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Tumor autocrine motility factor induces hyperpermeability of endothelial and mesothelial cells leading to accumulation of ascites fluid☆

Tatsuyoshi Funasaka,[a] Arayo Haga,[a,*] Avraham Raz,[b] and Hisamitsu Nagase[a]

[a] *Department of Hygienics, Gifu Pharmaceutical University, 5-6-1 Mitahora-Higashi, Gifu 502-8585, Japan*
[b] *Departments of Oncology, Pathology, and Radiation Oncology, Barbara Ann Karmanos Cancer Institute, Wayne State University, School of Medicine, 110 East Warren Avenue, Detroit, MI 48210, USA*

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Abstract

Accumulation of ascites fluid often observed in some solid tumors is one of the most devastating conditions of a patient's difficulty in responding to treatment, and to a decrease in the quality of life. Various factors are thought to be associated with the formation of cancer-induced fluid accumulation and hyperpermeability of a blood vessel is thought to go with this process. Here, we report a new factor that is involved in this process, e.g., autocrine motility factor (AMF). AMF is a tumor-related cytokine which stimulates the tumor cell locomotion and migration and promotes tumor cell invasion during metastasis. AMF secretion and its receptor (AMFR) expression in tumor cells are closely correlated with disease aggravation of convalescence. The response of endothelial or mesothelial cellular morphological alternation to AMF leads to motile enhancement and vascular permeability. Tumor AMF induces gaps in an endothelial or mesothelial monolayer by stimulating a cellular movement, and accelerates the ascites accumulation. And treatment experiment with anti-AMF antibody succeeded in the reduction of the ascites accumulation, which renders AMF to the target molecule. It is suggested that AMF is one of the significant factors which relates to various pathological malignancies induced by tumor mass, and understanding of its function could benefit prognosis and treatment.

Keywords: Autocrine motility factor; Autocrine motility factor receptor; Ascites fluid; Vascular permeability; Cell motility

The ascites and pleural effusion are one of the well-known pathologic conditions of some solid tumors such as gastric, liver, or lung cancer. The malignant accumulation obstructs a treatment, and brings a remarkable decline in the patient's quality of life. The accumulation of ascites fluid is often observed when tumor cells invade into peritoneal cavity, but the process is poorly understood. At least three pathological features are associated with tumor ascites formation: (i) a reduced lymphatic recovery system, which is associated with the obstruction of the draining lymphatics by tumor cells [1–3]; (ii) angiogenesis, which is observed in ascites tumor-bearing peritoneal walls, and some angiogenic inhibitors significantly reduce the ascites accumulation and neovascularization [4,5]; and (iii) microvessels' hyperpermeability of peritoneal cavity lining [6–8].

Many factors relating to tumor-associated hyperpermeability of microvessels have been reported, many of them include: inflammatory mediators, such as prostaglandin [9], bradykinin [10,11], histamine [12], leukokinins [13], and nitric oxide [14]; cytokines, such as tumor necrosis factor and interleukin-2 [15], transforming growth factor α [16], and vascular endothelial growth factor (VEGF), also known as vascular permeability

☆ *Abbreviations*: AMF, autocrine motility factor; AMFR, AMF receptor; HUVECs, human umbilical vein endothelial cells; PHI, phosphohexose isomerase; RT-PCR, reverse transcription PCR; VEGF, vascular endothelial growth factor.

* Corresponding author. Fax: +81-58-237-5979.
E-mail address: arayo@gifu-pu.ac.jp (A. Haga).

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factor [7,17–20]. These factors are presumed to act alone or compositely on endothelial cells when induction of vascular permeability occurs. It is well established that endothelial cells function as barriers for the high molecular plasma proteins and lipid inflows across the blood vessel wall. Breakdown of endothelial monolayer leads to increased vascular permeability and edema development often observed in inflammatory diseases. For example, when the endothelial cells are exposed to histamine or bradykinin, intercellular junctions of the cell monolayer open, leading to a local leakage of serum proteins [21].

Autocrine motility factor (AMF) is a cell motility-stimulating factor associated with cancer development. AMF was originally identified as a tumor self-producing factor which was able to enhance the directed and random migration [22]. Exo-AMF stimulates tumor cell motility with binding to its cell surface receptor as 78 kDa glycoprotein (AMFR) [23,24]. Receptor stimulation by AMF triggers signal transduction pathway involving a pertussis toxin-sensitive G-protein activation [25], inositol trisphosphate production [26], protein kinase C activation [27,28], and receptor phosphorylation [29]. There are many reports that enhanced AMF level and AMFR overexpression are correlated with tumor progression and malignancy. AMF was genetically identified as a phosphohexose isomerase (PHI), which catalyzes the conversion of glucose 6-phosphate to fructose 6-phosphate intracellularly [30]. It is shown that tumor-secreted AMF retains the PHI enzymatic activity, and AMF activities are inhibited by specific enzymatic PHI inhibitors [30]. AMF/PHI does not have a signal leader sequence, and it is unclear how AMF/PHI is secreted by tumor cells. We have reported that serine phosphorylation of AMF/PHI participated in the secretion recently [31]. It was thought of previously that AMF acted on cancer cells in an autocrine manner; however, recent studies show that human vascular endothelial cells express AMFR, and the AMF promotes new blood vessel formation in the circumference of growing tumors. AMFR showed that AMFR mRNA is expressed by various organs and tissues, and it was suggested that tumor-secreted AMF may affect surrounding tissues [32]. The angiogenic effect of AMF was considered to be due to the AMF–AMFR signaling, namely cell motility stimulation [33].

Numerous gaps are observed in the endothelial or mesothelial monolayer from which permeability is accelerated. The cellular morphological changes and cell movement are the result of stimulated motility, and are associated with the formation of cellular gaps. Here, we tested the hypothesis that tumor-secreted AMF affects ascites accumulation by invading cancers, and thus we investigated the influence of tumor AMF on endothelial and mesothelial cells.

Materials and methods

Antibodies and reagents. The anti-AMFR rat monoclonal antibody (3F3A) was used as described before [23,24,29]. The anti-recombinant human AMF polyclonal antibody (anti-rhAMF) was obtained from immunized New Zealand white rabbit [33]. Recombinant human or mouse AMF (rhAMF or rmAMF) was created as a glutathione S-transferase (GST) fusion protein [31,33]. The horseradish peroxidase (HRP)-conjugated goat anti-rabbit or rat antibody was purchased from American Qualex (San Clemente, CA). 3, 3′-Diaminobenzidine tetrahydrochloride (DAB) was purchased from GIBCO BRL (Rockville, MD). TRIzol was purchased from Life Technologies (Grand Island, NY). AMV Reverse Transcriptase First-strand cDNA Synthesis Kit was purchased from Life Sciences (St. Petersburg, FL). Taq DNA polymerase was purchased from Promega (Madison, WI). Endothelial cell growth supplement (ECGS) was purchased from Collaborative Research (Bedford, MA). FITC-labeled dextran and Evans blue dye were purchased from Sigma (St. Louis, MO).

Animals and cells. Male BALB/c mice and Wistar rats were purchased from SLC (Hamamatsu, Japan). Two different strains of Ehrlich ascites tumor cells were used. One was obtained from the Cancer Cell Repository, Research Institute for Tuberculosis and Cancer, Tohoku University (Sendai, Japan) and grows in mice by passaging weekly intraperitoneal (i.p.) injection in 6–8-week-old BALB/c mice [34]. When 5.0×10^6 cells of this strain are i.p. inoculated into BALB/c mice, abdominal tumors appeared within 6–8 days with abundant ascites formation and the treated mice die 2 weeks later. The other one was obtained from the Japanese Collection of Research Bioresources (Osaka, Japan). Since it grows poorly in mice, this strain was passaged in vitro in Roswell Park Memorial Institute 1640 (RPMI 1640) medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) [35]. Human umbilical endothelial cells (HUVECs) were purchased from Cell Application (San Diego, CA) and cultivated in RPMI 1640 medium supplemented with 20% heat-inactivated FBS and ECGS. Mouse mesothelial cells were isolated by the reported method described by Akedo et al. [36]. Briefly, cells were isolated from normal male BALB/c mouse peritoneums or diaphragms by trypsinization and inoculated into a 35 mm culture dish and placed with RPMI 1640 medium containing 10% heat-inactivated FBS. All strains were maintained at 37 °C in an air–5.0% CO_2, incubator under mycoplasma-free conditions.

RT-PCR analysis. Total RNAs of Ehrlich cell lines or excised mouse parietal peritoneal wall and diaphragm were extracted with TRIzol reagent according to the standard acid–guanidium–phenol–chloroform method. The cDNAs were synthesized by a reverse transcriptase as recommended in manufacturer's protocol. The sequences of oligonucleotide primers are as follows: 5′-ACC CCT CAT GGT GAC TGA AG (forward) and 5′-GGT CTG GAC AGG GAT GAG AA (reverse) for mouse AMF detection; 5′-CGT GTG GGT TCT GGT GAA TA (forward) and 5′-AAG AAC GAA GGC AGG AGT TG (reverse) for mouse AMFR detection; 5′-AGA GAG GTA TCC TGA CCC TGA AGT A (forward) and 5′-CAT AGA GGT CTT TAG GGA TGT CAA C (reverse) for mouse β-actin detection as a housekeeping gene. The PCR condition with Taq DNA polymerase was as follows: 94 °C for 5 min as an initial denaturation; 30 cycles of 94 °C for 30 s, 55°C for 30 s, and 72 °C for 2 min, followed by 72 ° C for 10 min as the final extension. PCR-amplified products were electrophoresed in 1.0% agarose gel and stained with ethidium bromide.

Western blot analysis. Subconfluent cultured Ehrlich ascites cells in 10 cm culture dishes were washed twice with PBS and then recultured with 10 ml serum-free medium for 24 h. The centrifuged (3000 rpm at 4 °C for 20 min) Ehrlich supernatants were 100-fold concentrated with Ultrafree-CL tubes (30,000 NMWL, MILLIPORE). The 4×10^6 Ehrlich cells were lysed in 500 μl of the Triton X-100 lysis buffer, and sonicated for 15 s on ice. The lysates were clarified by centrifugation (15,000 rpm at 4 °C for 30 min). Protein concentration of each sample was determined using a commercialized kit (Bio-Rad, Hercules, CA).

Fifty μg of the supernatant or the lysate proteins was separated in 10% SDS–polyacrylamide gel. The proteins in the gel were electrophoretically transferred to 0.2 μm PVDF membrane (Bio-Rad) at 170–80 mA for 2 h. The blotted membrane was blocked with 5% skim milk for 1 h at room temperature. The membrane was then incubated with a rabbit anti-AMF antibody (1:500 dilution of 750 μg/ml IgG) and HRP-conjugated goat anti-rabbit antibody (1:1000 dilution) for 1 h at room temperature, respectively. The antibodies were detected by the DAB stain method.

Cell motility assay. Cell motility was determined by the phagokinetic track assay [37]. 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. 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 cells were seeded on each coverslip. After 24 h of culture, phagokinetic tracks were visualized using dark-field illumination and at least 50 microscopic fields were measured.

Immunofluorescent detection. Cells seeded on coverslips were fixed with 3.5% paraformaldehyde/PBS for 10 min at room temperature, or with ice-cooled (−80 °C) methanol and incubated for 30 min at −20 °C for the cell surface or permeabilized immmunofluorescent stain, respectively. The fixed cells were washed three times with PBS and labeled with 3F3A for 30 min at room temperature. After incubation, the cells were washed and incubated with 5.0% FITC-conjugated goat anti-rat secondary antibody for 30 min at room temperature in the dark. Following washing, the cells were then mounted on glycerol, and fluorescent images were visualized with a laser scanning microscope (LSM 510; Carl Zeiss).

Immunohistochemistry. Mouse parietal peritoneal wall and diaphragm tissues removed from normal or ascites tumor-bearing animals were excised, fixed in Bouin's solution, embedded in paraffin, and sliced into 5-μm-thick sections. Deparaffinized sections were incubated with 0.3% H_2O_2/methanol at room temperature for 30 min to prevent the endogenous-peroxidases. After blocking with 3.0% BSA, the first 3F3A antibody and 10% HRP-conjugated goat anti-rat secondary antibody were allowed to react at room temperature for 15 min. Stained AMFRs were developed according to the DAB stain methods.

Measurement of endothelial cell permeability. Cells were plated onto Transwell apparatus (Costar) with collagen-coated micropore membranes (0.4 μm) and grown to confluency. The endothelial or mesothelial monolayer was then washed twice with PBS, and 100 μl of medium containing 1 mg/ml FITC-dextran was added to the monolayers. After incubation, the fluorescence of the FITC-dextran molecules transversing the monolayers was determined by CytoFluor 2350 Fluorescence Measurement System (MILLIPORE). The data are expressed as follows:

$$\begin{aligned}\text{permeability index}(\%) = {} & [\text{experimental clearance}] \\ & - [\text{spontaneous clearance}] \\ & / [\text{clearance of filter alone}] \\ & - [\text{spontaneous clearance}] \times 100.\end{aligned}$$

Vascular permeability assay. A Miles-type assay [38] with a slight modification was used to determine the in vivo vascular permeability. The back of an anesthetized 7-week-old male Wistar rat was shaved, and 1 ml/rat of 0.5% Evans blue dye was injected intravenously. Ten minutes later, 200 μl of mAMF or control PBS was injected subcutaneously (s.c.) into the shaved area. The skins containing extravasated dye spots were removed after 20 min and cut into 10 mm square pieces. The extravasated dye was quantified by the extraction method reported by Katayama et al. [39]. A piece of skin was soaked into 1 ml of 1 N KOH at 37 °C for overnight and 9 ml of 0.6 N H_3PO_4: acetone = 5:13 was added. The solution was shaken vigorously for 2 min, centrifuged at 3000 rpm for 30 min, and absorbance of the supernatant was measured at 620 nm.

Results

AMF expression by Ehrlich ascites tumor cells and AMF receptor expression on the host tissues

There are many types of substrains among Ehrlich mouse ascites tumor cell lines. Two different strains used here are, "Ehrlich +" which grows well in mice and induces abundant ascites [34], and "Ehrlich −" which grows poorly in mice and did not develop ascites [35]. "Ehrlich +" cells are of 100% lethality in mouse, almost at any i.p. inoculum number. Morphological differences of these lines are shown in Fig. 1A, "Ehrlich +" cells are small and round whereas "Ehrlich −" cells exhibit a fibroblast-like appearance. Secreted-AMF levels from "Ehrlich +" cells were markedly higher than those of "Ehrlich −" cells (Fig. 1B), in spite of the same levels of AMF mRNA (Fig. 1C). Digital analyzing data suggest that "Ehrlich +" cells secrete ten times more AMF compared with "Ehrlich −" cells. High expression of AMFR mRNA was also detected in "Ehrlich +" cells (Fig. 1A). These results suggest that AMF might play a role in ascites fluid accumulation.

The extravasation of waters and circulating molecules from blood vessels to peritoneal lining tissues, such as peritoneal wall or diaphragm, is an essential process during ascites fluid accumulation. To open the gaps, the endothelial cells need to change the shape or to locomote. The ligand–receptor interaction on the cell surface is the initial step for AMF-stimulating cell motility signaling. Our previous study showed that normal human vascular endothelial cells express AMFR which was responsible for AMF motile stimulation [33]. If AMF secreted from "Ehrlich +" cells may affect the host, it is expected that the peritoneal wall and diaphragm cells should express AMFR. The parietal peritoneal walls and diaphragms of mice bearing Ehrlich ascites tumors or control mice were removed and subjected to immunohistochemistry staining and RT-PCR for AMFR expression. As shown in Fig. 2, AMFR was expressed at the peritoneal walls and diaphragms of normal control animals, with increased expressions in Ehrlich tumor cell-induced ascites fluid-bearing mice. This enhanced expression implies that ligand (AMF) secreted from Ehrlich interacts with host cells' receptor during the ascites formation phase.

Motile effect of AMF on mesothelial cells

A typical AMFR expression of mesothelial cell is shown in Fig. 3A, and induction of expression was observed when AMF was exposed to these cells (Fig. 3A,2). There was no difference in the distribution of intracellular AMFR (data not shown). The mesothelial cell motile response to AMF was tested by phagokinetic track assay (Fig. 3B). mAMF significantly

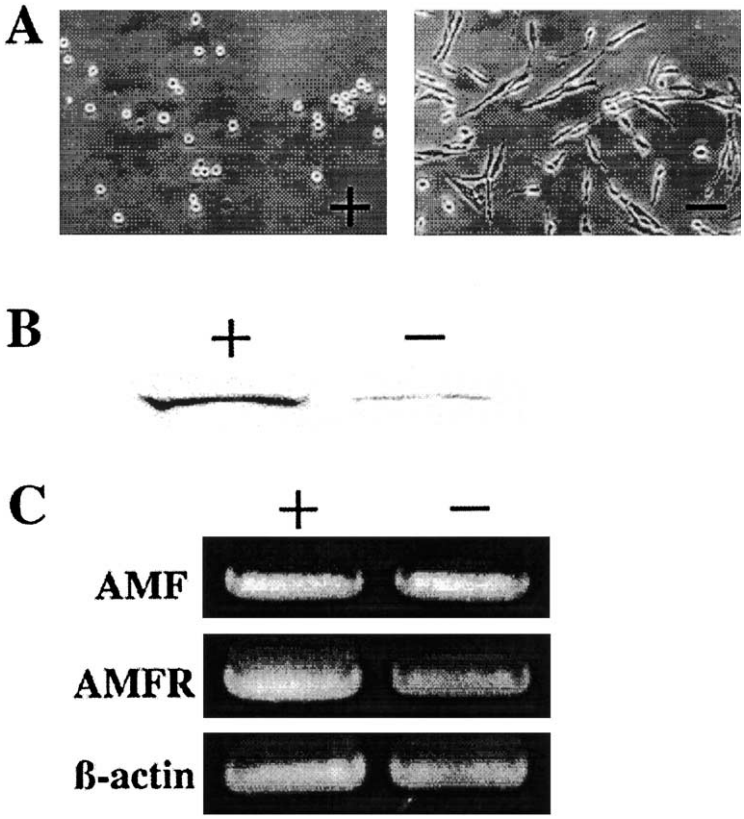

Fig. 1. Two strains of Ehrlich ascites tumor cells. (A) Morphology of cultured Ehrlich ascites tumor cells. + indicates the "Ehrlich +" cells, high accumulation of ascites fluid in mice; − indicates the "Ehrlich −" cells, growing poorly in mice. (B) Western blot analysis of the AMF expression. Each cell sample was electrophoresed, transferred to a PVDF membrane, and visualized as described under "Materials and methods". (C) RT-PCR analysis of AMF and AMFR mRNA in Ehrlich ascites tumor cell line.

stimulated mesothelial cell locomotion, with an optimal dose of 1 pM similarly to those reported previously [30,31,33]. Mesothelial cell locomotion was also elevated when a conditioned medium of "Ehrlich +" cells was added (data not shown). These "Ehrlich +" cell-stimulated motilities were blocked by anti-AMF antibody (Fig. 3C). The effective dose of antibody was in the same range of AMF added for stimulation, which is shown in Fig. 3B. The peritoneal wall cells showed a higher response than diaphragmatic cells in both the reactions.

Permeability of mesothelial cell monolayer was significantly enhanced by 10 pM or more mAMF (Fig. 3D). The high response to AMF was also observed in peritoneal wall cells in this assay, with rapid morphological changes of the mesothelial cells (Fig. 3E).

Induction of vascular permeability by AMF

Hyperpermeability of microvessels lining peritoneal cavity is considered to be responsible for the malignant ascites accumulation. Our previous study showed that AMF interacts with vascular endothelial cells and prompts locomotion and angiogenesis [33]. Thus, the effect of AMF on vascular permeability was tested. Various amounts of purified mAMF were applied to the Miles permeability assay, a well-known method as an estimation of vascular permeability, and the result is shown in Fig. 4. The mAMF exerted a permeability-enhancing effect, 100 μg or more mAMF made positive response significantly. A four-time rise in vascular leakage as compared with the controls was observed at a dose of 1 or 10 μg mAMF.

The effect of AMF on endothelial permeability was also assayed in vitro. One pM or more hAMF addition to the HUVECs monolayer for 24 h induced a significant increase of FITC-labeled dextran permeability (Fig. 5A). And, AMF was added to tightly confluenced cultured HUVEC monolayer, the cell–cell contact retracted and numerous gaps were formed (Fig. 5B). Morphological alteration of individual HUVECs and its locomotion enhancement in response to AMF seem to occur immediately after AMF-exposure (Fig. 5C). This pronounced alteration in cell shape was similar to that of AMFR expressing tumor cells, which is included in pseudopodia extension [40].

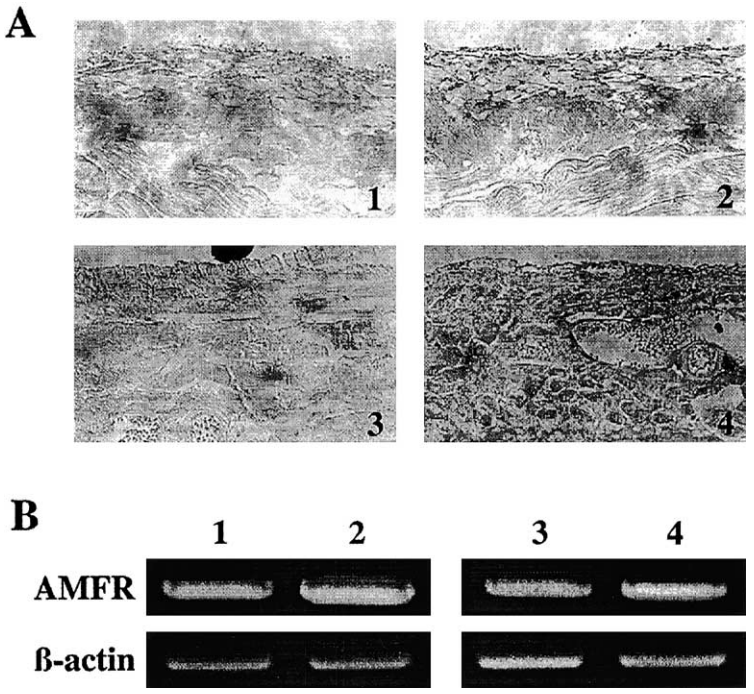

Fig. 2. AMF receptor expression in tissues lining peritoneal cavity on ascites formation phase. (A) Peritoneal wall [1,2] or diaphragm [3,4] of normal or "Ehrlich +" tumor-bearing mice at 14 days after i.p. injection of 5.0×10^6 cells. They are stained with anti-AMFR antibodies. 1, 3, normal; 2,4, tumor-bearing; magnification, 400×. (B) RT-PCR analysis of peritoneal wall [1,2] and diaphragm [3,4] AMFR expression. 1, 3, normal; 2, 4, tumor-bearing. β-actin was used as the housekeeping gene and indicated at each bottom.

The possibility of AMF targeting anti-tumor ascites therapy

The above in vitro and in vivo results suggest that AMF could be a critical molecule in tumor-induced ascites accumulation phase. Therefore, the curative value of AMF-targeting therapy was tested. The used anti-AMF antibody did not affect the Ehrlich cell proliferation in vitro (data not shown). The antibody was i.p. administered daily for eight times from the day 6 after "Ehrlich +" cell inoculation. On day 14, tumor volume was reduced by 25% than that of control group [(control group; 10.72 ± 0.441 ml/mouse, treatment group; 8.27 ± 0.464 ml/mouse) $P < 0.001$, Student's t test: Fig. 6].

Discussion

The availability of two variants of Ehrlich cell lines which exhibit different abilities for ascites accumulation allowed us to study the role of AMF in ascites formation. Since elevated-expression of AMF and AMFR is a characteristic associated with malignant tumor cells, we question whether the difference in the AMF secretion between the Ehrlich cell lines could be a significant factor in ascites accumulation. In addition we tested whether AMFR is expressed by the tissues lining the peritoneal cavity of mice.

The above results showing that AMFR is expressed by normal host cells and AMF affects host cells suggest that AMF-signaling enhances the permeability of endothelial or mesothelial monolayer. Because morphological changes and locomotion of cells forming the monolayer are expected due to AMF stimulation. AMF stimulates vascular leakage with s.c. injection of only a few hundred nanograms of protein. This enhanced vascular permeability was proved by in vitro cultured endothelial cells. The response to AMF associated with increase of permeability was due to morphological change of the cells and gap formation between adjacent cells. When the effect of AMF is compared with that of VEGF, one cannot escape the conclusion that AMF mimics VEGF. Since, the potency and end result of their two molecules seem to be similar [6,17] VEGF is the most investigated factor to induce vascular hyperpermeability, and to act as an angiogenic factor [17–20,41–43]. VEGF induces angiogenesis by directly affecting two well-known tyrosine kinase receptors, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1) of endothelial cells [44–46], probably by both direct and indirect mechanisms. VEGF was originally recognized as a growth factor of vascular endothelial cells [47], and it is unclear why VEGF should promote the vascular permeability. It is possible that VEGF stimulates the function of vesiculovacuolar organelles, which are endothelial cell cytoplasmic structures that are involved in the

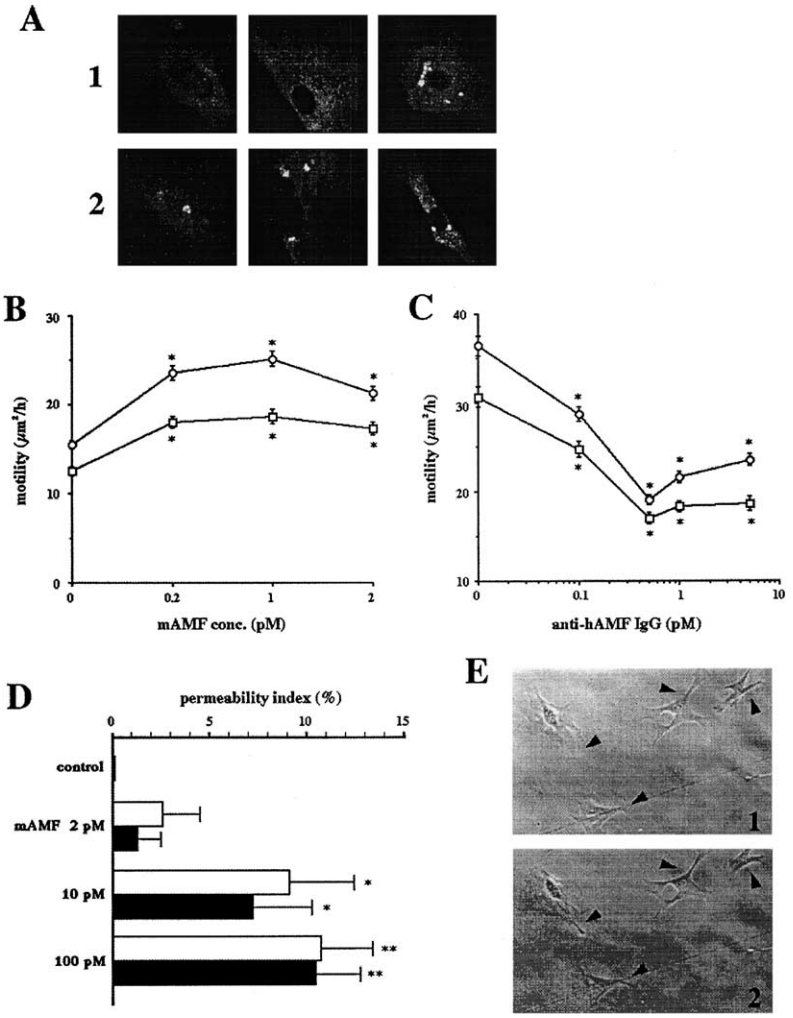

Fig. 3. Mesothelial cells motility and monolayer permeability with AMF. (A) Cells prepared from peritoneal wall were exposed with or without one pM mAMF for 24 h. Then the cells were fixed, processed for immunofluorescent staining to detect the surface AMFR as described in "Materials and methods" and photographed with magnification, 1000×. 1, untreated cells; 2, AMF-exposed cells. (B) Locomotion of mesothelial cells prepared from peritoneal lining tissues, peritoneal wall (-○-) or diaphragm (-□-). Motile area with or without AMF was measured by phagokinetic track assay. The values are expressed as the mean ± SEM. $^{*}P < 0.005$ by Student's t test. (C) Inhibitory effect of anti-AMF IgG on the stimulated motility by exposure of Ehrlich cell conditioned media. Peritoneal wall (-○-) or diaphragm (-□-) cells motility was stimulated with conditioned medium of "Ehrlich +" cells, and stimulated motility was suppressed with various concentrations of purified anti-AMF IgG. The values are expressed as the mean ± SEM. $^{*}P < 0.005$ by Student's t test. (D) Permeability of mesothelial cells monolayers treated with various concentrations of mAMF for 24 h. □, peritoneal wall cell; ■, diaphragmatic cell. The values are means ± SD for triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's t test. (E) Morphological changes of mesothelial cells against mAMF-exposure. Cultured peritoneal wall cells were exposed to 1 pM mAMF and photographed. 1, 0 min; 2, 10 min after AMF addition, magnification, 200×.

transendothelial cell transport of circulating macromolecules such as plasma proteins [48,49], or VEGF promotes the transiently disruption of gap junctional communication of endothelial cells, which is one of the cell-to-cell junctions of vascularized tissues inside [50]. Though VEGF is an important molecule in malignant hyperpermeability and ascites accumulation with its multiple functions, the complexity of conditions in tumor-bearing hosts may suggest that more than one molecule is involved in this multistep process. Many tumor-related cytokines have been reported as hyperpermeability-inducing factors besides VEGF so far, for example, tumor necrosis factor causes the breakdown of the barrier function in an epithelial cell sheet [51], interleukin-6 increases the endothelial permeability due to contractile action on endothelial cells [52], etc. Generally, evaluation of cytokine function with in vivo studies must pay attention to the various secondary factors that are produced by surrounding tissues with cytokine stimulation in addition to the direct cytokines-effect itself. Therefore, if AMF–AMFR signaling brings by as yet unknown secondary reaction or alternation, it may be associated with the understanding of the above biological phenomenon. There are several AMF-inhibitors, since AMF is a PHI which acts on the Embden–Meyerhof pathway. The enzymic inhibitor such as carbo-

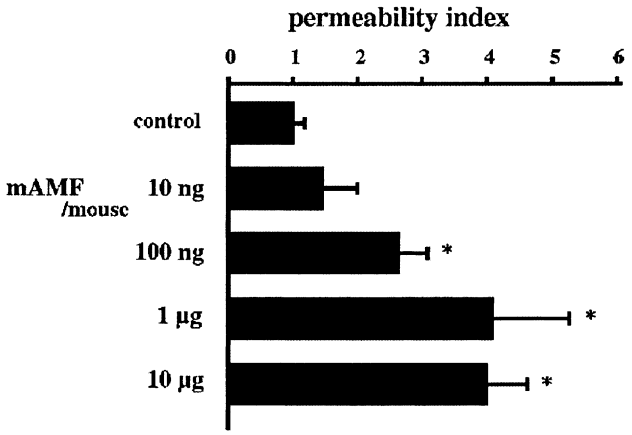

Fig. 4. Effect of mAMF on vascular permeability. Various amounts of mAMF were injected s.c. in the back of dye-pretreated rats ($n = 4$). The vascular leakage of dye from the plasma into the skin was quantified. The values are expressed as the mean ± SD. $^{*}P < 0.01$ by Student's t test.

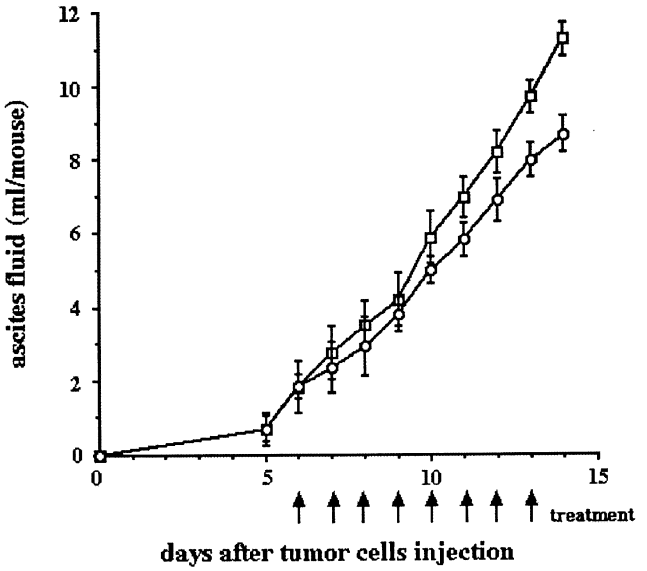

Fig. 6. Inhibitory effect of anti-AMF antibodies on the tumor ascites fluid standing. BALB/c mice (9-week-old male) were i.p. implanted with 5.0×10^6/0.2 ml PBS of "Ehrlich +" cells on day 0. Treatment started on the day 6 after cell inoculation. Mice were given 100 μg/0.2 ml PBS of anti-AMF IgG on days 6–13 everyday without drainage of ascites. Control animals were received PBS. After the ascites fluid was totally harvested, the volume of ascites fluid was determined [20]. -□-, PBS vehicle control; -○-, anti-AMF IgG treated. Each data value is the mean of five mice; *bars*, SD.

hydrate phosphates can inhibit the enhanced motility stimulated by AMF [30]. This may be applied in the future to the design of anti-metastatic or anti-angiogenic drugs.

In summary, the data indicate that AMF enhances vascular permeability due to gap formation of endothelial cell layer. And, AMF is able to affect directly host tissue cells and causes the loosening of a mesothelial cell sheet. AMF secreted by tumors causes the hyperpermeability of microvasculature supplying peritoneal lining tissues such as peritoneal wall and diaphragm, and hyperpermeability of mesothelium leads to extravasation of a plasma-rich exudate which extends subsequently. Or consecutive exposure of tumor-secreted AMF may enhance the permeability of preexisting microvessels lining tissues, and induction of angiogenesis,

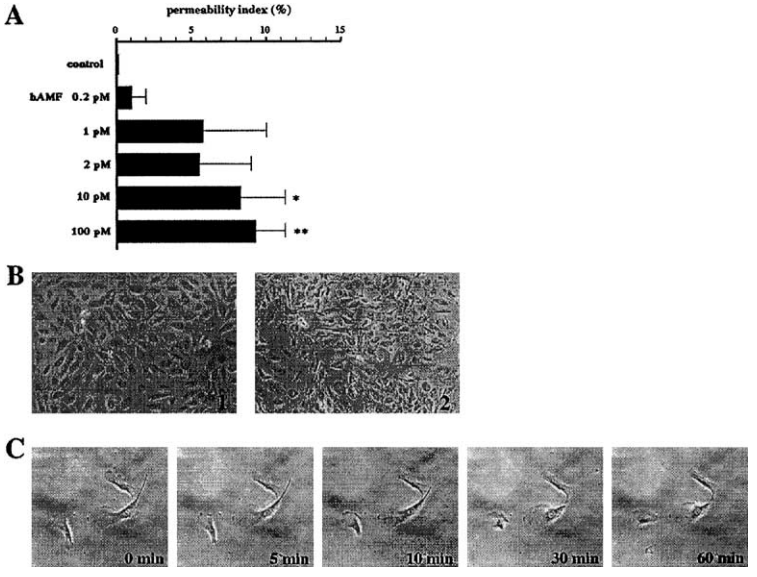

Fig. 5. Enhancing effect of hAMF on HUVECS monolayers permeability. (A) Permeability of HUVEC monolayers treated with various concentrations of hAMF for 24 h. The values are means ± SD for triplicate determinations. $^{*}P < 0.05$; $^{**}P < 0.01$ by Student's t test. (B) Gap formation of HUVECs monolayers. 10 pM of hAMF was exposed to tight confluent cultured HUVEC monolayer for 24 h and photographed. 1, No-exposure; 2, AMF-exposure. Magnification, 200×. (C) Morphological changes of HUVECs against 1 pM of hAMF-exposure. Magnification, 200×.

which contributes to the progressive growth of tumors, thus, mimicking the effect of VEGF.

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