Pertuzumab, a novel HER dimerization inhibitor, inhibits the growth of human lung cancer cells mediated by the HER3 signaling pathway

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A humanized anti-HER2 monoclonal antibody pertuzumab (Omnitarg, 2C4), binding to a different HER2 epitope than trastuzumab, is known as an inhibitor of heterodimerization of the HER receptors. Potent antitumor activity against HER2-expressing breast and prostate cancer cell lines has been clarified, but this potential is not clear against lung cancers. The authors investigated the *in vitro* anti-tumor activity of pertuzumab against eight non-small cell lung cancer cells expressing various members of the HER receptors. A lung cancer 11_18 cell line expressed a large amount of HER2 and HER3, and its cell growth was stimulated by an HER3 ligand, heregulin (HRG)-α. Pertuzumab significantly inhibited the HRG-α-stimulated cellular growth of the 11_18 cells. Pertuzumab blocked HRG-α-stimulated phosphorylation of HER3, mitogen-activated protein kinase (MAPK), and Akt. In contrast, pertuzumab failed to block epidermal growth factor (EGF)-stimulated phosphorylation of EGF receptor (EGFR) and MAPK. Immunoprecipitation showed that pertuzumab inhibited HRG-α-stimulated HER2/HER3 heterodimer formation. HRG-α-stimulated HER3 phosphorylation was also observed in the PC-9 cells co-overexpressing EGFR, HER2, and HER3, but the cell growth was neither stimulated by HRG-α nor inhibited by pertuzumab. The present results suggest that pertuzumab is effective against HRG-α-dependent cell growth in lung cancer cells through inhibition of HRG-α-stimulated HER2/HER3 signaling. (*Cancer Sci* 2007; 98: 1498–1503)

The HER family of receptor tyrosine kinases consists of four members: EGFR (also termed HER1/ErbB-1), HER2/ErbB-2/Neu, HER3/ErbB-3, and HER4/ErbB-4.[1] Binding of ligands leads to the homo- and heterodimer formation of the receptor tyrosine kinase.[2] There are numerous HER-specific ligands that generate signaling diversity within the cell.[3] EGF, amphiregulin, and TGF-α are known as a specific ligand of EGFR. HB-EGF, β-cellulin, and epiregulin have dual specificity for binding to EGFR and HER4. HRG-α binds HER3 and HER4.[4] No direct ligand for HER2 has been discovered. Dimerization consequently stimulates the intrinsic tyrosine kinase activity of receptors, and activates the downstream-signaling molecules such as MAPK, Akt, JAK, and STAT.[5,6]

Pertuzumab is a humanized monoclonal antibody and binds to the dimerization domain of HER2 distinct from the domain that trastuzumab binds to.[7] Therefore, pertuzumab is known as a dimerization inhibiter between HER2 and the other HER family receptors. A phase I trial of pertuzumab has been performed for advanced tumors,[8] and phase II studies of pertuzumab are underway. Two members of the HER family, HER2 and HER3, act as key oncogenes in breast cancer cells.[9,10] *In vitro* and *in vivo* anti-tumor activities of pertuzumab have been reported in breast tumors through the inhibition of the HER2/HER3 heterodimer formation.[11,12] In lung cancer cells, EGFR plays a crucial role in their biological behavior, but it is unclear whether pertuzumab inhibits the growth of the lung cancer cells mediated by HER family receptors.

The authors have focused on the growth inhibitory effect of pertuzumab against NSCLC cells expressing different types of HER receptors, and analyzed the mechanism of action of pertuzumab in response to the HER receptor ligand.

Materials and Methods

Reagents. Pertuzumab (Omnitarg, 2C4) was provided in sterile water at 25 mg/mL by Genentech, Inc. (South San Francisco, CA, USA) before use. All chemicals and reagents were purchased from Sigma (St Louis, MO, USA) unless noted otherwise.

Cell lines. The human NSCLC cell lines PC-7, PC-9, and PC-14 (Tokyo Medical University, Tokyo, Japan),[13,14] A549 (American Type Culture Collection, Manassas, VA, USA), and PC-3, Ma-1, Ma-24, and 11_18,[15] were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated FBS (Life Technologies, Rockville, MD, USA).

Cell stimulation and lysis. Cells were starved in serum free RPMI 1640 medium for 24 h and treated with EGF, TGF-α, HB-EGF, and HRG-α at 100 ng/mL for 10 min. Cells were washed twice with ice-cold PBS, and lysed with lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 5 mM sodium pyrophosphate, 50 mM NaF, 1 mM sodium vanadate, 4 mg/mL leupeptin, 4 mg/mL apoprotein, 1 mM PMSF). Protein concentration of the supernatants was determined by the BCA protein assay (Pierce, Rockford, IL, USA).

Immunoprecipitation. Cell lysates (1000 μg) were incubated with the anti-HER2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Protein G magnetic beads (New England BioLabs, Beverly, MA, USA) were added for 2 h. Beads were washed three times with lysis buffer, resuspended in SDS sample buffer with 2% β-mercaptoethanol, boiled, and separated using SDS-PAGE.

Western blotting. Cell lysates were electrophoretically separated on SDS-PAGE and transferred to a polyvinylidene difluoride

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Abbreviations: BCA, bicinchoninic acid; ECL, electrochemiluminescence; EDTA, ethylene diamine tetra-acetic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FBS, fetal bovine serum; HB-EGF, heparin-binding epidermal growth factor; HRG-α, heregulin-α; JAK, Janus kinase; MAPK, mitogen-activated protein kinase; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; NSCLC, non-small cell lung cancer; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; RPMI, Roswell Park Memorial Institute; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; STAT, signal transducer and activator of transcription; TGF-α, transforming growth factor-α.

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membrane (Millipore, Bedford, MA, USA). The membrane was probed with each antibody against EGFR and HER2 (Transduction Laboratory, San Diego, CA, USA), HER3 (Santa Cruz Biotechnology), phospho-EGFR (Tyr1068), phospho-HER3 (Tyr1289), MAPK, phospho-MAPK (Thr202/204), Akt, phospho-Akt (Ser473) (Cell Signaling, Beverly, MA, USA), phosphotyrosine (PY-20, Transduction Laboratory), and β-actin (Sigma) as the first antibody, followed by detection using a horseradish peroxidase-conjugated secondary antibody. The bands were visualized with ECL (Amersham, Piscataway, NJ, USA), and images of blotted patterns were analyzed with NIH image software (National Institutes of Health, Bethesda, MD, USA).

Growth inhibition assay. A 100-μL volume of cell suspension (5000 cells/well) in serum-free RPMI 1640 medium was seeded into a 96-well plate and 50 μL of each drug at various concentrations and 50 μL of EGF, TGF-α, HB-EGF, and HRG-α, at 100 ng/mL was added. Human IgG1 (Calbiochem, Cambridge, MA, USA) was used as isotype control. After incubation for 72 h at 37°C, 20 μL of MTS solution (Promega, Madison, WI, USA) was added to each well and the plates were incubated for a further 2 h at 37°C. The absorbance readings for each well were determined at 490 nm with a Delta-soft on a Macintosh computer (Apple, Cupertino, CA, USA) interfaced to a Bio-Tek Microplate Reader EL-340 (BioMetallics, Princeton, NJ, USA). For ligand-stimulated growth of cells, the experiment was performed in six replicate wells for each ligand and carried out independently three times. For growth inhibition of pertuzumab, the experiment was performed in three replicate wells for each drug concentration and carried out independently three times as described elsewhere.[16]

Results

HRG-α dependent cell growth in lung cancer cells. Ligand-dependent cell growth of lung cancer cells was examined (Fig. 1). The addition of EGF, TGF-α, and HB-EGF increased the cell growth of the PC-3, 11_18, and A549 cells, but not that of the PC-7, PC-9, PC-14, Ma-1, and Ma-24 cells. HRG-α addition significantly increased the growth of the 11_18 cells (390% of control, $P < 0.01$ by t-test) and Ma-24 cells (204% of control, $P < 0.01$ by t-test), but did not influence the growth of any other cells. These findings suggest that the growth of the 11_18 and Ma-24 cells is depending upon HRG-α.

Pertuzumab inhibits HRG-α-dependent cell growth of the 11_18 and Ma-24 cells. Pertuzumab inhibited cell growth stimulated by HRG-α (IC_{50} = 0.12 μg/mL) but not stimulated by EGF, TGF-α, and HB-EGF in the 11_18 cells (IC_{50} > 100 μg/mL; Fig. 2). Pertuzumab also inhibited HRG-α dependent cell growth in the Ma-24 cells (IC_{50} = 39.8 μg/mL). Isotype control human IgG1 had no effect on ligand-dependent growth in the 11_18 and Ma-24 cells (data not shown). The growth of the other cells was not affected by exposure to pertuzumab (data not shown). This finding suggests that pertuzumab selectively inhibits HRG-α-dependent cell growth.

Ligand-stimulated phosphorylation of HER receptors. The expression levels of the HER receptors in the pertuzumab-sensitive (11_18 and Ma-24 cells) and pertuzumab-resistant cell (PC-9 cells) lines were determined using western blotting (Fig. 3a). Comparison of the protein expression levels of EGFR revealed high to moderate expression in the PC-9 and Ma-24 cells. EGFR was also detected in the 11_18 cells, although the expression in this

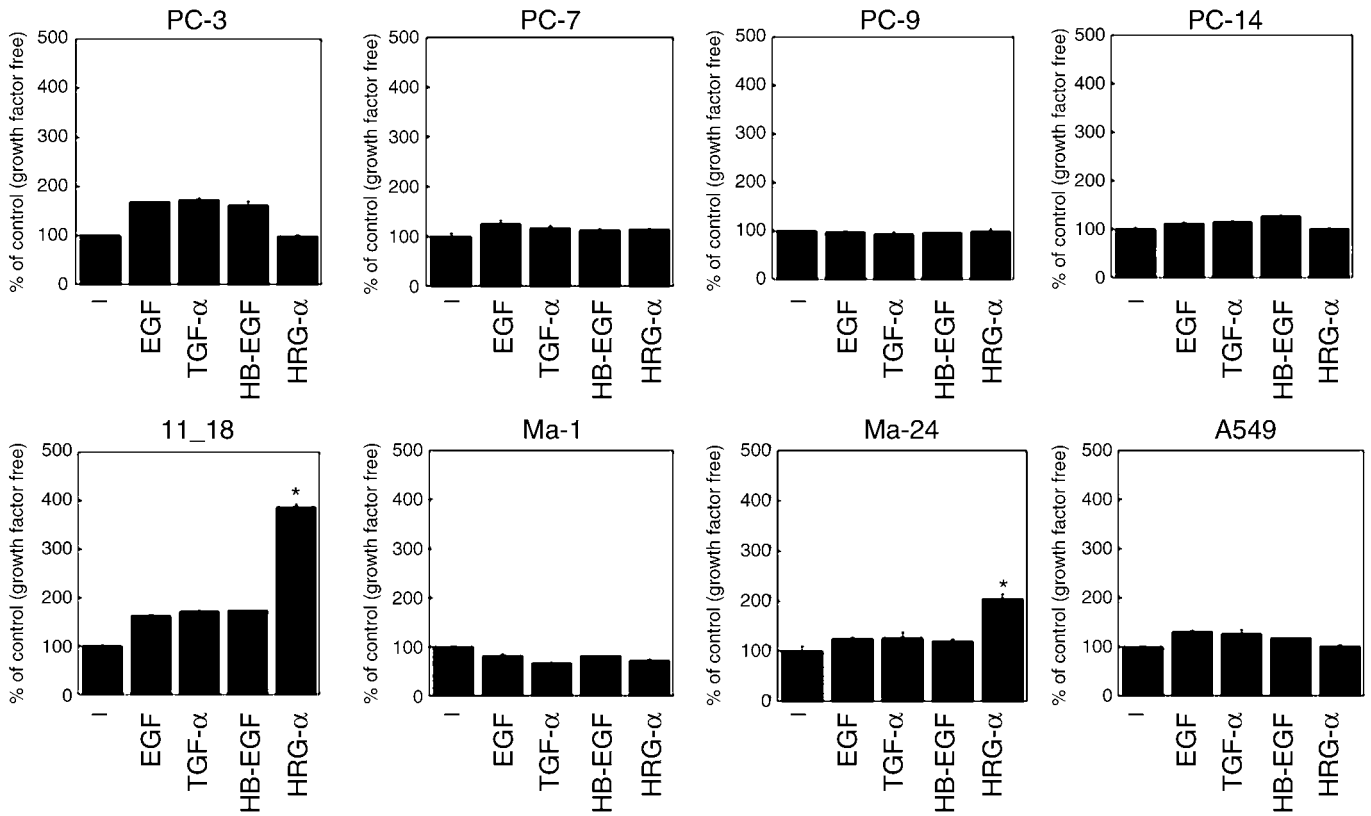

Fig. 1. Ligand-dependent cell growth in the lung cancer cells. Non-small cell lung cancer cells were stimulated with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), and heregulin (HRG)-α. After incubation for 72 h, cell growth was determined using the MTS assay. The growth of cells was presented as the percentage of absorbance compared with ligand-untreated cells. Error bars represent SE. *Significant difference ($P < 0.01$; t-test) compared to the ligand-non-stimulated cells. Data shown are representative of at least three independent experiments with similar results.

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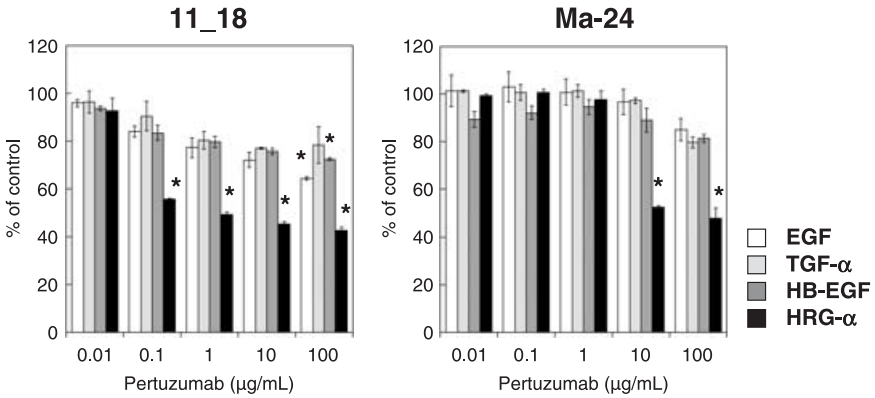

Fig. 2. Growth inhibitory effect of pertuzumab in the lung cancer cells. The lung cells were exposed to pertuzumab (0.01–100 μg/mL) for 72 h in serum free medium with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), or heregulin (HRG)-α. The viability was determined using the MTS assay. Result are presented as the percentage of absorbance compared with pertuzumab-untreated cells. Error bars represent SE. *Significant difference ($P < 0.01$; *t*-test) compared to pertuzumab-untreated cells. Data shown are representative of at least three independent experiments with similar results.

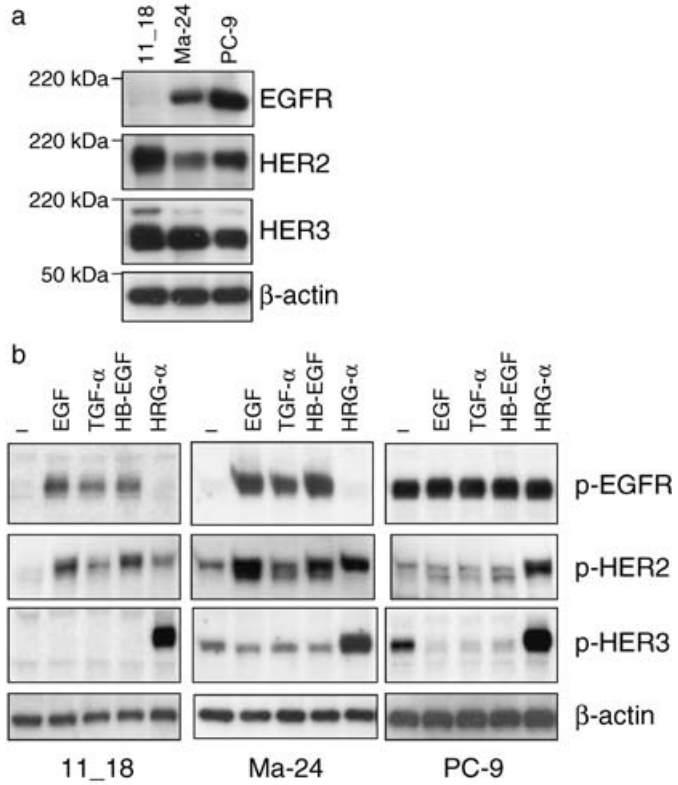

Fig. 3. Expression and phosphorylation of HER receptors in non-small cell lung cancer cells. (a) Expression of epidermal growth factor receptor (EGFR), HER2, and HER3 was detected using western blot analysis. Each lane contained 20 μg protein. β-Actin was used as a loading control. (b) The cells were stimulated with or without 100 ng/mL of epidermal growth factor (EGF), transforming growth factor (TGF)-α, heparin-binding epidermal growth factor (HB-EGF), and heregulin (HRG)-α for 10 min. Phosphorylation of EGFR and HER3 was detected using western blot analysis. Phosphorylation of HER2 was detected using immunoprecipitation followed by western blotting. β-Actin was used as a loading control. Data shown are representative of at least two independent experiments with similar results.

cell line was weak. The expression levels of HER2 were higher in the PC-9 and 11_18 cells than in the Ma-24 cells, which only expressed moderate levels of this receptor. All three cell lines showed strong expression of HER3. HER4 could not be detected in any of the three cell lines (data not shown). In contrast, these lung cancer cell lines expressed different types of EGFR mutations; the PC-9 cells had a 15-base deletion mutant (delE746-A750, exon 19), the 11_18 cells had a L858R point mutation (exon 21) of EGFR, and the Ma-24 cells had a E709G point mutation (exon 18) of EGFR. No mutations were detected in exons 19–21 of HER2 (data not shown).

Next, the ligand-stimulated phosphorylation of the HER receptors in the lung cancer cells after serum starvation was examined (Fig. 3b). While the ligands for EGFR (EGF, TGF-α, and HB-EGF) phosphorylated cellular EGFR in the 11_18 and Ma-24 cells, the EGFR in the PC-9 cells was hyperphosphorylated even under the non-stimulated condition, because PC-9 cells express an active mutant of EGFR. These results suggest that the EGF/TGF-α or HB-EGF–EGFR signals are active in lung cancer cells. The ligands for HER3 (HRG-α) specifically phosphorylated HER3 in the 11_18, Ma-24, and PC-9 cells. Phosphorylation of HER2 was analyzed by immunoprecipitation using an anti-HER2 antibody followed by western blotting for phosphotyrosine. The ligands for EGFR and HER3 phosphorylated HER2 in the 11_18 and Ma-24 cells, whereas only HRG-α but not the other ligands specifically phosphorylated HER2 in the PC-9 cells. These findings also suggest that the HRG-α–HER3 signal is active in lung cancer cells.

Pertuzumab blocks HRG-α but not EGF-stimulated signals. An inhibitory effect of pertuzumab on HRG-α-dependent cell growth in the 11_18 cells was demonstrated. To examine the effect of pertuzumab on signal transduction of both EGFR and HER3 in this cell line, the 11_18 cells were exposed to pertuzumab (0.2–200 μg/mL for 6 h) (Fig. 4a,b). HRG-α-stimulated phosphorylation of HER3 was dose-dependently inhibited by exposure to pertuzumab in the 11_18 cells, whereas EGFR phosphorylation was not stimulated by HRG-α stimulation (data not shown). MAPK and Akt were phosphorylated by HRG-α stimulation and these were inhibited by pertuzumab dose-dependently in the 11_18 cells. In contrast, EGF-stimulated phosphorylation of EGFR and MAPK was not inhibited by pertuzumab in the 11_18 cells. Phosphorylation of Akt was not detected by addition of EGF in the 11_18 cells. EGF did not phosphorylate HER3 and pertuzumab did not affect it (data not shown). Taken together, these results showed that pertuzumab inhibited HRG-α-stimulated phosphorylation of HER3, MAPK, and Akt, but not EGF-stimulated EGFR phosphorylation signaling.

HER3 is phosphorylated in response to HRG-α in the PC-9 cells as observed in the 11_18 cells, but the growth of the PC-9 cells was not increased by HRG-α (Figs 1,3b). To clarify the phosphorylation-inhibitory potential of pertuzumab, the effect of pertuzumab on signal transduction of the PC-9 cells was examined (Fig. 4c). When the PC-9 cells were stimulated by the addition of HRG-α, HER3 was phosphorylated in the PC-9 cells, but phosphorylation of HER3 was not inhibited by pertuzumab (20 and 200 μg/mL for 6 h). EGFR expressed in the PC-9 cells is constitutively active and pertuzumab failed to affect

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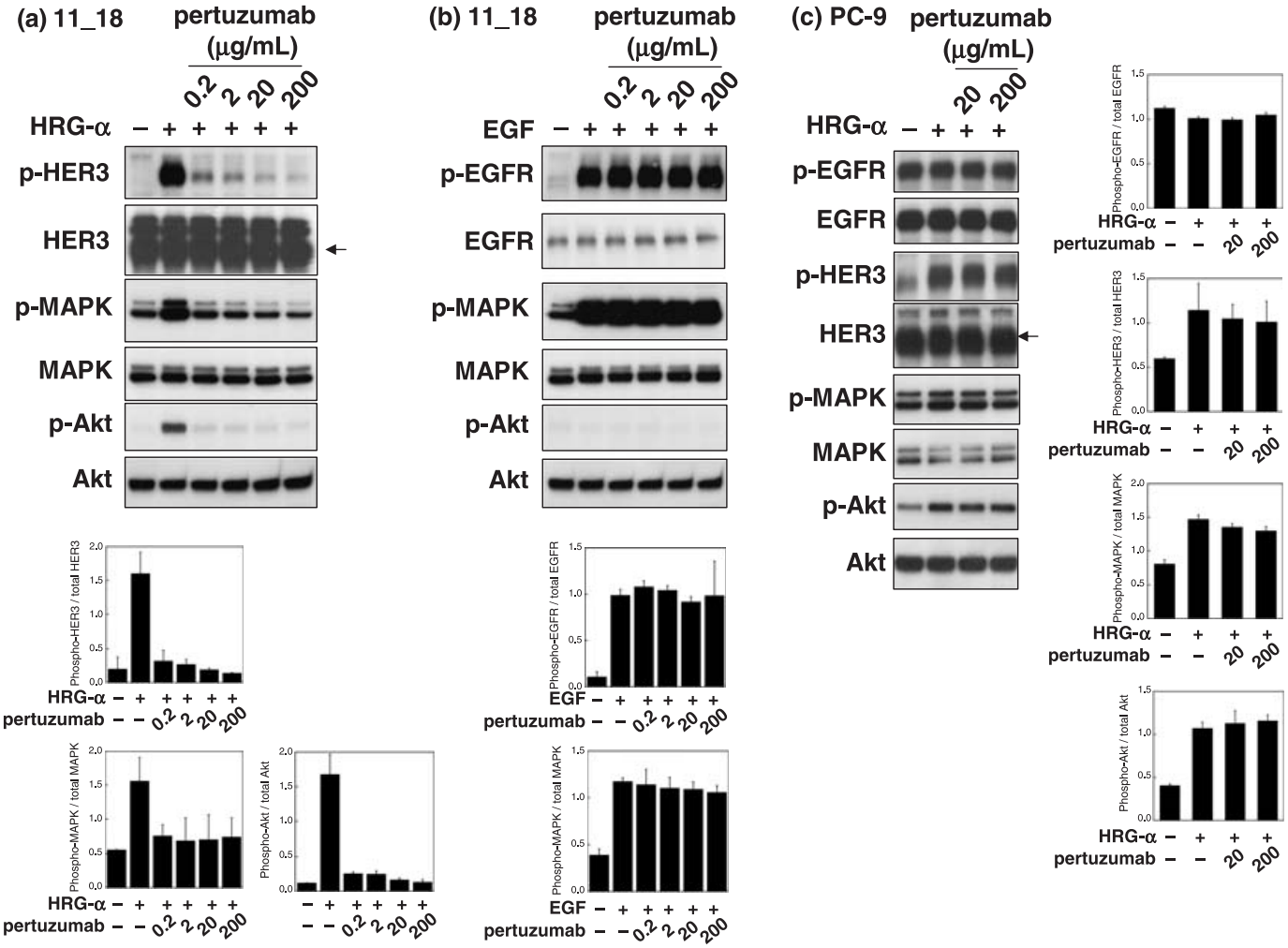

Fig. 4. Effect of pertuzumab on epidermal growth factor receptor (EGFR) and HER3 phosphorylation and their downstream signaling pathways. The 11_18 and PC-9 cells were exposed to pertuzumab for 6 h and stimulated with either heregulin (HRG)-α or epidermal growth factor (EGF) for 10 min. Cell lysate were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotted for indicated antibodies. The intensities of bands were quantified by densitometer. (a) HRG-α-stimulated 11_18 cells. (b) EGF-stimulated 11_18 cells. (c) HRG-α-stimulated PC-9 cells. Data shown are representative of at least two independent experiments with similar results. MAPK, mitogen-activated protein kinase.

the phosphorylation level of the EGFR. Phosphorylation of MAPK and Akt was detected by the addition of HRG-α, but these were not inhibited by pertuzumab. These results suggest that pertuzumab is unable to affect HRG-α-stimulated phosphorylation of HER3 in the PC-9 cells.

To clarify the effect of pertuzumab on HER2 phosphorylation and HER2/HER3 heterodimer formation, cell lysates were immunoprecipitated with anti-HER2 antibody (Fig. 5a,b). HRG-α stimulation increased HER2/HER3 heterodimer formation in the 11_18 cells, and pertuzumab decreased HRG-α-stimulated heterodimer formation. EGFR/HER2 heterodimer formation could be barely detected by HRG-α stimulation because of slight expression of EGFR in the 11_18 cells. In the case of EGF stimulation, HER2/HER3 heterodimer was not increased in the 11_18 cells. These findings suggest that pertuzumab inhibits HER2/HER3 heterodimerization by HRG-α stimulation. The HRG-α-stimulated phosphorylation of HER2 was inhibited by pertuzumab in the 11_18 cells. In contrast, the EGF-stimulated phosphorylation of HER2 was not inhibited. These data suggest that pertuzumab inhibits HRG-α stimulated phosphorylation in 11_18 cells. In the PC-9 cells, HRG-α stimulated HER2/HER3 heterodimer formation could be detected without any ligand stimulation, and pertuzumab diminished HRG-α-stimulated heterodimer formation (Fig. 5c). Phosphorylation of HER2 was increased by HRG-α stimulation, but not inhibited by pertuzumab in PC-9 cells. EGFR/HER2 heterodimer formation could be detected without any ligand stimulation, but pertuzumab did not affect it. Based on these results, it is speculated that the cell growth of the PC-9 cells is predominantly dependent on active EGFR signaling, and phosphorylation of HER3 is maintained by active mutant EGFR.

Discussion

Overexpression of HER3 was observed in the lung cancer cell lines and the HER3 was phosphorylated by the HER3 ligand in these cells. These results suggest that HER3 signaling is active in some types of lung cancer cells. Recently it was reported that high HER3 expression was associated with decreased survival.[17] A relationship between lung cancer metastasis and the expression of HER3 as well as EGFR and HER2 has been reported.[18] These bodies of evidence suggest that HER2/HER3 signaling is activated in a subpopulation of lung cancers and that HER2 and HER3 play an important role in the biological behavior of these lung cancers. Both HER2 and HER3 are therefore considered as a possible important target in the therapeutic strategy against lung cancer, just as they are in breast cancers.

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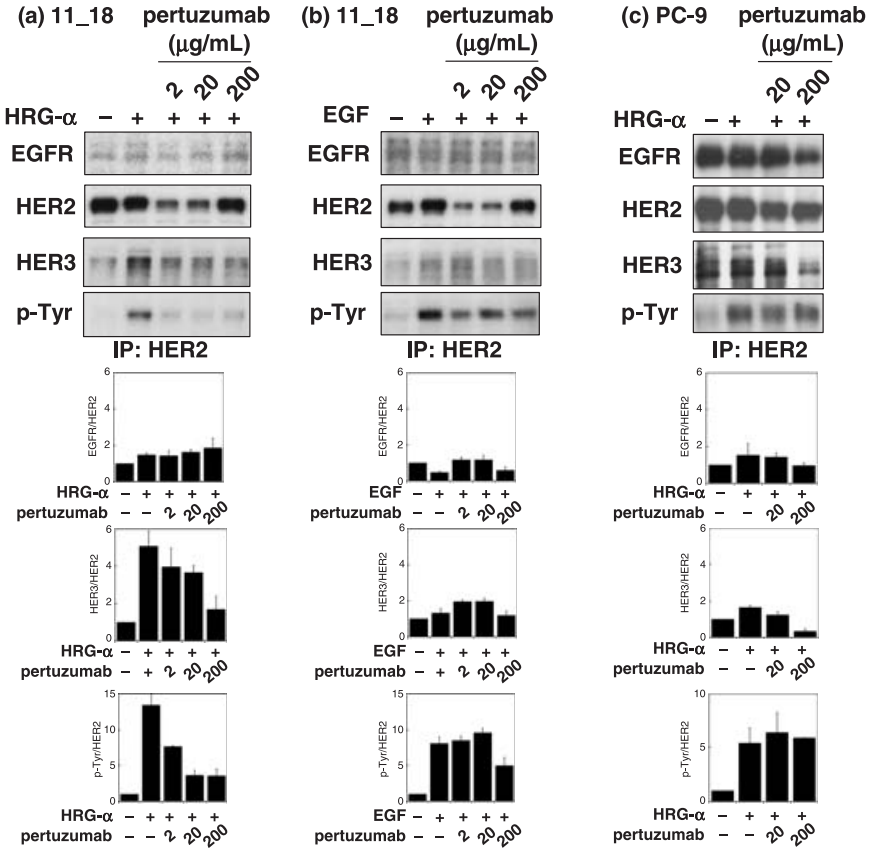

Fig. 5. Effect of pertuzumab on heterodimer formation. The 11_18 and PC-9 cells were exposed to pertuzumab for 6 h and stimulated with either heregulin (HRG)-α or epidermal growth factor (EGF) for 10 min. Cell lysates were immunoprecipitated with anti-HER2 antibody, separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and blotted for indicated antibodies. The intensities of bands were quantified by densitometer. (a) HRG-α-stimulated 11_18 cells. (b) EGF-stimulated 11_18 cells. (c) HRG-α-stimulated PC-9 cells. Data shown are representative of at least two independent experiments with similar results.

HER3 lacks kinase activity because of several base substitutions in motifs that are essential to tyrosine kinase and heterodimerization with HER2 or EGFR is essential for its signal transduction. Therefore co-expression of HER3 and its partners are determinants for the cellular sensitivity against pertuzumab in cancer cells. The present results showed that HER2/HER3 heterodimers are detected by HRG-α stimulation and these data are consistent with previous reports.[19] In contrast, the authors monitored the downstream phosphorylation signal, and demonstrated that HRG-α, but not EGF, phosphorylated Akt in the 11_18 cells. This finding allows us to speculate that HRG-α stimulation leads to Akt phosphorylation through HER2/HER3 heterodimerization.[20–22]

Recently, EGFR mutations have been reported in lung cancers and it was of great interest to clarify the relationship between the EGFR mutation and sensitivity to EGFR-targeted tyrosine kinase inhibitors.[23–25] The PC-9 cells express the deletional mutant EGFR (delE746-A750 in exon 19 of EGFR),[16,23,26,27] and their EGFR was constitutively phosphorylated under non-stimulated conditions (Fig. 3a). The authors speculate that the cell growth of the PC-9 cells is predominantly dependent on active EGFR signaling. In Fig. 3b, treatment with EGF and TGF-α seemed to decrease the phosphorylation of HER3 in PC-9 cells. Unfortunately, we could not conclusively explain this phenomenon. PC-9 cells express deletional EGFR and form EGFR homodimers in the absence of ligand stimulation. At the same time, phospho-HER3 was also detected under these conditions, suggesting that heterodimers of EGFR–HER3 were also formed. Ligand stimulation may alter the balance between homodimers and heterodimers, causing a reduction in HER3 phosphorylation, although there is not any evidence to support this hypothesis. In contrast, the phosphorylation of EGFR in the 11_18 cells that express a different type of mutant EGFR (L858R in exon 21 of EGFR),[26] was not constitutive. This finding may be explained by the differences between deletion mutant EGFR and L858R; constitutive active in the deletion mutant versus hyper-response to ligand stimulation in L858R.[28] Engelman *et al*. suggested that the mutant EGFR is used to couple HER3 in gefitinib-sensitive NSCLC cell lines.[29] The expression level of EGFR in the 11_18 cells was much lower than in the PC-9 cells, and a similar extent of HER3 expression was observed in these cell lines (Fig. 3a). The authors have demonstrated the differential inhibitory effect of pertuzumab against 11_18 and the PC-9 cells. Pertuzumab inhibited HER2/HER3 heterodimer formation and phosphorylation in the 11_18 cells, considering that mutant EGFR do not influence HER3 signals in the 11_18 cells. HER3 phosphorylation in the PC-9 cells was also increased by HRG-α stimulation. Although pertuzumab decreased HER2/HER3 heterodimer formation, it failed to inhibit HRG-α-stimulated HER3 phosphorylation, speculating that an active mutant EGFR transactivates HER3 in the PC-9 cells.

Several EGFR-targeted small inhibitors and antibodies have been under clinical evaluation in the treatment of lung cancer. An EGFR-targeted tyrosine kinase inhibitor, erlotinib, has been clinically applied as a second or third-line single agent therapy in NSCLC patients who have failed standard chemotherapy.[30] Anti-EGFR monoclonal antibodies such as cetuximab and ABX-EGF have been examined in a clinical study.[31] In addition to EGFR, HER2 and HER3 are also considered as important targeting molecules in lung cancers. The present results indicated that pertuzumab effectively inhibited signaling within HER2 and HER3, and may thus be effective in lung cancers expressing HER2 and HER3. To confirm the pertuzumab-sensitive population of lung cancer cells, experiments using small interfering RNA for mutant EGFR will be necessary in future studies.

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In conclusion, the authors have demonstrated that pertuzumab inhibits HRG-α-stimulated cell growth in lung cancer cells through the inhibition of HRG-α-stimulated HER3 signaling. It was further demonstrated that pertuzumab exerts an antiproliferative activity against lung cancer cells expressing HER2 and HER3. The next step will be to examine the clinical relevance of the occurrence of heterodimer formation between HER2 and the other HER receptors in lung cancer.

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