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Involvement of Phosphatidylinositol 3-kinase (PI3K) Pathway in Invasion and Motility H-ras-transformed MCF10A Cells

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Many studies have identified the phosphatidylinositol 3-kinase (PI3K) as a key regulator for various cellular functions including cell survival, growth and motility. We have previously shown that H-ras, but not N-ras, induces invasiveness and motility in human breast epithelial cells (MCF10A), while both H-ras and N-ras induce transformed phenotype. In the present study, we wished to investigate the functional role of PI3K pathway in H-ras-induced invasive phenotype and motility of MCF10A cells. Activation of PI3K in the parental, H-ras- and N-ras MCF10A cells was examined by detecting phosphorylation of Akt, a downstream molecule of PI3K, by Western blot analysis. Marked activation of Akt was detected not only in H-ras MCF10A cells but also in non-invasive/non-motile N-ras MCF10A cells at comparable levels. We then further investigated the functional significance of PI3K activation in invasion and motility by using known PI3K inhibitors, LY294002 and wortmannin. Treatment of LY294002 and wortmannin significantly inhibited invasive phenotype and motility of H-ras MCF10A cells, suggesting that the activation of PI3K pathway is not sufficient, but may be required for H-ras-induced invasion and motility. Prominent downregulation of MMP-2 and MMP-9 were observed in H-ras MCF10A cells treated with LY294002 in a dose-dependent manner. The results provide evidence that PI3K pathway is critical for H-ras-mediated upregulation of MMPs in MCF10A cells, resulting in phenotypic conversion of non-invasive MCF10A cells to an invasive phenotype.

Key Words: H-ras, MCF10A cells, PI3K, Akt, Invasion, Motility

INTRODUCTION

The ras proto-oncogene encodes guanine nucleotide-binding proteins that play an essential role in diverse cellular response, including cell proliferation and differentiation.[1] One of the most frequent defects in human cancer is the uncontrolled activation of the ras-signaling pathways.[2] Ras expression has been suggested to be a marker of tumor aggressiveness in breast cancer.[3] Recent evidences suggest a key role of Phosphatidylinositol 3-kinase (PI3K) pathway in regulation of various cellular responses. PI3K is activated either by G-protein-coupled receptors in response to extracellular stimuli[4] or by a direct

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interaction with the small GTPse Ras.[5,6] PI3K regulates cell proliferation, differentiation and apoptosis.[7~11] Akt is a downstream target of PI3K. Akt binds to membrane-associated phosphatidylinositol-3,4,5- triphosphate and phosphatidylinositol-3,4-biphosphate generated by PI3K.[12~14] Akt has been implicated in a variety of cellular functions, such as survival, transcription, and translation.[14,15]

We have previously shown that H-ras, but not N-ras, induces an invasive phenotype[16] and motility[17] of MCF10A human breast epithelial cells while both H-ras and N-ras induce transformed phenotype. In the present study, we wished to investigate the functional role of PI3K pathway in H-ras-induced invasive phenotype and motility of MCF10A cells. Here, we report that activation of PI3K is not sufficient, but may be required for H-ras-induced invasion and migration.

MATERIALS AND METHODS

1) Cell lines

The development and characterization of the MCF10A, H-ras MCF10A and N-ras MCF10A cells has been previously described.[16] Briefly, retroviral vectors containing a mutant H-ras (pBW1423) and a mutant N-ras (pBW1775) (provided by Dr. Douglas Lowy) were transfected into the amphotropic package cells line GP+envAm12 (provided by Dr. Arthur Bank). MCF10A cells were infected with the viral media and selected in the presence of 400μg/ml G418. More than 100 NeoR colonies were pooled together and named H-ras MCF10A and N-ras MCF10A, respectively. The cells were cultured in DMEM/F12 supplemented with 5% horse serum, 0.5 μg/ml hydrocortisone, 10μg/ml insulin, 20 ng/ml EGF, 0.1μg/ml cholera enterotoxin, 100 units/ml penicillin-streptomycin, 2 mM L-glutamine and 0.5 μg/ml fungizone.

2) Immunoblot analysis

Equal amounts of protein extracts in SDS-lysis buffer were subjected to 12% SDS-PAGE analysis and electrophoretically transferred to nitrocellulose membrane. Anti-Akt and anti-phosphorylated Akt antibodies were purchased from New England Biolabs, MA. Enhanced chemiluminescence (ECL, Amersham-Pharmacia, Buckinghamshire, UK) system was used for detection.

3) *In vitro* invasion assay

In vitro invasion assay was performed using 24-well transwell as previously described.[16] The lower side of the filter was coated with type I collagen, and the upper side was coated with Matrigel. Lower compartment was filled with serum free media containing 0.1% BSA. Cells were placed in the upper part of the Transwell plate, were incubated for 17 hr, 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.

4) *In vitro* wound migration assay

Cells were pretreated with mitomycin C (25μg/ml) for 30 min before injury line was made. The injury line was made with a tip with 2 mm in width on the cells plated in culture dishes at 90% confluency. After rinsed with PBS, cells were allowed to migrate in complete media and photographs were taken (×40) at indicated time points.

5) MTT assay

Cells (1×10^4) cultured in a 96-well plate were treated with LY294002 and wortmannin for 0～24 hr. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml) was added to the media and the cells were further incubated for 4 hr. After 100μl of DMSO, absorbance of each well was read at 540 nm with micro-ELISA reader (Molecular Devices, Sunnyvale, CA, USA). Percent of cell survival was defined as the relative absorbance of zero hour.

6) Gelatin zymography

Cells were cultured in serum-free DMEM/F12 medium for 48 hr. Conditioned medium was collected and centrifuged at 3,000 rpm for 10 min to remove cell debris. The protein concentration was measured using BCA protein assay reagents (Pierce, IL, USA). Equal amounts of conditioned media were mixed with 2× laemmli non-reducing sample buffer, incubated for 15 min at room temperature, and then electrophoresed on 10% SDS-PAGE gels containing 1 mg/ml gelatin. After electrophoresis, the gels were washed with 2.5% Triton X-100 three times for 30 min, rinsed for 15 min with a 50 mM Tris-HCl buffer (pH 7.6) containing 5 mM $CaCl_2$, 0,02% Brij-35, 0.2% sodium azide, and incubated overnight at 37°C. The gels were stained with 0.5% Coomassie Brilliant Blue R-250 solution containing 10% acetic acid and 20% methanol for 40 minutes and destained with 10% acetic acid. Areas of gelatinase activity were detected as clear bands against the blue-stained gelatin background. Relative band intensities were determined by quantitation of each band with an Image Analyzer (Vilber Lourmet, France).

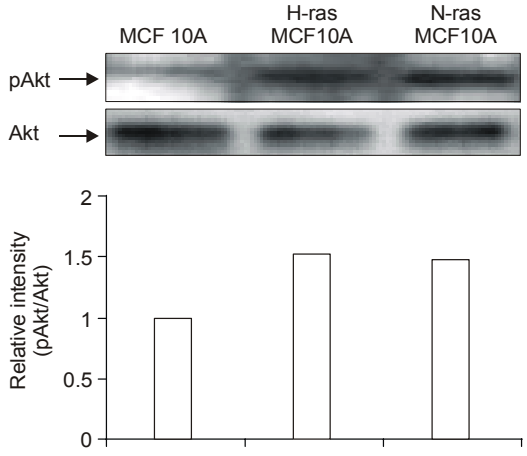

Fig. 1. H-ras and N-ras activate Akt in MCF10A cells. Activations of Akt in the cell lines were determined by Western blot analysis of whole cell lysates using antibodies against phospho-specific Akt (pAkt) and total Akt.

RESULTS AND DISCUSSION

We first wished to investigate whether PI3K is activated by H-ras and/or N-ras in MCF10A cells. Activation of PI3K was examined by detecting phosphorylation of Akt, a downstream molecule of PI3K, by Western blot analysis. Shown in Fig. 1 are activation of Akt in the parental, H-ras and N-ras MCF10A cells. Phosphorylated Akt (pAkt) level was increased in both H-ras- and N-ras MCF10A cells compared to the parental MCF10A cells while the amount of total Akt was about the same in these cell lines. Since PI3K is activated not only in H-ras MCF10A cells but also in non-invasive/non-motile[16,17] N-ras MCF10A cells, these findings suggest that activation of PI3K is not sufficient for H-ras-induced invasiveness and motility in breast epithelial cells.

Activation of PI3K in H-ras- and N-ras MCF10A cells was efficiently inhibited by specific inhibitors of PI3K, LY294002 and wortmannin. Treatment of 10 μM LY294002 or 20 nM wortmannin for 30 min prominently inhibited pAkt levels of H-ras- and N-ras MCF10A cells while total Akt levels were not affected (Fig. 2). The result show that both LY294002 and wortmannin can be used to inhibit PI3K pathway effectively in this experimental system.

PI3K and Akt have been shown to promote invasive phenotype in many cell systems.[20,21] To evaluate

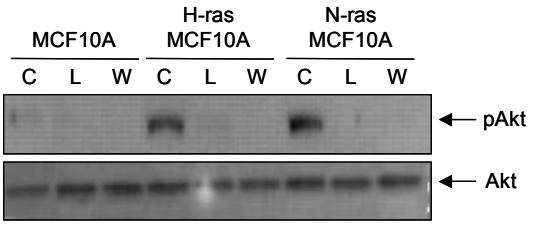

Fig. 2. PI3K inhibitors efficiently inhibit Akt in H-ras- and N-ras MCF10A cells. Western blot analysis was performed on whole cell lysates prepared from the cells treated with 10μM LY294002 (L) or 20 nM wortmannin (W) for 30 min. Control cells (C) were treated with DMSO.

whether PI3K is critical for H-ras-induced invasiveness, we examined the effect of the PI3K inhibitors on the invasive phenotype of H-ras MCF10A cells by performing *in vitro* invasion assay. As shown in Fig. 3A, treatment of 10μM LY294002 or 20 nM wortmannin significantly reduced the number of invaded cells. Because *in vitro* invasiveness depends on the number of viable cells, we tested the viability of the cells treated with LY294002 or wortmannin to exclude the possibility that the observed inhibition of H-ras-induced invasion was due to a cytotoxic effect. Survival of the treated cells for up to 24 hr was comparable to that of cells at 0 hr as determined by MTT assay (Fig. 3B), indicating that the inhibition of invasion was not due to a cytotoxic effect of LY294002 or wortmannin. The results show that PI3K activity is required for H-ras-induced invasive phenotype in MCF10A cells.

We then investigated the role of PI3K in the migratory property of H-ras MCF10A cells by *in vitro* wound migration assay. H-ras-induced cell migration was significantly inhibited in the presence of 10μM LY294002 or 20 nM wortmannin (Fig. 4). The data indicate that activation of PI3K is essential to migrative as well as invasive properties induced by H-ras in MCf10A cells. Consistently, it has been demonstrated that PI3K increases cell motility in a variety of cell systems including HT1080 fibrosarcoma cells.[22] It should be noted, however, that activation of PI3K pathway is not sufficeint for induction of invasiveness and motility in MCF10A cells.

Invasive phenotype of cancer cells is often associated with increased expression of MMP-2 and/or MMP-9, which can degrade type IV collagen, the major structural collagen of the basement membrane.[24] We previously showed that the H-ras-induced invasive phenotype is associated more closely with the levels of MMP-2 expression than the MMP-9 level in human breast epithelial cells.[16] Since the present study shows that PI3K activation is critical for H-ras-mediated invasion, we asked whether PI3K mediate H-ras induction of MMP-2 and/or MMP-9 expression. To determine the functional role of PI3K activation in MMP expression, a dose-response study was conducted on conditioned medium of H-ras MCF10A cells treated with LY294002 for 48 hr. As shown in

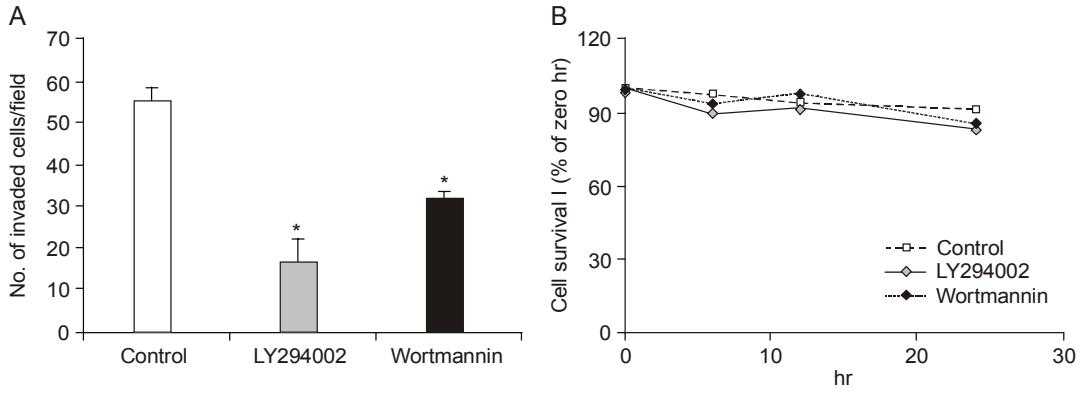

Fig. 3. H-ras-induced invasive phenotype is inhibited by PI3K inhibitors. *A*, H-ras MCF10A cells pretreated with 10μM LY294002 or 20 nM wortmannin for 30 min were subjected to *in vitro* invasion assay. Fifty thousand viable cells were incubated in a transwell chamber for 17 hr. The number of invaded cells per field was counted (×400) in thirteen fields. The results presented were means S.E. of triplicates. *Statistically different from control at $p<0.05$. *B*, H-ras MCF10A cells (1×10^4) were treated with 10μM of LY294002 or 20 nM wortmannin for 0~24 hr. MTT assay was performed and the cytotoxicity was determined by relative absorbance normalized to zero hr. The results presented were means±S.E. of triplicates.

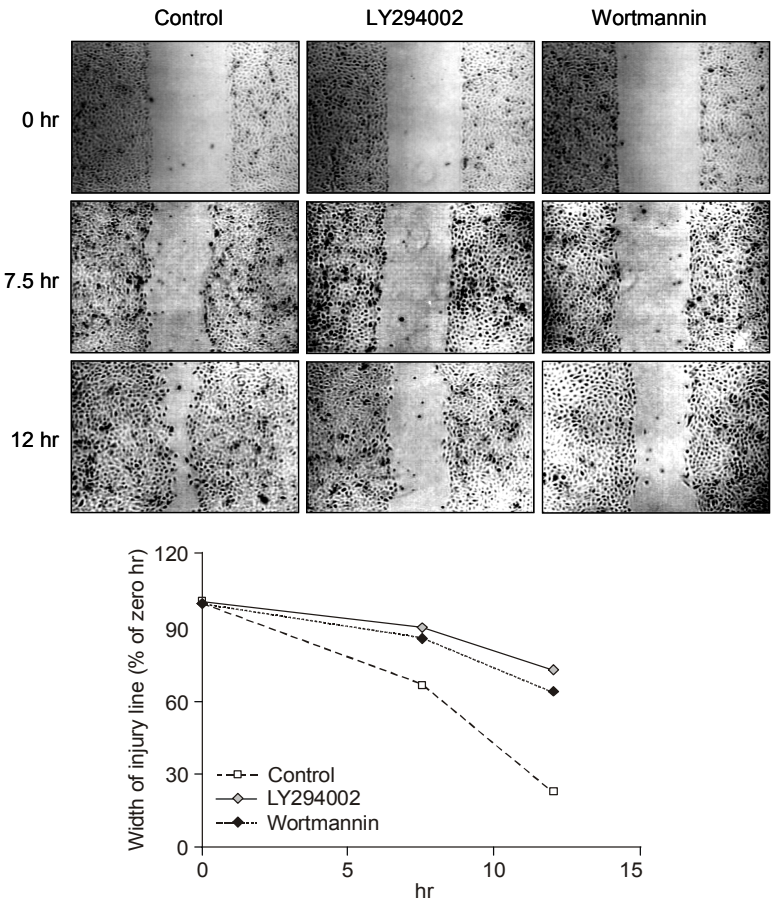

Fig. 4. H-ras-induced cell motility is inhibited by PI3K inhibitors. H-ras MCF10A cells pretreated with 10μM LY294002 or 20 nM wortmannin for 30 min were subjected to *in vitro* wound migration assay. Injury line (2 mm in width) was made on the cells plated in culture dishes at 90% confluency. Cells were rinsed with PBS and allowed to migrate in complete media containing 10μM LY294002 or 20 nM wortmannin. Cellular migration was observed with inverted microscope (40) at indicated time points. Width of injury line was measured and plotted as % of zero hr quantification.

Fig. 5, treatment of LY294002 resulted in a significant downregulation of MMP-2 and to a lesser extent, MMP-9 in a dose-dependent manner. These findings demonstrate that activation of PI3K is required for enhanced MMP-2 expression and to a lesser degree, MMP-9 expression in MCF10A cells.

The effect of PI3K activation on MMP expression is cell type-dependent. Previous studies have shown that Akt promotes MMP-9 production in HT1080 cells[22] and MMP-2 activity in mouse mammary epithelial cells.[23] In H-ras MCF10A breast epithelial cell system, both MMP-2 and MMP-9 were down-regulated when the PI3K pathway was inhibited. Taken together, we demonstrate that activation of PI3K pathway is not sufficient, but required for H-ras-induced invasive phenotype and migration in MCF10A cells. We also propose that PI3K pathway is critical for H-ras-mediated upregulation of MMPs in MCF10A cells, resulting in phenotypic conversion of non-invasive MCF10A cells to an invasive phe-

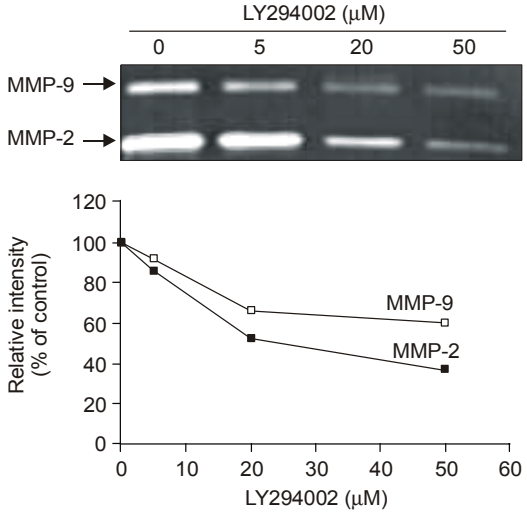

Fig. 5. A PI3K inhibitor downregulates MMP-2 and MMP-9 in H-ras MCF10A cells. Cells were treated with various concentrations of LY294002 for 48 hr. The levels of secreted MMP-2 (72 kDa) and MMP-9 (92 kDa) were determined by gelatin zymogram assay and relative band intensities were plotted for quantification.

notype.

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