Nonclinical Studies Addressing the Mechanism of Action of Trastuzumab (Herceptin)

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HER2 is a ligand-less member of the human epidermal growth factor receptor or ErbB family of tyrosine kinases. In normal biological systems, HER2 functions as a co-receptor for a multitude of epidermal growth factor-like ligands that bind and activate other HER family members. HER2 overexpression is observed in a number of human adenocarcinomas and results in constitutive HER2 activation. Specific targeting of these tumors can be accomplished with antibodies directed against the extracellular domain of the HER2 protein. One of these antibodies, 4D5, has been fully humanized and is termed trastuzumab (Herceptin; Genentech, San Francisco, CA). Treatment of HER2-overexpressing breast cancer cell lines with trastuzumab results in induction of $p27^{KIP1}$ and the Rb-related protein, p130, which in turn significantly reduces the number of cells undergoing S-phase. A number of other phenotypic changes are observed in vitro as a consequence of trastuzumab binding to HER2-overexpressing cells. These phenotypic changes include downmodulation of the HER2 receptor, inhibition of tumor cell growth, reversed cytokine resistance, restored E-cadherin expression levels, and reduced vascular endothelial growth factor production. Interaction of trastuzumab with the human immune system via its human immunoglobulin G_1 Fc domain may potentiate its antitumor activities. In vitro studies demonstrate that trastuzumab is very effective in mediating antibody-dependent cell-mediated cytotoxicity against HER2-overexpressing tumor targets. Trastuzumab treatment of mouse xenograft models results in marked suppression of tumor growth. When given in combination with standard cytotoxic chemotherapeutic agents, trastuzumab treatment generally results in statistically superior antitumor efficacy compared with either agent given alone. Taken together, these studies suggest that the mechanism of action of trastuzumab includes antagonizing the constitutive growth-signaling properties of the HER2 system, enlisting immune cells to attack and kill the tumor target, and augmenting chemotherapy-induced cytotoxicity.

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THE EPIDERMAL growth factor (also known as HER or ErbB) family of receptor tyrosine kinases are important mediators of cell growth, differentiation, and survival.[1,2] As shown in Fig 1, the receptor family is composed of four distinct members, including epidermal growth factor receptor (EGFR; HER1 or ErbB1), HER2 (*neu* or ErbB2), HER3 (ErbB3), and HER4 (tyro2 or ErbB4). Within a given tissue, these receptors are rarely, if ever, expressed alone, but are found in various combinations.[3] At present there are nine ligands that bind directly to EGFR, HER3, or HER4. HER2 also becomes activated as a result of ligand binding to other HER family members. Furthermore, blocking the association of HER2 with these receptors inhibits ligand-mediated signaling.[4-8] Thus, HER2 is an essential component of these receptor complexes, although no ligand has been characterized at the molecular level that interacts with HER2 alone.

Recently, the HER receptors have been studied either alone or in specific combinations.[9-11] Ligand-receptor complexes that include HER2 appear to be more potent than other receptor combinations. Features of HER2-containing complexes that may account for enhanced receptor signaling include the conversion from a low-affinity to a high-affinity growth factor binding site,[12] which results from a decrease in ligand dissociation from the complex.[5] Second, the internalization rate of HER2 receptor complex is nearly an order of magnitude lower than that observed for EGFR.[13] The higher-affinity binding state and the decreased internalization rate may result in a receptor–ligand complex that resides longer on the plasma membrane and prolongs the duration of receptor signaling. Third, HER2 is a very active tyrosine kinase[14] that also can be constitutively activated by mutation or overexpression. In addition to transphosphorylating other HER receptors, activated HER2 phosphorylates PLCγ,[15] activates the Ras/Raf/MEK/MAPK pathway,[16-18] and associates with the cytoplasmic tyrosine kinases src[19-21] and CHK.[22] Heterodimerization of HER receptors[23-25] appears to increase the diversity of cellular

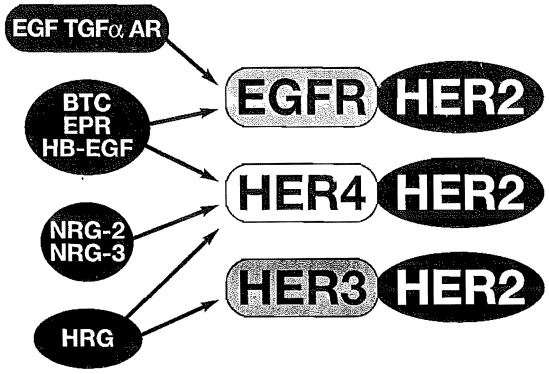

Fig 1. Schematic representation of the human epidermal growth factor receptor (HER) family and their ligands.[5]

responses to a variety of proteins that serve as ligands for these receptors.[9,10]

Activation of nonmutated HER2 occurs through self-association and by transactivation with other related receptor family members. In breast cancer, gene amplification results in HER2 protein levels in tumor cells that are 10- to 100-fold greater than that found in the adjacent normal breast epithelium.[26] Since HER2 is localized in the plasma membrane, its diffusion is limited to two dimensions; thus, even moderate overexpression of HER2 can lead to a constitutively activated receptor.[27] Soon after the HER2 gene was cloned, and well before its role as a common subunit was appreciated, compelling clinical data were generated demonstrating that HER2 gene amplification predicts a poor clinical outcome.[28] Although these data were controversial at first, at present the general consensus is that HER2 overexpression is a negative prognostic indicator for breast cancer.[29,30] Subsequent studies have shown that HER2 overexpression correlates with shorter disease-free interval and overall survival.[31] Moreover, comprehensive studies have shown that the presence of this molecular alteration is predictive of aggressive disease regardless of disease stage or node status.[32-34]

A role for HER2 as an oncogene was first appreciated in rodent systems. The activated form of the rodent homolog of the HER2 gene, c-*neu*, was initially identified as an extremely potent transforming oncogene in DNA isolated from rats treated with ethylnitrosourea.[35] This version of the *neu* oncogene contained a point mutation in the transmembrane domain, which resulted in the *neu* protein existing in a constitutively activated state. To date, no analogous mutations have been found in human tumors.[28,29,36,37] Rather, the molecular alteration that occurs in human cancers is overexpression of the normal gene product. Overexpression generates a constitutively activated receptor and leads to transformation of both NIH3T3 cells and human breast cells, suggesting that this alteration may play a pathogenic role in tumorigenesis.[38-41] Additionally, female transgenic animals that are engineered to overexpress the nonmutated *neu* oncogene in their breast tissue develop breast tumors after puberty with a high degree of penetrance.[42] Taken together, these data suggest that HER2 overexpression has an active role in neoplastic transformation.

TARGETING HER2-OVEREXPRESSING CANCERS

A number of approaches have been used to therapeutically target HER2-overexpressing cancers.[43] A common approach, based on similar studies with the closely related EGFR, has been the generation of antibodies that inhibit the growth of cells which possess activated HER2/*neu* receptors.[44-49] Analysis of one of these panels of anti-HER2 monoclonal antibodies led to the identification of the murine monoclonal, 4D5.[50] This antibody recognizes an extracellular epitope (amino acids 529 to 627) in the cysteine-rich II domain that resides very close to the transmembrane region. Treatment of breast cancer cells with 4D5 partially blocks heregulin activation of HER2-HER3 complexes, as measured by receptor phosphorylation assays.[6] To allow for chronic human administration, murine 4D5 was fully humanized to generate trastuzumab (Herceptin; Genentech, San Francisco, CA). Solution-phase binding studies determined that trastuzumab binds to the recombinant HER2 extracellular domain with an affinity (*Kd*) of 0.1 nmol/L, which is threefold greater than 4D5.[51]

IN VITRO EFFECTS OF ANTI-HER2 MONOCLONAL ANTIBODIES

All the antibodies generated against HER2 were assessed for their ability to inhibit the anchorage-dependent growth of human cancer cell lines. Several of these antibodies exhibited antiproliferative activity toward cell lines that expressed higher-than-normal levels of HER2. This observation was

also extended to include assays performed in soft agar or in an anchorage-independent format, and similar results were obtained.[52] The most potent growth-inhibitory antibody of this panel was 4D5. Cell cycle analysis following treatment of SK-BR-3 breast cancer cells, which express high levels of HER2, is shown in Fig 2. Trastuzumab or 4D5 significantly reduces the percentage of SK-BR-3 cells undergoing S-phase and increases the percentage of cells in G0/G1. In contrast, HER2 antibody treatment does not affect the cell line MCF-7, which expresses normal amounts of HER2. The effect of these antibodies on the cell cycle is dose-dependent, with maximal inhibitory activity occurring at antibody concentrations greater than 1 μg/mL.[53] To address the molecular basis for growth arrest, SK-BR-3 cells were treated with trastuzumab; cell lysates were then probed by immunoblot analysis with reagents specific for known inhibitors of the cell cycle. As shown in Fig 3, treatment of SK-BR-3 cells with trastuzumab resulted in marked induction of the CDK2 kinase inhibitor, $p27^{KIP1}$. Moreover, a similar induction of the retinoblastoma-related protein, p130, was

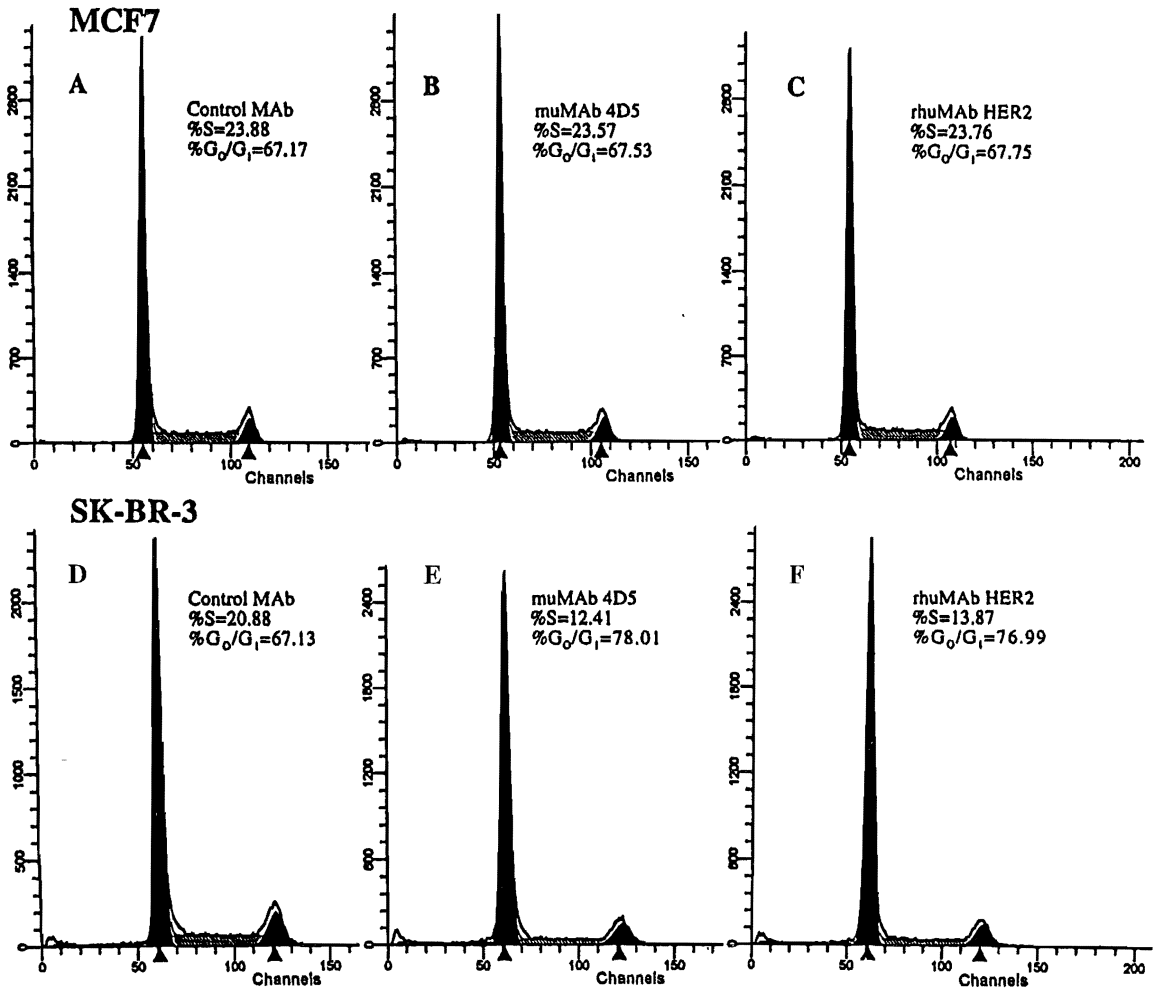

Fig 2. MCF-7 (A-C) or SK-BR-3 (D-F) cells were plated at a density of 2 × 10⁶/dish in 60 mm × 15 mm culture dishes and allowed to adhere overnight. Monolayers were washed with phosphate-buffered saline (PBS) and incubated with media containing trastuzumab or control antibodies at a concentration of 10 μg/mL. Following a 72-hour incubation, cells were trypsinized, washed with PBS, fixed in ice-cold methanol, and stored at -20°C. Fixed cells were then washed twice with PBS and allowed to incubate with RNAse 100 μg/mL (Worthington Biochemical, Lakewood, NJ) for 30 minutes at 37°C. Following centrifugation, nuclei were subjected to propidium iodide 50 μg/mL (Molecular Probes, Inc, Eugene, OR) staining in PBS. Samples were analyzed by flow cytometry (Epics Elite; Coulter Corporation, Miami, FL) using Modfit LT software (Verity Software House, Sunnyvale, CA).

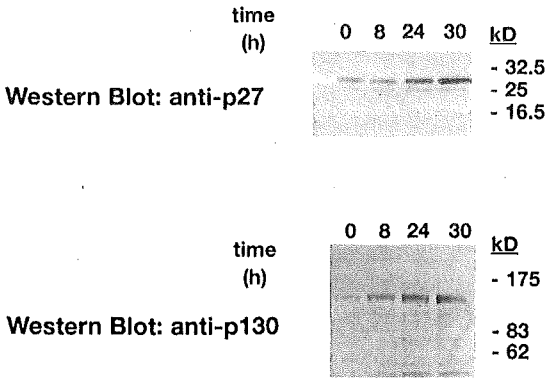

Fig 3. Induction of the cell cycle inhibitor $p27^{KIP1}$ and the retinoblastoma-related p130 protein in SK-BR-3 cells treated with trastuzumab. Cells (1.5×10^5 cells/well) were plated in six-well dishes and grown to approximately 50% confluence in medium containing 10% fetal bovine serum. The medium was removed and treatments (trastuzumab, 16 μg/mL) were prepared in fresh medium containing 10% fetal bovine serum and added to the culture dishes. Incubations were stopped by the addition of 0.5 mL sodium dodecyl sulfate sample buffer (5% sodium dodecyl sulfate, 2% β-mercaptoethanol, 10% glycerol, 50 mmol/L Tris HCl, pH 6.8). Samples (20 μg of protein/lane) were electrophoresed on an 8% to 16% Tris-glycine gel (Novex, San Diego, CA). Protein was transferred from the gels to nitrocellulose. Western blot analysis and detection were done following the manufacturer's instructions (ECD; Amersham, Buckinghamshire, UK). Primary antibodies used for $p27^{KIP1}$ and the retinoblastoma-related p130 protein immunoblots were rabbit polyclonals (SC-317 and SC-528, respectively; Santa Cruz, Santa Cruz, CA) and were used at 0.1 μg/mL. Both proteins were detected with an anti-rabbit horseradish peroxidase-conjugate secondary antibody used at a titer of 1:10,000 (ALI 3404; Biosource International, Carmarillo, CA).

also observed. These data are consistent with the observation that treatment of HER2-overexpressing cells is antiproliferative and that cytostasis results from an inhibition of cell cycle progression.

Cells that overexpress HER2 are intrinsically resistant to the cytotoxic effects of tumor necrosis factor-α.[54] This observation suggests that HER2 overexpression may potentiate tumorigenesis by inducing tumor cell resistance to host defense mechanisms. Furthermore, when HER2-overexpressing cells are treated with the murine monoclonal 4D5, they become sensitized to tumor necrosis factor-α treatment.[49]

Molecules involved in cell adhesion are also thought to play a critical role in malignant progression. One of these molecules, E-cadherin, plays a central role in maintaining epithelial cell morphology.[55] A reduction in E-cadherin synthesis can be correlated with increased invasiveness.[56] Overexpression of HER2 in normal human breast epithelial cells reduces morphogenesis in vitro.[57] These HER2 transfectants express significantly lower levels of E-cadherin as well as the α2 integrin subunit. Treatment of these HER2 transfectants with 4D5 restores E-cadherin and α2 integrin to normal levels.[58] Taken together, these data suggest that HER2 overexpression affects adhesion molecule synthesis, which in turn may contribute to the metastatic process and malignant progression. Targeting HER2 overexpression with monoclonal antibodies such as 4D5 and trastuzumab may be effective in controlling metastatic potential.

Angiogenesis is a critical survival function for solid tumors.[59,60] Vascular endothelial growth factor is one of the more important mediators of tumor angiogenesis.[61] Recently, it has been demonstrated that treatment of HER2-overexpressing tumor cells with 4D5 decreases vascular endothelial growth factor production.[62] Suppression of angiogenesis may enhance the activity of anti-HER2 monoclonal antibody therapy in vivo.

AGONIST PROPERTIES OF TRASTUZUMAB

Several reports have suggested that binding of 4D5 to HER2 activates the receptor, as monitored by an increase in tyrosine autophosphorylation.[63] In addition, c-fos induction[64] and increases in inositol polyphosphate and diacyl glycerol synthesis[65] following 4D5 treatment also have been reported. These agonistic properties are difficult to reconcile the potent cytostatic activity of 4D5 or trastuzumab on the growth of human tumor cells that overexpress HER2.[6,52]

With recent recognition of the role of HER2 as a shared signaling subunit with other HER receptor family members, the ligand-like properties of trastuzumab have been re-examined. MCF-7 cells express normal/low levels of HER2 and are not growth inhibited by trastuzumab treatment. MCF-7 cells were treated with trastuzumab, 4D5, or recombinant heregulin (rHRG). Cell lysates were prepared using denaturing (sodium dodecyl sulfate) or nondenaturing (nonionic detergent, Triton X-100) conditions. These latter conditions are frequently used for examination of receptor activation or co-immunoprecipitation of mediators involved in signal transduction. As shown in Fig 4, treatment of MCF-7 cells with rHRG results in HER2 tyrosine phosphorylation, regardless of the manner in which the cells are lysed. In con-

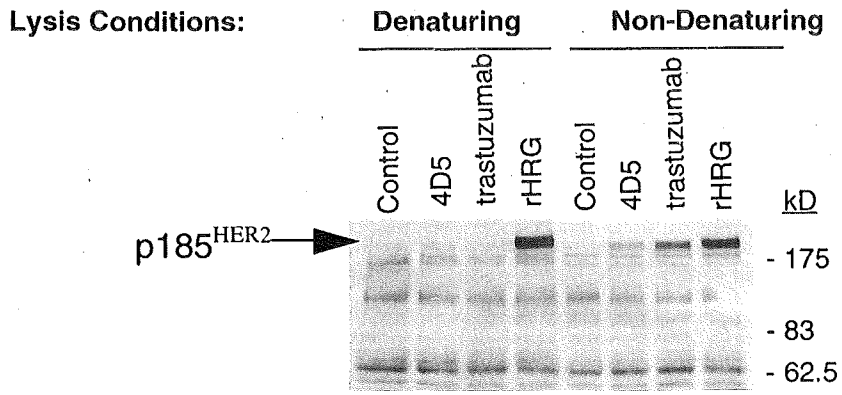

Fig 4. Phosphotyrosine analysis of HER2. MCF-7 cells were seeded in 24-well plates at 1.5 × 10^5 cells/well and allowed to grow for 2 days. Before treatment, cells were transferred to medium without serum. Cells were treated for 30 minutes at room temperature with 4D5 (16 μg/mL), trastuzumab (16 μg/mL), or rHRG (300 pmol/L). Cell lysates were then prepared using denaturing or nondenaturing conditions and allowed to remain at room temperature for 3 hours. For nondenaturing conditions, lysates were prepared in 0.5% Triton X-100, 50 mmol/L Hepes, 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 2 mmol/L $NaVO_4$, and 5 KIU/mL aprotinin. Sodium dodecyl sulfate-polyacrylamide electrophoresis buffer was used for denaturing conditions. Samples were prepared for electrophoresis by adding an equivalent volume of 5% sodium dodecyl sulfate and 2% β-mercaptoethanol to each sample and then heated at 100°C for 5 minutes. Following sodium dodecyl sulfate-polyacrylamide electrophoresis, proteins were transferred to nitrocellulose membranes and probed with anti-phosphotyrosine monoclonal antibody (#E120H; Transduction Labs, San Diego, CA) linked to horseradish peroxidase used at a final concentration of 0.125 ng/mL.

trast, 4D5 or trastuzumab results in HER2 tyrosine phosphorylation only when lysates are prepared in nonionic detergent. Although biochemically interesting, this phosphorylation event does not appear to be physiologic. A similar artifact has been reported for anti-EGFR monoclonal antibodies.[66] When similar experiments are performed with SK-BR-3 cells, which overexpress HER2 and are growth inhibited by trastuzumab treatment, the results are confounded by a high degree of basal tyrosine phosphorylation that exists on HER2. Taking this high baseline phosphotyrosine level into account, trastuzumab treatment of SK-BR-3 cells results in a modest but reproducible increase in tyrosine phosphorylation content, which is in agreement with previous published studies.[63]

To assess whether trastuzumab treatment causes further activation of the HER2 signal transduction pathway, the common adapter molecule, shc, was examined. MCF-7 or SK-BR-3 cells were treated with trastuzumab, 4D5, or rHRG, and cell lysates were then prepared using denaturing conditions. Immunoprecipitates of shc were prepared and immunoblots were then probed with anti-phosphotyrosine antibody to determine the level of shc tyrosine phosphorylation. As shown in Fig 5, rHRG treatment of either MCF-7 or SK-BR-3 cells results in a rapid increase in shc phosphorylation that is sustained for approximately 120 minutes. In contrast, neither trastuzumab nor 4D5 treatment causes a significant increase in shc phosphorylation. Mitogen-activated protein kinases are common mediators of growth factor receptor signal transduction pathways. To further investigate potential differences in downstream signaling, mitogen-activated protein kinase activation was examined after exposure to trastuzumab or rHRG. As shown in Fig 6, strong mitogen-activated protein kinase activation occurs in either MCF-7 (Fig 6, top) or SK-BR-3 cells (Fig 6, bottom) only after rHRG treatment. These data suggest that while trastuzumab or 4D5 may cause autophosphorylation under some conditions, downstream signaling pathways are not significantly activated.

Other detailed investigations have been undertaken with well-characterized panels of anti-HER2 monoclonal antibodies in an attempt to correlate agonistic activities of these antibody panels with the desired biological outcome, ie, cytostasis.[47,48,67,68] To date, no strict correlation exists between any one measured biochemical parameter and antiproliferative activity. Rather, a number of attributes emerge as components in assessing anti-HER2 cytostatic activity. These include antibody affinity, bivalency, pH dependence of binding, downmodulation, internalization, and the ability to block HER2 association with other HER family members. It appears that the more potent antipro-

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