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ErbB Receptor Activation, Cell Morphology Changes, and Apoptosis Induced by Anti-Her2 Monoclonal Antibodies

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A panel of mAbs were generated against the purified soluble form of erbB2/Her2 receptor, corresponding to the extracellular region of the receptor, and examined for their ability to mimic the receptor ligand. Some of the mAbs strongly induced tyrosine phosphorylation of 180-185 kDa proteins, including not only Her2 but also Her3 and Her4 receptors, when they were expressed on the surface of breast cancer cells. These mAbs do not cross-react with Her3 or Her4 as demonstrated by competition study. Receptor phosphorylation was also observed with the cell lines transfected with Her2 or a chimeric receptor consisting of the extracellular domain of Her2 and the transmembrane and cytoplasmic domains of epidermal growth factor receptor. Selected mAbs were tested for their ability to change cell morphology, and one specific mAb, mAb74, induced cell morphology changes and apoptosis.

There have been numerous studies showing that high expression of erbB2/Her2 tyrosine kinase receptor correlates with poor prognosis in patients with breast cancer (1,2,3,4). Her2 is a member of the epidermal growth factor (EGF) receptor subfamily, which includes EGF receptor, and Her3 and Her4 receptors (5,6). EGF, transforming growth factor-α, amphiregulin, heparin binding EGF and betacellulin are known as ligands of the EGF receptor. Neu differentiation factor (NDF) or heregulin and other structurally related ligands including p25 from MDP-activated macrophage conditioned media, NAF, p75 from SKBR3 conditioned media, NEL-GF, ARIA and GGF all have been shown to increase tyrosine phosphorylation of the Her2 receptor and, therefore, were initially assumed to be ligands for the Her2 receptor (7,8,9,10,11,12,13,14). There is now convincing evidence that NDF neither binds directly to Her2 nor stimulates its kinase activity (15) but rather binds to Her3 or Her4 and stimulates tyrosine phosphorylation of these receptors (6,16,17).

The conventional approach to circumvent the absence of ligand is to generate a ligand-like monoclonal antibody (mAb). In fact, several groups have generated anti-Her2 mAbs using cells expressing high levels of $p185^{Her2}$ for immunizations (18,19,20,21). The $p185^{Her2}$ overexpressing cells possibly coexpress $p180^{Her3}$ and /or $p180^{Her4}$, and therefore the mAbs using those cells as an antigen may cross-react to $p180^{Her3}$ and/or $p180^{Her4}$. In addition, Her2 expressed on

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Abbreviations used: EGF, epidermal growth factor; NDF, neu differentiation factor; MDP, muramyl dipeptide; NAF, neu protein-specific activating factor; NEL-GF, neu erbB2 ligand-growth factor; ARIA, acetylcholine receptor inducing activity; GGF, Glial growth factor; mAb, monoclonal antibody; CHO, Chinese hamster ovary; sHer2, soluble Her2 receptor; HEG, a chimeric receptor consisting of the extracellular domain of Her2 and the transmembrane and intracellular domains of EGF; EGFR, EGF receptor; PBS, Dulbecco's phosphate-buffered saline; HRP, horseradish peroxidase; SDS, sodium dodecyl sulfate; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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cell surface may have limited accessibility of epitopes compared to its free molecule in solution. Although these mAbs induced increased tyrosine phosphorylation in Her2 overexpressing cells, they were not fully characterized in terms of the binding to each of Her2, Her3 or Her4 or in terms of the kinase activation in Her2 transfected cells in which no other members of EGFR family exist. Using Her2 specific mAbs, which were extensively characterized and will be described elsewhere, we show here their ability to induce kinase activation using various cell lines either overexpressing Her2, or cell lines transfected with Her2 or chimeric receptor (HEG or Her2-EGFR). We also show here one specific mAb that induces cell apoptosis and morphologic change.

MATERIALS AND METHODS

Cells. SKBR3 and MDAMB453 cells were grown in DMEM and RPMI1640, respectively, supplemented with 10% heat-inactivated fetal bovine serum (FBS) and 2mM glutamine. Her2/CHO cells were prepared by co-transfection of dihydrofolate reductase-deficient CHO cells with two vectors: pJT2 carrying the genes coding for Her2 and dihydrofolate reductase (dhfr) in pDRa2. Her2-transfected CHO cells were grown in selective medium without nucleosides (DMEM containing 5% dialyzed FBS, 2mM glutamine and 0.1mM non-essential amino acids). A hematopoietic cell line, 32D, was transfected with a chimeric receptor consisting of the Her2 extracellular ligand binding region and EGFR intracellular and transmembrane regions (designated HEG), or with a full length Her2. Construction of a chimeric receptor cDNA and gene transfection were carried out as described (22). HEG/32D, Her2/32D and 32D cells were grown in RPMI, supplemented with 10% heat inactivated FBS and 1 ng/ml IL-3. Her2/MCF7 were grown in MEMa containing 10% inactivated FBS, 0.1mM non-essential amino acids and 1mM sodium pyruvate.

Assay of receptor tyrosine phosphorylation. Adherent cells (SKBR3 or MDAMB453) were grown in 48 well plates and washed with DMEM 2-3 times. Suspension cells (32D, Her2/32D or HEG/32D) were pelleted by centrifugation and washed with PBS. mAb solution or ligand solution was added to the well or to the pelleted tube and incubated for 5 min at 37°C. The solution was removed and the cells were solubilized with SDS sample buffer. The samples, with or without immunoprecipitation, were subjected to SDS-PAGE followed by Western blotting and probing with anti-phosphotyrosine antibody.

Cell morphologic change. Cells were seeded in 5 cm dishes to about 20% confluency and mAbs added after 18 h. After 5 days, cells were observed with light microscopy, photographed, and counted.

Cell apoptosis assay. Cells were seeded in 8-well Chamber Slides (Nunc) at 60-70% confluency and after 18 h, culture media was changed to 1% FBS-containing media with or without mAb. On day one, cells were fixed with 4% neutral-buffered formalin (NBF) followed by 3 washes with PBS. After cells were dried, apoptosis assay was done using a modified TUNEL (terminal deoxynucleotidyl transferase, TdT, mediated dUTP-biotin nick end-labeling) method (23). TUNEL detects 3′-OH DNA ends generated by DNA fragmentation, after labeling digoxigenin-conjugated dUTP with TdT followed by incubating with HRP-conjugated anti-digoxigenin. Bound HRP was detected with the substrate, 3-amino-9-ethylcarbazole (Sigma). Most of the reagents used were from Apop Tag in situ apoptosis detection kit (Oncor). HRP-conjugated antibodies were from Boehringer Mannheim.

RESULTS AND DISCUSSION

Twelve clones of anti-sHer2 mAbs were tested for stimulation of receptor tyrosine phosphorylation in SKBR3 cells. As shown in Figure 1a, mAb74, 52, 58 and 83 at 250 nM strongly stimulated the tyrosine phosphorylation of 180-185 kDa proteins in SKBR3 cells in which both Her2 and Her3 were identified. Stimulation was much weaker for mAb42b, 86, 80 and 73, and not much different from the basal level.

Dose dependence of stimulation was examined for mAb74 and 83, as shown in Figure 1b. As the concentration of mAb74 was increased from 10 nM to 250 nM, the phosphorylation increased dose-dependently, approaching a level observed with 2 nM NDFα2. mAb83 also exhibited dose-dependent increase in tyrosine phosphorylation, but to a much smaller extent than the level with mAb74 when compared at the same concentration.

Next, specificity of Her2 stimulation by the mAbs was tested by competition experiments. SKBR3 cells were incubated with 250 nM mAb83, 74 and 50 in the presence of increasing concentration of sHer2. As shown in Fig. 2-a it is evident that tyrosine phosphorylation was dose-dependently inhibited by sHer2. As expected, 10 nM sHer2 exhibited little inhibition

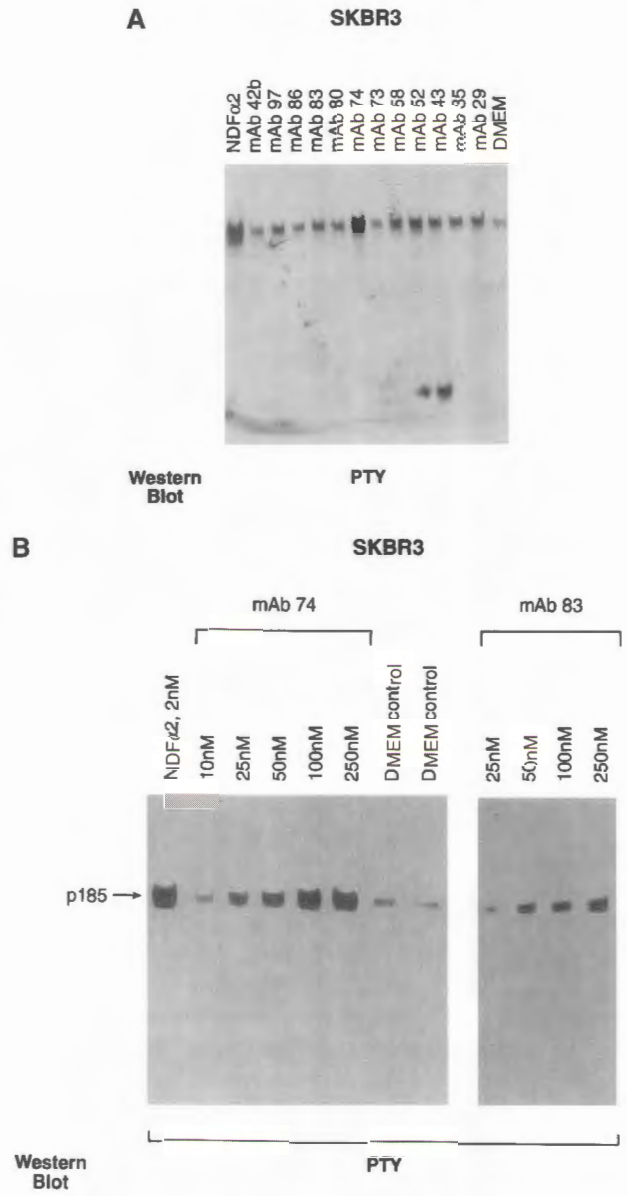

FIG. 1. Her2 and Her3 tyrosine phosphorylation induced by mAb stimulation in SKBR3. SKBR3 cells were seeded in a 48-well plate for 5 min at 37°C for 18 h before mAb stimulation. Cells were solubilized with SDS sample buffer. Solubilized samples were electrophoresed on 6% polyacrylamide gels, followed by Western blotting and probing with anti-phosphotyrosine. (a) All mAb concentrations were 250 nM in DMEM and 2 nM NDFa were used as a positive control. (b) mAb dose dependence of tyrosine phosphorylation.

while 1.3 or 2.5 μM sHer2 showed a nearly complete suppression of stimulation by these mAbs. Competition with sHer3 was tested with SKBR3 stimulated by mAb52. sHer3 concentration was varied from 1.5 nM to 1.6 μM in the presence of 250 nM mAb52. As shown in Figure 2-b, the phosphorylation was not inhibited with sHer3 even at the highest concentration.

Although these mAbs are highly specific for Her2, both Her2 and Her3 in SKBR3 cells and

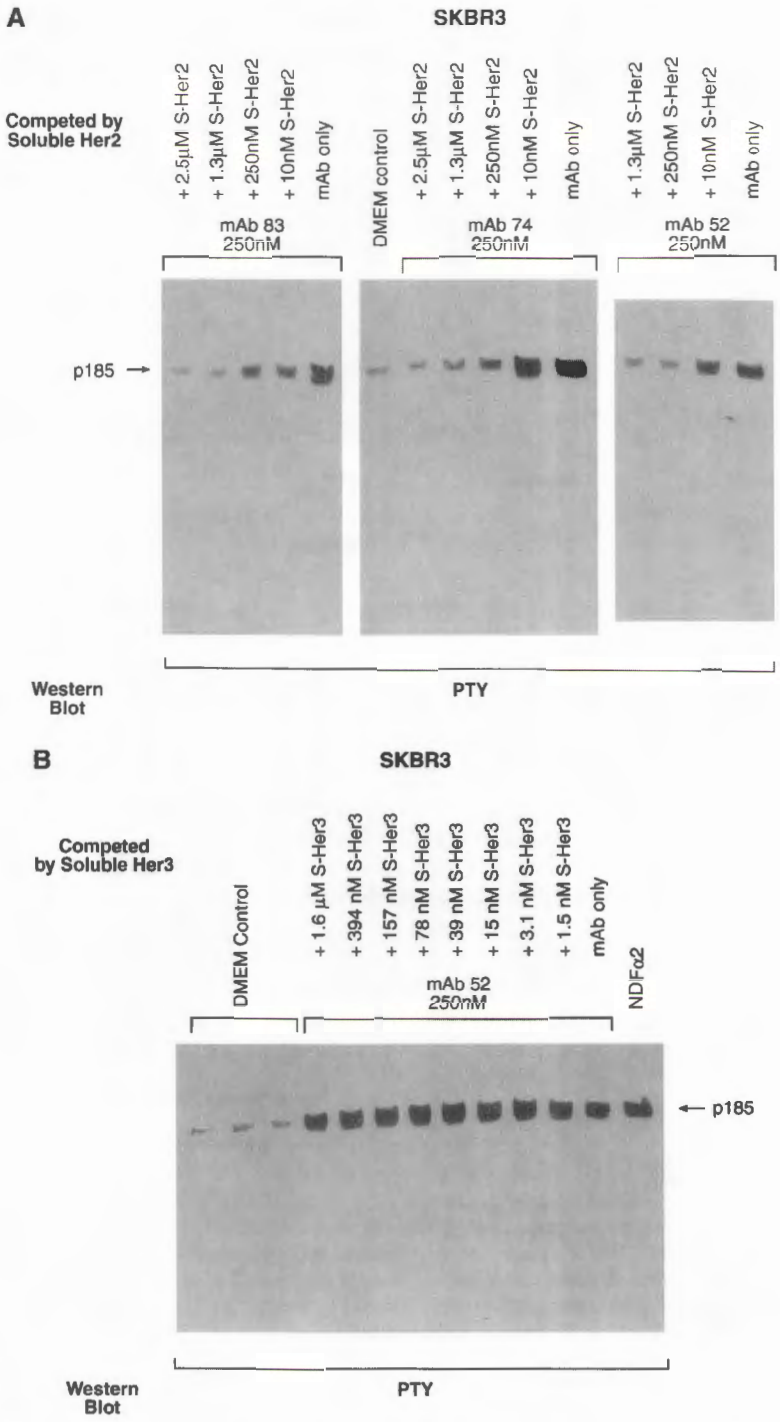

FIG. 2. Inhibition by soluble receptor of receptor tyrosine phosphorylation induced by mAb. Phosphorylation assay is similar to that described in Figure 1. Cells were incubated with 250 nM mAb with different concentrations of sHer2 (a) or sHer3 (b).

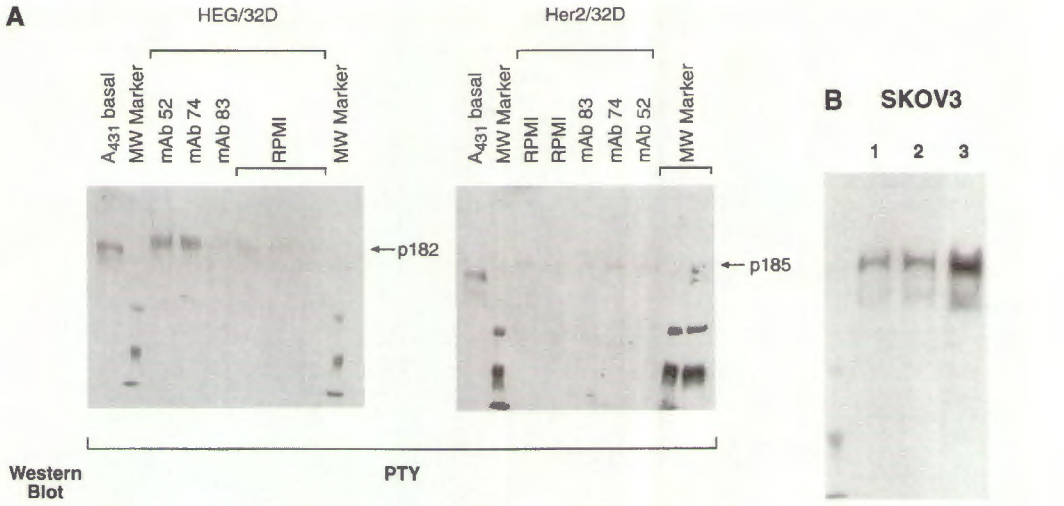

FIG. 3. Receptor tyrosine phosphorylation of cell lines. (a) Transfected cell lines Her2/32D and HEG/32D, induced by mAb stimulation. For phosphorylation assay, cells were pelleted by centrifugation, washed with PBS, and then incubated with 100 μl of 250 nM mAbs in RPMI for 5 min at 37°C, followed by quenching with the addition of 1 ml ice cold PBS and centrifugation at 4°C. Supernatant was removed and SDS sample buffer added to the centrifuged pellet. The sample was subjected to 6% SDS-PAGE followed by Western blotting and probing with anti-phosphotyrosine antibody. A_{431} basal phosphorylated sample was used as a positive control. (b) Wild type cells, SKOV3. Phosphorylation assay is similar to that described in Figure 1. Higher MW bands are p180. Lane 1, 2nM NDFa; Lane 2, DMEM control; Lane 3, 250nM mAb74.

all Her2, Her3 and Her4 in MDAMB453 cells were found to be phosphorylated when the cells were stimulated with mAb52 and immunoprecipitated with specific antibodies (data not shown).

A similar assay was done with transfected cell lines, Her2/CHO and Her2/32D to study direct interaction of the mAbs and cell-surface Her2. All the mAbs tested, mAb83, 74 and 52 failed at 250 nM to phosphorylate Her2 in the transfected cells, Her2/CHO (data not shown) and Her2/32D (Figure 3-a). As the transfection may have caused receptor inactivation and changed the interaction between mAb and Her2, we prepared a cell line, HEG/32D, transfected with a chimeric receptor (HEG), whose extracellular domain comes from Her2 and intracellular and transmembrane domains come from EGF receptor. As shown in Figure 3-a, when HEG/32D cells were stimulated with the same mAbs, HEG is phosphorylated over basal level. mAb74 showed the strongest activity among the three tested. These results show that Her2 kinase does not phosphorylate itself whereas EGFR kinase could autophosphorylate HEG upon mAb incubation under the experimental conditions used. However, when the expression of Her2 was increased in 32D, it was phosphorylated in the presence of mAb (data not shown). We also examined tyrosine phosphorylation in SKOV3 cells. SKOV3 cells that naturally overexpress Her2 were not phosphorylated by NDF/heregulin (24) but were phosphorylated by mAb74 as shown in Fig. 3-b. These results strongly suggest that the Her2 kinase was activated by homodimerization, and Her3/or Her4 kinase were not required for Her2 kinase activation.

Cell morphology change by mAb. Her2/ MCF7 cells were incubated with 250 nM mAb42b, mAb83 and mAb74. After 5 days incubation, mAb74 caused extensive cell death and a dramatic cell morphology change as shown in Figure 4-a,b,c,d. mAb83 caused a moderate cell morphology change and 42b resulted in little change. The viable cell number 5 days after mAb74 incubation was only 36% of the control grown without mAb incubation. mAb74 also induced cell morphology change in MDAMB453 (Fig. 4-e,f).

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