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Roles of Matrix Metalloproteinase-2 and -9 on the H-ras-Induced Invasive Phenotype in Human Breast Epithelial Cells and Human Fibrosarcoma Cells

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ABSTRACT : *One of the most frequent defects in human cancer is the uncontrolled activation of the ras-signaling pathways. Significant evidence has accumulated to directly implicate members of the matrix metalloproteinases (MMPs) in tumor invasion and metastasis formation. We have previously shown that MMP-9 expression was significantly enhanced in the ras-tranfected HT1080 human fibrosarcoma cells at the mRNA level. In the present study, we investigated the roles of MMP-2 and -9 on the H-ras-induced invasive phenotypes of MCF10A human breast epithelial cells and HT1080 human fibrosarcoma cells. We show that H-ras is able to induce or enhance a signaling pathway leading to the enhancement of an invasive phenotype in both MCF10A and HT1080 cells as determined by matrigel invasion assay. We then examined the effect of H-ras activation on the expression of MMP-2 and -9 by measuring enzymatic activities and mRNA levels. Our data clearly demonstrated that H-ras prominently induces expression of MMP-2 in MCF10A cells, while it efficiently upregulates MMP-9 in HT1080 cells. Taken together, these findings suggest that the correlation between ras-mediated invasiveness and enhanced expression of MMPs may be cell type-specific: MMP-9 is closely associated with the invasive phenotype induced by ras activation in fibrosarcoma cells, whereas MMP-2 is more likely associated with it in epithelial cells.*

Key Words : *Ras oncogene, Matrix metalloproteinase (MMP), Invasive phenotype, Human breast epithelial cells, Human fibrosarcoma cells*

I. INTRODUCTION

The number of barriers tumor cells must overcome to form metastases at distant sites suggests that multiple alterations may be required (Weinberg, 1989). Tumor progression involves alteration or activation of several growth-regulatory genes. Oncogene cooperation has been observed in the transformation of human breast epithelial cells (Thompson *et al.*, 1994). One of the most frequent defects in human cancer is the uncontrolled activation of the *ras*-signaling pathways (Barbacid, 1987). The *ras* proto-oncogene encodes guanine nucleotide-binding proteins that play an essential role in diverse cellular responses, including cell proliferation and differentiation (Boguski and McCormick, 1993).

Collagenase IV is a member of a family of matrix metalloproteinases (MMP) whose excessive secretion has been implicated in a variety of chronic human diseases (Wooley and Evanson, 1980). Significant evidence has accumulated to directly implicate members of the gene family of MMPs in tumor invasion and metastasis formation (Ura *et al.*, 1989; Liotta *et al.*, 1991; Kohn and Liotta, 1995). Numerous studies show a correlation between the levels of MMP-2 (72 kDa type IV collagenase, gelatinase A) and/or MMP-9 (92 kDa type IV collagenase, gelatinase B) and the invasive phenotypes of cancer cells (Ura *et al.*, 1989; Nakajima *et al.*, 1990).

In rat and human embryonic fibroblasts, H-*ras*-mediated transformation and invasiveness have been shown to be associated with enhanced expression of MMP-9 mRNA and protein (Bernhard *et al.*, 1994). A correlation between malignant transformation by c-H-*ras* oncogene and enhanced expression of mRNA for MMP-9 has also been suggested in

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KMS-6 fibroblasts (Sato *et al.*, 1992). We have previously demonstrated that MMP-9 expression was significantly enhanced in the *ras*-transfected HT1080 human fibrosarcoma cells at mRNA level (Moon *et al.*, 1996). However, the role of MMPs in *ras*-mediated invasiveness in human epithelial cells needs to be investigated, especially since *ras*-mediated cellular responses differ between epithelial cells and fibroblasts (Oldham *et al.*, 1996).

In the present study, we investigated the roles of MMP-2 and -9 on the H-*ras*-induced invasive phenotypes of MCF10A human breast epithelial cells and HT1080 human fibrosarcoma cells. We studied induction of invasiveness by H-*ras* transfection in MCF10A and HT1080 cells. We then detected the enzymatic activities and mRNA levels of MMP-2 and -9 in the *ras*-transfected cells in order to elucidate a possible correlation between the induction of invasive phenotype by H-*ras* and the expression of MMP (s) in these cell systems.

II. MATERIALS AND METHODS

1. Cell lines and Culture Condition

The development and characterization of human breast epithelial cell line MCF10A has been described elsewhere (Soule *et al.*, 1990; Tait *et al.*, 1990). MCF10A cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/*ml* hydrocortisone, 10 μg/*ml* insulin, 2 ng/*ml* EGF, 0.1 g/*ml* cholera enterotoxin, 2 mM L-glutamine, 100 units/*ml* penicillin-streptomycin and 0.5 μg/*ml* fungizone. MCF10A cells transfected with H-ras (H-*ras* MCF10A) were provided by Dr. Yong Chen. The human fibrosarcoma HT1080 cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 100 units/*ml* penicillin-streptomycin. HT1080 cells were transfected with v-H-*ras* as previously described (Moon *et al.*, 1996) and named H-*ras* HT1080.

2. *In vitro* Invasion Assay

In vitro invasion assay was performed as described previously (Albini *et al.*, 1987) with a slight modification using a 24-well transwell unit with polycarbonate filters, 6.5 mm in diameter and a pore size of 8.0 μm (Corning Costar, Cambridge, MA). The lower side of the filter was coated with 10 μ*l* of 0.5 mg/*ml* type I collagen, and the upper side was coated with 10 μ*l* of 0.5 mg/*ml* reconstituted basement membrane substance (Matrigel: Collaborative Research, Lexington, KY). The coated filters were air-dried for 1 hour prior to the addition of the cells. The lower compartment contained 600 μ*l* of DMEM/F12 containing 0.1 mg/*ml* BSA. Fifty thousand cells were resuspended in 100 μ*l* of DMEM/F 12 and placed in the upper part of a transwell plate. Cells were incubated for 17 hours in a humidified atmosphere of 5% CO_2 at 37°C. Cells were fixed with methanol and stained with hematoxylin for 10 min followed briefly by eosin. Cells on the upper surface of the filter were mechanically removed by wiping with a cotton swab, and 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 assay. Each sample was assayed in triplicate.

3. Gelatin Zymography

Control and transfected MCF10A cells and HT1080 cells were incubated for 24 hr with serum-free medium. After the incubation, supernatants were collected, centrifuged at 3,000 rpm for 10 min to eliminate cell debris and the total protein content was determined using bicinchoninic acid protein assay reagents (Pierce, IL). The zymography assay was performed as previously described (Mackay *et al.*, 1992) with a slight modification. Ten % SDS-PAGE gels were co-polymerized with 0.1% gelatin and samples were electrophoresed. After electrophoresis, gels were washed twice (2× 15 min) in 2.5% triton ×-100 to remove SDS and incubated for 18 hr at 37°C in 40 mM Tris, 200 mM NaCl, 10 mM $CaCl_2$, pH 7.5, which permits enzymatic activity. After staining with 0.1% Coomassie brilliant blue, areas of lysis were observed as white bands against a blue backgound.

4. Northern Blot Analysis

Total RNA was isolated by a modification of the

method of Chomczynski and Sacchi (1987). The Northern blot analysis was performed as previously described (Moon *et al.*, 1996) with a slight modification. The level of MMP-2 and MMP-9 were detected using the MMP-2 and MMP-9 cDNA probes prepared as follows. The 1.5 kb *EcoRI-BamHI* fragment or 1.2 kb *PstI-EcoRI* fragment DNA probe was excised from MMP-2 (Collier *et al.*, 1988) or MMP-9 (Wilhelm *et al.*, 1989) cDNA clones (provided by Dr. Kyu-Won Kim). As house-keeping probes, glyceradehyde-3-phosphate dehydrogenase and β-actin genes were used. The DNA fragments were labeled with [α-^{32}P]dCTP (3,000 Ci/mmol, Amersham, Buckinghamshire, England) using the multiprime DNA labeling system (Amersham).

III. RESULTS

1. Inducing Effect of H-*ras* on Invasiveness of MCF10A and HT1080 Cells

To investigate the effect of H-*ras* activation on induction of an invasive phenotype, we examined the ability of control and H-*ras* transfected MCF10A breast epithelial cells and HT1080 fibrosarcoma cells to invade through a reconstituted basement membrane (Matrigel) in a transwell chamber. While the parental MCF10A cells failed to migrate across the filter, H-*ras* MCF10A cells invaded through the matrigel and migrated across the filter (Fig. 1). The HT1080 fibrosarcoma cells, which have been reported to be highly metastatic, showed intrinsic invasiveness (Fig. 2). In the *ras*-transfected HT1080 cells, the invasive ability was significantly enhanced: the number of H-*ras* HT1080 cells migrated through matrigel per field were increased by 50.3% of the parental HT1080 cells, showing that the *ras* activation also enhanced the invasive potential of the fibrosarcoma cells. The data clearly demonstrate that H-*ras* is able to induce or enhance a signaling pathway leading to the induction of an invasive phenotype in both MCF10A and HT1080 cells.

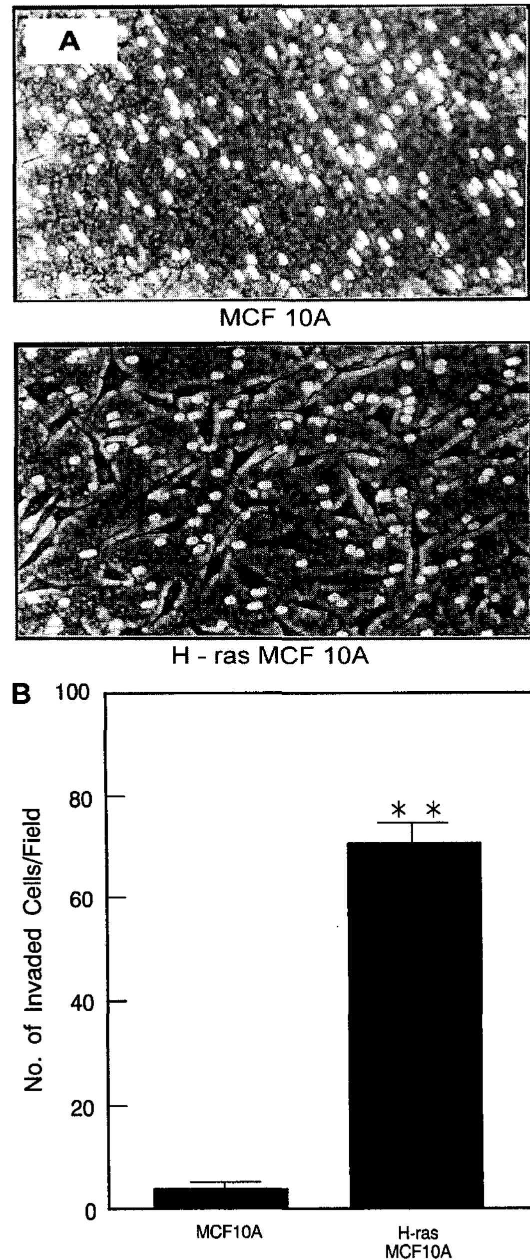

Fig. 1. *In vitro* invasion assay of parental and H-*ras*-transfected MCF10A cells. ***A.*** Light microscopic examination of the cells (×100). ***B.*** Invasive activity of the cells. The cultured cells (5×10^{4} cells) were incubated in a transwell chamber for 17 hr. The number of invaded cells per field were counted and the mean values were determined under ×400 light microscopy. Experiments were performed triplicate. Bar is mean number of invaded cells per field and line is SE for triplicate determinations. **, Statistically different from parental MCF10A cells, at $p<0.01$.

2. Differential Effect of H-*ras* Activation on Enzymatic Activities of MMP-2 and -9

Since enhanced expression of type IV collagenolytic activities have been detected in *ras*-activated cells, we investigated the effect of H-*ras* activation on expression of MMP-2 and -9. As shown in Fig. 3A, both 92 kDa (MMP-9) and 72 kDa (MMP-2) protei-

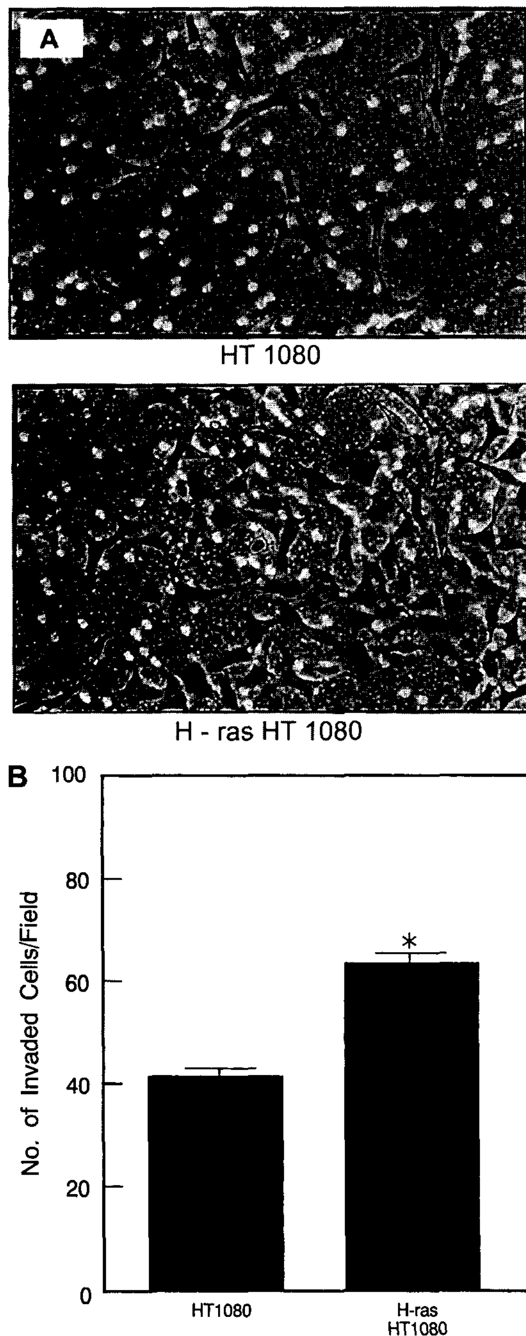

Fig. 2. *In vitro* invasion assay of parental and H-*ras*-transfected HT1080 cells. ***A.*** Light microscopic examination of the cells (× 100). ***B.*** Invasive activity of the cells. The cultured cells were incubated in a transwell chamber for 17 hr. The number of invaded cells per field were counted and the mean values were determined under ×400 light microscopy. Experiments were performed triplicate. Bar is mean number of invaded cells per field, and line is SE for triplicate determinations. *, Statistically different from parental HT1080 cells, at $p<0.05$.

nase activities were greatly increased in H-*ras* MCF10A cells while the parental MCF10A cells

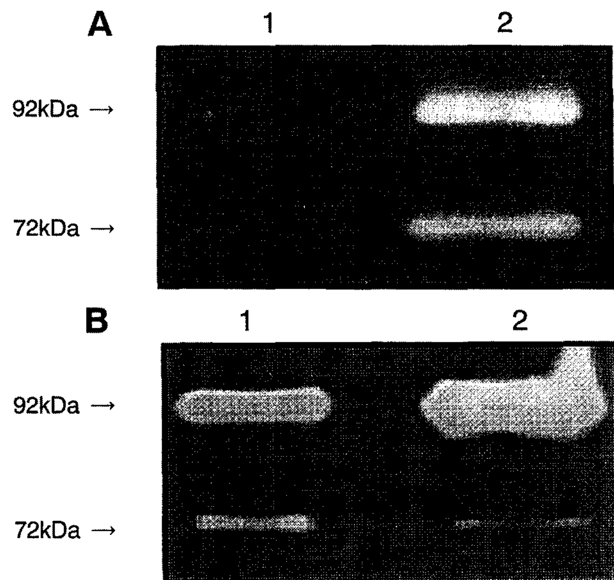

Fig. 3. Zymogram analysis for gelatinolytic activity in conditioned media. ***A.*** MCF10A cells. ***B.*** HT1080 cells. After the cells were cultured in serum-free media for 24 hrs, the conditioned media were collected, centrifuged and equal amounts of protein (10 g) were subjected to gelatin zymography. 1, parental cells; 2, H-*ras*-transfected cells.

showed little activities. The HT1080 cells showed intrinsic proteinase activities of MMP-9 and MMP-2. Upon the H-*ras* transfection, the MMP-9 proteinase activity was prominently increased while the MMP-2 activity was not (Fig. 3B). Considering that the intensity of MMP-2 activity in the H-*ras* HT1080 cells was even less than that shown in the parental HT1080, the relative increase in MMP-9 by *ras* transfection is thought to be substantial. The result suggests that H-*ras* differentially modulate enzymatic activities of MMP-2 and MMP-9 in MCF10A breast epithelial cells and HT1080 fibrosarcoma cells.

3. Differential Effect of H-*ras* Activation on Expression of MMP-2 and -9

The mRNA levels of MMPs in the control and H-*ras* transfected cells were examined by Northern blot analysis. In MCF10A cells, the expression of MMP-2 was upregulated by H-*ras* activation (Fig. 4). We have previously shown (Moon *et al.*, 1996) that MMP-9 expression was significantly enhanced in the *ras*-tranfected HT1080 human fibrosarcoma cells compared with the parental HT1080 cells, while the expression of MMP-2 was not greatly

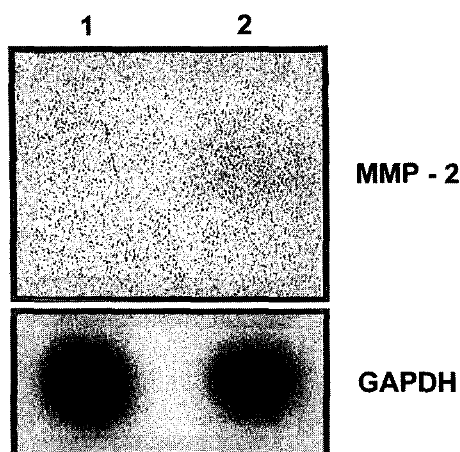

Fig. 4. mRNA level of MMP-2 in parental or ras-transfected MCF10A cells. Northern blot analysis of total RNA (20 μg) from cell cultures was performed. The blots were hybridized to the ^{32}P-labeled MMP-2 or glyceradehyde-3-phosphate dehydrogenase (GAPDH) probes. 1, parental cells; 2, H-ras-transfected cells.

affected. These data clearly demonstrate that H-*ras* prominently induces expression of MMP-2 in MCF10A cells, while it efficiently upregulates MMP-9 in HT1080 cells.

IV. DISCUSSION

One of the most frequent defects in human cancer is the uncontrolled activation of the *ras*-signaling pathway (Barbacid, 1987). Since elevated levels of p21 *ras* protein and aberrant function of the ras signaling transduction pathway have been observed in human breast carcinomas (Clair *et al.*, 1987; Clark and Der, 1995), the role for the *ras* oncogene in breast cancer needs to be investigated. In this study, we studied the effect of H-*ras* transfection of MCF10A cells which are spontaneously immortalized from human fibrocystic mammary tissue and have characteristics for normal breast epithelial cells (Soule *et al.*, 1990; Tait *et al.*, 1990). Since *ras*-mediated cellular responses differ between epithelial cells and fibroblasts (Oldham *et al.*, 1996), we wished to compare the role for *ras* oncogene in MCF10A human breast epithelial cells and HT1080 fibrosarcoma cells. *In vitro* invasion assay showed that H-*ras* was able to induce an invasive phenotype in MCF10A cells. When H-*ras* was transfected into the HT1080 cells, which are intrinsically metastatic (Cha *et al.*, 1996), the invasive ability was significantly enhanced. Our data clearly demonstrate that the *ras*-signaling induced the invasive phenotype of both breast epithelial cells and fibrosarcoma cells.

There have been considerable efforts to examine the involvement of the MMPs in the metastatic and invasive phenotype of cells (Reviewed in Aznavoorian *et al.*, 1993; Kohn and Liotta, 1995). The correlation between the enhanced expression of MMPs and *ras* oncogene has been reported (Collier *et al.*, 1988; Ura *et al.*, 1989). In MCF10A cells, H-*ras* activation greatly increased the enzymatic activities of both 92 kDa (MMP-9) and 72 kDa (MMP-2) proteins. Since we have recently found that N-*ras* MCF10A cells, which have more prominent induction of MMP-9, did not show any invasiveness (Moon *et al.*, 1998), *ras*-induced MMP-9 is unlikely to be responsible for the induction of an invasive phenotype in humen breast epithelial cells. Northern blot analysis showed that H-*ras* upregulated MMP-2 mRNA, suggesting a possible role for MMP-2 in H-*ras*-induced invasiveness in the MCF10A cells.

Unlike MCF10A cells, HT1080 cells showed the intrinsic gelatinolytic activities of MMP-2 and -9. The H-*ras* transfection prominently increased the activity of MMP-9 while it did not affect that of MMP-2. Northern blot analysis showed that the level of MMP-9 mRNA was significantly induced by H-*ras* whereas MMP-2 level was not affected. This is consistent with the previous finding of Sato *et al.* (1992) on the enhanced expression of mRNA for MMP-9, but not MMP-2 in c-H-*ras*-transformed KMS-6 fibroblasts.

The MMP-9 promoter has several transcription factor binding motifs including AP-1, NFκB, Ets, PEA3, and SP-1 sites and the stimulation of the MMP-9 promoter by ras has been found to require multiple elements including closely spaced PEA3/ets and AP-1 sites (Gum *et al.*, 1996). The promoter of MMP-2 is notably distict from that of the other MMP family members (Benbow and Brinckerhoff, 1997). It lacks common regulatory elements including the adjacent AP-1 and PEA3 elements. Therefore, the activation of MMP-2 by *ras* in MCF10A cells is likely to be mediated by a totally different pathway from that is seen in MMP-9 activation.

Taken together, the correlation between *ras*-

mediated invasiveness and enhanced expression of MMP appears to be cell type-specific. Our results strongly suggest that MMP-9 is closely associated with the invasive phenotype induced by ras activation in fibrosarcoma cells, whereas MMP-2 is more likely to be associated with it in epithelial cells. In order to provide a more direct evidence, it remains to be investigated whether antisense oligonucleotides which can efficiently block MMP-2 or -9 affect the invasive phenotype of these cells.

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