Monoclonal Antibody against the Ectodomain of E-Cadherin (DECMA-1) Suppresses Breast Carcinogenesis: Involvement of the HER/PI3K/Akt/mTOR and IAP Pathways

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Abstract

**Purpose:** Although targeted therapies against HER2 have been one of the most successful therapeutic strategies for breast cancer, patients eventually developed acquired resistance from compensatory upregulation of alternate HERs and mitogen-activated protein kinase–phosphoinositide 3-kinase (PI3K)/Akt/mTOR signaling. As we and others have shown that the soluble ectodomain fragment of E-cadherin exerts prooncogenic effects via HER1/2–mediated binding and activation of downstream prosurvival pathways, we explored whether targeting this ectodomain [DECMA-1 monoclonal antibody (mAb)] was effective in the treatment of HER2-positive (HER2⁺) breast cancers.

**Experimental Design:** MMTV-PyMT transgenic mice and HER2⁺/E-cadherin–positive MCF-7 and BT474 trastuzumab-resistant (TtzmR) cells were treated with the DECMA-1 mAb. Antitumor responses were assessed by bromodeoxyuridine incorporation, apoptosis, and necrosis. The underlying intracellular prooncogenic pathways were explored using subcellular fractionation, immunoprecipitation, fluorescence microscopy, and immunoblotting.

**Results:** Treatment with DECMA-1 mAb significantly delayed tumor onset and attenuated tumor burden in MMTV-PyMT mice by reducing tumor cell proliferation and inducing apoptosis without any detectable cytotoxicity to mice or end-organs. In vitro treatment of MCF-7 and BT474 TtzmR cells reduced proliferation and induced cancer cell apoptosis. Importantly, this inhibition of breast tumorigenesis was due to concomitant downregulation, via ubiquitin-mediated degradation through the lysosome and proteasome pathways, of all HER family members, components of downstream PI3K/Akt/mTOR prosurvival signaling and suppression of inhibitor of apoptosis proteins.

**Conclusions:** Our results establish that the E-cadherin ectodomain-specific mAb DECMA-1 inhibits Ecad⁺/HER2⁺ breast cancers by hindering tumor growth and inducing apoptosis via downregulation of key oncogenic pathways involved in trastuzumab resistance, thereby establishing a novel therapeutic platform for the treatment of HER2⁺ breast cancers.

Introduction

Breast cancer, a heterogeneous disease with multiple subgroups and molecular signatures, remains the second leading cause of cancer-related deaths in women (1). One of the most successful strategies in the treatment of breast cancers involves the administration of monoclonal anti-bodies directed against epitopes of the human epidermal growth factor receptor (HER) family that are abundant on tumor cells. In this regard, trastuzumab (Herceptin), a humanized monoclonal antibody (mAb) against the extracellular domain of HER2 has revolutionized the care of HER2-positive (HER2⁺) cancers, an aggressive subtype representing 20% to 25% of breast cancers (1). However, despite encouraging clinical trials, targeted mAb therapies for HER2⁺ breast cancers have only had a modest efficacy due to the development of cancer cell resistance (1, 2).

Several key cell survival pathways have been suggested to contribute to cancer cell resistance, including signaling by other HER family members (HER1, 3, and 4), hyperactivation of the phosphoinositide 3-kinase (PI3K)/Akt and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signaling axis, and dysregulation of the inhibitor of apoptosis proteins (IAP; refs. 1, 2). It is well documented that trastuzumab efficiently blocks...
Translational Relevance

In the last decade, significant improvements in the prognosis of patients with HER2-positive (HER2⁺) breast cancer have been achieved using HER2-targeted therapies. However, despite encouraging results, the majority of patients develop acquired resistance due to extensive cross-talk and feedback loops associated with alternate HER family members and downstream MAPK–PI3K/Akt/mTOR pathways. Our current study shows in vivo and in vitro proof-of-concept that an antibody against the ectodomain of E-cadherin selectively induces apoptosis and reduces tumor growth by downregulating all HER family members, and components of the MAPK–PI3K/Akt/mTOR and inhibitor of apoptosis protein pathways, thereby successfully overcoming multiple resistance pathways. More importantly, we show that this occurs without any detectable cytotoxicity to mice, end-organs, or a panel of normal human epithelial or mesenchymal cells in culture. These findings provide a rationale for the development and clinical testing of antibodies directed against the ectodomain of E-cadherin that may, in combination with trastuzumab or other combination therapies, improve the survival of patients with HER2⁺ tumors.

HER2–HER2 homodimer signaling, but has little effect on HER1, HER3, or HER4 homodimers or heterodimers (1, 2). Along these lines, the trastuzumab-resistant (Tzmr) BT474 cell line exhibited higher levels of endogenous phospho-HER1 and HER1/HER2 heterodimers, the latter of which was not inhibited by trastuzumab treatment (3). This is consistent with clinical reports, in which patients with HER2-overexpressing tumors that became resistant to trastuzumab responded to the HER1/HER2 inhibitors lapatinib and HKI-272 (4, 5). HER3 contains 6 PI3K-binding sites, which make the HER2/HER2 heterodimers among the most potent activators of the PI3K/Akt pathway (6). Studies have shown that sustained MAPK and PI3K/Akt signaling is integral to trastuzumab-induced resistance (7). Notably, constitutive PI3K/Akt signaling prevented cell-cycle arrest and apoptosis mediated by trastuzumab, and BT474 Tzmr clones showed enhanced phospho-Akt and Akt kinase activity (8, 9). Not surprisingly, preclinical studies in HER2-amplified cell lines and xenograft models showed that the bispecific mAb pertuzumab, which blocks ligand-induced HER2/HER3 dimerization, effectively disrupted HER2–HER2 heterodimers, leading to inhibition of downstream MAPK and PI3K signaling and significant antitumor activity (9). Combination therapy with trastuzumab and pertuzumab also exhibited enhanced tumor activity in models of trastuzumab resistance, suggesting that both drugs have complimentary mechanisms of action (10). Interactions of the HER family, with other major gene families regulating cell survival, such as the IAPs also confer resistance to apoptosis in breast cancer cells (11, 12).

Constitutive overexpression of survivin, the smallest IAP family member downstream of PI3K, was shown to be indispensable for survival of HER2⁺ breast cancer cells that exhibited intrinsic cross-resistance to multiple HER1/2 inhibitors (13). Moreover, coexpression of HER1 and HER2 enhanced survivin levels, resulting in enhanced resistance to etoposide-induced apoptosis (11). Therefore, it is clear that HER2⁺ cancers may benefit from therapeutic approaches that simultaneously block multiple HER receptor family members.

Prior studies have shown a clear interaction between the HER receptor family and E-cadherin, a transmembrane protein that mediates calcium-dependent homophilic cell–cell adhesions. HER1 [EGF receptor (EGFR)] activation was shown to disrupt adherens junctions and chronic HER1 stimulation enhanced the E-cadherin transcriptional repressors, TWIST and Snail (14, 15). Conversely, in lung and breast cancer cell lines, inhibition of HER1 signaling increased intact E-cadherin and restored adherens junctions (16, 17), suggesting an inverse correlation between E-cadherin and HER family members. Recent studies in our laboratory, as well as others, also show a direct interaction between the shed E-cadherin ectodomain fragment, sEcad, and the HER receptors (18, 19). sEcad is derived from the proteolytic cleavage of the extracellular domain of E-cadherin, which contains 5 subdomains, termed EC1-5 (20). This ectodomain shedding, mediated by matrix metalloproteinases (MMP) or members of the ADAMs (a disintegrin and metalloproteinases), leads to the release of an 80-kDa sEcad fragment into the extracellular space (21). sEcad is constitutively shed at low levels in normal epithelial cells but is significantly elevated in breast cancer patients and correlates with tumor size, clinical response to chemotherapy, and predicts a shorter disease-free interval (22). In prostate, skin, and ovarian cancer cells, addition of a purified 80-kDa sEcad fragment or recombinant sEcad chimERIC protein (rhEcad/Fc) destabilized adherens junctions, enhanced tumor cell proliferation, migration, and invasion (18, 19, 21, 23, 24). This sEcad-induced invasion was shown to be MMP-2-, MMP-9-, and/or MT1-MMP–dependent in lung and skin cancer cells (18, 25). Although the mechanisms for these prooncogenic effects have yet to be elucidated, we recently showed endogenous and exogenous sEcad binding to HER1, HER2, and the insulin-like growth factor-1 receptor (IGF-IR) in human skin squamous cell carcinoma (SCC) specimens and skin cancer cells (18). Similarly, Najy and colleagues showed endogenous sEcad binding to HER2 and HER3 in E-cadherin–positive MCF-7 breast cancer cells, and exogenous sEcad associating with HER2 in E-cadherin–negative SKBR3 cells (19). The latter resulted in HER2–HER3 heterodimerization, HER3 phosphorylation, and ERK activation. Consistent with these results, exogenous sEcad promoted cell survival and acted as a potent antiapoptotic protein, via HER1, Akt, and ERK signaling in serum-starved transformed Madin–Darby canine kidney (MDCK) cells and in polarized epithelial acini (26). Furthermore, exogenous sEcad induced the phosphorylation of HER1, HER2, and IGF-IR in PAM212...
and CC4A skin cancer cells, and depending on the cell-type, resulted in the activation of downstream MAPK–PI3K/mTOR signaling (18). Therefore, although an inverse relationship exists between intact E-cadherin and HERs, it is clear that the shed ectodomain sEcad fragment binds to HER family members and nurtures the growth and survival of various cancers, showing a potential important role of targeting sEcad and sEcad-HER receptor interactions in breast carcinogenesis.

In this study, we set out to explore whether a commercially available mAb (DECMAl-1) that specifically targets epitopes within the extracellular domain of E-cadherin, is able to suppress breast carcinogenesis in vivo and in vitro. We show that DECMAl-1 mAb treatment exhibits potent anti-cancer activity and effectively downregulates HER1-4 family members and multiple downstream resistance pathways, without untoward cytotoxicity to normal cells, tissues or mice. Our results suggest that antibodies targeting specific extracellular domains of E-cadherin may provide a novel therapeutic platform for patients with HER2+ breast cancer that develop de novo or acquired resistance.

Materials and Methods

Animal studies and in vivo treatments

MMTV-PyMT mouse breeders were obtained from The Jackson Laboratory and mated according to the vendor’s specifications. When mice reached 47 days of age, virgin female mice were randomly assigned to different groups (n = 5, each group) to receive weekly intraperitoneal injections of 1 mg/kg DECMAl-1 (US885; Sigma; custom order), rat immunoglobulin G1 (IgG1; 0116-14; Southern Biotech; custom order), or equivalent volumes of saline (200 μL). Palpable tumors were monitored twice weekly from 40 days until sacrifice at 90 days of age. At the end of the protocol, mice were given 2 mg/mouse of bromodeoxyuridine (BrdUrd) by intraperitoneal injection 2 hours before sacrifice and organs were collected and mammary tumors were excised, weighed and either formalin-fixed or flash-frozen at −20°C until further use.

Patients and tissues

HER2+ human breast cancer specimens were obtained from the National Cancer Institute (NCI) Cooperative Human Tissue Network (CHTN) and from Proteogenex. Tumors from CHTN included 7 HER2+-infiltrating ductal carcinomas with an age range of 52 to 77 years and corresponding adjacent normal breast tissues or normals from reduction mammoplasty tissues (n = 7).

Cells and reagents

Human MCF-7, BT474-parental, MCF-10A, primary epidermal keratinocytes (PHK), and primary epidermal melanocytes (Mel) were purchased from American Type Culture Collection (ATCC), and cultured according to ATCC recommendations. TzimR BT474 cells were generously provided by Dr. Kute (Wake Forest University, Winston-Salem, NC) and grown in 10% FBS RPMI-1640 medium (ATCC). Primary antibodies used in this study were as follows: for immunoprecipitation, EGFR (Ab-15) and HER2 (Ab-17; Thermo Fisher Scientific); for Western blot analysis and immunostaining, EGFR (4267), HER2 (2165), HER3 (4754), HER4 (4795), pPI3K (4228), pAkt (4060), Akt (4691), pERK1/2 (4370), ERK1/2 (5695), pmTOR (5536), mTOR (2983), pRaptor (2083), p4E-BP1 (2855), pp70S6K (9234), and survivin (2808; Cell Signaling Technology); E-cadherin (H-108), β-actin (C4) and p53 (c-11 and FL-393; Santa Cruz Biotechnology); G3PDH (AM4300; Ambion), ubiquitin (13-1600; Zymed); E-cadherin C-terminus (clone 36/E-cadherin; BD Transduction Laboratories). For in vitro studies of MCF-7 cells, DECMA-1 (mAb, U3254; Sigma) and IgG (0116-14; Southern Biotech; equivalent sodium azide) were used, respectively. For parental BT474 in vitro cultures, DECMA-1 (US885; Sigma; custom order) and IgG (0116-14; Southern Biotech; custom order) were used.

Immunoprecipitation, subcellular fractionation, and Western blotting

Protein extraction in cells was conducted on ice using total protein extraction buffer: 20 mmol/L Tris pH 7.5, 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% NP40, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), and protease inhibitor cocktail (Sigma). Protein concentration was measured using a BCA Protein Assay Kit (Pierce). Protein samples (10–100 μg) were denatured at 95°C (unless otherwise stated) and subsequently separated by 4% to 15% SDS-PAGE. After transfer to nitrocellulose membrane and blocking with 5% nonfat milk, samples were probed with primary antibodies. Western blot analysis images were captured using HP Scanjet G4050 and analyzed relative to G3PDH or actin using NIH Scion Image. Subcellular fractionation was conducted using the BioVision FractionPREP Cell Fractionation System (BioVision Inc.), as per the manufacturer’s instructions. IAP family members, in the presence or absence of DECMA-1, were analyzed using a Human Apoptosis Array kit (RD Systems). Immunoprecipitation assays were conducted by harvesting cells with immunoprecipitation lysis buffer (20 mmol/L Tris–HCl pH 7.5, 137 mmol/L NaCl, 100 mmol/L NaF, 10% glycerol, 1% Nonidet P-40, 1 mmol/L PMSF, and protease inhibitor cocktail; Sigma). After brief sonication, lysates were cleared by centrifugation at 4°C. Supernatants were precleared and incubated with EGFR/HER1 (Ab-15) or HER2 (Ab-17)–specific antibodies for 4 hours and protein A/G plus agarose beads (Santa Cruz Biotechnology; sc-2003) for 2 hours at 4°C. The immunocomplexes were washed 3 times, boiled in sample buffer [60 mmol/L Tris–Cl pH 6.8, 2% SDS (v/v), 10% glycerol (v/v), 5% β-mercaptoethanol (v/v), and 0.01% bromophenol blue (v/v)], and loaded on SDS-PAGE for protein analysis.

Assessment of apoptosis, cell number, and proliferation

Cellular apoptosis was quantitatively determined using the Cell Death Apoptosis Detection ELISA Plus Kit
(Roche), which is based on the detection of DNA–histone complexes in the form of mono- and oligo-nucleosomes, according to the manufacturer’s instructions. Briefly, cells were treated in the presence or absence of various concentrations of DECMA-1 or rat IgG1 for 24 to 48 hours. Cells were harvested by trypsination, counted, and lysates were subjected to the ELISA. In vitro and in vivo apoptosis was further analyzed by in situ detection of fragmented DNA, using the DeadEnd Fluorometric TUNEL System (Promega), the ApoTag Peroxidase In situ Apoptosis Detection Kit (Millipore), and the human Apoptosis Array Kit (ARY009; R&D Systems), according to the manufacturer’s instructions. The ApoTag Kit was conducted on deparaffinized 5-μm thick sections treated with proteinase K (20 μg/mL) at room temperature. For cell counting, the cells were trypsined and counted on a hemocytometer. For tumor BrdUrd analyses, tissues were stained using an anti-BrdUrd (ab2284; Abcam) antibody, as previously described (27). The proliferation of cells was monitored by a cell proliferation ELISA BlrdUrd (colorimetric) kit (Roche), according to the manufacturers protocol.

Immunofluorescence

Cells cultured on chamber slides (no. 177437; Nalge Nunc International) were fixed with 4% formaldehyde, blocked in PBS containing 1% (w/v) bovine serum albumin (BSA), and incubated in p53 (Santa Cruz Biotechnology) or HER1-4 antibodies (4267, 2165, 4754, 4795; Cell Signaling Technology). Formalin-fixed and paraffin-embedded MMTV-PyMT mouse breast tumors and human breast tumor sections were incubated with antigen retrieval reagent (Dako), and then blocked in 1% BSA PBS and probed with DECMA-1 (U5885; Sigma), as previously described (18, 27).

Histologic analysis and immunohistochemistry

Paraffin-embedded tumors were fixed, sectioned at 5 μm, deparaffinized and stained with H&E according to standard protocols. H&E-stained slides were evaluated by a board-certified pathologist (C. Tornos, Stony Brook University, Stony Brook, NY). For immunohistochemical analyses, tissues were stained using anti-HER1, HER2, HER3 HER4, p53, ERK1/2, Akt, mTOR, and survivin (Cell Signaling Technology) antibodies, as previously described (18, 27).

Statistical analysis

Comparisons between groups were made using ANOVA followed by post hoc analysis using the Student–Newman–Keuls or Dunnett’s method unless otherwise stated. Statistical significance is indicated in figures as *, P < 0.05; **, P < 0.01; or ***, P < 0.001.

Study approval

This study was conducted in accordance with NIH guidelines for the use of experimental animals. Protocols were approved by the Institutional Animal Care and Use Committee and the use of human tissues was according to the Institutional Review Board at Stony Brook University (Stony Brook, NY).

Results

Targeted mAb therapy against the ectodomain of E-cadherin (DECMA-1) suppresses breast tumorigenesis in MMTV-PyMT transgenic mice by inhibiting tumor growth and inducing apoptosis

The in vivo efficacy of DECMA-1 was tested in the HER2+ MMTV-PyMT transgenic mouse model of breast cancer, in which mammary-targeted overexpression of the polyomavirus middle T antigen (PyMT) leads to the rapid development of palpable tumors that progress to aggressive adenocarcinomas with metastasis to the lungs that are similar to human ductal carcinomas of the breast (28). Mice were treated intraperitoneally once weekly with saline, IgG, or DECMA-1 (1 mg/kg in 200 μL saline) beginning at 47 days of age (just before the development of carcinomas in our study) until 90 days of age when the mice were sacrificed. Treatment with the DECMA-1 mAb significantly delayed tumor onset and tumor numbers compared with saline or IgG control mice (Fig. 1A and B). In these tumors that developed, pathologic analysis by H&E staining of histologic sections revealed that control-, IgG-, and mAb-treated tumors were poorly differentiated (tumor grade of 3, architectural grade of 3, and nuclear grade of 3), except in 1 of 5 DECMA-1–treated mice in which the tumor was moderately differentiated (tumor grade 2, architectural grade 2, and nuclear grade 1; Fig. 1B).

Immunohistochemical analyses of these tumors were then conducted, so as to determine the potential mechanisms for the decreased tumorigenesis after DECMA-1 mAb therapy. Consistent with decreased tumor burden, there was a statistically significant decrease in BrdUrd staining in mAb-treated tumors versus controls (Fig. 1C). In contrast, apoptosis was significantly increased in tumors from mice treated with DECMA-1 mAb compared with all control groups as measured by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL) analyses (Fig. 1D). Taken together, these data show that targeted mAb therapy against the membrane proximal region of the ectodomain of E-cadherin significantly delayed tumor growth and tumor burden by reducing proliferation, but more importantly by inducing tumor cell death.

Anti-E-cadherin ectodomain-specific mAb inhibits cell proliferation, induces cell death, and upregulates wild-type p53 expression in vitro and in vivo

Because DECMA-1 mAb treatment suppressed breast carcinogenesis in vivo by decreasing MMTV-PyMT tumor growth and inducing apoptosis, we next tested whether this treatment would induce similar effects on MCF-7 and the E-cadherin/HER2+ BT474 TtzmR cell lines that express varying levels of HER2 amplification. DECMA-1–treated MCF-7 and TtzmR cells exhibited a significant decrease in cell numbers (Fig. 2A) and cellular proliferation (Fig. 2B) compared with control groups. Blebbing of the cellular
membrane began to appear after incubation with the mAb for 24 to 48 hours and resulted in approximately 70% loss of attached cells (Fig. 2C), suggesting induction of programmed cell death and/or necrosis. ELISA quantification of histone-associated DNA fragments confirmed the phase contrast images and showed a significant DECMA-1 mAb-mediated induction of apoptosis 48 hours following treatment (Fig. 2D), but no effects on necrosis (data not shown). As p53 activation is important in the induction of apoptosis, we next investigated whether the effects of DECMA-1 treatment involved p53. The intensity of p53 immunohistochemical staining and Western blotting in tumors from DECMA-1–treated MMTV-PyMT mice was dramatically increased compared with saline- or IgG–treated mice (Fig. 3A). Interestingly, DECMA-1 also remarkably increased p53 expression in MCF-7 cells as shown by immunoblotting and immunofluorescence staining (Fig. 3B and C), whereas it decreased the mutant p53 expression in BT474 TtzmR cells (Fig. 3B). Because one potential mechanism for these DECMA-1–induced effects seemed to be through alteration of p53 expression, we next determined the effect of DECMA-1 on apoptosis in both wild-type p53 (MCF-7) and mutant p53 (parental BT474) cell lines. Our results show that DECMA-1 treatment significantly increased apoptosis, regardless of p53 mutation status (Fig. 3D).

**Ectodomain-specific E-cadherin mAb downregulates HER signaling pathways and IAP family members**

Because the HER family of receptors are integral to the growth and progression of mammary cancers, we next used...
the MMTV-PyMT breast cancer mouse model and the E-cadherin/HER2+ MCF-7 cell line to assess the effects of DECMA-1 on HER receptor regulation, as well as effects on other downstream cancer cell resistance pathways, including PI3K/Akt/mTOR, MEK/ERK, and the IAPs. First, to confirm that DECMA-1 colocalizes with HER2, the preferred dimerization partner for the HER family of receptors, we conducted double-label immunofluorescence staining on MMTV-DECMA-1–treated tumor specimens. Punctate HER2 immunostaining was visible in the cytoplasm of cells and colocalized with DECMA-1, suggesting interaction and internalization (Fig. 4A). Immunohistochemical and Western blot analyses of HER1 and HER2 expression levels in resected tumors exhibited decreased HER1/2 immunostaining and decreased HER1/2 levels compared with control or IgG-treated mice (Fig. 4B). Moreover, in DECMA-1–treated mice, resected tumors exhibited a decrease in ERK1/2, Akt, and mTOR immunostaining and a decrease in the expression levels of pMEK1/2, pPI3K, and p4E-BP1, but not pp70S6K (Fig. 4C and D). As HER receptor signaling involves IAP family members (11, 12), we next evaluated the expression level of the IAP family member survivin in the resected tumors by immunohistochemistry and Western blotting. In saline- and IgG-treated mice, survivin immunostaining was predominately nuclear, whereas in DECMA-1–treated mice a clear reduction in survivin immunoreactivity was noted.
Degradation of HER receptors by ectodomain-specific E-cadherin mAb DECMA-1

As endocytic trafficking and ubiquitin-mediated degradation of cell surface receptors potentially provides an important mechanism of HER receptor regulation (29), we next assessed whether DECMA-1 decreased HER expression levels by inducing their internalization and degradation. HER1–4 receptor immunofluorescence in control MCF-7 cells showed predominantly plasma membrane localization, whereas mAb treatment induced HER receptor downregulation and aggregation into intracellular vesicular structures (Fig. 5A). In addition, using equal immunoprecipitation (IP) products for HER1 and HER2 (minimal protein was recovered from DECMA-1–treated...
lysosome and ubiquitin–proteasome pathways play an important role in the ectodomain-specific E-cadherin mAb-induced HER receptor degradation and apoptosis in E-cadherin/HER2+ breast cancer cells.

DECA-1 binds to human and mouse mammary tumors but does not exert toxicity to normal tissues and cells

Because skin toxicity is a common drug-related adverse event observed in patients with cancer treated with HER-directed mAb therapies (30), we next evaluated the binding specificity of different extracellular-domain–specific antibodies to intact E-cadherin or the shed E-cadherin ectodomain, on normal human skin specimens lysed in PBS under nonreduced and reduced conditions by Western blotting. In normal human skin tissues, lysed in PBS under either reduced or nonreduced conditions, DECA-1 predominately bound to sEcad with minimal binding to FL-Ecad (Fig. 6A). In contrast, an antibody against the C-terminal region of E-cadherin mainly recognized FL-Ecad, whereas SHE78-7 and HEC1-1, which were generated against EC1 and EC2, respectively, bound both FL-Ecad and sEcad by immunoblotting (Fig. 6A). To determine whether DECA-1 binds intact E-cadherin and/or the shed soluble approximately 80-kDa ectodomain fragment in tumors, we next evaluated binding levels of DECA-1 in human and mouse breast cancer specimens lysed in PBS by immunoblotting. In both human and mouse specimens, DECA-1 bound both intact E-cadherin (120 kDa) and the soluble shed fragment (80 kDa), albeit at much higher levels in the latter (Fig. 6B and C). Furthermore, immunofluorescence microscopy of Triton-X–treated tissue sections showed both membrane and cytosolic staining for DECA-1, confirming the immunoblot results (Fig. 6B and C). In our in vitro studies, TUNEL analyses in both normal human breast cells (MCF-10A) and cancer cells (MCF-7) showed an increase in apoptosis in breast cancer cells, but no change in apoptosis in normal mammary cells (Fig. 6D). Moreover, DECA-1 treatment of confluent human normal mammary epithelial cells (E-cadherin–positive; MCF-10A; Mam), fibroblasts (E-cadherin–negative; Fib), primary human epidermal keratinocytes (E-cadherin–positive; PHK), and melanocytes (E-cadherin–positive; Mel) also exhibited no significant induction of apoptosis compared with IgG controls as assessed by a Cell Death ELISA Assay (Fig. 6E). Because rapidly dividing cells of the intestine have been shown to be significantly impacted in conditional E-cadherin knockout models, and gastrointestinal side effects are common with HER-targeted therapies (31, 32), we next verified that the intestinal architecture is not altered upon prolonged DECA-1 treatment. In accordance with these in vitro findings, DECA-1–treated MMTV-PyMT mice exhibited no overt signs of toxicity (i.e., grooming, vocalization, hunched posture, or change in appetite or body weight) and no cytotoxicity was observed in excised intestine (Fig. 6F) or in heart, liver, lung, and kidney sections by H&E analysis (data not shown). Collectively, these data show that DECA-1 binds predominately to the approximately

**Figure 5.** HER degradation induced by DECA-1 treatment is dependent on the proteasome, lysosome, and ubiquitin pathways. A, immunofluorescence images showing HER1–4 staining in MCF-7 cells in the presence or absence of DECA-1 (mAb). The arrows indicate staining of HER1–4 in vesicular structures. B, membrane and cytosolic HER1 and HER2 fractions in the presence or absence of DECA-1 (mAb). The arrows indicate staining of intact HER1 and HER2, respectively. C, analysis of apoptosis in MCF-7 cells treated with DECA-1 (mAb); 20 μg/mL in the presence or absence of 10 μmol/L chloroquine (CQ) or 2.5 μmol/L of acetyl-leu-leu-norleu-al (ALLN). ***P < 0.001 versus no mAb, chloroquine, or ALLN group. ***, P < 0.001 versus mAb alone group, n = 3. D, 10 μmol/L chloroquine or 2.5 μmol/L ALLN rescue HER1–4 expression levels following mAb treatment. Scale bar, 200 μm.
80-kDa ectodomain of E-cadherin in human and mouse tumors and normal tissues, and produces no untoward toxic effects on normal cells, tissues or mice.

Discussion

Targeted monoclonal antibodies have become attractive therapeutic drug candidates due to their potential for tumor-specific targeting and low toxicity profiles. Accordingly, trastuzumab, a recombinant humanized mAb against the extracellular domain of HER2, was approved by the U.S. Food and Drug Administration (FDA) for the treatment of patients with HER2 metastatic breast cancers. However, a large percentage of patients who are initially responsive to HER-targeted therapies experience tumor recurrence and
become refractory to therapy (1, 2, 3). Extensive cross-talk between multiple HER receptors and downstream key survival-signaling pathways, have been suggested to contribute to this drug resistance (1, 2, 3). Accordingly, bispecific antibodies that simultaneously neutralize HER2 and HER3, or combinations of targeted mAb that inhibit HER1 and HER2 alone or target these receptor tyrosine kinases in combination with downstream MAPK or PI3K/Akt/mTOR inhibitors are currently being actively pursued in clinical trials (33, 34).

The tumor microenvironment is enriched with factors that nurture the growth and survival of tumors, and as such, is an indispensable player in malignant growth. Contained within this functional space are abundant proteases that facilitate the proteolytic cleavage of membrane proteins into bioactive soluble ectodomain fragments, such as sEcad. The sEcad fragment is increased in the serum of patients with cancer (35), and acts in a paracrine or autocrine manner to stimulate tumor cell growth and survival (18, 26). Accordingly, levels of sEcad in patients with breast cancer correlates with tumor size, response to chemotherapy, and predict a shorter disease-free interval (22). Reports further show elevated sEcad expression levels in primary gastric and prostate tumors, skin cancers, and metastatic foci (18, 36, 37). This distinct prooncogenic property of sEcad is further supported by studies in ovarian, skin, prostate, and transformed MDCK cancer cell lines, whereby recombinant sEcad disrupted E-cadherin–mediated cell–cell adhesions and enhanced migration and invasion, whereas immunodepletion of sEcad from the conditioned media reversed these effects (18, 21, 23, 24). Although the mechanisms of action of sEcad are not well understood, both HER2 overexpression and enhanced sEcad predict for a poor clinical outcome in breast cancer, making it tempting to speculate that the 2 interact. Along these lines, we and others have shown that sEcad binds to HER family members to increase tumor growth and cell survival (18, 26). Specifically, we have shown that endogenous and exogenous sEcad binds to HER1, HER2, and IGF-IR in human skin SCC specimens and mouse SCC cell lines (18). Similarly, Nayi and colleagues showed endogenous sEcad binding preferentially to HER2 and HER3, enhancing HER2–HER3 heterodimerization, HER3 phosphorylation, and activating downstream ERK1/2 in MCF-7 breast cancer cells (19). In normal MDCK cells, exogenous sEcad promoted cell survival via activation of HER1, PI3K, Akt, and ERK1/2 signaling (26). Taken together, the abundance of sEcad in tumor tissues and bodily fluids, together with its prooncogenic properties, HER binding and correlation with disease-free survival and therapeutic response, makes sEcad a novel candidate protein for drug targeting.

In this study, we evaluated the feasibility and efficacy of targeting sEcad in HER2+ breast cancers by using a commercially available mAb against the ectodomain of E-cadherin, DECMA-1, which may equally bind cell surface bound intact E-cadherin, sEcad, or potentially sEcad–HER interactions. Here, we show that DECMA-1 mAb therapy, significantly delayed tumor onset and tumor burden in MMTV-PyMT mice by retarding cancer cell growth and inducing apoptosis selectively in cancer cells without toward cytotoxicity toward normal cells and tissues. These results were validated in cell culture studies, wherein DECMA-1 treatment in E-cadherin–HER2+ MCF-7 and BT474 Tzmr cells inhibited breast cancer proliferation and induced apoptosis. These results are in line with prior publications showing that DECMA-1 significantly reduced cell numbers, inhibited proliferation, and induced apoptosis in PAM212 SCCs (38, 39). Moreover, DECMA-1 was shown to increase apoptotic cell death in MCF-7 cells and decrease Bcl-2 gene transcription (40). Others reported a significant inhibition of oral SCC multicellular aggregate proliferation, induction of apoptosis, and decreased Bcl-2 expression after administration of SHE78-7, a commercially available antibody against the EC1 domain of E-cadherin (41). Potentiation of TPA-induced apoptosis by SHE78-7 was further documented in LNCaP prostate and SUM185 breast cancer cells (42) and intraperitoneal administration of SHE78-7 was shown to significantly prolong survival and prevent peritoneal bleeding (indicative of advanced disease) compared with controls in an HT29 colorectal xenograft model (43). Finally, Tomlinson and colleagues showed that 5-day injections of HEC-D1 (EC2 domain mAb) resulted in a decrease in both the size and number of pulmonary lymphovascular emboli using a MARY-X human inflammatory breast carcinoma xenograft model (44).

Because the tumor suppressor p53 is well known to activate apoptosis, and disruption of this process can promote tumor progression and chemoresistance, we next sought to determine if these DECMA-1–induced effects were p53 mediated. Interestingly, in MMTV-PyMT tumors and HER2+ MCF-7 cells (wild-type p53), DECMA-1 treatment significantly increased wild-type p53 expression, but in BT474 Tzmr cells decreased mutant p53 levels. Importantly, one of the gain-of-function effects of mutant p53 is apoptotic resistance to proapoptotic stimuli, including growth factors deprivation, γ-irradiation, and anticancer agents (45). Accordingly, Mirzayans and colleagues showed that siRNA-mediated knockdown of mutant p53 in BT474 cells significantly increased cell death via apoptosis (46), which is consistent with our findings. Taken together, alteration of p53 expression is likely involved in DECMA-1–induced apoptosis, yet whether the latter is p53-dependendent needs further study.

To gain a better understanding of the putative mechanisms by which DECMA-1 administration suppressed the development of mammary carcinomas, we next examined whether DECMA-1 modulated the HER family of receptors. Importantly, the most intriguing and clinically relevant finding of this study is the demonstration that DECMA-1 colocalized with HER2 in MMTV-PyMT mAb-treated tumors and downregulated many of the compensatory pathways that trastuzumab-treated cells eventually develop so as to sustain cell proliferation and enhance survival. That is, one of the most important strategies to improve the
efficacy of HER-targeted therapies is to inhibit multiple HER receptors, thereby interfering with the horizontal cooperatively that exists between these receptors that currently limit the success of agents that target individual receptors. Specifically, simultaneous use of trastuzumab, pertuzumab, and the HER tyrosine kinase inhibitor gefitinib inhibited HER2-overexpressing xenografts more effectively than any of these drugs used as a single agent or in dual combinations (47). Here, our mechanistic studies reveal a previously undiscovered pathway, wherein DECMA-1 mAb treatment downregulated all HER1–4 family members, with the HER1 and HER2 family members being endocytosed and degraded by the ubiquitin–proteasome and lysosome pathways. However, downregulation of all HER family members may not be sufficient, as acquired drug resistance can still occur through reactivation of downstream prosurvival signaling pathways.

The PI3K/Akt/mTOR and MAPK pathways, along with IAP family members, are highly dysregulated in breast cancer (2), making them well-validated targets for cancer treatment. However, with extensive cross-talk and many feedback loops, results of single-agent inhibitors have only had modest effects. This was shown by Serra and colleagues, who showed that sole PI3K targeting in HER2-overexpressing breast cancers activated alternate compensatory pathways resulting in ERK dependency (48). Moreover, Carracedo and colleagues, showed that inhibition of mTORC1, using rapamycin, resulted in MAPK activation through a PI3K feedback loop in prostate cancer (49). Here, we show that DECMA-1 mAb treatment suppressed many of the complex and redundant downstream pathways involved in trastuzumab resistance. Specifically, membrane fractionation of mAb-treated MCF-7 cells resulted in the downregulation of ERK1/2 as well as Akt, mTOR, the mTOR-binding protein Raptor and the mTOR substrate 4E-BP1. Consistent with the in vitro findings, PI3K/Akt/mTOR signaling was also significantly reduced in tumors from mAb-treated MMTV-PyMT mice. Recent studies also show that IAPs are indispensable for survival of HER2− breast cancer cells. Xia and colleagues showed that the HER1/HER2 inhibitor, lapatinib, markedly reduced survivin expression and induced apoptosis through its inhibition of PI3K signaling in HER2-overexpressing BT474 cells (12). Similarly, HER2+ tumors from patients treated with lapatinib exhibited a marked inhibition of survivin expression levels (11, 12). In contrast, trastuzumab had little effect on steady-state survivin levels in trastuzumab-sensitive BT474 cells, but in the HER2− Tzmr SIM190PT cell line, it induced the upregulation of both survivin and XIAP (12, 50). In this study, we further show that DECMA-1 therapy downregulated the inhibitor of apoptosis protein family members survivin, XIAP, and c-IAP-1, many of which are essential for tumor cell survival and are also upregulated in Tzmr breast tumors (12).

Because targeted mAb based therapies, are not without untoward side effects and E-cadherin forms homotypic cell–cell adhesions in normal epithelial cells, we next evaluated DECMA-1 binding in human and mouse breast tumor specimens versus potential off-target binding in normal tissues. Importantly, Tsuiji and colleagues showed that the EC3–EC5 domains of E-cadherin are much less accessible to mAb binding than EC1–EC2, and suggest that this masking may be due to the conformational state of cadherins or by other moieties (51). In the current study, we show that DECMA-1 binds predominately to the approximately 80-kDa ectodomain fragment in human breast tumor specimens and both intact E-cadherin and the 80-kDa protein in mouse MMTV-PyMT tumors. However, in nonreduced and reduced normal human skin tissue lysates (homogenized in PBS), DECMA-1 predominaently bound the ectodomain fragment. This contrasted to the EC1 and EC2 antibodies, SHE78-7 and HECD-1, which bound both intact E-cadherin and the ectodomain fragment versus the cytosolic E-cadherin antibody, which only bound the former. It would be tempting to speculate that the monomeric conformation of E-cadherin in tumor cells, versus the homodimeric conformation of E-cadherin in normal cells, may potentially unmask the epitopes necessary for this tumor-specific DECMA-1 binding. This lack of DECMA-1–induced cytotoxicity was further confirmed in an array of normal human cells. Moreover, in MMTV-PyMT mice there were no apparent signs of distress and histopathologic analysis of end-organs, including heart, liver, kidney, and intestine showed no morphologic differences between treated mice and healthy controls.

Breast cancer is a heterogeneous disease, wherein multiple resistance mechanisms may coexist in each individual patient with late-stage metastatic disease. Therefore, we propose that in addition to targeting each of the HER family members and/or MAPK, PI3K/Akt/mTOR, or IAP pathway alterations individually or in combination, researchers may effectively overcome resistance by further administration of a targeted mAb against the ectodomain of E-cadherin. Our findings suggest that this mAb regimen, together with existing therapies, may have a more significant impact in delaying or preventing further progression, resistance, and impending metastatic progression for patients with E-cadherin−positive yet invasive HER2+ breast cancers without untoward cytotoxicity to normal tissues and cells.

Disclosure of Potential Conflicts of Interest
R. Clarke has honoraria from Speakers Bureau of SigmaXi Distinguished Lecturer. No potential conflicts of interest were disclosed by the other authors.

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Grant Support
This work was supported by a Komen Career Catalyst Grant KG081308 (to S.M. Brouxhon).

References


Monoclonal Antibody against the Ectodomain of E-Cadherin (DECMA-1) Suppresses Breast Carcinogenesis: Involvement of the HER/PI3K/Akt/mTOR and IAP Pathways

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