P < 0.01$). TUNEL-positive apoptotic cells were significantly increased by *Reg IV* knockdown in MKN45 cells. In contrast, PCNA indices were not affected by *Reg IV* knockdown.

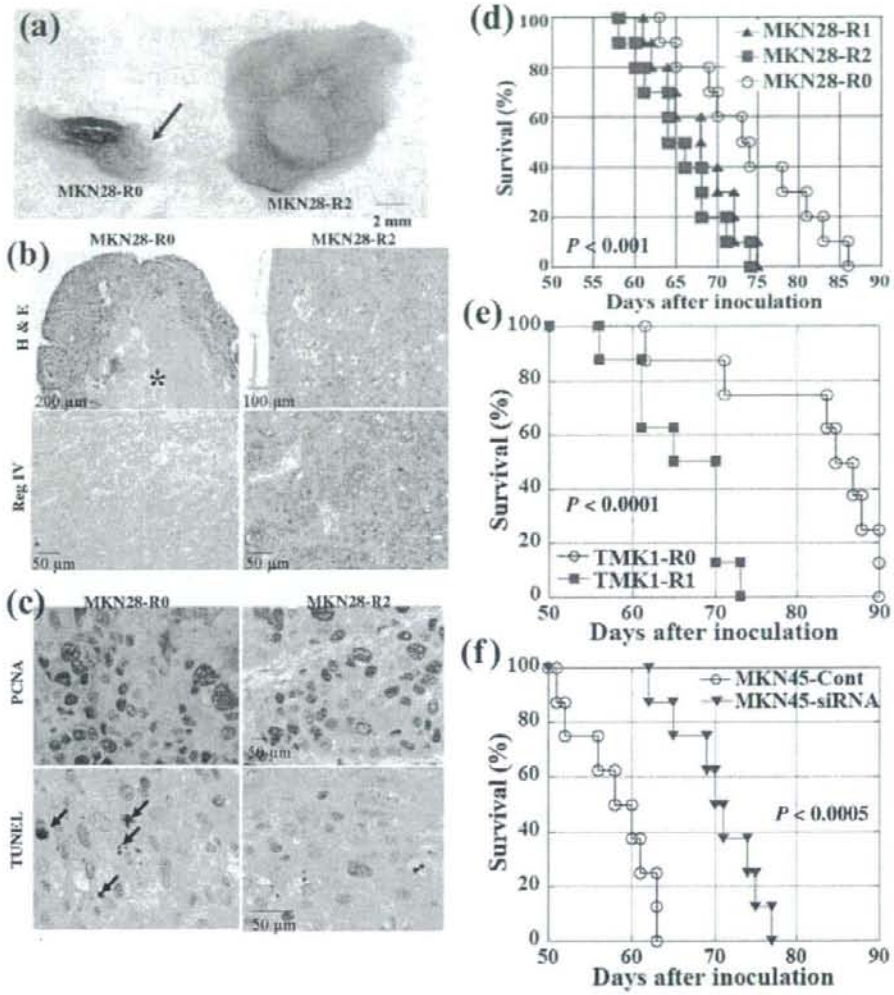


Figure 3. Peritoneal tumours of Reg IV transfectants. (a) Macroscopic appearance of MKN28-R0 cell tumour (at the mesocolon, arrow) and MKN28-R2 tumour (at the abdominal wall). (b) Histological findings using haematoxylin and eosin staining (upper panels). Necrotic area (asterisk). Immunostaining of Reg IV (lower panels). Immunoreactivity was observed in cytoplasm in MKN28-R2 cells. (c) Immunostaining of PCNA and TUNEL assay in MKN28-R2 and MKN28-R0 tumours. Arrow, TUNEL-positive apoptotic cells. (d–f) Survival of mice inoculated with MKN28-R1, MKN28-R2 and MKN28-R0 cells (d), TMK1-R0 and TMK1-R1 (e), and MKN45 treated with *Reg IV*-siRNA (MKN45-siRNA) or siRNA mixture (MIN45-Cont) (f) were calculated by Kaplan–Meier model and compared by Cox proportional hazard model. Survival of mice inoculated with MKN28-R1/MKN28-R2, TMK1-R1 and MKN45-Cont were significantly worse than those of mice inoculated with MKN28-R0, TMK-R0, MKN45-siRNA ($P < 0.001$, $P < 0.0001$, $P < 0.0005$, respectively).

Survival of mice burdened with peritoneal tumours of Reg IV-transfected gastric cancer cells

The survival of a further set of mice inoculated with *Reg IV*-transfected MKN28 and TMK1 cells or *Reg IV* siRNA-treated MKN45 cells into the peritoneal cavity was analysed (Fig. 3d–f). Mice inoculated with MKN45 cells were continuously administrated liposome-encapsulated siRNA into the peritoneal cavity. Survival of mice inoculated with MKN28-R1 and MKN28-R2 cells, or

Table 2. PCNA and TUNEL indices in peritoneal metastasis of *Reg IV* transfectants in mice

	Immunohistochemistry		
Cell line	Reg IV grade[a]	PCNA (%)	TUNEL (%)
MKN28			
R0	0	84 ± 5	6.2 ± 1.6[b,c]
R1	7	87 ± 5	0.8 ± 0.8[b]
R2	8	83 ± 4	0.5 ± 0.7[c]
TMK1			
R0	0	58 ± 7	8.6 ± 2.4[d]
R1	8	65 ± 7	2.3 ± 1.8[d]
MKN45			
Control	8	76 ± 8	3.7 ± 0.5[d]
siRNA	0	72 ± 9	10.6 ± 2.3[d]

[a]According to Allred grading. Grade 0, no staining; Grade 7, intermediates immunoreactivity was found in all cells; Grade 8, strong immunoreactivity was found in all cells.
[b,c]$P < 0.0001$, [d]$P < 0.01$.

TMK1-R1 cells was significantly worse than of those of mice inoculated with MKN28-R0 and TMK-R0 cells ($P < 0.001$ and $P < 0.0001$, respectively). In contrast, mice inoculated with *Reg IV* siRNA-treated MKN45 cells showed significantly better survival than those inoculated with siRNA mixture-treated MKN45 cells ($P < 0.0005$). All mice died from extended peritoneal tumours which lead to malnutrition.

Reg IV levels in peritoneal lavage fluid and serum of Reg IV transfectants-inoculated nude mice and human gastric cancer patients

Next, we detected Reg IV protein in peritoneal lavage fluid and serum from *Reg IV*-transfected MKN28 and TMK1 cells or siRNA-treated MKN45 cells (Fig. 4a,b). Reg IV protein levels in peritoneal lavage fluid from mice inoculated with MKN28-R1, MKN28-R2 and TMK1-R1 cells increased 12.3, 19.6 and 1.5 times, respectively, higher than that from mice inoculated with MKN28-R0 or TMK1-R0 cells. In contrast, Reg IV protein levels in peritoneal lavage fluid from mice inoculated with *Reg IV* siRNA-treated MKN45 cells were 9% of that in mice inoculated with siRNA mixture-treated MKN45 cells. Reg IV protein levels in serum from mice inoculated with MKN28-R1, MKN28-R2 and TMK1-R1 increased 2, 3.4 and 12 times, respectively, higher than that in mice inoculated with MKN28-R0 or TMK1-R0 cells. In contrast, Reg IV protein levels in serum from mice inoculated with *Reg IV* siRNA-treated MKN45 cells were 0.6% of that in mice inoculated with siRNA mixture-treated MKN45 cells.

Table 3. Reg IV expression in metastatic gastric cancer

		Reg IV expression	
	n	Positive	Negative
At operation			
Peritoneal metastasis (+)	43	29[a]	14
Peritoneal metastasis (–)	21	1[a,b]	20
Peritoneal recurrence	21	18[b]	3
Total	85	48	37

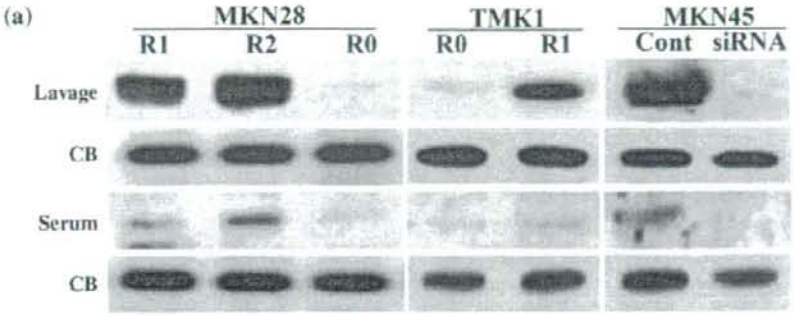
[a,b]$P < 0.0001$ (Fisher's exact test).

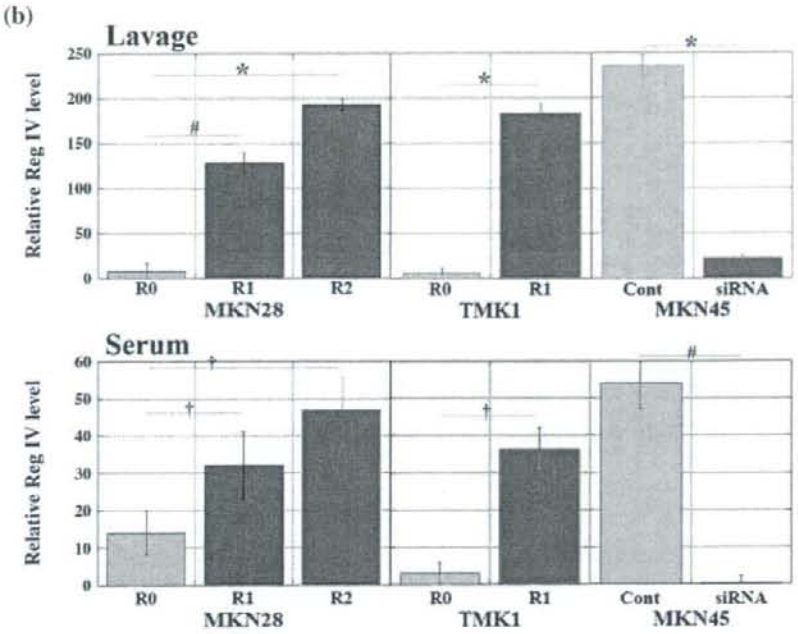
We next examined expression of Reg IV in 85 human gastric cancer patients with metastasis to the lymph nodes, liver or peritoneum (Table 3, Fig. 4c). Reg IV expression was detected in 30 of 64 (47%) gastric cancers at the time of surgery. In these cases, Reg IV was detected in 29 of 43 (67%) peritoneal metastasis-positive cases at the operation, whereas 1 of 21 (5%) peritoneal metastasis-negative cases showed Reg IV expression ($P < 0.0001$). In 21 peritoneal recurrent cases, 18 (86%) were positive for Reg IV. In 48 Reg IV-positive cases, 29 showed peritoneal metastasis at the time of operation. In contrast, the 18 out of the 48 cases showed peritoneal recurrence after the operation despite no peritoneal metastasis at the operation. It suggested that Reg IV-positive peritoneal lavage might be a marker for peritoneal recurrence.

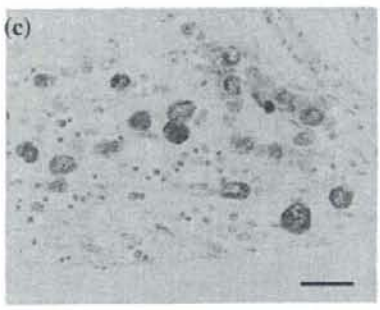
We further detected Reg IV protein in peritoneal lavage fluid from 14 gastric cancer cases, which were found to be invading into the serosa (Fig. 4c). In 8 out of the 14 cases, Reg IV protein, keratin mRNA, and cancer cells were detected in the peritoneal lavage fluids (Fig. 4d). PCR examination of epithelial cell-specific keratin was also positive in the above 8 cases, which supported the evidence that cancer cells existed in the lavage fluid. In 6 cytology-negative cases, Reg IV protein was detected in 2 cases in which keratin was detected. The other 4 cases were negative for Reg IV protein, cytology and keratin.

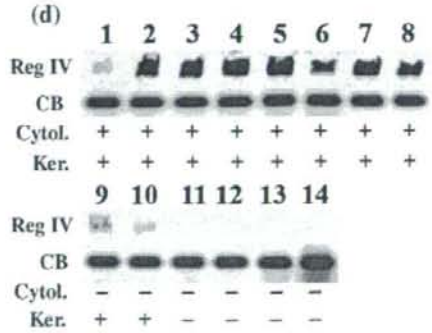
Discussion

Our data have shown that Reg IV increased expression levels of anti-apoptotic BCL-2, BCL-XL and survivin, and phosphorylation levels of AKT in *Reg IV*-transfected MKN28 and TMK1 gastric cancer cells. Moreover, *Reg IV* knockdown decreased these apoptotic factors in *Reg IV*-expressing MKN45 cells; Reg IV protein levels paralleled apoptotic factors in these cells. Although NO is a strong inducer of apoptosis, increments of anti-apoptotic factors reduced NO-induced cytotoxicity in these cells (36). Anti-apoptotic property of Reg IV has been reported in several studies, and this gives cancer cells a









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survival advantages for progression and metastasis (24,27,29,37).

We have previously confirmed that EGF and the receptor formed autocrine and paracrine loops in MKN28 and TMK1 cells (38). MKN28 and TMK1 cells showed high phosphorylation levels of EGFR and Reg IV is reported to be associated with phosphorylation of EGFR (27). Our data confirmed increased phophorylation levels of EGFR in *Reg IV*-transfected MKN28 and TMK1 cells, whereas cell population growth and invasive capacity were not enhanced in the transfectants. Reg IV enhanced phosphorylation of EGFR, however, downstream signals might be preferentially associated with cell survival but not with growth and invasion in the *Reg IV*-transfected MKN28 and TMK1 cells. *Reg IV* knockdown inversely suppressed EGFR phosphorylation in MKN45 cells but the intracellular signalling pathway of Reg IV needs to be elucidated.

Reg IV-transfected and TMK1 cells produced peritoneal metastasis with larger diameter tumours and higher multiplicity than those of control cells. Proliferative activity of transfectant tumours was not different from that of the control cell tumours, whereas transfectant tumours had reduced necrosis and apoptosis in comparison to control tumours. These findings suggest that anti-apoptotic property of Reg IV renders more pronounced potential for peritoneal metastasis of MKN28 and TMK1 cells. Moreover, remarkable progression of metastatic tumours worsened survival of mice inoculated with the *Reg IV*-transfected MKN28 cells than that of mice inoculated with control cells. In contrast, *Reg IV* knockdown significantly suppressed peritoneal metastasis of MKN45 cells. Tumourigenicity was still sustained in *Reg IV* siRNA-treated MKN45 cells. *In vivo* knockdown using liposome encapsulation of siRNA might be less efficient than that of *in vitro* treatment (34). MKN45 cells express c-*met* at high levels with gene amplification, which is associated with the scirrhous phenotype and peritoneal metastasis (7,9,10).

Our previous report shows that Reg IV expression is not associated with peritoneal metastasis in the overall gastric cancer cases (33). However, examination of metastatic gastric cancer shows that Reg IV expression is significantly associated with peritoneal metastasis and peritoneal recurrence. In establishment of peritoneal metastasis, several mechanisms are proposed. Cell-to-cell adhesion between cancer cells and peritoneal mesothelial cells is an initial step of peritoneal metastasis, expression of CD44 and β1 integrin and intercellular adhesion molecule-1 playing a role in cancer cell adhesion to mesothelial cells (39,40). However, CD44 expression is silenced in MKN28 cells and also in the *Reg IV*-transfected MKN28 cells (data not shown) (41). The anti-apoptotic property of cancer cells is emphasized in formation of peritoneal metastasis (15–18). In our data, *Reg IV* transfectants showed up-regulation of several anti-apoptotic proteins: Bcl-2, Bcl-XL, survivin, and phosphorylated AKT. *Reg IV* transfectants acquired resistance to NO-induced apoptosis. TMK1 cells, which are sensitive to various apoptotic inducers, show the anti-apoptotic phenotype after *Reg IV* transfection (35). Reg IV is associated with anti-apoptotic phenotype in MKN45 cells, which carry wild-type p53 differently from MKN28 and TMK1 cells (41) and the anti-apoptotic property is not specific to peritoneal metastasis; however, enhanced survival potential might be a relevant advantage for peritoneally disseminated cancer cells to form metastatic foci.

Because Reg IV is a small secretory protein, its detection in ascites might be expected as a marker for peritoneal metastasis (22). We examined Reg IV protein in peritoneal lavage of gastric cancer cell-inoculated mice. Reg IV in peritoneal lavage fluid was at higher levels in *Reg IV*-transfected MKN28 and TMK1 cells than in control cells. In contrast, lavage Reg IV was significantly lower in mice inoculated with *Reg IV*-knocked down MKN45 cells. We then examined Reg IV protein in peritoneal lavage fluid of gastric cancer patients at the operation. Reg IV protein was detected in all cases with macroscopical and cytological peritoneal metastasis. Moreover, all cases with keratin mRNA-positive ascites showed Reg IV protein in the ascites. Ascites keratin detected by RT-PCR is a sensitive marker for scanty cancer cells in ascites in cytologically metastasis-negative cases (42). These findings suggest that ascites Reg IV might be a sensitive marker for peritoneal metastasis of gastric cancer.

In the present study, we have reported the pivotal role of Reg IV in peritoneal metastasis of gastric cancer. Reg IV is expected to be a marker for early detection of peritoneal metastasis and a prognostic marker for gastric cancer.

Figure 4. Reg IV protein levels in peritoneal lavage fluids and serum of Reg IV-transfected gastric cancer cells and human gastric cancer cases. (a) Reg IV protein levels were examined in peritoneal lavage fluids and serum of *Reg IV*-transfected MKN28 and TMK1 cells and *Reg IV* siRNA-treated MKN45 cells by immunoblotting (CB: loaded protein detected by Coomassie blue). (b) Reg IV signals of mice lavage fluids and serum were semiquantified. Reg IV signal of peritoneal lavage fluid of MKB28-R0 inoculated mice was set to 10. Error bar: standard deviation. (c) Immunohistochemistry of Reg IV in serosa-invading human gastric cancer. Signet ring cells showed strong Reg IV immunoreactivity. Bar: 50 μm. (d) Reg IV protein levels were examined in peritoneal lavage fluids from human gastric cancers by immunoblotting. Cytol., cytological examination of the lavage fluid; +, cancer cell positive; –, cancer cell negative; Ker., PCR examination of keratin in the lavage fluid.

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Vascular Endothelial Growth Factor C Stimulates Progression of Human Gastric Cancer via Both Autocrine and Paracrine Mechanisms

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Abstract **Purpose:** Vascular endothelial growth factor (VEGF)-C induces lymphangiogenesis by activating the VEGF receptor (VEGFR)-3, which is expressed by lymphatic endothelial cells. VEGFR-3 has also been detected on several malignant cells, but the significance of VEGFR-3 expression on malignant cells remains unclear. In this study, we examined the expression and function of VEGFR-3 in gastric carcinoma cells.

Experimental Design: We examined the expression of VEGFR-3 by four human gastric carcinoma cell lines and in 36 surgical specimens of gastric carcinoma. We also used cDNA microarrays to examine the effect of VEGF-C on gene expression in VEGFR-3-expressing KKLS cells. To stimulate VEGF-C/VEGFR-3 signaling in an autocrine manner, the VEGF-C expression vector was transfected into KKLS cells, and stable transfectants were established. These cells were then transplanted into the gastric walls of nude mice.

Results: Two of the four gastric carcinoma cell lines expressed *VEGFR-3* mRNA. In 17 of 36 gastric carcinoma specimens, VEGFR-3-specific immunoreactivity was detected on tumor cells. *In vitro* treatment of KKLS cells with VEGF-C stimulated cell proliferation and increased expression of mRNAs encoding cyclin D1, placental growth factor, and autocrine motility factor. Following inoculation of VEGF-C-transfected and control cells into the gastric walls of nude mice, tumor growth of the VEGF-C-transfected cells was greatly accelerated in comparison with that of control cells. Greater angiogenesis and lymphangiogenesis were also detected in VEGF-C-transfected tumors than in control tumors.

Conclusions: Gastric carcinoma cells express VEGF-C and VEGFR-3. VEGF-C may play a role in the progressive growth of human gastric carcinoma through both autocrine and paracrine mechanisms.

The process of cancer metastasis is sequential and selective and consists of a series of interlinked independent steps (1). To produce a metastatic lesion, tumor cells must complete all of the steps, which include angiogenesis, motility, invasion, survival in the circulation, adhesion, extravasation, and proliferation (2, 3). Several sets of growth factors and their cognate receptors have been reported to be important in the regulation of metastasis. For example, cancer cells attach to the vascular endothelium via adhesion molecules, invade the tissue via motility factors, grow in the tissue with the help of growth factors, and establish their blood supply by the participation of angiogenic factors. Thus, disruption of a growth factor/receptor axis is a current strategy for the development of anticancer drugs (4).

The vascular endothelial growth factor (VEGF) family includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF; refs. 5, 6). The importance of VEGF and VEGF receptor (VEGFR) expression in tumor angiogenesis and lymphangiogenesis is supported by several lines of evidence. VEGF-A is one of the most potent stimulators of angiogenesis identified thus far, affecting endothelial cell proliferation and motility and vascular permeability (7). VEGF-A exerts its angiogenic functions through activation of the tyrosine kinase receptors VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1/KDR), which are expressed primarily by vascular endothelial cells (8). However, VEGFRs are also expressed by a wide variety of cancer cell lines. VEGF-A and VEGFR-1/2 are coexpressed in a number of cancers, including cancers of the breast (9), prostate (10), colon (11), and pancreas (12, 13), suggesting that VEGF-A may directly influence tumor cell growth via an autocrine mechanism.

VEGF-C induces lymphangiogenesis by activating VEGFR-3, which is expressed by lymphatic endothelial cells (14). We

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Translational Relevance

Many studies in experimental models of cancer have shown that the vascular endothelial growth factor (VEGF)-C/VEGF receptor-3 (VEGFR-3) signaling system is a key regulator of tumor lymphangiogenesis. In the present study, we showed that tumor cells express not only VEGF-C but also VEGFR-3 in human gastric carcinoma. *In vitro* and *in vivo* experiments showed VEGF-C acts as a growth factor for carcinoma cells, in addition to acting as a lymphangiogenic or angiogenic factor. Therefore, VEGF-C may play a role in the progressive growth of human gastric carcinoma through both autocrine and paracrine mechanisms. We propose that interruption of the VEGF-C/VEGFR-3 axis may be a therapeutic approach for controlling disease progression. Reagents that block this pathway could provide benefit for patients with gastric carcinoma in the clinic.

reported previously that expression of VEGF-C correlates with lymph node metastasis in several malignancies, including esophageal (15), gastric (16), and colorectal (17) carcinomas. VEGFR-3 has also been detected on malignant cells, including lung adenocarcinoma (18), head and neck carcinomas (19), prostate carcinoma (20), and leukemia cells (21). These observations suggest that VEGF-C may directly affect cancer cells. Su et al. (18) reported that the VEGF-C/VEGFR-3 axis plays an important role in promoting invasion and metastasis of human lung adenocarcinoma cells. However, unlike the well-characterized axes of VEGF-A and VEGFR-1/2, the biological significance of the activation of the VEGF-C/VEGFR-3 axis in epithelial tumor cells is not well understood. The expression status and significance of VEGFR-3 on gastric carcinoma cells remain unclear. In the present study, to characterize the VEGF-C/VEGFR-3 axis in gastric carcinoma, we examined the expression and function of VEGFR-3 on gastric carcinoma cell lines and tissue specimens.

Materials and Methods

Patients and tumor specimens. Thirty-six patients who underwent surgical resection for gastric carcinoma without preoperative treatment at Hiroshima University Hospital, Hiroshima, Japan, were enrolled in this study. The patient group comprised 32 men and 4 women; median age was 66 y. Informed consent was obtained from all patients for participation in the study. Paraffin-embedded archival specimens from the patients were examined by immunohistochemistry. Pathology reports and clinical histories were reviewed for accurate staging at the time of surgery. Criteria for staging and histologic classification were those proposed by the Japanese Research Society for Gastric Cancer (22). All patients had invasive gastric carcinoma in which the tumor invasion was beyond the submucosa (21 patients stage II; 15 patients stage III).

Cell cultures. Four cell lines established from human gastric carcinomas were maintained in RPMI 1640 (Nissui Co.) with 10% fetal bovine serum (FBS; MA BioProducts). The TMK-1 cell line (a poorly differentiated adenocarcinoma) was provided by Dr. E. Tahara of Hiroshima University. The KKLS cell line (an undifferentiated carcinoma) was provided by Dr. Y. Takahashi of Chiba University, Chiba, Japan. The other two cell lines (MKN-1, from an adenosquamous carcinoma, and MKN-28, from a well-differentiated adenocarcinoma) were obtained from the Health Science Research Resources Bank, Osaka, Japan.

Cell proliferation assays. *In vitro* growth was measured with a Cell Proliferation Biotrak ELISA System, version 2 (Amersham Biosciences), according to the manufacturer's instructions to determine whether recombinant human VEGF-C (rhVEGF-C; R&D Systems) would stimulate proliferation of KKLS cells. Cells were seeded in a 96-well plate at a density of 1×10^4 cells per well and incubated overnight in 200 μL culture medium containing 10% FBS. After incubation for 24 h, cells were cultured in serum-free culture medium containing 10 μmol/L bromodeoxyuridine with or without rhVEGF-C for 24 h, and cell proliferation was measured in a plate reader (Microplate Manager 5.2.1; Bio-Rad) at 450 nm.

Microarray analysis. KKLS cells were cultured in RPMI 1640 without FBS for 6 h and then cultured with or without rhVEGF-C (20 ng/mL) for 8 h. These cells were collected and stored at -80°C until use. Microarray analysis was done with the Human Cancer CHIP (version 4, Takara Shuzo), which contains 637 human cancer-related genes (listed on the home page of Takara Shuzo[5]) spotted on glass plates. A fluorescent probe synthesized by reverse transcription of 1 μg of the above mRNA with 50 U AMV reverse transcriptase (Takara Shuzo) was added to each reaction mixture. Cy3- and Cy5-labeled probes were prepared from mRNAs isolated from control cells and rhVEGF-C-treated cells, respectively; both were mixed in the reaction buffer [6× SSC, 0.2% SDS, 5× Denhardt's solution, 0.8 mg/mL poly(dA), and 1 mg/mL yeast tRNA]. The mixture was hybridized to the cDNA CHIP at 65°C overnight. The CHIP was washed twice with 2× SSC/0.2% SDS solution at 55°C for 30 min and then with the same solution at 65°C for 5 min. Finally, the CHIP was washed with 0.05× SSC at room temperature for 10 min. Signals on the hybridized CHIP were visualized and quantified with the Scan-Array 5000 (GSI Lomonids) and normalized to the averaged signals of housekeeping genes. Genes were excluded from further investigation when the intensities of both Cy3 and Cy5 were below 1,000 fluorescence units. Those with Cy3/Cy5 signal ratios >2.0 were regarded as up-regulated.

Semiquantitative and quantitative reverse transcription-PCR. Total RNA was extracted from gastric carcinoma cell lines with an RNeasy Kit (Qiagen) according to the manufacturer's instructions. cDNA was synthesized from 1 μg total RNA with a first-strand cDNA synthesis kit (Amersham Biosciences). After reverse transcription of RNA into cDNA, quantitative reverse transcription-PCR (RT-PCR) was done with a LightCycler-FastStart DNA Master SYBR-Green I Kit (Roche), and semiquantitative RT-PCR was done with an AmpliTaq Gold Kit (Roche) according to the manufacturer's recommendations. Quantitative RT-PCR was used to monitor gene expression and was done with a LightCycler system and LightCycler Data Analysis Software ver. 3.5 (Roche) in accordance with standard procedures. PCR reactions were carried out in triplicates. To correct for differences in both RNA quality and quantity between samples, the data were normalized to those of *β-actin*. Primers for PCR were designed with specific primer analysis software (Primer Designer, Scientific and Educational Software), and the specificity of the sequences was confirmed by FASTA (EMBL Database). Primer sequences, annealing temperatures (Ta), and PCR cycles were as follows: VEGF-C forward, GAGGAGCAGTTACGGTCTGT and VEGF-C reverse, GTAGCTCGTGCTGGTGTTCA (*VEGF-C* PCR product, 371 bp; Ta, 59°C; 28 cycles); VEGFR-3 forward, GGTTCCTCCAGGATGAAGAC and VEGFR-3 reverse, CAAGCAGTAACGCCAGTGTC (*VEGFR-3* PCR product, 505 bp; Ta, 62°C; 28 cycles); AMFR forward, ACTCTCCTGTCCCTGGACCT and FLT-4 reverse, TCATTGTTGACAGCCAGCTC (*AMFR* PCR product, 218 bp; Ta, 59°C; 35 cycles); AMF forward, CGCCCAACCAACTCTATTGT and AMF reverse, GGTAGAAGCGTCGTGAGAGG (*AMF* PCR product, 213 bp; Ta, 59°C; 40 cycles); PlGF forward, ATGTTCAGCCCATCCTGTGT and

[5] http://bio.takara.co.jp/catalog/DNAChip.Down-load.htm

PlGF reverse, CTTCATCTTCTCCCGCAGAG (*PlGF* PCR product, 201 bp; Ta, 59°C; 40 cycles); GAPDH forward, ATCATCCCTGCCTCTACTGG and GAPDH reverse, CCCTCCGACGCCTGCTTCAC (*GAPDH* PCR product, 188 bp; Ta, 55°C; 28 cycles); and β-actin forward, GGACTTCGAGCAAGAGATGG and β-actin reverse, AGCACTGTGTTGGCGTACAG (*β-actin* PCR product, 234 bp; Ta, 55°C;35 cycles). After amplification, PCR products were resolved on 5% nondenaturing polyacrylamide gels in Tris-borate-EDTA buffer. RT-PCR in the absence of reverse transcriptase showed no specific bands.

Immunohistochemical staining. Immunohistochemistry for VEGF-C, VEGFR-3, Lyve1, and Ki-67 was done on formalin-fixed, paraffin-embedded tissues cut into serial 4-μm sections. After deparaffinization and rehydration, tissue sections for staining of VEGF-C and VEGFR-3 were pretreated by microwave twice for 5 min in Dako REAL Target Retrieval Solution (Dako). Immunohistochemical staining of CD31 was done on fresh-frozen specimens cut into 8-μm sections, mounted on positively charged slides and stored at -80°C. Frozen tissue sections were fixed in cold acetone for 10 min. VEGF-C was detected with a goat antihuman polyclonal antibody (1:200; R&D Systems), and VEGFR-3 was detected with a goat antihuman polyclonal antibody (1:50; R&D Systems). Lyve1 was detected with the rat antimouse monoclonal antibody (1:20; R&D Systems), and Ki-67 was detected with the mouse antihuman polyclonal antibody (MIB-1; 1:25, Dako). CD31 was detected with the rat antimouse polyclonal antibody (1:200; Pharmingen). The primary antibodies were applied to the slides and incubated overnight in humidified boxes at 4°C. After incubation for 1 h at room temperature with peroxidase-conjugated corresponding secondary antibodies, a positive reaction was detected by exposure for 5 to 10 min to stable 3,3'-diaminobenzidine. Slides were counterstained with hematoxylin for visualization of the nucleus.

Gene transfection and cloning of transfected cell lines. A full-length *VEGF-C* cDNA (a 1.9-kbp *Eco*RI-*Eco*RI fragment) was inserted into the *Eco*RI-*Eco*RI site of pBR322 (Invitrogen). The resultant plasmid was digested with *Xho*I-*Bam*HI and cloned into the *Xho*I-*Bam*HI site of the pEGFP-N1 expression vector (BD Biosciences) to yield the *VEGF-C* expression vector. Expression of *VEGF-C* cDNA was under the control of the cytomegalovirus promoter. The KKLS cell line was transfected with either the *VEGF-C* expression vector or the pEGFP-N1 vector alone with Lipofectin (Life Technologies) according to the manufacturer's instructions. After transfection, cells were grown in selective medium (10% FBS-RPMI 1640 containing 800 μg/mL G418). The selective medium was changed every 3 d. G418-resistant clones (KKLS/VEGF-C and KKLS/EGFP) were selected and then expanded for additional studies. G418 was from Sigma.

ELISA for VEGF-C protein. For generation of conditioned media, tumor cells were plated at 1.0×10^5/mL per 10-cm dish (Becton Dickinson Labware), and the supernatants were collected after 48 h, centrifuged to remove floating cells, and stored at -80°C. We used the Quantikine Human VEGF-C Immunoassay (R&D Systems) according to the manufacturer's instructions to measure VEGF-C levels.

Western blot analysis. Expression of VEGF-C, VEGFR-3, and cyclin D1 was evaluated by Western blot analysis. The phosphorylation status of VEGFR-3 or Akt was also evaluated by Western blot analysis. To evaluate VEGFR-3 or Akt phosphorylation of KKLS cells stimulated with VEGF-C, cells were seeded in 10-cm dishes (1.5×10^5 cells per dish) in growth medium and allowed to attach overnight before the growth medium was replaced with serum-free medium for an additional 24-h incubation. The cells were then stimulated with or without VEGF-C (20 ng/mL) for 5 or 10 min at 37°C. To evaluate VEGFR-3 or Akt phosphorylation in KKLS cells transfected with expression vector, cells

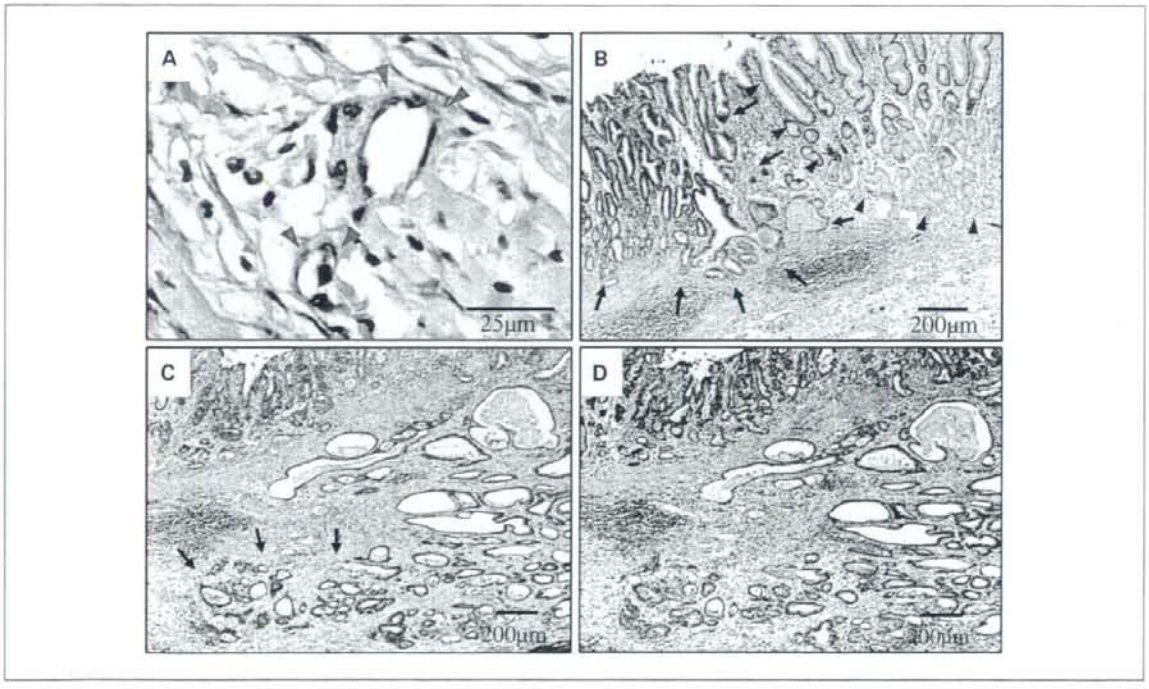

Fig. 1. Expression of VEGFR-3 and VEGF-C in human gastric carcinoma tissues. Immunoreactivity of VEGFR-3 was detected on tumor cells in 17 of 36 (47.2%) gastric carcinoma specimens. *A*, staining of VEGFR-3 on lymphatic vessels (*red arrowhead*). *B*, homogeneous staining of VEGFR-3 (*black arrow*) was observed in 7 of 17 specimens. Normal mucosa did not show VEGFR-3 staining (*black arrowhead*). *C*, in 10 of 17 specimens, VEGFR-3 expression was heterogeneous. Immunoreactivity for VEGFR-3 was more intense at the deepest invasive site (*black arrow*) than at the central portion or superficial part. *D*, VEGF-C immunoreactivity was detected on tumor cells.

were seeded in 10-cm dishes (1.5×10^5 cells per dish) in growth medium and allowed to attach overnight before the growth medium was replaced by serum-free medium for an additional 24-h incubation. After three washes with cold PBS containing 1 mmol/L sodium orthovanadate, cells were lysed. Protein samples of the cell lysates (total protein 20 μg) or 1 μL of culture medium were separated by SDS-PAGE and transferred to nitrocellulose transfer membranes (Whatman GmbH). After being blocked with 1% or 3% skim milk in TBS, the membranes were incubated with primary antibodies, namely, polyclonal goat antihuman VEGF-C antibody, polyclonal goat antihuman VEGFR-3 antibody, polyclonal mouse antihuman cyclin D1 (Dako), polyclonal rabbit antihuman phopho-VEGFR-3 antibody (Calbiochem), polyclonal rabbit antibody to phospho-Akt (phosphorylated at Ser473, Cell Signaling Technology), or β-actin (Sigma), and were diluted in TBS at 4°C overnight. The membranes were then washed in TBS-T (0.1% Tween 20 in TBS) and incubated with the secondary antibodies specific for each primary antibody at room temperature for 1 h. To confirm equivalent protein loading, membranes were stripped and reprobed with anti-Akt antibody (Cell Signaling Technology). The immune complexes were visualized with enhanced chemiluminescence with an ECL Plus Kit (Amersham Life Science).

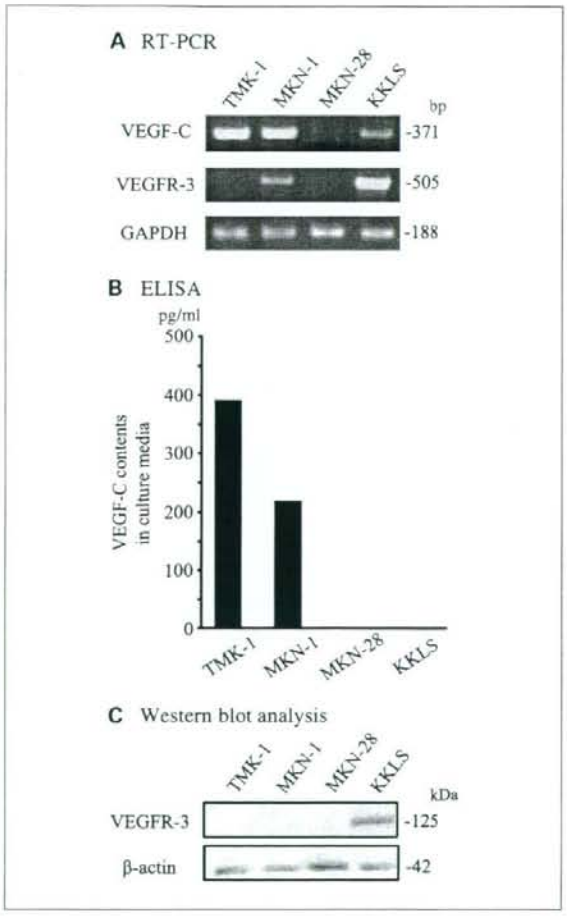

Fig. 2. Expression of VEGF-C and VEGFR-3 in gastric carcinoma cell lines. *A*, *VEGF-C* and *VEGFR-3* expression by gastric cell lines was examined by RT-PCR. *B*, culture supernatants were assayed for VEGF-C by ELISA. *C*, VEGFR-3 protein expression was analyzed by Western blotting.

Immunofluorescence staining for pVEGFR-3. To confirm the activation of VEGFR-3 by VEGF-C, KKLS cells were cultured in RPMI 1640 without FBS for 24 h and then stimulated with or without rhVEGF-C (20 ng/mL) for 10 min. Tumor cells were stained with pVEGFR-2, 3 (1:1,000; Calbiochem).

Animal models. Male athymic BALB/c nude mice were obtained from Charles River Japan. The mice were maintained under specific pathogen-free conditions and used at 5 wk of age. This study was carried out after permission was granted by the Committee on Animal Experimentation of Hiroshima University.

Orthotopic (gastric mucosa) xenograft model. For implantation, subconfluent KKLS/VEGF-C and KKLS/EGFP cells were harvested by brief treatment with 0.25% trypsin and 0.02% ethylenediamine tetraacetic acid, and resuspended to a final concentration of 2.0×10^7 cells/mL Hanks' solution. Using a 30-gauge needle attached to a 1-mL syringe, cells (1.0×10^6/0.05 mL) were implanted into the gastric walls of nude mice under observation with a zoom stereomicroscope. After 4 wk, the mice were sacrificed, and the tumors were resected for study. The tumors were fixed in 10% buffered formalin or formalin-free IHC Zinc Fixative (Pharmingen) for immunohistochemistry.

Quantitation of lymphatic vessel density, microvessel density, and Ki-67 labeling index. Lymphatic vessel density and microvessel density were determined from counts of Lyve1-positive vessels and CD31-positive vessels, respectively. Vessel counts were assessed by light microscopy in immunohistochemistry-stained areas of the intratumoral and peritumoral regions containing the highest numbers of capillaries and small venules (23). Highly vascularized areas were first identified by scanning tumor sections at low power (×40 and ×100). The vessel count was determined for six such areas at ×400 (×40 objective and ×10 ocular), and the mean of the six counts was calculated. A vessel lumen was not necessary for a structure to be defined as a blood microvessel (23). In slides immunolabeled for Lyve1, only vessels with typical morphology (including a lumen) were counted as lymphatic vessels because of occasional weak antibody cross-reactivity with fibroblasts (24). The Ki-67 labeling index was determined by light microscopy at the site of the greatest number of Ki-67–positive cells. The sites were identified by scanning tumor sections at low power (×40). For the Ki-67 labeling index, the number of positive cells among ~1,000 tumor cells was calculated as a percentage. Staining of cells was evaluated by two independent observers (M.K. and Y.K.) blinded to the patient's status.

Statistical analysis. Results are expressed as mean ± SE. Fischer's exact test or χ^2 test was used for the analysis of categorical data. Wilcoxon/Kruskal-Wallis analysis was used for comparison of continuous variables. A P value of <0.05 was considered statistically significant.

Results

Immunolocalization of VEGFR-3 in human gastric carcinoma tissues. We analyzed 36 human gastric cancer specimens with a polyclonal antibody specific for human VEGFR-3. We detected VEGFR-3-specific immunoreactivity on lymphatic endothelial cells (Fig. 1A). In 17 of 36 (47.2%) gastric carcinoma specimens, VEGFR-3-specific immunoreactivity was detected on tumor cells (Fig. 1B and C). Immunoreactivity for VEGFR-3 was more intense at the site of deepest invasion than at the central portion or superficial part of the tumor (Fig. 1C). In 12 of 36 (33.3%) gastric carcinomas, tumor cells expressed both VEGF-C and VEGFR-3 (Fig. 1C and D). VEGFR-3 immunoreactivity was not detected in normal gastric epithelial cells (Fig. 1B).

Expression of VEGF-C and VEGFR-3 in gastric carcinoma cell lines. We next analyzed VEGF-C and VEGFR-3 expression in gastric carcinoma cell lines. Gastric carcinoma cell lines constitutively expressed *VEGF-C* mRNA at various levels. Two

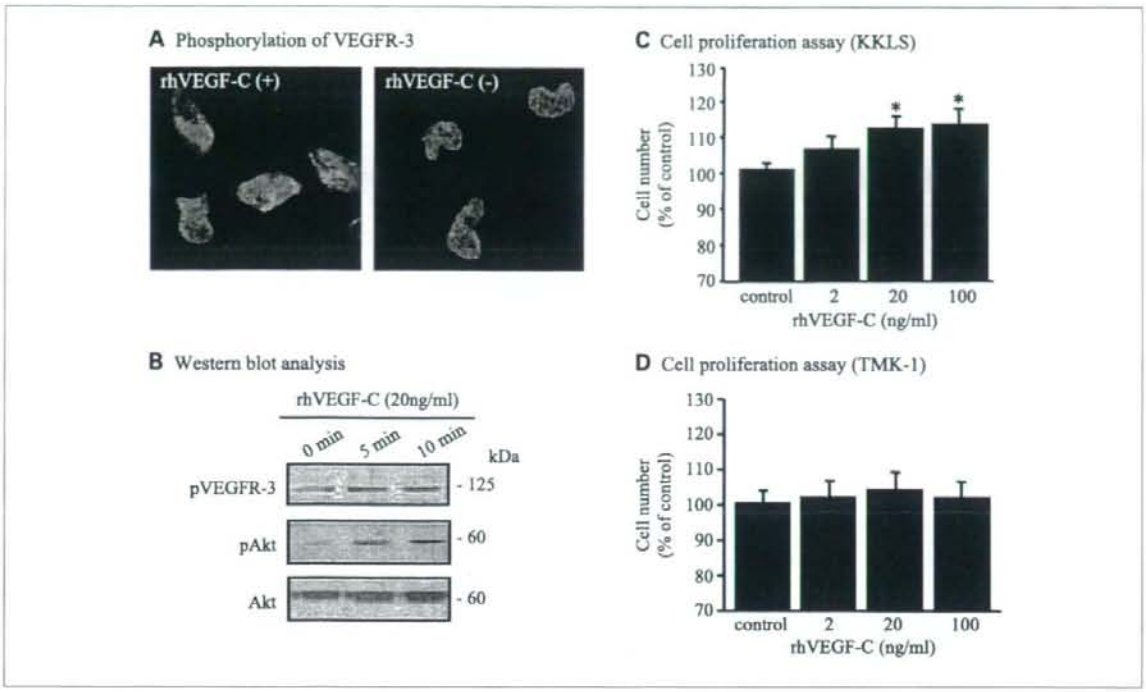

Fig. 3. Effect of VEGF-C on KKLS cells. *A*, VEGF-C-dependent phosphorylation of VEGFR-3. KKLS cells were cultured in medium without FBS for 24 h and then stimulated with or without rhVEGF-C (20 ng/mL) for 10 min. Tumor cells were stained with pVEGFR-2, 3 (*red*). *B*, VEGF-C increases Ser473 phosphorylation of Akt in KKLS cells. KKLS cells were maintained in serum-free medium for 24 h and then treated with 20 ng/mL VEGF-C for the indicated times. Equal amounts of protein (20 μg/lane) were then subjected to Western blot analysis with phospho-specific Akt antibody. Reprobing of the blot with anti-Akt antibody confirmed the equivalent loading of lanes. KKLS (*C*) or TMK-1 (*D*) cells were incubated with rhVEGF-C (2, 20, or 100 ng/mL) for 24 h, and cell proliferation was measured with a cell proliferation ELISA system. Asterisks denote a statistically significant difference compared with control values. * $P < 0.05$; bars, SE.

of the four gastric carcinoma cell lines (KKLS and MKN-1) expressed *VEGFR-3* mRNA. Of these two cell lines, the KKLS cell line overexpressed *VEGFR-3* mRNA (Fig. 2A). Expression of VEGF-C and VEGFR-3 by gastric carcinoma cells was confirmed at the protein level. Culture supernatants were assayed for VEGF-C by ELISA (Fig. 2B). VEGFR-3 protein expression was analyzed by Western blotting (Fig. 2C). KKLS cells expressed high levels of VEGFR-3 protein, but the amount of VEGF-C protein was below the limit of detection, which was not consistent with the level of mRNA. For this reason (high endogenous VEGFR-3 and low endogenous VEGF-C in KKLS cells), we used KKLS cells for further studies.

VEGF-C stimulates proliferation of KKLS cells. To investigate the possibility of autocrine tumor cell growth stimulation by VEGF-C, we treated KKLS cells with rhVEGF-C and analyzed phosphorylation of VEGFR-3, Akt, and mitogen-activated protein kinase in KKLS cells and the effect on cell proliferation. VEGF-C treatment induced phosphorylation of VEGFR-3 and Akt in KKLS cells (Fig. 3A and B) and increased cell proliferation of KKLS cells in a dose-dependent manner (Fig. 3C). Phosphorylation of mitogen-activated protein kinase was not detected (data not shown). In contrast, VEGF-C had no effect on cell proliferation of TMK-1 cells (VEGFR-3 negative cell line; Fig. 3D).

VEGF-C up-regulates expression of genes associated with disease progression in KKLS cells. To study the downstream effector genes of VEGF-C/VEGFR-3 signaling, we did microarray analysis using the Human Cancer CHIP (Takara Shuzo). Expression of various mRNAs in untreated KKLS cells was compared with expression in KKLS cells treated with rhVEGF-C for 8 hours. Under the highly stringent conditions we used, 52 genes were classified as genes showing significantly increased expression in response to VEGF-C. Among these genes, we confirmed the increased expression of cyclin D, PlGF, and autocrine motility factor receptor (AMFR) by quantitative RT-PCR or Western blotting. Treatment with VEGF-C increased expression of *AMFR* mRNA in a dose-dependent and time-dependent manner (Fig. 4A). We also investigated expression of the mRNA encoding AMF, the ligand of AMFR. Treatment with VEGF-C also increased expression of *AMF* mRNA (Fig. 4B). Levels of *PlGF* mRNA were similarly increased in VEGF-C-treated KKLS cells (Fig. 4C). In addition, VEGF-C increased expression of cyclin D1 protein in a dose-dependent manner (Fig. 4D).

Transfection of the VEGF-C gene into KKLS cells. To stimulate autocrine VEGF-C/VEGFR-3 signaling, a *VEGF-C* expression vector was transfected into KKLS cells. After transfection with the VEGF-C expression vector or the control vector (pEGFP-N1), we selected a stable clone (KKLS/VEGF-C) that overexpressed *VEGF-C* and a control clone (KKLS/EGFP) for subsequent assays. Overexpression of *VEGF-C* mRNA and VEGF-C protein was confirmed by quantitative RT-PCR (Fig. 5A) and Western blotting,

respectively (Fig. 5B). Phosphorylation of Akt in KKLS/VEGF-C cells was also confirmed by Western blotting (Fig. 5B).

In vitro *and* in vivo *proliferation of VEGF-C-transfected gastric carcinoma cells.* Under culture conditions of 0.5% FBS, cell proliferation was stimulated by transfection with *VEGF-C* expression vector (Fig. 5C). To investigate the role of the VEGF-C/VEGFR-3 axis in an animal model, we implanted KKLS/VEGF-C and control cells into the gastric walls of nude mice. At the end of the 4-week experimental period, *in vivo* growth of KKLS/VEGF-C cells was significantly greater than that of control cells (Fig. 5D).

We next used immunohistochemistry for Lyve1, CD31, and Ki-67 to investigate lymphatic vessel density, microvessel density, and Ki-67 labeling index. Greater lymphangiogenesis (Fig. 6A) and angiogenesis (Fig. 6B) were observed in mice implanted with VEGF-C-transfected KKLS cells than in those implanted with control KKLS cells. Statistical analysis showed that the number of Ki-67–positive cells, as well as lymphatic vessel density and microvessel density, was significantly higher in the KKLS/VEGF-C tumors than in the control tumors ($P < 0.01$; Fig. 6C).

Discussion

Until recently, studies of VEGF family members have focused primarily on their functions as paracrine stimulators of angiogenesis or lymphangiogenesis. Promotion of tumor metastasis by VEGF-C is reported to be due to the induction of tumor lymphangiogenesis via effects of activated VEGFR-3 on lymphatic endothelial cells (25). Association of VEGF-C with tumor lymphangiogenesis and with lymph node metastasis has been observed in many human carcinomas, including thyroid, prostate, esophageal, gastric, colorectal, and lung cancers (15, 16, 26, 27). Furthermore, several studies have shown that overexpression of VEGF-C induces lymphangiogenesis and promotes tumor metastasis in mouse tumor models (28, 29).

After the discovery of VEGFRs on malignant cells, it was reported that VEGF-A can act as an autocrine growth factor for various types of cancer cells (13, 30, 31). Unlike the well-characterized VEGF-A/VEGFR-2 axis, there may be many undefined functions and molecular mechanisms involved in

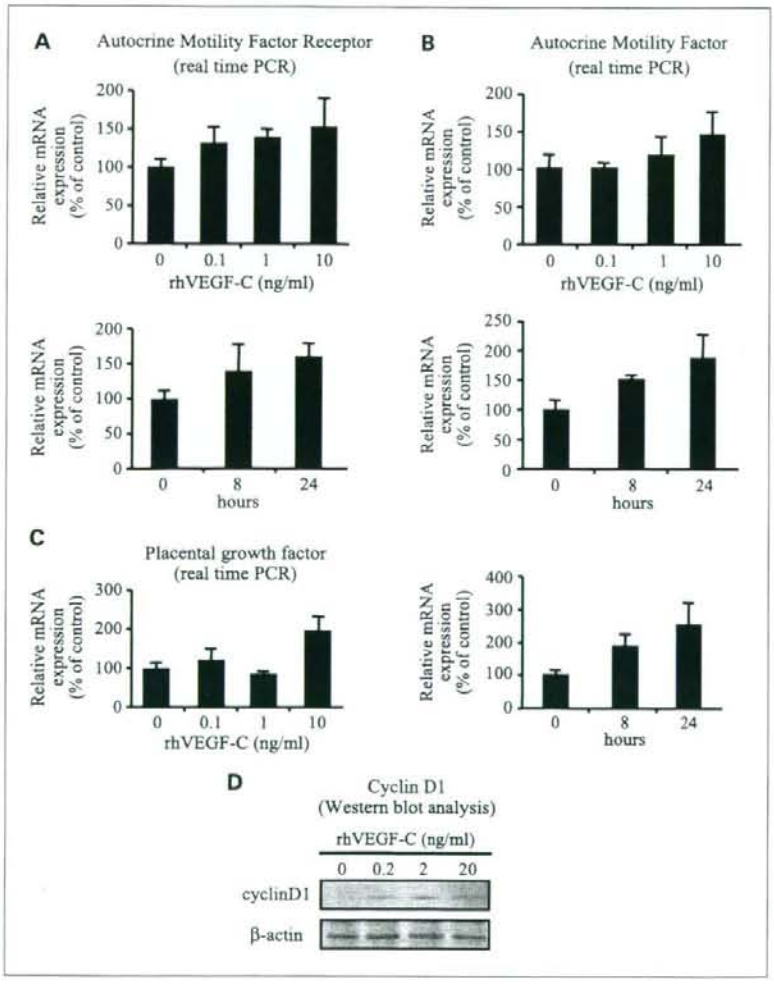

Fig. 4. KKLS cells were treated with various concentrations of rhVEGF-C for 8 h, and the expression of *AMFR* (*A*), *AMF* (*B*), and *PlGF* (*C*) mRNA was determined by RT-PCR. Expression of cyclin D1 protein was examined by Western blot analysis (*D*). PCR reactions were carried out in triplicates. Data for RT-PCR were normalized to those of *β-actin*. Bars, SE.

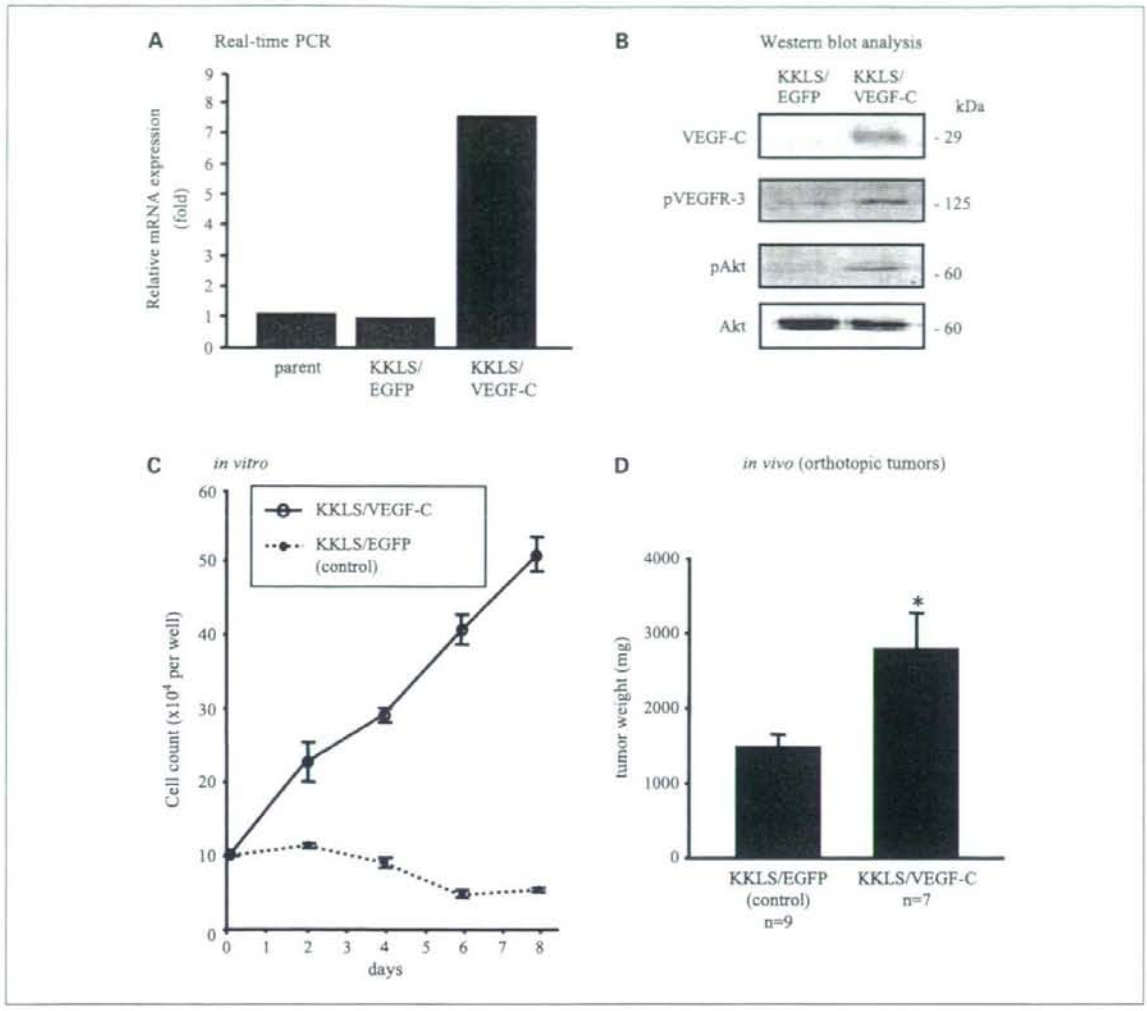

Fig. 5. Establishment of a clonal cell line overexpressing VEGF-C, and *in vitro* and *in vivo* growth of VEGF-C-transfected gastric carcinoma cells. *A*, expression of *VEGF-C* mRNA was examined by RT-PCR. *B*, Western blot analyses of VEGF-C in culture medium of transfected cells and phosphorylation of VEGFR-3 and Akt in lysates of transfected cells. *C*, cells (1×10^5) were seeded in 24-well plates and cultured in medium containing 0.5% FBS. Cell number was determined in triplicate cultures. *D*, orthotopic (gastric mucosa) xenograft model. Tumor weights at 4 wk after implantation of a VEGF-C overexpressing clone (KKLS/VEGF-C) or cells transfected with the corresponding vector control (KKLS/EGFP). * $P < 0.05$; bars, SE.

tumor progression mediated by the VEGF-C/VEGFR-3 axis. Results regarding the expression of VEGFR-3 on tumor cells are controversial. Some studies did not detect expression of VEGFR-3 on tumor cells (32–34), whereas other studies found expression of VEGFR-3 on tumor cells (18–21, 35–37). These contradictory findings suggest that expression of VEGFR-3 on cancer cells may differ between malignancies or cell lines.

Expression of VEGF-C and VEGFR-3 correlates significantly with poor prognosis of specific types of cancer (18, 19, 21, 37–41). Marchio et al. (40) reported that tyrosine phosphorylation of VEGFR-3 is increased in Kaposi sarcoma cells treated with recombinant VEGF-C protein, and they found that activation of the VEGF-C/VEGFR-3 axis in Kaposi sarcoma cells is involved in the regulation of cellular functions, such as proliferation and migration. The VEGF-C/VEGFR-3 axis has also been found to play a role in the growth of malignant mesothelioma cells (41) and leukemic cells (21). It has become clear that the VEGF-C/VEGFR-3 axis plays an important role in promoting invasion and metastasis of human lung adenocarcinoma cells (18). It has also been reported, however, that inhibition of VEGFR-3 signaling by the soluble fusion protein VEGFR-3-immunoglobulin did not change the growth of lung cancer cells (42). These findings indicate that the effects and interactions of the VEGF-C/VEGFR-3 system in cancer biology are complex and may differ between malignancies. Previously, we reported that VEGF-C immunoreactivity is associated not only with lymphatic invasion and lymph node metastases but also with greater depth of tumor invasion in gastric cancer (16).

This fact suggests that VEGF-C may directly influence tumor cell growth or motility via an autocrine mechanism. Therefore, we conducted the present study to clarify whether gastric cancer cells express functional VEGFR-3 and to evaluate the biological significance of VEGFR-3 expression in gastric cancer progression.

In the present study, we found that VEGFR-3 is expressed by tumor cells as well as lymphatic endothelial cells in gastric carcinoma tissues. Approximately half of our gastric cancers (17 of 36) contained tumor cells that expressed VEGFR-3 protein. On the basis of the lack of VEGF-C and VEGFR-3 expression in normal gastric epithelial cells, we concluded that *de novo* expression of VEGF-C and VEGFR-3 seems to be associated with the process of malignant transformation. Treatment of cultured KKLS cells with VEGF-C induced tyrosine phosphorylation of VEGFR-3 and then increased proliferation. It also induced expression of cyclin D1, PlGF, AMF, and AMFR. PlGF and AMF are growth factors known to regulate angiogenesis and tumor cell motility, respectively. We previously reported that VEGF-C expression is higher at the site of deepest

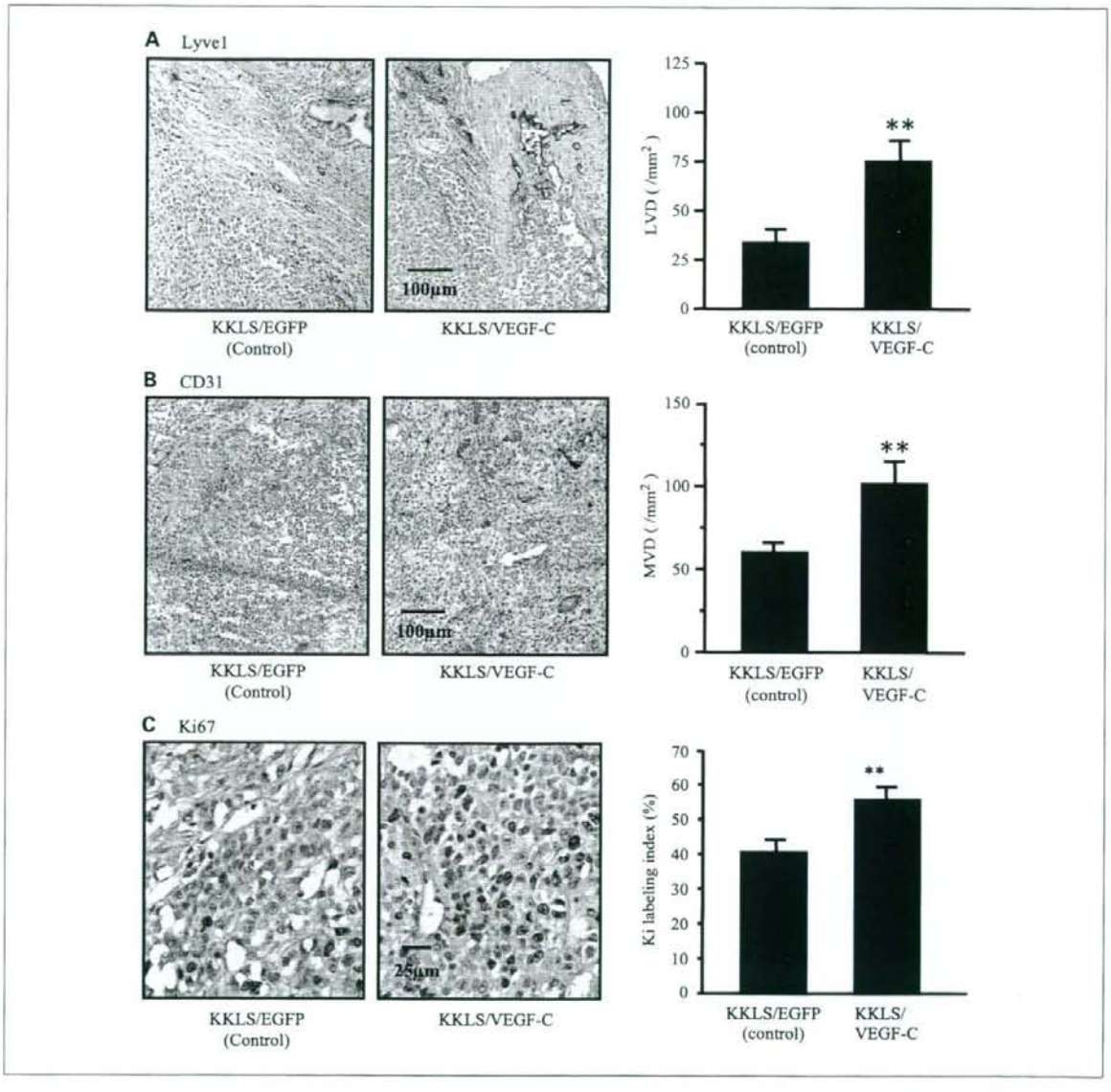

Fig. 6. Immunohistochemistry for Lyve1 (*A*), CD31 (*B*), and Ki-67 (*C*) in KKLS cells growing in the stomach of nude mice. VEGF-C-transfected KKLS tumors showed increased density of Lyve-1– and CD31-positive vessels and higher numbers of Ki-67–positive cells than control tumors. Right panels, quantification of Lyve-1–positive vessels, CD31-positive vessels, and Ki-67–positive cells in these tumors. ** $P < 0.01$; bars, SE.

penetration of the invasive tumor than in the superficial portions (16). In the present study, heterogeneous intratumoral staining was observed for VEGFR-3 and VEGF-C, with the highest levels of expression at the invasive edges. Expression of PlGF and AMF/AMFR induced by VEGF-C/VEGFR-3 signaling may play a role in progression of gastric cancer cells to an aggressive phenotype.

Makinen et al. proved the critical role of VEGF-C/VEGFR-3 signaling in the growth and survival of lymphatic endothelial cells (43). They found VEGFR-3 induces a protein kinase C–dependent p42/p44 mitogen-activated protein kinase activation and wortmannin-sensitive phosphorylation of Akt. However, the biochemical signaling pathways activated via VEGFR-3 are unknown in tumor cells. In the present study, we found treatment with VEGF-C resulted in phosphorylation of Akt but not mitogen-activated protein kinase in KKLS cells. Further studies using Akt inhibitor will be needed to clarify whether Akt indeed plays a role as a signaling molecule of the VEGF-C/VEGFR-3 axis.

To stimulate VEGF-C/VEGFR-3 signaling in an autocrine manner, we transfected a VEGF-C expression vector into KKLS cells, established stable transfectants, and transplanted VEGF-C–transfected cells and control cells into the gastric walls of nude mice (orthotopic site). Lyve1-positive vessel-like structures were found at a much higher density in KKLS/VEGF-C tumors than in control tumors. These findings are consistent with the published direct evidence for the role of VEGF-C in tumor lymphangiogenesis (28, 29). However, VEGF-C secreted by the tumor did not promote lymphatic metastasis in our present experiments. Lymph node metastasis was not observed in the KKLS/VEGF-C or the control mice. For lymphatic metastasis, tumor cells must complete multiple steps, which include lymphangiogenesis, motility, invasion, survival in the circulation, adhesion, extravasation, and proliferation (2, 3). He et al. (42) reported that tumors of lung carcinoma cells overexpressing VEGF-C contain more lymphatic vessels than vector-transfected tumors but do not have increased metastatic ability. Therefore, lymphangiogenesis induced by VEGF-C may not be the only metastasis rate-limiting factor. To survive in the lymph circulation and colonize lymph nodes, another growth factor, such as VEGF-A (44) or platelet-derived growth factor-BB (45), may be needed.

In addition to lymphangiogenesis, we observed greatly accelerated angiogenesis and *in vivo* growth of KKLS/VEGF-C cells compared with that of control cells in the present study. VEGF-C and VEGF-D can exert angiogenic activity through VEGFR-2 (46), and previous data clearly link VEGFR-2 to angiogenesis and progression of gastric cancer (47). On the other hand, Mandriota et al. reported that VEGF-C induces lymphangiogenesis, but not angiogenesis in double-transgenic mice (48). Although there is no explanation for the discrepancy, this might be due to differences in VEGF-C proteolytic processing in the different models. The stepwise proteolytic processing of VEGF-C generated several VEGF-C forms with increased activity towards VEGFR-3, but only the fully processed VEGF-C could activate VEGFR-2 (49). VEGFR-3 has been detected in both blood vessels and lymphatic vessels in tumor tissues (27, 50, 51). There are several studies that showed that antibody interference with VEGFR-3 function can inhibit tumor growth by inhibiting neoangiogenesis in various human tumor xenografts in immunocompromised mice (34, 52). We found in the present study that VEGF-C induces the expression of PlGF by KKLS cells. Expression of PlGF by KKLS cells may also play a role in tumor angiogenesis.

Beside roles in lymphangiogenesis and angiogenesis, we identified an additional role of VEGF-C/VEGFR-3 in tumor growth. Treatment with VEGF-C increased expression of cyclin D1 (a cell cycle regulator) and stimulated growth of KKLS cells *in vitro*, and we found a higher number of Ki-67–positive cells in KKLS/VEGF-C tumors than in vector control tumors. He et al. (53) reported that treatment of ectopic xenografts of lung carcinoma cells with VEGFR-3-immunoglobulin inhibits tumor growth without a reduction in microvessel density. These findings support the existence of autocrine stimulation of tumor growth by the VEGF-C/VEGFR-3 axis.

In summary, our results show that VEGF-C is an important growth factor, in addition to acting as a lymphangiogenic or angiogenic factor. Thus, we propose that interruption of the VEGF-C/VEGFR-3 axis may be a therapeutic approach for controlling disease progression.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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Gene expression profiling with microarray and SAGE identifies PLUNC as a marker for hepatoid adenocarcinoma of the stomach

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Gastric cancer is one of the most common malignancies worldwide. In this study, we screened for genes upregulated in gastric cancer by comparing gene expression profiles from serial analysis of gene expression and microarray and identified the palate, lung, and nasal epithelium carcinoma-associated protein (*PLUNC*) gene. Immunostaining for PLUNC in 140 gastric cancer cases revealed strong and extensive staining of PLUNC in hepatoid adenocarcinoma of the stomach, whereas 7% of conventional gastric cancer cases showed focal immunostaining of PLUNC. Gastric hepatoid adenocarcinoma is an extrahepatic tumor characterized by morphologic similarities to hepatocellular carcinoma. To investigate the utility of PLUNC immunostaining in the diagnosis of gastric hepatoid adenocarcinoma, six cases of gastric hepatoid adenocarcinoma (six primary tumors and two associated liver metastases) were studied further. PLUNC staining was observed in all six primary hepatoid adenocarcinomas. PLUNC staining was observed in both the hepatoid adenocarcinoma and tubular/papillary adenocarcinoma components of primary tumors, although PLUNC staining was preferentially localized in tubular/papillary adenocarcinoma components. Staining of PLUNC was also detected in both liver metastases. PLUNC staining was not observed in 52 cases of primary hepatocellular carcinoma or in normal adult or fetal liver. These results indicate that PLUNC is a novel marker that distinguishes gastric hepatoid adenocarcinoma from primary hepatocellular carcinoma.
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Keywords: hepatoid adenocarcinoma; hepatocellular carcinoma; PLUNC; microarray; SAGE; gastric cancer

According to the World Health Organization, gastric cancer is the fourth most common malignancy worldwide, with approximately 870 000 new cases occurring every year. Mortality due to gastric cancer is second only to that due to lung cancer.[1] Cancer develops as a result of multiple genetic and epigenetic alterations.[2–4] Better knowledge of the changes in gene expression that occur during gastric carcinogenesis may lead to improvements in diagnosis, treatment, and prevention. Identification of novel biomarkers for cancer diagnosis and novel targets for treatment are the major goals in this field.[5] To identify potential molecular markers for cancer and to better understand the development of cancer at the molecular level, comprehensive gene expression analysis may be useful. Among the comprehensive methods used to analyze transcript expression levels, array-based hybridization[6] and serial analysis of gene expression (SAGE)[7] are currently the most common approaches.

We previously performed SAGE of four primary gastric cancers.[8] From the SAGE data, we identified several gastric cancer-associated genes;[9] however, these alterations cannot completely explain the pathogenesis of gastric cancer. In addition, although gene expression profiles from SAGE and microarray have better correlations for genes with high-fold changes, the gene expression profiles from these methods show relatively poor correlations among genes with low-fold changes, suggesting that SAGE data may not yield a comprehensive gene expression profile.[10] In our previous study, the invasion/metastasis-associated genes identified by SAGE

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were quite different from those identified by microarray.[11] Therefore, we performed gene expression profiling using Affymetrix GeneChip Human Genome U133Plus 2.0 arrays of one gastric cancer sample previously analyzed by SAGE and identified several candidate gastric cancer-associated genes. Among these candidate genes, the palate, lung, and nasal epithelium carcinoma-associated protein (*PLUNC*, also known as lung-specific X protein, *LUNX*) gene is upregulated in human gastric cancer. However, little is known about the relation of PLUNC to human gastric cancer.

PLUNC was originally identified in the nasal epithelium of mouse embryo and the trachea and bronchi of adult mouse lung.[12] The human *PLUNC* gene shows a similar expression pattern, including localization to the tracheal epithelium.[13] *PLUNC* was also identified as a marker of non-small-cell lung carcinoma.[14] Immunohistochemical analysis of PLUNC in lung cancer revealed that PLUNC is commonly expressed in adenocarcinoma, mucoepidermoid carcinoma, and bronchoalveolar carcinoma and is absent from small cell carcinoma and squamous cell carcinoma.[15] Although PLUNC is a major secreted protein product in the upper respiratory tract,[15] the biologic function of PLUNC is poorly understood.

In this study, we examined the expression and distribution of PLUNC in human gastric cancer by immunohistochemistry. The relation between staining for PLUNC and clinicopathologic characteristics was examined. In addition, because we observed frequent immunostaining of PLUNC in hepatoid adenocarcinoma of the stomach, we also performed PLUNC immunostaining of primary hepatocellular carcinoma to investigate the potential utility of PLUNC immunostaining in the diagnosis of gastric hepatoid adenocarcinoma.

Materials and methods

Tissue Samples

In total, specimens from 144 cases of primary gastric cancer and 52 cases of primary hepatocellular carcinoma were collected. Patients were treated at the Hiroshima University Hospital. The histologic classification was based on the World Health Organization system. Tumor staging was according to the TNM classification system.[16] Because written informed consent was not obtained, for strict privacy protection, identifying information for all samples was removed before analysis. This procedure was in accordance with the Ethical Guidelines for Human Genome/Gene Research of the Japanese Government. For microarray analysis, one primary gastric cancer sample (Case P208T, 60-year-old man, T4N3M0, stage IV, poorly differentiated adenocarcinoma) and corresponding non-neoplastic mucosa were used. This gastric cancer sample was analyzed previously by SAGE for comprehensive gene expression profiling.[6] For quantitative reverse transcription (RT)-polymerase chain reaction (PCR) analysis, four gastric cancer samples and corresponding non-neoplastic mucosa samples were used. The samples were obtained during surgery at the Hiroshima University Hospital. We confirmed microscopically that the tumor specimens were predominantly (>50%) cancer tissue. Samples were frozen immediately in liquid nitrogen and stored at −80°C until use. Samples of normal brain, spinal cord, heart, skeletal muscle, lung, stomach, small intestine, colon, liver, pancreas, kidney, bone marrow, spleen, peripheral leukocytes, and trachea were purchased from Clontech (Palo Alto, CA, USA). For western blot analysis, lysates from normal adult stomach and trachea were purchased from Clontech. For immunohistochemical analysis, we used archival formalin-fixed, paraffin-embedded tissues from 144 patients who had undergone surgical excision of gastric cancer (65 women and 79 men; age range, 38–90 years; mean, 68 years). Thirty-nine of the 144 patients had early gastric cancer, and 105 had advanced gastric cancer. Early gastric cancer is limited to the mucosa or the mucosa and submucosa, regardless of nodal status. Advanced gastric cancer is a tumor that has invaded beyond the submucosa.[17] Primary gastric cancers exhibiting a hepatoid component were regarded as hepatoid adenocarcinomas. Out of 144 primary gastric cancers, 6 cases were histologically classified as hepatoid adenocarcinoma. Out of six hepatoid adenocarcinoma cases, liver metastasis samples were available for two cases. Two patients with liver metastasis were serologically negative for HBV and HCV infection and they did not show any clinical or echographic signs of cirrhosis. Both patients had no history of alcohol abuse. In addition, we used archival formalin-fixed, paraffin-embedded tissues from 52 patients who had undergone surgical excision of hepatocellular carcinoma (8 women and 44 men; age range, 44–82 years; mean, 64 years; stage I, 31 cases; stage II, 16 cases; stage III, 5 cases). Normal adult ($n=2$, 45 and 57 years) and fetal ($n=2$, 10 and 18 gestational weeks) livers were obtained at autopsy.

Microarray Analysis

One primary gastric cancer sample (P208T) and corresponding non-neoplastic mucosa were analyzed by genome-wide microarray, as described previously.[18] Here, we used Affymetrix GeneChip Human Genome U133Plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA). Each transcript on this array is represented by a set of 11 probe pairs, called the probe set. The array contains >54 000 probe sets, representing 47 400 transcripts, including 38 500 genes. Five micrograms of total RNA was used to prepare antisense biotinylated RNA with One-cycle Target Labeling and Control Reagent (Affymetrix) as per the manufacturer's instructions. In brief, first-stranded cDNA was synthesized with a T7-RNA polymerase

promoter-attached oligo(dT) primer followed by second-stranded cDNA synthesis. This cDNA was purified and served as a template in the subsequent *in vitro* T7-transcription (IVT). The IVT reaction was carried out in the presence of T7 RNA polymerase and biotinylated UTP for cRNA production. The biotinylated cRNAs were then cleaned up and fragmented. The fragmented, biotinylated cRNA was hybridized to the array (45°C for 16 h). The procedures for staining, washing, and scanning of arrays were carried out as per the instructions in the Affymetrix technical manual. The expression value (average difference, AD) of each probe was calculated with GeneChip Operating Software Version 1.1 (Affymetrix). The mean of AD values in each experiment was 1000 to reliably compare variable multiple arrays.

Quantitative RT-PCR

Total RNA was extracted with an RNeasy Mini Kit (Qiagen, Valencia, CA, USA), and 1 μg of total RNA was converted to cDNA with a First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ, USA). Quantitation of *PLUNC* mRNA levels in human tissue samples was done by real-time fluorescence detection, as described previously.[19] *PLUNC* primer sequences were 5′-CAG TTGCCT TCT CTC CGA GG-3′ and 5′-CAT GGG ATG TTA CAC ACGCC-3′. PCR was performed with an SYBR Green PCR Core Reagents Kit (Applied Biosystems, Foster City, CA, USA). Real-time detection of the emission intensity of SYBR Green bound to double-stranded DNA was performed with an ABI PRISM 7700 Sequence Detection System (Applied Biosystems), as described previously.[20] *ACTB*-specific PCR products were amplified from the same RNA samples and served as internal controls.

Western Blotting

Western blotting was performed as described previously.[21] Lysates (40 μg) were solubilized in Laemmli sample buffer by boiling and then subjected to 12% SDS-polyacrylamide gel electrophoresis followed by electrotransfer onto a nitrocellulose filter. The filter was incubated with the primary antibody against PLUNC (goat polyclonal, dilution 1:500; R&D Systems, Abingdon, UK). Peroxidase-conjugated anti-goat IgG was used in the secondary reaction. Immunocomplexes were visualized with an ECL Western Blot Detection System (Amersham Biosciences). β-Actin (Sigma, St Louis, MO, USA) was also stained as a loading control.

Immunohistochemistry

Formalin-fixed, paraffin-embedded samples were sectioned, deparaffinized, and stained with H&E to ensure that the sectioned block contained tumor cells. Adjacent sections were then stained immunohistochemically. Antigen retrieval was done by microwave heating in citrate buffer (pH 6.0) for 30 min for PLUNC, HepPar1, cytokeratin 19 (CK19), cytokeratin 20 (CK20), and polyclonal carcinoembryonic antigen (p-CEA). After peroxidase activity was blocked with 3% H_2O_2–methanol for 10 min, sections were incubated with normal goat serum (Dako Cytomation, Carpinteria, CA, USA) for 20 min to block nonspecific antibody-binding sites. Sections were incubated with the primary antibodies against PLUNC (the same antibody used in western blotting to PLUNC, dilution 1:50), alpha-fetoprotein (AFP) (C3, 1:20; Novocastra, Newcastle, UK), HepPar1 (OCH1E5, 1:20; Dako Cytomation), CK19 (RCK108, 1:50; Dako Cytomation), CK20 (Ks20.8, 1:50; Dako Cytomation), and p-CEA (1:1000; Dako Cytomation) for 1 h at room temperature, followed by incubations with biotinylated anti-goat, anti-rabbit, or anti-mouse IgG and peroxidase-labeled streptavidin for 10 min each. Staining was completed with 10 min incubation with the substrate–chromogen solution. The sections were counterstained with 0.1% hematoxylin. The staining results were recorded in semiquantitative fashion as follows: 0, absence of staining; 1+, any tumor cell stained to 10% of tumor cells stained; 2+, 11–50% of tumor cells stained; and 3+, more than 50% of the tumor cells stained.

Statistical Methods

Associations between clinicopathologic parameters and PLUNC expression were analyzed by χ^2-test. Kaplan–Meier survival curves were constructed for PLUNC-positive and PLUNC-negative patients. Survival rates were compared between PLUNC-positive and PLUNC-negative groups. Differences between survival curves were tested for statistical significance by log-rank test.[22] A *P*-value of less than 0.05 was considered statistically significant.

Results

Comparison of Gene Expression Profiles from Microarray and SAGE

The gene expression profiles obtained from the gastric cancer sample (P208T) and corresponding non-neoplastic gastric mucosa sample were compared. To identify ideal biomarkers for gastric cancer, we focused on genes that showed significantly increased expression in gastric cancer. The top 20 genes that showed higher expression in the gastric cancer sample than in the corresponding non-neoplastic gastric mucosa sample by microarray analysis are listed in Table 1. The gene showing the greatest increase in expression in the gastric cancer sample by microarray was *PLUNC*. Increased expression of the *MAGE* genes