Novel roles of the autocrine motility factor/phosphoglucose isomerase in tumor malignancy

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Abstract

Autocrine motility factor (AMF) stimulates cell motility in an autocrine manner and is related to tumor malignancy. AMF is a multifunctional molecule, also known as phosphoglucose isomerase and neuroleukin. Signal cascades of the AMF-stimulated motility and novel functions of this protein contributing to tumor malignancy have been presented recently. AMF stimulation activated small Rho-like GTPases and subsequently induced actin fiber rearrangement, which was removed by the C3 exoenzyme, a specific inhibitor of Rho. The expression of Jun N-terminal kinase (JNK)1, JNK2 and the Rho GDP dissociation inhibitor- β was upregulated by AMF. The addition of AMF to culture medium stimulated the motility of the endothelial cells and the formation of tube-like structures in collagen gels. Highly AMF-expressing HT1080 cells induced aggressive angiogenesis *in vivo*. The expression of fms-like tyrosine kinase (Flt)-1, a vascular endothelial growth factor (VEGF) receptor, was enhanced in AMF-expressing tumors dependent on protein kinase C and phosphatidylinositol 3 kinase (PI3K) activation; meanwhile kinase insert domain-containing receptor, another receptor of VEGF, was not. Permeability of mesothelial and endothelial cell monolayers was increased by AMF, and numerous gaps were observed in the monolayers after treatment with AMF. AMF gene transfection transformed NIH3T3 cells to proliferate quickly and acquire anti-apoptosis ability induced by serum deprivation in a PI3K-dependent manner. The anti-apoptotic effect of AMF has been described by other authors who have shown that the AMF over-expressing cells were resistant to mitomycin-C-induced apoptosis showing regression of Apaf-1 and caspase-9 dependent on PI3K and MAP kinase. These novel functions of AMF makes it a likely target for cancer therapy.

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Introduction

Autocrine motility factor (AMF) was originally purified from the conditioned medium of human A2058 melanoma cells as a 55 kDa cytokine that stimulates the random or directed motility of cells in an autocrine manner (Liotta *et al.* 1986). This protein is elevated in the serum or urine of patients with malignant tumors such as gastrointestinal, kidney, breast, colorectal and lung carcinomas, thereby being useful as a tumor marker (Baumann & Brand 1988, Baumann *et al.* 1990, Filella *et al.* 1991, Patel *et al.* 1995).

Molecular cloning and sequencing have identified AMF as a phosphoglucose isomerase (PGI) (Watanabe

et al. 1996), neuroleukin (NLK) (Chaput *et al.* 1988, Faik *et al.* 1988) and maturation factor (MF) (Xu *et al.* 1996), suggesting that this cytokine is multifunctional and a member of the ectoenzyme/exoenzyme family. PGI is an ubiquitous cytosolic enzyme that plays a critical role in both Embden–Meyerhof glycolytic and glucogenetic pathways, catalyzing the reversible inter-conversion of glucose-6-phosphate to fructose-6-phosphate (Harrison 1974). Specific PGI inhibitors inhibit not only PGI activity but also suppress the cell motility stimulated by AMF (Watanabe *et al.* 1996). Crystal structure analysis and site-directed mutagenesis studies

have revealed that the regions responsible for PGI enzymatic activity overlap AMF cytokine active sites (Tanaka *et al.* 2002). Since hereditary non-spherocytic hemolytic anemia associated with PGI deficiency was first reported in 1968 (Baughan *et al.* 1968), this anemia has been found in many patients with PGI mutations, which are not, however, common (Kanno *et al.* 1996, Beutler *et al.* 1997). NLK is a neurotrophic factor that supports the survival but not the proliferation of embryonic spinal and sensory neurons, but it does not affect sympathetic or parasympathetic neurons (Gurney *et al.* 1986a). The expression of NLK and its receptor is increased in the hippocampus of rats when maze learning and is reduced in aged rats with learning deficits (Luo *et al.* 2002). A point mutation of NLK is associated with mental retardation (Schröter *et al.* 1985, Kugler *et al.* 1998). MF mediates the differentiation of human myeloid leukemia HL-60 cells to terminal monocytic cells (Xu *et al.* 1996). Furthermore, this protein can induce immunoglobulin secretion by cultured human peripheral blood mononuclear cells (Gurney *et al.* 1986b), affect the differentiation of osteoblastic mouse cell MC3T3-E1 (Zhi *et al.* 2001) and is defined as the antigen that provokes arthritis of the K/BxN T cell receptor transgenic mouse with many features similar to rheumatoid arthritis in humans (Matsumoto *et al.* 1999).

Interestingly, AMF has no signal peptide essential for the classical or endoplasmic reticulum (ER)/Golgi-dependent secretory pathway and is predominantly secreted from some kinds of tumor cells (Liotta *et al.* 1986, Silletti *et al.* 1991, Watanabe *et al.* 1991a, Watanabe 1994, Niinaka *et al.* 1998) or T cells stimulated with lectins such as Concanavalin A, phytohemagglutinin or pokeweed mitogen (Gurney *et al.* 1986b). We have reported that the transfection of the AMF gene into normal or non-AMF-secreting tumor cells augments the release of AMF (Tsutsumi *et al.* 2003, Yanagawa *et al.* 2004). In addition, the cells secreting AMF express higher mRNA levels of AMF than do normal cells (Niinaka *et al.* 1998), and secreted AMF is phosphorylated (Haga *et al.* 2000). Phosphorylation or an excess amount of this protein in the cytosol may induce secretion of AMF although the details of the secretion mechanism still remain uncertain.

Secreted AMF stimulates cell motility via binding to a seven transmembrane glycoprotein of 78 kDa, AMF receptor (AMFR)/gp78 (Silletti *et al.* 1991, Shimizu *et al.* 1999). AMFR was found on the surface of B16-F1 melanoma cells cultured as spheroids on a non-adhesive substrate (Nabi & Raz 1987) and, recently, has been identified as a really interesting new gene (RING) finger-dependent ubiquitin protein ligase (E3) of the ER (Fang *et*

al. 2001). This binding reaction is followed by internalization of the receptor, stimulating pertussis toxin (PT)-sensitive G protein (Watanabe *et al.* 1991b), inositol phosphate production (Kohn *et al.* 1990) and receptor phosphorylation (Watanabe *et al.* 1991a). Analysis using specific kinase inhibitors has revealed that AMF-induced cell motility is dependent on protein kinase C (PKC) and tyrosine kinase, but not protein kinase A (PKA) (Timar *et al.* 1993, Kanbe *et al.* 1994). AMFR internalizes its ligand via two pathways, a caveolae-mediated pathway to the smooth ER tubules (Benlimame *et al.* 1998) and a clathrin-dependent pathway where the AMF/AMFR complex is delivered to the multivesicular body and AMF recycles to cell surface fibronectin fibrils (Le *et al.* 2000).

In addition to the facts mentioned above, some novel and important functions of AMF that contribute to tumor malignancy have been found recently. We describe their mechanisms and the relationship between AMF and the molecules related to motility, angiogenesis and anti-apoptosis.

The small Rho-like GTPases related to the motility induced by AMF

The family of small Rho-like GTPases is critical for mediating a number of cellular processes such as proliferation, secretion and an extensive reorganization of the cytoskeleton, alterations of which are associated with a migratory phenotype (Ridley *et al.* 1992, Hall 1998, 1990). We therefore examined the roles of small Rho-like GTPases, RhoA, Rac1 and cdc42, in the cell motility stimulated by AMF (Tsutsumi *et al.* 2002). The levels of active RhoA, Rac1 and cdc42 in AMF-stimulated versus resting human malignant melanoma A375 cells were analyzed. AMF stimulation increased active RhoA and Rac1 levels in a time- and dose-dependent manner without elevation of total RhoA and Rac1 protein levels; meanwhile, active cdc42 levels did not change with AMF stimulation. Furthermore, responding to AMF stimulation, both Jun-N-terminal kinase (JNK)1 and JNK2 which are downstream proteins of small Rho-like GTPases were also increased in a dose-dependent manner.

Rho is essential for both the formation and maintenance of actin stress fiber (Ridley & Hall 1992, Nobes & Hall 1995). We have analyzed the distribution and organization of the molecules using immunofluorescence (Tsutsumi *et al.* 2002). AMF added to the conditioned medium effectively enhanced the motility of cells and reorganization of the actin molecules of A375 melanoma cells. This cytoskeletal rearrangement, the formation of heavy bundles of stress fiber-like structures transversing the cells, was also observed in human fibrosarcoma HT1080 cells stimulated by AMF. Next, we observed

Figure 1 Signals of the molecules related to the motility induced by AMF stimulation. The motility of some tumor cells is enhanced by AMF. AMF stimulation via its receptor AMFR activates small Rho-like GTPase, Rac1 and RhoA in a time- and dose-dependent manner in human malignant melanoma A375 cells; meanwhile, cdc42 does not respond to AMF. Subsequent signals upregulate JNK1 and JNK2, both of which are downstream proteins of small Rho-like GTPases and induce actin fiber rearrangement, the formation of heavy bundles of stress fiber-like structures transversing the cells. AMF overexpression also enhances GDI- β expression which regulates small Rho-like GTPases in the putative negative feedback mechanisms against excess signals from AMF.

the cytoskeletal changes induced by AMF in the presence of the C3 exoenzyme, a specific inhibitor of Rho. C3 exoenzyme inhibited stress fiber formation accompanied by a decrease in active RhoA, indicating that AMF-induced cytoskeletal rearrangement is dependent on Rho activation. Torimura *et al.* (2001) also demonstrated that, in human hepatoma cell lines, AMF/PGI enhanced Rho activity, which was slightly blocked by the function-blocking antibody for the integrin- β 1 subunit.

We recently presented evidence that overexpression of AMF in mouse fibrosarcoma Gc-4 PF cells using the adenovirus vector enhances the expression of Rho GDP dissociation inhibitor- β (GDI- β) (Yanagawa *et al.* 2004). GDI- β , a member of the Rho GDI superfamily, regulates the small Rho family GTPases suppressing the GDP dissociation rate (Scherle *et al.* 1993, Dirac-Svejstrup *et al.* 1997). The role of GDI- β in invasion and metastasis is still

controversial. In ovarian carcinoma, upregulation of GDI- β is associated with progression of the tumor (Tapper *et al.* 2001), while this protein is also reported as an invasion and metastasis suppressor in bladder cancers (Seraj *et al.* 2000, Gildea *et al.* 2002, Harding *et al.* 2002). Upregulation of GDI- β might be induced as a negative signal in the putative feedback mechanisms against excess signals from AMF. Figure 1 represents the signals of the molecules related to the AMF-induced motility.

The effect of AMF on endothelial cells

Angiogenesis is a crucial process for tumor growth and metastasis (Liotta *et al.* 1974, Folkman *et al.* 1989) and has three main steps: enzymatic degradation of the basement membrane; endothelial cell migration; and

endothelial cell multiplication. First, we focused on 'endothelial migration' and examined the effect of AMF on endothelial cells (Funasaka *et al.* 2001). For this experiment, we used human umbilical vein endothelial cells (HUVECs) and detected AMFR with immunofluorescence. The addition of AMF to the cells led to augmented expression of AMFR presumably caused by positive feedback. This phenomenon is observed in tumor cells (Niinaka *et al.* 1998). Phagokinetic assay revealed that the HUVECs' motility was increased approximately 2-fold by AMF stimulation. To accomplish the process of angiogenesis, migrating endothelial cells need to proliferate and undergo morphogenesis such as the formation of capillary-like tubes. AMF did not affect proliferation of HUVECs *in vitro* with the dose effective for motility stimulation. To investigate the changes in the structure formed by endothelial cells *in vitro*, we cultured HUVECs on collagen gels with or without AMF and measured the total length of a network of the cells elongated and anastomosed with each other. This tube-like structure, related to angiogenesis, was 2.5-fold more in the culture with AMF than in that without AMF.

We used two ways to evaluate the *in vivo* angiogenic activity of AMF. The first one was the Matrigel plug assay according to the report by Passaniti *et al.* (1992). Matrigel in liquid form was mixed with/without AMF and injected subcutaneously into mice. AMF increased the cells infiltrating into the Matrigel that formed capillary-like tube structures in a dose-dependent manner. In another assay, the effect of AMF on tumor-induced angiogenesis was investigated using a diffusion chamber which contained HT1080 cells and which was transplanted into a mouse dorsal sac (Abe *et al.* 1993). We prepared two cell lines, HT1080 cells transfected with the AMF gene and a control counterpart. The cells transfected with the AMF gene secreted four-fold more than did the control cells. In addition, in this assay, AMF contributed to the formation of capillary blood vessels. We concluded that AMF is capable of affecting endothelial cells and inducing angiogenesis.

The crosstalk between AMF–AMFR and vascular endothelial growth factor (VEGF)–VEGF receptor (VEGFR) signals

Many angiogenic factors such as acidic-fibroblast growth factor (FGF), basic FGF, tumor necrosis factor (TNF)- α , TGF- β and VEGF are needed as either a stimulator or an inhibitor for the various steps in the angiogenesis process (Folkman & Klagsbrun 1987, Folkman 1990, Folkman & Shing 1992, Koch *et al.* 1992, Risau 1997). Among them, VEGF is the prime regulator of angiogenesis and affects endothelial cells

specifically (Leung *et al.* 1989). VEGF acts via two tyrosine-phosphorylating receptors, fms-like tyrosine kinase (Flt-1) (Shibuya & Yamaguchi 1990) and KDR (Terman *et al.* 1991), the expression of which is limited to the endothelium (de Vries *et al.* 1992, Quinn *et al.* 1993). We therefore proceeded to the next investigation, the signal crosstalk between VEGF–VEGFR and AMF–AMFR (Funasaka *et al.* 2002). HT1080 cells known to secrete VEGF expressed more VEGF and AMFR at the mRNA level in the medium with AMF than in control medium. With regard to the VEGFRs, AMF increased the expression of Flt-1 in HUVECs in a dose-dependent manner while not that of KDR. To confirm the *in vivo* Flt-1 expression in response to tumor-secreted AMF, we implanted a diffusion chamber including HT1080 cells transfected with AMF into mice and evaluated the expression with immunohistochemical staining. Increased Flt-1 expression was found on the blood vessels surrounding the diffusion chamber.

Next, the biological function of the Flt-1 induced by AMF was examined. The HUVECs were exposed to AMF to enhance the Flt-1 expression and the motile response to VEGF of the pretreated cells was measured in three assays, the phagokinetic track assay (Albrecht-Buehler 1977), the wound healing assay (Silletti *et al.* 1993) and the Transwell migration assay (Repesh 1989). VEGF significantly stimulated the untreated cells' haptotactic and chemotactic motility but not the chemokinetic one as previously reported (Kumar *et al.* 1998). Meanwhile, the chemokinetic random locomotion of AMF-treated cells was also increased by VEGF in other motility assays. We considered that the augmented Flt-1 expression by AMF enhanced the sensitivity of endothelial cells to VEGF stimulation. This result is compatible with the fact that proliferative signals of VEGF in endothelial cells mainly depend on KDR, on the other hand, migrational activities depend on Flt-1 (Kanno *et al.* 2000, Soker *et al.* 2001).

Furthermore, the intracellular signal transduction of AMF-induced Flt-1 expression in HUVECs was investigated using various inhibitors, a non-isoform-selective PKC inhibitor GF109203X, a PKA inhibitor H89, a phosphatidylinositol 3 kinase (PI3K) inhibitor wortmannin, a MAP kinase (MAPK) inhibitor PD98059, a tyrosine kinase inhibitor genistein and a G regulatory subunit of adenylate cyclase inhibitor PT. Among them, GF109203X and wortmannin could inhibit Flt-1 expression induced by AMF while the others had no suppressive effect, which indicated that AMF-induced Flt-1 expression in endothelial cells is dependent on the activation of PKC and PI3K in endothelial cells. Meanwhile, the AMF–AMFR pathway leading to tumor cell motility is regulated not only by PKC

Figure 2 Interaction between AMF–AMFR and VEGF–VEGFR signal in the tumor and host endothelial cells. Secreted AMF stimulates tumor cells in an autocrine manner and enhances the production of VEGF which regulates endothelial cells via its receptor, Flt-1 and KDR. Host endothelial cells show augmented expression of Flt-1 responding to AMF stimulation in PKC- and PI3K-dependent manners. Increased Flt-1 contributes to endothelial locomotion responding to VEGF. In addition, AMF stimulation via AMFR also enhances endothelial cell motility. VEGF enhanced by AMF signals induces endothelial cell proliferation via KDR. These results indicated that AMF can change the sensitivity of endothelial cells to VEGF and induce angiogenesis in both autocrine and paracrine manners.

(Timer *et al.* 1993) but also by tyrosine kinase (Kanbe *et al.* 1994) and PT-sensitive G protein (Watanabe *et al.* 1991b). We present the interaction between AMF–AMFR and VEGF–VEGFR signals in the tumor and host endothelial cells (Fig. 2).

AMF involvement in the accumulation of ascites fluid

Tumor ascites are produced due to various reasons such as lymphatic obstruction (Feldman *et al.* 1972), neovascularization of the parietal peritoneum (Garrison *et al.* 1987) and hyperpermeability of microvessels lining the peritoneal cavity (Senger *et al.* 1983, Garrison *et al.* 1987). The cellular morphological changes and movements are associated with the formation of cellular gaps in the endothelial monolayer which accelerates permeability (Maruo *et al.* 1992, Suarez & Ballmer-Hofer 2001).

Thus we investigated the influence of AMF on the permeability of endothelial or mesothelial cells and ascites accumulation (Funasaka *et al.* 2002a). In the study, we used two types of Ehrlich mouse ascites tumor cell line, one of which grows well in mice and induces abundant ascites (Dunham & Stewart 1953) and another which grows poorly and does not develop ascites (Boone *et al.* 1965). The former secreted AMF ten times more than the latter, which may suggest that AMF plays an important role in ascites accumulation. To examine the change in host cells by the AMF secreted from Ehrlich cells, we isolated mesothelial cells from a mouse (Akedo *et al.* 1986) and tested their motilities. Both recombinant AMF and a conditioned medium of Ehrlich cells stimulated mesothelial cell locomotion, which was inhibited by anti-AMF antibody. The permeability of the mesothelial cell monolayer on micropore membranes was increased by the addition of recombinant AMF. The permeability of the

Figure 3 Signal pathways with regard to apoptosis resistance induced by AMF overexpression. AMF-expressing cells are resistant to apoptosis induced by serum deprivation or mitomycin-C. AMF signals activate PI3K which activates Akt/PKB, the signal of which inactivates BAD and caspase-9, thereby involving the suppression of apoptosis induced by serum deprivation. Another experiment shows AMF overexpression which suppresses the expression of Apaf-1 and caspase-9 which are important for apoptosis initiation, activating PI3K and MAPK and causing mitomycin-induced apoptosis resistance. There is a possibility that the increase of PGI enzymatic activity may cause hyper-metabolism of glucose which is related to malignant tumors and affects the apoptosis pathways.

HUVEC monolayer was also enhanced by AMF and the monolayer formed numerous gaps and was retracted. The enhancement of vascular permeability by AMF was also confirmed with the Miles' *in vivo* permeability assay (Miles & Miles 1952). In addition, VEGF is known to increase microvessel permeability although its mechanism still remains to be defined (Dvorak *et al.* 1995, Luo *et al.* 1998). Taken together, these results indicated that AMF directly or indirectly enhances the permeability of endothelial and mesothelial layers and subsequently leads to accumulation of ascites.

Anti-apoptotic effect related to AMF overexpression

Many growth factors are known to transform normal cells and promote cell survival (Harrington *et al.* 1994, Bardelli *et al.* 1996, Bikfalvi *et al.* 1997, Parrizas *et al.* 1997, Descamps *et al.* 2001). We transfected the AMF gene into mouse fibroblast NIH3T3 cells and established the cell line overexpressing AMF (Tsutsumi *et al.* 2003). This cell line secreted AMF, although the parental or vector-only transfected control NIH3T3 cells did not,

changed its morphology from a flat appearance to a fusiform one and acquired aggressive invading ability. Interestingly, the AMF-transfected cells inoculated into mice formed tumors whereas control cells failed to do so. In addition, the proliferation rate of the AMF-transfected cells was two-fold greater than that of the controls *in vitro*. Notably, these transfected cells were resistant to the induction of apoptosis evoked by serum deprivation which led the control cells to die. Thus, we examined the anti-apoptotic function of AMF using this cell line. To determine the apoptosis pathways affected by AMF overexpression, two pharmacological inhibitors, Ly294002 (PI3K inhibitor) and PD98059 (MAPK inhibitor) were used. DNA fragmentation, an indicator of apoptosis, was detected in AMF-transfected cells under treatment with Ly294002 but not with PD98059, which means that the AMF-induced apoptosis resistance is mainly dependent on PI3K. PI3K produces phosphoinositol phospholipid second messengers that activate Akt/protein kinase B (PKB) the signal of which phosphorylates the Bcl-2 family member BAD (Datta *et al.* 1997) and caspase-9 (Cardone *et al.* 1998) and is involved in apoptosis suppression (Franke *et al.* 1997, Khwaja *et al.* 1997). The activation of Akt/PKB is provoked by phosphorylation of both Thr308 and Ser473 (Alessi *et al.* 1996). Western blot analysis using phosphospecific antibody against Akt/PKB revealed that its phosphorylation was detected only in AMF-transfected cells and not in controls. This phosphorylation was abrogated by inhibition of PI3K. In conclusion, AMF overexpressing NIH3T3 cells acquired aggressive invasion ability, tumorigenicity and apoptosis resistance via PI3K-mediated activation of Akt/PKB.

Haga *et al.* (2003) also reported apoptosis protection by AMF using the AMF-transfected HT1080 cells and the Ehrlich cells that originally overexpressed and secreted AMF, both of which were the same as the cell lines that we used. Their experiment also showed that overexpression of AMF contributed to morphological changes and an increase in cell motility. To induce apoptosis, mitomycin C, an apoptosis-inducible anti-cancer drug, was used and AMF overexpressing cells were resistant to this drug. They used a DNA chip to compare the gene expression of AMF-transfected HT1080 with that of MOCK cells and detected some genes that disappeared including caspase-9 and apoptotic protease activating factor 1 (Apaf-1). Interacting with cytochrome c and 2-deoxy-ATP, Apaf-1 processes and activates pro-caspase-9 and then active caspase-9 cleaves and activates pro-caspase-3, initiating a cascade of apoptosis (Purring-Koch & McLendon 2000). Haga *et al.* (2003) were able to detect active caspase-3 in MOCK cells but not in AMF-transfected HT1080 cells. PKC inhibitor GF109203X,

PI3K inhibitor wortmanin and MAPK inhibitor PD98059 were able to recover the expression of Apaf-1 and caspase-9 in the AMF-transfected HT1080 cells; meanwhile, in the Ehrlich cells, only GF109203X was an effective helper of mitomycin-C-induced apoptosis. However, a cocktail of GF109203X, wortmanin and PD98059 induced apoptosis on the Ehrlich cells more effectively than did GF109203X alone.

These two sets of data indicated that AMF plays an important role in inducing cell survival against the apoptosis signal. However, it remains to be discussed which pathways overexpressed the AMF effect to acquire anti-apoptotic ability. The sensitivity of the inhibitors may be different among the cells or AMF may display different effects on different apoptosis inducers (serum deprivation versus mitomycin-C). In addition, there is a possibility that the increase in PGI enzymatic activity by overexpression of this protein may result in hypermetabolism of glucose which is related to malignant tumors (Warburg 1956, Di Chiro *et al.* 1982) and affect the apoptosis pathways. The signal pathways with regard to apoptosis resistance induced by AMF overexpression are presented in Fig. 3.

Conclusion

AMF is a multifunctional protein and recent studies have revealed that, in addition to motility stimulation, this protein plays important roles in the behavior of malignant tumors, such as angiogenesis, ascites accumulation and anti-apoptotic effects. Disrupting AMFR or neutralizing AMF can be considered to be a molecular targeting cancer therapy. Meanwhile, we must remember that the complete block of AMF may be lethal for normal cells because this protein is essential for glycolysis. Romagnoli *et al.* (2003) reported that antisense against AMF induced apoptosis in normal neuronal cells. Advanced studies with regard to the mechanisms of overexpression and secretion of this protein are needed to block the signals related to malignancy.

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