Biochemical and Biophysical Research Communications **285,** 118–128 (2001)
doi:10.1006/bbrc.2001.5135, available online at http://www.idealibrary.com on IDEAL®

Tumor Autocrine Motility Factor Is an Angiogenic Factor That Stimulates Endothelial Cell Motility

Tatsuyoshi Funasaka,* Arayo Haga,*[,1] Avraham Raz,† and Hisamitsu Nagase*
**Department of Hygienics, Gifu Pharmaceutical University, Gifu 502, Japan; and †Oncology, Pathology and Radiation Oncology, Barbara Ann Karmanos Cancer Institute, Wayne State University, School of Medicine, Detroit, Michigan 48210*

Received May 25, 2001

Autocrine motility factor (AMF) is a type of tumor-secreted cytokine which primarily stimulates tumor cell motility via receptor-mediated signaling pathways, and is thought to be connected to tumor progression and metastasis. Using *in vivo* models, we showed that critical neovascularization responded to a biological amount of AMF. This angiogenic activity was fixed by specific inhibitors against AMF. AMF stimulated *in vitro* motility of human umbilical vein endothelial cells (HUVECs), inducing the expression of cell surface AMF receptor localizing a single predominant perinuclear pattern closely correlated with its motile ability. AMF also elicited the formation of tube-like structures mimicking angiogenesis when HUVECs were grown in three-dimensional type I collagen gels. We further immunohistochemically detected AMF receptors on the surrounding sites of newborn microvessels. These findings suggest that AMF is a possible tumor progressive angiogenic factor which may act in a paracrine manner for the endothelial cells in the clinical neoplasm, and it will be a new target for anti-angiogenic treatment. © 2001 Academic Press

Key Words: **AMF; gp78; cell motility; tumor angiogenesis; metastasis.**

Metastasis is an important clinical parameter in the prognosis of patients who develop malignant tumors. It generally occurs via the vascular or lymphangial system on distant organs such as the liver or lung metastasis of colon cancer; therefore, metastasis is closely related to the vascular system. Another important relation between metastasis and blood vessels is angiogenesis. For solid tumors of more than several millimeters in diameter, nutrition and oxygen supplies are essential from tumor-generated new blood vessels. Therefore, solid tumors cannot grow without the induction of angiogenesis (1). Tumor growth is accelerated with the induction of angiogenesis, invading into the surrounding host tissue, and disseminating to distant organs. There are reported to be correlations between tumor vascular density and clinical malignancy in numerous malignant tumors, and an unfavorable prognosis for tumors with a high density of vessels (2–4).

Molecular mechanisms of angiogenesis have been investigated since angiogenesis was recognized to play a serious role in solid tumor progression. It is well known that the processes of neovascularization are postulated to synchronize with the up-regulation or down-regulation of several angiogenic factors (5). Tumor angiogenesis is promoted by angiogenic-stimulating factors alone or in combination, such as vascular endothelial growth factor (VEGF), platelet-derived endothelial cell growth factor (PD-ECGF), and interleukin 8 (IL-8) which are overexpressed in solid tumors (6–10). The angiogenic factors modulate the single or multiple phases among the three processes of angiogenesis: (i) enzymatic degradation of the basement membrane; (ii) endothelial cell migration; and (iii) endothelial cell multiplication. The mechanisms of angiogenesis have been elucidated with the demonstration of the roles of factors in each process. We focused on "endothelial cell migration," as so far little investigation has been done on this aspect.

Autocrine motility factor (AMF) is a major cell motility-stimulating factor associated with the development of tumors. AMF was originally purified from the conditioned medium of human A2058 melanoma cells as a cytokine with an estimated size of 55 kDa and stimulates the motility of the producing cells directly or at random (11). The stimulation of tumor cell motility with AMF is induced by AMF-binding to the cell surface of the 78 kDa glycoprotein AMF receptor gp78 (12, 13). Receptor stimulus with AMF leads to a pertussis toxin-sensitive G-protein activation (14), inositol

Abbreviations used: AMF, autocrine motility factor; E4P, erythrose 4-phosphate; HUVECs, human umbilical vein endothelial cells; PHI, phosphohexose isomerase.

[1] To whom correspondence should be addressed at Department of Hygienics, Gifu Pharmaceutical University, 5-6-1 Mitahora-Higashi, Gifu 502-8585, Japan. Fax: (058) 237-5979. E-mail: arayo@gifu-pu.ac.jp.

0006-291X/01 $35.00

trisphosphate production (15), protein kinase C activation (16, 17), and receptor phosphorylation (18). These alterations of intracellular metabolism are considered to promote cell locomotion. There have been many reports that enhanced expression of AMF and AMF receptor is correlated with progression of malignant tumors. AMF has been purified from the culture media of various tumor strains as an autocrine-type motility factor, and its mechanism, including interaction with its receptor gp78, has been investigated.

The remarkable property of AMF is identified with phosphohexose isomerase/neuroleukin (PHI/NLK), which catalyzes conversion of glucose 6-phosphate to fructose 6-phosphate (19). Furthermore, it is recognized that AMF has PHI enzymatic activity. AMF activities are inhibited with specific PHI inhibitor, and commercial PHI has AMF activities. AMF secretion has never been found on normal cells, although AMF has molecular similarities to the PHI family which is a kind of enzyme associated with intracellular saccharic metabolism. However, it is presumed that the AMF/PHI extracellular receptor may be expressed on normal cells due to PHI distribution. Quite recently, Shimizu *et al.* recognized the expression of the AMF receptor mRNA in various mouse normal tissues (20). This possibly suggests that tumor AMF may react to normal cells through its gp78 or an unknown receptor on the membrane and affect and control its motile conditions. That is, AMF not only stimulates AMF-producing tumor cell motility in an autocrine manner, but also acts as a paracrine factor to vein endothelial cells to induce angiogenesis with cell motility stimulation, and may facilitate metastasis through these effects on the metastasis phase. Thus, we examined whether AMF may exhibit angiogenic activity by focusing on endothelial cell motility, which is a previously unrecognized biological property of AMF.

MATERIALS AND METHODS

Antibodies and reagents. The anti-gp78 rat monoclonal antibody (3F3A) was used in the form of either ascites fluid or concentrated hybridoma supernatant (12, 13, 18). The anti-recombinant human AMF polyclonal antibody was obtained from immunized New Zealand white rabbit serum by conventional methods. The fluorescein isothiocyanate (FITC)-conjugated goat anti-rat antibody was purchased from Biochemical Technologies Inc. (Stoughton, MA). The horseradish peroxidase (HRP)-conjugated goat anti-rabbit or rat antibody was purchased from American Qualex (San Clemente, CA). Type I collagen was obtained from Funakoshi (Tokyo, Japan), and reconstituted basement membrane Matrigel was purchased from Collaborative Research Inc. (Bedford, MA). Erythrose 4-phosphate (E4P) was purchased from Sigma (St. Louis, MO). 3,3′-Diaminobenzidine tetrahydrochloride (DAB) was purchased from GIBCO BRL (Rockville, MD).

Cell culture. Human umbilical endothelial cells (HUVECs) obtained from Cell Application, Inc. (San Diego, CA), were maintained in RPMI 1640 medium supplemented with 20% heat-inactivated fetal bovine serum (FBS) and endothelial cell growth supplement (ECGS; Collaborative Research Inc., Bedford, MA). Human fibrosarcoma HT-1080 cells were cultured with Dulbecco's modified Eagle medium (DMEM) containing 10% FBS. These lines were maintained at 37°C in an air–5.0% CO_2 incubator under mycoplasma-free conditions.

AMF mutant lines and recombinant human AMF. Cloned AMF cDNA and ribozyme against AMF mRNA (21) were inserted into the mammalian expression vector pBK-CMV (Amersham Pharmacia Biotech, Uppsala, Sweden) at an *Eco*RI site and transfected to HT-1080 cells. Three stable transfectant lines, AMF-rich, AMF-knockout and mock, were maintained with 500 μg/ml G-418. Expression and secretion of AMF protein were confirmed by Western blot analysis using serum-free conditioned media from these three clones. The AMF secretion activity of AMF-rich transfectant line was 4.0-fold higher than that of the control counterpart (mock), which showed the same level as parental cells.

Recombinant human AMF (rhAMF) was created as a glutathione S-transferase (GST) fusion protein (22). AMF cDNA was inserted into *Escherichia coli* expression vector pGEX-6P (Amersham Pharmacia Biotech) and GST-free AMF was purified by using recombinant protein purification system (Amersham Pharmacia Biotech). rhAMF was recognized as a single band on the silver-stained gel of sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).

Cell motility assay. Cell motility was analyzed by using a phagokinetic track assay (23). Coverslips were coated with a uniform layer of 1.0% bovine serum albumin (BSA) by means of fixing with 100% ethanol and warm air-drying. The treated coverslips were then embedded with colloidal gold particles and placed onto six multiwell plates (SUMILON) with DMEM containing AMF. Then 2.0×10^3 HUVECs were seeded on each coverslip. After 24 h of culture, phagokinetic tracks were visualized using dark-field illumination and at least 50 gold particle-free fields were measured.

Immunofluorescent detection. HUVECs were seeded on coverslips and cultured with or without AMF for 24 h. These cells on the coverslips were then fixed with 3.5% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min at room temperature or ice-cooled (−80°C) methanol for 30 min at −20°C for the cell surface or permeabilized immmunofluorescent stain. The cells were washed three times with PBS and incubated with 10% 3F3A in PBS for 30 min at room temperature. After incubation with primary antibody, the coverslips were washed and incubated in the dark with 5.0% FITC-conjugated goat anti-rat antibody in PBS as a secondary antibody for 30 min at room temperature. The coverslips were washed extensively and then mounted on slides with glycerol, and fluorescent images were visualized using a Laser Scanning Microscope (LSM 510; Carl Zeiss Co., Ltd.).

In vitro angiogenesis assay. The assay for *in vitro* tube formation of HUVECs was performed by the reported method with slight modification (24). HUVECs suspended in RPMI 1640 containing 1.0% FBS were cultured for 24 h on the surface of 0.2% type I collagen prepared with exposure of RPMI 1640 containing 1.0% FBS poured onto collagen gel for 1 h in 24 multiwell plates (SUMILON). After the medium was removed at the end of the incubation, an equivalent volume of 0.2% collagen solution was overlaid and the plates were allowed to stand for 1 h to solidify the collagen. Then 1.0% FBS–RPMI 1640 with or without AMF was added to the well and cultured. The medium was changed every other day and morphological changes which resembled a tubular structure in the gel were observed and photographed under a phase-contrast microscope after a 10-day culture.

In vivo Matrigel plug assay. Matrigel plug assay according to the report by Passaniti *et al.* (25) was performed to examine the potential effect of AMF on angiogenesis *in vivo.* Matrigel (10 mg/ml) in liquid form at 4°C was mixed with AMF and injected subcutaneously into the abdominal region of BALB/c mice (0.5 ml volume each). Matrigel rapidly forms a plug at body temperature after injection, and the

holding factor allows slow release and long exposure to surrounding tissues. After 14 days, the Matrigel plugs were removed, fixed in Bouin's solution and embedded in paraffin. Histological sections of the gel were stained with hematoxylin and eosin. The ingrowth of blood vessels was microscopically observed.

Tumor angiogenesis assay. HT-1080 AMF-transfectant lines were suspended in PBS at a concentration of 1.0×10^6 cells/ml, and 0.2 ml of this suspension was injected into a diffusion chamber consisting of a ring (Millipore) covered with 0.45-μm-pore filters (Millipore) on both sides. The chamber containing tumor cells was inserted into a dorsal air sac of BALB/c mice made by injecting air. The mice were sacrificed, the skins contacted to the chamber were carefully removed on day 3 or 5, and the newly formed capillary networks were observed and photographed.

Immunohistochemistry. The resected skins showing neovascularization were fixed in Bouin's solution, embedded in paraffin, and sliced into 5-μm-thick sections. To prevent endogenous peroxidases, the deparaffinized sections were incubated with 0.3% H_2O_2 in methanol at room temperature for 30 min. After blocking with 3.0% BSA in PBS, the slides were exposed to rabbit anti-rhAMF or 3F3A in PBS at room temperature for 15 min. They were then reacted with a 10% HRP-conjugated goat anti-rabbit or rat antibody at room temperature for 15 min and developed according to the DAB stain methods.

RESULTS

AMF Stimulates Endothelial Cell Motility

AMF was originally identified as a cytokine that enhances tumor cells motility in an autocrine manner and was identified with PHI/NLK, which is a consequential intracellular molecule (11, 19). AMF is recognized as a secretory cytokine only from tumor cells, but may have an effect on normal tissues that should have AMF receptor gp78, as PHI universally exists in normal tissues. Therefore, we questioned the effect of AMF on normal cells, especially endothelial cells. The effect of AMF on endothelial cell motility was evaluated by phagokinetic analysis of overnight cultures of HUVECs. The average motile area of the untreated HUVECs was 15.7 μm^2/h, and the motility response was stimulated by AMF treatment (Fig. 1). AMF stimulated the motility of HUVECs approximately 2.0-fold at an AMF concentration of 50 pg/ml to that without stimulation ($P < 0.001$, Student's t test). The stimulation by AMF on HUVECs motility was at the same level as for tumor cells and the effective dose was similar to that on tumor cells. However, AMF did not affect the endothelial cell proliferation *in vitro* with the effective dose against HUVEC motility (data not shown).

AMF Receptor Expression on Endothelial Cells Surface

Tumor cell locomotion enhanced by AMF results from the binding of AMF to gp78. Such AMF-stimulated motility of HUVECs raises the question of whether motility stimulation is dependent on the interaction between AMF and gp78-like tumor cells. To examine the distribution of gp78 on the cell surface or intracellular expression of HUVECs, cells were immunofluorescently stained with anti-gp78 antibodies. As shown in Fig. 2, gp78 was not detected on the surface of untreated cells, while AMF exposed cells exhibited gp78 expression, which closely correlated with their motility response. Furthermore, the gp78 expression on the cell surface was localized predominantly in a single perinuclear pattern with motility enhancement having some similarity to high-locomotive tumor cell localization (12, 14). There was little difference in the distribution of intracellular gp78, although it was expressed with scattered vesicles (data not shown).

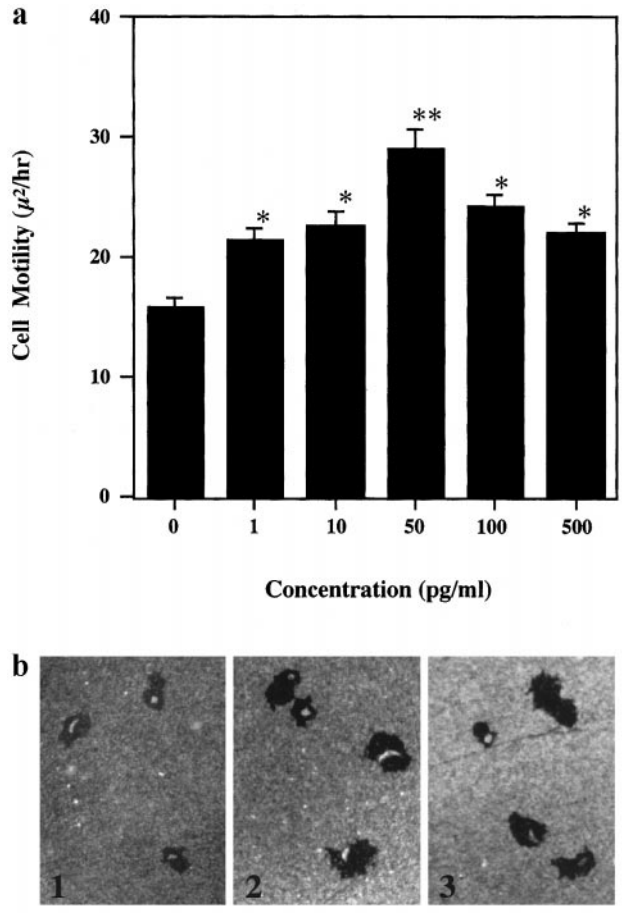

FIG. 1. Motility response of HUVECs stimulated by AMF exposure. (a) HUVECs were plated on colloidal gold cover slides in culture medium with or without various concentrations of AMF. After 24 h, the cells were fixed and the migration of at least 50 fields was photographed and each motile area was calculated by NIH Image software. Values are expressed as the mean ± SEM. $*P < 0.01$; $**P < 0.001$ by Student's t test. (b) Typical images of this assay. 1, control HUVECs; 2, 50 pg/ml; 3, 100 pg/ml AMF-treated HUVECs, respectively. Magnification, ×40.

Promotive Ability of AMF to Organize Capillary Tubes of Endothelial Cells

To accomplish the process of angiogenesis, migrating endothelial cells must undergo morphogenesis such as

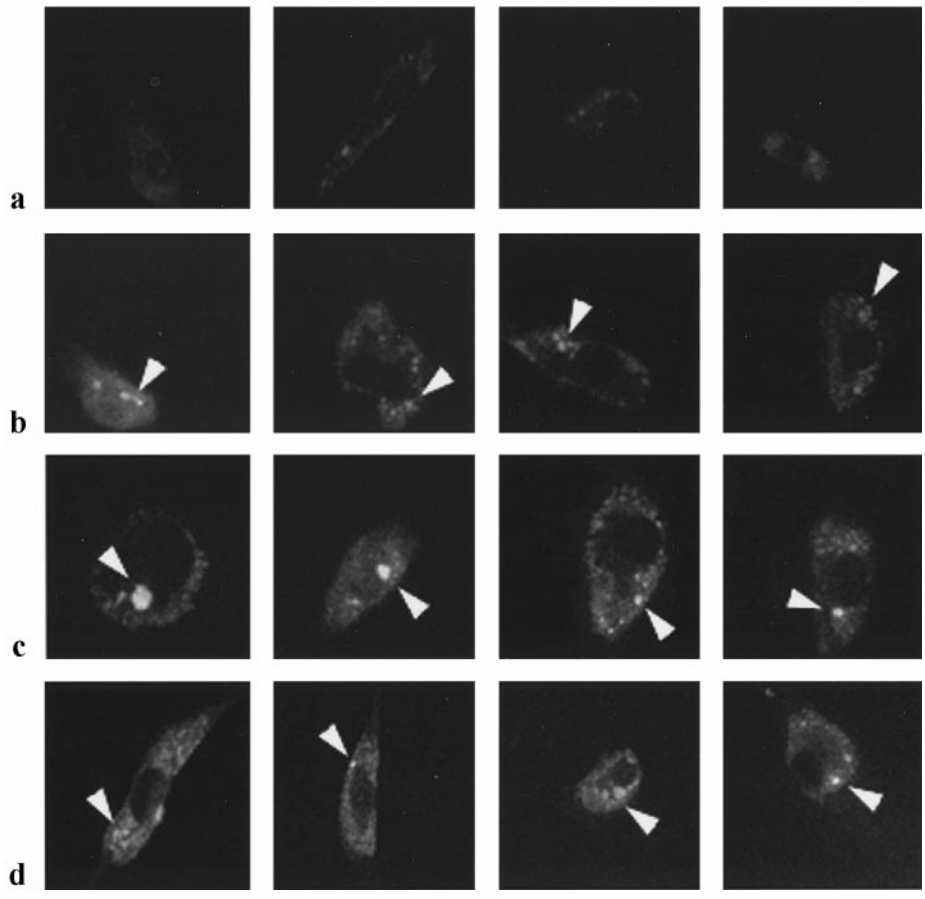

FIG. 2. gp78 expression on HUVECs surface after the exposure with AMF. HUVECs were exposed to various concentrations of AMF for 24 h. The cells were fixed, processed for immunofluorescent staining to detect the surface gp78 as described under Materials and Methods and photographed with magnification, ×1000. Arrow indicates the corresponding location of gp78 fluorescence on the cells. a, untreated with AMF; b, 10 pg/ml; c, 50 pg/ml; d, 100 pg/ml AMF exposure, respectively.

formation of capillary-like tubes. We investigated the possibility that AMF affects tubular morphogenesis *in vitro*. As described under Materials and Methods, HUVECs were plated onto collagen gels, and cultured with or without AMF. After 10 days of culture, AMF induced a network of branched and associated elongated cells which often anastomosed with one another (Fig. 3b). While HUVECs maintained in the control medium at the same time constituted tube-like structures in the collagen gels, the structures were obviously less than that of AMF-treated cells. The exposure of 50 pg/ml of AMF caused about 2.5-fold higher *in vitro* angiogenesis than at the control level (Fig. 3a).

In Vivo Angiogenic Effect of AMF

To examine the potential effect of AMF on angiogenic activity *in vivo*, we used two different assays. One was the Matrigel plug assay; Matrigel impregnated with samples were injected subcutaneously into mice. By this assay, we tested whether AMF would be able to promote new vessel formation in Matrigel plugs. Mice injected with Matrigel containing AMF (1, 10, 100 or 1000 ng) were sacrificed for histological analysis of the Matrigel plugs after 14 days. Microscopic histological analysis of hematoxylin and eosin-stained plugs showed little cellularity on the border of the control plugs (Fig. 4a), but a large number of invaded cells and vessels were recognized in the plugs containing 10 ng AMF (Fig. 4b). As for the plugs with 100 ng of AMF, even more invaded cells and considerable tubular structures were recognized (Fig. 4c). Histological examination revealed half-maximal and maximal angiogenic responses of AMF at 10 and 100 ng, respectively.

In the other assay, we investigated the contribution of AMF on tumor-induced angiogenesis. In this *in vivo* model, a diffusion chamber containing tumor cells was transplanted into a mouse dorsal air sac. Extensive capillary networks developed in the dorsal subcutis in contact with transplanted chamber. The AMF mutants of human fibrosarcoma HT-1080, AMF-high expression or AMF-knockout, were transplanted into the chamber, and the effect of AMF was assayed (Fig. 5). The

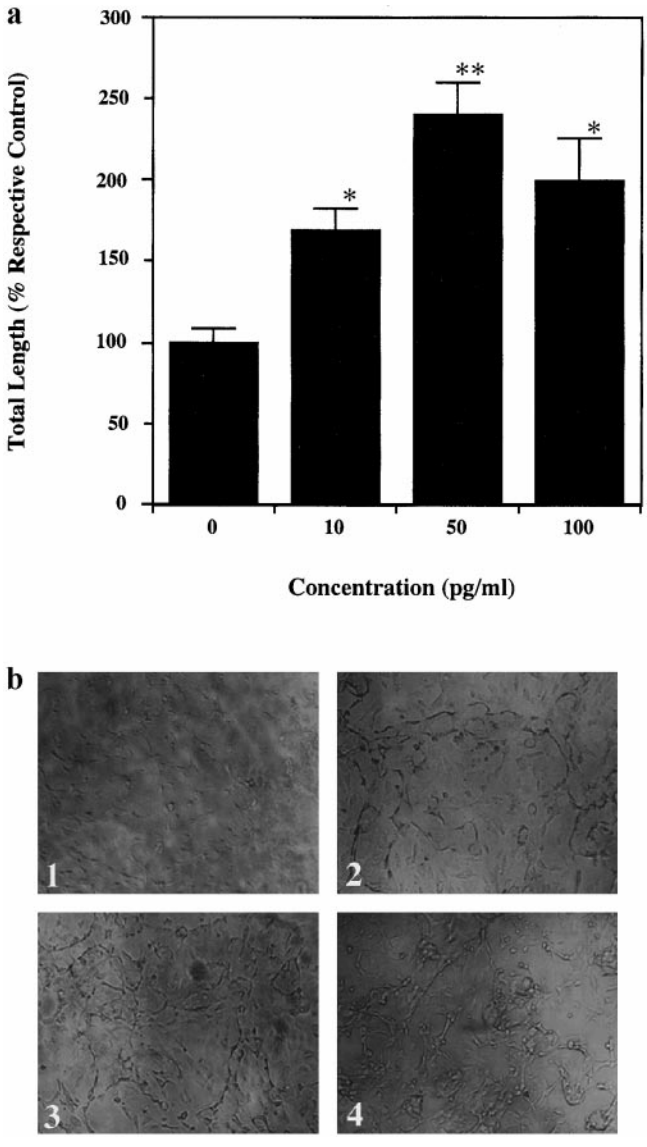

FIG. 3. Effect of AMF on *in vitro* tube-like structure formation of HUVECs. (a) Cells were seeded onto type I collagen gel in culture medium with or without different concentrations of AMF. After 10 days culture, the formed twigs were photographed under phase-contrast microscope with magnification, ×100 and its 10 random fields were determined with NIH image software and the total length per field was calculated. Values are expressed as the mean ± SD. $*P < 0.01$; $**P < 0.001$ by Student's t test. (b) Typical images of HUVECs tube formation used for analyzing. 1, control (without AMF); 2, 10 pg/ml; 3, 50 pg/ml; 4, 100 pg/ml AMF addition, respectively.

presence of HT-1080 cells (control-transfectant line) in the chamber promoted the development of capillary networks, tumor neovasculatures, in the dorsal subcutis in contact with the implanted chamber on day 5 (Fig. 5b-1). Newly developed capillary blood vessels were observed in mice which carried AMF-high expression tumor cells after 3 days in addition to the preexisting microvessels, but AMF-knockout tumor cells exhibited reduction of angiogenic responses on day 5 (Fig. 5a-3 and 5b). As expected, the AMF and its receptor gp78 expression were found on the edge of newborn microvessels by the immunohistochemistrical analysis for new blood vessels in mice carrying the AMF-high expression tumor (Fig. 6).

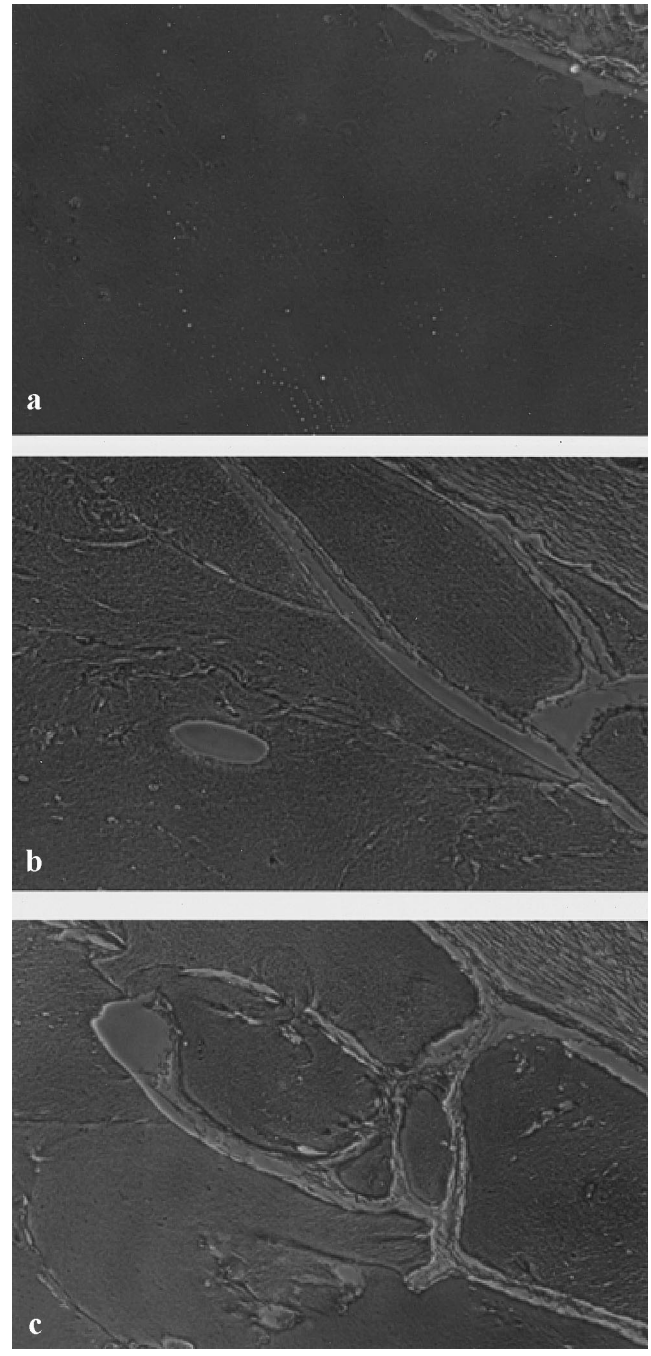

FIG. 4. Microscopic morphology of Matrigel plugs. Matrigel in the presence or absence of AMF was injected into female BALB/c mice subcutaneously ($n = 3$). Plugs were removed after 14 days and paraffin-embedded. 5-μm-thick sections of plugs were subjected to hematoxylin and eosin stain. a, control (Matrigel without AMF); b, 10 ng; c, 100 ng AMF containing Matrigel, respectively. Pictures shown as typical images visualized under magnification, ×200.

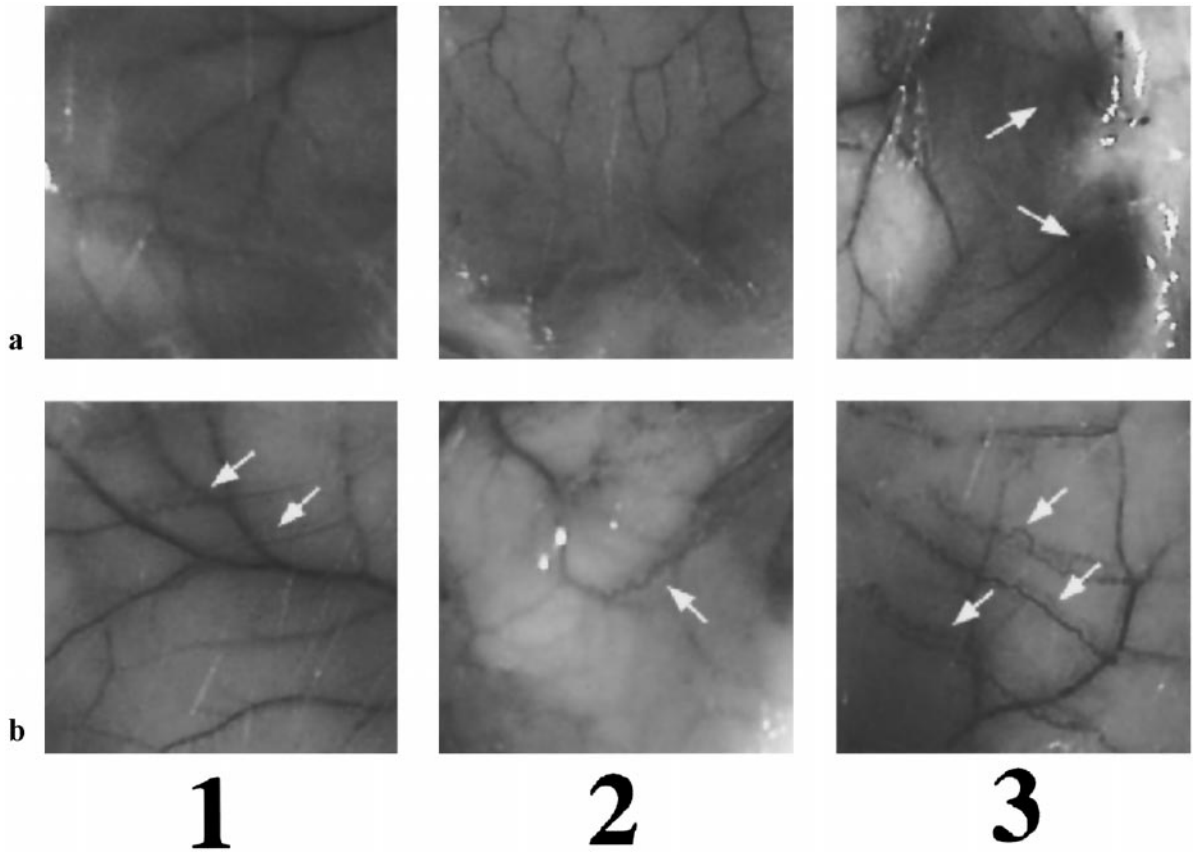

FIG. 5. *In vivo* angiogenesis induced by AMF. AMF mutants of human fibrosarcoma HT-1080 were injected into a diffusion chamber and implanted into a BALB/c mice ($n = 3$). A network of newly formed blood vessels on the skins contacted to chamber on day 3 (a) or day 5 (b) was observed (indicated as an arrow) and photographed by digital images of 1.4 Million Pixels CCD, Olympus Optical Co., Ltd. Pictures are the capillary networks developed inside the rings implanted with chamber containing control HT-1080 mutant cells (1), AMF-knockout HT-1080 mutant cells (2), or AMF-high-expression HT-1080 mutant cells (3).

We also assessed the suppression of angiogenesis with AMF inhibitors *in vivo* by using the same dorsal air sac assay in mice. E4P, which is known as a PHI enzymic inhibitor (19), or column-purified polyclonal IgG against AMF was used as the inhibitor. Subcutaneous administration of the inhibitor near the tumor chamber reduced the development of tumor neovascularization (Fig. 7). Mice given the AMF inhibitor developed microvessels with fragile structures compared to the control; furthermore, many tiny bleeding spots were observed with 500 μg/mouse/day of E4P or 25 μg/mouse/day of anti-AMF IgG treatment.

DISCUSSION

Angiogenesis, formation of capillary blood vessels leading to neovascularization, is an organic reaction caused by endothelial cell growth and migration from pre-existing blood vessels (26), and is essential to form a circulatory system or fabric at the embryonic phase in vertebrate (27, 28). It is also associated with an array of pathologic processes including inflammatory disease, diabetic retinopathy and wound-healing. Capillary blood vessels are arranged in a series of cellular processes, that is, endothelial cells can migrate, propagate, organize to lumen and form new capillaries in response to appropriate angiogenic signals (29). A number of angiogenic factors have been identified, such as vascular endothelial growth factor (VEGF), basic-, acidic-fibloblast growth factor (bFGF, aFGF), platelet-derived endothelial cell growth factor (PD-ECGF), transforming growth factor β (TGFβ), tumor necrosis factor (TNF), angiogenin and interleukin 8 (IL-8) (26, 30, 31).

Our results in this report demonstrate a new previously unrecognized function of AMF as a tumor angiogenic factor. When Matrigel containing AMF was subcutaneously injected into mice, vigorous positive angiogenic response was recognized. In contrast to the control plugs without AMF which were noninvaded and actually acellular, Matrigel plugs impregnated with AMF had numerous infiltrated cells and organized to form capillary-like tubular structures. Furthermore, the structures possessed a cellular assembly

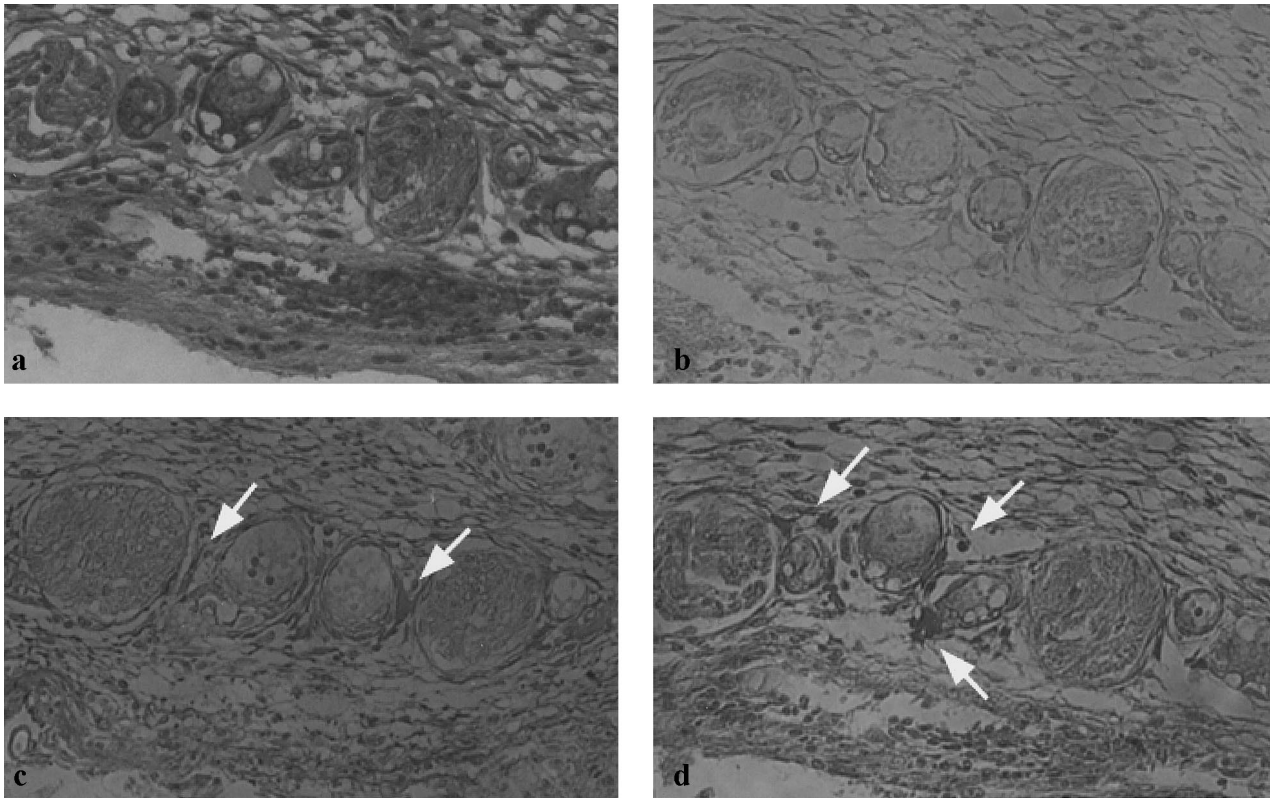

FIG. 6. Immunohistochemical localization of AMF and AMF receptor on the angiogenesis phase. The skins containing tumor-induced new blood vessels demonstrated in Fig. 5 were paraffin-embedded and sliced into 5-μm-thick sections. Each section was incubated with anti-AMF or gp78 antibody as described under Materials and Methods. a, hematoxylin and eosin staining; b, immunohistochemically background; c, AMF observation; d, gp78 observation, respectively. Arrow indicates each positive staining. Magnification, $\times$400.

which appears to be endothelial cells in the blood vessels. The histological properties of this kind of angiogenic response were similar to those induced by other angiogenic factors. Therefore, we have come to the conclusion that AMF is a tumor-derived angiogenic factor for the surrounding tissues.

We further investigated whether AMF could modulate angiogenesis with an *in vivo* model; i.e., a diffusion chamber method with AMF overexpression or AMF-knockout variable tumor cells. An assay of this kind would be able to estimate the angiogenic effect against incessant exposure to various tumor-secreted proteins and factors. Neovascularization was occurred at an early stage and its vessels image was apparent when endothelium was revealed much tumor AMF. Little neovasculature seemed to be developed against the AMF-knockout tumor. Furthermore the angiogenic effect derived from this AMF-rich tumor was fixed by anti-AMF polyclonal antibody or E4P which is known as a specific enzymic inhibitor of PHI. It is well known that malignant tumor cells secrete various angiogenic factors. Extensive research has shown that the VEGF-system plays an important part in most cases as a tumor angiogenic factor, and other investigations suggested that some cytokines like PD-ECGF or IL-8 are closely related to cancerous angiogenesis (32–34). Our results reveal that AMF also plays an essential role and contributes substantially to tumor angiogenesis. Many clinical studies demonstrated that excessive enzymic activity of PHI identified as AMF is found in the serum of patients who had malignant tumors with abundant small blood vessels such as kidney, breast and colorectal carcinoma, etc. (35–37). The presence of AMF/PHI molecules seems to be implicated in tumor malignancy and its progression, including various type of angiogenesis (38).

Tumor cell autocrine motility factor (AMF) was originally distinguished as a cell motility modulating factor of tumor cells (11). Secreted AMF has not hitherto been observed in normal cells and tissues despite the expression of the AMF receptor gene in various mouse tissues, because research on AMF was limited to cancerous investigations. However, AMF is hypothesized to participate in physiolosical phenomena through its cell motile stimulation activity by a molecule identified as PHI/NLK which acts at the intracellular saccharic metabolism. Experiments using *in vitro* assays revealed that HUVECs locomotion was promoted by AMF-exposure. The effective dose for HUVECs motile stimulation well corresponded to those of previous re-

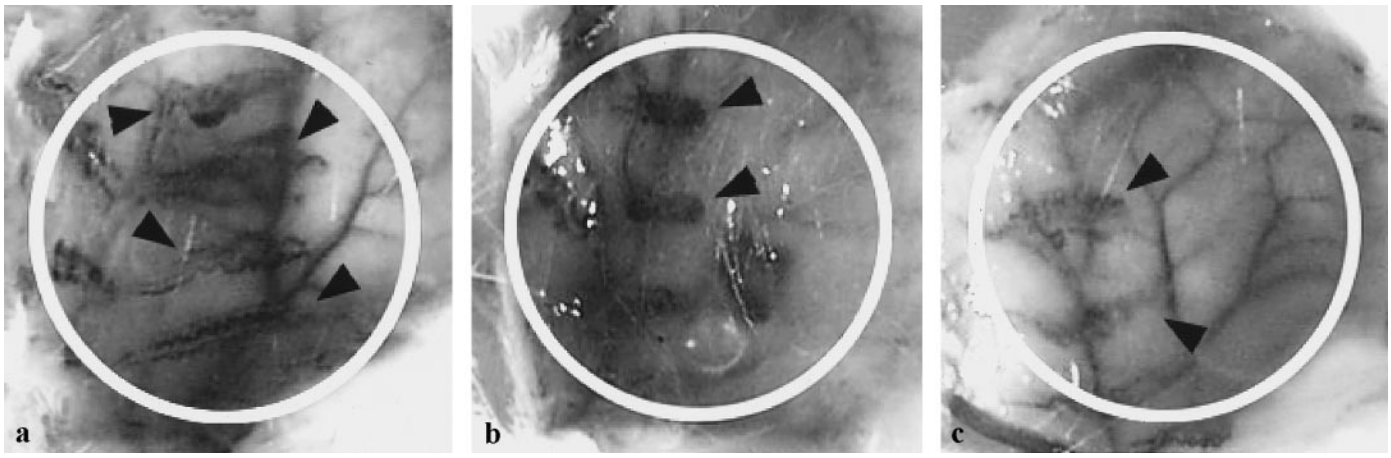

FIG. 7. Inhibition of *in vivo* tumor-induced angiogenesis by AMF inhibitors treatment. 4.5×10^5 of HT-1080 transfectant cells which were recognized as AMF high expression were placed into diffusion chamber with 0.45-μm-pore filters. The chamber was inserted in the back of BALB/c mice ($n = 3$). 500 μg E4P/mouse/day or 25 μg column-purified rabbit polyclonal antibody against AMF/mouse/day was subcutaneously administered near the chamber every 3 days before sacrifice. 5 days after the chamber implantation, the mouse dorsal skin was resected. The capillary blood vessels inside the chamber were photographed by the same methods as described in the legend to Fig. 5. Typical pictures for neovascularization of PBS vehicle control (a), E4P-treated (b), and anti-AMF IgG-treated (c) mice are shown. Arrowheads show typical tumor-associated neovascularization.

ports on various types of tumor cells. The AMF receptor gp78 was observed at a predominant perinuclear domain on HUVECs surface, and it closely correlated with enhanced motility. The expression of gp78 as such a single intensive pattern on the membrane surface of HUVECs was in good agreement with previous observations using high-metastatic tumor lines (12, 14). It is suggested that internalization of gp78 (endocytosis) and return to leading lamella on cytomembrane (exocytosis) might occur after AMF-bounding, and a single predominant domain would appear along the cell edge in the AMF-stimulated high-metastatic tumors (14, 39). Such distinct gp78 distribution between the perinuclear domain on HUVECs and the verge place on malignant cells is unclear. The mechanism and control of endocytosis or exocytosis of gp78 on normal cells may be different from those of tumor cells. Furthermore, AMF stimulated the growth of tubular HUVECs in collagen three-dimensional gels *in vitro*. This result also suggests that HUVECs migration was promoted by AMF via binding to its receptor, i.e., gp78 exists on the HUVECs membrane and its expression is promoted by AMF stimulation. The mechanism of the enhanced angiogenesis observed in mouse model is conceived to be similar to that of HUVECs experiments, and histochemical findings of gp78 expression of the surrounding neovascularizated capillary vessels also support our assumption. Intracellular signalling pathways consist of a complicated informational network involving a number of signal transduction molecules. Cell locomotion is thought to be a final resolution of various signal transduction cascades. Promotion of tumor cell motility initiated by the AMF–gp78 system is believed to be a series of subcellular processes including serine/threonine autophosphorylation of gp78, activation of protein kinase C-dependent signal transmission and cytoskeletal rearrangements (40). The essential cytokine receptors and cytokines for neovascularization are practically tyrosine kinase-dependent receptors and recognizable ligands. Since endothelial cells responded against the AMF stimulation similarly to the tumor cells reported previously, the binding behavior of AMF to gp78 on endothelial cells surface may activate the identical signal transduction pathway, or multiplicative effects with other or unknown angiogenic cytokines related signaling may strongly induce the neovascular formation *in vivo*.

The respective systems of *in vivo* and *in vitro* assays are different. Agreement between the *in vivo* and *in vitro* actions of cytokines at angiogenic response is not universal. TNF and TGFβ stimulate angiogenesis in *in vivo* systems (41–43), but TNF suppresses ansiogenesis *in vitro* as an inhibitor of endothelial growth (41, 42) and TGFβ exhibits the endothelial proliferation and motility (44, 45). It was considered that the angiogenic effect of TNF and TGFβ *in vivo* are a secondary function resulting macrophage/monocyte mobilization and gathering, because it would be able to up-regulate the secretion of cell mitogenic factors such as VEGF (46, 47). The regulation mechanisms of these various angiogenic cytokines are also modulated or controlled by the surrounding extracellular environment. Other studies suggest that TGFβ promotes the capillary-like tube formation in collagen gel (48, 49). TNF can stimulate the endothelial cell migration (41), and the stimulation is significantly enhanced when interleukin 6 (IL-6) known as a cytokine transiently expressed in rodent endothelium during physiologic angiogenesis

(50) which inhibits its proliferation (51) coexists (41, 52). These findings indicate that the complexities of the angiogenic phenomena and physiologic angiogenesis are due to expression and interaction of coordinated multiple cytokines. Generally, the angiogenic effect of cytokines in the *in vivo* studies are considered to be due to various secondary factors which are produced from the surrounding tissues by the cytokines stimulation in addition to the direct cytokines effect itself. AMF shows the angiogenic effect in the respective *in vivo* and *in vitro* assays. The anti-AMF antibodies could fix the neovascularization in the mouse model, and AMF-exposure also incited the endothelial cells to express the AMF receptor on the membrane in the *in vitro* system. These results suggest that AMF directly acts against the endothelium and it would be able to perform an important role in clinical tumor angiogenesis. Amplified angiogenesis observed in the two different types of *in vivo* assays clearly shows the direct effect of AMF on endothelial cells; i.e., AMF stimulates and enhances the endothelial migration. Enhancement of *in vitro* endothelial cell locomotion and tube formation also support this *in vivo* observation. AMF had been recognized as a kind of autocrine-type cytokine that acted against only tumor cells as the name implies. However, our new findings in this report suggest that AMF can affect normal surrounding tissues in a paracrine mannner. The gp78 expression on the HUVECs surface which is responsible for AMF stimulation, and immunohistchemical observation of the gp78 on the outside of the newborn blood vessel wall also suggest that AMF secreted by tumor cells will be able to stimulate the migration of various normal cells. This possibility also means that AMF affects the conditions of other cancers such as the development of ascites and pleural effusion due to increased capillary permeability. AMF has not hitherto been detected from normal cell lines although AMF has closely high homology sequence with PHI; therefore, AMF is thought to be some special form of PHI in cancer cells. Recent research suggests that some cell motile stimulating factors are really an ecto/exo-enzyme, such as the T-cell chemotactic factor is a CAP37/azurocidin which is a kind of serine protease (53) or autotaxin known as a tumor cell motility factor having high homology against phosphodiesterase (54). It is unclear why these intracellular enzymes would be secreted, showing an extracellular cytokine-like function in cancer cells. It may be a kind of carcinogenic mutation or it may act as an orphan ligand in wholesome bodies. In any case, it is conceivable that they have important roles in the regulation and control of homeostasis for both normal and neoplastic tissues.

Angiogenesis is a critical physiological reaction including various essential and important processes such as embryogenesis, wound healing and inflammation. Morbid angiogenesis such as tumor-inducing neovascularization will initiate and promote metastasis; therefore, to fix the angiogenesis is necessary in these cases. AMF will be a new target as an anti-angiogenic effect because the cell motile stimulating activity of AMF will be blocked by an antibody or well-known specific inhibitors. Also, AMF may be an effective and safe treatment factor for some diseases which need the formation of new blood vessels such as cerebral hemorrhage. In this paper, we have demonstrated new biological functions as well as further possibilities of tumor-secreted AMF.

REFERENCES

1. Folkman, J. (1990) What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.* **82,** 4–6.
2. Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N., Moore, D. H., Meli, S., and Gasparini, G. (1992) Tumor angiogenesis: A new significant and independent prognostic indicator in early-stage breast carcinoma. *J. Natl. Cancer Inst.* **84,** 1875–1887.
3. Gasparini, G., and Harris, A. L. (1995) Clinical importance of the determination of tumor angiogenesis in breast carcinoma: Much more than a new prognostic tool. *J. Clin. Oncol.* **13,** 765–782.
4. Vermeulen, P. B., Gasparini, G., Fox, S. B., Toi, M., Martin, L., McCulloch, P., Pezzella, F., Viale, G., Weidner, N., Harris, A. L., and Dirix, L. Y. (1996) Quantification of angiogenesis in solid human tumors: An international consensus on the methodology and criteria of evaluation. *Eur. J. Cancer* **32A,** 2474–2484.
5. Morelli, D., Lazzerini, D., Cazzaniga, S., Squicciarini, P., Bignami, P., Maier, J. A., Sfondrini, L., Menard, S., Colnaghi, M. I., and Balsari, A. (1998) Evaluation of the balance between angiogenic and antiangiogenic circulating factor in patients with breast and gastrointestinal cancers. *Clin. Cancer Res.* **4,** 1221–1225.
6. O'Brien, T., Cranston, D., Fuggle, S., Bicknell, R., and Harris, A. L. (1995) Different angiogenic pathways characterize superficial and invasive bladder cancer. *Cancer Res.* **55,** 510–513.
7. Hanahan, D., and Folkman, J. (1996) Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* **86,** 353–364.
8. Risau, W. (1997) Mechanisms of angiogenesis. *Nature* **386,** 671–674.
9. Koukourakis, M. I., Giatromanolaki, A., O'Byrne, K. J., Comley, M., Whitehouse, R. M., Talbot, D. C., Gatter, K. C., and Harris, A. L. (1997) Platelet-derived endothelial cell growth factor expression correlates with tumor angiogenesis and prognosis in non-small-cell lung cancer. *Br. J. Cancer* **75,** 477–481.
10. Dosquet, C., Coudert, M. C., Lepage, E., Cabane, J., and Richard, F. (1997) Are angiogenic factors, cytokines, and soluble adhesion molecules prognostic factors in patients with renal cell carcinoma? *Clin. Cancer Res.* **3,** 2451–2458.
11. Liotta, L. A., Mandler, R., Murano, G., Katz, D. A., Gordon, R. K., Chiang, P. K., and Schiffmann, E. (1986) Tumor cell autocrine motility factor. *Proc. Natl. Acad. Sci. USA* **83,** 3302–3306.
12. Nabi, I. R., Watanabe, H., and Raz, A. (1990) Identification of B16-F1 melanoma autocrine motility-like factor receptor. *Cancer Res.* **50,** 409–414.
13. Silletti, S., Watanabe, H., Hogan, V., Nabi, I. R., and Raz, A. (1991) Purification of B16-F1 melanoma autocrine motility factor and its receptor. *Cancer Res.* **51,** 3507–3511.

14. Watanabe, H., Nabi, I. R., and Raz, A. (1991) The relationship between motility factor receptor internalization and the lung colonization capacity of murine melanoma cells. *Cancer Res.* **51,** 2699–2705.
15. Kohn, E. C., Liotta, L. A., and Schiffmann, E. (1990) Autocrine motility factor stimulates a three-fold increase in inositol trisphosphate in human melanoma cells. *Biochem. Biophys. Res. Commun.* **166,** 757–764.
16. Silletti, S., Timar, J., Honn, K. V., and Raz, A. (1994) Autocrine motility factor induces differential 12-lipoxygenase expression and activity in high- and low-metastatic K1735 melanoma cell variants. *Cancer Res.* **54,** 5752–5756.
17. Timar, J., Silletti, S., Bazaz, R., and Honn, K. V. (1993) Regulation of melanoma cell motility by the lipoxygenase metabolite 12(S)-HETE. *Int. J. Cancer* **55,** 1003–1010.
18. Watanabe, H., Carmi, P., Hogan, V., Raz, T., Silletti, S., Nabi, I. R., and Raz, A. (1991) Purification of human tumor cell autocrine motility factor and molecular cloning of its receptor. *J. Biol. Chem.* **266,** 13442–13448.
19. Watanabe, H., Takehana, K., Date, M., Shinozaki, T., and Raz, A. (1996) Tumor cell autocrine motility factor is the neuroleukin/phosphohexose isomerase polypeptide. *Cancer Res.* **56,** 2960–2963.
20. Shimizu, K., Tani, M., Watanabe, H., Nagamachi, Y., Niinaka, Y., Shiroishi, T., Ohwada, S., Raz, A., and Yokota, J. (1999) The autocrine motility factor receptor gene encodes a novel type of seven transmembrane protein. *FEBS Lett.* **456,** 295–300.
21. Niinaka, Y., Paku, S., Haga, A., Watanabe, H., and Raz, A. (1998) Expression and secretion of neuroleukin/Phosphohexose isomerase/maturation factor as autocrine motility factor by tumor cells. *Cancer Res.* **58,** 2667–2674.
22. Haga, A., Niinaka, Y., and Raz, A. (2000) Phosphohexose isomerase/autocrine motility factor/neuroleukin/maturation factor is a multifunctional phosphoprotein. *Biochim. Biophys. Acta* **1480,** 235–244.
23. Albrecht-Buehler, G. (1977) The phagokinetic tracks of 3T3 cells. *Cell* **11,** 359–404.
24. Montesano, R., Orci, L., and Vassalli, P. (1983) *In vitro* rapid organization of endothelial cells into capillary-like networks is promoted by collagen matrices. *J. Cell Biol.* **97,** 1648–1652.
25. Passaniti, A., Taylor, R. M., Gage, W. R., Pili, R., Guo, Y., Long, P. V., Haney, J. A., Pauly, R. R., Grant, D. S., and Martin, G. R. (1992) A simple, quantitative method for assessing angiogenesis and antiangiogenic agents using reconstituted basement membrane, heparin and FGF. *Lab. Invest.* **67,** 519–528.
26. Folkman, J., and Klagsbrun, M. (1987) Angiogenic factors. *Science* **235,** 442–447.
27. Risau, W., Sariola, H., Zerwes, H. G., Sasse, J., Ekblom, P., Kemler, R., and Doetschman, T. (1988) Vasculogenesis and angiogenesis in embryonic-stem-cell-derived embryoid bodies. *Development* **102,** 471–478.
28. Wang, R., Clark, R., and Bautch, V. L. (1992) Embryonic stem cell-derived cystic embryoid bodies from vascular channels: An *in vitro* model of blood vessel development. *Development* **114,** 303–316.
29. Polverini, P., Coltran, R., Gimbrone, M., and Uranue, E. (1977) Activated macrophages induce vascular proliferation. *Nature* **269,** 804–806.
30. Folkman, J., and Shing, Y. (1992) Angiogenesis. *J. Biol. Chem.* **267,** 10931–10934.
31. Koch, A. E., Polverini, P. J., Kunkle, S. L., Harlow, L. A., DiPietro, L. A., Elner, V. M., Elner, S. G., and Strieter, R. M. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis. *Science* **258,** 1798–1801.
32. Kendall, R. L., and Thomas, K. A. (1993) Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. *Proc. Natl. Acad. Sci. USA* **90,** 10705–10709.
33. Kim, K. J., Li, B., Winer, J., Armanini, M., Gillett, N., Phillips, H. S., and Ferrara, N. (1993) Inhibition of vascular endothelial cell growth factor-induced angiogenesis suppresses tumor growth *in vivo. Nature* **362,** 841–844.
34. Millauer, B., Shawver, L. K., Plate, K. H., Risau, W., and Ullrich, A. (1994) Glioblastoma growth inhibited *in vivo* by a dominant-negative Flk-1 mutant. *Nature* **367,** 576–579.
35. Baumann, M., Kappl, A., Lang, T., Brand, K., Siegfried, W., and Paterok, E. (1990) The diagnostic validity of the serum tumor marker phosphohexose isomerase (PHI) in patients with gastrointestinal, kidney, and breast cancer. *Cancer Invest.* **8,** 351–356.
36. Bodansky, O. (1954) Serum phosphohexose isomerase in cancer. II. As an index of tumor growth in metastatic carcinoma of the breast. *Cancer* **7,** 1200–1226.
37. Filella, X., Molina, R., Jo, J., Mas, E., and Ballesta, A. M. (1991) Serum phosphohexose isomerase activities in patients with colorectal cancer. *Tumor Biol.* **12,** 360–367.
38. Schwartz, M. K. (1976) Laboratory aids to diagnosis—Enzymes. *Cancer* **37,** 542–548.
39. Nabi, I. R., Watanabe, H., and Raz, A. (1992) Autocrine motility factor and its receptor: Role in cell locomotion and metastasis. *Cancer Metastasis Rev.* **11,** 5–20.
40. Timar, J., Raso, E., Fazakas, Z. S., Silletti, S., Raz, A., and Honn, K. V. (1996) Multiple use of a signal transduction pathway in tumor cell invasion. *Anticancer Res.* **16,** 3299–3306.
41. Leibovich, S. J., Polverini, P., Shepard, H. M., Wiseman, D. M., Shively, V., and Nuseir, N. (1987) Macrophage-induced angiogenesis is mediated by tumor necrosis factor-alpha. *Nature* **329,** 630–632.
42. Frater-Schroder, M., Risau, W., Hallmann, R., Gautschi, P., and Bohlen, P. (1987) Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth *in vitro,* is angiogenic *in vivo. Proc. Natl. Acad. Sci. USA* **84,** 5277–5281.
43. Roberts, A. B., Sporn, M. B., and Assoian, R. K. (1986) Transforming growth factor type β: Rapid induction of fibrosis and angiogenesis *in vivo* and stimulation of collagen formation *in vitro. Proc. Natl. Acad. Sci. USA* **83,** 4167–4171.
44. Heimark, R. L., Twardzik, D. R., and Schwartz, S. M. (1986) Inhibition of endothelial regeneration by type-β transforming growth factor from platelets. *Science* **233,** 1078–1080.
45. Muller, G., Behrens, J., Nussbaumer, U., Bohlen, P., and Birchmeier, W. (1987) Inhibitory action of transforming growth factor β on endothelial cells. *Proc. Natl. Acad. Sci. USA* **84,** 5600–5604.
46. Pertovaara, L., Kaipainen, A., Mustonen, T., Orpana, A., Ferrara, N., Saksela, O., and Alitalo, K. (1994) Vascular endothelial growth factor is induced response to transforming growth factor-β in fibroblastic and epithelial cells. *J. Biol. Chem.* **269,** 6271–6274.
47. Sunderkotter, C., Steinbrink, K., Goebeler, M., Bhardwaj, R., and Sorg, C. (1994) Macrophages and angiogenesis. *J. Leukocyte Biol.* **55,** 410–422.
48. Madri, J. A., Pratt, B. M., and Tucker, A. M. (1988) Phenotypic modulation of endothelial cells by transforming growth factor-β depends upon the composition and organization of the extracellular matrix. *J. Cell Biol.* **106,** 1375–1384.
49. Sankar, S., Mahooti-Brooks, N., Bensen, L., McCarthy, T. L., Centrella, M., and Madri, J. A. (1996) Modulation of transforming growth factor-β receptor levels on microvascular endothelial cells during *in vitro* angiogenesis. *J. Clin. Invest.* **97,** 1436–1446.
50. Motro, B., Itin, A., Sachs, L., and Keshet, E. (1990) Pattern of interleukin 6 gene expression *in vivo* suggests a role for this

cytokine in angiogenesis. *Proc. Natl. Acad. Sci. USA* **87,** 3092–3096.

51. May, L. T., Torcia, G., Cozzolino, F., Ray, A., Tatter, S. B., Santhanam, U., Sehgal, P. B., and Stern, D. (1989) Interleukin-6 gene expression in human endothelial cells: RNA start sites, multiple IL-6 proteins and inhibition of proliferation. *Biochem. Biophys. Res. Commun.* **159,** 991–998.
52. Rosen, E. M., Liu, D., Setter, E., Bhargava, M., and Goldberg, I. D. (1991) Interleukin-6 stimulates motility of vascular endothelium. *In* Cell Motility Factors (Goldberg, I. D., and Rosen, E. M., Eds.), pp. 194–205, Birkhauser, Basel.
53. Chertov, O., Michiel, D. F., Xu, L., Wang, J. M., Tani, K., Murphy, W. J., Longo, D. L., Taub, D. D., and Oppenheim, J. J. (1996) Identification of defensin-1, defensin-2, and CAP37/azurocidin as T-cell chemoattractant proteins released from interleukin-8-stimulated neutrophils. *J. Biol. Chem.* **271,** 2935–2940.
54. Murata, J., Lee, H. Y., Clair, T., Krutzsch, H. C., Arestad, A. A., Sobel, M. E., Liotta, L. A., and Stracke, M. L. (1994) cDNA cloning of the human tumor motility-stimulating protein, autotaxin, reveals a homology with phosphodiesterases. *J. Biol. Chem.* **269,** 30479–30484.