MMP9 induction by vascular endothelial growth factor receptor-1 is involved in lung-specific metastasis

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Summary

The molecular mechanism of tissue-specific metastasis in tumors endogenously expressing members of the vascular endothelial growth factor (VEGF) family is not yet clear. Here we demonstrate that MMP9 is specifically induced in premetastatic lung endothelial cells and macrophages by distant primary tumors via VEGFR-1/Flt-1 tyrosine kinase (TK) and that it significantly promotes lung metastasis. In a genetic approach using mice, suppression of MMP9 induction by deletion of either VEGFR-1TK or MMP9 markedly reduced lung metastasis. Furthermore, the MMP9 levels in endothelial cells of normal lung lobes from patients carrying distant tumors were significantly elevated as compared with those from patients without tumors. Thus, a block of MMP9 induction via VEGFR-1 inhibition could be useful for the prevention of tumor metastasis in lung.

Introduction

Vascular endothelial growth factor (VEGF) and its receptor (VEGFR) family including VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk-1), and VEGFR-3 (Flt-4) are well known to be a crucial regulatory system for normal and pathological angiogenesis (Mustonen and Alitalo, 1995; Ferrara and Davis-Smyth, 1997; Risau, 1997; Shibuya, 2001). VEGFR-1 (as well as VEGFR-2 and VEGFR-3) is structurally related to the Fms/Kit/PDGFR family and contains an extracellular domain carrying seven immunoglobulin (Ig)-like sequences and a cytoplasmic tyrosine kinase domain with a long kinase insert. VEGFR-1 is expressed as a full-length tyrosine kinase receptor and in some cases as a soluble form that carries only the extracellular domain (Shibuya et al., 1990; Matthews et al., 1991; Terman et al., 1992; Kendall and Thomas, 1993; Kondo et al., 1998). VEGFR-1 and VEGFR-2 are specifically expressed on vascular endothelial cells (Jakeman et al., 1992; Eichmann et al., 1993; Kaipainen et al., 1993; Quinn et al., 1993; Yamane et al., 1994). As an exception, the *VEGFR-1* mRNA has been shown to be expressed in monocytes/macrophages, which are involved in the migration toward VEGF (Barleon et al., 1996; Clauss et al., 1996). In addition, other cell types were recently reported to express VEGFR-1 (Gerber et al., 2002; Hattori et al., 2002).

VEGFR-2 is a major positive signal transducer for angiogenesis through its strong tyrosine kinase activity. VEGFR-1 has unique biochemical activity with a 10-fold higher affinity to VEGF than VEGFR-2 but with much weaker tyrosine kinase activity (Park et al., 1994; Mustonen and Alitalo, 1995; Seetharam et al., 1995; Sawano et al., 1996, 1997; Ferrara and Davis-Smyth, 1997; Takahashi et al., 1999). We previously showed using VEGFR-1TK$^{-/-}$ mice that VEGFR-1 has a dual function in angiogenesis, acting in a positive or negative manner under different biological conditions. VEGFR-1 has a negative regulatory function for physiological angiogenesis in the embryo, possibly with its strong VEGF-trapping activity (Hiratsuka et al., 1998).

In solid tumor angiogenesis, nutrition supplied by blood vessels is necessary for growth once the tumor is over a few millimeters in diameter (Folkman, 1992; Hanahan and Folkman, 1996). VEGFR-1 can also act as a potential positive regulator using its tyrosine kinase during tumor angiogenesis when the VEGFR-1-specific ligand is expressed at abnormally highly levels (Hiratsuka et al., 2001). However, in the case of spontaneous metastasis, the metastatic tumors in the wild-type grew faster

SIGNIFICANCE

The mechanism of tissue-specific tumor metastasis is still not clearly elucidated. Here we propose a crucial role for the VEGF-receptor/MMP system not only in angiogenesis but also in tumor metastasis via a novel mechanism. We demonstrate that the primary tumors themselves determine the specific site of metastasis by selectively inducing MMP9 expression in lung endothelial cells and macrophages, thereby promoting the invasion of tumor cells, preferentially to the lung. The induction of MMP9 expression is dependent on the VEGFR-1 tyrosine kinase. This study also suggests that macrophages play an important role in the upregulation of MMP9 expression in lung endothelial cells. Thus, more preventable medication for lung metastasis could be possible by VEGFR-1 tyrosine kinase inhibitor and MMP inhibitor.

than in VEGFR-1TK$^{-/-}$ mice in spite of no significant difference in the growth rate of the primary tumors between the two genotypes. This raised an intriguing possibility that the difference might be due to a difference in tumor migration or tumor invasion that was not directly related to the tumor angiogenic phase.

Matrix metalloproteinases (MMPs), which can degrade extracellular matrix components, play a critical role in tissue remodeling during development in pathological process, including inflammation, tissue repair, tumor invasion, and metastasis (Stetler-Stevenson, 1999; Egeblad and Werb, 2002). Recently, MMPs such as MMP9/gelatinaseB, MMP2/gelatinaseA, MMP3/stromelysin1, and MMP7/matrylysin have emerged as regulators of angiogenesis and tumor progression (Brooks et al., 1996; Wilson et al., 1997; Cockett et al., 1998; Itoh et al., 1998; Vu et al., 1998; Sternlicht et al., 1999; Coussens et al., 2000). Among MMPs, MMP9 is of particular interest, because it is thought to be a tumor angiogenic factor that signals through the VEGF-VEGFR system (Bergers et al., 2000). In the present study, we have focused on the MMP9 expression in a target organ during the premetastatic phase and have demonstrated that an elevation of MMP9 in the premetastatic lung by a distant primary tumor is dependent on the tyrosine kinase of VEGFR-1 and that this elevation promotes lung metastasis.

Results

MMP9 induction in lung stimulated by a distant primary tumor depends on the tyrosine kinase of VEGFR-1

The growth rate of lung metastatic tumors is different between the wild-type and VEGFR-1TK$^{-/-}$ mice. We previously showed that the growth rates of Lewis lung carcinoma (LLC) and melanoma (B16) in primary subcutaneous sites were not significantly different between wild-type and VEGFR-1TK$^{-/-}$ mice (Hiratsuka et al., 2001). Similarly, implanted highly spontaneous metastatic 3LL-LLC (3LL) tumors exhibited indistinguishable growth rates in primary sites between the two genotypes. However, the number of spontaneous metastatic tumor nodules in the lungs of the wild-type was found to be 3-fold higher than that in VEGFR-1TK$^{-/-}$ mice (Figures 1A and 1B). Since no significant difference in the lung metastasis was found between the two genotypes when LLC cells were directly injected intravenously into mice without primary tumors (Figure 1C), we hypothesized that distant primary tumors have a crucial effect on premetastatic lung tissues via tyrosine kinase of VEGFR-1. To separate the premetastatic phase from the lung metastatic phase, we replaced the highly metastatic 3LL-LLC cells with a LLC cell line that lacks spontaneous metastatic ability but elicits metastasis after intravenous injection. We detected fluorescence-stained tumor cells only in primary tumor tissues but not in lungs for up to 1.5 months. In addition, no PCR products for the vector sequence inserted into tumor cells were found in the lungs (Figure 1D), indicating that there were no detectable micrometastases to lungs.

As an experimental model, we defined the premetastatic phase as a period until the implanted LLC had grown to 1.5 cm in diameter at subcutaneous sites. Alternatively, for the metastatic phase, we intravenously injected LLC cells into the tumor-bearing mice to examine the organs that the tumor cells prefer to invade (Figure 1E).

Lung colonization of tumor cells injected through the tail vein is widely used as a model system for detecting metastasis. Our system is similar to that, but one step improved, since we used tumor-bearing mice as well as healthy mice for tumor cell colonization in the variety of tissues. One merit of this "experimental" metastasis model is that we could use a fixed number of tumor cells for i.v. injection, and thus we could simply ask the difference or similarities on the latter half of metastatic process (colonization and growth of tumor cells in the tissues).

MMP9 was specifically induced in the lungs of tumor-bearing wild-type but not VEGFR-1TK$^{-/-}$ mice. To determine whether any factors linked to VEGFR-1 might have an effect on the premetastatic lungs, we examined lung tissues obtained from wild-type and VEGFR-1TK$^{-/-}$ mice with or without LLC tumors implanted subcutaneously on their backs. Lung tissues were histologically indistinguishable among the normal wild-type and the tumor-implanted wild-type and VEGFR-1TK$^{-/-}$ mice (data not shown). To identify the candidate molecules that play key roles in lung metastasis, we next examined the gene expression in various tissues, including lung, for VEGFR-1-related ligands, VEGF-R family, MMPs, TGF-β (Gohongi et al., 1999), and others. As shown in Figure 1F, among MMPs, MMP9 expression was specifically increased in the lungs of wild-type mice after tumor implantation. Moreover, this increase was dependent on the tyrosine kinase of VEGFR-1, since the MMP9 expression in VEGFR-1TK$^{-/-}$ mice carrying LLC tumors was not elevated.

We also analyzed various MMPs at protein levels and confirmed that only the MMP9 protein was elevated in the tumor-bearing wild-type mice (data not shown). Therefore, we next examined whether MMP9 has a critical role in determining the lung-specific metastasis of tumor cells.

To assess whether other tumor cell lines also stimulate the lung MMP9 level from a distant site in vivo, we used gelatin zymography to check the lung tissues of nude mice carrying various tumors. Figure 2A shows that melanoma, colon cancer (C26), Ehrlich tumor, and angioma (F2) implanted subcutaneously induced an increase of pro-MMP9 in lung, a proenzyme form of MMP9. No induction of MMP9 was found in livers, kidneys, or spleens, although the spleens consistently carried pro-MMP9 possibly derived from macrophages.

To confirm that the upregulation of lung MMP9 by primary tumors depends on the tyrosine kinase of VEGFR-1, we used LLC and B16 melanoma (B16) cells that can be implanted in the genetic background of VEGFR-1TK$^{-/-}$ mice. As shown in Figure 2B, the pro-MMP9 in premetastatic lungs from wild-type mice was three to five times higher than that from VEGFR-1TK$^{-/-}$ mice. An activated form of MMP9 was also induced at low levels in tumor-bearing wild-type mice but not in tumor-bearing VEGFR-1TK$^{-/-}$ mice. These primary tumors did not stimulate another gelatinase, MMP2 in lungs (Figure 2B). Even in the spontaneous metastatic situation using 3LL-LLC, the MMP9 induction occurred at very early stage, possibly prior to lung metastasis, at day 12 (Figure 2C).

VEGF and PlGF stimulated lung MMP9 through VEGFR-1 in organ culture. Both LLC and B16 tumors express VEGF and VEGF-B, ligands for VEGFR-1 (Hiratsuka et al., 2001). Therefore, we next examined whether VEGF could stimulate MMP9 expression through the tyrosine kinase of VEGFR-1 using an organ culture system in vitro. Addition of exogenous VEGF or placenta growth factor (PlGF), a ligand specific for VEGFR-1 (Maglione et al., 1991; Kendall and Thomas, 1993; Sawano et al., 1996; Barleon et al., 1997), into the organ culture medium increased the induction of MMP9. However, VEGF-E (Ogawa et al., 1998),

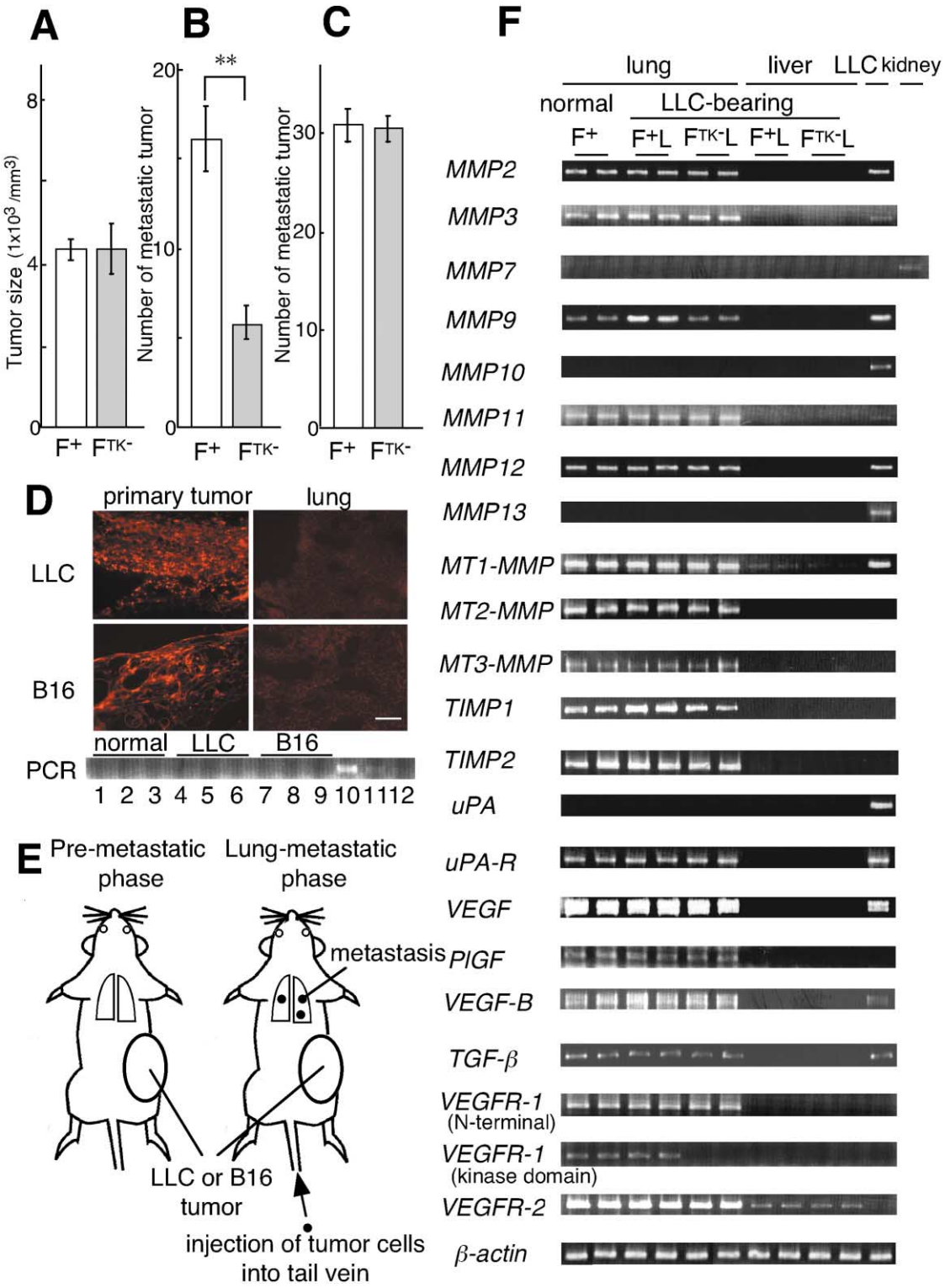

Figure 1. Induction of lung MMP9 in tumor-bearing wild-type (F^+) but not in VEGFR-1 $TK^{-/-}$ (F^{TK-}) mice

A–C: Tumor growth rates in primary sites (**A**) and spontaneous lung metastasis (**B**) in 3LL-LLC-bearing mice. Lung metastasis of LLC without primary tumors (**C**). Student's t test, $p < 0.01$. **D:** Top: No metastases were detected from primary tumors to the lungs in wild-type or VEGFR-$1TK^{-/-}$ mice. Fluorescence-stained, retrovirus vector-inserted LLC and B16 cells were detected in tumor tissues but not in the lungs on day 14. The bar equals 100 μm. Bottom: No bands were found in the lungs from normal (lanes 1–3), LLC-bearing (lanes 4–6), or B16-bearing (lanes 7–9) mice using PCR analysis. Lung tissues containing tumor cells (lane 10, 100 cells; lane 11, 50 cells; lane 12, 0 cell) were used as a positive control. **E:** An experimental metastatic model. Premetastatic phase is the period of tumor growth at a primary site. Metastatic phase is the period after intravenous tumor cells injection. **F:** Screening of genes in various tissues and tumors obtained from LLC-bearing wild-type (F^+L) and VEGFR-$1TK^{-/-}$ ($F^{TK-}L$) mice.

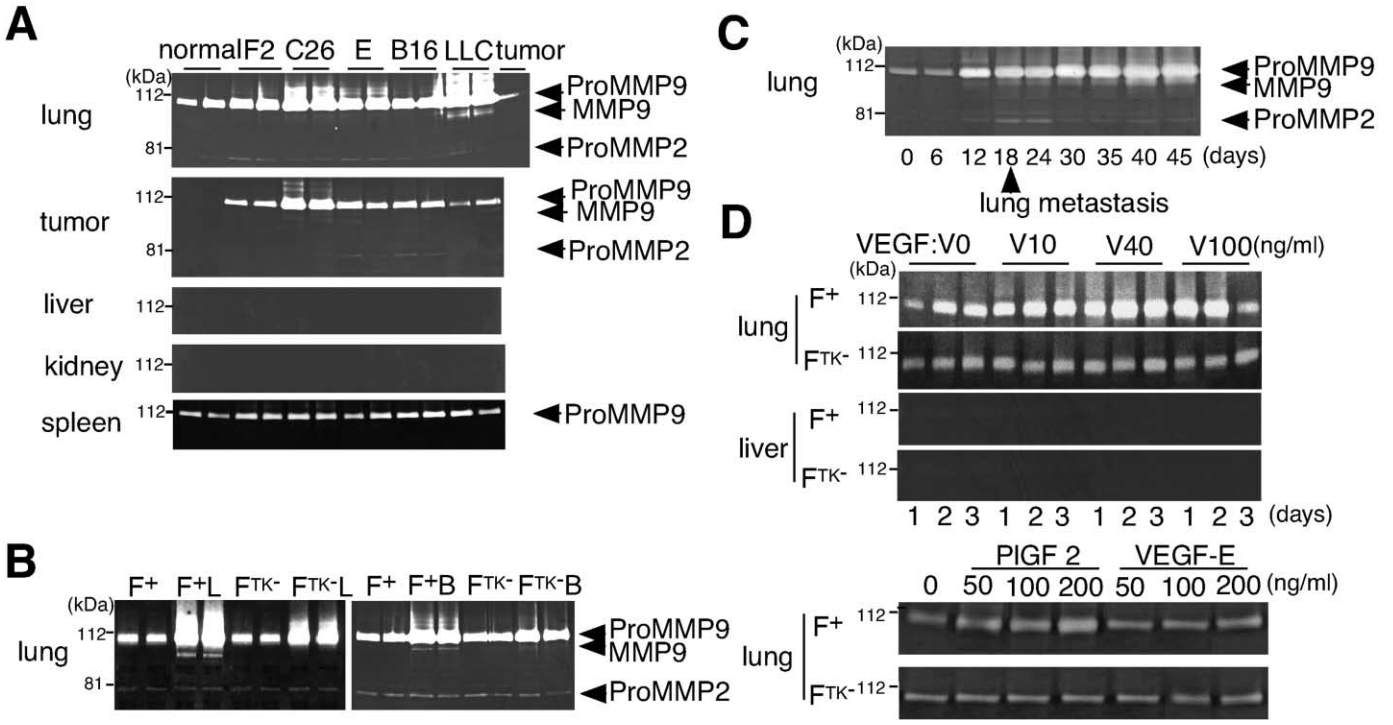

Figure 2. Reduced induction of lung MMP9 in tumor-bearing VEGFR-1TK$^{-/-}$ mice using gelatin zymography

A: Various tissues were obtained from Angioma (F2), colon cancer (C26), Ehrlich tumor (E), melanoma (B16), or LLC-bearing nude mice. **B:** MMP9 in wild-type (F^{+}), VEGFR-1TK$^{-/-}$ (F^{TK-}), LLC-bearing wild-type (F^{+}L) and VEGFR-1TK$^{-/-}$ (F^{TK-}L), and B16-bearing wild-type (F^{+}B) and VEGFR-1TK$^{-/-}$ (F^{TK-}B) mice. **C:** MMP9 in the lungs of 3LL-LLC-bearing mice. **D:** Organ culture with the VEGF family. Specimens of lungs were cultured with VEGF for 1, 2, or 3 days. Additionally, lung tissues were cultured with PlGF2 or VEGF-E for 2 days.

which is a specific ligand for VEGFR-2, did not stimulate MMP9 in the lungs of either genotype (Figure 2D). TGF-β and TNF, expressed in LLC and B16 tumors, slightly stimulated MMP9, but not in a VEGFR-1-dependent manner (data not shown). These results indicate that the lung MMP9 could be induced by VEGF or PlGF signaling through the tyrosine kinase of VEGFR-1 but not VEGFR-2 in vitro.

Macrophages cooperate in the endothelial MMP9 induction

To define the cells in which MMP9 was induced by primary tumors, we examined lung tissues by immunohistochemistry using an anti-mouse MMP9 antibody. We found that the MMP9 was induced in endothelial cells, as characterized by the mouse CD31 (data not shown) and VE-cadherin staining, in tumor-bearing wild-type but not VEGFR-1TK$^{-/-}$ mice (Figure 3A). In addition, the numbers of Mac1-MMP9 double-positive lung macrophages were 1.5- to 2-fold higher in tumor-bearing wild-type mice than those in tumor-bearing VEGFR-1TK$^{-/-}$ mice (Figure 3A, bottom, and Table 1).

For further analyses, we collected lung endothelial cells using anti-mouse CD31 antibody beads. By staining, these CD31-positive cells included 93% of VE-cadherin-positive cells and about 7% of Mac1-positive cells (data not shown). These cells expressed *VEGFR-2*, a marker for endothelial cells, but neither *VEGF*- nor *PDGF*-receptor, markers for epithelial cells and smooth muscle cells, respectively, using RT-PCR. The *MMP9* mRNA was expressed in these CD31-positive cells specifically from tumor-bearing wild-type mice but not from others. Consistently, the proteolytic activity of MMP9 was also found in these cells from tumor-bearing wild-type mice, but it was only faint in those from tumor-bearing VEGFR-1TK$^{-/-}$ mice (Figure 3B). Moreover, the purified lung endothelial cells expressed VE-cadherin in both genotypes, but MMP9 was detectable only in tumor-bearing wild-type mice by immunohistochemistry (Figure 3C).

To analyze the relationship between the MMP9 induction in endothelial cells and the primary tumor-activated macrophages, we cocultured these cells in vitro for 2 days. Wild-type lung endothelial cells cultured on the alveolar macrophages obtained from tumor-bearing wild-type mice highly expressed MMP9. In contrast, the same endothelial cells of wild-type on the macrophages from tumor-bearing VEGFR-1TK$^{-/-}$ mice showed little MMP9 induction (Figure 4). The macrophages at lower layer expressed some amounts of MMP9 (see Figure 4 legend). These results suggest that primary tumors induce endothelial cell MMP9 through an interaction between endothelial cells and lung macrophages via a VEGFR-1-dependent mechanism.

The number of macrophages carrying MMP9 increased 3- to 4-fold in the tumor-bearing wild-type mice compared to those in healthy mice (Table 1), and the lung endothelial cells in the same tumor-bearing mice upregulated MMP9 in cooperation with macrophages (Figures 3 and 4). Based on these results and the proportion of macrophages and endothelial cells in the lung, we suggest that about one-half of MMP9 increase in the

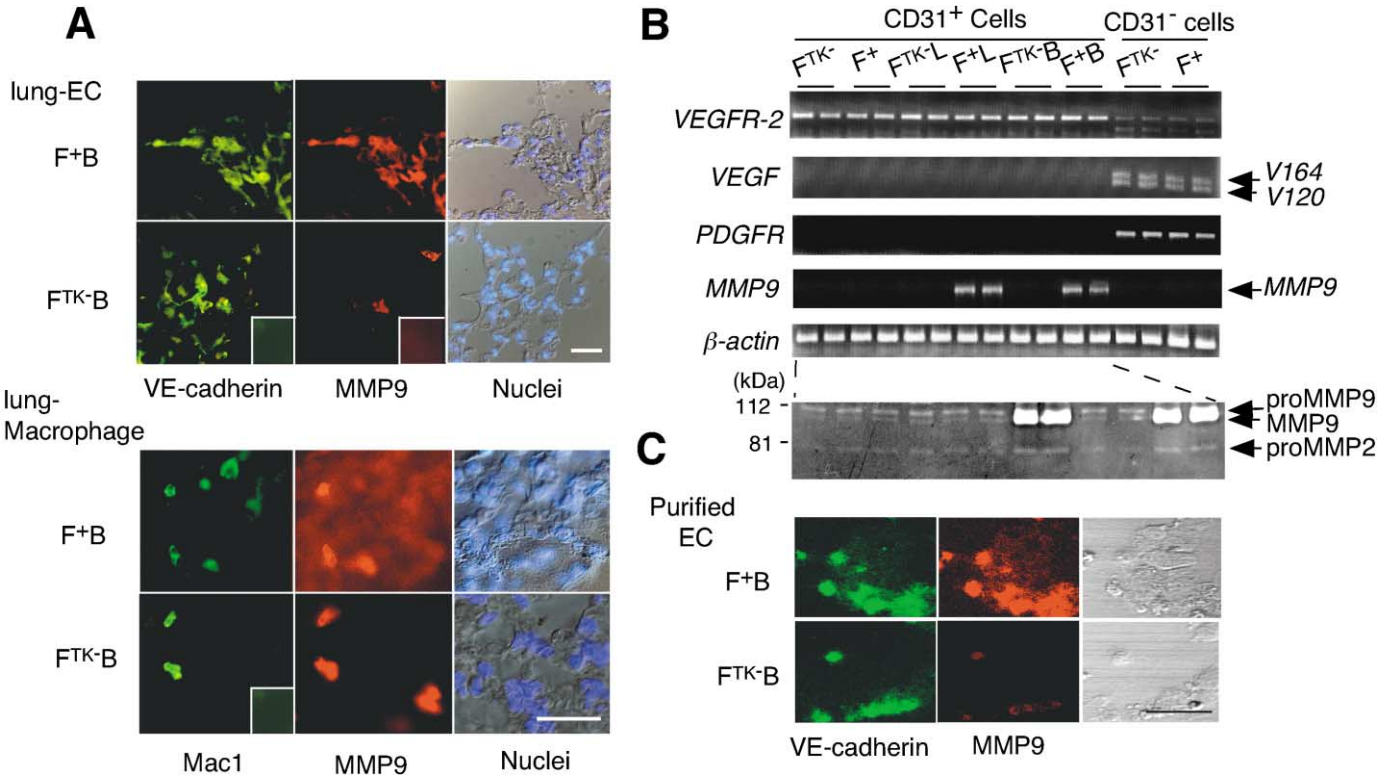

Figure 3. Suppression of lung MMP9 in endothelial cells and macrophages from tumor-bearing VEGFR-1TK$^{-/-}$ mice

A: Immunohistochemistry of lung tissue from B16-bearing wild-type (F^+B) or VEGFR-1TK$^{-/-}$ ($F^{TK-}B$) mice using anti-VE-cadherin, anti-MMP9, or anti-Mac1 antibody. VE-cadherin (FITC), Mac1 (FITC), and MMP9 (Rhodamin) signals were detected by using fluorescence microscope. Overlapped figure of nuclear staining (DAPI) with Nomarski. Normal rat or rabbit IgG-stained sections were shown as negative control (bottom right). The bar equals 50 μm. **B:** RT-PCR analysis of lung CD31-positive cells collected from wild-type and VEGFR-1TK$^{-/-}$ mice implanted with normal, LLC, or B16 cells (top). Gelatin-substrate zymography of lung CD31-positive cells also indicates the suppression of MMP9 induction in tumor-bearing VEGFR-1TK$^{-/-}$ mice (bottom). **C:** MMP9 in lung CD31-positive cells (93% VE-cadherin-positive, 7% Mac1-positive cells). VE-cadherin-positive cells (endothelial cells) express a high level of MMP9 in B16-bearing wild-type (F^+B) but not in VEGFR-1 TK$^{-/-}$ ($F^{TK-}B$) mice. The bar equals 50 μm.

Table 1. The numbers of MMP9- or Mac1-positive cells in lung tissues

Genotypes of mice with (LLC or B16) or without tumors (norm)	MMP9-positive cells[a]	Mac1-positive cells (/mm^2)
Flt-1TK$^{-/-}$(F^{TK-})-norm	28 ± 2[b]	30 ± 2
Wild (F^+)-norm	30 ± 2	33 ± 2
F^{TK-}-LLC	68 ± 7[c]	78 ± 7[c]
F^+-LLC	122 ± 18	175 ± 11
F^{TK-}-	61 ± 5[c]	62 ± 5[c]
F^+-B16	92 ± 7	114 ± 8
Flt-1TK$^{-/-}$/MMP9$^{-/-}$mice ($F^{TK-}M^-$)-norm	0	43 ± 2
Flt-1TK$^{+/+}$/MMP9$^{-/-}$mice (F^+M^-)-norm	0	44 ± 3
Flt-1TK$^{+/+}$/MMP9$^{+/+}$mice (F^+M^+)-norm	39 ± 4	41 ± 3
$F^{TK-}M^-$-LLC	0	95 ± 8[c]
F^+M^--LLC	0	198 ± 6
F^+M^+-LLC	161 ± 13	207 ± 21
$F^{TK-}M^-$-B16	0	51 ± 2[c]
F^+M^--B16	0	100 ± 9
F^+M^+-B16	85 ± 9	120 ± 19

The cells were counted from one out of five sections per one mouse. Three mice of each genotype were used for the analysis.
[a] MMP9-positive cells except for endothelial cells. Most of the MMP9-positive cells were Mac1-positive cells (Macrophage-like cells).
[b] Mean ± S.D.
[c] Significantly different from tumor-bearing wild-type mice. $p < 0.05$.

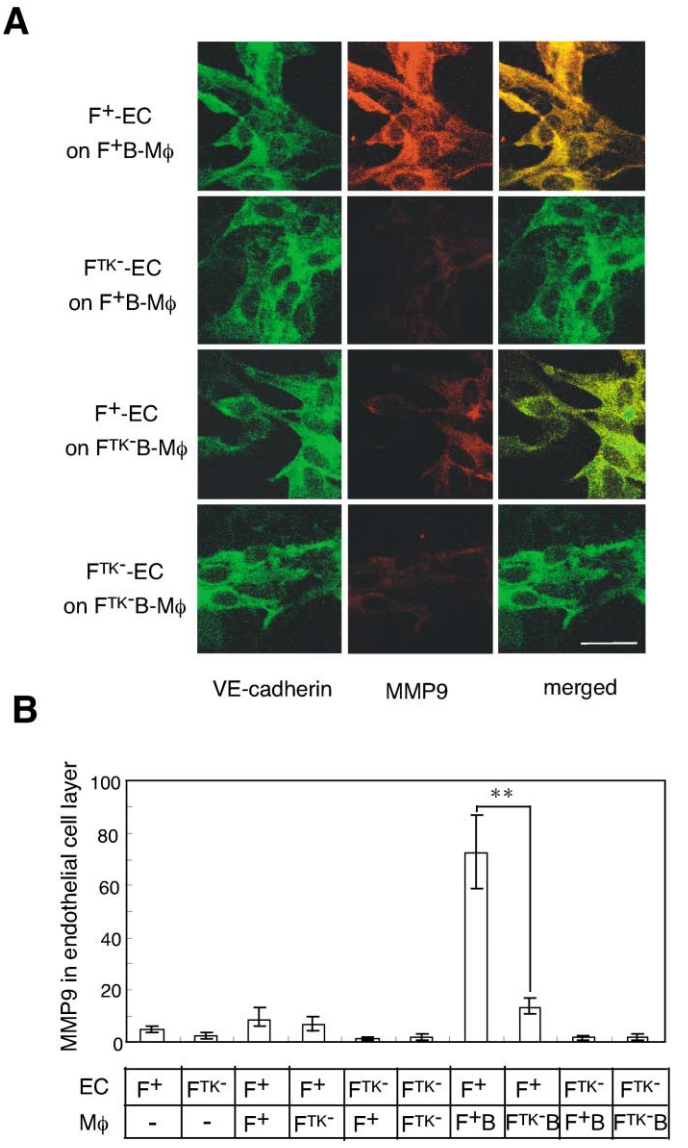

Figure 4. MMP9 induction in lung endothelial cells is cooperatively regulated by macrophages via VEGFR-1 in vitro

A: Endothelial cells (EC) from normal wild-type (F^+) or VEGFR-1$TK^{-/-}$ (F^{TK-}) mice cocultured with alveolar macrophages (Mϕ) from B16-bearing mice (F^+B or $F^{TK-}B$). Endothelial cells were plated on the macrophage layer and further cultured in a medium containing 10 ng/ml VEGF for 2 days (see Experimental Procedures). The bar equals 20 μm. **B:** Summary of the MMP9 quantification in endothelial cells. The signal intensities of macrophage MMP9 ranged from 50 to 60 arbitrary units. Mean ± S.D. (Student's t test, $p < 0.01$)

lung (Figure 2B) is derived from endothelial cells and another half from macrophages.

MMP9 regulates lung-specific metastasis via VEGFR-1

To address the functional significance of the VEGFR-1 tyrosine kinase-dependent stimulation including MMP9 induction on lung metastasis, we monitored fluorescence-marked tumor cells injected intravenously into the tumor-bearing mice. Remarkably, 2 days after the i.v. injection, the number of metastatic tumor cells in the lungs of tumor-bearing wild-type mice was three times higher than that of the other three groups including wild-type without primary tumors and VEGFR-1$TK^{-/-}$ mice with or without primary tumors (Figure 5A). In contrast, the number of tumor cells that had invaded other tissues such as liver, spleen, and kidney were essentially the same in tumor-bearing mice of both genotypes, and these cells were unable to proliferate from the small colonies by day 7 (Figure 5A).

Furthermore, to assess whether VEGFR-1-dependent lung MMP9 induction by primary tumors has any direct effects on metastasis in vivo, we adopted a genetic approach, utilizing mice carrying disruptions of the VEGFR-1TK and/or MMP9 genes (Itoh et al., 1999). We investigated lungs from the premetastatic and metastatic phases in wild-type (F^+M^+), VEGFR-$1TK^{+/+}$/$MMP9^{-/-}$ (F^+M^-), and VEGFR-$1TK^{-/-}$/$MMP9^{-/-}$ ($F^{TK-}M^-$) mice using the same strategy as above. Since no difference in the growth rates of the primary tumors was found among F^+M^+, F^+M^-, and $F^{TK-}M^-$ mice, we were able to examine the invasion phenotype without the need to consider differences in size of primary tumors. To monitor the metastatic phase, we injected fluorescence-marked tumor cells from the tail vein into F^+M^+, F^+M^-, and $F^{TK-}M^-$ mice in which tumors had already been implanted on their backs. Interestingly, the number of tumor cells in the lungs from tumor-bearing F^+M^- mice significantly decreased compared to F^+M^+ mice on day 2 after i.v. injection (Figure 5B). These results suggest that the elevation of MMP9 stimulated by the primary tumors is a key regulator of lung metastasis.

MMP9 and VEGFR-1 upregulate the invasion of tumor cells in vitro

To examine tumor cell invasion to the lung more directly and to avoid any uncertain factors in vivo, we developed a simple in vitro system for the migration of tumor cells into a thin slice of lung tissues from mice with or without tumors. To easily measure the number of cells that invaded the lung specimens, we used LLC and B16 tumor cells carrying a retrovirus vector that could be detected by PCR analysis in this chemotaxis assay (Figure 6A). Stronger PCR bands reflecting a greater number of invading tumor cells were found in lung tissues from tumor-bearing wild-type mice compared to those from tumor-bearing VEGFR-$1TK^{-/-}$ mice (Figure 6B, top). These invasions were inhibited to basal levels by 100 μM batimastat (BB94), a MMP inhibitor (Figure 6B, top; Prontera et al., 1999). We also detected more intensive bands in tumor-bearing F^+M^+ mice than in tumor-bearing F^+M^- and $F^{TK-}M^-$ mice, indicating that the invasion of tumor cells into lung tissues in vitro depends on the amount of MMP9 induced in lung (Figure 6B, bottom).

Human MMP9 of endothelial cells is upregulated in lung tissues from patients with various tumors

To assess whether distant primary tumors stimulate the lung endothelial cell MMP9 in humans, we examined the expression of MMP9 in healthy regions of lung from patients who carried tumors in other organs apart from the lungs. At first, we microscopically checked lung lobes to obtain healthy regions without tumor metastasis, atelectasis, or inflammation. As a control, we detected very low levels of MMP9 in VE-cadherin-positive lung endothelial cells from patients without tumors. In contrast, intensive staining of MMP9 on endothelial cells was found in lung tissues obtained from patients with tumors, such as hepatocel-

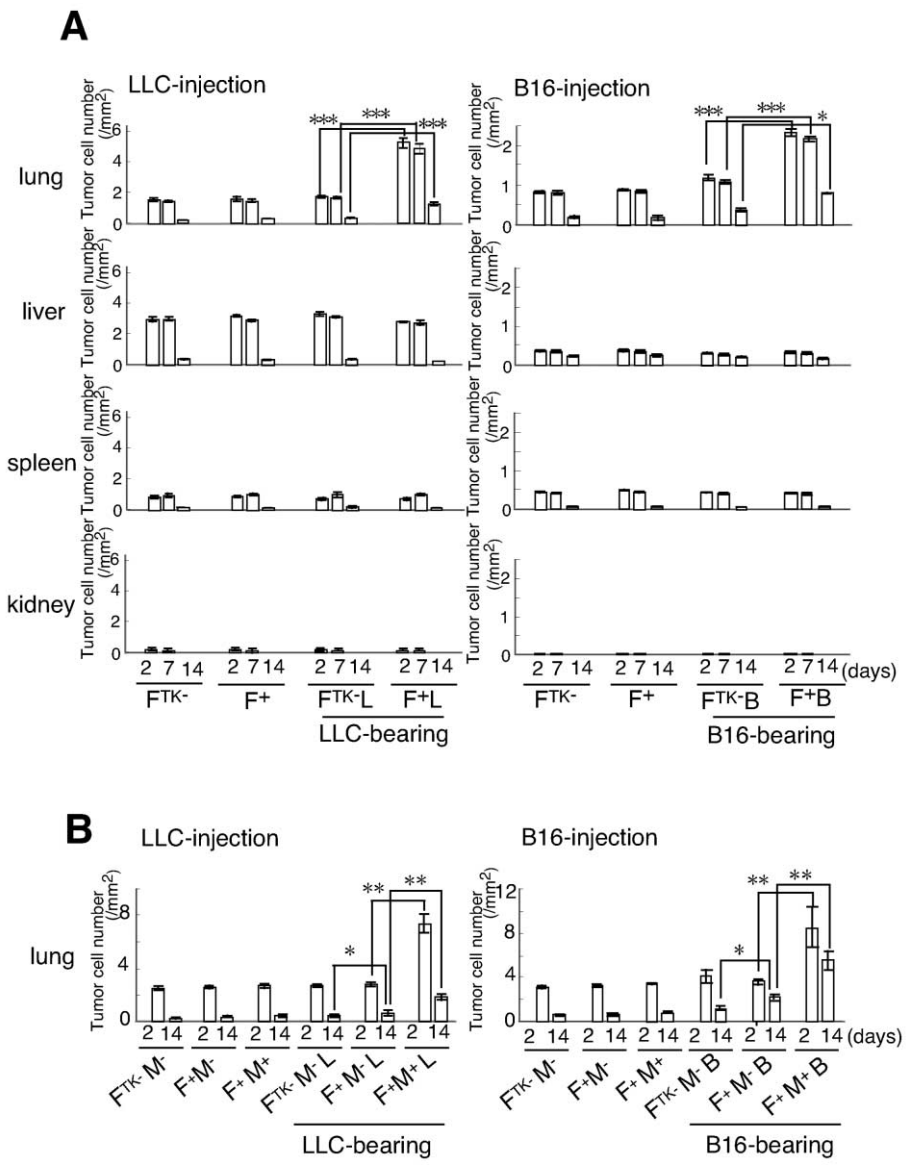

Figure 5. Decreased infiltration of injected tumor cells into lung in tumor-bearing VEGFR-1TK$^{-/-}$ or MMP9$^{-/-}$ mice

A: Number of invaded tumor cells on day 2, 7, and 14 after i.v. injection into normal wild-type (F^+), VEGFR-1TK$^{-/-}$ (F^{TK-}), tumor-bearing wild-type (F^+L or F^+B), and tumor-bearing VEGFR-1TK$^{-/-}$ ($F^{TK-}L$ or $F^{TK-}B$) mice. Before assay, blood was replaced to PBS (−) to avoid a contamination of tumor cells in blood. **B:** Number of invaded tumor cells in lung on day 2 and 14 after i.v. injection of fluorescence-stained LLC or B16 cells into normal or tumor-bearing VEGFR-1TK$^{-/-}$/MMP9$^{-/-}$ ($F^{TK-}M^-$), VEGFR-1TK$^{+/+}$/MMP9$^{-/-}$ (F^+M^-), and VEGFR-1TK$^{+/+}$/MMP9$^{+/+}$ (F^+M^+) mice. Fluorescence-stained cells were counted from ten sections per tissue. Mean ± S.D. (Student's t test, $p < 0.05$, $p < 0.01$, $p < 0.005$).

lular carcinoma, pancreatic cancer, esophageal cancer, colon cancer, cholangiocellular carcinoma, gastric cancer, malignant melanoma, malignant lymphoma, and ovarian cancer (Figures 7A–7N). We calculated the average MMP9 intensities of lung endothelial cells stained with an anti-human MMP9 antibody to compare between patients with and without tumors. A significant difference was found in the intensity of MMP9 per single endothelial cell between tumor-bearing (90 ± 17, mean ± S.D.) and non-tumor-bearing (19 ± 3) patients (Figure 7O). Finally, we detected a few small metastatic nodules in lung lobes other than the examination sites in 77% of patients with primary tumors.

Discussion

Metastasis is the result of many sequential steps, such as migration from primary sites to blood or lymphatic vessels, nonrandom chemotaxis to preferential target organs, invasion from vessels to secondary sites, proliferation supported by the metastatic environment, and tumor growth over a few millimeters in diameter with angiogenesis (Folkman, 1992; Hanahan and Folkman, 1996). The organ preference of metastasis is established by original migration and invasion of tumor cells to specific metastatic sites (Nicolson, 1993; Yeatman and Nicolson, 1993). Although some molecules have been considered as candidates governing the organ preference in the chemotaxis and chemoinvasion (Nicolson, 1993), it has been an open question as to whether any cytokines from the primary tumors might have an effect on the organ specificity of metastasis.

Chemokine ligands and receptors, the chemotactic factors preexisting in target organs and tumor cells, have recently been reported to decide the organ preference at the phase of migration to the target organs (Muller et al., 2001). On the other hand, cytokines secreted from primary tumors are capable of effecting distant metastatic sites because several factors such as angiostatin (O'Reilly et al., 1994), endostatin (O'Reilly et al., 1997),

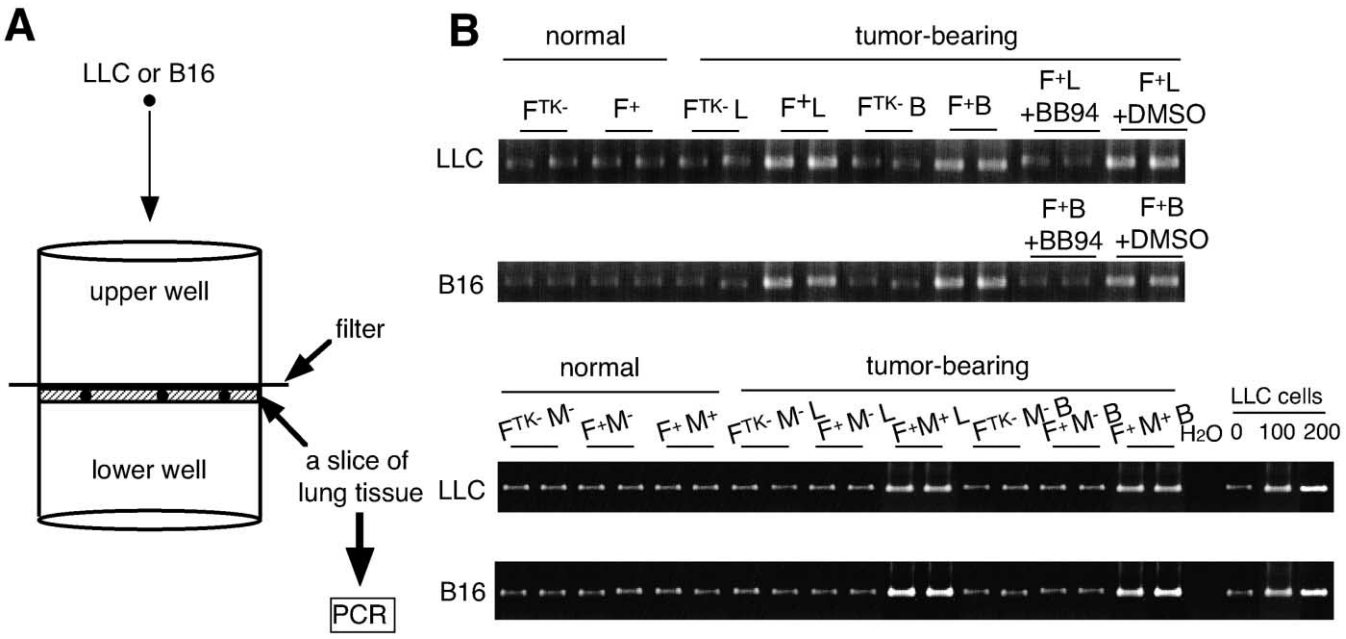

Figure 6. Reduced invasion of tumor cells into lung tissues from tumor-bearing VEGFR-1TK$^{-/-}$, MMP9$^{-/-}$, or VEGFR-1TK$^{-/-}$/MMP9$^{-/-}$ mice in vitro

A: Scheme of the invasion assay. **B:** Semiquantitative PCR analysis for invasion of tumor cells. Lung specimen from normal as well as LLC or B16 tumor-bearing wild-type (F^+) and VEGFR-1TK$^{-/-}$ (F^{TK-}) mice were used. DMSO is negative control for BB94 (top). Lung tissues from normal VEGFR-1TK$^{-/-}$/MMP9$^{-/-}$ ($F^{TK-}M^-$), VEGFR-1TK$^{+/+}$/MMP9$^{-/-}$ (F^+M^-), and VEGFR-1TK$^{+/+}$/MMP9$^{+/+}$ (F^+M^+) mice as well as tumor-bearing $F^{TK-}M^-$, F^+M^-, and F^+M^+ mice were used. For the PCR positive control, 0, 100, and 200 LLC cells plus lungs were used (bottom).

and TGF-β (Gohongi et al., 1999) from primary tumors nonspecifically inhibited angiogenesis in metastatic sites.

Now, we have shown that the tumors themselves aggressively determine a specific metastatic site by selectively inducing an invasive factor in a distant premetastatic site. More specifically, the MMP9 induced by primary tumors in lung endothelial cells and macrophages promotes the invasion of tumor cells preferentially into the lung tissue, and this MMP9 induction was dependent on the VEGFR-1 tyrosine kinase. Our results also suggest that macrophages play an important role in the MMP9 upregulation in lung endothelial cells.

The MMP9-induced lung is the target of circulating tumor cells

The VEGF-VEGF receptor system is a strong angiogenic factor during tumor angiogenesis (Ferrara and Davis-Smyth, 1997; Shibuya, 2001). In addition, we have shown that premetastatic sites without angiogenesis are stimulated by primary tumors through the ligand-receptor system of the VEGFR-1 pathway. Many tumors express VEGF and VEGF-B as well as PlGF occasionally (Hatva et al., 1996; Luo et al., 1998; Olofsson et al., 1998; Salven et al., 1998; Donnini et al., 1999). Although cytokines are secreted from primary tumors in serum, only lung endothelial cells had the ability to induce MMP9 secretion in vivo. At first, we examined the expression of the VEGF family in lungs derived from mice with and without tumors. However, no clear induction of any VEGF family member was found in lungs under stimulation by primary tumors. Accordingly, we supposed that the response to the VEGF family in endothelial cells might vary among endothelial cells from different organs, because VEGF induced MMP9 specifically in lungs but not in livers from organ cultures in vitro.

Our preliminary result using RT-PCR showed that *VEGFR-1* is more highly expressed in purified lung endothelial cells than in those of liver, indicating that MMP9 might be more inducible in lungs than other organs because of the difference in signaling through VEGFR-1. Moreover, as indicated in Figure 4, primary tumor-stimulated lung macrophages could efficiently cooperate with endothelial cells to upregulate MMP9 via VEGFR-1 at least in vitro. Thus, we think that the lung has unique conditions where the activated macrophages and endothelial cells closely interact and activate each other in tumor-bearing mice.

MMP9 elevation in lung endothelial cells of patients with primary tumors

We have shown that MMP9 protein was increased 4.5-fold in normal lung endothelial cells of all patients who carried primary tumors in organs other than the lungs as compared to those of patients without tumors. This result indicates that the primary tumors might stimulate MMP9 in the premetastatic lung in human. Furthermore, metastasis to a few lung lobes was actually found in 77% of patients with primary tumors. We can rule out the possibility that these small lung metastatic nodules might cause MMP9 induction in other lung lobes because it was reported that MMP9 expression was absent in neighboring nontumoral parenchyma in the presence of existing primary tumors without distant metastasis in the lung (Nawrocki et al., 1997). These data support the theory derived from the mouse model that primary tumors induce MMP9 in lung to increase the chance of metastasis. However, it remains to be established which

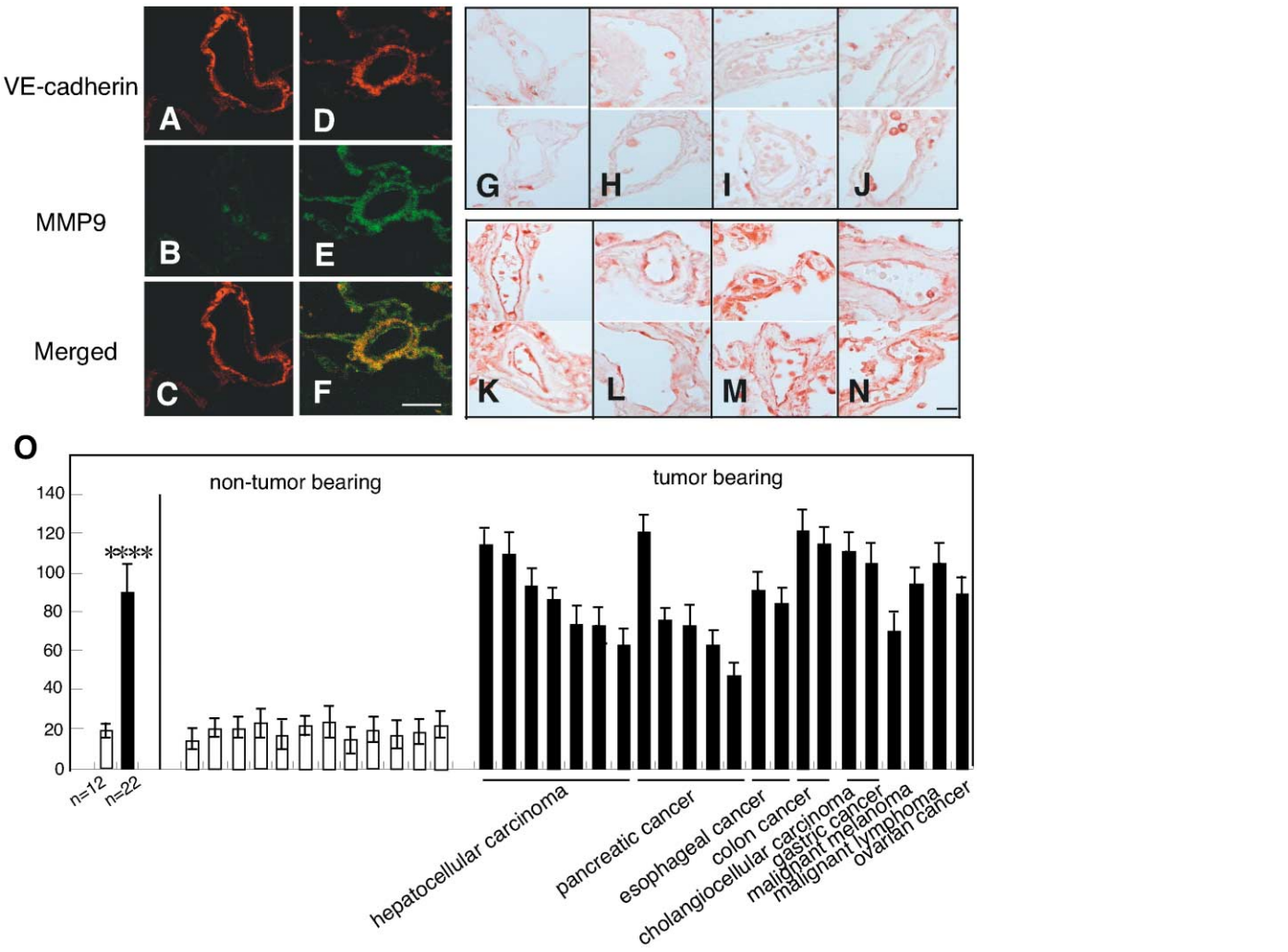

Figure 7. Increased MMP9 in endothelial cells from patients with various tumors

A–N: Immunohistochemistry of anti-VE-cadherin (**A** and **D**) and anti-MMP9 antibodies (**B**, **E**, **G–N**) in lung tissues of non-tumor-bearing (**A–C** and **G–J**) and tumor-bearing (**D–F** and **K–N**) patients. Representative staining is shown in tissues from eight patients (**G–N**). The bars equal 50 μm (**A–F**) and 10 μm (**G–N**). **O:** Summary of the MMP9 quantification in endothelial cells. Lung tissues from patients with no tumors and patients with various tumors. Mean ±S.D. (Student's t test, $p < 0.0001$)

types and stages of human primary tumors efficiently raise the MMP9 levels in lung.

Anticancer drugs could be safer and more effective by selecting a metastatic target organ. The results presented in Figures 5 and 6B suggest that more effective approaches to prevent lung metastasis could be developed by combination of a VEGFR-1 TK inhibitor and/or an MMP9 inhibitor.

Experimental procedures

Animals

Generation of the VEGFR-1TK homozygous null animals whose genetic background is 50% of 129 and 50% of C57BL/6 mice has been reported previously (Hiratsuka et al., 1998). MMP9 homozygous null mice (Itoh et al., 1999) were bred for five generations into C57BL/6 before intercrossing with VEGFR-1TK$^{-/-}$ mice. The litters of all genotypes derived from the crossing of VEGFR-1TK$^{+/-}$ and MMP9$^{+/-}$ mice were almost indistinguishable phenotypically and histologically.

Cells

Tumor cells used are 3LL-LLC, high-metastasis type mouse Lewis lung carcinoma; LLC, no metastasis type (from s.c. transplantation); B16, no metastasis variant (from s.c. transplantation) mouse melanoma; F2, mouse angioma; and C26, mouse colon cancer.

RT-PCR analysis

Total RNA from homogenized tissue samples was extracted and reverse transcribed using mouse-specific primers. Specific primers for MMP2, MMP11, MMP13, MT1-MMP, MT2-MMP, TIMP-1, TIMP2, VEGFR-1 (N), VEGFR-1 (K), VEGF, and β-actin were designed as previously described (Hiratsuka et al., 1998; Luo et al., 1998; Kim et al., 2000). In addition, the appropriate primers were used to amplify MMP3, MMP7, MMP9, MMP10, MMP12, MT3-MMP, uPA, uPA-R, PlGF, VEGF-B, TGF-β, and VEGFR-2.

Tumor cell labeling and metastasis assay in vivo

Lewis lung carcinoma (LLC) and B16 melanoma (B16) cells were double marked by infecting with a murine pSRaMSVtk-neo retrovirus vector and by labeling with a PKH26 fluorescent staining kit (Zynaxis). For the metastasis assay, 1×10^5 B16 or LLC cells labeled with fluorescence were intravenously

injected at 2 or 3 weeks after the subcutaneous implantation of 1×10^7 tumor cells into the mouse back. At the time points indicated, the lungs were collected from the mice after perfusion to eliminate circulating tumor cells. PCR analysis for the retrovirus vector sequence using a specific primer was carried out to detect tumor cells.

Gelatin zymography

Specimens derived from the mice were incubated in a sample buffer containing 2% sodium dodecyl sulfate (SDS) and 2% glycerol without β-mercaptoethanol at room temperature for 30 min. Samples were loaded onto 7.5% SDS-PAGE gels containing gelatin (Sigma, St. Louis, MO) at 1 mg/ml. Gels were washed three times in 50 mM Tris-HCl, 150 mM NaCl (pH 7.6), 10 mM $CaCl_2$, and 5 mM $ZnCl_2$ for more than 12 hr at 37°C without shaking before being subjected to Coomassie brilliant blue staining and destaining.

Culture

For organ culture, 2 mm^2 specimens of tissue were cultured in 2% FCS-DMEM containing mouse VEGF164 (R&D, Minneapolis, MN), PlGF2 (R&D), or VEGF-E (Ogawa et al., 1998).

For coculture of endothelial cells and macrophages, 1×10^3 lung endothelial cells were cultured on 1×10^3 alveolar macrophages derived from tumor-bearing mice with 2% FCS-DMEM containing 10 ng/ml VEGF164. Alveolar macrophages were collected intratracheally with 1% BSA-PBS from anesthetized mice.

Immunohistochemistry and quantitative analysis of the endothelial cells intensity

Mouse lung tissue sections were immunohistochemically stained with an anti-mouse MMP9 antibody (Betsuyaku et al., 2000), as well as antibodies against mouse VE-cadherin (Pharmingen, San Diego, CA) as the endothelial cell-specific marker or Mac1/CD11b (Serotec, Oxford, UK) as the macrophage-specific marker. Human lung sections were incubated with anti-human MMP9 antibody (Anawa, Swiss) or anti-human VE-cadherin antibody (Pharmingen). The signal intensity in the endothelial cells, which ranged from 0 to 250 on an arbitrary unit, was calculated using Adobe Photoshop. Mean intensity per 1 endothelial cell was calculated by dividing the total MMP9 signal in all endothelial cells by the number of endothelial cells in five sections.

Isolation of endothelial cells

Lung endothelial cells were collected using a modified method (Dong et al., 1997). In brief, minced mouse lungs were digested in collagenase at 37°C for 90 min and then filtered through a sterile 58 μm nylon mesh. Washed cells were separated into the cell layer containing endothelial cells using Histpaque (Sigma). Collected cells were incubated with anti-mouse CD31 or VE-cadherin rat antibody, and this was followed by Dynabeads (Dynal, Oslo, Norway) coated with anti-rat-IgG antibody.

In vitro invasion assay

Tumor cell invasion was evaluated using a chemotaxis Boyden chamber (Neuroprobe). The upper and lower wells were separated by a 5 μm pore size polyvinylpyrrolidone-free polycarbonate filter (Nucreopore, Costar, Cambridge, MA), and thin slices of mouse organ tissue were attached to the bottom of the filter. A 50 μl aliquot of the cell suspension (5×10^4 cells/well) was seeded in each of the upper wells. The tissue slices were then collected for PCR after a 3 hr incubation at 37°C with 5% CO_2. BB94 (kindly supplied by Dr. P. Brown, British Biotech) was used as an inhibitor against MMPs.

Human tissue samples

Tissue samples of human lungs taken from patients within the last 2 years for the purpose of autopsy were obtained with informed consent. The quality of lung tissues was confirmed using an anti-human VE-cadherin antibody, an endothelial cell-specific marker. The normal portions of the lungs from patients with various primary and metastatic tumors were used as samples. The normal control group included patients with dissecting aneurysm, acute cardiac infarction, cerebral infarction, cerebral hemorrhage, and degenerative neuron disorders. Samples were microscopically examined to eliminate the cases that had abnormal changes such as micrometastasis, inflammation, atelectasis, and congestion. The cases that had a clinical history of blood vessel disorders such as diabetes (Jin et al., 2001) and athrosclerosis were also excluded.

Statistical analysis

For statistical analysis, the data were expressed as mean ± S.D. and were analyzed using Student's t test. A p value less than 0.05 was considered significant.

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