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Targeted Antireceptor Therapy with Monoclonal Antibodies Leads to the Formation of Inactivated Tetrameric Forms of ErbB Receptors

Keiji Furuuchi,* Alan Berezov,* Toru Kumagai,*† and Mark I. Greene2*

mAbs capable of disabling heterodimeric kinase complexes of the epidermal growth factor receptor (EGFR) and human EGFR type 2/neu have therapeutic relevance to various human cancers. In this study, we demonstrate that in addition to the dimer, EGFR and human EGFR type 2 can associate as homo- and heterotetramers. EGF-induced phosphorylation of the tetramers was significantly lower than that of the dimers, indicating that the tetrameric receptor complexes have impaired signaling activity. Targeting v-erb-b2 erythroblast leukemia viral oncogene homolog (erbB) receptors with mAbs promoted erbB tetrameric assembly, suggesting that a component of the antitumor activity may be mediated by the ability of Abs to shift the equilibrium from active dimeric to impaired tetrameric receptor complex states. This study suggests a novel therapeutic approach to disable signaling of erbB and potentially other receptors in tumors by biologic agents capable of inducing receptor tetramerization.  The Journal of Immunology, 2007, 178: 1021–1029.

A major development in cancer immune therapy was the demonstration that mAbs could reverse the malignant phenotype of v-erb-b2 erythroblastic leukemia viral oncogene homolog (erbB) transformed cells (1–5), a basic observation that has now reached the clinic (6, 7). The erbB receptor family consists of the four receptor tyrosine kinases—ErbB1 (epidermal growth factor receptor (EGFR)), ErbB2 (p185Her2/neu, human EGFR type 2 (HER2)), ErbB3, and ErbB4—that play a fundamental role in cellular proliferation, development, and differentiation (8–12). Overexpression of EGFR and HER2 has been implicated in the development and malignancy of various human neoplasms, including breast, lung carcinoma, and glioblastoma as well as other malignant diseases (13–15).

Previously, we (1–4) and others (16, 17) have shown that targeting erbB receptors with ectodomain-binding mAbs led to a reversal of the malignant phenotype. One mechanism that was found to accompany the reversal of the malignant phenotype was down-regulation of a proportion of the p185Her2/neu homomeric complexes and this was considered to be an important component of the therapeutic response. However, we noted that not all complexes (~40%) were actually down-regulated from the cell surface by the targeting monoclonals although the overall kinase activity of the cell was diminished. Moreover, cells were not induced to undergo apoptosis by this process and could recover malignant growth when Ab was removed. Furthermore, EGFR mAbs that prevent EGF ligand binding have also been shown to be clinically useful (18) which represents another mechanism of disabling erbB receptors by preempting ligand binding.

In addition to loss of receptors through down-modulation that was seen in vitro, other mechanisms of targeted therapy became apparent in vivo. Initially, we found that Ab-dependent cellular cytotoxicity (19) was a component of in vivo activity but was not essential and forms of the Ab that were unable to effectively bind to FcRs could still mediate tumor growth retardation in vivo. Ravetch et al. and colleagues (20–22) later found that other Abs targeting some human tumor Ags (CD20 and HER2/neu) expressed on a limited panel of transformed cells required Ab-dependent cellular cytotoxicity activity for optimal in vivo activity. Therefore, understanding details of optimal ways to disable erbB receptors with monoclonals and further clarification of the mechanism of homo- and heterodimeric associations between erbB receptors has therapeutic importance (1, 14, 23–26) and will also help in future therapies.

Currently, little is known regarding the possible role of higher ordered erbB oligomeric species in transformed cells. Clayton et al. (27) studied cancer cells overexpressing EGFR or p185Her2/neu receptors microscopically and suggested that erbB molecules might undergo higher orders of aggregation into tetramers that would be active and possess higher catalytic activity than that of the dimer (27–29). Others have also suggested that active tetramers might develop in this receptor system (27–29). We initially predicted the possible existence of erbB receptor tetramers by computer-assisted modeling, although our calculations predicted a reduction of the catalytic activity of EGFR homo- or heterotetramers because of the likely improper alignment of the putative EGFR and p185neu tetrameric components (30).

Despite the obvious need for studying oligomerization between members of the erbB receptor family, research in this area has been

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3 Abbreviations used in this paper: erbB, v-erb-b2 erythroblastic leukemia viral oncogene homolog; EGFR, epidermal growth factor receptor; HER2, human EGFR type 2; VSVG, vesicular stomatitis virus glycoprotein; IB, immunoblotting; IP, immunoprecipitation; AHNP, anti-HER2 peptide; Sbd, subdomain.

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limited by lack of an established biochemical protocol to analyze high molecular-mass tetrameric complexes (~700 kDa) that have relatively weak interreceptor interactions. To address this issue, we have recently developed a new approach to detect oligomerization of EGFR and p185Her2/neu with reproducible biochemical techniques. Using a sequential immunoprecipitation technique and using ultra-thin gels, we demonstrated that EGFR and HER2/p185Her2/neu form tetrameric complexes (31).

In this study, we have studied the ability of mAbs to affect the kinase activity of the complexes in transiently transfected and stable cell lines and growth in vitro. Our studies indicate that kinase-disabled tetrameric forms physiologically assemble at the cell surface. The early appearance of kinase-disabled forms suggests that even before receptor internalization, signal attenuation has begun. We have analyzed the effects of monoclonal anti-HER2 Abs on oligomerization of EGFR/HER2 receptors. In addition, we studied the initial mAb that were shown to target the rodent form of p185 neu and which cross-reactively bind to p185Her2/neu and found that Ab treatment leads to the formation of kinase-disabled tetrameric forms. As predicted from previous studies from our laboratory and others (15, 32–34), using mixtures of ectodomain-binding Abs further enhanced the formation of tetrameric receptor complexes. Finally, we propose a hypothesis that argues for the existence of tetramers as part of the dynamic process of receptor formation, signal attenuation, trafficking, and function.

Materials and Methods
Expression vectors
A schematic representation of the expression vectors used in this study is shown in Fig. 1 (35). Briefly, the pMVEGFR is a full-length human EGFR cDNA expression vector containing vesicular stomatitis virus glycoprotein (VSVG) in PMV expression vector (Roche). A series of CDNA of p185 neu were generated into pSecTagB plasmid (Invitrogen Life Technologies) with double Myc-His-tagged peptide, which allowed us to collect and detect these exogenous p185 neu species with certainty. The pTNeu expression vector has a single point mutation (V664G) in the pNeu vector (35). pNotNeu lacking extracellular subdomain IV (SbdIV) is identical with N901 (36).

Cell lines and transfection
Cos7 and 293T (no expression of ErbB2, ErbB3, and ErbB4, low level of EGFR), A431 (human lung cancer, EGFR; 2.6 × 10^5/cell), SKBR3 (human breast cancer, EGFR; 1 × 10^5/cells, HER2; 1 × 10^5/cells), and NE99 (ErbB receptor null NR6 stable transfectant with human wild-type EGFR containing the VSVG tag, EGFR; 2.8 × 10^5/cell) cell lines were used in this study. Cos7 and 293T cells were transfected with plasmids by using the DOTAP reagent (Roche) according to the manufacturer’s protocol.

Detection of oligomers
Following EGF stimulation (50 ng/ml) or serum-free medium for 10 min, cells were incubated with or without 2 mM nonpermeable bis(sulfosuccinimidy)suberate (BS3) cross-linker (Pierce) in PBS at 4°C for 45 min. After the reaction was terminated in saline solution with 10 mM Tris-HCl (pH 7.5) and 0.1 M glycine, the cells were lysed in radioimmunoprecipitation assay buffer (35). All cell lysates prepared in this study were precleared with protein G agarose to eliminate proteins with affinity to protein G agarose. In studies of isolation of hetero-oligomeric complexes (see Fig. 3A), sequential immunoprecipitation procedure was performed as we have reported previously (31). For detection of homo-oligomers of truncated p185 neu (see Fig. 3B), precleared protein lysates were collected by immunoprecipitation with the anti-His (Santa Cruz Biotechnology) Ab and eluted in SDS sample buffer with 10 mM DTT at 65°C for 15 min. Otherwise, the samples were immunoprecipitated with either anti-His Ab (Santa Cruz Biotechnology) or anti-EGFR Ab 1005 (Santa Cruz Biotechnology). All immunoprecipitated proteins were eluted in SDS sample buffer with 200 mM DTT at 65°C for 15 min and subjected to 3.7% SDS-PAGE analysis (31).

Laser-scanning microdensitometry analysis
Laser-scanning microdensitometry analysis based on NIH Image software was used to determine the signal density in this study. Relative amounts of erbB receptor dimers and tetramers were evaluated by comparison with the intensity of the monomeric receptor band which was taken as 100% in Figs. 2, 4, 6A, and 6B, and intensities of the tetramer bands were divided by four to account for molecular mass differences between the receptor forms. Averaged phosphotyrosine-protein ratios shown in arbitrary units were evaluated based on the phosphotyrosine content concentration of the corresponding complexes of ErbB receptors. We have used the averaged ratio of phosphotyrosine content against the concentration of whole EGFR (immunoprecipitation [IP] with EGFR and immunoblotting [IB] with VSVG) and Neu (IP with histidine and IB with Myc) in Fig. 2B. Time zero concentrations and phosphotyrosine-protein ratios were taken as 1.0 in Figs. 5A, 6B, and 6C.

Ab treatment
SKBR3 cells cultured in serum-free medium for 24 h were incubated with mouse monoclonal anti-HER2 Ab 4D5 (for domain IV) (Genentech), IgG1, κ (Sigma-Aldrich) (10 μg/ml; 66.7 nM) as a control Ab, a single chain of scFv of 7.16.4 Ab (133.4 nM), anti-HER2 peptide (AHNP; mimic some function of 4D5 Ab binding to the C-terminal loop of HER2 receptor) (133.4 nM) (37), or anti-HER2/neu Abs (7.16.4, which are thought to recognize extracellular domain IV; 2C4, which recognizes extracellular domain II; 1E1, which appears to recognize the extracellular domain II) (single Ab; 10 μg/ml, mixture; 5 μg/ml each) for 2 h and then treated in the presence of EGF (50 μg/ml) or serum-free medium for 10 min and lysated as described above.

Western blot analysis
We used mouse mAbs against VSVG-tagged peptide (Roche), Myc-tagged peptide (Invitrogen Life Technologies), HER2 (Cell Signaling), and EGFR (Ab13, Neo Marker) and anti-phosphotyrosine Abs specific for EGFR (pY1173) (sc-12351; Santa Cruz Biotechnology), for HER2 (pY1248) (sc-12352-R; Santa Cruz Biotechnology) and any phosphorylated tyrosine residues (PY99; Santa Cruz Biotechnology) for Western blotting analyses. We performed immunoprecipitation with anti-Myc Ab (Roche) and anti-polyhistidine Ab (G18; Santa Cruz Biotechnology) for exogenous p185 neu (pNeu, pTNeu, p4Neu, and pNex), and rabbit anti-EGFR Ab (1005; Santa Cruz Biotechnology) and rabbit anti-HER2 Ab (Cell Signaling Technology) for stable cell lines. Primary Abs were detected using goat anti-mouse or goat anti-rabbit, HRP-conjugated secondary Abs (Jackson ImmunoResearch Laboratories) and ECL detection (Amersham Biosciences).

Results
Detection of higher molecular mass receptor protein
To overcome difficulties of separating huge molecular masses ranging from 680 to 740 kDa, we used a low concentration (3.7%) SDS polyacrylamide gel with chemically cross-linked α2-macroglobulin as a molecular mass indicator in this study, as described previously (31). In addition, to enrich for receptor proteins and

FIGURE 1. Schematic representation of the expression vectors used in this study. SP, signal peptide; I-IV, subdomains I-IV, respectively; TM, transmembrane domain; TK, tyrosine kinase domain; Myc, Myc epitope; His, polyhistidine tag; V664G, a single-point mutation in the transmembrane region.
minimize the influence of background products, we developed several tagged fusion protein expression plasmids, which allowed us to define tetrameric forms with certainty (Fig. 1) (31).

We first investigated the relationship between the assembly or transition from dimeric to oligomeric forms and receptor expression densities. Graded and equivalent amounts (1.25, 2.5, and 5.0 μg) of pEGFR and pNeu plasmids were transiently cotransfected into Cos7 cells (Fig. 2). As shown in Fig. 2, A and C, left, heterotetramers were observed even when low doses of vectors were transduced (lanes 5 and 6). The very low doses of plasmids encode physiologic levels of receptors as judged by precipitation of cell surface receptor proteins and by flow cytometry (data not shown). Molecular mass densitometric analysis demonstrated that the averaged relative amount of heterodimers compared with the amount of monomer (taken as 100%) ranged between 10 and 18%, and similarly, that of heterotetramer was between 4.0 and 4.6% (Fig. 2, A and C, lanes 1, 3, and 5). The monomeric forms of the receptors in these gels arise from incompletely cross-linked complexes followed by separation upon gel filtration and were described previously.

Monomers that are released from these complexes that are not covalently cross-linked are then detected by immune blotting (23, 38).

It is noteworthy that the ratio of heterotetramer to heterodimer presented in Fig. 2 revealed a relatively consistent proportion even at low receptor levels similar to the ratio observed when large amounts of receptors were present. It is also notable that tetramerization can be detected in the absence of EGF. These consistent ratios indicate that heterotetramerization is a physiologic event in cells, rather than a receptor number-dependent event.

Our next goal was to estimate receptor tyrosine kinase activity of the tetrameric receptor forms. To that end, we measured the average phosphotyrosine content of dimers and tetramers in Cos7 cells first using an anti-polyhistidine Ab (Fig. 2, B and C, right) and also using an anti-VSVG Ab as an immunoprecipitation agent (data not shown). Compared with the heterodimeric receptor forms, the phosphotyrosine-protein ratio of the heterotetramers was 3- to 4-fold lower for different receptor densities, consistent with the decreased signaling activity of the observed tetramers with respect to the active dimers (Fig. 2B).

**FIGURE 2.** Oligomerization of erbB receptors in transiently transfected Cos7 cells. Cos7 cells transfected with indicated plasmid (EGFR, pMVEGFR; Neu, pNeu) in a various amounts (1.25, 2.5, and 5.0 μg) were treated with EGF (+) or without EGF (−). IP and IB were performed using indicated Abs. Each experiment was repeated multiple times. Representative raw data are shown (C) and signal density of each oligomer species was determined by laser-scanning microdensitometry analysis (A and B). A, Relative amounts and phosphotyrosine contents of erbB receptor dimers and tetramers at different receptor densities. B, Averaged phosphotyrosine-protein ratios. Shown in arbitrary units as a ratio of phosphotyrosine content to concentration based on the densitometric analysis. O, Higher oligomer complex; T, tetramer; D, dimer; M, monomer.
The stoichiometry and specificity of formation of the heterotetrameric complexes of the EGFR family members

The stoichiometry and specificity of the heterotetrameric complexes was next analyzed to exclude the possibility that proteins other than the EGFR family members were components of the complex. Because the molecular masses of the EGFR and HER2 receptors were similar, it was impossible to distinguish between homo- and heteromeric complexes formed between wild-type receptors. To resolve this problem, we used sequential immunoprecipitation to isolate oligomeric complexes developed by deletion mutants of p185\(^{\text{c-neu}}\) (Nex, 115 kDa) lacking most of their intracellular domains (36), allowing us to easily distinguish different receptor combinations by molecular mass. We minimized the influence of other ErbB species by using Cos7 cells and by using anti-tag-specific Abs against exogenous ErbB receptors for purification and detection. The expected molecular masses were 625 kDa for the 1:3, 570 kDa for the 2:2, and 515 kDa for the 3:1 Nex/EGFR complexes, respectively. Another advantage of using the pNex plasmid was reduced uncertainty about the final predicted molecular mass, because the pNex receptor complexes were predicted molecular mass that could be visualized under weak reducing condition (10 mM DTT). Clearly, homotetrameric complexes were probed with anti-Myt Ab, T; Tetramer; D, dimer; M, monomer. The details were described in Materials and Methods. Each experiment was repeated multiple times and representative data are shown.

FIGURE 3. The stoichiometry of the heterotetrameric complex of EGFR families and truncated p185\(^{\text{c-neu}}\). A, The stoichiometry of the heterotetrameric complex of EGFR families. Cos7 cells transfected with indicated plasmid (EGFR, pMEV; Neu, pNeu; Nex, pNex; −, No plasmid) were treated with EGF (+) by following sequential IP using anti-His Ab first and then anti-EGFR Abs (lanes 1 and 2) or EGFR Ab alone (lane 3). IB were performed using indicated Abs. B, Detection of high-ordered homo-oligomers of p185\(^{\text{c-neu}}\). Cos7 cells transfected with indicated plasmid (Neu, pNeu; Tneu, pTnue; Δ4Neu, pΔ4Neu) were directly lysated under weak reducing condition. Homo-oligomers of p185\(^{\text{c-neu}}\) were probed with anti Myc Ab, T, Tetramer; D, dimer; M, monomer. The details were described in Materials and Methods. Each experiment was repeated multiple times and representative data are shown.

Detection of tetramers of truncated p185\(^{\text{c-neu}}\)

We were also concerned that the cross-linking process in some way created the tetramers. To investigate whether the EGFR family members normally associate as tetramers, we used transient transfection using truncated p185\(^{\text{c-neu}}\) in Cos7 cells (Fig. 1) and prepared cell lysates without chemical cross-linkers (Fig. 3B). Interestingly, truncated p185\(^{\text{c-neu}}\) developed tetramers of the predicted molecular mass that could be visualized under weak reducing condition (10 mM DTT). Clearly, homotetrameric complexes form on the cell surface by the large interfaces with hydrogen bonds and hydrophobic forces in a physiologic rather than non-specific process (Fig. 3B). We also observed that the phosphotyrosine content of the tetramers was lower than that of the dimers as detected by the anti-phosphotyrosine Ab for HER2 (pY1248) and pan phosphotyrosine Ab PY99 (data not shown). Similarly, we have confirmed and detected tetramer formation of other truncated p185\(^{\text{c-neu}}\) forms in cells (data not shown).

FIGURE 4. Detection of oligomeric EGFR in a stable cell line NE99. Oligomerization of ErbB receptors in stable cell line NE99 expressing EGFR alone was analyzed. Lysates of cells stimulated with EGF (+) or without EGF (−) were IP with anti-EGFR and oligomers of ErbB receptors were visualized by anti-VSVG Ab (IB). Each experiment was repeated multiple times and representative data are shown (C) and signal density of each oligomer species was determined. A, Relative amounts and phosphotyrosine contents of erbB receptor dimers and tetramers. B, Phosphotyrosine-protein ratios. Shown in arbitrary units as a ratio of phosphotyrosine content to concentration based on the densitometric analysis. T, Tetramer; D, dimer; M, monomer.
To examine whether the tetramer ensembles occurred in other cells, we have transiently transfected either pNeu or pEGFR plasmids into the 293T (data not shown). As in Cos7 cells, we observed tetramers of p185\textsuperscript{c-neu} and EGFR in 293 T cells. In addition, both tetrameric forms (p185\textsuperscript{c-neu} and EGFR) were less kinase active than the dimeric forms of the receptors. Therefore, we concluded that EGFR and p185\textsuperscript{c-neu} form tyrosine kinase impaired homo- and heterotetramers in transiently transfected cells.

Detection of higher ordered EGFR complexes in various stable cell lines

To confirm that receptor tetramerization is a general phenomena that is not limited to the transiently transfected Cos7 and 293T cells (27–29), we tested whether erbB receptors can also form tetramers in cell lines, which stably express wild-type EGFR. To address this issue, we have tested cell lines with different expression levels of EGFR, namely the NE99 and A431 lines. NE99 express one-tenth the level of receptor numbers compared with the dimers (Fig. 4A). Upon EGF stimulation (Fig. 4C, lane 1), the molar amount of the formed homotetramers was \( \sim 6 \)-fold lower than that of the homodimers (Fig. 4A). However, the phosphotyrosine content in the homotetramers (Fig. 4C, lane 3) was \( \sim 24 \)-fold lower than in the homodimers (Fig. 4A). Therefore, the phosphotyrosine-protein ratio (and thus signaling activity) of the tetrameric receptor forms (Fig. 4C, lanes 1 and 3) was \( \sim 4 \)-fold lower than that of the dimers (Fig. 4B).

We detected homodimeric and heterotetrameric EGFRs in the case of the human carcinoma cell line (A431) (data not shown). We also detected the homotetramers in both sets of cells in the absence of EGF. The phosphotyrosine-protein ratio for the tetramers in that cell line was about one-half of the corresponding value for the dimers (data not shown).

Summarizing the data obtained for NE99 and A431 cells, we conclude that the oligomerization pattern of erbB receptors is similar in both of these cell lines. In addition, it is noteworthy that the phosphotyrosine content of homotetramers in both cell lines was remarkably lower than that of homodimers, indicating that higher order oligomers of EGFR such as tetramers have an impaired signaling activity compared with the dimers when ectopically transfected or when overexpressed within a cell.

Anti-p185\textsuperscript{HER2/neu} mAbs induce catalytically inactivated oligomeric forms of EGFR and HER2

We have to this point, demonstrated that the EGFRs naturally form tetramers and dimers and moreover that the phosphotyrosine content in tetramers is lower than in dimers. However, the biological significance of the tetrameric receptor forms remained unclear.

Recently, an anti-p185\textsuperscript{HER2/neu} Ab that reverses the malignant phenotype has been used as a kinase-inhibiting immunotherapy for human tumors overexpressing HER2 receptor. These HER2-disabling mAbs act in a similar manner as earlier mAb forms (7,16,4 mAb) that established targeting erbB receptors as a rational therapeutic approach (1–3, 39). Effects of mAbs on higher ordered receptor homo- or hetero-oligomer formation have not been studied.

Therefore, we tested the effect of murine anti p185\textsuperscript{HER2/neu} mAbs (mAb 4D5 which binds p185\textsuperscript{Her2/neu}) on erbB receptor oligomerization in SKBR3 cells for various times (Fig. 5, A and B). Representative data are shown (Fig. 5, C and D). We found that mAb 4D5 induced tetrameric species of EGFR and p185\textsuperscript{Her2/neu} in a time-dependent manner. Molar ratios of each heterooligomeric species were compared with that of control untreated cells. These studies demonstrated that the amount of tetramers increased time dependently even after 15 min, and further increased over the incubation period of 240 min (Fig. 5A). In contrast, the amount of both the dimer and monomer decreased. The residue-specific phosphotyrosine content of each species was compared with that of untreated control cells. The pattern of specific phosphorylation was...
FIGURE 6. Mixture of anti-HER2 mAbs induces impaired tetramers of erbB receptors while monovalent fragments of Abs have limited effects. A. Monovalent ScFv fragment of mAb 4D5 and AHNP peptide are incapable of developing tetrameric forms of EGFRs. B. Effect of combination of mAbs 4D5 and 2C4 on tetramer/dimer concentrations, phosphorylation activity, and time course of formation of dimeric and tetrameric receptors. C. Effect of combination of mAbs 4D5 and 1E1 on tetramer/dimer concentrations, phosphorylation activity, and time course of formation of dimeric and tetrameric receptors. Tetrameric species were demonstrated by 2 h of therapy. Mixtures of 4D5 and 2C4 as well as mixtures of 4D5 and 1E1 synergistically enhance formation of inactive tetrameric forms of erbB receptors. Error bars (SE) are shown for each time point. Each experiment was repeated and representative data are presented.

A mixture of anti-p185HER2/new mAbs synergistically induces impaired tetramers of erbB receptors while monovalent forms of Abs cannot

mAb 4D5 treatment induced the induction of catalytically impaired erbB receptor complexes on the cell surface. However, it was unclear whether the monovalent forms of Abs or Ab mimetics could do the same. To address this issue, we have tested the effect of rat anti-p185c-neu Ab (7.16.4, cross-reacting to p185c-neu and p185HER2/new), a ScFv fragment of mAb 7.16.4 and an AHNP (37, 40–42). It is noteworthy that 7.16.4 binds to p185c-neu and to a lesser extent (~70 times less) to human p185HER2/new. Although mAb 7.16.4 could induce disabled tetrameric complexes of human erbB receptors, it is notable that the ScFv of 7.16.4 fragment was unable to induce tetramers to any extent. Moreover, AHNP, a monovalent peptide structural mimetic of the 4D5 CDR3, as well as control mouse monoclonal IgG1 Ab, also could not induce tetramers (Fig. 6A). The induction of erbB receptor tetramers was considered as a dominant event triggered by bivalent mAbs.

Previously, we have demonstrated that the mixtures of Abs reactive with two distinct regions on the p185Her2/new molecule resulted in synergistic antitumor effects in vivo (4, 15). However, the contribution of tetramerization of erbB receptors in cells treated with two mAbs has not been studied. To address this issue, we treated SKBR3 cells with mixtures of mAbs targeting the ectodomain of HER2 receptors (2C4 and 1E1) (Fig. 6, B and C). We found a mixture of mAbs synergistically enhanced the formation of phosphorylation impaired tetramer complexes of the erbB receptors using a combination of Abs (4D5 and 2C4 and 4D5 and 1E1).

Moreover, in distinction to the time pattern of tetramer formation induced by mAb 4D5 alone (Fig. 5), we observed more rapid induction of the tetramers of erbB receptors by combination of the two Abs (Fig. 6, B and C, right). In addition, the tetramers observed after even a brief 15-min treatment with a mixture of Abs had significantly diminished kinase activity (data not shown).

In separate studies, we have found that using the 4D5 on T6-17 cells expressing only HER2/new induced kinase inactive homotetrameric species (data not shown). Therefore, heterotetramers and homotetramers are created after disabling erbB kinase complexes with targeted mAbs in different cell types. Both constitutively formed and Ab-induced oligomeric forms of erbB receptors are significantly less active than dimeric forms as deduced from the residue-specific tyrosine phosphorylation patterns. In addition, we studied the effect of mAb 4D5 on the proliferation of SKBR3 cells

inversely proportional to the density of tetramers while it was directly proportional to dimer density (Fig. 5B). It is noteworthy that the phosphotyrosine content of the 4D5-induced tetramers was lower than that of the dimers.
and verified that mAb 4D5 had an inhibitory effect on cell growth (data not shown).

Discussion

We have identified a new mechanism by which anti-receptor Abs lead to attenuation of receptor signaling. The studies document that some monoclonal anti-p185 Abs lead to apparently misaligned and disabled receptor tetramers with diminished kinase activity. In addition to mAbs being able to induce nonfunctional tetrameric species of p185, it is also apparent that the Abs act to promote a normal physiologic process that occurs in the cell to attenuate signaling. These studies are of translational relevance because mAbs can reverse the malignant phenotype of erbB-transformed cells (1–7, 9, 15).

Currently, it is widely accepted that dimeric forms of erbB receptors are the predominant physiologically relevant species for active signaling (38, 43, 44) and indeed, active dimers of erbB receptors are responsible for the malignant phenotype. However, it was not clear whether tetrameric forms of erbB receptors possessed biologically relevant functional activity. In this study, we have demonstrated that full-length EGFR and HER2 receptors can proportionally associate as low activity tetramers in transiently transfected and stable cell lines expressing different erbB receptor numbers and ratios. The obtained results support our hypothesis that formation of erbB receptor tetramers represents a physiologically relevant event in all studied cell lines and rules out the notion that higher receptor number or density of the ErbB receptors promotes higher oligomerization (9). Second, we studied the biologically relevant functional activity of the tetrameric receptors by examining their phosphorylation status, which is highly dependent on receptor orientation for kinase activation (30, 36, 38, 43, 45). Thus, the fact that the phosphotyrosine of dimers and tetramers was measurable even though diminished suggests that they are indeed physiologically relevant functional units. Indeed, nanometer scale fluorescence resonance energy transfer technology strongly supports our observation (27). Our observations further indicate that, contrary to a proposed model, tetramers are the most active species (27–29); higher ordered erbB tetrameric forms are less active than dimeric forms. Third, we have demonstrated that immunotherapeutic use of mAbs targeting the ErbB receptors induces formation of catalytically impaired oligomeric receptors. Taken together, these data suggest that there are significant biological implications of the observed oligomeric receptor complexes and also provide a rationale for combining different anti-erbB Abs as an immunotherapeutic for malignant tumors.

Because tetrameric complexes have not been detected in the existing crystal structures of extracellular domains of erbB receptors, information regarding the tetramerization site on the receptor surface is not known. However, this study is consistent with our earlier prediction that the biological role of the higher oligomerization of erbB receptors might be attenuation of receptor signaling due to structural constraints on certain autophosphorylated residues in the tyrosine kinase domain (30). One potential interreceptor contact that could lead to receptor tetramerization is the N-terminal part of subdomain II (SbdII) that is involved in formation of a dimer in the crystal structure of erbB3 (46). In the receptor tetramerization scheme proposed in Fig. 7, this site is assumed to be a contact interface between the dimeric receptors for both locked (Fig. 7A) and extended (Fig. 7B) dimeric complexes. It could also be involved in the interaction between extended and locked dimers forming a hybrid tetramer.

The tetramerization of ligand-bound active dimeric complexes leads to a conformation in which receptor ectodomains are oriented at an angle to the cell membrane resulting in partial misalignment of the intracellular kinase domains and reduction of signaling activity (Fig. 7B). This orientation would only be possible if there was a flexible hinge region between the cysteine-rich part of SbdIV and the transmembrane domain. Because this region is disordered in all crystal structures of erbB receptors solved so far, it is likely to represent a flexible surface and therefore able to allow significant degrees of freedom for the orientation of receptor ectodomains. The speculative model presented in Fig. 7 shows one possible arrangement of the tetramer that is consistent with our experimental data. Structural studies will be necessary to elucidate the actual conformation of the tetrameric receptor complexes. Collectively, our studies indicate that tetramerization may trap the active dimers in an autoinhibitory conformation effectively reducing their signaling activity (Fig. 7B).
mAbs against p185 neu or HER2 have been developed to induce regression of tumors overexpressing p185 neu. The details of the mechanism of the ErbB2 targeting Abs such as herceptin is poorly characterized. Our findings derived from the analysis of SKBR3 which is often used as a typical breast cancer cell line in experimental studies. We have demonstrated that mAbs induced catalytically impaired hetero-oligomerization (Figs. 5 and 6) and simultaneously inhibited cell proliferation of SKBR3 using the same concentration of the mAb 4DS (10 µg/ml) (data not shown). Thus, erbB heterotetramerization induced by herceptin (which is the humanized form of 4DS) represents one of the processes that accounts for its antitumor activity (48, 49). It is notable that herceptin failed to cause down-regulation of ErbB receptors (which is the humanized form of 4D5) represents one of the processes that accounts for its antitumor activity (48, 49). It is notable that herceptin failed to cause down-regulation of ErbB receptors via an endocytosis pathway in SKBR3 cells (50).

Because the tetrameric and oligomeric erbB receptor forms described in this study have been shown to possess significantly lower signaling activity than dimeric erbB receptor forms, as deduced from the residue-specific tyrosine-phosphorylation patterns, our results suggest a novel approach for disabling receptor signaling. The therapeutic agents that can induce oligomerization or stabilize the already formed oligomers are likely to inhibit receptor activity by increasing the number of low activity oligomeric complexes. This effect may be achieved by mAbs or by dimeric or tetrameric forms of small molecules capable of binding and cross-linking erbB receptors into functionally impaired oligomeric ensembles. We have found, in agreement with our previous studies (4, 19) and others (51, 52), that a simple combination of two Abs reactive to distinct epitopes is most effective to create tetrameric forms of inactivated homomers or even heteromers (Fig. 6).

Our studies thus identify that signal attenuation on the cell surface occurs in part as a consequence of kinase inactive tetramer formation from receptor dimers. This may occur directly by receptor misalignment or may be aided by other molecules such as SIRPs as reported earlier (32). Most importantly, receptor tetramerization is a biologically relevant step in receptor signal attenuation. Finally, these studies also suggest that signals that cause erbB receptors to aggregate into a low activity tetramer may be immunotherapeutically important.

Disclosures

The authors have no financial conflict of interest.

References

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