Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer

Kenji Shimizu,[1] Hajime Kubo,[2, 4] Koji Yamaguchi,[3] Kazuhiko Kawashima,[1] Yoshihide Ueda,[1] Koichi Matsuo,[1] Masaaki Awane,[1] Yasuyuki Shimahara,[1] Arimichi Takabayashi,[1] Yoshio Yamaoka[1] and Seiji Satoh[1, 4]

[1]Department of Gastroenterological Surgery, Graduate School of Medicine, Kyoto University, 53 Shogoin Kawaharacho, Sakyo-ku, Kyoto 606-8507; [2]Molecular & Cancer Research Unit, HMRO, Graduate School of Medicine, Kyoto University, Yoshida Konoe-cho, Sakyo-ku, Kyoto 606-8501; and [3]First Department of Surgery, Sapporo Medical University School of Medicine, South 1, West 16 Chuo-ku, Sapporo 060-8543

(Received November 25, 2003/Revised February 16, 2004/Accepted February 23, 2004)

In gastric cancer, lymph node metastasis is one of the major prognostic factors and forms the basis for surgical removal of local lymph nodes. Recently, several studies have demonstrated that overexpression of lymphangiogenic growth factor VEGF-C or VEGF-D induces tumor lymphangiogenesis and promotes lymphatic metastasis in mouse tumor models. We examined whether these processes could be inhibited in naturally metastatic tumors by blocking of their cognate receptor VEGFR-3 signaling pathway. Using a mouse orthotopic gastric cancer model which has a high frequency of lymph node metastasis, we estimated lymphatic vessels in gastric cancers by immunostaining for VEGFR-3 and other specific lymphatic markers, LYVE-1 and prox-1. Then we systemically administered anti-VEGFR-3 blocking antibodies. This treatment resulted in the inhibition of regional lymph node metastasis and reduction of lymphatic vessel density in the primary tumors. In addition, increased density of LYVE-1-positive lymphatic vessels of primary tumors was closely correlated with lymph node metastasis in human samples of gastric cancer. Anti-lymphangiogenesis by inhibiting VEGFR-3 signaling could provide a potential strategy for the prevention of lymph node metastasis in gastric cancer. (Cancer Sci 2004; 95: 328–333)

Gastric cancer is one of the leading causes of cancer deaths worldwide.[1)] The extent of regional lymph node metastasis is an important indicator of tumor aggressiveness and forms the basis for surgical ablation of the local lymph nodes.[2)] Lymphatic capillaries are considered to provide entrance into the lymphatic vascular system when the tumor cells migrate to the lymph nodes.[3)] The identification of three lymphatic endothelial markers, LYVE-1,[4, 5)] podoplanin[6)] and prox-1,[7)] has recently enabled the precise observation of lymphatic vessels in tumor tissues. Prox-1, homeobox transcriptional factor, was shown to be the master gene for the differentiation of lymphatic endothelial cells. Moreover, vascular endothelial growth factor-C (VEGF-C) and VEGF-D, which are related to the major angiogenic factor VEGF, have been identified as major regulators of the development of lymphatic vessels (lymphangiogenesis).[8, 9)] Their cognate receptor, VEGFR-3, is exclusively expressed by the endothelial cells of lymphatic vessels during embryogenesis and in adult tissues.[10)]

Correlation of VEGF-C expression with lymph node metastasis has also been observed in a variety of human cancers, including thyroid, prostate, gastric, colorectal and lung cancers.[11–15)] Furthermore, a correlation between the lymphatic vessel density (LVD) and lymph node metastasis has been reported in human head and neck squamous cell carcinomas and breast cancers.[16, 17)] On the other hand, some authors did not observe this correlation in lung and ovarian cancers.[18, 19)] Recent reports have demonstrated that overexpression of VEGF-C or VEGF-D induces tumor lymphangiogenesis and promotes lymphatic metastasis in mouse tumor models.[20–22)] Soluble VEGFR-3 has been shown to inhibit VEGF-C-induced tumor lymphangiogenesis.[23)] To investigate whether blocking the activity of endogenous VEGF-C or VEGF-D in naturally metastatic tumors can inhibit lymphangiogenesis and lymph node metastasis, we have analyzed a mouse orthotopic gastric cancer model, which has a high frequency of lymph node metastasis.

Materials and Methods

Cell culture. AZL5G,[24)] a human gastric cancer cell line, was cultured in RPMI1640 supplemented with 10% fetal bovine serum and antibiotics.

Human samples. Surgical tissue samples of gastric cancers were obtained from 30 gastric cancer patients (12 without regional lymph node metastasis [node-negative], and 18 with regional lymph node metastasis [node-positive]). The median age was 63 years (range, 33–82); the group included 19 men and 11 women. All 30 patients had advanced gastric cancer (T2 or T3 tumor), in which the tumor invasion is beyond the submucosa.

Immunohistochemistry. Immunohistochemistry was performed on frozen sections using the DAB chromogen. Frozen tissues were sectioned (5–7 μm thickness), mounted and air-dried. After having been fixed in cold acetone and washed with PBS, the sections were incubated in 0.03% hydrogen peroxide for 15 min to inactivate endogenous peroxidase. Slides were then incubated with antibodies against human LYVE-1 (1:500),[4)] mouse LYVE-1 (1:500, kind gifts from Dr. David Jackson),[5)] prox-1 (1:200),[7)] human VEGFR-3 (1 μg/ml, kind gifts from Kari Alitalo),[25)] mouse VEGFR-3 (1 μg/ml),[26, 27)] human CD31 (1:500, DAKO, Carpinteria, CA), mouse CD31 (1:500, PharMingen, San Diego, CA) or human VEGF-C (1:200, Santa Cruz Biotechnology, CA). Positive reactions were visualized by incubating the slides with stable DAB after treatment with the corresponding peroxidase-conjugated secondary antibodies. The tyramide signal amplification (TSA-indirect, NEN Life Science, Boston, MA) method was used to enhance the staining according to the manufacturer's instructions. The sections were counterstained with hematoxylin.

Animal experiments. For implantation, subconfluent AZL5G cells were harvested with trypsin-EDTA and resuspended to a final concentration of 1×10^8/ml PBS. Five-week-old male BALB/c-*nu/nu* mice (CLEA Japan, Tokyo) received upper-middle laparotomy under general anesthesia with Nembutal. Using a 30-gauge needle attached to a 1 ml syringe, cells (1×10^7 cells/0.1ml) were injected orthotopically under the serosal membranes in the greater curvature of the antrum of the mice. The mice were sacrificed according to the institutional guidelines on postoperative days 7 to 42.

S. S. and H. K. contributed equally.

[4]To whom reprint requests should be addressed.
E-mail: sesami@kuhp.kyoto-u.ac.jp E-mail: kuboflt@kuhp.kyoto-u.ac.jp

Abbreviations: VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; LYVE-1, lymphatic vessel endothelial hyaluronan receptor-1: LVD, lymphatic vessel density.

For antilymphangiogenesis treatment after the implantation of tumor cells, the mice were randomly assigned to either the control or treatment group. Control rat IgG (600 μg/mouse, control group, Chemicon, Temecula, CA) or monoclonal anti-VEGFR-3 antibodies (600 μg/mouse, treatment group)[26, 27] were injected subcutaneously every 3 days from postoperative days 7 to 42. Primary tumors were excised and weighed, and the numbers of regional lymph node metastases were counted.

Semi-quantitative RT-PCR analysis of VEGF-C. Total RNA from the primary tumors and normal stomachs of mice was extracted using TRIZOL (Life Technologies, Rockville, MD) according to the manufacturer's protocol. First-strand cDNA was prepared from total RNA by reverse transcriptase (Superscript II, Life Technologies) using oligo(dT) primers. For the detection of *VEGF-C* and *GAPDH* gene expression, the following oligomers (identical in both human and mouse sequences) were used in the PCR; VEGF-C (5′-AGTTTTGCCAATTCACACTTC-CTG-3′ and 5′-GTCATTGGCAGAAAACCAGTCTT-3′), GAPDH (5′-AGTCCATGCCATCACTGCCA-3′ and 5′-CTT-ACTCCTTGGAGGCCATG-3′).

Quantification of the vessel density. The immunostained slides were first scanned at ×40 magnification to identify the areas of highest vascular density within the tumor (so-called vascular hot-spots). The number of vessels was counted in five fields of hot-spots per slide at ×200 magnification. The mean of the vessel counts in minimally three sections of the sample was recorded as the vessel density of the tumor. Lymphatic vessel density (LVD) and microvessel density (MVD) were determined from the counts of LYVE-1-positive vessels and CD31-positive LYVE-1 negative vessels, respectively. All samples were evaluated by two observers (K. S., K. K.).

Statistical analysis. Statistical analysis was performed using the Stat-View 5.0 software (SAS Institute, Inc., NC). The χ^2 test was used to compare the numbers of mice with lymph node metastases between groups. The number of regional lymph node metastases and the primary tumor weight in each group was evaluated using the Mann-Whitney *U* test. A *P* value of less than 0.05 was considered significant. The correlation between vessel counts and regional lymph node metastasis was evaluated using the unpaired Student's *t* test.

Results

Lymphatic vessel density is correlated with the frequency of regional lymph node metastasis in human gastric cancers. To examine the relationship between lymphatic vessel density (LVD) in the hot-spots in the primary tumors and regional lymph node metastasis in human gastric cancer, we carried out immunohistochemistry for LYVE-1 in frozen sections obtained from 30 advanced gastric cancer patients. We closely matched two populations of patients with lymph node metastasis [node-positive]

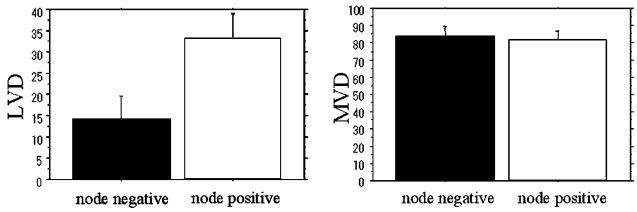

Fig. 1. Correlation of LVD and MVD with regional lymph node metastasis in human gastric cancer. We compared two populations of advanced gastric cancer patients with lymph node metastasis [node-positive] (n=18) and without lymph node metastasis [node-negative] (n=12), closely matched for age, gender, tumor type, thickness and invasion level, by means of immunohistochemistry for LYVE-1 and CD31. LVD was significantly correlated with the frequency of lymph node metastasis (P<0.05). On the other hand, CD31-positive LYVE-1-negative MVD did not correlate with the frequency of regional lymph node metastasis (P=0.7). The graphs represent mean±SEM.

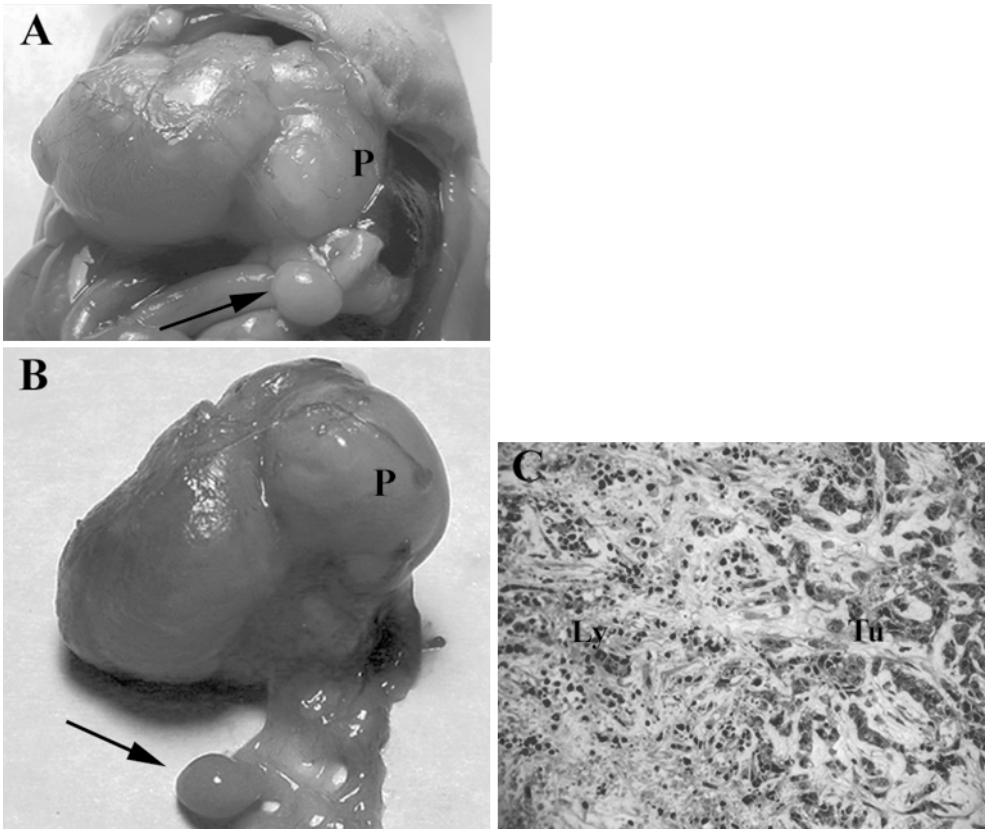

Fig. 2. Gross and histological appearance of primary AZL5G gastric tumors and lymph node metastases. A: Primary gastric tumor (P) and grossly enlarged regional lymph node metastasis (arrow) 42 days after orthotopic implantation. B: Resected gastric primary tumor (P) and lymph node metastasis (arrow). C: H&E staining of metastatic lymph node. Tu; AZL5G tumor cells, Ly; lymphoid cells. C; ×200.

(n=18) and without lymph node metastasis [node-negative] (n=12) for age, gender, tumor type, thickness and invasion level. LVD was significantly correlated with the frequency of lymph node metastasis (node-negative vs. node-positive; 14.2±4.0 vs. 32.7±5.7; mean±SEM, P<0.05, Fig. 1 A). On the other hand, CD31-positive LYVE-1-negative MVD did not cor-

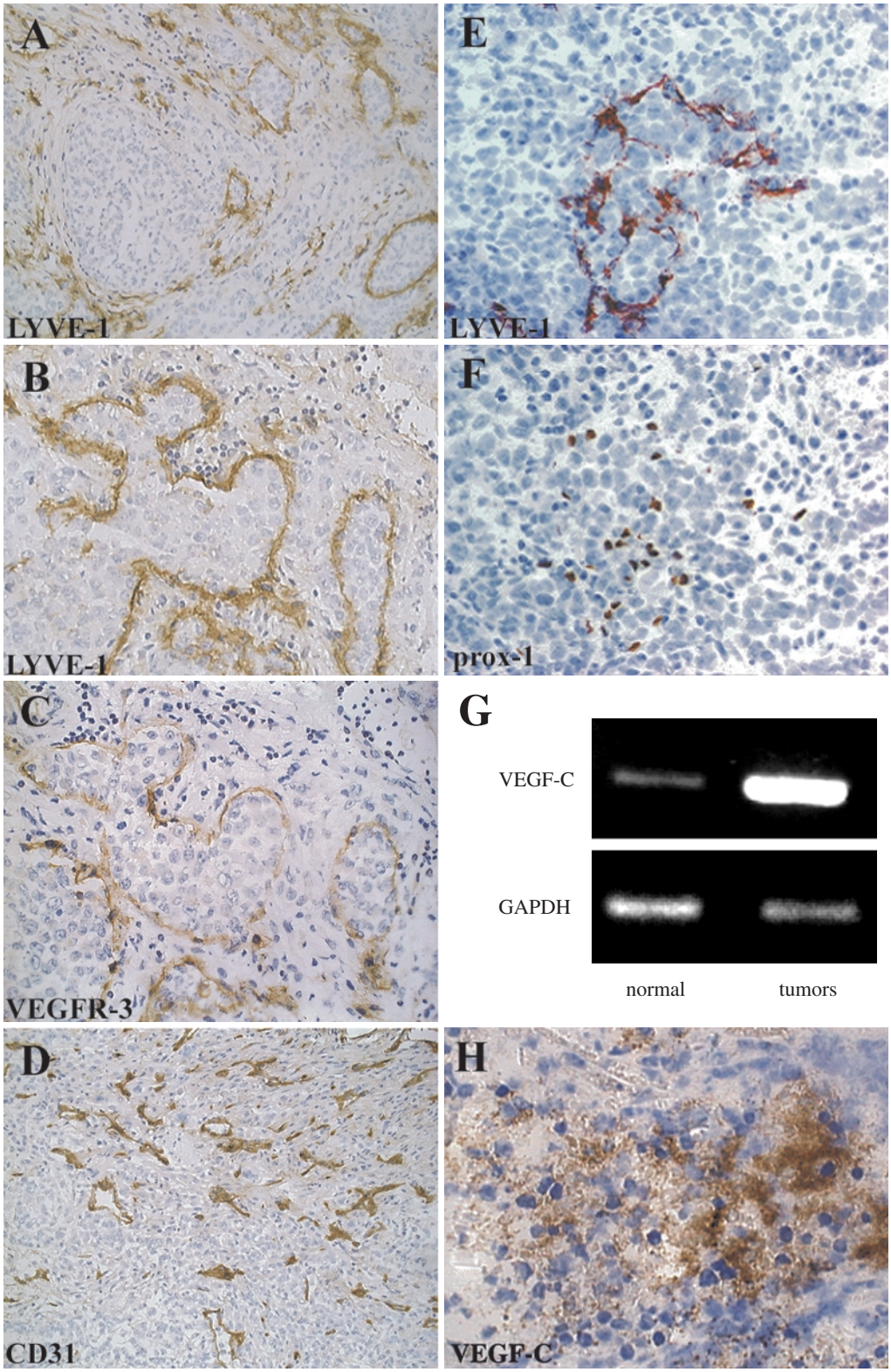

Fig. 3. Histology and *VEGF-C* gene expression in the AZL5G primary gastric tumors. A: Immunohistochemistry for LYVE-1 in the primary tumors. B, C: Immunohistochemistry for LYVE-1 (B) and VEGFR-3 (C) in serial sections. LYVE-1-positive vessels in the primary tumors are also positive for VEGFR-3. D: Immunostaining for CD31. E, F: Immunostaining for LYVE-1 (E), and prox-1 (F) in serial sections of primary tumors. All LYVE-1-positive vessels also stain for prox-1. Note the dot-like pattern of prox-1 immunostaining (F). G: Semi-quantitative RT-PCR for VEGF-C mRNA. GAPDH served as an internal control. H: Immunostaining for VEGF-C. A; ×200, B–F; ×400, H; ×800.

relate with the frequency of regional lymph node metastasis (node-negative vs. node-positive; 80.0±5.8 vs. 82.8±5.3; mean±SEM, P=0.7, Fig. 1B). Prox-1, homeobox transcriptional factor, was shown to be the master gene for the differentiation of lymphatic endothelial cells,[7] and is a reliable marker for lymphatics. We confirmed that all LYVE-1-positive vessels also expressed prox-1 in the human samples (data not shown).

Orthotopic implantation of the gastric cancer cell line AZL5G is a mouse model with a high frequency of lymph node metastasis. To evaluate the role of tumor lymphatic vessels in regional lymph node metastasis, we used a mouse orthotopic gastric cancer model, which has a high frequency of lymph node metastasis. Orthotopically implanted human gastric cancer cell line AZL5G cells formed primary gastric tumors with regional lymph node metastases after postoperative day 42 (arrows in Fig. 2, A and B). Enlarged lymph nodes were histologically confirmed to be occupied by tumor cells (Fig. 2C). The frequency of lymph node metastasis was 17 out of 23 (74%).

Numerous LYVE-1-positive lymphatic vessels were observed in the primary tumors (Fig. 3A). LVD was similar to those of node-positive human gastric cancer tissues (33.3±5.6; mean±SEM). LYVE-1-positive lymphatic vessels also expressed VEGFR-3 in the serial sections (Fig. 3, B and C). CD31 marked all vessels and showed a similar pattern to those in human gastric cancer tissues (Fig. 3D and data not shown). We confirmed that all LYVE-1-positive vessels also expressed prox-1 in this model (Fig. 3, E and F). The expression of VEGF-C mRNA, expressed by the AZL5G cells *in vitro*, was remarkably upregulated in the primary tumor tissue in comparison with the normal tissue as determined by semi-quantitative RT-PCR (Fig. 3G). VEGF-C protein was detected in the cytoplasm of tumor cells (Fig. 3H). These results suggested that the AZL5G gastric cancer model has similarities with human gastric cancers both macroscopically and histologically.

Systemic administration of anti-VEGFR-3 antibody inhibits lymph node metastasis. To assess the usefulness of inhibition of VEGFR-3 signaling to prevent lymph node metastasis in gastric cancer, we administered anti-mouse VEGFR-3 antagonistic antibody (clone AFL4) subcutaneously in the orthotopic gastric cancer model. We have previously used AFL4 in a glioblastoma model and in mouse corneal assay, and it has inhibited tumor angiogenesis and corneal lymphangiogenesis at maximum with 600 μg/mouse, while 200 μg/mouse has shown less effect.[26, 27] Therefore, we chose to use 600 μg/mouse for subsequent experiments. In the control group, the number of node-positive mice was 12 out of 16 (75%) 42 days after tumor implantation. By contrast, in the AFL4-treated group, the number of node-positive mice was only 3 out of 16 (19%) (Fig. 4, Table 1, P<0.01). In addition, the average number of metastatic lymph nodes per node-positive mouse in the AFL4-treated group was significantly smaller than that in the control group (Table 1, P<0.01). No difference in gross appearance or weight of the primary tumor was observed between the control group and AFL4-treated group (Fig. 4, Table 1). Also, no toxic effects of the antibody administration were apparent.

Anti-VEGFR-3 antibodies inhibit lymphangiogenesis but not angiogenesis in gastric cancer. To determine the relationship between LVD or blood vessel density (MVD) and the occurrence of regional lymph node metastasis, we examined the numbers of LYVE-1-positive vessels and CD31-positive LYVE-1 negative blood vessels in the hot-spots in the primary tumors by immunostaining. LVD was significantly decreased in the AFL4-treated group in comparison with the control group (10.5±1.5 vs. 32.8±3.6; mean±SEM, P<0.05) (Fig. 5, A–C). In contrast, MVD was not different between the AFL4-treated groups and control (56.8±4.7 vs. 55.4±4.4; mean±SEM, P=0.84) (Fig. 5, D–F).

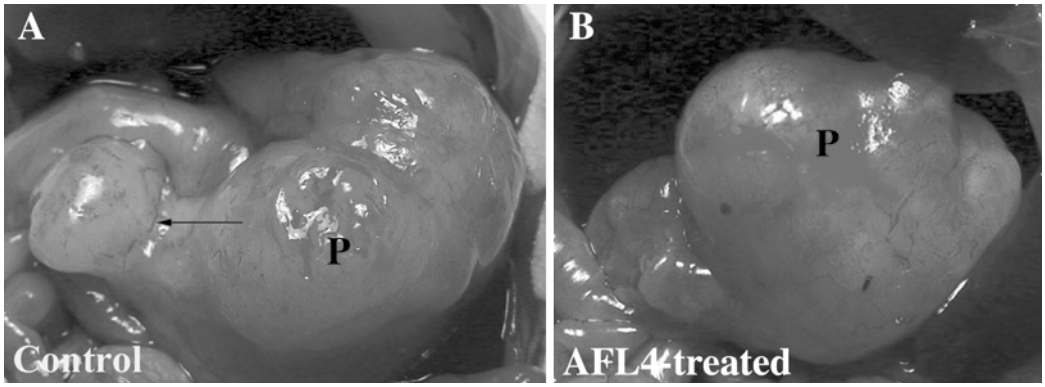

Fig. 4. Inhibition of metastatic spread of orthotopic gastric AZL5G tumors by systemic administration of anti-VEGFR-3 antibodies (AFL4). Mice with orthotopic gastric tumors were analyzed on day 42 after tumor cell inoculation. Control rat IgG (600 μg/mouse, control group) or anti-VEGFR-3 antibodies (600 μg/mouse, AFL4-treated group) were injected subcutaneously every 3 days between postoperative days 7 to 42. A: Control mouse (P; primary tumor, arrow; metastatic lymph node). B: AFL4-treated mouse (P; primary tumor). For the statistics, see Table 1.

Table 1. Effect of anti-VEGFR-3 antibodies on lymph node metastasis and tumorigenicity in a mouse orthotopic gastric cancer model

	Number of mice	Number of node positive mice[2)]	Number of positive lymph nodes[3)]	Weight of primary tumors (g)[4)]
Control	16	12 (75%)	2.4±0.6 (n=12)	2.6±0.4
AFL4-treated[1)]	16	3 (19%)[5)]	1.0±0.0 (n=3)	2.8±0.5

1) Systemic administration of control IgG or AFL4 was performed at 600 μg/dose every 3 days.
2) Metastasis to the regional lymph nodes was determined by histological examination of frozen sections stained with H&E.
3) Mean±SEM of the number of metastatic lymph nodes per mouse.
4) Mean±SEM of weight of primary tumor per mouse.
5) P<0.01, in comparison with control group.

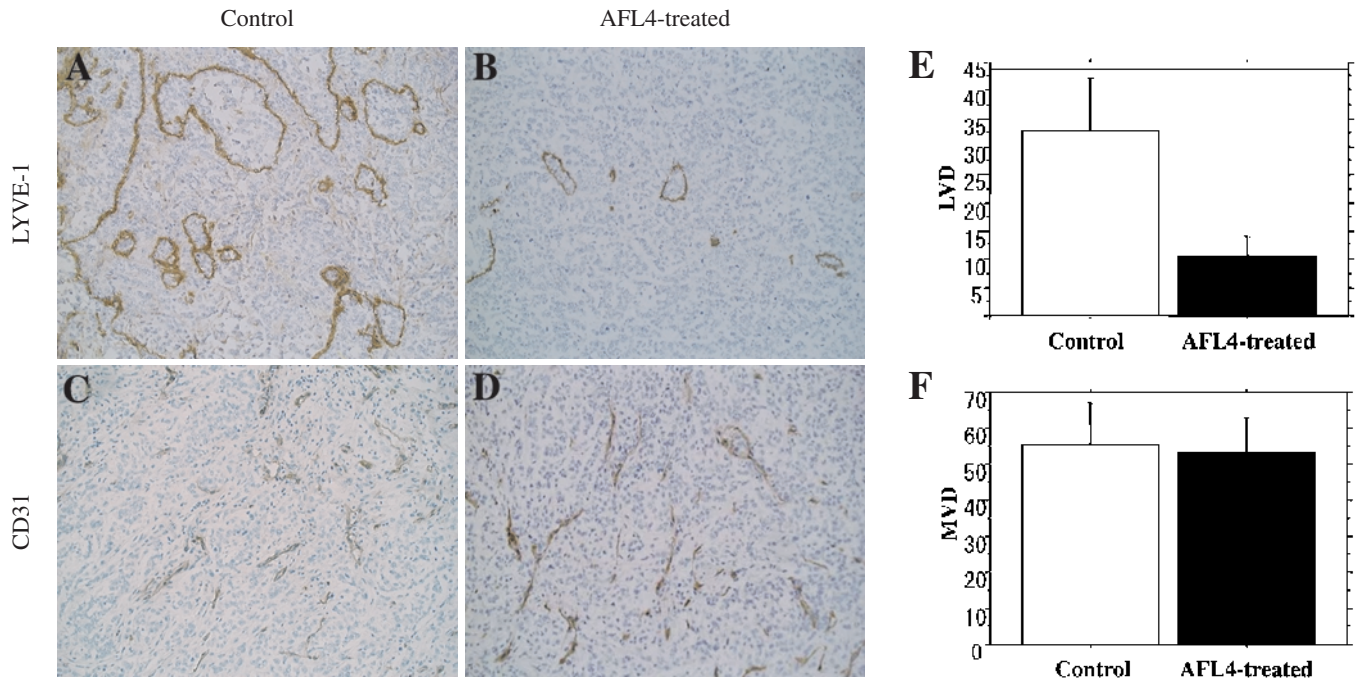

Fig. 5. Assessment of lymphatic and blood vessel density in control and AFL-4-treated mice. Immunohistochemistry of primary tumors for LYVE-1 (A, B) and CD31 (C, D) and schema of the vessel counts (E, F). Compared with the control group, the number of LYVE-1-positive lymphatic vessels (LVD) in the primary tumors in the AFL4-treated group is dramatically decreased (E, $P<0.05$). In contrast, CD31-positive LYVE-1-negative microvessel density (MVD) was not significantly different between the control group and the AFL4-treated group (F, $P=0.84$). A, B, C and D; ×200. E and F: The relative vessel numbers were compared by means of Student's *t* test. The graphs represent mean±SEM.

Discussion

In this study, we first found that the increased density of LYVE-1-positive lymphatic vessels of primary tumors was closely correlated with lymph node metastasis in human samples of gastric cancer. In addition, we systemically administered anti-VEGFR-3 blocking antibodies in an orthotopic gastric cancer model with a high frequency of lymph node metastasis. Consequently, this treatment inhibited lymph node metastasis by decreasing LVD. Antilymphangiogenesis by inhibiting VEGFR-3 signaling could provide a potential strategy for the prevention of lymph node metastasis in gastric cancer.

The lymphatic vessel marker LYVE-1 was reported to be also expressed by blood vessels in the liver, lung and in some tumors. In contrast, homeobox transcriptional factor, *prox-1*, was shown to be the master gene for the differentiation of lymphatic endothelial cells.[7] At present, it is definitely the most reliable marker for lymphatics. However, we hardly detect the lymphatic structure by immunostaining for prox-1, because it is localized at the nucleus of lymphatic endothelial cells. We then compared the pattern of immunostaining for LYVE-1 and prox-1 and found that all LYVE-1-positive vessels are also positive for prox-1 in tumors from a mouse orthotopic gastric cancer model. Therefore, we used LYVE-1 as a specific marker for tumoral lymphatic vessels in this study.

Correlation of VEGF-C expression with lymph node metastasis has been observed in human gastric cancer.[13] However, the correlation between LVD and lymph node metastasis has not yet been investigated. We found that the elevation of LVD is significantly associated with the frequency of lymph node metastasis in human gastric cancer. LYVE-1-positive lymphatic endothelial cells also expressed VEGFR-3 in serial sections of human gastric cancers (data not shown). These results suggest that the development of lymphatics via the VEGF-C/VEGFR-3 signal pathway may promote lymphatic invasion in human gastric cancer. On the other hand, in the human surgical samples, we failed to detect a clear increase in PCNA+ or Ki67+ lymphatic vessels in node-positive cases compared with blood vessels (data not shown). This may be due to the fact that the lymphatic vessels had already stabilized by the time the tumor samples were harvested.

While this manuscript was in preparation, He *et al*. reported that adenoviral administration of soluble VEGFR-3 suppresses lymph node metastasis from a subcutaneously implanted lung cancer cell line.[28] Soluble VEGFR-3 can block activation of the corresponding cellular receptor by sequestering the ligands, VEGF-C or VEGF-D. However, VEGF-C has the potential to bind and activate VEGFR-2 in addition to VEGFR-3, and thus it could not be determined whether or not VEGFR-3 signaling was specifically responsible for the effects on metastasis. In comparison with soluble VEGFR-3, anti-VEGFR-3 antibodies block only VEGFR-3 signaling. The importance of VEGFR-3-positive tumor lymphatic vessels in metastasis was shown by the finding that systemic administration of antagonistic anti-VEGFR-3 antibodies inhibited lymph node metastasis in a human gastric cancer xenograft model in mice, via a mechanism that also decreased LVD. We have previously reported that in mouse corneal assay, anti-VEGFR-3 antibodies inhibit lymphangiogenesis.[27] Accordingly, our results provide direct evidence supporting the hypothesis that inhibition of VEGFR-3 signaling can block tumor lymphangiogenesis and subsequently inhibit lymph node metastasis. In this study, we also tried 200 μg/mouse of anti-VEGFR-3 antibodies, but this dose could not inhibit lymph node metastasis completely, in agreement with the previous findings.[26, 27]

In previous reports, VEGFR-3 was upregulated in angiogenic blood vessels in several human cancers.[29, 30] Also, we have shown previously that blocking anti-VEGFR-3 antibodies can destabilize tumor blood vessels in a glioblastoma model.[26] In the AZL5G orthotopic gastric cancer model, however, we de-

tected VEGFR-3 only in the endothelium of lymphatic vessels, but not blood vessels. In this model, anti-VEGFR-3 antibodies inhibited lymph node metastasis by suppressing lymphangiogenesis without affecting tumor angiogenesis or tumor growth. As a result, this model could be useful to understand the impact of tumoral lymphatics on lymph node metastasis by tumor lymphatic vessel ablation.

Orthotopic tumor implantation models have been recognized to be more clinically relevant than ectopic implantation models from the viewpoint of the tumor microenvironment. In our analysis, the orthotopic model was similar to human gastric cancers macroscopically and histologically. In some respects, however, our mouse model differs from human gastric cancer. First, the tumor cells mainly exist in the submucosal region in our mouse model, whereas they start to develop from the mucosa in the human cancers. Second, VEGFR-3 was expressed on blood vessels in human gastric cancer tissues, but not expressed on the tumor blood vessels in our mouse model (data not shown). Hence, the establishment of additional *de novo* models may help in understanding better the role of the VEGFR-3 system in tumor progression.

We have demonstrated that blockade of VEGFR-3 signaling might have therapeutic potential for gastric cancer. Such a therapy should reduce the incidence of metastasis after treatment, although it is unlikely to affect the growth of preexisting metastases. We could not investigate the effect of the therapy on the survival time of tumor-bearing mice in the present study. Evaluation of the effect on prognosis would require clinically relevant studies, including the resection of primary tumors.

We thank Drs. Kari Alitalo, Pirjo Laakkonen and Tatina V. Petrova, University of Helsinki, for the antibodies for prox-1, LYVE-1 and human VEGFR-3, and Dr. David G. Jackson, University of Oxford, for the antibodies for human and mouse LYVE-1. This work was supported by grants provided by the Ministry of Education, Culture, Sports, Science and Technology of Japan (GN12470261 and GN13357013), the Public Trust Fund for the Promotion of Surgery, the Ichiro Kanehara Foundation, the Princess Takamatsu Cancer Research Fund and the Yamanouchi Foundation for Metabolic Disorders.

1. Parkin MD, Pisani P, Ferlay J. Estimate of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999; **80**: 827–41.
2. Adachi Y, Shiraishi N, Suematsu T, Shiromizu A, Yamaguchi K, Kitano S. Most important lymph node information in gastric cancer: multivariate prognostic study. *Ann Surg Oncol* 2000; **7**: 503–7.
3. Liotta LA. Cancer cell invasion and metastasis. *Sci Am* 1992; **266**: 54–9, 62–3.
4. Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 1999; **144**: 789–801.
5. Prevo R, Banerji S, Ferguson DJ, Clasper S, Jackson DG. Mouse LYVE-1 is an endocytic receptor for hyaluronan in lymphatic endothelium. *J Biol Chem* 2001; **276**: 19420–30.
6. Breiteneder-Geleff S, Soleiman A, Kowalski H, Horvat R, Amann G, Kriehuber E, Diem K, Weninger W, Tschachler E, Alitalo K, Dontscho KD. Angiosarcomas express mixed endothelial phenotypes of blood and lymphatic capillaries. Podoplanin as a specific marker for lymphatic endothelium. *Am J Pathol* 1999; **154**: 385–94.
7. Wigle TJ, Oliver G. Prox1 function is required for the development of the murine lymphatic system. *Cell* 1999; **17**: 769–78.
8. Joukov V, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996; **15**: 290–8.
9. Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 1998; **95**: 548–53.
10. Kaipainen A, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 1995; **92**: 3566–70.
11. Bunone G, Vigneri P, Mariani L, Buto S, Collini P, Pilotti S, Pierotti MA, Bongarzone I. Expression of angiogenesis stimulators and inhibitors in human thyroid tumors and correlation with clinical pathological features. *Am J Pathol* 1999; **155**: 1967–76.
12. Tsurusaki T, Kanda S, Sakai H, Kanetake H, Saito Y, Alitalo K, Koji T. Vascular endothelial growth factor-C expression in human prostatic carcinoma and its relationship to lymph node metastasis. *Br J Cancer* 1999; **80**: 309–13.
13. Yonemura Y, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K, Sasaki T. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 1999; **5**: 1823–9.
14. Akagi K, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, Sugimachi K. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 2000; **83**: 887–91.
15. Niki T, Iba S, Tokunou M, Yamada T, Matsuno Y, Hirohashi S. Expression of vascular endothelial growth factors A, B, C, and D and their relationships to lymph node status in lung adenocarcinoma. *Clin Cancer Res* 2000; **6**: 2431–9.
16. Beasley NJ, Prevo R, Banerji S, Leek RD, Moore J, van Trappen P, Cox G, Harris AL, Jackson DG. Intratumoral lymphangiogenesis and lymph node metastasis in head and neck cancer. *Cancer Res* 2002; **62**: 1315–20.
17. Schoppmann SF, Birner P, Studer P, Breiteneder-Geleff S. Lymphatic microvessel density and lymphovascular invasion assessed by anti-podoplanin immunostaining in human breast cancer. *Anticancer Res* 2001; **21** 4A: 2351–5.
18. Padera TP, Kadambi A, Tomaso E, Carreira CM, Brown EB, Boucher Y, Choi NC, Mathisen D, Wain J, Mark EJ, Munn LL, Jain RK. Lymphatic metastasis in the absence of functional intratumoral lymphatics. *Science* 2002; **196**: 1883–6.
19. Birner P, Schindl M, Obermair A, Plank C, Breitenecker G, Kowalski H, Oberhuber G. Lymphatic microvessel density in epithelial ovarian cancer: its impact on prognosis. *Anticancer Res* 2000; **20**: 2981–5.
20. Skobe M, Hawighorst T, Jackson DG, Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K, Detmar M. Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 2001; **7**: 192–8.
21. Stacker SA, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* 2001; **7**: 186–91.
22. Mattila MM, Ruohola JK, Karpanen T, Jackson DG, Alitalo K, Harkonen PL. VEGF-C induced lymphangiogenesis is associated with lymph node metastasis in orthotopic MCF-7 tumors. *Int J Cancer* 2002; **98**: 946–51.
23. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, YlaHerttuala S, Jaattela M, Alitalo K. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001; **61**: 1786–90.
24. Yamaguchi K, Ura H, Yashima T, Shishido T, Denno R, Hirata K. Eastablishment and characterization of a human gastric carcinoma cell line that is highly metastatic to lymph nodes. *J Exp Clin Cancer Res* 2000; **19**: 113–20.
25. Jussila L, Valtola R, Partanen TA, Salven P, Heikkila P, Matikainen MT, Renkonen R, Kaipainen A, Detmar M, Tschachler E, Alitalo R, Alitalo K. Lymphatic endothelium and Kaposi's sarcoma spindle cells detected by antibodies against the vascular endothelial growth factor receptor-3. *Cancer Res* 1998; **58**: 1599–604.
26. Kubo H, Fujiwara T, Jussila L, Hashi H, Ogawa M, Shimizu K, Awane M, Sakai Y, Takabayashi A, Alitalo K, Yamaoka Y, Nishikawa SI. Involvement of vascular endothelial growth factor receptor-3 in maintenance of integrity of endothelial cell lining during tumor angiogenesis. *Blood* 2000; **96**: 546–53.
27. Kubo H, Cao R, Brakenhielm E, Makinen T, Cao Y, Alitalo K. Blockade of vascular endothelial growth factor receptor-3 signaling inhibits fibroblast growth factor-2-induced lymphangiogenesis in mouse cornea. *Proc Natl Acad Sci USA* 2002; **99**: 8868–73.
28. He Y, Kozaki K, Karpainen T, Koshikawa K, Yla-Herttuala S, Takahashi T, Alitalo K. Suppression of tumor lymphangiogenesis and lymph node metastasis by blocking vascular endothelial growth factor receptor 3 signaling. *J Natl Cancer Inst* 2002; **94**: 819–25.
29. Partanen TA, Alitalo K, Miettinen M. Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. *Cancer* 1999; **86**: 2406–12.
30. Valtola R, Salven P, Heikkila P, Taipale J, Joensuu H, Rehn M, Pihlajaniemi T, Weich H, deWaal R, Alitalo K. VEGFR-3 and its ligand VEGF-C are associated with angiogenesis in breast cancer. *Am J Pathol* 1999; **154**: 1381–90.