Trastuzumab (Herceptin), a Humanized Anti-HER2 Receptor Monoclonal Antibody, Inhibits Basal and Activated HER2 Ectodomain Cleavage in Breast Cancer Cells

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ABSTRACT

HER2 is a ligand-less tyrosine kinase receptor of the ErbB family that is frequently overexpressed in breast cancer. It undergoes proteolytic cleavage that results in the release of the extracellular domain and the production of a truncated membrane-bound fragment, p95. We show that HER2 shedding is activated by 4-aminophenylmercuric acetate (APMA), a well-known matrix metalloprotease activator, in HER2-overexpressing breast cancer cells. The HER2 p95 fragment, which appears after APMA-induced cleavage, is phosphorylated. We analyzed 24 human breast cancer specimens, and a phosphorylated M95,000 HER2 band could be detected in some of them, which indicated that the truncated receptor is also present in vivo. The activation of HER2 shedding by APMA in cells was blocked with batimastat, a broad-spectrum metalloprotease inhibitor. Trastuzumab (Herceptin; Genentech, San Francisco, CA), a humanized monoclonal antibody directed against HER2 ectodomain, which has been shown to be active in patients with HER2-overexpressing breast cancer, inhibited basal and induced HER2 cleavage and, as a consequence, the generation of phosphorylated p95. This inhibitory effect of trastuzumab was not shared by 2C4, an antibody against a different epitope of the HER2 ectodomain. The inhibition of basal and APMA-induced cleavage of HER2 by trastuzumab preceded antibody-induced receptor down-modulation, which indicated that the effect of trastuzumab on cleavage was not attributable to a decrease in cell-surface HER2 induced by trastuzumab. We propose that the inhibition of HER2 cleavage and prevention of the production of an active truncated HER2 fragment represent a novel mechanism of action of trastuzumab.

INTRODUCTION

The HER (or ErbB) family of transmembrane tyrosine kinase receptors is composed of four members, HER1 to HER4 (1). HER2, a ligand-less M185,000 receptor encoded by the neu proto-oncogene, is overexpressed in 25–30% of human breast cancer and has been associated with enhanced tumor aggressiveness and a high risk of relapse and death (2). Recent evidence indicates that HER2 amplifies the signal provided by other receptors of the ErbB family by heterodimerizing with them (3). The important biological role of HER2 in the signaling network that drives epithelial cell proliferation and transformation, together with its extracellular accessibility and its overexpression in some human tumors led to considering HER2 as an appropriate target for tumor-specific therapies. Several MAbs directed against HER2 ectodomain that specifically inhibit the growth of tumor cell lines overexpressing HER2 have been developed. One of them, 4D5 (4), was humanized and the resulting antibody was termed trastuzumab (Herceptin; Ref. 5). Trastuzumab has antitumor activity against HER2-positive human breast tumor cells in laboratory models (6) and is active for the treatment of women with HER2-overexpressing breast cancers (7–9). On the basis of trastuzumab clinical efficacy, this antibody was approved in 1998 for clinical use for HER2 overexpressing metastatic breast cancer. Trastuzumab seems to exert its antitumor effects by several mechanisms that are not yet completely understood (10). In HER2 overexpressing cells, trastuzumab markedly down-regulates HER2 expression by accelerating receptor endocytosis and degradation (11) and inhibits cell cycle progression by inducing the formation of p27Kip1/Cdk2 complexes (10, 12). Trastuzumab also induces antibody-dependent cell-mediated cytolysis against HER2 expressing tumor cells in animal models. This process is regulated by antibody receptors FcγRIII and FcγRIIB on myeloid cells (10, 13). Other additional mechanisms that have been proposed include suppression by trastuzumab of angiogenesis (14) and metastasis (10).

The full-length HER2 receptor (p185) undergoes a slow proteolytic cleavage in HER2-overexpressing tumor cells in culture, and the resulting M95,000 receptor ECD can be detected in the conditioned medium (15–17). Proteolytic cleavage also generates a M95,000 NH2-terminally truncated membrane-associated fragment with both in vitro and in vivo antitumor effects by several mechanisms that are not yet completely understood. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1754 solely to indicate this fact. The abbreviations used are: MAb, monoclonal antibody; APMA, 4-aminophenylmercuric acetate; BB-94, batimastat; ECD, extracellular domain; TACE, tumor necrosis factor α converting enzyme.
metalloproteases TIMP-1, a matrix metalloproteinase inhibitor that does not inhibit TACE (31), prevents HER2 shedding (30).

The study presented here aimed to further characterize HER2 shedding, and specially to determine the possible effects of trastuzumab on this process. We found that APMA, a mercurial compound used to activate matrix metalloproteases in *vitro*, induced shedding of HER2, which resulted in the generation of a phosphorylated p95 intracellular-bound receptor fragment. Trastuzumab was able to effectively block basal and induced HER2 cleavage, and this property was not shared by 2C4, another antibody against the HER2 ectodomain. Finally, analysis of human breast tumors revealed the presence of some of them of a phosphorylated HER2 fragment with a *M*~r~ of ~95,000, which indicated that a truncated phosphorylated receptor is also present in *vivo*. Our results suggest that the inhibition of HER2 cleavage by trastuzumab could contribute to the antitumor properties of this antibody.

**MATERIALS AND METHODS**

**Materials.** APMA was purchased from Sigma Chemical Co. (St. Louis, MO). Pervanadate was prepared according to Codony-Servat (30). Trastuzumab and antibody 2C4 were kindly provided by Genentech, Inc. (South San Francisco, CA) and BB-94 by British Biotech Pharmaceuticals, Ltd. (Oxford, United Kingdom).

**Tumor Cell Lines.** The two breast adenocarcinoma cell lines used in this study, BT-474 and SK-BR-3, were obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DMEM/F12 supplemented with 10% fetal bovine serum, 2 mM glutamine and, in the case of BT-474, 10 µg/ml insulin (all from Life Technologies, Inc., Ltd., Paisley, United Kingdom).

**Western Blot Analyses for HER2 p185, p95, and ECD Detection.** Cells grown in 6-well dishes were washed with PBS and treated with various compounds in serum-free DMEM/F12 for variable periods of time, as indicated. Medium was removed, and cultures were washed twice with cold PBS and lysed in 0.15 M of RIPA B lysis buffer [20 mM sodium phosphate (pH 7.4), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 5 mM phenylmethyl-sulfonyl fluoride, 10 µg/ml aprotinin, 10 µg/ml leupeptin, and 250 µg/ml sodium vanadate]. After removal of cell debris by centrifugation, protein concentration in cell lysates was determined by Lowry assay (DC Protein assay, Bio-Rad, Hercules, CA). In some cases, medium were concentrated using 5K Ultrafree centrifugal filters (Millipore, Bedford, MA) and stored at −80°C.

**Protein Extraction and HER2 p185 and p95 Analysis from Tissue Specimens.** All of the breast tissues used in this study were surgical resection specimens obtained at the Vall d’Hebron Hospital following Institutional Guidelines. Paired tumor and histologically normal breast were analyzed from each patient (*n* = 24). About 0.2–0.4 g of tissue, which had been fresh-frozen and stored at −70°C, was minced on dry ice and resuspended in RIPA B buffer containing protease inhibitors and vanadate. Samples were homogenized using a polytron and were centrifuged at 15,000 × *g* for 10 min at 4°C. The supernatant was recovered, and protein concentration was determined by the Lowry assay. Western blot analyses were performed as described above. After developing with the chemiluminescent reagent, membranes were exposed to X-ray films for up to 15 min. The samples having a detectable p95 band were scored as p95 positive. Tumors showing an intense HER2 p185 band after a 1-s exposure to X-ray film were considered as HER2 overexpressors.

**RESULTS**

**APMA Induces HER2 Shedding.** Our previous data indicated that HER2 cleavage is catalyzed by a metalloproteinase (30). To find a specific way to induce receptor shedding, we assayed the effects of APMA, a well-known matrix metalloproteinase activator (32). Two HER2-overexpressing breast cancer cell lines were used, BT-474 and SK-BR-3. HER2 cleavage was analyzed by Western blot and by metabolic labeling followed by immunoprecipitation of cell lysates and medium; both techniques gave the same qualitative results. Exposure of cells to 0.25–1 mM APMA induced cleavage of HER2 p185, leading to the appearance of HER2 ECD in the culture medium and HER2 p95 in cells. This effect was time-dependent, reaching its peak after a 15-min incubation (Fig. 1A). APMA concentrations lower than 0.1 mM did not induce detectable HER2 cleavage after a 15-min incubation, whereas the effect of concentrations higher than 0.25 mM was almost dose-independent (Fig. 1B). The truncated p95 receptor was undetectable under basal conditions or at APMA concentrations that did not induce HER2 shedding, probably because of the small amounts of p95 present under these conditions. The APMA concentrations that were needed to activate HER2 shedding were similar to those used *in vitro* for matrix metalloprotease activation, which usually ranged around 1 mM (33, 34).

To further characterize the APMA-induced cleavage of HER2, we estimated the fraction of receptor that was cleaved in the presence of...
Labeled for 3 h with 500 μM APMA in 0.2 ml of serum-free medium, six-well plates were treated at 37°C with 1 mM APMA in 0.2 ml of serum-free medium, and cell lysates (20 μg of protein) were analyzed by Western blotting. Control cells were kept for 20 min in serum-free medium in the absence of the mercurial compound. B, effects of different APMA concentrations on HER2 shedding. Control cells were treated with APMA for 15 min in 0.2 ml of serum-free medium, and cell lysates (20 μg of protein) and medium (50 μl) were analyzed by Western blot. C, BT474 cells were labeled for 3 h with 500 μCi/m of [35S]-transferrin in methionine- and cysteine-free medium. The label was monitored 2 h in complete medium. Then, 1 mM APMA was added for the indicated times. D, effects of BB-94 on APMA-induced HER2 cleavage. BT474 cells were treated with APMA and/or BB-94 for 15 min in 0.3 ml of serum-free medium, and cell lysates (20 μg of protein) and medium (50 μl) were analyzed by Western blotting. BB-94 was added just before (≈30 s to 1 min) APMA. Preincubation with BB-94 for longer times was not deemed necessary because protease inhibitors act immediately, and long exposures to them may result in nonspecific effects (26). kDa, M, in thousands.

**Fig. 1. Effects of APMA and BB-94 on HER2 shedding in BT-474 cells.**, A, kinetics of APMA-induced HER2 ECD secretion and HER2 p95 production. Cells growing in six-well plates were treated at 37°C with 1 mM APMA in 0.2 ml of serum-free medium, and cell lysates (20 μg of protein) and medium (50 μl) were analyzed by Western blotting. Control cells were kept for 20 min in serum-free medium in the absence of the mercurial compound. B, effects of different APMA concentrations on HER2 shedding. Control cells were treated with APMA for 15 min in 0.2 ml of serum-free medium, and cell lysates (20 μg of protein) and medium (50 μl) were analyzed by Western blot. C, BT474 cells were labeled for 3 h with 500 μCi/m of [35S]-transferrin in methionine- and cysteine-free medium. The label was monitored 2 h in complete medium. Then, 1 mM APMA was added for the indicated times. D, effects of BB-94 on APMA-induced HER2 cleavage. BT474 cells were treated with APMA and/or BB-94 for 15 min in 0.3 ml of serum-free medium, and cell lysates (20 μg of protein) and medium (50 μl) were analyzed by Western blotting. BB-94 was added just before (≈30 s to 1 min) APMA. Preincubation with BB-94 for longer times was not deemed necessary because protease inhibitors act immediately, and long exposures to them may result in nonspecific effects (26). kDa, M, in thousands.

**Fig. 2. Effects of trastuzumab on HER2 shedding.**, A, effects of trastuzumab on basal HER2 shedding in BT-474 and SK-BR-3 cells. Cells were treated with trastuzumab in 0.3 ml of serum-free medium for 30 min. Media were concentrated to 50 μl and analyzed by Western blot. B, 2C4 does not inhibit basal HER2 shedding in BT-474 cells. Cultures were incubated with 2C4 or with trastuzumab for 2 h in 0.3 ml of serum-free medium, which was afterward concentrated to 50 μl and analyzed by Western blot. C, trastuzumab inhibits APMA-induced HER2 cleavage in BT-474 and SK-BR-3 cells. Cell lysates (20 μg of protein) and medium (50 μl) were analyzed by Western blotting. APMA and/or trastuzumab was added to cultures; and after 15 min, cells were washed and medium (50 μl) and lysates (100 μg in the case of SK-BR-3 and 20 μg for BT-474 cells) were subjected to Western blot analysis. Media were assayed with no prior concentration. Film exposure times were 5 min for SK-BR-3 medium, 1 min for BT-474 medium, and 8 min for lysates.
be seen as early as 10 min after initiation of the treatment (data not shown). At this time point, there was no evidence of any detectable amount of HER2 internalization induced by trastuzumab. A time course experiment in biotin-labeled cells demonstrated that trastuzumab did not significantly reduce membrane HER2 after as much as 6 h and showed an effect only after a 24-h incubation of the cells with the antibody (Fig. 3B), whereas the inhibition of ECD release was observed at all times tested. Similar results were seen in metabolic-labeling experiments (data not shown). Because $\approx 20\%$ of cell surface HER2 was cleaved by APMA, if the inhibition of HER2 cleavage by trastuzumab was dependent on receptor internalization, this level of cell surface HER2 down-regulation ($\approx 20\%$) should have been detected in the two experimental approaches that assayed cell surface HER2 or in prior work (11). Taken together, these results show that trastuzumab reduces HER2 shedding well before it induces any detectable decrease in cell surface HER2, which supports the observation that it has a direct inhibitory effect on HER2 shedding that is not mediated by receptor down-modulation.

**HER2 p95 Is Tyrosine-phosphorylated in APMA-induced BT-474 Cells.** The tyrosine phosphorylation of p95 was examined by treating BT-474 cells with APMA and blotting the lysates with an antibody against phosphorylated HER2. A phosphorylated M$_r$ 95,000 band was apparent on APMA treatment, corresponding to the truncated HER2 receptor. The presence of trastuzumab inhibited the appearance of this phosphorylated band (Fig. 4). **HER2 p95 Is Present and Phosphorylated in Human Breast Tumors.** Twenty-four human breast tumors and paired histologically normal tissues were examined for HER2 proteins by Western blot (Fig. 5A). All of the tumors analyzed expressed higher full-length (p185) HER2 levels relative to normal breast tissue, albeit at different degrees. Immunohistochemistry revealed that the six tumors with the highest level of HER2 by Western blot had moderate or intense complete membrane staining in more than 10% of tumor cells and, therefore, could be considered positive for HER2 overexpression. In 14 of the tumors, a highly variable HER2 band M$_r$ $\approx$95,000 was detectable, which indicated that the cleavage of HER2 varies in different tumors. The same breast tumor samples were analyzed for tyrosine-phosphorylated HER2 proteins. A HER2 M$_r$ 185,000 phosphorylated band was apparent in all of the tumors analyzed, always elevated in comparison to paired normal samples. Five of the tumors also showed a phosphorylated band of M$_r$ 95,000 (Fig. 5B). These tumors were those expressing the highest levels of p95.
decrease in cell surface receptor levels. Overall, these data indicate that trastuzumab has a direct inhibitory effect on HER2 shedding, probably acting by steric hindrance, blocking the cleavage site of the receptor. In this respect, it is particularly interesting to note that the epitope recognized by trastuzumab is near the HER2 transmembrane domain, where the cleavage site of the receptor is located, whereas the epitope for MAb 2C4 is very discontinuous in the amino acid sequence and the two antibodies do not cross-block. Our results provide the first evidence that an antibody directed against the ECD of a membrane protein can block the proteolytic cleavage of the protein.

To find a specific way to activate HER2 shedding and further study this process, we assayed the effects of the general metalloproteinase activator APMA. APMA was found to induce HER2 shedding in breast cancer cells overexpressing this receptor in a time- and dose-dependent manner. The induction of HER2 cleavage by APMA could be completely blocked by BB-94, a broad-spectrum metalloprotease inhibitor. In a previous report, we had already demonstrated that BB-94 also blocks basal HER2 cleavage. Trastuzumab was also found to effectively block APMA-induced HER2 shedding and, therefore, HER2 ECD release and p95 generation. Hence, the antibody retains its ability to inhibit receptor cleavage even when the shedding machinery that acts on HER2 is up-regulated. The degree of effect of trastuzumab on released ECD versus the truncated intracellular fragment was difficult to compare because they are recognized by different antibodies. However, the production of cell-associated HER2 fragment was usually markedly reduced or suppressed concomitantly with the inhibition of ECD release. Further research is needed to identify possible physiological activators of HER2 shedding and to determine whether trastuzumab can also block their effects. In this respect, preliminary data suggest that heregulin, an ErbB ligand that is frequently expressed in breast tumors, stimulates HER2 cleavage.

Christianson et al. have reported that the membrane-bound HER2 p95 fragment present in breast tumor cell lines in basal conditions is phosphorylated and has kinase activity. We found that the p95 truncated fragment generated on APMA-induced HER2 cleavage in tumor cells in culture is also phosphorylated. Because APMA is a cystein reactive agent that targets other proteins in addition to metalloproteases, the explanation for the increased phosphorylation of full-length HER2, present after APMA treatment, may possibly include the targeting and inactivation of phosphotyrosine phosphatases. Another possibility, which would not exclude the prior one, is that the phosphorylated cytoplasmic p95 HER2 fragment that arises after receptor cleavage may dimerize with the full-length receptor and contribute to the increased phosphorylation of HER2 that follows APMA treatment. The presence of trastuzumab in the culture medium inhibited the appearance of the HER2 p95 phosphorylated band, as a consequence of the prevention of receptor shedding. It is attractive to hypothesize that the inhibition of HER2 cleavage by trastuzumab may contribute to its therapeutic value by preventing the formation of the potentially deleterious intracellular HER2 p95 kinase fragment that arises from receptor cleavage.

The ECD of several membrane-anchored proteins, including some tyrosine-kinase receptors such as HER2, can be released as a soluble fragment by the action of a cell surface endoproteolytic system. Substantial evidence has demonstrated that the ectodomain of this class of receptors acts as a molecular “brake” of the intracellular kinase activity. Some retroviral receptor oncogenes code for oncoproteins that lack most of the ECD and give rise to constitutively active membrane-bound receptor fragments. Moreover, the in vitro deletion of the ectodomain of several receptor tyrosine kinases including HER2 (23, 24), confers transforming potential to the mutated receptor. In the case of the neurotrophin receptor TrkA, ectodomain cleavage generates a cell-associated fragment with increased phosphotyrosine content and probably higher catalytic activity. Finally, ectodomain shedding of HER4 results in the formation of a membrane-truncated fragment that has tyrosine kinase activity, is tyrosine phosphorylated, and may act as a membrane-localized docking molecule for signaling molecules with SH2 domains. Taken together, these data suggest that proteolytic cleavage of the ECD constitutes a ligand-independent mechanism for the activation of tyrosine kinase receptors via the generation of a cell-bound receptor fragment with constitutive kinase activity. This mechanism could be relevant in the case of HER2 for several reasons. First, HER2 does not seem to have a natural ligand; second, receptor cleavage has been shown by us and others to be a regulated process; and third, presence of ECD in serum is associated with metastatic progression, as mentioned above. A full-length HER2 M, 185,000 phosphorylated band was present in the 24 tumors analyzed in the present study. A phosphorylated band of M, 95,000 was also apparent in tumor samples with the highest p95 levels. It is, therefore, possible that the truncated receptor is constitutively phosphorylated, but, because of the lower sensitivity of the antiphosphotyrosine HER2 antibody, the phosphorylation of this band can be detected with the antibody only in tumors expressing high levels of p95. This finding of a phosphorylated HER2 truncated receptor in human breast tumors supports the hypothesis that it may play a role in signal transduction in cancer cells.

In summary, we have shown that HER2 shedding can be induced in...
breast cancer cells in culture by APMA via the activation of a metallo-proteinase(s) that is inhibited by BB-94. A truncated membrane-bound phosphorylated HER2 p95 fragment appears on APMA-induced receptor cleavage. Trastuzumab, a MAb that is effective in the therapy of breast tumors that overexpressing HER2, has a direct inhibitory effect on basal and APMA-induced HER2 shedding. In contrast, 2C4, another MAB against HER2 ectodomain, does not show any effect on HER2 cleavage. Finally, a p95 HER2 fragment is present and phosphorylated in some human breast cancer tumors, which indicates that HER2 cleavage and the subsequent generation of a truncated fragment with potential signaling activity also occurs in vivo. Our findings suggest that the inhibition of HER2 shedding by trastuzumab may be one of the mechanisms responsible for the antitumor effects of this MAB.

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