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Biological Effects of Anti-ErbB2 Single Chain Antibodies Selected for Internalizing Function

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Two internalizing monovalent single chain antibody fragments (scFv), C6.5 and F5, that recognize distinct ErbB2 extracellular domain (ECD) epitopes, and their bivalent forms dbC6.5 and F5(scFv′)$_2$, were compared to the growth-inhibiting anti-ErbB2 antibody Herceptin/trastuzumab, in either its bivalent (Her) or monovalent (4D5Fab′) form, for their abilities to induce biological responses in the ErbB2-overexpressing breast cancer cells, SkBr-3. Assays compared internalization by receptor-mediated endocytosis, effects on cell cycling and culture growth, and interference with intracellular MAPK and PI3K signaling pathways. We found no correlation between ErbB2 epitope affinity or valency on degree of antibody-induced endocytosis, since all the scFv were able to internalize better than Her. Unlike Her, neither the monovalent or bivalent forms of the internalizing scFv had any sustained effect on cell growth. Basal levels of MAPK and PI3K signaling in SkBr-3 cells were not inhibited by up to 8 h scFv treatment, while decreased MAPK and PI3K signals were noted within 8 h of Her treatment. In summary, antibody-induced ErbB2-mediated endocytosis is not a surrogate marker for resultant biological response, as it shows no correlation with cell cycle, culture proliferation, or intracellular kinase signal induction by internalizing antibodies. Thus, the enhanced endocytotic property of scFv like C6.6 and F5 in conjunction with their absence of any growth or signaling impact on ErbB2-overexpressing cells favors their choice as ErbB2 targeting moieties for intracellular delivery of novel cancer therapeutics.

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The ErbB2 receptor tyrosine kinase (RTK) plays an important role in normal organ development as well as in human tumorigenesis, where it is found amplified and/or overexpressed in 20–30% of breast, ovarian and bladder adenocarcinomas and is associated with poor patient prognosis (1, 2). It is one of four ErbB2 family members constituting the Type I RTKs and, although it remains an orphan receptor, ErbB2 is the preferential binding partner of all ErbB receptors (3, 4). Given that ErbB2 is accessible at the cell membrane, modulates intracellular ErbB signaling, and is implicated in tumor progression, ErbB2 represents a promising target for antibody-mediated cancer therapy (2). A number of antibodies raised against the extracellular domain (ECD) of ErbB2 have led to differing responses when used to treat ErbB2-overexpressing cancer cells (5, 6). Exposure of such cells to the murine anti-ErbB2 monoclonal antibody (MoAb) 4D5 has been shown to inhibit breast cancer cell proliferation both in vitro and in vivo, and the humanized version of this MoAb, Herceptin/trastuzumab, recently received FDA approval for the treatment of advanced ErbB2-overexpressing breast cancers (2). As a result, there are now an ever increasing variety of antibody-based anti-ErbB2 therapeutics under clinical development (7).

At least two mechanisms have been identified for the direct tumoristatic effect of anti-ErbB2 antibodies like trastuzumab (2): receptor-mediated endocytosis with downmodulation of ErbB2 receptor levels, and disruption of ErbB2 heterodimers with inhibition of signaling by these ErbB receptor complexes. Single chain variable region antibody fragments (scFv) directed intracellularly against the ErbB2 receptor have also been shown to downmodulate receptor levels, inhibit receptor signaling, and induce cell cycle arrest in breast cancer cell lines overexpressing ErbB2 (8). More recently, extracellularly administered scFv selected from phage display libraries on the basis of their ability to

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bind and internalize the ErbB2 receptor have been developed as targeting moieties for newer generations of antibody-based anti-ErbB2 therapeutics (9–11). However, the cellular consequences following exposure to ErbB2 ECD-targeted scFv remain undefined despite their potential influences on the clinical efficacy or toxicity of scFv-based therapeutics. Thus, here we have compared the effects of both monovalent and bivalent forms of newly engineered ErbB2 ECD-targeted scFv on receptor-mediated endocytosis, intracellular kinase signaling, cell cycling and proliferation following treatment of a cultured human breast cancer line, SkBr-3, known to represent ErbB2-overexpressing breast cancers and respond in vitro to the cytostatic effects of Herceptin/trastuzumab.

MATERIALS AND METHODS

Monovalent and bivalent anti-ErbB2 scFv, and control anti-ErbB2 antibodies. Bacterial expression, purification, and characterization of the phage-selected anti-ErbB2 scFv, C6.5 and F5, has been described previously (9–13). In addition, bivalent F5(scFv′)$_2$ and the diabody form of C6.5 (14), dbC6.5, were similarly produced with the following modifications: bivalent C6.5 diabody was generated by shortening the linker region that promotes inter- rather than intramolecular pairing between V_H and V_L domains, while bivalent (F5(scFv′)$_2$ was formed by disulphide bridging the free cysteine residues at the V_L C-terminus of two F5 scFv molecules. Hereceptin/trastuzumab or the murine monoclonal, 4D5, and its monovalent fragment, 4D5Fab′, were commercially obtained or provided (Genentech Inc.) and used as anti-ErbB2 antibody comparison controls. C6.5 scFv, C6.5db, and F5 scFv affinity (K_d values) for ErbB2 was determined on human SKOV3 cells using flag tagged antibody fragment as previously reported (15, 16). Competitive epitope mapping was determined by surface plasmon resonance in a BIAcore1000 instrument (BIAcore Inc.) using 3000 RU of ErbB2 ECD coupled to a CM5 sensor chip (BIAcore Inc.) under a continuous flow rate of 15 μL/min, as previously described (15). These assays established that C6.5 and F5 (and their bivalent forms) and 4D5/trastuzumab recognize different epitopes within the ErbB2 ECD; furthermore, these scFv did not interfere with ErbB2 receptor heterodimerization as evidenced by the observation that F5 and C6.5 can be co-immunoprecipitated with heterodimeric complexes of ErbB1 and ErbB2 (data not shown).

Cell culture, growth factor and other antibody reagents. ErbB2-overexpressing SkBr-3 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Low ErbB2 expressing MCF-7 cells were maintained in 10% FCS supplemented DME-H-16 medium (with 1 g/L glucose, 10 μg/ml insulin). In all experiments 1×10^6 cells were transferred to 10 cm dishes and left overnight to attach before antibody or growth factor were applied. Antibody reagents used in the cellular response assays were commercially obtained (New England Biolabs, MA) and included anti-Akt (PKB), anti-phospho-Akt (Ser 473), anti-phospho-Erk1/Erk2 (p44/42; Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185). Recombinant heregulinβ growth factor (NDF) was also commercially obtained (Neomarkers, Fremont, CA).

Antibody internalization assay. The antibodies were labeled with ^{125}I using carrier-free [^{125}I]-NaI (Amersham) (1 mCi/mg of protein) and Iodo-Beads kit (Pierce, Rockford, IL) according to the manufacturer's instructions. Radioiodinated antibodies were purified by gel-filtration on Sephadex G-25. SkBr-3 cells were seeded in 12-well plates at 1.5×10^5 cells/well and incubated in triplicate with 5×10^6 cpm of radioiodinated antibodies in 1 ml of growth media at 37°C for 2 h. The cells were then rinsed 4 times with Hanks' balanced salt solution, and 2 times with 1 ml of ice-cold glycine buffer pH 2.5 (500 mM NaCl, 0.1 M glycine, pH 2.5). Following acid rinse, the cells were lysed with 2 ml of 100 mM TEA for 4 min at 4°C. Radioactivities of the rinses and of the acid-washed cell lysate was determined using Cobra 5003 gamma-counter (Packard Instruments). Radioactivity of the last Hanks' rinse was used to check for completeness of the removal of antibody not associated with the cells. The proportion of internalized antibody was determined as the ratio of cell lysate radioactivity (internalized antibody) to the sum of radioactivities of the cell lysate (internalized antibody) and acid rinses (surface-bound antibody).

Cell extraction and immunoblotting. Cells were lysed in NP40 lysis buffer (50 mM Hepes, pH 7.4, 1% NP40, 150 mM NaCl, 25 mM β-glycerol phosphate, 25 mM NaF, 5 mM EGTA, 1 mM EDTA, 10 μg/ml leupeptin and aprotinin, 1 mM PMSF) and cell debris was pelleted at 10,000*g* for 30 min. Cell extract protein concentrations were determined by the method of Bradford, proteins separated on 7.5–15% SDS-PAGE gels, blotted onto PVDF and detected by ECL as previously described (5a). Immunoblot signal intensities were quantified by image densitometry, with phospho-Erk1/2, phospho-PKB, and phospho-JNK signal intensities then normalized by total PKB signal intensity, as a measure of protein lane loading.

Cell cycle analysis. Trypsinized cells were resuspended in PI staining buffer (1 mM sodium citrate, pH 4.0, 1.5 mM NaCl, 5 mM EDTA, 5 mM EGTA, 0.1% NP40, 4 μg/ml propidium iodide, 80 μg/ml RNase A in PBS), placed on ice for 1 h. Cell cycle analysis was performed on a Becton Dickinson FACScan Flow Cytometer as previously described (5a).

Cell culture growth assays. SkBr-3 cells were seeded 1×10^4/well on 96-well plates. After incubation with respective antibodies for 3 days, the number of living cells was estimated using a cell growth assay (Promega CellTiter 96 AQ) and relative cell number was determined spectrophotometrically by crystal violet staining.

RESULTS AND DISCUSSION

Endocytosis and Cell Internalization by Anti-ErbB2 Antibodies Is Independent of Antibody Valency and ErbB2 Binding Affinity

Receptor-mediated endocytosis and internalization of a ligand-bound or otherwise activated receptor complex is an immediate cellular response closely linked to intracellular signaling (17). Ligand binding can induce receptor-mediated endocytosis, as is the case for EGF-bound EGFR, which then leads to either proteosomal degradation (receptor downmodulation) or surface recycling of the receptor. Antibodies causing ErbB2 internalization and downregulation have been associated with cytostatic growth inhibition (18, 19); however, due to the limited number of anti-ErbB2 antibodies studied, this is not a well defined mechanism (5, 19, 20). Therefore, the monovalent and bivalent scFv were tested for their ability to internalize by receptor-mediated endocytosis after administration and binding to the ErbB2-overexpressing breast cancer cell line, SkBr-3. Hereceptin/trastuzumab (Her) and the monovalent Fab′ fragment of the murine monoclonal Her precursor, 4D5, were used as positive control comparisons. As expected, internalization of the bivalent Her monoclonal exceeded that of the monovalent 4D5Fab′. Surprisingly, despite their lower ErbB2 affinity (K_d)

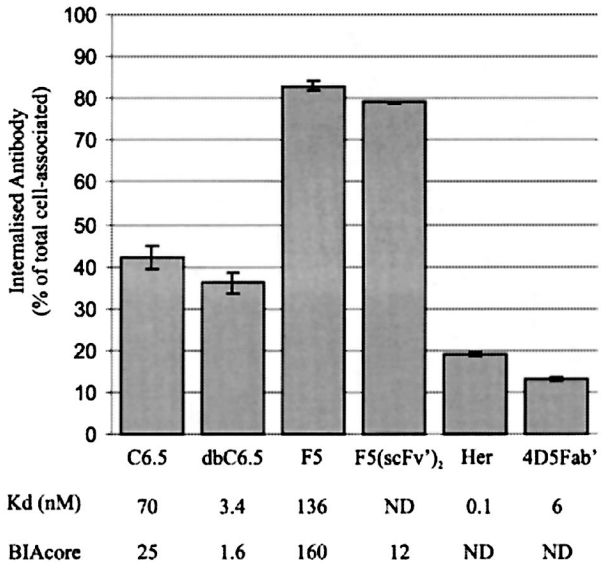

FIG. 1. Receptor-mediated internalization of anti-ErbB2 antibodies into SkBr-3 cells. Anti-ErbB2 scFv: monovalent C6.5 and F5, bivalent dbC6.5 and F5(scFv′)$_2$, in comparison to the anti-ErbB2 control antibodies, Herceptin/trastuzumab (Her) and 4D5Fab′. Affinity constants determined on cells (K_d) and by BIAcore for ErbB2 are indicated below the graph. Cells were incubated with ^{125}I-labeled antibodies at 37°C for 2 h. The amounts of cell surface-bound and internalized antibodies were determined by acid rinse technique, and antibody affinity constants (K_d values) for ErbB2 were determined by flow cytometry on SKOV3 cells as described under Materials and Methods and have been previously reported (15, 16).

constants, both monovalent and bivalent forms of the C6.5 and F5 scFv internalized 2- to 4-fold better than the Her monoclonal (Fig. 1). Given the order of magnitude K_d differences between these antibodies including their monovalent and bivalent forms, it is evident that the degree of receptor-mediated internalization by anti-ErbB2 antibodies is not necessarily dependent on either antibody K_d or valency, with the monovalent and low affinity binding anti-ErbB2 scFv F5 capable of the highest level of intracellular uptake. Further analysis indicated that while all the scFv could initiate ErbB2 internalization in SkBr-3 cells, F5 was significantly more efficient at ErbB2-mediated internalization than the next most efficient antibody, C6.5, across all antibody concentrations tested, suggesting that F5 acts either as a monovalent ErbB2 ligand or otherwise stimulates ErbB2 endocytosis in an epitope-specific manner.

Cell Cycle and Growth Inhibition Do Not Correlate with Cell Internalization by Anti-ErbB2 Antibodies

Anti-ErbB2 monoclonals that bind with high affinity to cellular ErbB2 can have diverse effects on cell cycling and growth of ErbB2-overexpressing cells (5, 18–21). Treatment with Her or 4D5, as well as other cytostatic anti-ErbB2 monoclonals, has now been shown to alter ErbB2 phosphorylation and inhibit growth of ErbB2-overexpressing cells in a cyclinE/cdk2-dependent manner (18, 22). In contrast, other anti-ErbB2 antibodies also known to alter ErbB2 phosphorylation can stimulate cellular proliferation (20). Figure 2A demonstrates that 4D5 treatment of SkBr-3 cells produces an accumulation of cells in the G1 phase of the cell cycle after 24 h of treatment, consistent with its known cytostatic effect (22). In contrast, neither the C6.5 nor F5 monovalent scFv had any significant affect on the SkBr-3 cell cycle profile relative to untreated control cells, while the bivalent forms of these scFv caused an apparent mitogenic cell cycle push reflected by an increase in the proportion of S-phase cells after 24 h of treatment (Fig. 2A). Growth assays measuring the antibody responses of these same cells after 3 days of continuous exposure again confirmed the marked growth inhibitory effect of 4D5, but also showed that neither monovalent nor bivalent forms of C6.5 and F5 scFv result in any significant growth altering effects on ErbB2 overepressing cells (Fig. 2B). Thus, the increased number of cells in the S-phase of the cell cycle observed by FACS analysis after 24 h treatment with the bivalent scFv appears as a transient cellular response and is not sustained long enough to produce a significant proliferative response. However, this transient response does suggest that ErbB2-overexpressing cells react somewhat differently to monovalent vs. bivalent forms of the same scFv.

Internalizing scFv Do Not Inhibit the Same Cell Signaling Pathways Affected by Cytostatic Anti-ErbB2 Monoclonals

Various mitogen activated protein kinase (MAPK, JNK) and phosphatidylinositol 3′-kinase (PI3K) signaling pathways have been shown to be involved in ErbB2-mediated transformation (8, 22–24), and these same pathways are inhibited by cytostatic anti-ErbB2 monoclonals in ErbB2-overexpressing cells like SkBr-3 (18, 22). While low ErbB2 expressing cells like MCF-7 cannot be growth inhibited by cytostatic anti-ErbB2 monoclonals like Her, these same antibodies can interrupt PI3K and JNK signaling without affecting other MAPK pathways (e.g., Erk) when MCF-7 cells are growth stimulated by heregulin/neuregulin/neu differentiation factor (NDF) (24). Thus, we evaluated the signaling effects of 30 min and 8 h treatments of SkBr-3 and MCF-7 cells (in the absence or presence of NDF) with monovalent and bivalent scFv, as compared to a comparable and cytostatic dose of Her. As expected, none of the antibodies had any detectable effect on the basal MAPK or PI3K signaling in MCF-7 cells (data not shown). In contrast and as shown in Fig. 3, all antibodies had variable degrees of an early (30 min) and transient agonist-like effect on basal MAPK signaling, measured by increased levels of phospho-Erk1/

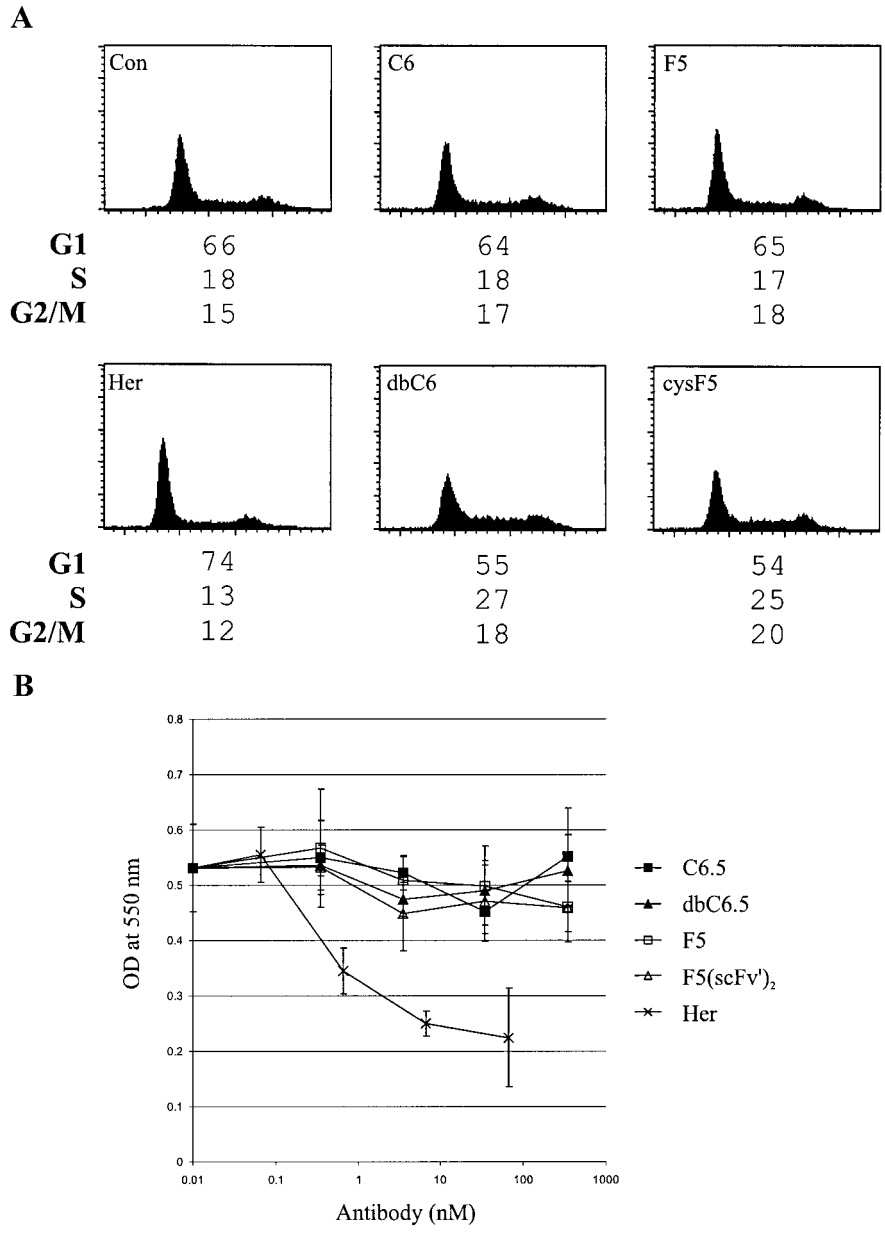

FIG. 2. (A) Cell cycle profiles of SkBr-3 cells in response to 24 h treatment with anti-ErbB2 scFv (C6.5, F5, dbC6.5, F5(scFv′)$_2$), anti-ErbB2 control antibody (Her), or control buffer (Con). The percentage of cells in each phase of the cell cycle (G1, S and G2/M) are shown below each profile. (B) Effect of monovalent and bivalent anti-ErbB2 scFv and control anti-ErbB2 antibody (bivalent 4D5 monoclonal) on SkBr-3 culture growth, with relative cell number quantitated spectrophotometrically (as described under Materials and Methods) after 3 days of continuous exposure to the indicated antibody concentrations.

Erk2 and phospho-JNK. Only the bivalent scFv demonstrated a more sustained (8 h) MAPK activation, particularly with regard to phospho-Erk1/Erk2 and consistent with their transient cell cycle stimulatory effect and S-phase push (Fig. 2). Notably, basal levels of MAPK and PI3K signaling in SkBr-3 were not significantly impaired by up to 8 h treatment with monovalent or bivalent scFv while declines down to 10% of control levels were noted in SkBr-3 phospho-JNK and phospho-PKB signals within 8 h of treatment with an identical and cytostatic dose of Her. Thus, the enhanced degree of ErbB2 internalization showed by monovalent and bivalent scFv over Her was not clearly reflected in their effects on SkBr-3 basal kinase signaling; and only the cytostatic Her monoclonal exhibited any inhibiting effect on basal JNK or PI3K signaling in these ErbB2-overexpressing cells.

Ligand activation of heterodimeric ErbB2 receptor complexes (e.g., ErbB2/ErbB3, ErbB2/ErbB4) by NDF can induce tumor growth in low ErbB2 expressing cells

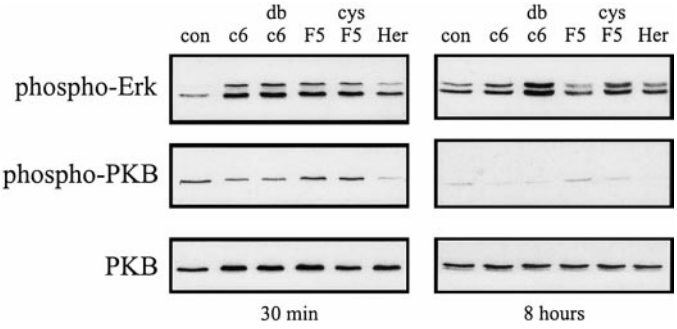

FIG. 3. Time-dependent responses in the MAPK and PI3K signaling pathways upon exposure of SkBr-3 cells to monovalent or bivalent scFv. SkBr-3 cells were treated with identical protein concentrations (10 μg/ml) containing the indicated scFv control anti-ErbB2 bivalent antibody (Her), or buffer only (con) for the indicated times (30 min, 8 h). As described under Materials and Methods, cell extracts were prepared and immunoblotted with antibodies recognizing the activated and phosphorylated products of these signaling pathways (phospho-Erk1/Erk2, phospho-JNK, phospho-PKB), or with an antibody to total PKB (phosphorylated plus unphosphorylated forms) to normalize signal intensities for gel protein loading.

like MCF-7; some of these receptor mediated signaling pathways, but not the cellular growth response, can be inhibited by pretreatment of these cells with an ErbB2 specific monoclonal like Her (24). While also capable of activating these same ErbB mediated signals in SkBr-3 cells, NDF has been paradoxically shown to induce partial differentiation and growth arrest of this ErbB2-overexpressing cell line (18, 25). As shown in Fig. 4, NDF-induced cell signaling in both SkBr-3 and MCF-7 cells occurs by MAPK (increased phospho-Erk1/Erk2 and phospho-JNK levels) and PI3K (increased phospho-PKB levels) transduction pathways. To explore the impact of anti-ErbB2 scFv on NDF-induced signaling, SkBr-3 and MCF-7 cells were pretreated for 8 h with scFv before being given a 15 min stimulatory dose of NDF. While the Her monoclonal was able to substantially block basal PI3K and JNK signaling in SkBr-3 cells (Fig. 3), it had only a partial ability to block NDF-induced SkBr-3 PI3K and JNK signaling (Fig. 4A). Consistent with an earlier report (12), Her also partially blocked NDF-induced PI3K and JNK signaling in the low ErbB2 expressing MCF-7 cells (Fig. 4B), despite the inability of these cells to internalize detectable levels of Her or any of the anti-ErbB2 scFv (data not shown). NDF-induced JNK signaling in both low (MCF-7) and high (SkBr-3) ErbB2 expressing cells appeared most sensitive to Her and scFv pretreatment; however, despite evidence of differential antibody recognition by the two cell lines the biological significance of this observation remains unknown since no consistent pattern emerged destinguishing Her from C6.5 or F5 scFv (in either monovalent or bivalent form) on NDF-induced JNK signaling.

Thus, while there appears to be a differential sensitivity to Her and monovalent or bivalent anti-ErbB2 scFv by both low and high ErbB2 expressing cells, antibody internalization by receptor-mediated endocytosis can only be detected in ErbB2-overexpressing cells where the degree of antibody internalization has no apparent influence on intracellular signaling or cell growth. A striking consistency among all the above results is this absolute lack of correlation between the varying degrees of anti-ErbB2 antibody internalization, their early or delayed effects on basal and NDF-induced cell signaling, and their more sustained effects on cell cycling and culture proliferation. The dramatic 2- to >4-fold enhanced endocytotic property of scFv like C6.5 and F5 over the clinically proven internalizing monoclonal Her, or its 4D5Fab′ fragment, favors the choice of these scFv as ErbB2 targeting moieties for intracellular delivery of novel therapeutics (10). Furthermore, clinical studies involving Her have indicated notable cardiac toxicity both as a single agent and in combination with doxorubicin (dox) (26). ErbB2 signaling appears to play a critical role in myocardial development and maintenance (27), thus the antiproliferative effects of Her on ErbB2 signaling may account for the observed cardiotoxicity (28). Therefore, the absence of any independent signaling or growth-altering effect by the scFv on ErbB2-overexpressing tumor cells, suggests that scFv-mediated treatment may not cause adverse cardiac side-effects seen with Her treatment, providing an additional rationale for the use of these agents to target ErbB2-overexpressing cancer cells.

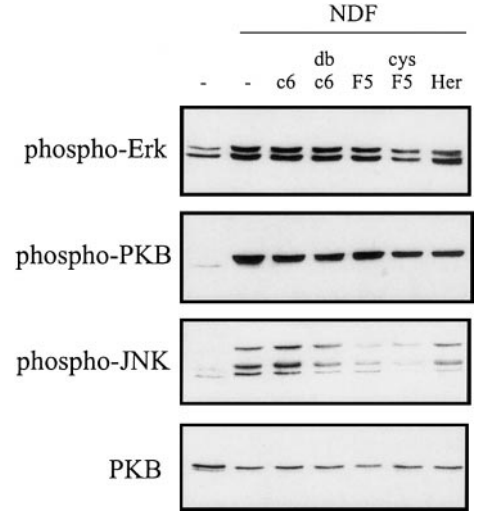

FIG. 4. Effects of scFv pretreatment on SkBr-3 cell responses to the ErbB growth factor, NDF. Cells were pretreated with identical concentrations (10 μg/ml) of the indicated scFv, control anti-ErbB2 antibody (Her), or buffer only (−) for 8 h before 15 min treatment with NDF (1 nM). As described in the legend to Fig. 3, cell extracts were prepared and immunoblotted with antibodies recognizing the phosphorylated products of the MAPK (phospho-Erk1/Erk2, phospho-JNK) and PI3K (phospho-PKB) signaling pathways, or with an antibody to total PKB (phosphorylated plus unphosphorylated forms) to normalize signal intensities for gel protein loading.

REFERENCES

1. Hynes, N. E., and Stern, D. F. (1994) *Biochim. Biophys. Acta* **1198,** 165–184.
2. Benz, C., and Tripathy, D. (2000) *J. Women's Cancer* **2,** 33–40.
3. Graus-Porta, D., Beerli, R. R., Daly, J. M., and Hynes, N. E. (1997) *EMBO J.* **16,** 1647–1655.
4. Olayioye, M. A., Neve, R. M., Lane, H. A., and Hynes, N. E. (2000) *EMBO J.* **19,** 3159–3167.
5. Harwerth, I. M., Wels, W., Schlegel, J., Muller, M., and Hynes, N. E. (1993) *Br. J. Cancer* **68,** 1140–1145.
6. Klapper, L. N., Vaisman, N., Hurwitz, E., Pinkas-Kramarski, R., Yarden, Y., and Sela, M. (1997) *Oncogene* **14,** 2099–2109.
7. Kraus, W. C., Park, J. W., Kirpotin, D. B., Hong, K., and Benz, C. C. (2000) *Breast Dis.* **11,** 113–124.
8. Neve, R. M., Sutterluty, H., Pullen, N., Lane, H. A., Daly, J. M., Krek, W., and Hynes, N. E. (2000) *Oncogene* **19,** 1647–1656.
9. Becerril, B., Poul, M. A., and Marks, J. D. (1999) *Biochem. Biophys. Res. Commun.* **255,** 386–393.
10. Nielsen, U. B., Kirpotin, D. B., Pickering, E. M., Hong, K., Park, J. W., Shalaby, M. R., Shao, Y., Benz, C. C., and Marks, J. D. (2000), submitted.
11. Nielsen, U. B., and Marks, J. D. (2000) *Pharm. Sci. Tech. Today* **263,** 551–567.
12. Schier, R., Marks, J. D., Wolf, E. J., Apell, G., Wong, C., McCartney, J. E., Bookman, M. A., Huston, J. S., Houston, L. L., Weiner, L. M., *et al.* (1995) *Immunotechnology* **1,** 73–81.
13. Park, J. W., Kirpotkin, D., Hong, K., Shalaby, R., Shao, Y., Nielsen, U. B., Marks, J. D., Papahadjopoulos, D., and Benz, C. C. (2000) submitted.
14. Adams, G. P., Schier, R., Marshall, K., Wolf, E. J., McCall, A., Weiner, L. M., and Marks, J. D. (1998) *Brit. J. Cancer.* **77,** 1405–1412.
15. Poul, M.-A., Becerril, B., Nielsen, U. B., Morisson, P., and Marks, J. D. (2000) *J. Mol. Biol.* **301,** 1149–1161.
16. Nielsen, U. B., Adams, G. P., Weiner, L. M., and Marks, J. D. (2000) *Cancer Res.* in press.
17. Trowbridge, I. S., Collawn, J. F., and Hopkins, C. R. (1993) *Annu. Rev. Cell Biol.* **9,** 129–161.
18. Le, X. F., McWatters, A., Wiener, J., Wu, J. Y., Mills, G. B., and Bast, R. C., Jr. (2000) *Clin. Cancer Res.* **6,** 260–270.
19. Hudziak, R. M., Lewis, G. D., Winget, M., Fendly, B. M., Shepard, H. M., and Ullrich, A. (1989) *Mol. Cell Biol.* **9,** 1165–1172.
20. Hurwitz, E., Stancovski, I., Sela, M., and Yarden, Y. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 3353–3357.
21. Stancovski, I., Hurwitz, E., Leitner, O., Ullrich, A., Yarden, Y., and Sela, M. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 8691–8695.
22. Lane, H. A., Beuvink, I., Motoyama, A. B., Daly, J. M., Neve, R. M., and Hynes, N. E. (2000) *Mol. Cell Biol.* **20,** 3210–3223.
23. Adam, L., Vadlamudi, R., Kondapaka, S. B., Chernoff, J., Mendelsohn, J., and Kumar, R. (1998) *J. Biol. Chem.* **273,** 28238–28246.
24. Dankort, D. L., and Muller, W. J. (2000) *Oncogene* **19,** 1038–1044.
25. Peles, E., Bacus, S. S., Koski, R. A., Lu, H. S., Wen, D., Ogden, S. G., Levy, R. B., and Yarden, Y. (1992) *Cell* **69,** 205–216.
26. Cobleigh, M. A., Vogel, C. L., Tripathy, D., Robert, N. J., Scholl, S., Fehrenbacher, L., Wolter, J. M., Paton, V., Shak, S., Lieberman, G., and Slamon, D. J. (1999) *J. Clin. Oncol.* **17,** 2639–2648.
27. Lee, K. F., Simon, H., Chen, H., Bates, B., Hung, M. C., and Hauser, C. (1995) *Nature* **378,** 394–398.
28. Ewer, M. S., Gibbs, H. R., Swafford, J., and Benjamin, R. S. (1999) *Semin. Oncol.* **26,** 96–101.