Inhibition of lymphangiogenesis-related properties of murine lymphatic endothelial cells and lymph node metastasis of lung cancer by the matrix metalloproteinase inhibitor MMI270

Eliane Shizuka Nakamura,[1] Keiichi Koizumi,[1] Mitsuo Kobayashi[1] and Ikuo Saiki[1, 2]

[1]Division of Pathogenic Biochemistry, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, 2630 Sugitani, Toyama 930-0194

(Received September 12, 2003/Revised November 10, 2003/Accepted November 19, 2003)

Based on a previous report on the effect of a matrix metalloproteinase (MMP) inhibitory compound, MMI270, in regulating tumor-induced angiogenesis, as well as recent findings concerning functional correlations among tumor metastasis, angiogenesis and lymphangiogenesis, we investigated the anti-metastatic efficacy of MMI270 in a murine model of lymph node metastasis of lung cancer, and analyzed whether this inhibitor could also regulate lymphangiogenesis-related properties of murine lymphatic endothelial cells (LECs) and invasive properties of Lewis lung cancer (LLC) cells. The observation that MMI270 led to a significant decrease in the weight of tumor-metastasized lymph nodes of mice led us to test its anti-lymphangiogenic and anti-invasive effects *in vitro*. Murine LECs were characterized by an *in vitro* tube formation assay, by semi-quantitative RT-PCR assay to examine the expression of mRNAs for flt-4, Flk-1, Tie-1, Tie-2, CD54/ICAM1, vWF, MMPs and uPA, and by western blotting to confirm the protein expression of flt-4 and CD31/PECAM. This is the first report on the expression of MMP-2, MMP-9 and MT1-MMP in murine LECs, as well as on the inhibition of their enzymatic activity, and of the invasive ability and tube-forming property of LECs by an MMP inhibitor. Furthermore, MMI270 was shown to strongly inhibit the activity of MMP-2 and -9 produced by LLC cells and the invasion of these cells through Matrigel. In summary, the present results indicate that MMI270, apart from its anti-tumor angiogenic application, might be useful as an anti-metastatic drug, on the basis of its downregulatation of both the lymphangiogenesis-related properties of LECs and the invasive properties of LLC cells *in vitro*. (Cancer Sci 2004; 95: 25–31)

Blood and lymphatic vessels develop in parallel, but independently, and together form a circulatory system that allows the passage of fluid and delivers molecules within the body.[1)] It is known that the spread of cancer cells to regional lymph nodes through the lymphatic system is the first step in the dissemination of various cancers, including cancers in the breast,[2)] lung and gastrointestinal tract.[3)] In clinical terms, lymphatic metastasis significantly influences the prognosis of cancer patients and therefore is an important part of tumor staging, especially in gynecological and breast cancers.[4)]

Much of the current knowledge on the identification of molecules that regulate the development and growth of lymphatic vessels (lymphangiogenesis) was derived from extensive research into the molecular mechanisms that control blood vessel growth.[5)] Among the known lymphangiogenic molecules, three members of the vascular endothelial growth factor cytokine family (VEGF-C, VEGF-D and VEGF-A) have been reported to induce both angiogenesis and lymphangiogenesis under appropriate circumstances[1, 6–8)] and, recently, direct evidence that VEGF-C-induced lymphangiogenesis could promote tumor metastasis[9–10)] was reported.

Based on these findings, we hypothesized the existence of other molecules that might play a common role in the processes of angiogenesis, lymphangiogenesis and metastasis. We previously reported that a synthetic matrix metalloproteinase (MMP) inhibitor, namely MMI270, downregulated the properties of murine hepatic sinusoidal endothelial (HSE) cells *in vitro*, and inhibited tumor-induced angiogenesis *in vivo*.[11)] MMI270 (formerly CGS 27023A, Novartis Pharmaceuticals, Inc.) is a novel synthetic hydroxamate-type MMP inhibitor that shows a broad-spectrum inhibition of the enzymatic activities of MMP-1, MMP-9, MMP-2, MMP-3 and MMP-13 at nanomolar concentrations *in vitro*. This compound significantly reduced the tumor burden compared with that in controls and enhanced the activity of cytotoxic and hormonal agents.[12)] Recently, the first phase I and pharmacological study of MMI270 in patients with advanced solid cancer demonstrated that MMI270 led to disease stabilization for more than 90 days in 19 of 92 patients without causing myelotoxicity or tumor regression. Furthermore, the recommended dose for phase II studies was 300 mg twice/day.[13)]

Here we investigated the anti-metastatic efficacy of MMI270 in a murine model of lymph node metastasis of lung cancer, and analyzed whether this inhibitor could also regulate the lymphangiogenesis-related properties of murine lymphatic endothelial cells (LECs) and the invasive properties of Lewis lung cancer (LLC) cells. The results suggest a possible common involvement of MMPs in the processes of angiogenesis, lymphangiogenesis and metastasis.

Materials and Methods

Materials. MMI270, *N*-hydroxy-2(*R*)-[(4-methoxysulfonyl)(3-picolyl)-amino]-3-methyl butanamide hydrochloride monohydrate, MW 393.43 (free base), was a kind gift from Novartis Pharma K.K. For the *in vitro* experiments, it was dissolved in dimethylsulfoxide (DMSO). For the *in vivo* metastasis experiments, MMI270 was prepared as a suspension containing 50% polyethylene glycol 300 in DMSO. Flt-4 (M-20) and anti-mouse CD31 (clone 390) antibodies were purchased from Santa Cruz Biotechnology, Inc. and eBioscience, Inc., respectively.

Animals. Specific pathogen-free C57BL/6 mice (7-week-old females) were purchased from Japan SLC. The mice were maintained in the Laboratory for Animal Experiments, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, under laminar air flow conditions. This study was con-

[2]To whom all correspondence should be addressed.
E-mail: byosei@ms.toyama-mpu.ac.jp

Abbreviations: LECs, lymphatic endothelial cells; LLC, Lewis lung cancer; MMP, matrix metalloproteinase; uPA, urokinase-type plasminogen activator; flt-4, fms-like tyrosine kinase receptor 4; CD31/PECAM, platelet endothelial cell adhesion molecule; CD54/ICAM1, intercellular adhesion molecule 1; vWF, von Willebrand factor.

ducted in accordance with the standards established by the Guidelines for the Care and Use of Laboratory Animals of Toyama Medical and Pharmaceutical University.

Lymph node metastasis model. The induction of lymph node metastasis was performed by the orthotopic intrapulmonary implantation of LLC cells, as described previously,[14] with some modifications. Briefly, log-phase cell cultures of LLC cells were harvested with 1 m*M* EDTA in phosphate-buffered saline (PBS), washed twice with serum-free EMEM, and resuspended at a cell density of 15×10^4 cells/ml in PBS containing 500 μg/ml of Matrigel. Animals were anesthetized with ether. The left chest was swabbed with 70% ethanol and a small skin incision to the left chest wall (approx. 5 mm in length) was made at about 5 mm posterior to the scapula. Sub-skin fat and muscles were separated from costal bones. The left lung motion was observed through the pleura, and a 29-gauge needle attached to a 0.5-ml insulin syringe was inserted directly through the intercostal space into the lung to a depth of 3 mm. Tumor cells (3×10^3) were resuspended in 20 μl of PBS containing 10 μg of Matrigel to prevent the suspension from leaking out of the lung, and were then injected into the lung parenchyma. A cotton-tipped applicator was pressed on the site of puncture as the needle was withdrawn to stop any bleeding. After confirming that the animals had recovered from bradycardia and displayed stable spontaneous respiration, they were returned to their cages.

Administration of MMI270 using Alzet osmotic pumps. After 3 days of intrapulmonary implantation of LLC cells, the mice (n=10) were implanted intraperitoneally with Alzet omostic pumps (Alzet mini-osmotic pump, Model 2004: DURECT Corp.). For the control group, the pumps were filled with a solution containing DMSO (2 ml) and PEG 300 (2 ml) that had been strongly mixed using a vortex mixer. For the MMI270-treated group, a solution containing 0.224 g of MMI270 and 2 ml of DMSO was prepared and vigorously mixed; then, 2 ml of PEG 300 was added and mixed until a clear suspension was obtained.

Primary culture of lymphatic endothelial cells (LECs). LECs were derived from lymphangiomas induced by intraperitoneal injection of incomplete Freund's adjuvant (IFA), as previously reported[15] with some modifications. Briefly, each mouse was injected intraperitoneally with 200 μl of an emulsion containing IFA (Sigma-Aldrich) and PBS (1v:1v). A total of 5 mice were thus treated. After 11 days, the mice were given another injection of IFA, and after 21 days they were sacrificed for tumor removal. The tumors developed as solid white tumor masses in the peritoneal cavity along the thoracic duct on both abdominal surfaces of the diaphragm and the upper surface of both lobes of the liver. The tumors were then explanted, mechanically disrupted in HuMedia-EG2 (Kurabo) and incubated in PBS containing 0.1% collagenase type I (Worthington) and trypsin-EDTA at 37°C for 30 min. Digestion was stopped by washing with HuMedia-EG2 containing 10% fetal calf serum (FCS). The cells were centrifuged at 1000 rpm for 5 min, resuspended in medium supplemented with 10% FCS, and plated in dishes previously coated with "Attachment Factor" (Cell Systems Corp.). Once confluence was reached, the cells were used in appropriate experiments or cultivated until they reached the third passage.

Tumor cells and endothelial cells. Lewis lung cancer (LLC) cells were maintained as monolayer cultures in EMEM supplemented with 10% FCS. Human chronic myelogenous leukemia K-562 cells were cultured as cell suspensions in RPMI1640 supplemented with 10% FCS. Hepatic sinusoidal endothelial (HSE) cells were seeded in culture flasks previously coated with "Attachment Factor" and maintained as monolayer cultures in DMEM/F12 supplemented with bovine endothelial mitogen (Biomedical Technologies, Inc.) (2 μg/ml) and 5% heat-inactivated FCS.

***In vitro* growth inhibition assay.** Cell growth was assessed using a WST-1 Cell Counting Kit (Wako Pure Chemical Ind., Ltd.). Briefly, LLC cells or LECs (1×10^4) respectively suspended in EMEM or HuMedia-EG2 (100 μl) containing 0.1% BSA were seeded in each well of a 96-well plate. In the case of LECs, the plate was previously coated with "Attachment Factor." After 24 h of incubation, various concentrations of MMI270 (100 μl) were added to the wells and the cultures were incubated for a further 24 h. WST-1 solution (10 μl) was added to each well and the cultures were incubated at 37°C for 2 h. The absorbance at 450 nm was measured in an immunoreader (Immuno Mini NJ-2300: Nippon Inter-Med. KK).

Tube formation assay. LECs were seeded in 60-mm-diameter culture dishes and, under sub-confluent conditions, the medium was replaced with fresh medium containing 0.2% FCS and various concentrations of MMI270. After 24 h of incubation, the cells were washed once with PBS, released with trypsin-EDTA and centrifuged at 1000 rpm for 5 min. The cell suspensions (4×10^4 cells) were then prepared in 0.2% FCS-supplemented HuMedia-EG2 (500 μl) and seeded in a 48-well plate that had been pre-coated with 100 μl of Matrigel (10 mg/ml). The formation of tube-like structures was monitored every hour for 6 h by means of observation under a microscope. After 6 h of observation, different fields of each well were photographed at 10× magnification. The photographs were printed out and, from each printed image the length of tube-like structures was measured with a graduated ruler. The extent of MMI270-induced inhibition of tube formation was examined by comparing the length of tube-like structures with that of the structures formed in the untreated control wells.

Invasion assay. The invasive properties of LLC cells and LECs were assayed in Transwell cell culture chambers with a membrane filter, as described previously.[16] Briefly, the chambers were attached to membrane filters (8.0-μm pore size: Nucleopore). The filters were precoated with 2 μg of fibronectin (Iwaki Glass) on their lower surfaces and dried at room temperature. Reconstituted BM Matrigel (Collaborative Research Co.) was applied to the upper surfaces of the filters (10 μg/50 μl/filter) and dried in the same way. The prepared filters (Matrigel/fibronectin-coated filters) were washed extensively in PBS and dried before use. Exponentially growing cells were harvested using 1 m*M* EDTA in PBS, washed twice with FCS-free medium and resuspended in 0.1% BSA medium at a density of 1×10^6 cells/ml with two concentrations of MMI270. One-hundred-microliter aliquots of the cell suspension were added to the upper compartment and incubated for 24 h at 37°C in a 5% CO_2/95% air atmosphere. The cells that invaded the lower surface were quantified by colorimetric measurement using the crystal violet staining method and an immunoreader. The extent of MMI270-induced inhibition of invasion was examined by comparing the absorbance values measured at 590 nm for the treated and untreated wells, and then expressed as a percentage in relation to the control.

Gelatin zymography. LLC cells suspended in EMEM supplemented with 10% FCS were firstly seeded in 25-cm^2 culture flasks. LECs were cultured as primary cultures in 60-mm-diameter dishes. While the cells were still sub-confluent, the media of both types of cells were replaced with DMEM/F12 medium (4 ml) containing 0.1% BSA, and the cells were cultured for a further 24 h. The culture supernatants were collected and centrifuged at 1000 rpm for 5 min to remove the debris. The conditioned media thus obtained were collected and stored at −80°C until use. In the case of LECs, because there was only a small amount of total protein in the culture supernatants, we concentrated the samples using a Centricon concentrator (Centricon YM-10: Amicon USA) with centrifugation at 5000g for 30 min. The prepared samples of LLC and LECs were then mixed with non-reducing sample buffer (62.5 m*M* Tris-HCl, pH 6.8,

10% glycerol, 0.00125% bromophenol blue, 2% SDS) and electrophoresed at 4°C on a 7.5% polyacrylamide gel containing 0.1% SDS and 0.1% gelatin. After electrophoresis, the gel was washed twice with rinsing buffer (50 m*M* Tris-HCl, pH 7.5, 2.5% Triton X-100, 5 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, 0.05% NaN_3) at room temperature for 1 h to remove SDS. Strips of the gel were incubated at 37°C for 24 h with the reaction buffer for assaying gelatinolytic activity (50 m*M* Tris-HCl, pH 7.5, 5 m*M* $CaCl_2$, 1 μ*M* $ZnCl_2$, 0.05% NaN_3) in the presence of various concentrations of MMI270, according to a procedure similar to that described in a previous report.[17] The gel strips were stained with staining solution (0.1% Coomassie Brilliant Blue, 10% acetic acid, 10% isopropanol). After the gel strips were destained with destaining solution (10% acetic acid, 10% isopropanol), the locations of the enzymes could be visualized as clear bands on the blue background. Gelatinolytic activity was quantified using a Master Scan Gel Analysis System (Scanalytics).

Reverse transcription-polymerase chain reaction (RT-PCR). The mRNA expression of MMPs, urokinase-type plasminogen activator (uPA) and a lymphatic endothelial marker by LECs and HSE cells was evaluated by semi-quantitative RT-PCR. Briefly, total RNAs from primary cultures of LECs or HSE cell cultures were extracted using Trizol reagent (Invitrogen Corp.). First-strand complementary DNA (cDNA) was prepared from RNA template (1 μg) using oligo$(dT)_{18}$ primer and SuperScript II reverse transcriptase (Invitrogen Corp.). The RT-reaction profile was 42°C for 50 min, followed by 70°C for 15 min. PCR amplification was performed by denaturation at 94°C for 30 s, annealing at 60 or 62°C for 1 min, and extension at 72°C for 1 min and 45 s, using template cDNA and a TaKaRa "Ex *Taq*" HS PCR kit (TaKaRa Shuzo Co., Ltd.). The sequences of primers were as follows: MMP-2 sense and antisense, 5′-CCT GAT GTC CAG CAA GTA GAT GC-3′ and 5′-TTA AGG TGG TGC AGG TAT CTG G-3′[18]; MMP-9 sense and antisense, 5′-TTC TCT GGA CGT CAA ATG TGG-3′ and 5′-CAA AGA AGG AGC CCT AGT TCA AGG-3′[18]; MT1-MMP sense and antisense, 5′-CCT GCA TCC ATC AAT ACT ACT GC-3′ and 5′-GCG TCT GAA GAA GAA GAC AGC-3′[18]; GAPDH sense and antisense, 5′-GGT GAA GGT CGG TGT CAA CGG ATT T-3′ and 5′-GAT GCC AAA GTT GTC ATG GAT GAC C-3′[18]; uPA sense and antisense, 5′-CGA ATA CTA CAG GGA AGA C-3′ and 5′-GAC ATT TTC AGG TTC TTT GG-3′; flt-4 sense and antisense, 5′-TTG GCA TCA ATA AAG GCA G-3′ and 5′-CTG CGT GGT GTA CAC CTT A-3′[15]; vWF sense and antisense, 5′-TGG TCC GCT ATG TCC AAG GT-3′ and 5′-TCT TAT TGA AGT TGG CTT CA-3′[15]; Flk-1 sense and antisense, 5′-GAC AAG ACA GCG ACT TGC-3′ and 5′-AAT TGT GTA TAC TCT GTC A-3′[15]; Tie-1 sense and antisense, 5′-GTG CTG GTC GGA GAG AAC CTG GCC T-3′ and 5′-AGG TGA AGT TCT CAA ACA GCG ACA T-3′[15]; Tie-2 sense and antisense, 5′-AGT TGG TGA AAA ACT ACA TAG-3′ and 5′- GGT AAA CTT CTC ATA CAG TGT GGT-3′[15]; ICAM1 sense and antisense, 5′-TTT TGC TCT GCC GCT CTG GAG-3′ and 5′-TAC ACA TTC CTG GTG ACA TTC-3′.[19] All these primers were confirmed to yield the expected products under these conditions. The PCR products were electrophoresed on 1.5% agarose gels and detected by ethidium bromide staining.

Western blotting analysis. HSE cells, LECs and K-562 cells were cultured in medium containing 10% FCS for 24 h. The cells were then washed with PBS (−) and lysed in sample buffer (25 m*M* Tris-HCl, pH 6.8, 5% w/v glycerol, 1% w/v SDS, 0.05% w/v bromophenol blue). Cell lysates were resolved by SDS-PAGE and transferred to Immobilon-P nylon membrane (Millipore). The membrane was treated with Block-Ace (Dainippon Pharmaceutical Co., Ltd.) for 2 h and probed with primary antibodies flt-4 and anti-mouse CD31 (1:200 and 1:100 dilutions, respectively). The antibodies were detected using horseradish peroxidase-conjugated anti-rabbit and anti-rat IgG (DAKO) (1:1500 dilution) and visualized with the ECL system (Amersham Biosciences).

Statistical analysis. The significance of differences between groups was determined by applying Student's two-tailed *t* test. A *P* value lower than 0.05 was considered to be significant.

Results

Inhibition of lymph node metastasis by MMI270. Lymph node metastasis was induced by the orthotopic intrapulmonary implantation of LLC cells into C57BL/6 mice,[14] and the inhibitory effect of MMI270 was analyzed by intraperitoneal implantation of an Alzet osmotic pump with a continuous-releasing function. Although the intraperitoneal administration of MMI270 did not cause a significant decrease in the weight of metastasized lymph nodes (data not shown), implantation of an osmotic pump containing this inhibitor led to clear inhibition of the lymph node metastasis (Fig. 1, A and C). The weight of primary tumors was lower in the MMI270-treated group than in the control group, although this difference was not statistically significant (Fig. 1, B and C). No significant changes in body weight during the course of the study were observed.

Characterization of murine lymphatic endothelial cells (LECs). To confirm the endothelial characteristics of the LECs induced by the intraperitoneal injection of IFA into C57/BL6 mice, we investigated the ability of these cells to form tube-like structures *in vitro*. LECs were seeded in Matrigel-coated wells and monitored for the formation of capillary tubes during a 6-h period. Fig. 2A shows the primary culture of LECs, with a classical cobblestone morphology, while Fig. 2B shows a well-branched

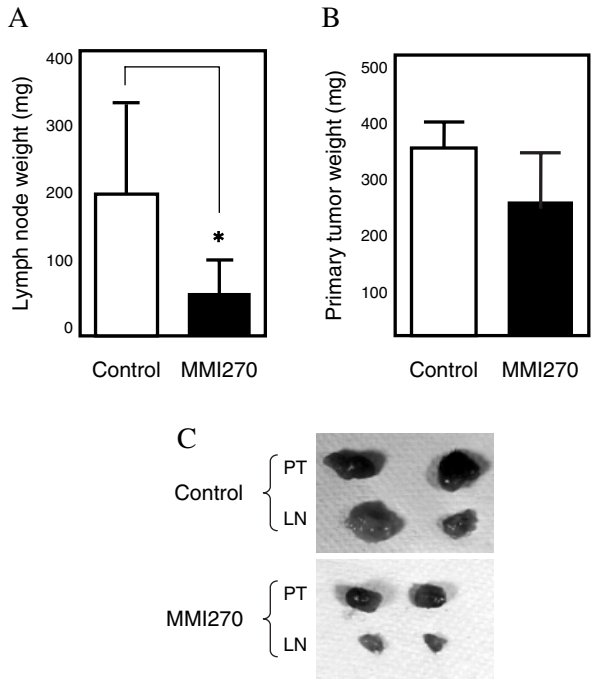

Fig. 1. Inhibition by MMI270 of lymph node metastasis induced by orthotopic intrapulmonary implantation of LLC cells. LLC cell suspension containing Matrigel was orthotopically implanted into the left lungs of C57BL/6 mice. On day 3 after the tumor implantation, Alzet osmotic pumps were implanted intraperitoneally, allowing the release of MMI270 or control solution for 15 days. On day 18 after tumor implantation, the weight of metastasized tumor at the mediastinal lymph node (A) and that of the primary tumor (B) were measured, and photographed (C). The data are expressed as the mean±SD of a group of 5 mice. * $P<0.05$ compared with the untreated control by Student's two-tailed *t* test. PT, primary tumor; LN, lymph nodes.

network with formation of long capillary tubes after culture on Matrigel. LECs were also characterized for expression of the lymphatic endothelial cell marker flt-4. Using semi-quantitative RT-PCR and western blot analyses, we confirmed the mRNA and protein expressions of flt-4 (approx. 150 kDa) in LECs, as previously reported,[15] and the lack of expression of this marker in HSE cells, that we used as a negative control (Fig. 2, C and D). In Fig. 2C, K-562 cells were used as a positive control for the expression of flt-4 according to the recommendations of the antibody manufacturer. We also confirmed the expression of endothelial cell markers in both LECs and HSE cells using western blotting (CD31/PECAM, 130–140 kDa) and RT-PCR (Flk-1, Tie-1 and Tie-2,[15] besides ICAM1) (Fig. 2, C and D). Expression of vWF mRNA by HSE cells and LECs was not observed (Fig. 2C), in agreement with reported data.[15] Furthermore, the expression of MMP-2, MMP-9, MT1-MMP and uPA mRNAs was also detected in LECs and HSE cells (Fig. 3A).

Inhibition by MMI270 of invasion, tube formation and gelatinolytic activity of MMP-2 and MMP-9 produced in LECs. The direct treatment of electrophoresed conditioned media of LECs with various concentrations of MMI270 caused a marked inhibition of the gelatinolytic activities of MMP-2 and -9. The samples of conditioned media from LECs treated with 1 μg/ml of inhibitor showed complete inhibition and almost complete inhibition of MMP-9 and MMP-2 activity, respectively (Fig. 3B).

MMI270 inhibited the growth of LECs at concentrations higher than 1 mg/ml (data not shown). Therefore, we used lower, non-cytotoxic doses of MMI270 to examine its effect on the invasion of these cells, and we observed that, at 100 μg/ml, it significantly inhibited the invasion of LECs (Fig. 4A) after a 24-h incubation. These results were representative of two independent experiments.

We also investigated the inhibitory effect of MMI270 on the ability of LECs to form tube-like structures, an ability which is considered to be crucial to lymphangiogenesis. Fig. 4B shows that addition of the inhibitor at 50, 100 or 200 μg/ml led to the decrease of total length of tube-like structures formed by LECs, in relation to the control. The results are representative of two independent experiments.

Inhibition by MMI270 of LLC cell invasion and gelatinolytic activity of MMP-2 and MMP-9 in the cells. The direct treatment of electrophoresed conditioned media of LLC cells with various concentrations of MMI270 led to inhibition of the gelatinolytic activities of MMP-2 and -9 to levels similar to those observed in LECs. At 1 μg/ml of MMI270, we detected complete inhibition and almost complete inhibition of MMP-9 and MMP-2 activity, respectively (Fig. 5A). When the effects of non-cytotoxic doses of MMI270 were tested on the invasion of LLC cells, we observed a significant inhibition at 1 μg/ml of both invasion (Fig. 5B) and migration (data not shown) after a 24-h incubation.

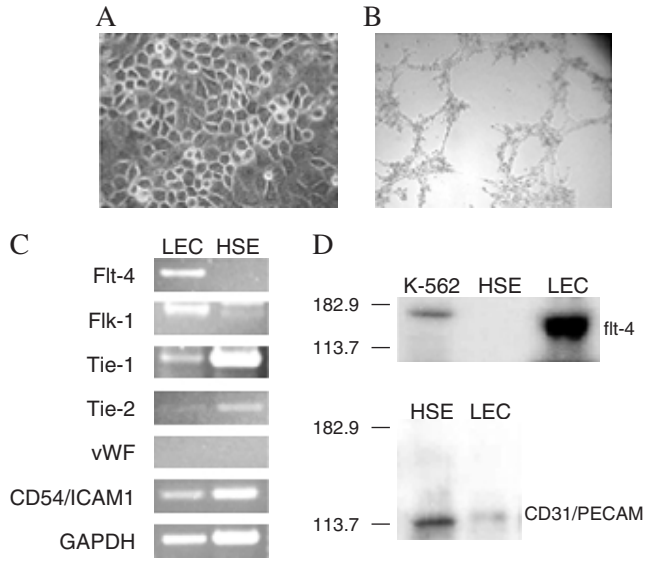

Fig. 2. Characterization of murine lymphatic endothelial cells (LECs) derived from lymphangiomas induced by intraperitoneal injection of IFA (200 μl of an emulsion containing IFA and PBS, 1v:1v). (A) Primary culture of LECs after 9 days of culture. (B) Formation of tube-like structures. Cell suspensions (4×10^4 cells) were seeded in 48-well plates previously coated with Matrigel. The formation of tube-like structures was monitored every hour for 6 h, visualized and photographed. (C) Expression patterns of mRNAs for lymphatic endothelial cell marker flt-4 and endothelial markers Flk-1, Tie-1, Tie-2, CD54/ICAM1 and vWF in cultured LECs and HSE cells. The PCR products for flt-4, Flk-1, Tie-1, Tie-2, ICAM1 and vWF (788, 654, 430, 427, 287 and 360 bp, respectively) were amplified for 35 cycles, while the product for GAPDH (502 bp) was amplified for 24 cycles. All products were electrophoresed on 1.5% agarose gels. (D) Protein expression of flt-4 in LECs and endothelial marker CD31/PECAM in LECs and HSE cells. Cells were cultured in medium containing 10% FCS for 24 h, washed with PBS (–) and then lysed in sample buffer. Whole cell lysates were immunoblotted with anti-flt-4 and anti-CD31 antibodies, detected using HRP-conjugated anti-rabbit and anti-rat IgG, respectively, and then visualized with the ECL system. The migration positions of markers are shown in kilodaltons.

Discussion

Clinical and pathological observations suggest that the transport of many carcinoma cells via the lymphatics is the most common pathway of initial dissemination, with patterns of spread via afferent lymphatics following the routes of natural drainage.[20] Moreover, the extent of lymph node metastasis is the most important factor influencing the prognosis for non-small

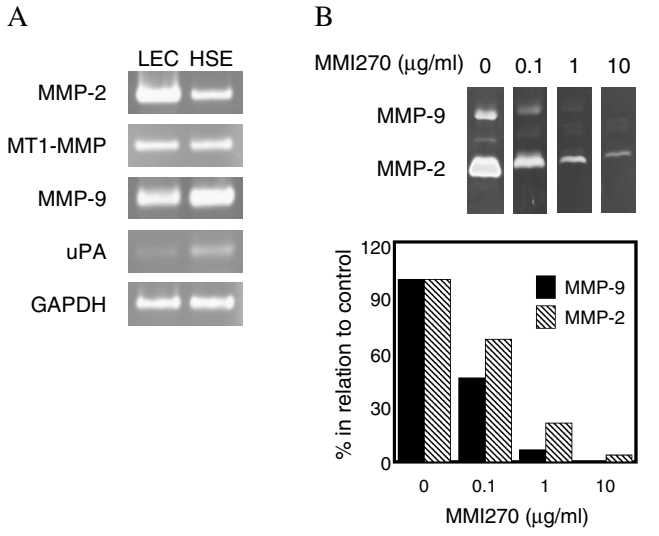

Fig. 3. mRNA expression of MMPs and uPA in LECs and HSE cells, and inhibition by MMI270 of the gelatinolytic activity of MMPs produced in LECs. (A) The expression patterns of mRNAs for various MMPs and uPA in LECs and HSE cells are shown. The PCR products for MMP-9, MMP-2, MT1-MMP and uPA (414, 760, 600 and 202 bp, respectively) were amplified for 35 cycles, while the product for GAPDH (502 bp) was amplified for 24 cycles. All products were electrophoresed on 1.5% agarose gels. (B) Direct effect of MMI270 on the gelatinolytic activity of MMP-2 and MMP-9 produced by LECs. Conditioned media of cells that were pre-incubated in 0.1% BSA-containing DMEM/F12 medium for 24 h were collected and subjected to electrophoresis in a gelatin-embedded SDS-polyacrylamide gel. After electrophoresis, strips of the gel were incubated with various concentrations of inhibitor for 24 h. The gel strips were then stained with Coomassie Brilliant Blue. The locations of the enzymes were visualized as clear bands on the blue background.

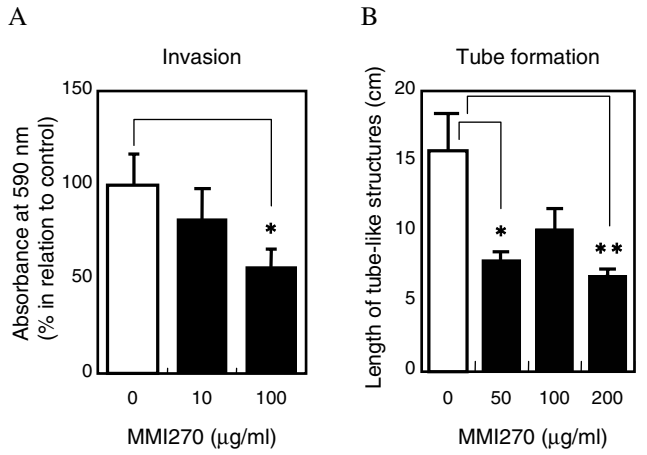

Fig. 4. Inhibition by MMI270 of invasion and tube formation of LECs. (A) For the invasion assay, cells were mixed with the indicated concentrations of MMI270 and added to the upper compartment of Transwell chambers. After 24 h of incubation, the cells that had invaded the lower surfaces were fixed with 30% methanol and stained with 0.5% crystal violet in 20% methanol for 5 min. The crystal violet dye retained on the filters was extracted with 30% acetic acid and the absorbance at 590 nm was measured colorimetrically. The data are expressed as a percentage in relation to the control group (quadruplicate cultures in each group). * $P<0.002$, compared with the untreated control by Student's two-tailed *t* test. (B) For the tube formation assay, cell suspensions were seeded in a 48-well plate previously coated with Matrigel. The formation of tube-like structures was monitored every hour for 6 h. The extent of inhibition of tube formation was analyzed by measuring the total length of tube-like structures visualized in the photographs of the cells. * $P=0.05$, ** $P<0.05$ compared with the untreated control by Student's two-tailed *t* test.

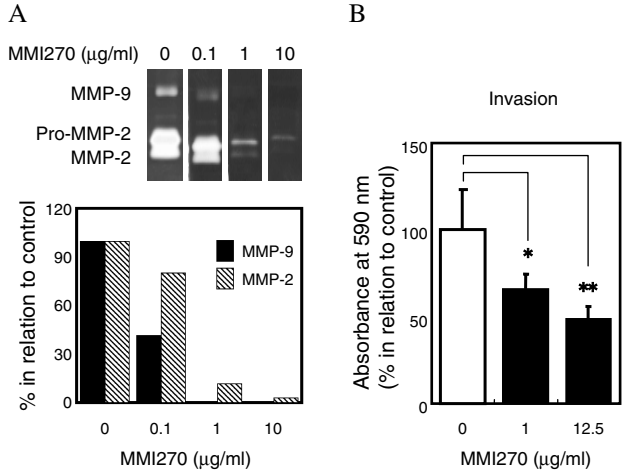

Fig. 5. Inhibition by MMI270 of the gelatinolytic activity of MMPs produced in LLC cells and invasion of LLC cells. For the gelatin zymography assay (A) and the invasion assay (B) we used procedures similar to those applied for LECs. (B) The data are expressed as a percentage in relation to the control group (quadruplicate cultures in each group). * $P<0.02$, ** $P<0.002$, compared with the untreated control by Student's two-tailed *t* test.

cell lung cancer (NSCLC). The 5-year survival rate after resection for patients with pN_2 disease is only 23%.[21] Therefore, the control of lymph node metastasis is a fundamental requirement during the treatment of lung cancer patients.

We have previously reported the efficacy of a synthetic MMP inhibitor, MMI270, in regulating *in vitro* capillary tube-like formation of murine HSE cells, and *in vivo* tumor-induced angiogenesis.[11] Based on that study and on recently reported findings concerning functional correlations between angiogenesis and lymphangiogenesis,[1, 6–8] we further aimed to investigate the anti-metastatic efficacy of MMI270 and to examine whether it could also regulate lymphangiogenesis-related properties of murine LECs and invasive properties of LLC cells, based on the hypothesis of a common involvement of MMPs in the processes of angiogenesis, lymphangiogenesis and metastasis.

Firstly, we investigated the effect of MMI270 on the mediastinal lymph node metastasis of LLC cells produced by the orthotopic intrapulmonary implantation of tumor cells. This model has been useful in the evaluation of anticancer drugs[14, 22, 23] and in the characterization of differential patterns of metastasis of human non-small cell lung cancers.[24] The continuous administration of MMI270 for 15 days using an osmotic pump led to a significant decrease in the weight of tumor-metastasized lymph nodes. Although a decreasing tendency in the primary tumors of the MMI270-treated group in relation to the control group was noted, this difference was not statistically significant (Fig. 1, B and C). The inhibitory effect of MMI270 on the lymph node metastasis of LLC cells (Fig. 1A) was further supported by *in vitro* assays showing inhibition of the gelatinolytic activities of MMP-2 and MMP-9 produced in LLC cells (Fig. 5A) and inhibition of the invasion of LLC cells (Fig. 5B).

Secondly, taking into account the lack of information concerning the participation of MMPs in lymphangiogenesis, despite numerous studies on angiogenesis, we attempted to investigate the functions of MMPs in murine LECs.

The establishment of primary cultures of LECs from lymphangiomas induced by the intraperitoneal injection of IFA into mice[15] was recently described as a reproducible and convenient source of lymphatic endothelial cells that possess *in vitro* propagating ability and undergo spontaneous differentiation, leading to formation of vessel-like structures. In order to characterize the LECs obtained in the present study, we initially observed the formation of tube-like structures by these cells (Fig. 2B) when they were seeded on a Matrigel-coated plate. We subsequently confirmed the protein and mRNA expression of the lymphatic endothelial cell marker flt-4 in the LECs,[15] but not in endothelial HSE cells. Flt-4 is known to be a useful marker of lymphatic endothelial cells, since, within the mature endothelia, it is exclusively expressed by lymphatic vessels.[25, 26] In addition, we observed the absence of mRNA expression of vWF in the lymphatic endothelial cells. This is in good agreement with previous reports indicating that vWF is not expressed in the lymphatic endothelium or in the lymph node HEVs (high endothelial venules) in humans.[20, 27] Although vWF is frequently used as a biochemical marker for endothelial cells, there are marked differences among the different murine tissues in the expression of vWF mRNA. In this regard, endothelial cells of vessels in the lung showed abundant expression of vWF mRNA, whereas endothelial cells of vessels in liver showed little or no expression of vWF mRNA.[28] Therefore, the lack of expression of vWF mRNA in HSE cells could be explained by a similar tissue distribution of vWF in the murine model. Furthermore, the protein expression of CD31/PECAM and mRNA expression of other endothelial markers such as Flk-1, Tie-1, Tie-2 and ICAM1 was confirmed in both LECs and HSE cells.

Concerning MMPs secreted by LECs, we detected for the first time mRNA expression of MMP-2, MMP-9 and MT1-MMP in these cells using a semi-quantitative RT-PCR assay. In addition, we detected the expression of uPA mRNA in LECs (Fig. 3A). The overexpression of uPA and uPA receptor has been correlated with lymphatic metastasis of lung cancer[29, 30] and uPA expression in bovine large vessel lymphatic endothelial cells was described in previous work.[31–33]

Concerning MMPs secreted by endothelial cells, recent findings have indicated that they play a critical role during angio-

genesis. When endothelial cells were cultured on Matrigel, the formation of tubular networks was increased by the addition of recombinant MMP-2 (gelatinase A) and decreased when neutralizing antibody or TIMP-2 was added.[34] The importance of MMP-9 (gelatinase B) in angiogenesis was shown in gelatinase B-deficient mice, which exhibited an abnormal pattern of skeletal growth plate vascularization and ossification.[35] Moreover, MT1-MMP (MMP-14) activation of MMP-2 and the functional role of this process in endothelial cell organization were demonstrated in an *in vitro* angiogenesis model of microvascular endothelial cells cultured within a collagen matrix.[36] MMPs have also been reported to correlate with the uPA/plasmin system, both in *in vivo* studies showing a close immunolocalization of the two systems[37–39] and in *in vitro* studies in which plasmin was able to activate several MMPs, such as MMP-1, MMP-3 and MMP-9.[40–42]

Once we had characterized the lymphatic endothelial features of LECs isolated in our study, we further investigated the effects of MMI270 on several lymphangiogenesis-related properties of these cells: 1) MMP-degrading activity; 2) invasion; and 3) tube formation.

The expression of MMP-2 and MMP-9 in LECs was detected by gelatin zymography, and the gelatinolytic activities of these MMPs were inhibited by MMI270 in a concentration-dependent manner. At low doses of the MMP inhibitor, a correspondence was observed between the almost complete inhibition of MMP-9 expressed in LECs (Fig. 3B) and endothelial HSE cells in our previous study.

The invasive property of endothelial cells during the process of angiogenesis is closely associated with expression of MMPs.[43] When we analyzed the invasive potential of LECs *in vitro*, we observed their ability to invade Matrigel and the inhibition of this invasion by the addition of MMI270 (Fig. 4A). Thus, we have observed for the first time the invasive ability of LECs and its relationship with MMPs expressed by the cells, demonstrating the common features shared by lymphatic endothelial cells and endothelial cells. Given the small number of reports dealing with the lymphangiogenic and angiogenic functions of other classes of metalloproteinases, such as ADAMs and astacin-like enzymes, we plan to investigate the expression and function of these enzymes in LECs in a future study.

Moreover our observation that the ability of LECs to form tube-like structures was inhibited by the MMP inhibitor (Fig. 4B) showed that these cells might act in a similar way to endothelial HSE cells during the formation of vessels through which tumor cells are transported from the primary tumor site to the target organ of metastasis. Accordingly, we suppose that MMPs secreted by LECs might be important in degrading the tissues near the original sites where the LECs form capillaries, subsequently allowing the tumor cells to spread via the lymphatics.

In the context of anticancer therapy, the inhibition of lymphangiogenesis and lymphatic metastasis represents a new potential target. So far, studies on lymphangiogenesis have been restricted to the VEGF family of cytokines (VEGF-D, VEGF-A), and some therapeutic approaches such as the application of soluble VEGFR-3 in transgenic mice[44] and in VEGF-C-overexpressing MCF-7 tumors[45] have been tested. However, the present results suggest that MMP inhibition might also be evaluated as an alternative approach for the regulation of lymphatic metastasis. This would be consistent with our results indicating that MMPs might mediate lymphangiogenesis and tumor metastasis. In the future, this could be confirmed by means of immunohistochemical studies and quantification of specific lymphatic markers by real-time quantitative PCR.

In summary, the present study showed that MMI270 inhibited the lymph node metastasis of orthotopically implanted LLC cells, in addition to regulating lymphangiogenesis-related properties of lymphatic endothelial cells (MMPs-degrading activity, invasion and tube formation). This suggests that MMPs might be mediators of lymphangiogenesis, angiogenesis and tumor metastasis.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology, Japan: a Grant-in-Aid for Cancer Research (No. 14030028), a Grant-in-Aid for Leading Regional Utilizing Potential of Regional Science and Technology, and a Grant-in-Aid for the 21st COE Program.

1. Jussila L, Alitalo K. Vascular growth factors and lymphangiogenesis. *Physiol Rev* 2002; **82**: 673–700.
2. 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.
3. Jackson DG. New molecular markers for the study of tumour lymphangiogenesis. *Anticancer Res* 2001; **21**: 4279–83.
4. Schoppmann SF, Horvat R, Birner P. Lymphatic vessels and lymphangiogenesis in female cancer: mechanisms, clinical impact and possible implications for anti-lymphangiogenic therapies. *Oncol Rep* 2002; **9**: 455–60.
5. Baldwin ME, Stacker SA, Achen MG. Molecular control of lymphangiogenesis. *Bioessays* 2002; **24**: 1030–40.
6. Cao Y, Linden P, Farnebo J, Cao R, Eriksson A, Kumar V, Qi JH, Claesson-Welsh L, Alitalo K. Vascular endothelial growth factor C induces angiogenesis *in vivo*. *Proc Natl Acad Sci USA* 1998; **95**: 14389–94.
7. Oliver G, Detmar M. The rediscovery of the lymphatic system: old and new insights into the development and biological function of the lymphatic vasculature. *Genes Dev* 2002; **16**: 773–83.
8. Nagy JA, Vasile E, Feng D, Sundberg C, Brown LF, Detmar MJ, Lawitts JA, Benjamin L, Tan X, Manseau EJ, Dvorak AM, Dvorak HF. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med* 2002; **196**: 1497–506.
9. Mandriota SJ, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 2001; **20**: 672–82.
10. 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.
11. Nakamura ES, Koizumi K, Yamaura T, Saiki I. Anti-tumor angiogenic effect of a matrix metalloproteinase inhibitor MMI-270. *Anticancer Res* 2003; **23**: 411–8.
12. Wood JM, Muller M, Schnell C, Cozens RM, O'Reilly T, Cox D, Ganu V, Melton R, Parker DT, MacPerson LJ, Nakajima M, Reich R. CGS 27032A, a potent and orally active matrix metalloproteinase inhibitor with antitumor activity. *Proc. Am Assoc Cancer Res* 1998; **39**: 83.
13. Levitt NC, Eskens FA, O'Byrne K, Propper DJ, Denis LJ, Owen SJ, Choi L, Foekens JA, Wilner S, Wood JM, Nakajima M, Talbot DC, Steward WP, Harris AL, Verweij J. Phase I and pharmacological study of the oral matrix metalloproteinase inhibitor, MMI-270 (CGS27023A), in patients with advanced solid cancer. *Clin Cancer Res* 2001; **7**: 1912–22.
14. Doki Y, Murakami K, Yamaura T, Sugiyama S, Misaki T, Saiki I. Mediastinal lymph node metastasis model by orthotopic intrapulmonary implantation of Lewis lung carcinoma cells in mice. *Br J Cancer* 1999; **79**: 1121–6.
15. Mancardi S, Stanta G, Dusetti N, Bestagno M, Jussila L, Zweyer M, Lunazzi G, Dumont D, Alitalo K, Burrone OR. Lymphatic endothelial tumors induced by intraperitoneal injection of incomplete Freund's adjuvant. *Exp Cell Res* 1999; **246**: 368–75.
16. Saiki I, Murata J, Watanabe K, Fujii H, Abe F, Azuma I. Inhibition of tumor cell invasion by ubenimex (bestatin*) in vitro*. *Jpn J Cancer Res* 1989; **80**: 873–8.
17. Sawada S, Murakami K, Yamaura T, Mitani N, Tsukada K, Saiki I. Therapeutic and analysis model of intrahepatic metastasis reflects clinical behavior of hepatocellular carcinoma. *Jpn. J. Cancer Res*. 2002; **93**: 190–7.
18. Sawada S, Murakami K, Murata J, Tsukada K, Saiki I. Accumulation of extracellular matrix in the liver induces high metastatic potential of hepatocellular carcinoma to the lung. *Int J Oncol* 2001; **19**: 65–70.
19. Tsuchiya Y, Sawada S, Yoshioka I, Ohashi Y, Matsuo M, Harimaya Y, Tsukada K, Saiki I. Increased surgical stress promotes tumor metastasis. *Surgery* 2003; **133**: 547–55.
20. Pepper MS. Lymphangiogenesis and tumor metastasis: myth or reality? *Clin Cancer Res* 2001; **7**: 462–8.

21. Mountain CF. Revisions in the international system for staging lung cancer. *Chest* 1997; **111**: 1710–7.
22. Mitani N, Murakami K, Yamaura T, Ikeda T, Saiki I. Inhibitory effect of berberine on the mediastial lymph node metastasis produced by orthotopic implantation of Lewis lung carcinoma. *Cancer Let* 2001; **165**: 35–42.
23. Ichiki K, Mitani N, Doki Y, Hara H, Misaki T, Saiki I. Regulation of activator protein-1 activity in the mediastinal lymph node metastasis of lung cancer. *Clin Exp Metastasis* 2001; **18**: 539–45.
24. Yamaura T, Murakami K, Doki Y, Sugiyama S, Misaki T, Yamada Y, Saiki I. Solitary lung tumors and their spontaneous metastasis in athymic nude mice orthotopically implanted with human non-small cell lung cancer. *Neoplasia* 2000; **2**: 315–24.
25. 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.
26. Veikkola T, Alitalo K. VEGFs, receptors and angiogenesis. *Semin Cancer Biol* 1999; **9**: 211–20.
27. Schlingemann RO, Rietveld FJ, Kwaspen F, van de Kerkhof PC, de Waal RM, Ruiter DJ. Differential expression of markers for endothelial cells, pericytes and basal lamina in the microvasculature of tumors and granulation tissue. *Am J Pathol* 1991; **138**: 1335–47.
28. Yamamoto K, de Waard V, Fearns C, Loskutoff DJ. Tissue distribution and regulation of murine von Willebrand factor gene expression *in vivo*. *Blood* 1998; **92**: 2791–801.
29. Oka T, Ishida T, Nishino T, Sugimachi K. Immunohistochemical evidence of urokinase-type plasminogen activator in primary and metastatic tumors of pulmonary adenocarcinoma. *Cancer Res* 1991; **51**: 3522–5.
30. Nagayama M, Sato A, Hayakawa H, Urano T, Takada Y, Takada A. Plasminogen activators and their inhibitors in non-small cell lung cancer. Low content of type 2 plasminogen activator inhibitor associated with tumor dissemination. *Cancer* 1994; **73**: 1398–405.
31. Pepper MS, Wasi S, Ferrara N, Orci L, Montesano R. *In vitro* angiogenic and proteolytic properties of bovine lymphatic endothelial cells. *Exp Cell Res* 1994; **210**: 298–305.
32. Liu N-F, He Q-L. The regulatory effects of cytokines on lymphatic angiogenesis. *Lymphology* 1997; **30**: 3–12.
33. Pepper MS, Mandriota SJ, Jeltsch M, Kumar V, Alitalo K. Vascular endothelial growth factor (VEGF)-C synergises with basic fibroblast growth factor and VEGF in the induction of angiogenesis *in vitro*, and alters endothelial cell proteolytic properties. *J Cell Physiol* 1998; **177**: 439–52.
34. Schnaper HW, Grant DS, Stetler Stevenson WG, Fridman R, D'Orazi G, Murphy AN, Bird RE, Hoythya M, Fuerst TR, French DL. Type IV collagenase(s) and TIMPs modulate endothelial cell morphogenesis *in vitro*. *J Cell Physiol* 1993; **156**: 235–46.
35. Vu TH, Shipley M, Bergers G, Berger JE, Helms JA, Hanahan D, Shapiro SD, Senior RM, Werb Z. MMP-9/gelatinase B is a key regulator of growth plate angiogenesis and apoptosis of hypertrophic chondrocytes. *Cell* 1998; **93**: 411–22.
36. Haas TL, Davis SJ, Madri JA. Three-dimensional type I collagen lattices induce coordinate expression of matrix metalloproteinases MT1-MMP and MMP-2 in microvascular endothelial cells. *J Biol Chem* 1998; **273**: 3604–10.
37. Newman KM, Jean-Claude J, Li H, Scholes JV, Ogata Y, Nagase H, Tilson MD. Cellular localization of matrix metalloproteinases in the abdominal aortic aneurysm wall. *J Vasc Surg* 1994; **20**: 814–20.
38. Walter H, Kawashima A, Nebelung W, Neumann W, Roessner A. Immunohistochemical analysis of several proteolytic enzymes as parameters of cartilage degradation. *Pathol Res Pract* 1998; **194**: 73–81.
39. Carmeliet P, Moons L, Lijnen R, Baes M, Lemaitre V, Tipping P, Drew A, Eeckhout Y, Shapiro S, Lupu F, Collen D. Urokinase-generated plasmin activates matrix metalloproteinases during aneurysm formation. *Nat Genet* 1997; **17**: 439–44.
40. Okumura Y, Sato H, Seiki M, Kido H. Proteolytic activation of the precursor of membrane type 1 matrix metalloproteinase by human plasmin. A possible cell surface activator. *FEBS Lett* 1997; **402**: 181–4.
41. Santibanez JF, Martinez J. Membrane-associated procollagenase of leukemic cells is activated by urokinase-type plasminogen activator. *Leuk Res* 1993; **17**: 1057–62.
42. Murphy G, Stanton H, Cowell S, Butler G, Knauper V, Atkinson S, Gavrilovic J. Mechanisms for pro matrix metalloproteinase activation. *APMIS* 1999; **107**: 38–44.
43. Westermarck J, Kahari V-M. Regulation of matrix metalloproteinase expression in tumor invasion. *FASEB J* 1999; **13**: 781–92.
44. Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S, Yla-Herttuala S, Alitalo K. Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* 2001; **7**: 199–205.
45. Karpanen T, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo V. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer R*es 2001; **61**: 1786–90.