Fibronectin Induces Pro-MMP-2 Activation and Enhances Invasion in H-Ras-Transformed Human Breast Epithelial Cells

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Abstract – Interactions between tumor cells and the extracellular matrix (ECM) strongly influence tumor development, affecting cell survival, proliferation and migration. Fibronectin, a major component of ECM, has been shown to interact with integrins especially the $\alpha 5 \beta 1$ integrin. Cell invasion and metastasis are often associated with matrix metalloproteinases (MMPs) which are capable of digesting the different components of the ECM and basement membrane. MMP-2 is produced as a latent pro-MMP-2 (72 kDa) to be activated, resulting the 62 kDa active MMP-2. In this study, we investigated the effect of fibronectin on activation of pro-MMP-2 and the cellular invasiveness in H-Ras-transformed MCF10A human breast epithelial cells. Here we show that fibronectin induces activation of pro-MMP-2 and up-regulation of MT1-MMP and TIMP-2 in H-Ras MCF10A cells. These results demonstrate that H-Ras MCF10A cells secrete high levels of active MMP-2 when cultured with fibronectin, suggesting a possible interaction between the ECM network and H-Ras MCF10A cells to generate active MMP-2 which is important for proteolysis and ECM remodeling. Invasive and migratory abilities of H-Ras MCF10A cells were enhanced by fibronectin. Fibronectin up-regulated the expression of $\beta 1$ integrin which may play a role in cellular responses exerted by fibronectin. Since acquisition of pro-MMP-2 activation can be associated with increased malignant progression, this study provides a mechanism for the cell surface-matrix degrading effect of fibronectin which will be crucial to breast cell invasion and migration.

Keywords: Fibronectin, Active MMP-2, H-Ras MCF10A, Breast cell invasion

INTRODUCTION

Interactions between the tumor cell and the extracellular matrix (ECM) strongly influence tumor growth, cell survival, proliferation, migration and invasion (Juliano, 2002). During cancer development, the ECM of the tissue in which the tumor grows is extensively remodeled both by degradation of preexisting ECM molecules and by the neo-synthesis of ECM components (Kaspar *et al.*, 2006). Cell adhesive interactions with ECM proteins such as fibronectin are mediated by specific cell surface receptors, called integrins. Fibronectin is a multifunctional extracellular glycoprotein which has at least two independent cell adhesion sites with different receptor specificities (Akiyama *et al.*, 1995). One of the principal fibronectin binding integrins is integrin $\alpha 5 \beta 1$, which interacts with the

central cell adhesive region of fibronectin for maximal binding (Akiyama *et al.*, 1995).

The process of tumor cell invasion and metastasis requires degradation of connective tissue associated with basement membrane and interstitial connective tissue (Stetler-Stevenson *et al.*, 1993; Yu *et al.*, 1996). Matrix metalloproteinases (MMPs), a family of zinc dependent endopeptidases, are capable of digesting the components of the ECM and basement membrane. Mounting evidence strongly implicates MMPs, particularly MMP-2, in the process of tumor, including a positive correlation between MMP-2 expression and invasive potential (Stetler-Stevenson *et al.*, 1993; Curran and Murray *et al.*, 1999; Kleiner and Stetler-Stevenson *et al.*, 1999).

MMP-2 is produced as a latent pro-MMP-2 (72 kDa) requiring activation for catalytic activity, a process that is accomplished by proteolytic removal of the propeptide domain, resulting the 62 kDa active MMP-2 (Strongin *et al.*, 1995). It has been demonstrated that pro-MMP-2 forms a

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trimolecular complex with membrane type 1 (MT1)-MMP and tissue inhibitor of metalloproteinase (TIMP)-2, and then to be cleaved by an adjacent MT1-MMP molecule on the cell surface (Strongin *et al.*, 1995; Seiki, 2002; Sato *et al.*, 2005).

Mounting evidence from laboratories including ours suggests unique roles of the Ras family members in tumor aggressiveness of breast cancer (Clair *et al.*, 1987; Watson *et al.*, 1991; Clark and Der, 1995). We previously showed that H-Ras induced invasive and migratory phenotypes in MCF10A human breast epithelial cells and H-Ras-induced invasive phenotype was associated closely with the expression of MMP-2 (Moon *et al.*, 2000; Kim *et al.*, 2003; Song *et al.*, 2006). Active form of MMP-2 (62 kDa), however, was not detected in H-Ras MCF10A cells when cultured in polystyrene dishes (Moon *et al.*, 2000). We have recently demonstrated that H-Ras induces pro-MMP-2 activation in MCF10A cells when cultured in two-dimensional gel of type I collagen (Kim *et al.*, 2007). Since fibronectin has been shown to be associated with cellular invasiveness in various cancer cells including breast carcinoma, papillary thyroid carcinoma and colon cancer cells (Arihiro *et al.*, 1993; Ryu *et al.*, 1999; Ito *et al.*, 2004; Ding *et al.*, 2008), the present study investigates the effect of fibronectin on pro-MMP-2 activation in H-Ras MCF10A cells. Here we show that fibronectin induces activation of pro-MMP-2 and invasive and migratory abilities of H-Ras MCF10A cells.

MATERIALS AND METHODS

Cell culture

Establishment and culture condition of H-Ras MCF10A cells were described previously (Moon *et al.*, 2000). The cells were cultured in DMEM/F12 medium supplemented with 5% horse serum, 0.5 µg/ml hydrocortisone, 10 µg/ml insulin, 20 ng/ml epidermal growth factor, 0.1 mg/ml cholera toxin, 2 mM L-glutamine and 0.5 µg/ml amphotericin B and 100 unit/ml penicillin-streptomycin.

In vitro invasion assay

In vitro invasion assay was performed using 24-well transwell as previously described (Kim *et al.*, 2003). The lower side of the filter was coated with type I collagen, and the upper side was coated with fibronectin (5 µg/ml). Lower compartment was filled with serum-free media containing 0.1% BSA. Cells were placed in the upper part of the Transwell plate, incubated for 17 h, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The invasive phenotypes were determined by

counting the cells that migrated to the lower side of the filter with microscopy at ×400. Thirteen fields were counted for each filter, and each sample was assayed in triplicate.

Transwell migration assay

In vitro Transwell migration assay was performed using a 24-well transwell unit with polycarbonate filters as previously described (Kim *et al.*, 2003). The lower side of the filter was coated with type I collagen (Sigma Chemical Co). Lower compartment was filled with serum-free media containing 0.1% BSA. Soluble fibronectin and 2.5×10^4 cells were placed in the upper part of the transwell were plate, incubated for 17 h, fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. The migrative phenotypes were determined by counting the cells that migrated to the lower side of the filter with microscopy at ×400. Thirteen fields were counted for each filter and each sample was assayed in triplicate.

Gelatin zymography

Cells were cultured in serum-free DMEM/F12 medium containing soluble fibronectin. Conditioned medium was collected and centrifuged at 3,000 rpm for 10 min to remove cell debris. Gelatinolytic activity of the conditioned medium was determined by gelatin zymogram assay as previously described (Moon *et al.*, 2000; Kim *et al.*, 2003). Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background.

Immunoblot analysis

Immunoblot analysis was performed as previously described (Kim *et al.*, 2003). Anti-MT1-MMP, anti-TIMP-2, anti-α5 and anti-β1 antibodies were purchased from R&D Systems Inc. Enhanced chemiluminescence (ECL, Amersham-Pharmacia) system was used for detection. Relative band intensities were determined by quantitation of each band with an Image Analyzer (Vilber Lourmet, France).

RESULTS

Fibronectin activates pro-MMP-2 in H-Ras MCF10A cells

To examine the effect of fibronectin on the activation of MMPs, conditioned media of H-Ras MCF10A cells treated with fibronectin were subjected with gelatin zymogram assay. As shown in Fig. 1, an active form of MMP-2 (62 kDa) was detected when the cells were treated with 5 µg/ml of fibronectin for 24 h. The results demonstrate that fibronectin induces activation of pro-MMP-2 in H-Ras MCF10A cells.

Fig. 1. Fibronectin induces pro-MMP-2 activation in H-Ras MCF10A cells. Gelatin zymogram assay was performed on the conditioned media cultured with the indicated concentration of fibronectin for 24 h. Gelatinolytic activities of secreted MMP-2 and MMP-9 were determined by gelatin zymogram assay.

Fig. 2. Fibronectin up-regulates MT1-MMP and TIMP-2 in H-Ras MCF10A cells. Cell lysates were prepared from the cells cultured on 5 µg/ml fibronectin and analyzed for the expression of MT1-MMP (57 KDa) and TIMP-2 (25 KDa) by immunoblot analysis. β-actin was used as a loading control. The immunoblots shown are representative of three blots from independent experiments. Relative band intensities from three independent blots were determined by using an Image analyzer. *, **Statistically different from control at $p < 0.05$ and $p < 0.01$, respectively.

MT1-MMP and TIMP-2 are up-regulated by fibronectin

It has been reported that MT1-MMP and TIMP-2 form a 'receptor' complex that binds pro-MMP-2, resulting in the proteolysis of bound MMP-2 by an adjacent free MT1-MMP (Itoh *et al.*, 2001). We investigated the effect of fibronectin on the expressions of MT1-MMP and TIMP-2 in H-Ras MCF10A cells. Immunoblot analyses to detect MT1-MMP and TIMP-2 were performed on cell lysates of H-Ras MCF10A cells treated with fibronectin. As shown in Fig. 2, significant increases in MT1-MMP and TIMP-2 were observed in H-Ras MCF10A cells treated with 5 µg/ml of fibronectin for 24 h. These data suggest that MT1-MMP and TIMP-2 may be involved in the activation of pro-MMP-2 induced by fibronectin.

Invasion and migration of H-Ras MCF10A cells were increased by fibronectin

Since MMPs play important roles in cellular invasive and

Fig. 3. Fibronectin enhances invasive and migratory phenotypes of H-Ras MCF10A cells. The number of invaded or migrated cells per field was counted ($\times 400$) in thirteen arbitrary visual fields. The results represent means $\pm$ SE of triplicates. *, **Statistically different from control at $p < 0.05$ and $p < 0.01$, respectively.

Fig. 4. Fibronectin up-regulates integrin β1 in H-Ras MCF10A cells. Cells were cultured on 5 µg/ml fibronectin and analyzed for the expression of α5 and β1 integrins by immunoblot analysis. β-actin was used as a loading control. The immunoblots shown are representative of three blots from independent experiments. Relative band intensities from three independent blots were determined by using an Image analyzer. *Statistically different from control at $p < 0.05$.

migratory phenotypes, we then investigated the effect of fibronectin on H-Ras MCF10A cell invasion and migration. Treatment of H-Ras MCF10A cells with 5 µg/ml of fibronectin for 17 hr caused significant increases in invasive and migratory abilities of H-Ras MCF10A cells (Fig. 3), indicating the enhancing effect of fibronectin on H-Ras-induced invasion and migration of human breast epithelial cells.

Fibronectin induces expression of β1 integrin

Integrins α5 and β1 are known to be major receptors for fibronectin. We examined if expression of these integrins was affected by fibronectin by immunoblot analysis. The expression level of α5 integrin was strong in H-Ras MCF10A cells while the basal level of β1 integrin was very weak (Fig. 4). Treatment of H-Ras MCF10A cells with 5 µg/ml of fibronectin for 24 h significantly induced the ex-

pression of $\beta 1$ integrin whereas the level of $\alpha 5$ integrin was not significantly altered. These results demonstrate that fibronectin up-regulates the expression of $\beta 1$ integrin in H-Ras MCF10A cells which may play a role in cellular responses exerted by fibronectin.

DISCUSSION

Invasion of the cancer cells into the surrounding matrix is crucial to metastasis (Liotta *et al.*, 1980). An essential part of invasion includes degradation of the ECM components such as type I collagen, fibronectin and laminin and the basement membrane by members of the MMP family (Ura *et al.*, 1989; Fidler, 1990). We have previously shown that type I collagen induces activation of MMP-2 only in the invasive H-Ras MCF10A cells but not in the non-invasive N-Ras MCF10A cells (Kim *et al.*, 2007).

This study investigates the effect of fibronectin on pro-MMP-2 activation in H-Ras MCF10A cells. Here, we show that fibronectin induces activation of pro-MMP-2 and enhances the invasive/migratory phenotypes in H-Ras MCF10A cells. These results suggest that H-Ras MCF10A cells interact with the ECM network to generate active MMP-2 which is important for proteolysis and ECM remodeling.

In consistent with our results, involvement of fibronectin in MMP activation and cell invasion has been demonstrated in various cell systems. Cultivation of cells in the presence of fibronectin results in increased extracellular protease activity and activation of MMP-2/-9 in MCF-7 human breast cancer cells (Munshi and Stack, 2006; Das *et al.*, 2008). Activation of MMP-2 was also observed in HT-1080 fibrosarcoma cells grown in the presence of fibronectin (Stanton *et al.*, 1998). Fibronectin deposited in the extracellular matrix of tumors strongly promotes the migration and intracerebral invasion of glioma cells (Enam *et al.*, 1998; Ohnishi *et al.*, 1998).

Proteolytic activation of MMP-2 involves a coordinated interplay of pro-MMP-2, MT1-MMP and TIMP-2 (Goldberg *et al.*, 1989; Hernandez-Barrantes *et al.*, 2000; Wang *et al.*, 2000). Our data showed that the cellular level of MT1-MMP and TIMP-2 increased when H-Ras MCF10A cells were cultured with fibronectin, suggesting the role of MT1-MMP and TIMP-2 in MMP-2 activation of H-Ras MCF10A cells.

Cellular interaction with fibronectin is mediated largely through integrin receptors (Akiyama *et al.*, 1995). The role of $\alpha 5 \beta 1$ integrin and fibronectin interaction in tumor cell invasiveness has been investigated in several studies (Brakebusch *et al.*, 2002; Danen *et al.*, 2002). Our results

show that the expression level of $\beta 1$ integrin was increased by fibronectin treatment while $\alpha 5$ integrin level was not significantly altered. Since the basal level of $\alpha 5$ is high and that of $\beta 1$ integrin is very low of H-Ras MCF10A cells, fibronectin treatment may result in a more balanced expression of these integrins. Involvement of other integrins in fibronectin-induced pro-MMP-2 activation is currently under investigation.

In the present study, we demonstrate that fibronectin induces activation of pro-MMP-2 in H-Ras MCF10A cells. These results suggest that in an *in vivo* environment, H-Ras MCF10A cells may secrete active MMP-2 which is likely to remodel ECM and enhance invasion and migration. Since the activation of pro-MMP-2 can be associated with increased malignant progression, this study provides a mechanism for the cell surface-matrix degrading effect of fibronectin which will be crucial to breast cell invasion and migration.

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