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The Role of Multicellular Aggregation in the Survival of ErbB2-positive Breast Cancer Cells during Extracellular Matrix Detachment*

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Background: Cancer cells evade death caused by extracellular matrix (ECM)-detachment to facilitate metastasis.
Results: ErbB2-expressing cancer cells form aggregates during ECM-detachment that promote survival signaling through EGFR.
Conclusion: Multicellular aggregation in ErbB2 positive cancer cells promotes survival by preventing EGFR degradation.
Significance: Disrupting aggregation or inhibiting EGFR may be effective strategies to eliminate ErbB2-expressing cancer cells during ECM-detachment.

The metastasis of cancer cells from the site of the primary tumor to distant sites in the body represents the most deadly manifestation of cancer. In order for metastasis to occur, cancer cells need to evade anoikis, which is defined as apoptosis caused by loss of attachment to extracellular matrix (ECM). Signaling from ErbB2 has previously been linked to the evasion of anoikis in breast cancer cells but the precise molecular mechanisms by which ErbB2 blocks anoikis have yet to be unveiled. In this study, we have identified a novel mechanism by which anoikis is inhibited in ErbB2-expressing cells: multicellular aggregation during ECM-detachment. Our data demonstrate that disruption of aggregation in ErbB2-positive cells is sufficient to induce anoikis and that this anoikis inhibition is a result of aggregation-induced stabilization of EGFR and consequent ERK/MAPK survival signaling. Furthermore, these data suggest that ECM-detached ErbB2-expressing cells may be uniquely susceptible to targeted therapy against EGFR and that this sensitivity could be exploited for specific elimination of ECM-detached cancer cells.

The overwhelming majority of cancer-related deaths (90%) are a direct result of the metastasis of cancer cells from the primary tumor to distant sites (1–3). Metastasis is an inherently inefficient process as a significant percentage of cells that escape the primary tumor are not successful in colonizing secondary sites. It is currently understood that a significant contributor to this inefficiency is the induction of cell death, particularly in cells that lack attachment to the extracellular matrix (ECM)[4] (4). Caspase-dependent programmed cell death that is caused by ECM-detachment is known as anoikis, and anoikis resistance is an important factor in determining the success of cancer cells in navigating the metastatic cascade (5, 6). In addition to anoikis, recent studies have discovered multiple, distinct cellular alterations that can impact the survival of ECM-detached cancer cells in an anoikis-independent fashion, suggesting that cancer cells may need to utilize a multifaceted approach to survive in the absence of ECM-attachment (7–14).

The activation of oncogenic signaling in cancer cells is of paramount importance to developing anoikis resistance and to rectifying other cellular alterations that compromise cell viability in absence of ECM-attachment (9). In particular, overexpression of the ErbB2 oncogene has been linked to the survival of ECM-detached cells in a number of different contexts (12, 13, 15–17). While these studies have unveiled distinct mechanisms by which ErbB2 can promote anchorage-independent survival, it remains unclear if ErbB2 can promote survival through additional molecular mechanisms. Given the tremendous biological heterogeneity in ErbB2-positive breast tumors, it seems likely that the ability of ErbB2 to promote the survival of ECM-detached cells is not solely limited to the aforementioned studies (18).

Among the signaling pathways associated with ErbB2 that have yet to be investigated during the survival of ECM-detached cancer cells are those regulating cell-cell adhesion. Signaling from the ErbB2 receptor has been shown to impinge upon key molecules that determine the nature and efficacy of cell-cell contacts in a number of settings (19). In addition, circulating tumor cells (which lack normal attachment to ECM) have often been discovered as multicellular aggregates. This is particularly true in malignancies like inflammatory breast cancer and epithelial ovarian cancer (20, 21), which are cancers that

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[4] The abbreviations used are: ECM, extracellular matrix; DAPI, 4,6-diamidino-2-phenylindole; DNECAD, dominant-negative E-cadherin; EMT, epithelial-mesenchymal transition; MET, mesenchymal to epithelial transition; CTC, circulating tumor cell; MC, methylcellulose; EGFR, epidermal growth factor receptor.

are oftentimes driven by ErbB2-mediated signaling. Furthermore, the oncogene TrkB, which has been shown to stimulate survival signaling pathways (including those also downstream of ErbB2) in a fashion that blocks anoikis and promotes metastasis (22), can enhance multicellular aggregation during ECM-detachment (23).

These data have motivated us to examine the relationship between ErbB2 and multicellular aggregation during ECM-detached conditions. In this study, we have discovered that ErbB2-induced multicellular aggregation is critical to the inhibition of anoikis. Interestingly, we have found that this multicellular aggregation during ECM-detachment promotes ErbB2/EGFR-mediated activation of ERK/MAPK by preventing EGFR from being internalized and trafficked to the lysosome. Furthermore, our data suggest that ErbB2 and E-cadherin-expressing cancer cells may be uniquely susceptible to therapies antagonizing EGFR activity during ECM-detachment.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-10A cells (ATCC) and derivatives were cultured in Dulbecco's Modified Eagle Medium/F12 supplemented with 5% horse serum (Invitrogen), 20 ng/ml epidermal growth factor (EGF), 10 μg/ml insulin, 500 μg/ml hydrocortisone, 100 ng/ml cholera toxin, and 1% penicillin/streptomycin. BT474 cells (ATCC), and derivatives were cultured in RPMI-1460 medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin. SKBR3 cells (ATCC) were cultured in McCoy's 5A medium supplemented with 10% fetal bovine serum (Invitrogen) and 1% penicillin/streptomycin.

Reagents—1% weight to volume (*w*/*v*) methylcellulose was made by dissolving methylcellulose in line-specific medium. The following reagents were used at the doses indicated in the figure legends: U0126 (EMD Millipore), chloroquine diphosphate (Sigma-Aldrich), and Z-VAD-FMK (Apex Bio). Plates for detachment assays were made by coating with 6 mg/ml poly-(2-hydroxyethyl methacrylate) (poly-HEMA) as previously described (12).

Caspase Activity Assays—Caspase activity was measured after 48 h by the Caspase-Glo 3/7 Assay System according to the manufacturer's instructions (Promega). Cells were plated at a density of 13,333 cells per well in 96-well poly-HEMA-coated plates. Representative data from at least three biological replicates are shown.

Cell Viability Assays—Cellular viability was measured after 48 h using the Cell Titer Glo Assay according to the manufacturer's instructions (Promega). Cells were plated at a density of 13,333 cells per well in 96-well poly-HEMA coated plates. Representative data from at least three biological replicates are shown.

Ethidium Homodimer Assay—Ethidium homodimer (Life Technologies) assays were measured after 72 h with an excitation of 528 nm and an emission of 671 nm. Cells were plated at a density of 13,333 cells per well in 96-well poly-HEMA-coated plates. Representative data from at least three biological replicates are shown.

Western Blot Analysis—Cells were plated at a density of 400,000 cells per well in 6-well poly-HEMA-coated plates. After 48 h, images were acquired, and cells were harvested, washed twice with ice-cold PBS, and then lysed in 1% Nonidet P-40 supplemented with protease inhibitors (leupeptin (5 μg/ml), aprotinin (1 μg/ml), and PMSF (1 mM), and the Halt® Phosphatase Inhibitor Mixture (Thermo Scientific)). Lysates were collected after spinning at 14,000 rpm and normalized by BCA Assay (Pierce Biotechnology). Samples were subjected to polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (10). Representative data from at least three biological replicates are shown.

Measurement of Aggregate Size—Cells were plated at a density of 400,000 cells per well in 6-well poly-HEMA coated plates. After 48 h, images were acquired using a light box and camera and measured using the ImageJ analysis suite Fiji.

Antibodies—The following antibodies were used for Western blotting: Myc (Cell Signaling 2272), α-tubulin (Cell Signaling 2144), β-tubulin (Cell Signaling 2146) Total EGFR (Cell Signaling 4267), pEGFR (Y1173) (Cell Signaling 4407), Total Bad (Cell Signaling 9239), pBad S112 (Cell Signaling 5284), Bim (Cell Signaling 2933), Bcl-2 (Millipore 05–729), Mcl-1 (Millipore MABC43), Bcl-XL (Cell Signaling 2762), β-actin (Sigma A1978), pErk1&2 (pTpY$^{185/187}$) (Invitrogen 368800), Total Erk1&2 (Cell Signaling 9102), cytochrome *c* (BD Pharmigen 556433), E-cadherin (AbCam ab40772), and ErbB2 (Dako A0485). The following antibodies were used for immunofluorescence: Total EGFR (Cell Signaling 4267) and LAMP1 (BD Pharmigen 555798). E-cadherin (Invitrogen 135700) was used for E-cadherin engagement and reconstituted according to manufacturer's instructions.

Utilization of Retrovirus to Generate Stable Cell Lines—VSV-psuedotyped retroviruses were produced as previously described (12). MCF-10A cells were plated at 4×10^5 cells and infected with retrovirus. Stable populations of MCF-10A:ErbB2, MCF-10A:MEKDD, and MCF-10A:Bcl-2 were obtained by selection with 2 μg/ml puromycin (Invivogen). Stable populations of MCF-10A:DNECAD cells were obtained by selection with 10 μg/ml blasticidin (24).

Immunoprecipitation—Cells were plated at a density of 400,000 cells per well in 6-well poly-HEMA-coated plates. After 48 h, cells were harvested, washed twice with ice-cold PBS, and lysed in lysis buffer (1% Triton X-100, 50 mM NaCl, 1 mM EDTA, 20 mM HEPES) supplemented with leupeptin (5 μg/ml), aprotinin (1 μg/ml), PMSF (1 mM), and the Halt® Phosphatase Inhibitor Mixture (Thermo Scientific). Lysates were collected following a spin at 14,000 rpm and normalized by BCA Assay (Pierce Biotechnology). Samples were precleared with Protein A-Sepharose Fast Flow beads (GE Healthcare) for 1 h and treated with 1:50 ErbB2 antibody (Dako) for 48 h at 4 °C. Proteins were captured with Protein A-Sepharose Fast Flow beads blocked with 2% BSA (Millipore). Proteins were washed three times with wash buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Nonidet P-40, leupeptin (5 μg/ml), aprotinin (1 μg/ml), PMSF (1 mM), Halt Phosphatase Inhibitor Mixture)), eluted with SDS sample buffer, and analyzed by immunoblot. Representative data from at least three biological replicates are shown.

Cytochrome c Release Assay—Cytosolic cell extracts free of mitochondria were prepared as described previously (25).