Mitogen-activated Protein/Extracellular Signal-regulated Kinase Kinase (MEK) Inhibitors Restore Anoikis Sensitivity in Human Breast Cancer Cell Lines with a Constitutively Activated Extracellular-regulated Kinase (ERK) Pathway

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Abstract
Anchorage-independent growth is a hallmark of oncogenic transformation. We reported that the mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) inhibitor U0126 inhibited anchorage-independent growth of Ki-ras-transformed rat fibroblasts, whereas HBC4 cells, unsensitized by MEK inhibitors, were more selective for the ERK pathway. Removal of anchorage substantially sensitized p70S6K to PD98059 in MDA-MB231 cells, whereas p70S6K in suspended HBC4 cells remained fairly refractory. U0126 was either without effect or less inhibitory on p70S6K in MDA-MB453 and SKBR3, two cell lines in which anoikis sensitivity was not induced. Thus, susceptibility of the p70S6K pathway to MEK inhibitors appeared to be an important determinant of anoikis sensitivity. The results indicate that concurrent inhibition of MEK-ERK and mTOR-p70S6K pathways induces apoptosis in MDA-MB231 and HBC4 cells when cells are deprived of anchorage but not when anchored. Inhibitors of MEK-ERK and mTOR-p70S6K pathways may provide a therapeutic strategy to selectively target neoplasms proliferating at ectopic locations, with acceptable effects on normal cells in their proper tissue context.

Introduction
Growth parameters of normal and transformed cell lines in culture differ in various aspects. Notably, whereas normal cells generally require integrin-mediated adhesion to appropriate extracellular matrix for growth and survival, tumor cells can proliferate without cell-substratum interactions. Anchorage-independent growth is the best in vitro correlate to tumorigenicity (1, 2), and the difference between normal and tumor cells in anchorage dependence in vitro likely reflects their nature in vivo. Survival, growth, and differentiation of normal cells are permitted only when the cells are in their proper tissue context within the organism. On the other hand, tumor cells have the competence for activity outside of their original environment.

We anticipate that compounds that reverse anchorage independence would target tumor cells growing in defiance of positional restrictions while minimally influencing normal cells in their appropriate locations. The MEK inhibition by U0126 suppressed anchorage-independent growth of Ki-ras-transformed rat fibroblasts (3). Because most malignant cancers in humans originate in epithelial tissues, we examined the effects of U0126 on growth properties of human epithelial tumor cells. Of eight breast cancer cell lines tested, U0126 inhibited anchorage-independent growth of two lines with constitutively activated ERK.

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3 The abbreviations used are: MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; ERK, extracellular signal-regulated kinase; mTOR, mammalian target of rapamycin; HEMA, 2-(hydroxyethyl methacrylate); MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylytetrazolium bromide; PI3K, phosphatidylinositol 3-kinase.
Many normal cell types, including epithelial cells, undergo apoptosis upon detachment from their proper substratum, a phenomenon termed anoikis (4–6). This process prevents displaced and shed cells from colonizing outside of their appropriate environment and is tightly controlled to maintain homeostasis and tissue integrity. In the course of malignant transformation, cells acquire resistance to anoikis. We show that U0126 inhibits anchorage-independent growth of human breast cancer cell lines by reestablishing susceptibility to anoikis. Apoptotic response was confined exclusively to nonanchored cells, and anchored cells remained viable.

In Ki-ras-transformed rat fibroblasts, U0126 inhibited the mTOR-p70S6K pathway in addition to the ERK pathway. Experiments with PD98059 and rapamycin suggested that restoration of the normal phenotype depended on simultaneous blockade of both ERK and p70S6K pathways. Here we present data that induction of anoikis sensitivity in human breast cancer cell lines also requires simultaneous inhibition of the two signaling pathways. Susceptibility of the p70S6K pathway to MEK inhibitors varied among the tumor cells analyzed and appeared to underlie the sensitivity to anoikis. Our data demonstrate the potential of MEK inhibitors as a means to target malignant cells growing outside of their correct tissue context and have implications for predicting efficacy of MEK inhibitors. The results also indicate that usefulness of MEK inhibitors may be overlooked if evaluated alone by growth inhibition assay on solid support.

Materials and Methods

Materials. U0126 was from Promega Corp. (Madison, WI), and PD98059 and rapamycin were from Calbiochem (San Diego, CA). Concentrated stock solutions of inhibitors were prepared in DMSO. polyHEMA was from Sigma Chemical Co. (St. Louis, MO). Antibodies against ERK1 and ERK2 were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphospecific antibodies and antibodies against S6 ribosomal protein and cleaved caspase 3 were from Cell Signaling Technology (Beverly, MA). SYBR Green I was from Molecular Probes (Eugene, OR).

Cell Culture and Measurement of Anchorage-independent Growth. Human breast cancer cell lines MCF-7, MDA-MB453, SKBR3, and ZR75-1 were purchased from the American Type Culture Collection. BSY1, HBC4, HBC5, and MDA-MB231 were obtained from Dr. T. Yamori (Japanese Foundation for Cancer Research, Tokyo, Japan). Anchorage-independent growth was measured on polyHEMA-coated or uncoated tissue culture plastic 35-mm dishes and incubated for 24 h. Cells were treated with inhibitors for 24–48 h, washed with PBS, and lysed with 10 mM Tris-HCl (pH 7.4), 10 mM EDTA, and 0.5% Triton X-100. The low molecular weight DNA-containing fraction was isolated by centrifugation at 10,000 × g for 10 min. Supernatants containing equal amounts of protein were treated with 20 μg/ml RNase A for 1 h and then with 20 μg/ml proteinase K for 30 min at 37°C. DNA was 2-propanol precipitated, run on a 2% agarose gel, and visualized by SYBR Green I staining.

Immunoblotting Analysis. Cells were seeded as above, treated with inhibitors for 6–24 h, washed with cold PBS, fixed for 10 min with 10% cold trichloroacetic acid, and lysed with 80 μl of 9 M urea and 2% Triton X-100 and 20 μl of 10% lithium dodecyl sulfate. Lysates were neutralized with 2 μl Tris, sonicated to reduce viscosity and normalized for protein. Equal amounts of protein were electrophoresed through SDS-PAGE, immunoblotted, and detected by enhanced chemiluminescence.

Results

U0126 Inhibits Anchorage-independent Growth of Human Breast Cancer Cell Lines with Elevated ERK Activity. We reported that the MEK inhibitor U0126 (9) selectively suppressed anchorage-independent growth of Ki-ras-transformed rat fibroblasts (3). To explore the potency of U0126 on human cancer cells of epithelial origin, we examined the inhibitor’s effects on anchorage independence of human breast cancer cell lines. The cell lines displayed different responses to the inhibitor. Of the eight lines tested, the growth of MDA-MB231 and HBC4 was suppressed by U0126 on the nonadhesive polyHEMA-coated surface at concentrations lower than on plastic (Fig. 1). Other cell lines did not exhibit increased sensitivity to U0126 in polyHEMA-coated plates. Neither Adriamycin nor etoposide showed any selectivity between the two surfaces in any of the cells (data not shown).

To search for correlation between U0126 responsiveness and ERK activity, we examined the status of ERK phosphorylation/activation in the breast cancer cells by immunoblotting. As shown in Fig. 2, MDA-MB231 and HBC4, the two cell lines that showed increased sensitivity to U0126 when nonanchored, had higher levels of phosphorylated ERK than the other lines. Total ERK was similarly expressed in all cell lines. Hoshino et al. (10) reported that ERK is constitutively activated in MDA-MB231 and HBC4. These cells showed high ERK activity, even in the absence of growth factor stimulation.

U0126 Sensitizes MDA-MB231 and HBC4 Cells to Anoikis. Most normal cell types undergo apoptosis upon detachment from the matrix, a phenomenon referred to as anoikis. Evidently, tumor cells must acquire anoikis resistance to grow independently of anchorage. We explored the possibility that U0126 selectively inhibits anchorage-independent growth of MDA-MB231 and HBC4 cells by rendering these cells anoikis sensitive.

Exposure of MDA-MB231 and HBC4 cells to U0126 at 10 μM for 24 h led to DNA nucleosomal fragmentation when cells were deprived of anchorage but not when attached (Fig. 3A). Furthermore, other features of apoptotic cell death

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(caspase 3 cleavage and morphology) were also observed, exclusively in nonanchored cells (Figs. 6 and 7). Nucleosomal DNA ladder was not observed in MDA-MB453 or SKBR3 (Fig. 3B) or in other cell lines (not shown), even at 50 μM U0126. Adriamycin induced apoptosis, irrespective of anchorage in every cell line tested (Fig. 2 and not shown). The results indicate that U0126 induced anoikis sensitivity in human breast tumor cells with a constitutively activated ERK pathway, thereby selectively inhibiting anchorage-independent growth in polyHEMA-coated plates.

**PD98059 Renders MDA-MB231 Cells Anoikis Sensitive, But Induction of Anoikis Sensitivity in HBC4 Cells Requires Further Addition of Rapamycin.** We reported that simultaneous blockade of ERK and p70S6K pathways is necessary to reverse Ki-ras-mediated transformation in rat fibroblasts (3). U0126 inhibited both pathways and restored the normal phenotype. Another MEK inhibitor, PD98059 (11), only marginally influenced the p70S6K pathway and required addition of the mTOR-p70S6K pathway inhibitor rapamycin (12–15) to inhibit anchorage-independent growth of Ki-ras-transformed fibroblasts. Transformation by Ha-ras, on the other hand, appeared to be normalized by PD98059 alone.

We examined whether PD98059 by itself would inhibit anchorage-independent growth of human breast cancer cell lines. As shown in Fig. 4, PD98059 selectively inhibited anchorage-independent growth of MDA-MB231 cells. However, the compound did not inhibit anchorage-independent growth of HBC4 cells (Fig. 4) or other breast cancer cells (not shown).
shown). Consistent with the above results, PD98059 induced anoikis sensitivity in MDA-MB231 cells but not in HBC4 cells, as determined by nucleosomal DNA fragmentation, caspase 3 activation, and morphology (Figs. 5–7). These features of apoptosis were not observed in attached MDA-MB231 cells. We anticipated that the combination of PD98059 and rapamycin would mimic actions of U0126 and render HBC4 cells anoikis sensitive. As shown in Figs. 5–7, the combination of PD98059 and rapamycin sensitized HBC4 cells to apoptosis in suspension. Again, apoptotic features were detected only in nonanchored, and anchored cells remained viable. Rapamycin by itself did not activate any apoptotic response in MDA-MB231 or HBC4 cells, anchored or nonanchored, whereas Adriamycin induced apoptosis in both cell lines regardless of anchorage.

**Induction of Anoikis Sensitivity in MDA-MB231 and HBC4 Cells Requires Concurrent Inhibition of ERK and p70S6K Pathways.** The above results raised the possibility that inhibition of the ERK pathway alone is sufficient for anoikis execution in MDA-MB231 cells. Alternatively, the p70S6K pathway of MDA-MB231 cells might be more susceptible to PD98059 than that of HBC4 cells. To study the biochemical events that underlie induction of anoikis in MDA-MB231 and HBC4 cells, we examined the effects of the MEK inhibitors on activation of ERK and p70S6K pathways in anchored versus nonanchored cells by monitoring phosphorylation of ERK and S6 ribosomal protein. In contrast to ERK phosphorylation, all of the cell lines showed similar basal S6 ribosomal protein phosphorylation levels, which were not influenced by anchorage (not shown).

U0126 at 10 μM for 6 h essentially abolished ERK phosphorylation in both MDA-MB231 and HBC4 cells, anchored or nonanchored (Fig. 8). U0126 also significantly reduced S6 ribosomal protein phosphorylation, although requiring somewhat longer treatment in HBC4 cells. On the other hand, PD98059 in anchored cells was more selective for the ERK pathway and only slightly reduced phospho-S6 ribosomal protein. Removal of anchorage appeared to sensitize the p70S6K pathway to PD98059 in both cell lines but with greater impact on MDA-MB231. Whereas S6 ribosomal protein in suspended MDA-MB231 cells underwent complete dephosphorylation, a noticeable level of phosphorylated form was still detected in HBC4 cells. Rapamycin selectively inhibited the mTOR pathway of both cell lines, with and without anchorage, and did not influence phosphorylation status of ERK. Combination of PD98059 and rapamycin inhibited both ERK and p70S6K activity in all cellular settings. Adriamycin had no effect on ERK or S6 ribosomal protein phosphorylation. Thus, MDA-MB231 and HBC4 cells underwent apoptosis in situations in which ERK and p70S6K pathways were both blocked and deprived of anchorage. Separate interference of these pathways had little effect on survival, and anchored cells remained viable even when both pathways appeared to be fully inhibited.

We also examined the effect of U0126 on the phosphorylation status of S6 ribosomal protein in MDA-MB453 and SKBR3 cells. As shown above, U0126 does not sensitize these cells to anoikis. S6 ribosomal protein phosphorylation of MDA-MB453 was completely insensitive to U0126, and that of SKBR3 was considerably refractory (Fig. 9). Rapamycin, however, still inhibited S6 ribosomal protein phosphorylation in these cells. The combination of U0126 and rapamycin blocked both ERK and p70S6K pathways but did not induce anoikis sensitivity in MDA-MB453 and SKBR3 (not shown). The results suggest that susceptibility of p70S6K to MEK inhibitors may be one of the determinants of anoikis sensitivity.
Discussion

The important finding of this study is that in certain mammary epithelial cell lines, MEK inhibitors trigger apoptosis when cells are deprived of anchorage but not when attached. This phenomenon resembles anoikis, a form of apoptosis that occurs in various cell types upon detachment from the extracellular matrix. Anoikis is an important physiological mechanism to prevent ectopic survival of displaced cells, and its strict control is essential for maintenance of homeostasis and proper tissue organization. Acquisition of resistance to anoikis is an essential step in development of the malignant phenotype (16), and inhibitors of anoikis resistance may provide a therapeutic strategy to selectively target malignant cells proliferating at inappropriate locations.

The MEK inhibitor U0126 induced anoikis sensitivity in MDA-MB231 and HBC4, two human breast cancer cell lines with constitutively activated ERK. U0126 did not trigger any apoptotic response in anchored cells. Another MEK inhibitor PD98059 also rendered MDA-MB231 cells anoikis sensitive but had to be combined with rapamycin to induce anoikis sensitivity in HBC4 cells. Additional experiments suggested that concurrent inhibition of ERK and mTOR-p70S6K pathways was essential for induction of anoikis sensitivity by MEK inhibitors. U0126 and PD98059 both pronouncedly inhibited activation of ERK but differed in their effects on the p70S6K pathway. Whereas U0126 blocked the p70S6K pathway in both MDA-MB231 and HBC4 cells, PD98059 showed a strong effect only in nonanchored MDA-MB231 cells. U0126 was either without effect or less inhibitory on p70S6K in MDA-MB453 and SKBR3, two cell lines in which anoikis sensitivity was not induced. Thus, susceptibility of the p70S6K pathway to MEK inhibitors appeared to be an important determinant of anoikis sensitivity.
Induction of Anoikis Sensitivity by MEK Inhibitors

It is unclear how p70S6K is inhibited. We examined the effects of the recently introduced MEK inhibitor PD184352 (17) on anchorage independence of human tumor cell lines. This compound proved to be effective in inhibiting anchorage-independent growth of HBC4 cells and also inhibited the p70S6K pathway in this cell line.4 U0126, PD98059, and PD184352 are structurally dissimilar, and the p70S6K pathway blockade by these compounds is conceivably a consequence of MEK inhibition. Compared with PD98059, U0126 has considerably higher affinity to all forms of MEK (9). Although both compounds appeared to significantly suppress ERK, a subtle difference in the level of residual activity probably accounts for the greater impact of U0126 on p70S6K in a wider range of cells. On the basis of the inability of PD98059 to suppress p70S6K in many cells, we speculate that inhibition of the p70S6K pathway by MEK inhibitors requires absolute or near complete shutoff of ERK signals.

Inhibitors of the p70S6K pathway may synergize to block malignant growth when total ERK inhibition is not attainable.

In general, p70S6K has been considered to be controlled through a mechanism that is dependent on PI3K and AKT but independent of ERK (18–21). However, recent reports demonstrate that MEK inhibitors can suppress p70S6K in several cellular settings. Effects of MEK inhibitors and PI3K inhibitors on p70S6K vary in different systems and also depend on activating stimuli. For example, in human embryonic kidney 293 cells, activation of p70S6K by phorbol ester was sensitive to PD98059 and U0126 but insensitive to the PI3K inhibitor wortmannin (22). By contrast, in the same cells, activation by insulin was sensitive to wortmannin but insensitive to MEK inhibitors, p70S6K activation by nitric oxide in rat adventitial fibroblasts and by angiotensin II in rat vascular smooth muscle cells was sensitive to both MEK and PI3K inhibitors (23). Thus, there appears to be at least two pathways that regulate p70S6K activity, one that requires PI3K and another that depends on the MEK-ERK module. These two systems may function simultaneously within a cell and may cross-talk at various levels to influence each other, generating further complexity in mechanisms of p70S6K control. Our data indicate that diverse modes of p70S6K control exist in human cancers, and that in some cases ERK is the major upstream regulator.

As shown in Fig. 1, there were distinct differences in response to U0126 among the human breast cancer cell lines. Susceptibility to conventional antitumor agents such as Adriamycin and etoposide was not as diverse. They suppressed both anchorage-dependent and -independent growth of all the cell lines assessed at comparable concentrations. These in vitro results can be extrapolated to various effects of MEK inhibitors in clinical situations. Tumors that rely heavily on ERK for growth and survival are expected to be extremely susceptible to MEK inhibitors, whereas some tumors may be totally unresponsive. Prognosis of sensitivity from biochemical and genetic profiles will be of particular importance in clinical application of MEK inhibitors.

However, oncogenic growth involves irregularity of several pathways, and reciprocal interdependence and cross-talk of multiple pathways would add further complication in predicting consequences of MEK-ERK intervention. To gain insight into determinants of susceptibility to MEK inhibitors, we have extended our study to a large panel of human cancer cell lines. MEK inhibitors proved to be effective in suppressing anchorage-independent growth of several other cancer cell lines.

Fig. 8. Immunoblot analysis of phosphorylation level of ERK and S6 ribosomal protein in anchored or nonanchored MDA-MB231 (A) and HBC4 (B) cells treated with inhibitors. MDA-MB231 cells were treated for 6 h. HBC4 cells were treated for 6 h (for ERK analysis) or 15 h (for S6 ribosomal protein analysis). Concentrations used were: U0126, 10 μM; PD98059, 20 μM; rapamycin, 25 μM; Adriamycin, 1 μg/ml. Cell lysates were electrophoresed through 10% SDS-PAGE, followed by immunoblotting using phospho-ERK or phospho-S6 ribosomal protein-specific antibodies. Filters were stripped and reprobed with antibodies against total ERK or S6 ribosomal protein. PD, PD98059; RPM, rapamycin; ADM, Adriamycin; pERK, phosphorylated ERK; pS6R, phosphorylated S6 ribosomal protein.

Fig. 9. Immunoblot analysis of phosphorylation level of S6 ribosomal protein in anchored or nonanchored MDA-MB453 and SKBR3 cells treated for 24 h with U0126 (U) or rapamycin (R). Concentrations used were: U0126, 10 μM; rapamycin, 25 μM. Cell lysates were electrophoresed through 10% SDS-PAGE followed by immunoblotting using anti phospho-S6 ribosomal protein antibody. pS6R, phosphorylated S6 ribosomal protein; C, vehicle control.

* H. Fukazawa and Y. Uehara, unpublished observation.
lines, notably of colon. Although preliminary results suggested that tumors vulnerable to MEK inhibitors generally have elevated ERK, the correlation was not perfect, and some tumors with high levels of phosphorylated ERK were completely refractory. It is likely that tumors acquire anoikis resistance through various mechanisms, and even tumors with constitutively activated ERK may depend primarily on other pathways to evade anoikis. We anticipate that MEK inhibitors will still be useful in treatment of such tumors in combination with other targeted therapies and are currently testing formulas containing MEK inhibitors and other signal transduction inhibitors.

In summary, we provide data that MEK inhibitors induce anoikis sensitivity in human breast cancer cell lines with high levels of activated ERK. Preclinical assessment of targeted therapies has been suggested to require nontraditional approaches. Our results indicate that a simple growth inhibition assay on solid support may underestimate the efficacy of MEK inhibitors. We propose incorporation of anoikis assays in assessment of agents that target signaling molecules to help prevent overlooking unexpected activities.

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