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Type I Collagen-induced Pro-MMP-2 Activation is Differentially Regulated by H-Ras and N-Ras in Human Breast Epithelial Cells

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Tumor cell invasion and metastasis are often associated with matrix metalloproteinases (MMPs), among which MMP-2 and MMP-9 are of central importance. We previously showed that H-Ras, but not N-Ras, induced invasion of MCF10A human breast epithelial cells in which the enhanced expression of MMP-2 was involved. MMP-2 is produced as a latent pro-MMP-2 (72 kDa) to be activated resulting the 62 kDa active MMP-2. The present study investigated if H-Ras and/or N-Ras induces pro-MMP-2 activation of MCF10A cells when cultured in two-dimensional gel of type I collagen. Type I collagen induced activation of pro-MMP-2 only in H-Ras MCF10A cells but not in N-Ras MCF10A cells. Induction of active MMP-2 by type I collagen was suppressed by blocking integrin α2, indicating the involvement of integrin signaling in pro-MMP-2 activation. Membrane-type (MT)1-MMP and tissue inhibitor of metalloproteinase (TIMP)-2 were up-regulated by H-Ras but not by N-Ras in the type I collagen-coated gel, suggesting that H-Ras-specific up-regulation of MT1-MMP and TIMP-2 may lead to the activation of pro-MMP-2. Since acquisition of pro-MMP-2 activation can be associated with increased malignant progression, these results may help understanding the mechanisms for the cell surface matrix-degrading potential which will be crucial to the prognosis and therapy of breast cancer metastasis.

Keywords: MMP-2 activation, MT1-MMP, Ras, TIMP-2

Introduction

An essential part of invasion and metastasis includes degradation of the basement membrane and the stromal extracellular matrix by members of the matrix metalloproteinase (MMP) family (Ura *et al*., 1989; Fidler, 1990). Of MMP family members, MMP-2 (72 kDa type IV collagenase, gelatinase A) and MMP-9 (92 kDa type IV collagenase, gelatinase B) have been shown to play critical roles in tumor invasion and metastasis formation (Tryggvason, 1993; Yoon *et al*., 2003; Rhee *et al*., 2007). MMP-2 is produced as a latent pro-MMP-2 (72 kDa) requiring activation for catalytic activity, a process that is usually 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 trimolecular complex with membrane type I (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).

During metastasis, invasive cells must traverse tissue barriers comprised largely of type I collagen, the main component of the interstitial fibrillar collagen network. This process depends on the ability of tumor cells to degrade the surrounding collagen matrix and then migrate through the matrix defects (MacDougall and Matrisian, 1995; Stetler-Stevenson *et al*., 1993). It has been reported that type I collagen activates pro-MMP-2 in various cell types such as neoplastic human breast cells, ovarian carcinoma cells and human palmar fascia fibroblasts (Azzam *et al*., 1992; Tomesek *et al*., 1997; Ellerbroek *et al*., 1999). Culturing a variety of cell types within a three-dimensional gel of type I collagen stimulates cellular activation of pro-MMP-2 (Haas *et al*., 1998; Ellerbroek *et al*., 1999).

Elevated levels of Ras expression are associated with tumor aggressiveness in breast cancer, including the degrees of invasion to fat tissues and tumor recurrence (Clair *et al*., 1987; Watson *et al*., 1991; Clark and Der, 1995). Mounting evidence

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from laboratories including ours suggests unique roles of the Ras family members in normal and pathological conditions. We previously showed that H-Ras, but not N-Ras, induced invasive and migratory phenotypes in MCF10A human breast epithelial cells, whereas both induced transformed phenotype (Moon *et al*., 2000; Kim *et al*., 2003). H-Ras-induced invasive phenotype is associated more closely with the expression of MMP-2 in human breast epithelial cells (Moon *et al*., 2000), rather than MMP-9 expression. We also showed that transcriptional activation of MMP-2 was responsible for H-Ras-induced invasive and migratory phenotypes of MCF10A cells (Song *et al*., 2006). Active form of MMP-2 (62 kDa), however, was detected neither in H-Ras MCF10A nor in N-Ras MCF10A cells when cultured in polystyrene dishes (Moon *et al*., 2000).

The present study investigates if H-Ras and/or N-Ras induces pro-MMP-2 activation of MCF10A cells when cultured in two-dimensional gel of type I collagen. Here, we show that pro-MMP-2 is activated by type I collagen only in H-Ras MCF10A cells, but not in N-Ras MCF10A cells. We further show that H-Ras enhances expression of MT1-MMP and TIMP-2 in two-dimensional gel of type I collagen while the expression level of these proteins were not altered by N-Ras. This study demonstrates that type I collagen induces pro-MMP-2 activation in a H-Ras-specific manner for which H-Ras-induced up-regulation of MT1-MMP and TIMP-2 may be responsible.

Materials and Methods

Cell lines. Development and characterization of H-Ras MCF10A and N-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 μg/ml cholera enterotoxin, 100 units/ml penicillin-streptomycin, 2 mM L-glutamine and 0.5 μg/ml ampotericin B.

Cell culture on two-dimensional gel of type I collagen. Type I collagen gel was prepared as previously described (Guo and Piacentini, 2003). Briefly, 1 ml of 2 mg/ml type I collagen (Trevigen, Gaithersburg, MD, USA) was added in a 6-well culture plate, and the plate was incubated at 37°C for 2 hr to allow polymerization of gel. Cell suspension in complete media (2 ml) was added onto the polymerized type I collagen gel. Cells remained attached to the type I collagen gel plates for the duration of incubation.

Preparation of whole cell lysates from type I collagen gel. Cells overlaid with type I collagen were recovered using a detergent extraction method as previously described (Lee *et al*., 1997). Collagen gel containing embedded cells was centrifuged at 13,000 rpm to remove as much water as possible, then suspended in a large volume of ice-cold PBS, mixed well, and centrifuged again. The wash procedure was performed five times. The entire lattice was then mixed with a chilled lysis buffer and forced several times through a syringe with a 21-gauge needle. After a centrifugation at 13,000 rpm for 15 min, supernatants corresponding to the whole cell lysates were collected.

Gelatin zymography. Cells were cultured in serum-free DMEM/F12 medium on polystyrene dish or type I collagen gel. 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 and anti-TIMP-2 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).

Results

Type I collagen activates pro-MMP-2 only in H-Ras MCF10A cells. To examine if type I collagen activates pro-MMP-2, conditioned media of H-Ras MCF10A and N-Ras MCF10A cells cultured in two-dimensional gel of type I collagen were subjected to gelatin zymogram assay. Gelatinolytic activities corresponding to pro-MMP-9 (92 kDa) and pro-MMP-2 (72 kDa) were detected in conditioned media from H-Ras MCF10A and N-Ras MCF10A cells cultured in type I collagen-coated gel (Fig. 1). Active form of MMP-2 (62 kDa) appeared in H-Ras MCF10A cells cultured for 24 hr and it increased in a time-dependent manner. In contrast, active MMP-2 band was not detected in N-Ras MCF10A cells cultured in the same condition. Given that neither H-Ras MCF10A nor N-Ras MCF10A cells showed activation of pro-MMP-2 when the cells were cultured in polystyrene dish (Moon *et al*., 2000), the data demonstrate that type I collagen induces activation of pro-MMP-2 in H-Ras MCF10A, but not in N-Ras MCF10A cells.

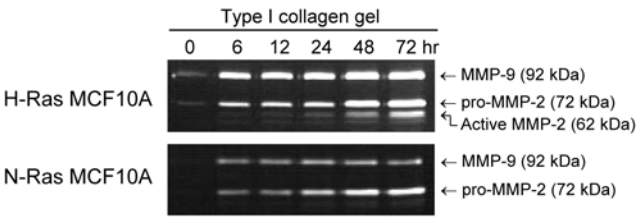

Fig. 1. Type I collagen induces activation of pro-MMP-2 in H-Ras MCF10A cells. Conditioned media were collected from cells cultured on polystyrene dish or type I collagen gel at indicated time points. Gelatinolytic activities of secreted MMP-2 and MMP-9 were determined by gelatin zymogram assay.

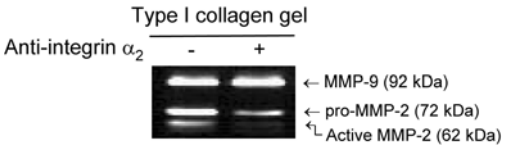

Fig. 2. Pro-MMP-2 activation is inhibited by blocking integrin α2 in H-Ras MCF10A cells cultured on type I collagen gel. H-Ras MCF10A cells were cultured on type I collagen gel with anti-integrin α2 antibody for 72 hr. Gelatinolytic activities was determined by gelatin zymogram assay.

Integrins α1β1 and α2β1 are known to be major receptors for type I collagen (Carloni *et al*., 1996). Blocking antibodies to α1 and α2 inhibited cell-ECM interactions (Yang *et al*., 2003). In an attempt to evaluate the involvement of integrin in type I collagen-induced activation of pro-MMP-2, H-Ras MCF10A cells were incubated with an antibody against integrin α2 for 72 hr and the conditioned media were subjected to gelatin zymogram assay. As shown in Fig. 2, active form of MMP-2 was almost completely abolished by blocking integrin α2. A slight decrease in pro-MMP-2 level was observed while MMP-9 was not affected by anti-integrin α2 antibody. The results indicate an essential role of integrin α2 in type I collagen-induced pro-MMP-2.

MT1-MMP is up-regulated by type I collagen only in H-Ras MCF10A cells. It has been hypothesized 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 (Stongin *et al*., 1995; Imai *et al*., 1996; Butler *et al*., 1998). A variation in either MT1-MMP or TIMP-2 status could result in the modulation of MMP-2 proenzyme processing (Haas *et al*., 1998; Gilles *et al*., 1997). In order to elucidate the mechanism for the differential regulation of type I collagen-induced pro-MMP-2 activation by H-Ras and N-Ras, we investigated the effect of type I collagen on the expression of MT1-MMP and TIMP-2 in H-Ras MCF10A and N-Ras MCF10A cells. Immunoblot analyses to detect MT1-MMP were performed on cell lysates of H-Ras MCF10A and N-Ras MCF10A cells cultured either on polystyrene dishes or on two-dimensional gel of type I collagen. An antibody against β-actin was used for loading control. As shown in Fig. 3, a significant increase in the ratio of active MT1-MMP (57 kDa) to β-actin was observed when H-Ras MCF10A cells were cultured on type I collagen for 6 hr and beyond whereas MT1-MMP expression was unchanged on the polystyrene dish. Up-regulation of MT1-MMP by type I collagen was maximally detected when the cells were cultured for 24 hr and 48 hr. In contrast, MT1-MMP level of N-Ras MCF10A cells was not up-regulated on two-dimensional

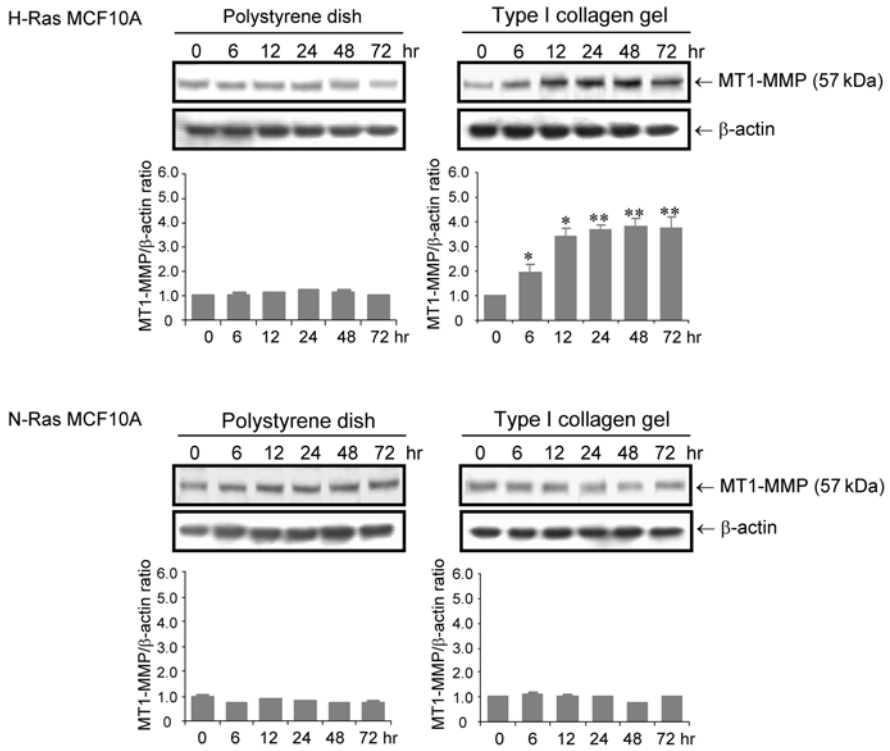

Fig. 3. MT1-MMP is up-regulated in H-Ras MCF10A cells cultured on type I collagen gel. Cell lysates were prepared from the cells cultured on polystyrene dish or type I collagen gel for indicated time points and analyzed for the expression of active MT1-MMP (57 kDa) by immunoblot analysis. Relative band intensities were determined by using an Image analyzer. *,**Statistically different from control at $p < 0.05$ and $p < 0.01$, respectively.

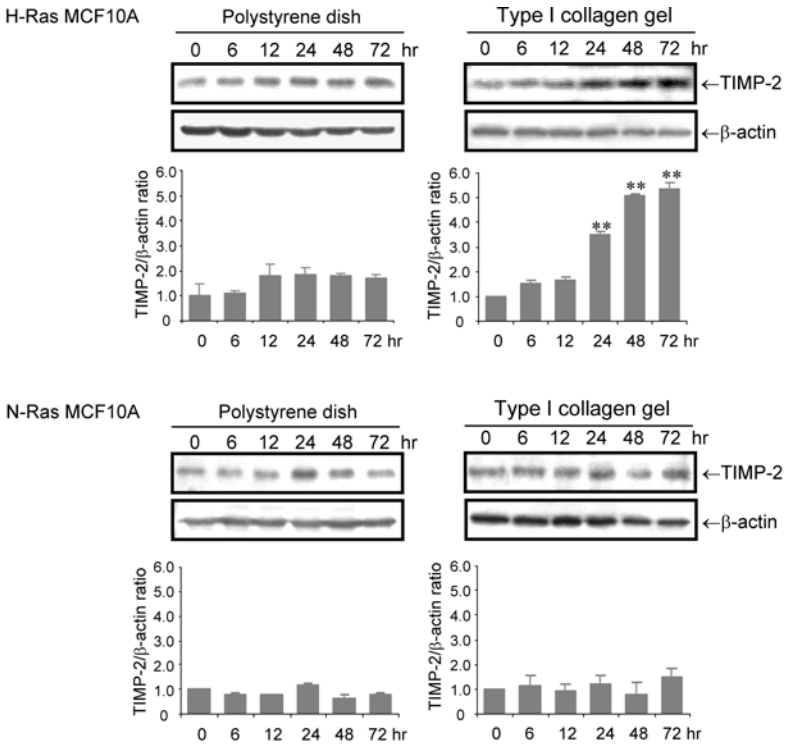

Fig. 4. TIMP-2 is up-regulated in H-Ras MCF10A cells cultured on type I collagen gel. Cell lysates were prepared from the cells cultured on polystyrene dish or type I collagen gel for indicated time points and analyzed for the expression of TIMP-2 (25 kDa) by immunoblot analysis. Relative band intensities were determined by using an Image analyzer. **Statistically different from control at $p < 0.01$.

gel of type I collagen. The data demonstrate that type I collagen up-regulates MT1-MMP in H-Ras MCF10A cells but not in N-Ras MCF10A cells.

Type I collagen up-regulates TIMP-2 only in H-Ras MCF10A cells. We then examined the effect of type I collagen on TIMP-2 expression in H-Ras MCF10A and N-Ras MCF10A cells. As shown in Fig. 4, a significant increase in the ratio of TIMP-2 to β-actin was observed when the H-Ras MCF10A cells were cultured in two-dimensional gel of type I collagen in a time-dependent manner with the maximal increase at 72 hr. Up-regulation of TIMP-2 was not detected in the same cells when cultured in polystyrene dishes. The expression level of TIMP-2 was not affected by type I collagen in N-Ras MCF10A cells. Taken together, the data presented in this study suggest that the type I collagen-activated pro-MMP-2 only in H-Ras MCF10A cells, but not in N-Ras MCF10A cells, may be due to the H-Ras-specific up-regulation of MT1-MMP and TIMP-2 in two-dimensional gel of type I collagen.

Discussion

Tumor cell invasion is a complex process involving matrix-degrading activity and migrating activity. Our previous studies showed that H-Ras-specific induction of invasive/migratory phenotypes was due, at least in part, to the enhanced expression of pro-MMP-2 by H-Ras. In the current study, we show that type I collagen, the major component of ECM, induces activation of MMP-2 only in the invasive H-Ras MCF10A cells but not in the non-invasive N-Ras MCF10A cells. H-Ras MCF10A cells cultured in two-dimensional gel of type I collagen secrete high levels of active MMP-2, raising the possibility that an *in vivo* interaction between the collagen network and H-Ras MCF10A cells may work to generate active MMP-2 which is important for focal proteolysis, ECM remodeling and tumor cell functions (Deryugina *et al*., 1998 and 2001). H-Ras-specific activation of pro-MMP-2 by type I collagen may contribute to the previously described *in vitro* invasiveness and migration of H-Ras MCF10A cells. Since *in vitro* invasion assay (Moon *et al*., 2000) and *in vitro* migration assay (Kim *et al*., 2003) use transwell filters coated with type I collagen, it is probable that pro-MMP-2 is activated while the H-Ras MCF10A cells are incubated inside the transwell filter coated with type I collagen and the resulting active MMP-2 may be responsible for the *in vitro* invasive and migratory phenotypes of the cells.

Considerable evidence supports a role for TIMP-2 and MT1-MMP in the activation of MMP-2. TIMP-2 plays a dual role in the regulation of MMP-2 activation, functioning both as an activator and an inhibitor of MMP-2 in a concentration-

dependent manner (Goldberg *et al*., 1989; Hernandez-Barrantes *et al*., 2000; Wang *et al*., 2000). At low concentrations, a TIMP-2-free MT1-MMP can effectively activate pro-MMP-2 whereas at high concentrations, all of the cell-surface MT1-MMP undergoes complex formation with TIMP-2, thereby inhibiting pro-MMP-2 activation (Strongin *et al*., 1995; Kinoshita *et al*., 1998). Our results demonstrated that cellular level of TIMP-2 increased when H-Ras MCF10A cells were cultured in two-dimensional gel of type I collagen. In addition, kinetic studies showed that type I collagen-induced TIMP-2 up-regulation coincided with pro-MMP-2 activation. When the cellular level of TIMP-2 was not sufficient at 6 and 12 hr of incubation in type I collagen gel (Fig. 4), MMP-2 activation did not occur in H-Ras MCF10A cells (Fig. 1), although these cells did express detectable amount of active MT1-MMP at the corresponding time points (Fig. 3). These data imply that the presence of cell-associated TIMP-2 is likely to be a prerequisite for an occurrence of MMP-2 activation in H-Ras MCF10A cells cultured in a type I collagen gel. The exact role of TIMP-2 in type I collagen-induced MMP-2 activation of H-Ras MCF10A cells, however, remains undefined. Up-regulation of TIMP-2 by type I collagen was observed when the cardiac fibroblasts were embedded in type I collagen lattices (Guo and Piacentini, 2003). In contrast, the level of TIMP-2 did not change when human skin fibroblasts and rat capillary endothelial cells were cultured in three-dimensional type I collagen lattices (Seltzer *et al*., 1994; Borg *et al*., 1997). These results suggest that the regulation of TIMP-2 by type I collagen is cell type-specific.

Cellular interaction with type I collagen is mediated largely through integrin receptors. It has been postulated that collagen stimulation occurs either directly or indirectly through integrin signaling (Ellerbroek *et al*., 1999; Theret *et al*., 1999). Here we show that integrin $\alpha 2$ is required for MMP-2 activation induced by type I collagen, suggesting a two-cell receptor-mediated event involving both MT1-MMP and integrin $\alpha 2$ for the completion of pro-MMP-2 activation in H-Ras MCF10A cells. Involvement of other integrins in type I collagen-induced pro-MMP-2 activation is currently under investigation.

Differential cellular functions exerted by H-Ras and N-Ras are supported by the demonstration that there are differences in the signal transduction pathways induced by Ras proteins (Carbone *et al*., 1991). Our previous study showed that H-Ras selectively activated the Rac/MKK6/p38 signaling pathway while Raf/MEK/ERKs and PI3K/Akt pathways were commonly activated by both H-Ras and N-Ras in MCF10A cells (Shin *et al*., 2005). The data presented in this study suggest that H-Ras-specific signaling to activate downstream effector molecules may lead to the induction of a set of gene expressions whose products are critical for type I collagen-induced MMP-2 activation. In endothelial cells, expressions of MT1-MMP and MMP-2 were attenuated by ERK1/2 inhibition but unaffected by either JNK or p38 inhibition (Boyd *et al*., 2005). Complex reciprocal effects of ERK1/2 and p38 in the regulation of MT1-MMP activity and pro-MMP-2 activation were demonstrated in an invasive cellular model of oral squamous cell carcinoma (Munshi *et al*., 2004). It would be worthwhile to further elucidate the functional role of these MAPKs in H-Ras-specific activation of pro-MMP-2 activation induced by type I collagen in MCF10A cells.

In the present study, we demonstrate for the first time that H-Ras and N-Ras differentially regulate pro-MMP-2 activation, MT1-MMP and TIMP-2 expression in two-dimensional gel of type I collagen. In an *in vivo* environment, H-Ras MCF10A cells may secrete active MMP-2 more efficiently than N-Ras MCF10A cells, which is likely to remodel ECM and affect tumor cell functions. These events involve a coordinated interplay of MMP-2, MT1-MMP, TIMP-2, cell receptor molecules and ECM components to spatially and temporally control focal proteolysis and locomotion of H-Ras MCF10A human breast epithelial cells. Since acquisition of pro-MMP-2 activation is associated with increased migration and invasiveness of cancer cells, this study may provide information on the mechanisms for the cell surface matrix-degrading potential to facilitate invasion and migration which will be crucial to the prognosis and therapy of breast cancer metastasis.

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