Tumor-inhibitory Antibodies to HER-2/ErbB-2 May Act by Recruiting c-Cbl and Enhancing Ubiquitination of HER-2

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Abstract

Overexpression of HER-2/ErbB-2, a homologue of the epidermal growth factor receptor, is associated with poor prognosis, and an ErbB-2-specific antibody is therapeutic when administered to patients with metastatic breast cancer. To understand the mechanism underlying immunotherapy, we concentrated on antibody- and epidermal growth factor-induced degradation of ErbB-2. We show that enhanced degradation is preceded by poly-ubiquitination of ErbB-2. This process necessitates recruitment of the c-Cbl ubiquitin ligase to tyrosine 1112 of ErbB-2. Consequently, maturation of this site retards antibody-induced degradation. Thus, the therapeutic potential of certain antibodies may be due to their ability to direct ErbB-2 to a c-Cbl-regulated proteolytic pathway.

Introduction

HER-2/ErbB-2 is a receptor tyrosine kinase that is a member of the EGFR-receptor family. By serving as a shared coreceptor for multiple stromal growth factors, ErbB-2 cooperates with ErbB-1 (EGF receptor) and the neuregulin receptors (ErbB-3 and ErbB-4; Ref. 1). Unlike low expression in normal adult cells, ErbB-2 is overexpressed in ~20–35% of human breast, gastric, lung, and prostate cancer. ErbB-2 overexpression in breast carcinomas is correlated with poor prognosis, especially of node-positive cases (reviewed in Ref. 2). Extensive work overexpression in breast carcinomas is correlated with poor prognosis, especially of node-positive cases (reviewed in Ref. 2). Extensive work on animal models has indicated that blocking ErbB-2 with mAbs can reduce the rate of tumor growth. These studies led to successful clinical trials of a humanized anti-ErbB-2 mAb (3). Furthermore, patients with metastatic breast cancer showed improved responses when treated with a combination of chemotherapy and the mAb (4). Two potential mechanisms may underlie immunotherapy: (a) mAbs can inhibit the interaction of ErbB-2 with other family members (5); and (b) they can accelerate ErbB-2 degradation (6, 7).

Accelerated degradation of ErbB-1 following EGF binding (“down-regulation”) involves receptor ubiquitination and sorting of internalized molecules in the early endosome (8). The specific ubiquitin ligase involved in this process has been recently identified as c-Cbl (9, 10), a major substrate of many tyrosine kinases (11). Because mAb-induced down-regulation of ErbB-2 is related to the process promoted by EGF binding to ErbB-1, we addressed the possibility that immunotherapy targets ErbB-2 to a c-Cbl-regulated ubiquitination/degradation pathway. Here, we show that a tumor-inhibitory mAb can enhance a Cbl-mediated process of ubiquitination and degradation of ErbB-2.

Materials and Methods

Materials. mAbs directed against ErbB-2 have been described previously (5). A polyclonal rabbit antiserum was raised against a synthetic peptide comprising the 15 COOH-terminal amino acids of the human ErbB-2. EGF (human, recombinant) and an anti-HA mAb were purchased from Boehringer Mannheim. Antibodies were radiolabeled with Na125I (Amersham) by using the chloramine T method. The isolation of a recombinant v-Cbl-GST protein and its subsequent binding to glutathione-agarose has been described previously (10). The lysis buffer contained 25 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Na-deoxycholate, 1% NP40, 0.1% SDS, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM NaVO4, 5 μg/ml leupeptin, and 5 μg/ml aprotinin. Washing solution contained 25 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol.

Construction and Transfection of Expression Vectors. To generate mutated ErbB-2 proteins, we used an oligonucleotide-directed mutagenesis method with a site-directed mutagenesis kit (QuickChange; Stratagene, La Jolla, CA). Tyrosine 1112 was mutated to a phenylalanine using the oligonucleotide 5’-CTCTACAGCGGTTCAGTGAGGACCC. The transmembranal valine 659 was replaced by a glutamic acid with the oligonucleotide 5’-GTCTTCTGGTGAG GGCATTCTGCTG. The double mutant EF was generated by introducing the V659E mutation into a receptor construct containing the Y1112F mutation. Expression vectors were introduced into CHO cells by using the Lipofectamine transfection method (Life Technologies, Inc., Bethesda, MD) and into HEK-293T cells by using the calcium phosphate precipitation method.

Determination of mAb Effect on Tumor Growth in Vivo. N87 cells (5 × 106) were injected s.c. into CD1/nude mice, followed by three i.p. injections of the mAbs on days 3, 7, and 10. Tumor parameters were measured once a week with calipers, and tumor volume was calculated according to the formula: tumor volume = length × width × height.

Receptor Down-Regulation. Cells grown in 24-well plates were incubated at 37°C for various time intervals with or without mAb L26 (20 μg/ml) in binding buffer (0.01% albumin in DMEM). Cells were then rinsed three times in ice-cold binding buffer. Surface-bound antibody molecules were removed by use of low pH wash (8). The level of receptor residing on the cell surface was then determined by incubating the cells at 4°C with a radiolabeled mAb L26 for 1.5 h.

Detection of Ubiquitinated Proteins. The ubiquitinated form of ErbB-2 was detected in immunoprecipitates prepared from cells that were cotransfected with a plasmid encoding a HA-tagged ubiquitin [a gift from Dirk Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany)] and the appropriate ErbB-2 construct. The receptor was immunoprecipitated from whole cell lysates with a rabbit serum. Ubiquitin levels were determined by immunoblotting with anti-HA antibodies.

Receptor Turnover. Confluent cell monolayers grown in 90-mm plates were biosynthetically labeled with 35S-methionine for 16 h. The cells were then chased at 37°C for different time intervals, in the presence or absence of mAb L26. ErbB-2 was immunoprecipitated and separated by gel electrophoresis. Labeled proteins were detected by directly exposing the dried gel to an X-ray film.

In Vitro Binding of v-Cbl to ErbB-2. HEK-293T cells grown in 90-mm plates were transfected with pcDNA3 plasmids encoding either ErbB-1 (1 μg) or ErbB-2 (2 μg). Twenty-four h later, cultures were split into two plates, and forty-eight h after transfection cells were treated with EGF (10 min at 37°C). Cell lysates were then prepared, cleared from debris, and incubated with glutathione-agarose beads that were precoupled to a v-Cbl-GST fusion protein.
After 2 h of incubation at 4°C, the beads were washed twice and bound proteins were dissociated in boiling sample buffer prior to gel electrophoresis.

Results and Discussion

The L26 mAb Down-Regulates ErbB-2 from the Surface of Human Cancer Cells and Inhibits Their Tumorigenic Growth in Mice. We have previously shown that antibodies directed at several distinct epitopes of ErbB-2 inhibit tumor growth in vivo (5). Although this suggests a variety of mechanisms underlying the inhibitory effect, the ability of mAbs to increase receptor turnover seems common to many tumor-inhibitory mAbs (6, 7). To elucidate the underlying mechanism, we selected antibody L26 as an example for mAbs capable of strong tumor inhibition. This previously described mAb (5) decreased the growth, in nude mice, of human gastric carcinoma cells (N87) overexpressing ErbB-2, in a dose-dependent manner (Fig. 1A). Mice receiving a total dose of 2 mg of purified L26 demonstrated an almost undetectable tumor growth along a period of 35 days. Because it has previously been shown that tumor-inhibitory mAbs can induce the internalization of antibody-receptor complexes whereas a noninhibitory mAb cannot (7), we tested the ability of L26 to down-regulate ErbB-2 from the surface of N87 cells. As shown in Fig. 1B, the amount of surface ErbB-2 declined significantly when cells were exposed to mAb L26, reaching 50% of the initial level within 2 h. This suggests that the enhanced receptor internalization caused by L26 treatment with a mAb that decreases the number of surface receptors available for the transmission of growth signals.

Antibody-induced Degradation of ErbB-2 Is Associated with an Increase in Receptor Ubiquitination. Treatment with a mAb that down-regulates ErbB-2 should be manifested in the total cellular amount of this oncoprotein. Using the same cellular system applied for in vivo studies, we could show that cells exposed to the tumor inhibitor gradually lost the expression of ErbB-2 as a function of time (Fig. 2A). Extending the treatment to 4 h caused the complete disappearance of the protein from the cellular pool. However, a similar regimen of N28, a mAb that cannot inhibit tumor growth (7), did not significantly affect the levels of ErbB-2.

Because several receptor tyrosine kinases, including ErbB-1 (8) and the platelet-derived growth factor receptor (12), undergo ligand-induced degradation through a mechanism that involves their polyubiquitination, we tested the possibility that mAb L26 can elevate ubiquitination of ErbB-2. To examine this possibility, we transiently expressed ErbB-2 in CHO cells together with a peptide-tagged ubiquitin. This revealed basal ubiquitination of ErbB-2 and a moderate increase on short treatment with L26, in receptor precipitates, as well as in a corresponding protein band in whole cell lysates (Fig. 2B). Because heterodimerization of ErbB-2 with ErbB-1 has been shown to
Cotransfection of WT or mutant ErbB-2 with c-Cbl vectors was performed in CHO cells. For stimulation, the WT form of ErbB-2 and a transforming mutant (V659E) were transfected into CHO cells. Alongside, plasmids encoding c-Cbl were also used. Twenty-four h later, receptor molecules were immunoprecipitated with an antibody (top), and their ubiquitination was detected by using an anti-HA antibody (bottom). Total cell proteins were examined for receptor expression 48 h later by immunoblotting with an anti-ErbB-2 antibody.

**A**

B: CHO cells were cotransfected with plasmids encoding the WT form of ErbB-2 or the transforming mutant (V659E), along with a c-Cbl vector (+) or the respective empty vector (−). Total cell proteins were examined for receptor expression 48 h later by immunoblotting (IB) with an anti-ErbB-2 antibody. A, an expression vector encoding the transforming mutant of human ErbB-2 (V659E) was transfected into CHO cells. Alongside, plasmids encoding a sequence-tagged ubiquitin, c-Cbl (+), or a corresponding empty vector (−) were also used. Twenty-four h later, receptor molecules were immunoprecipitated (IP) and their ubiquitination was detected by using an anti-HA antibody (top). Whole cell lysates were examined for the effect on receptor level (bottom). B, CHO cells were cotransfected with plasmids encoding the WT form of ErbB-2 or the transforming mutant (V659E), along with a c-Cbl vector (+) or the respective empty vector (−). Total cell proteins were examined for receptor expression 48 h later by immunoblotting (IB) with an anti-ErbB-2 antibody. C, amino acid sequence alignment of the predicted Cbl-binding sites of ErbB-1 (10), ErbB-2, and ZAP-70 (18) is shown. The consensus tyrosine and proline residues are shown in bold. The indicated amino acid numbering refers to the corresponding human proteins. D, HEK-293T cells were transfected with plasmids encoding ErbB-1 and ErbB-2 (either WT or Y1112F), and 24 h later cells were exposed for 10 min to EGF (100 ng/ml). Control cultures (Cont.) were mock-treated prior to cell lysis. Cell extracts were directly analyzed by IB with an anti-ErbB-2 antibody (bottom). Alternatively, extracts were first incubated with an immobilized v-Cbl-GST protein and association with ErbB-2 was analyzed by IB (top).

**B**

c-Cbl recruitment is involved in ubiquitination of ErbB-2. A, an expression vector encoding the transforming mutant of human ErbB-2 (V659E) was transfected into CHO cells. Alongside, plasmids encoding a sequence-tagged ubiquitin, c-Cbl (+), or a corresponding empty vector (−) were also used. Twenty-four h later, receptor molecules were immunoprecipitated (IP) and their ubiquitination was detected by using an anti-HA antibody (top). Whole cell lysates were examined for the effect on receptor level (bottom). B, CHO cells were cotransfected with plasmids encoding the WT form of ErbB-2 or the transforming mutant (V659E), along with a c-Cbl vector (+) or the respective empty vector (−). Total cell proteins were examined for receptor expression 48 h later by immunoblotting (IB) with an anti-ErbB-2 antibody. C, amino acid sequence alignment of the predicted Cbl-binding sites of ErbB-1 (10), ErbB-2, and ZAP-70 (18) is shown. The consensus tyrosine and proline residues are shown in bold. The indicated amino acid numbering refers to the corresponding human proteins. D, HEK-293T cells were transfected with plasmids encoding ErbB-1 and ErbB-2 (either WT or Y1112F), and 24 h later cells were exposed for 10 min to EGF (100 ng/ml). Control cultures (Cont.) were mock-treated prior to cell lysis. Cell extracts were directly analyzed by IB with an anti-ErbB-2 antibody (bottom). Alternatively, extracts were first incubated with an immobilized v-Cbl-GST protein and association with ErbB-2 was analyzed by IB (top). Note the up-smearing of ErbB-2, which is attributed to poly-ubiquitination of the WT form. E, ubiquitination effects of EGF (left) and a bivalent Fab fragment of the L26 mAb (right) were examined in CHO cells. For stimulation by EGF, the WT form of ErbB-2 and a Y1112F mutant were introduced along with a plasmid encoding ErbB-1. Antibody stimulation was tested in cells expressing the V659E mutant of ErbB-2, and a double mutant (V659E and Y1112F) denoted EF. Ubiquitination was detected in receptor immunoprecipitates (top), and the ErbB-2 level was determined in whole cell lysates (bottom).
following incubation at 37°C with or without the L26 mAb (20 μM) to undergo antibody-induced turnover. Receptor turnover was followed as described in Methods in time intervals. and ErbB-2 were compared for their ability to exhibit no electrophoretic up-smearing, suggesting a reduction in electrophoretic mobility (top left panel). This confirms the importance of tyrosine 1112 for antibody-induced degradation of ErbB-2. Similarly, stimulation by EGF decreased the level of the WT (Fig. 4B), but not of the Y1112F mutant (Fig. 4A). Monitoring ErbB-2 turnover by using metabolic labeling confirmed the short half-life of the V659E transforming mutant relative to the WT form (Fig. 4C). We then used this mutant, the ubiquitination in CHO cells of which was stimulated by mAb L26, to examine the involvement of tyrosine 1112 in antibody-induced ErbB-2 turnover. Treatment with mAb L26 rapidly decreased the amount of the V659E mutant (Fig. 4D, right), but double mutant EF molecules harboring a phenylalanine at the putative c-Cbl-docking site were not degraded after mAb treatment. This confirms the importance of tyrosine 1112 for antibody-induced degradation of ErbB-2 and suggests that c-Cbl directs mAb-stimulated ErbB-2 molecules to proteasomal/lysosomal degradation, as it does for other receptors on stimulation with their natural ligands.

Cbl proteins emerge as pivotal players in the process, leading to inactivation of tyrosine kinase signaling pathways (9, 10). Relative to other growth factor receptors, the coupling of c-Cbl to ErbB-2 seems very weak, which may explain why this interaction escaped detection by previous studies (17, 20). Nevertheless, the realization that breast cancer immunotherapy directs HER-2/ErbB-2 to this major pathway of negative regulation is consistent with several observations. For example, targeted inactivation of the c-cbl gene in mice resulted in mammary hyperplasia, perhaps because c-Cbl negatively regulates the action of many mammary gland-derived growth factors (21). Consistent with negative regulation by c-Cbl, infection of an ErbB-2/Neu-driven neuroblastoma with a c-cbl-
encoding retrovirus caused tumor retardation in mice.\textsuperscript{4} The exact mechanism allowing c-Cbl to enhance ubiquitination of target proteins is unknown, but similar to other ubiquitin ligase complexes, it may involve association with still unknown ancillary proteins. Predictably, detailed understanding of ErbB-2 ubiquitination and degradation by a c-Cbl-mediated process will yield improvements in immunotherapy. For example, drugs known to enhance ubiquitination and degradation of ErbB-2, such as geldanamycin and herbimycin A (19), may enhance c-Cbl-mediated immunotherapy. Likewise, mAbs that better recruit ErbB-2 to the c-Cbl-regulated endocytic pathway are expected to act as superior therapeutic agents. Future studies will have to resolve the intricate machinery leading to ErbB-2 degradation.

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References

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