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Advances in Brief

Epidermal Growth Factor Promotes MDA-MB-231 Breast Cancer Cell Migration through a Phosphatidylinositol 3′-Kinase and Phospholipase C-dependent Mechanism[1]

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Abstract

Epidermal growth factor receptor (EGFR) levels predict a poor outcome in human breast cancer and are most commonly associated with proliferative effects of epidermal growth factor (EGF), with little emphasis placed on motogenic responses to EGF. We found that MDA-MB-231 human breast cancer cells elicited a potent chemotactic response despite their complete lack of a proliferative response to EGF. Antagonists of EGFR ligation, the EGFR kinase, phosphatidylinositol 3′-kinase, and phospholipase C, but not the mitogen-activated protein kinases (extracellular signal-regulated protein kinase 1 and 2), blocked MDA-MB-231 chemotaxis. These findings suggest that EGF may influence human breast cancer progression via migratory pathways, the signaling for which appears to be dissociated, at least in part, from the proliferative pathways.

Introduction

EGFR[3] is one of the receptors most commonly associated with human tumors and has been shown to correlate with the progression of many tumor types including breast tumors (1, 2). Most often associated with aspects of tumor growth (*i.e.*, proliferation, apoptosis, and cell survival), little emphasis has been placed on the effects of EGF on breast cancer cell migration. The complex process of cell migration is a critical component of many normal and pathophysiological processes, and its central role in the progression of tumors from a noninvasive to an invasive and metastatic phenotype is well known (3). Previously, EGF has been shown to stimulate the migration of both normal and tumor cells, including normal mammary epithelial cells, fibroblasts, and renal and prostate carcinoma cells (4–7). In the current study, we characterized the migratory responses of MDA-MB-231 human breast cancer cells to EGF. Interestingly, these cells express EGFR but lack a proliferative response to EGF (8), and this is thought to be due to an activated K-ras. We further analyzed several major components of signaling pathways downstream of the EGFR. In particular, we examined the MAP kinases, PI3K, and PLC which have been demonstrated as having potential roles in cell migration (9–11).

Materials and Methods

Cell Culture. MDA-MB-231 cells originally derived from a pleural effusion were obtained from the American Type Culture Collection (Manassas, VA) and maintained by the Cell and Tissue Culture Core, Lombardi Cancer Center (Washington, D.C.). Cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum (RPMI 1640/FBS).

Inhibitors. PD153035 and AG1478, tyrosine kinase inhibitors with selectivity for the EGFR, were generous gifts from Dr. D. W. Fry (Parke-Davis Pharmaceutical Research, Ann Arbor, MI) and Dr. A. Levitzki (Jerusalem University, Jerusalem, Israel), respectively. The PI3k inhibitors wortmannin and LY294002 compound, the MEK inhibitor PD098059, and the PLC inhibitor U73122 were obtained from Calbiochem (Alexandria, Australia). The EGFR blocking antibody was purchased from Upstate Biotechnology, Inc. (Lake Placid, NY).

Chemomigration Assay. Cell migration was determined using a 48-well microchemotaxis chamber assay (Neuroprobe, Cabin John, MD) as described previously (12). Cell migration was quantified by the number of cells that migrated directionally through a collagen IV (40 μg/ml; Becton Dickinson, Bedford, MA)-coated 8-μm-pore polyvinyl pyrrolidone-free polycarbonate filter (Poretics, Livermore, CA) toward the chemoattractant (12). Briefly, cells (1×10^6 cells/ml) were resuspended in serum-free RPMI 1640 containing 0.1% BSA (RPMI 1640/BSA) and were either untreated or pretreated at room temperature with inhibitors (15 min) or antibodies (45 min). Recombinant human EGF (Becton Dickinson) was diluted to the indicated concentrations and added to the lower wells. Chambers were incubated at 37°C in a humidified incubator in an atmosphere of 5% CO_2:95% air for 4 h, after which the filters were removed, fixed, and stained with Diff-Quik (Baxer Scientific, McGaw Park, IL) and mounted on glass slides. Nonmigrated cells were removed by wiping with a cotton swab. At least four random fields of vision/well (×20 objective) were counted for quantitation of cell migration. Triplicate wells were performed in each assay, and the assay was repeated at least three times.

Proliferation Analysis. Cells (2×10^3) were plated in triplicate wells overnight in a 96-well plate with 200 μl of RPMI 1640/FCS. Each well was gently washed twice with unsupplemented RPMI 1640, and then either positive control (RPMI 1640/FBS), negative control (RPMI 1640/BSA), or test media (various dilutions of EGF in RPMI 1640/BSA) were added. Separate plates were set aside for WST-1 analysis (Roche, Castle Hill, Australia) on days 1, 3, and 5. Reduction of the WST-1 dye, due to mitochondrial activity, is linearly proportional to the cell number and was determined by analysis of absorption at 450 nm using a Dynatech ELISA plate reader (Dynatech International, Chantilly, VA).

ERK2 Activation Analysis. Cells were grown to 80% confluence and serum-starved in RPMI 1640/0.1% fetal bovine serum for 18 h, washed in PBS, and harvested with brief exposure to 0.05% trypsin and 0.02% EDTA. After trypsin inhibition with soybean trypsin inhibitor (0.5 mg/ml) for a period of 10 min, the cells were collected by centrifugation, washed once in RPMI 1640/BSA with soybean trypsin inhibitor and then washed once in RPMI 1640/BSA. The cells were then held in suspension in RPMI 1640/BSA during pretreatment with wortmannin or PD098059 in 0.1% DMSO (final concentration) or with vehicle alone (0.1% DMSO) for 15 min. The cells were then plated onto 6-well tissue culture plates that had been precoated overnight with

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[3] The abbreviations used are: EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; PI3K, phosphatidylinositol 3′-kinase; PLC, phospholipase C; ERK, extracellular signal-regulated protein kinase; MAP, mitogen-activated protein; MEK, MAP kinase kinase; PDGF, platelet-derived growth factor.

collagen IV (40 μg/ml) or poly-L-lysine (100 μg/ml) in PBS at 4°C. The plates were then incubated for 30 min or 4 h at 37°C in a humidified incubator with an atmosphere of 5% CO_2:95% air in either the presence or absence of EGF (10 ng/ml). After incubation, the plates were washed twice with ice-cold PBS, and the cells were lysed with 2× reducing sample buffer at 95°C, frozen on dry ice, and stored at −70°C until analysis. Samples were analyzed by 12% SDS-PAGE and immunoblotted with ERK2 monoclonal antibody IB3E9 (kindly provided by Mike Weber, University of Virginia, Charlottesville, VA).

Results and Discussion

In light of the observations regarding the prognostic significance of EGFR in human breast cancer and the effects of EGF on cell migration in a number of cell types, including normal mammary epithelial cells (4), we assessed the effects of EGF on the migration of MDA-MB-231 cells. The MDA-MB-231 cells demonstrated a characteristic bell-shaped chemomigratory curve toward EGF (1–100 ng/ml). Optimal migration of the cells was repeatedly observed at a concentration of 10 ng/ml EGF (Fig. 1*A*). Checkerboard analysis in which the amount of EGF was varied in both the top and bottom wells of the chamber demonstrated that the MDA-MB-231 cells predominantly display a directional motility (chemotaxis) toward EGF, with a minor component of random motility (data not shown). The specificity of this response to EGFR was demonstrated in that the migratory response could be inhibited to background levels by the EGFR-specific tyrosine kinase inhibitors AG1478 and PD153035 (Fig. 1*B*). Ligation of EGF to its receptor was also critical in that the migratory response could also be inhibited by an EGFR blocking antibody (Fig. 1*B*).

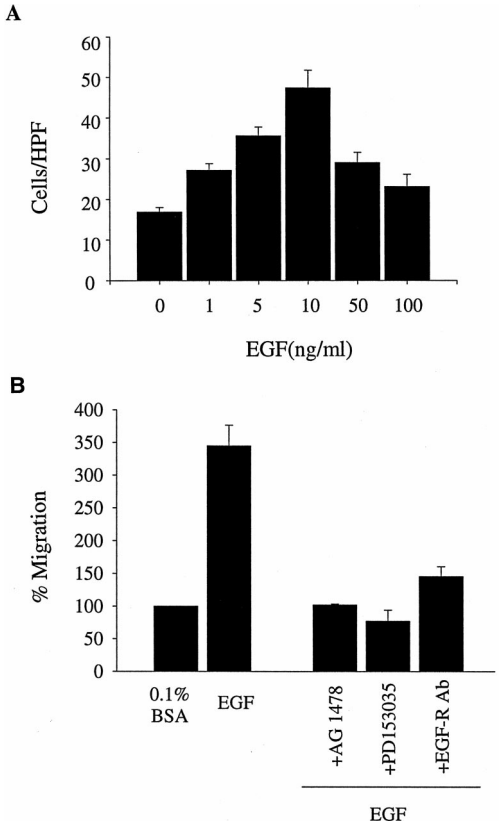

Fig. 1. EGF stimulates a strong migratory response in MDA-MB-231 cells. Analysis of the migratory response of the MDA-MB-231 cells to EGF (1–100 ng/ml) was performed using the 48 microchemotaxis chamber assay as described in "Materials and Methods." *A*, the MDA-MB-231 cells displayed a characteristic chemomigratory bell-shaped curve toward EGF, with optimal migration observed at 10 ng/ml EGF. *B*, the migration toward EGF (10 ng/ml) was inhibited to background migration levels when the cells were incubated at room temperature with the EGF-specific tyrosine kinase inhibitors AG1478 (2 μM) and PD153035 (1 μM) as well as an EGFR blocking antibody (0.4 μg/ml). The data are expressed as the mean ± SD of cell counts taken from 12 high-powered fields (four fields from each replicate well) or as the percentage of migration ± SD relative to unstimulated migration (*0.1% BSA*). The results are representative of at least three independent experiments (except for those of the PD153035 compound, which was performed on one occasion in triplicate).

Although EGF is potently mitogenic for many normal and tumor cells, during the course of this study, we were surprised to repeatedly find a lack of a proliferative response to EGF (data not shown). This finding supports previous work by Davidson *et al.* (8), who reported the lack of proliferation in these cells toward EGF, which was postulated to be due to an activating mutation in codon 13 of K-ras (8, 13). Further support for the role of activated K-ras in this proliferative insensitivity to EGF was recently provided by Kato *et al.* (14), who demonstrated that endometrial cancer cells lost their proliferative responsiveness to EGF when transfected with a codon 13-activated K-ras.

Because there was a clear dissociation between the migratory and proliferative responses to EGF in the MDA-MB-231 cell line, we used a number of standard approaches to identify the signal transduction pathways involved in the EGF-stimulated migratory response in these cells. Treatment of the cells with the PI3K inhibitor wortmannin (100 nM) resulted in a significant inhibition of both basal migration (from 100% to 44.20 ± 6.81%; $P < 0.01$) and EGF-induced migration (from 338.40 ± 15.15% to 117 ± 3.12%; $P < 0.01$; Fig. 2*A*). Similar levels of inhibition were also seen with another PI3K inhibitor, LY294002 (data not shown). PI3K involvement has previously been shown in PDGF-stimulated migration (15, 16); however, to our knowledge, it has yet to be demonstrated for EGF. The exact mechanism by which PI3K modulates cell migration has yet to be fully delineated. However, Azuma *et al.* (17) postulated that activation of PI3K via growth factor receptors causes the activation and recruitment of the small GTPase Rac to membrane sites where ruffling and motility are to occur. Activated Rac at the membrane recruits a multiprotein complex, leading to cycles of activation and inactivation of its downstream effector gelsolin, an actin-modifying protein. The activation and inactivation of gelsolin lead to the severing and partial dissolution of actin filament networks and the creation of nucleation sites for actin reassembly (17). Gelsolin has been previously shown to be critical for EGFR-mediated migration in fibroblasts (5).

The MAP kinase family members ERK1 and ERK2, which are known to be involved in EGF-mediated signaling and to lie downstream of PI3k in some signaling pathways, have been shown to be involved in growth factor- and integrin-mediated migration of cells (9, 11). Inhibition of ERK1 and ERK2 by the MEK inhibitor PD098059 (50 μM) resulted in the significant inhibition of the basal unstimulated migration of the MDA-MB-231 cells from 100% to 57.30 ± 9.00% ($P < 0.05$), whereas, the EGF-stimulated migration of the cells was not altered (Fig. 2*B*). Western blot analysis demonstrated that basal ERK2 and induced ERK2 were inhibited by treatment with the MEK inhibitor PD098059 (Fig. 3). Analysis of ERK2 activation on the control substrate poly-L-lysine demonstrated a high level of constitutively active ERK2 that was further enhanced by EGF stimulation. Due to this high basal level of activation of ERK2, any integrin-induced activation of ERK2 by plating the cells on collagen IV could not be distinguished. However, the PD098059 compound achieved effective inhibition of both basal and EGF-induced ERK2 activation. The lack of inhibition of EGF-stimulated ERK2 activation with the PI3K inhibitor wortmannin suggests that the MAP kinases do not lie downstream of PI3K in these cells (Fig. 3).

A pathway has recently been proposed by Klemke *et al.* (9) for the involvement of MAP kinases (ERK1 and ERK2) in the integrin-mediated haptotaxis of cells. Activation of MAP kinases leads to the subsequent phosphorylation and enhanced activity of myosin light chain kinase, leading to phosphorylation of myosin light chains and

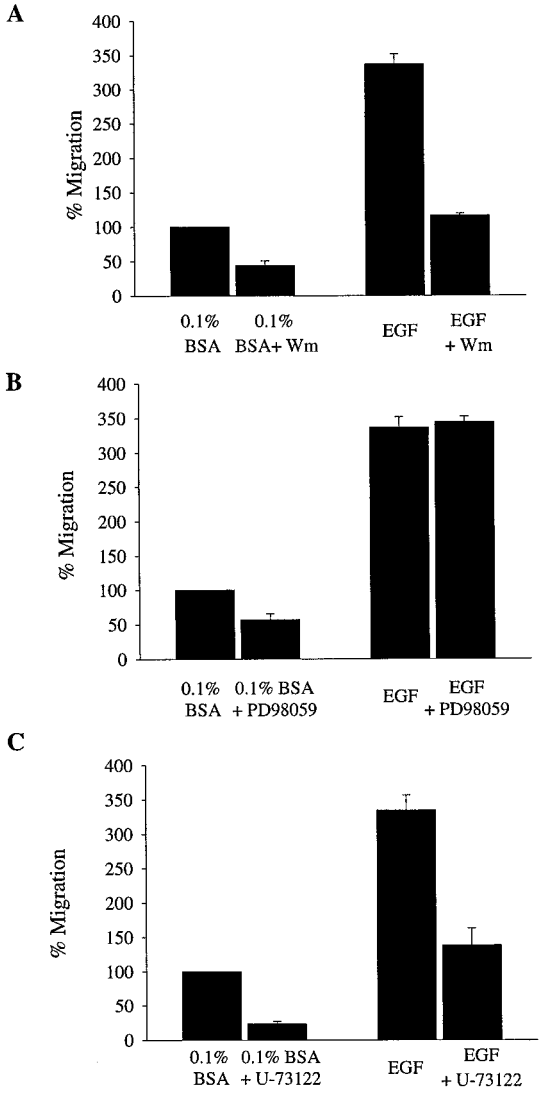

Fig. 2. Inhibition of EGF-stimulated migration with wortmannin and U73122. Cells were pretreated for 15 min at room temperature with the appropriate drug before migration analysis as described in "Materials and Methods." *A*, cells were treated with the PI3K inhibitor wortmannin (*Wm*; 100 nM), which significantly reduced the background migration ($P < 0.01$) and the EGF-stimulated migration ($P < 0.01$). *B*, cells were treated with MEK inhibitor PD98059 (50 μM), which significantly reduced the background migration ($P < 0.05$) but had no significant effect on the EGF-stimulated migration ($P > 0.05$). *C*, cells were treated with the PLC inhibitor U73122 (2 μM), which significantly reduced the background migration ($P < 0.006$) and the EGF-stimulated migration ($P < 0.01$). Results are expressed as the percentage of migration ± SD relative to the unstimulated migration (*0.1% BSA*) and are representative of at least three independent experiments.

resulting in enhanced cell migration. Furthermore, the involvement of MAP kinase in EGF-mediated migration of fibroblasts has been observed through its effects on disassembly and reorganization of cell-substratum adhesion (5). Our findings show a lack of involvement of the MAP kinases in the EGF chemotactic response of the MDA-MB-231 cells, further demonstrating that ERK1 and ERK2 do not lie downstream of PI3K in the EGF-stimulated migration of these cells. However, we do demonstrate its involvement, along with PI3k, in the basal migration of these cells on collagen IV. The mechanism by which MAP kinase is involved in the basal migration is uncertain; however, the presence of activated K-ras may induce a high basal migration through, in part, a MAP kinase pathway. Consistent with this is the observation of elevated baseline activation of ERK2. Previously, Ras has been shown to have a role in migration (18); thus, the K-ras mutation may not only affect the proliferative pathway but may also result in a high constitutive migration in the absence of chemoattractant (*i.e.*, 0.1% BSA) through a MAP kinase-mediated pathway. Alternative pathways on EGF stimulation may then supersede this, resulting in the lack of involvement of MAP kinase in the EGF-stimulated migration of the MDA-MB-231 cells.

In addition to PI3K and MAP kinases, PLCγ is another major signaling molecule that lies downstream of EGFR signaling. Pretreatment of the MDA-MB-231 cells with the PLC inhibitor U73122 (2 μM) resulted in a significant reduction of the basal migration from 100% to 23.40 ± 3.80% ($P < 0.006$). In addition, the EGF-stimulated migration was also significantly inhibited from 335.60 ± 22.00% to 138.60 ± 25.20% ($P < 0.01$; Fig. 2*C*). PLC involvement in growth factor-mediated migration has been demonstrated in relation to PDGF BB and insulin-like growth factor I-mediated migration in smooth muscle cells (19), as well as in the migration of fibroblasts in response to EGF stimulation (11). The activation of PLC by growth factor receptors leads to the hydrolysis of phosphatidylinositol-(4,5)-bisphosphate, and thus to the generation of diacylglycerol (DAG) and inositol triphosphate (IP3) which leads to the activation of protein kinase C and the release of intracellular stores of calcium, respectively. Both protein kinase C and calcium are effectors that have previously been reported to modulate processes required for cell motility (3). Mobilization of actin modifying proteins such as gelsolin from inactive sites to active cytoskeletal regions has also been linked to PLC activation, leading to cycles of actin dissolution and polymerisation, resulting in cell movement (20). Recent studies have indicated that the PI3K product phosphatidylinositol-(3,4,5)-P3 acts to directly and indirectly to enhance PLCγ-catalyzed breakdown of phosphatidylinositol-(4,5)-bisphosphate (21). We examined whether PLC may be downstream of PI3K in the EGF-induced migration in the MDA-MB-231 cells. Simultaneous inhibition of PI3K and PLC resulted in further inhibition of cell migration, indicating that PI3K and PLC may lie along divergent pathways in the MDA-MB-231 cells (data not shown).

In conclusion, we clearly demonstrate the potent and reproducible migratory responses in the MDA-MB-231 breast cancer cell line. These responses are chemotactic rather than chemokinetic, indicating that breast cancer cells may move along EGFR ligand gradients. This would be particularly relevant to the production of such ligands by stromal cells, as has been demonstrated during pregnancy in the rat (22). Interestingly, such migratory profiles can be seen in breast

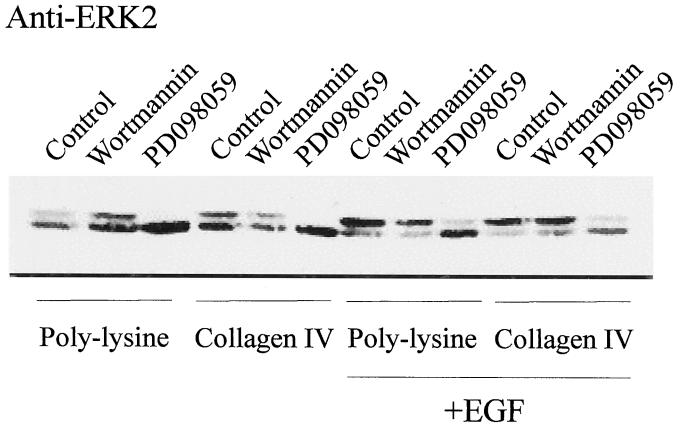

Fig. 3. Analysis of MAP kinase inhibition with PD098059. Cells were treated with vehicle alone (0.1% DMSO), wortmannin (100 nM), or PD098059 (50 μM) before being plated on the respective substrates in the absence or presence of EGF (10 ng/ml). Total cellular protein was obtained as described in "Materials and Methods" and analyzed by Western blot analysis with an ERK2-specific antibody. There was no significant difference between the results obtained from the 30-min incubation and those obtained from the 4-h incubation. The results here are representative of the 30-min incubation period.

cancer cells that have superseded growth factor proliferative signals, such as activated ras, and, as such, represent viable therapeutic targets. The characterization of signaling pathways for EGFR-mediated migration, which involves PI3k and PLC, appears consistent with migratory responses to other polypeptide growth factors (*e.g.*, PDGF and insulin-like growth factor I; Refs. 16, 19) and may provide more general opportunities to target for growth factor-stimulated migration in general.

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