Overexpression of autocrine motility factor in metastatic tumor cells: possible association with augmented expression of KIF3A and GDI- β

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Autocrine motility factor (AMF), which is identical to phosphohexose isomerase (PHI)/glucose-6-phosphate isomerase (GPI), a ubiquitous enzyme essential for glycolysis, neuroleukin (NLK), a neurotrophic growth factor, and maturation factor (MF) mediating the differentiation of human myeloid cells, enhances the motility and metastatic ability of tumor cells. AMF/PHI activity is elevated in the serum or urine in patients with malignant tumors. Here, we constructed an *amf/phi/nlk/mf* gene using adenovirus vector and transfected into two tumor cell lines. Overexpression of AMF/PHI/NLK/MF enhanced AMF secretion into the culture media in both tumor cell lines. However, upregulation of motility and metastatic ability was found only in metastatic fibrosarcoma cells expressing an AMF receptor, gp78, and was not found in gp78-undetectable osteosarcoma cells. Thus, not only serum AMF activity but also gp78-expression in tumor cells may be required for metastasis-related motility induction. With the use of microarray analyses, we detected two augmented genes, rho GDP dissociation inhibitor beta and kinesin motor 3A, as well as AMF itself. The RNA message and protein expression of these two molecules was confirmed to be upregulated, suggesting a possible association with AMF-induced signaling for cell motility and metastasis.

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One of the major features of malignancy is the invasive potential of the tumor cells. Liotta¹ proposed a three-step hypothesis for the mechanism of tumor cell invasion. The first is tumor cell attachment via cell surface receptors to the matrix, the second is local degradation of the matrix, and the third is tumor cell locomotion into the region of the matrix. A group of secreted cytokines inducing cell motile activity *in vitro* may initiate cell locomotion *in vitro*.² Among them, autocrine motility factor (AMF) stimulates cell motility in various types of tumor cells in an autocrine manner.^{3,4} We purified AMF from conditioned medium of murine metastatic fibrosarcoma, and demonstrated that AMF is

identical to phosphohexose isomerase (PHI), also designated as glucose-6-phosphate isomerase (GPI), which is a ubiquitous enzyme catalyzing the conversion of glucose-6-phosphate to fructose-6-phosphate.⁵ AMF/PHI has also been found to be identical to neuroleukin (NLK),^{6,7} a neurotrophic growth factor that supports the survival of spinal and sensory neurons,⁸ and maturation factor (MF) mediating the differentiation of human myeloid leukemic HL-60 cells to terminal monocytic cells,⁹ suggesting that this cytokine is multifunctional, probably dependent on the target cells. AMF/PHI activities have shown to be elevated in the serum or urine of patients with disseminated malignant tumors such as gastrointestinal, kidney, breast, colorectal, and lung carcinomas,^{10,11} thereby being useful as a tumor-dissemination marker. An immunohistochemical study has demonstrated that the prognosis of AMF/PHI-positive patients with pulmonary adenocarcinoma is poorer than that of negative patients.¹² Furthermore, AMF/PHI was

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found in rheumatoid joints,^{13,14} and was implicated as a synovial self-pathogenic antigen responsible for the onset of rheumatoid arthritis.^{15,16}

AMF binds to a unique 78 kDa glycoprotein receptor, and initiates gp78 phosphorylation.^{4,17} Anti-gp78 monoclonal antibody (3F3A) stimulates motility of metastatic tumor cell,¹⁸ and reveals the localization of gp78 on the leading lamella as well as the trailing edge of motile cells.¹⁹ The expression of gp78 is a predicting factor for prognosis in patients with various malignant neoplasms including esophageal, colorectal, bladder, gastric, and lung carcinoma.^{20–25}

The molecular pathway and regulatory mechanisms by which AMF manifests its effect on cells via its receptor have been elucidated in part. The signaling pathway involves receptor phosphorylation,⁴ and the inhibitory effect of pertussis toxin (PT) on the AMF-mediated cell motility suggests a regulatory role of G-binding protein.²⁶ It is also involved in the activation of phospholipases and the inositol cycle²⁷ with two downstream components, 12-lipoxygenases and protein kinase C.²⁸ Analysis of the protein kinase cascade(s) in the AMF-stimulated cell motility using specific kinase inhibitors reveals the involvement of protein kinase C and tyrosine kinase, but not kinase A, reiterating that the signaling is independent of the adenylate cyclase pathway.²⁹ Recently, AMF stimulation has been shown to result in stress-fiber formation, which is associated with activation of two family members of the Rho-like GTPases, RhoA and Rac1, but not Cdc42 activation. This association of the specific small GTPase Rho activation is accompanied by AMF-induced redistribution of the small GTPase from a uniform cellular distribution to the lamellipodia and filopodia at the leading edges of the cells' periphery.³⁰ Interestingly, transfection of AMF/PHI-tagged hemagglutinin into Cos7 cells reveals its intracellular localization within actin-rich pseudopodial domains.³¹ Torimura *et al*³² have demonstrated that AMF/PHI enhances Rho activity and phosphorylation of MEK1 and MEK2 in human hepatoma cell lines. Rho is one of the most important molecules in the AMF/PHI signal transduction.

In this study, to elucidate the metastatic mechanisms induced by AMF, we constructed an *amf/phi* transfection system using adenovirus, and then investigated its secretion, effects on cell motility, and metastasis. Furthermore, the expression of the molecules related to downstream reaction of AMF/PHI stimulation was analyzed with microarray analysis.

Materials and methods

Cells and Culture Conditions

To investigate the function of AMF/PHI as an autocrine cytokine, we used two cell lines able to grow in a protein-free condition. One was a murine

fibrosarcoma cell line, Gunma clone-4 protein-free (Gc-4 PF),⁵ in which the motility and metastatic capacity show high responses to AMF by increased expression of the receptor.¹⁸ Another was Dunn osteosarcoma protein free (DPF), a mouse osteosarcoma cell line.³³ Gc-4 PF cells were cultured in RPMI 1640 medium without serum. DPF cells were cultured in RPMI 1640 medium including trace elements (5 μ M FeCl₃, 5 μ M ZnSO₄, and 10 μ M sodium potassium tartrate) without serum. Both cells were incubated at 37°C in a humidified atmosphere of 5% CO₂ in air.

Construction of Recombinant Adenovirus

The cDNA of AMF/PHI was cloned by RT-PCR using mRNA extracted from BALB/cAnNCrj mouse liver as a template and the following primers: 5'-CTTCCGAGCACGTCTGC-3' and 5'-CTAGC TGGGGTGTGAAATACAG-3'. The PCR product was inserted into TA cloning vector pcR2.1 (Invitrogen Corp., Carlsbad, CA, USA), cut with *NotI* and *HindIII*, and inserted into pcDNA3 vector (Invitrogen Corp., Carlsbad, CA, USA) containing CMV promoter lesion. The whole sequence of the insert was analyzed and contained no mutation. An expression unit was excised with *NruI* and *SmaI* and inserted into the *SwaI* site of the cassette cosmid pAdex1cw. The DNA sample was packaged using Gigapack XL (Stratagene, La Jolla, CA, USA). After plating the transduced *Escherichia coli*, we could obtain a clone containing the desired insert. Recombinant adenovirus was constructed by homologous recombination in human embryonic kidney cell line 293 cells using the DNA samples and the *EcoT22I*-digested DNA-terminal protein complex (DNA-TPC). Viruses were propagated in the 293 cells and purified by two rounds of CsCl density centrifugation. We constructed two kinds of vectors, one of which contained *lacZ* gene (AdlacZ) and the other *amf/phi* gene (Adamf/phi). The cells were infected by viral solutions to cell monolayers and incubated at 37°C for 1 h with brief agitation every 20 min. Culture medium was added, and the infected cells were returned to the 37°C incubator.

β -Galactosidase Transduction Assay

To assess the efficiency of the adenovirus-mediated gene transfer, β -galactosidase activity of Gc-4 PF and DPF cells infected by adenovirus at 10 multiplicity of infection (MOI) and 100 MOI was examined 2 and 7 days after infection. At each point, cells were washed two times with PBS, fixed with 0.25% glutaraldehyde, washed four times with PBS, and stained in a 1 mg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal) solution consisting of 0.2 mM MgCl₂, 5 mM K₃[Fe(CN)₆] and 5 mM K₄[Fe(CN)₆] in PBS. β -Galactosidase-positive cells in each well were counted microscopically.

Immunoblot Analysis

Immunoblot analysis was performed to detect AMF/PHI and the receptor, gp78 in Gc-4 PF1 and DPF cells, as described previously.⁵ Briefly, cells were cultured on 100-mm dishes, infected by adenovirus at 10 and 100 MOI when they were confluent, and 200 μ l lysis buffer (PBS, 0.5% NP-40, 2 mM PMSF, 1 mM EDTA) was added 2 days after infection. The samples were incubated on ice for 30 min, centrifuged at 12 000 g for 5 min, and the supernatants were collected. At the same time, conditioned media were collected, dialyzed, lyophilized, and diluted with PBS. Samples were analyzed by SDS-PAGE in polyacrylamide slab gels at reducing condition for AMF receptor, gp78, and nonreducing condition for AMF, and electrotransferred to transfer membranes. The membranes were incubated with anti-AMF polyclonal antibody 1:400 in PBS containing 15% low-fat milk. Lactoperoxidase-labeled anti-rabbit antibody (Vector, Burlingame, CA, USA) was used for detection. ECL Western blotting system, an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Uppsala, Sweden), was then used to detect the antibody-specific proteins bound to the transfer membranes, according to the manufacturer's instructions.

To examine the upregulation of protein expression in response to *amf/phi* gene overexpression, transfected Gc-4 PF cells were lysed in NP-40 lysis buffer, 1% (v/v) NP-40/50 mM Tris, pH 8.0/150 mM NaCl, containing leupeptin (10 μ g/ml), aprotinin (10 μ g/ml), and phenylmethanesulfonyl fluoride (1 mM), incubated on ice for 30 min, centrifuged at 12 000 g for 5 min, and then the supernatants were collected. Subsequent steps were the same as mentioned above. Anti-GDI- β goat polyclonal antibody (Santa Cruz Biotechnology, Inc., CA, USA) and anti-KIF3A mouse antibody (BD Biosciences, San Jose, CA, USA) were used for detection of each molecule.

Phagokinetic Tracks

Gc-4 PF1 and DPF cells were infected by adenovirus at 10 and 100 MOI 2 days before assay. Uniform carpets of gold particles were prepared on coverslips coated with bovine serum albumin, as described previously.³⁴ Colloidal gold-coated coverslips were placed in 35-mm tissue culture dishes, and 3000 cells in suspension culture were added to the plates. After 24 h, the phagokinetic tracks were visualized using dark-field illumination in a Nikon inverted microscope. The area cleared of gold particles by at least 50 cells was measured using NIH image 1.62, and the standard error reflecting a 95% confidence level was calculated.

Experimental Pulmonary Metastasis

To examine the ability of experimental metastasis formation, 20 000 cells infected by adenovirus at 10

and 100 MOI were injected intravenously at the tail vein into C3H/He mice. Mice were killed 3 weeks later, and the lungs were examined for lung-colonizing nodules.

Oligonucleotide Microarray Analysis

Gc-4 PF1 cells were separated into three conditions; infected by *Adamf/phi* or adenovirus alone at 100 MOI where infection rate was almost 100%, or not infected. The isolated total RNA of each cell 2 days after transfection was used for synthesis of cDNA, which was labeled with Cy3 and hybridized with Atlas Glass Array Mouse 1.0 (Clontech Laboratories, Inc., Palo Alto, CA, USA) containing probe sets for 1081 mouse genes. The signal intensities from hybridized cDNA were quantified, and the ArrayGauge (Fuji film, Tokyo, Japan) analysis software was used to identify differentially expressed genes. The genes whose expression was up- and downregulated more than two-fold in response to adenovirus-mediated overexpression of AMF/PHI were selected and analyzed.

RT-PCR Analysis

RT-PCR was performed to confirm upregulation of RNA messages of the genes suggested to be upregulated in the microarray analysis. Total RNA was extracted from cells using Isogen (Wako, Osaka, Japan) 2 days after transfection. A measure of 3 μ g of total RNA treated with DNase was used as a template for cDNA synthesis. The products of RT reactions were used for PCR. Genes examined here involved rho GDP dissociation inhibitor beta (GDI- β) and kinesin motor 3A (KIF3A). The house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control. The number of cycles, 25 times for each gene, was selected to allow linear amplification of the cDNA under study. The primers sequences and their respective PCR fragment lengths were as follows: KIF3A, 5'-ACGCTGACGACATGGATAGAATCATG-3', 5'-CAGTTTGGAGTCCGATACGGCAC-3' (335bp); GDI- β , 5'-CAGACCCAACAGTTCCCAATGTGAC-3', 5'-CCATGAATGTGGCTTTATCCACTCTC-3' (249bp); and GAPDH 5'-CAAGATGCTGAAGGTCGCTGT-3', 5'-GGGTTTCTTACTCCTTGGAGG-3' (1013 bp). The abundance of the PCR products of interest was expressed in pixel intensities and divided for the abundance of the GAPDH signal amplified in parallel from the same cDNA sample.

Immunofluorescence Analysis Investigating KIF3A Distribution

Gc-4 PF1 cells plated for 24 h on glass coverslides were transfected with *Adamf/phi* gene or adenovirus alone at 100 MOI. After 48 h, cells were fixed with chilled methanol, incubated with anti-KIF3A antibody, and then the distribution was visualized

with FITC-conjugated rabbit anti-mouse IgG (CEDERLANE, Hornby, Ont., Canada). The coverslips bearing the stained cells were mounted in 90% glycerol in PBS for viewing and photomicrography.

Statistical Analysis

Phagokinetic and metastasis assays were analyzed with Bonferroni/Dunn's method as analysis of variance (ANOVA) and *post hoc* test.

Results

Adenovirus-mediated LacZ Expression in Gc-4 PF1 and DPF Cells

The proportions of β -galactosidase activity-positive Gc-4 PF1 and DPF cells infected AdlacZ at 100 MOI were both 100% at 2 days after infection (Figure 1a and e) and 95.8 ± 2.7 and 100% at 7 days after infection, respectively (Figure 1b and f). On the other hand, the proportions of positive cells at 10 MOI were 17.0 ± 6.1 and $54.5 \pm 9.8\%$ at 2 days after infection (Figure 1c and g) and 14.4 ± 2.6 and $53.0 \pm 10.8\%$ at 7 days after infection, respectively (Figure 1d and h). There were no positive cells when Adamf/phi, adenovirus alone, or nothing was transfected (data not shown).

Adenovirus-mediated Intracellular and Extracellular Expression of AMF/PHI and gp78 in Gc-4 PF and DPF Cells

The expression of intra- and extracellular AMF/PHI was detected in Gc-4 PF1 cells under all conditions (Figure 2a and b). Both intracellular and extracellular expressions were augmented by the infection with Adamf/phi at 100 MOI. Interestingly, dose dependency was evident in extracellular expression (Figure 2b). Although the intracellular AMF/PHI expression pattern in DPF cells was similar to that in Gc-4 PF1 cells (Figure 3a), the extracellular molecules were detected only in cells infected by Adamf/phi at 100 MOI (Figure 3b).

The expression of gp78 was found in Gc-4 PF cells as described previously,¹⁸ and was augmented by transfection with Adamf/phi in a dose-dependent manner (Figure 4a). In contrast, no expression of gp78 was observed in DPF cells even in the cells transfected by *amf/phi* gene at 100 MOI (Figure 4b), suggesting absence of the receptor for AMF in DPF cells.

Effects of Transfection of *amf/phi* Gene on Motility of Gc-4 PF1 and DPF Cells

As shown in Figure 5a, transfection of the *amf/phi* gene stimulates motility of Gc-4 PF1 cells in a dose-dependent manner. The motility of Gc-4 PF1 cells infected by Adamf/phi at 100 MOI was significantly

Figure 1 Adenovirus-mediated LacZ expression. Gc-4 PF1 ((a)–(d)) and DPF cells ((e)–(h)) were infected with AdlacZ at 100 MOI (a,b,e,f) and at 10 MOI (c,d,g,h). β -Galactosidase activity was examined 2 (a,c,e,g) or 7 days (b,d,f,h) after infection. Positive cells were depicted as blue cells. Bar indicates 20 μ m.

Figure 2 Effect of adenovirus-mediated *amf/phi* gene transfection on the expression of intracellular (a) and extracellular (b) AMF/PHI in Gc-4 PF1 cells.

higher than those not infected or infected by AdlacZ (ANOVA; $F(3, 125) = 9.128$, $P = 0.0003$, < 0.0001 , respectively), and cells infected by Adamf/phi at

Figure 3 Effect of adenovirus-mediated *amf/phi* gene transfection on the expression of intracellular (a) and extracellular (b) AMF/PHI in DPF cells.

Figure 4 Effect of adenovirus-mediated transfection of *amf/phi* gene on gp78 expression in Gc-4 PF1 (a) and DPF (b) cells.

10 MOI showed significantly higher motility than cells infected by AdlacZ ($P=0.0024$). In contrast, there was no change in the phagokinetic activity of DPF cells after the cells were transfected by *amf/phi* gene using adenovirus vector even at 100 MOI (Figure 5b).

Metastatic Responses of Gc-4 PF1 and DPF Cells to Transfection of *amf/phi* Gene

As shown in Figure 6, transfection of *amf/phi* gene resulted in an increase in the lung metastatic nodules of Gc-4 PF cells in a dose-dependent manner, while there was a negligible increase in the colonization of the AdlacZ-transfected cells. The metastatic ability of the cells infected by Adamf/phi at 100 MOI was significantly higher than that of untransfected and AdlacZ-transfected cells (ANOVA; $F(3, 28)=6.833$, $P=0.001$ and 0.0008 , respectively).

Figure 5 Effect of transfection of *amf/phi* gene on cell motility. Gc-4 PF1 (a) and DPF cells (b), transfected without or with Adamf/phi, or AdlacZ were plated on colloidal gold-coated coverslips. The area cleared of gold particles by at least 50 cells was photographed, and measured using NIH image 1.62.

Figure 6 Metastatic ability of Gc-4 PF1 cells induced by transfection of *amf/phi* gene. Mice were given i.v. injections of 2×10^4 Gc-4 PF1 cells transfected without or with Adamf/phi, or AdlacZ. The average number of lung-colonizing nodules is shown.

In contrast to Gc-4 PF cells, no metastatic pulmonary nodules were found after intravenous injection of DPF even after transfection of Adamf/phi at 100 MOI.

Identification of Genes Induced by *amf/phi* Gene Transfection by Microarray Analysis

Out of 1081 mouse-related genes, 14 and seven genes whose expression in the cells infected by Adamf/phi gene were increased and decreased more than two-fold compared with untransfected cells were, respectively, identified (Table 1). From them, three genes whose expression was more than two-fold compared with that of cells infected with adenovirus alone were selected. These were considered to be unaffected by adenovirus itself, and no suppressed genes were found in this selection. The identified genes were rho GDP dissociation inhibitor beta (GDI- β /GDID4/LyGDI; GenBank accession No. L07918), microtubule plus end-directed kinesin motor 3A (KIF3A; GenBank accession No. D12645), and phosphohexoseisomerase (PHI/AMF/NLK/MF; GenBank accession No. M14220). The enhanced rates of transfection of *amf/phi* gene against adenovirus alone were 2.15, 2.24, and 4.68, respectively.

Augmented Expression of GDI- β and KIF3A Transfected by *amf/phi* Gene Using RT-PCR and Immunoblot Analyses

RT-PCR analysis showed augmented expression of KIF3A after *amf/phi* gene transfection to be almost two-fold the mRNA level as compared with adenovirus transfection as well as nontransfection (Figure 7a). Immunoblot analysis also showed that a 27-kDa

band expression was augmented in *amf/phi* gene-transfected cells (Figure 7b), suggesting that overexpression of *amf/phi* gene induces protein production as well as RNA message of KIF3A. On the other hand, since augmented RNA message of GDI- β induced by *amf/phi* gene transfection was less than twice that of the control, we performed statistical analysis from three independent experiments. Densitometric analysis revealed that the intensity ratios of GDI- β expression against GAPDH expression in *amf/phi* gene-transfected, vector alone-transfected, and nontransfected cells were 1.122, 0.683, and 0.529, respectively, and that transfection of *amf/phi* resulted in significantly higher expression than the others (ANOVA; $F(2,9)=9.850$, $P=0.0114$, <0.0021 , respectively) (Figure 7c). Immunoblot analysis also showed again that an 80-kDa band was augmented in *amf/phi* gene-transfected cells (Figure 7d). These results confirmed those obtained from microarray analyses.

KIF3A Distribution in Gc-4 PF Cells

KIF3A is a microtubule-related protein,³⁵ and AMF receptor, gp78, is a family of microtubule-associated tubular organelles.³⁶ These findings prompted us to investigate the effect of *amf/phi* transfection on the KIF3A distribution. In the perinuclear area of untransfected cells, small puncta or tubulovesicular structures were abundant, as shown in neuron

Table 1 Up- and downregulated genes by infection of adenovirus including *amf/phi* gene in more than two-fold as compared with those in untransfected cells

Gene name	Rate	GenBank accession no.
<i>Upregulated genes</i>		
Homeobox protein 2.4 (HOX2.4)	3.11	X13721
Interferon regulatory factor 2 (IRF2)	2.19	J03168
myc proto-oncogene	2.76	X01023
Glucose-6-phosphate isomerase (GPI); phosphoglucose isomerase (PGI); phosphohexoseisomerase (PHI); neuroleukin (NLK)	4.02	M14220
Endothelin 3	2.16	U32330
Neuromodulin; growth-associated protein 43 (GAP43); protein F1; calmodulin-binding protein p57	2.43	J02809
Mothers against dpp homolog 7 (SMAD7; MADH7); MADH8	2.71	AF015260
Rho GDP dissociation inhibitor beta (GDI-beta; ARHGDI); GDP dissociation inhibitor D4 (GDID4)	2.69	L07918
Matrix metalloproteinase 14 (MMP14); membrane-type Matrix metalloproteinase 1 (MTMMP1)	3.23	X83536
Tissue inhibitor of metalloproteinase 3 (TIMP3); SUN	2.61	L19622
Microtubule plus end-directed kinesin motor 3A (KIF3A)	4.96	D12645
Alpha internexin neuronal intermediate filament protein (alpha-INX; INA)	2.19	L27220
Filensin; beaded filament structural protein in lens 1 (BFSP1)	2.24	Y13602
Unconventional myosin VI	3.45	U49739
<i>Downregulated genes</i>		
Groucho gene-related protein (GRG); amino enhancer of split protein (AES)	0.43	L12140
GATA-binding protein 4 (GATA4)	0.47	M98339
Ephrin A2 (EFNA2); eph-related receptor tyrosine kinase ligand 6 (EPLG6; LERK6); ELF1; CEK7 ligand (CEK7-L)	0.39	U14752
Wingless-related MMTV integration site 3a protein (WNT3A)	0.35	X56842
Radical fringe homolog (RFNG)	0.47	U94350
crk proto-oncogene	0.34	S72408
Transducin beta-2 subunit	0.29	U34960

Increased expression of genes more than two-fold as compared with that in cells transfected with adenovirus alone are depicted in bold.

Figure 7 RT-PCR and immunoblot analyses of Gc-4 PF1 cells transfected with *amf/phi* gene. mRNA ((a), (c)) and protein ((b), (d)) expressions of KIF 3A ((a), (b)) and GDI-β ((c), (d)) were investigated, as described in experimental procedures.

cells.³⁵ On the other hand, in some cells a diffuse distribution was depicted at the ruffled edges of the cells, including lamellipodia (Figure 8), showing a character unique to these metastatic fibrosarcoma cells. More cells with stained lamellipodia seemed to be found in *amf/phi*- than vector-transfected cells.

Discussion

In the present study, we constructed an *amf/phi/nlk/mf* transfection system using adenovirus vector, succeeded in obtaining a high efficiency of gene transfection, and produced a sustained gene expression for at least 1 week. This induction was observed in both cell lines in a dose-dependent manner, but more transduction efficiency seemed to be found in

Figure 8 Indirect immunofluorescent labeling of Gc-4 PF cell with the anti-KIF 3A mouse antibody. A diffuse distribution was depicted at the ruffled edges of the cells (arrows) in some AMF-transfected cells. Bar indicates 5 μm.

DPF cells at 10 MOI transfection than in Gc-4 PF cells (half vs one-fifth of total cells), although almost 100% of the cells were transfected at 100 MOI in both cell lines. Induction of RNA message of delivered *amf/phi* was clearly demonstrated by microarray analysis. Intracellular protein product was recognized by the anti-AMF pAb in a dose-dependent manner. This adenovirus vector-assisted gene delivery system may thus be useful for studying the mechanisms by which AMF/PHI stimulates disseminating ability in metastatic cells.

It is interesting that PHI, which catalyzes the conversion of glucose-6-phosphate to fructose-6-phosphate intracellularly, also stimulates extracellularly cell motility in an autocrine manner. The mechanisms of the secretion of AMF/PHI, which lacks a secretory signal peptide, have been elucidated in part. Haga *et al*³⁷ demonstrated that CK II-phosphorylation contributes to the secretion of AMF via a nonclassical pathway. Niinaka *et al*³⁸ have described that AMF/PHI is secreted into conditioned medium only in malignant cell lines while both malignant and normal cell lines express AMF/PHI, and that markedly higher RNA message expression of AMF/PHI is seen in malignant than in normal cell lines. In the present study, immunoblot analysis showed that AMF/PHI secretion was augmented by the *amf/phi* gene transfection in a dose-dependent manner in Gc-4 PF cells. Interestingly, AMF/PHI molecules appeared in conditioned medium following high-amount gene transfection even in DPF cells that did not secrete AMF/PHI before transfection. These results indicated that high production of *amf/phi* gene RNA message could allow the cells to secrete AMF/PHI even if the cells do not originally secrete AMF/PHI. Such quantitative factors may play an important role in the extracellular secretion of AMF/PHI molecule regardless of

the cell type, while qualitative factors such as biochemical modification have still not been fully elucidated.

Gc-4 PF1 cells exhibited an increased metastatic ability in response to the transfection of *amf/phi* gene in a transfection rate-dependent manner. This augmentation of metastatic ability corresponded well to the expression of both AMF/PHI and the receptor, gp78 in this metastatic tumor cell line. On the other hand, in nonmetastatic DPF cells, the metastatic ability was not obtained by gene transfection regardless of evident induction of extracellular AMF/PHI expression. It should be noted that in contrast to Gc-4 PF cells, no expression of gp78 was acquired even after *amf/phi* gene transfection in DPF cells. Probably, metastatic ability regulated by AMF/PHI may require the receptor, gp78 expression as well as AMF/PHI itself.

It is interesting to elucidate what phenotypes are responsible for augmented metastatic propensity in response to *amf/phi* gene overexpression. In the present study, augmented cell-motile activity was also found in Gc-4 PF cells in response to the *amf/phi* gene transfection in a transfection rate-dependent manner. In contrast, motile activity of DPF cells did not respond to the *amf/phi* gene transfection. Motility stimulated by AMF/PHI also requires AMFR expression, since gp78 expression was not induced by *amf/phi* gene transfection in DPF cells. Thus, motility may be most important in the phenotypes regulated by the autocrine AMF/PHI stimulation. Recently, on the other hand, Funasaka *et al*³⁹ have demonstrated that AMF/PHI enhances neovascularization and motile activity of human umbilical vein endothelial cells, which would initiate and promote metastasis. AMF/PHI also stimulates MMP-2 secretion and extracellular MMP-2 activation, being related to destruction of the basement membrane followed by metastasis and invasion in hepatoma cells.³² These mechanisms occurring in host organs also may contribute to AMF-regulated dissemination of tumor cells.

Rho GTPases play an important role in metastasis of cancer cells.^{40,41} The active form of the Rho family of small GTPases consists of a GTP-bound one, and inactive form GDP-bound. GTPase-activating proteins (GAPs) accelerate intrinsic GTP hydrolytic activity,⁴² guanine nucleotide exchange factors (GEFs) stimulating the replacement of GTP by GDP,⁴³ and guanine nucleotide-releasing factors (GRFs), enhancing the GDP dissociation rate. On the other hand, GDP dissociation inhibitors (GDIs) suppress the GDP dissociation rate.^{44,45} In the present study, microarray, RT-PCR, and Western blot analyses revealed that GDI- β , a member of the Rho GDI superfamily, was upregulated by *amf/phi* gene transfection. Rho GDI- β is preferentially expressed in hematopoietic cells,⁴⁵ and involved in T-cell antigen receptor-induced pathways.⁴⁶ Recently, the structural basis for the regulation of Rho proteins by the molecule has been elucidated.⁴⁷ The role of Rho

GDI- β in invasion and metastasis of cancer cells is still controversial at present. Tapper *et al*⁴⁸ demonstrated that upregulation of Rho GDI- β is associated with progression of ovarian carcinoma with large-scale survey in serous ovarian carcinoma by cDNA array analysis,⁴⁸ corresponding well to our present results. In contrast, however, a group of Theodorescu has reported the Rho GDI- β as an invasion and metastasis suppressor gene with the findings that the reduced expression is related to metastatic progression of bladder cancer.^{49–51} Interestingly, transfection of Rho GDI- β into metastatic cells resulted in suppression of both metastatic ability and *in vitro* motility, but did not affect *in vitro* growth, colony formation, or *in vivo* tumorigenicity.⁴⁹ Upregulation of GDI- β in the present study might be induced as a negative signal in the putative feedback mechanisms against excess signals from AMF/PHI. Further detailed experiments will be necessary to elucidate the exact role of the small G-binding protein.

Kinesin is known to be a microtubule-based motility protein that moves to the plus end of microtubules.⁵² Many kinds of kinesin-like motor protein have been identified, all of which have a homologous domain containing a putative ATP and microtubule-binding sites.⁵³ KIF3A is one of this superfamily, and forms a complex with KIF3B and kinesin superfamily-associated protein 3 (KAP3).⁵⁴ The role of microtubules in cell adhesion lies in the supporting kinesin-based transport to the adhesion sites, which control the dynamics of adhesive structures.⁵⁵ Adhesion site detachment leading to the retraction of a cell edge in motile cells is associated with microtubule-targeting events.⁵⁶ Recently, kinesin has shown to be required for the focal delivery of possible components to retard substrate adhesions' growth or promote their disassembly.⁵⁷ In the present study, *amf/phi*-transfected cells showing high motility exhibited not only augmented expression of KIF3A but also a tendency of distribution shift to the cell periphery. KIF3A may play an important role in the focal delivery of a component(s) that modulates the dynamics of adhesive structures at the focal contact, and then stimulate cell motility. Recently, activation of RhoA and Rac1, but not Cdc42 has been shown to be required for AMF/PHI signaling in association with c-Jun kinases.³⁰ These molecules corresponded well with components that are possible molecules specifically accumulated at microtubule tips as cofactors, together with cargo delivered by kinesin, in signal transduction events.⁵⁷ Small GTPase rho and the downstream effector molecules may be possible components to which KIF3A may be related, delivering to the cell periphery in association with microtubules in AMF/PHI-stimulated cells.

In conclusion, AMF/PHI overexpression induced AMF/PHI secretion. Augmentation of motility and metastatic ability required not only upregulation of intracellular expression of AMF/PHI followed by

secretion but also sufficient gp78 expression. Overexpression of AMF/PHI may be related to increases in GDI- β and KIF3A expression. AMF/PHI is a multipotential molecule. The responses may depend on the cell differentiation. Further investigations will be necessary in order to elucidate the exact role of this molecule in individually differentiated cells.

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