Therapy of an Animal Model of Human Gastric Cancer Using a Combination of Anti-erbB-2 Monoclonal Antibodies

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ABSTRACT

Amplification and/or overexpression of the erbB-2 gene have been demonstrated in 20–30% of adenocarcinomas of the breast (1–5), ovary (3), lung (6), and stomach (7). Two lines of evidence implicate erbB-2 overexpression in the pathogenesis of human neoplasia. First, overexpression has been linked with poor prognosis in breast (2–3, 8–11), ovarian (3, 12), stomach (13), and lung cancer (14), indicating that overexpression profoundly affects the cancer cell. Second, artificial overexpression of erbB-2 induces a transformed phenotype in NIH/3T3 fibroblasts (15, 16) as well as in mammary epithelial cells (17), suggesting that overexpression can contribute directly to the development of the malignant phenotype.

Because of extensive homology between gpl85erbB-2 and the epidermal growth factor receptor, it is widely assumed that the activation of growth signal transduction might proceed through similar mechanisms. One proposed mechanism involves receptor dimerization or oligomerization, which is thought to be an important step in the activation of the epidermal growth factor receptor intrinsic tyrosine kinase function (18, 19). In this study interference with receptor-receptor interactions was evaluated as a potential therapeutic approach to the treatment of cancers with erbB-2 overexpression. Previous studies have evaluated the use of single monoclonal antibodies directed against erbB-2 (20) and the related epidermal growth factor receptor protein (21) as potential therapeutic agents for the treatment of cancer.

Our studies focused on the synergistic effect of a combination of two anti-erbB-2 antibodies. We chose to evaluate combinations of monoclonal antibodies because binding to gpl85erbB-2 should induce complex lattices of highly constrained protein on the cell surface. We reasoned that these lattices might alter receptor function in a manner different from that of the binding of single monoclonal antibodies that can be expected to induce receptor dimerization. Our results show profound growth inhibition with the combination of two antibodies when compared to single-antibody treatment.

MATERIALS AND METHODS

Monoclonal Antibodies. Mice were immunized using a membrane preparation of N/erbB-2 cells (NIH/3T3 cells engineered to overexpress the human erbB-2 protein). Following tests of polyclonal antibody response using immunoprecipitation, three fusions were conducted using the myeloma cell line Ag8.653 and standard techniques. These fusions produced approximately 1500 hybridoma clones which were each screened using enzyme-linked immunosorbent assay. Membranes isolated from N/erbB-2 cells were bound to polystyrene plates, and culture medium was added to allow antibody-antigen interaction. Immunoglobulin binding was detected using a biotinylated goat antimouse antibody, streptavidin horseradish peroxidase, and o-phenylenediamine hydrochloride. Positive reacting hybridomas were picked and counter-screened using membranes from wild-type NIH/3T3 membranes. The monoclonal specificity was confirmed by immunoprecipitation analysis. Five hybridomas were picked with anti-erbB-2-specific reactivity and cloned by limiting dilution; two of these were designated as e21 and e23 and were used in this study. Ascites was prepared by administering injections of 10^7 hybridoma cells to pristane-primed mice. Antibodies were isolated in large amounts from ascites fluid and purified by high-performance liquid chromatography with a Gamma-bind Ultra column (Genex, Gaithersburg, MD). SDS-PAGE1 was run under nonreducing conditions using Coomassie blue staining with a single band at M, 180,000 observed, indicating a >98% purified preparation. From 1 ml of ascites approximately 8–15 mg of antibody were routinely purified.

Cell Lines and Tissue Culture. The human gastric tumor cell line used in these studies, N87, has been previously described (22) and was routinely subcultured in RPMI 1640 supplemented with 10% fetal bovine serum. The cell lines SK-BR-3, MDA-MB-468, and MDA-MB-231 (breast) and SK-OV-3 (ovarian) were routinely subcultured in improved minimal essential medium (IMEM) supplemented with 5% fetal bovine serum. Cultures were maintained in humidified incubators at 37°C in an atmosphere of 5% CO2 and 95% air. Cells were tested for Mycoplasma using a ribosomal RNA hybridization method (Gen-Probe, San Diego, CA).

Growth Inhibition Assays. A single cell suspension of 10,000 cells/well was plated in a serum-free defined media of RPMI 1640 containing bovine serum insulin (5 µg/ml), human transferrin (10 µg/ml), 17-β-estradiol (10 nM), sodium selenite (5 nM), and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid or in RPMI 1640 containing 2% fetal bovine serum. PBS, e21, e23, or a combination of e21 and e23 (0.1, 1, 10 µg/ml final concentration) was then added. The plates were

Received 8/29/91; accepted 3/11/92.

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1 Supported by Grant 2 R44 CA50077-02 from the Department of Health and Human Services.
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The abbreviations used are: SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; cDNA, complementary DNA.

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incubated for 5–7 days in a CO₂ incubator with humidity at 37°C. Cell viability was monitored by one of two different methods. The first, the MTT assay (23), is based on the ability of live cells to reduce a tetrazolium-based compound, MTT, to a purplish colored formazan product that can be measured spectrophotometrically. After 7 days, 50 μl of MTT reagent (0.1 mg) were added and allowed to incubate for 4 h at 37°C. Ninety % of the media was then removed, and the crystals were solubilized in 0.175 ml dimethyl sulfoxide with absorbance measured at 540 nm in a Molecular Devices Max kinetic microplate reader.

The second method involves the cell number measurement in monolayer cultures by crystal violet staining (24). Cells were plated as above and after 7 days cells were fixed by the addition of 20 μl of a 11% glutaraldehyde solution. After being shaken on a Bellco Orbital Shaker for 15 min the plates were washed three times with deionized water. Plates were then air-dried and stained by the addition of 100 μl of a 0.1% solution of crystal violet dissolved in 200 mM borate, pH 6.0. After being shaken for 20 min at room temperature, excess dye was removed by extensive washing with deionized water, and the plates were air-dried prior to solubilization in 100 μl of 1% acetic acid. Absorbance was measured at 590 nm in the microplate reader.

Antibody Specificity. Subconfluent SK-Br-3 monolayers were metabolically labeled with [35S]Cys (specific activity, 1000 Ci/mmol). Total cell proteins were immunoprecipitated with 10 μl of the indicated antibodies. The immune complexes were recovered by protein G-Sepharose (Genex) and analyzed by SDS-PAGE on an 8–16% Tris-glycine gel. The gel was exposed to film at −70°C overnight with an intensifying screen.

Western Blots. Cells or tumors were lysed in sample buffer which contained 0.125 M Tris-HCl, 4% SDS, 0.002% bromophenol blue, and 15% glycerol. Five % β-mercaptoethanol was added after the protein concentration was determined. Samples (10 μg total protein) were boiled for 3 min, fractionated by SDS-PAGE on 8–16% Tris-Glycine gel (Novex, Encinitas, CA), and transferred to nitrocellulose. Detection of gp185erbB-2 was performed with a monoclonal antibody (E2-4001; Molecular Oncology, Inc.) to the COOH-terminal portion of the protein.

Southern Blots. DNA was extracted from cell lines and human placenta tissue using guanidine thiocyanate and cesium gradient centrifugation. DNA (15 μg) was cleaved with restriction enzyme HindIII, separated by electrophoresis on a 1% agarose gel, transferred to nitrocellulose, and probed with a radioactive erbB-2 cDNA probe as previously described (25). The cDNA probe corresponds to the entire erbB-2 protein coding region.

In Vitro Antitumor Assay. Tumor cells (5 x 10⁶/mouse) were injected s.c. into the flanks of BNX (beige, nude, xid) mice. The day after cell inoculation treatment was begun which consisted of four trial groups (3 mice/group), each given 0.2-ml i.p. treatment injections twice a week. Tumor growth was monitored at least once a week and reported as an average relative tumor volume. The effect of treatment after the formation of small tumors was also carried out. Cells were injected using the same treatment protocol as above except that the treatment was begun 4 days after cell injection instead of 1 day after. Animal care was in accordance with institutional guidelines. Statistical analysis was carried out using a SAS Computer Package (SAS Institute, Cary, NC).

gp185erbB-2 Stability Assay. Subconfluent N87 cell monolayers were pulse-labeled 1 h with 20 μCi [35S]Cysteine and then chased with 5 mM Cys in the presence of antibody for 24 h. Total cellular protein was immunoprecipitated as described above using a monoclonal antibody directed against the COOH terminus of gp185erbB-2 coupled to Sepharose and analyzed by SDS-PAGE. The gel was exposed to film at −70°C overnight with an intensifying screen.

Tyrosine Phosphorylation. Cells were plated as in the protein stability assay. After 1 h, cells were processed, and proteins were extracted in sample buffer for electrophoresis as in the antibody specificity experiment. Following electrophoresis the proteins were electroblotted onto nitrocellulose paper and incubated with anti-phosphotyrosine IgG (monoclonal; Upstate Biotechnology, Inc.) and immunodetected using 125I-protein A. The gel was exposed to film at −70°C overnight with an intensifying screen.

RESULTS

Monoclonal antibodies directed against the extracellular domain of gp185erbB-2 were prepared by immunizing mice using a membrane preparation from NIH/3T3 cells engineered to overexpress the human erbB-2 protein (N/erbB-2 cells). Two of these, designated e21 and e23, were used in this study. Both antibodies specifically immunoprecipitated a single 35S-labeled protein of molecular weight 185,000 from SK-Br-3 cells (a breast cancer cell line which overexpresses gp185erbB-2 protein) (26) as shown in Fig. 1. No immunoprecipitation was detected in cells which do not overexpress the gp185erbB-2 protein (e.g., MDA-MB-468; data not shown).

Since the erbB-2 oncogene is overexpressed frequently in at least 20% of stomach cancers and these tumors have a poor clinical course, we investigated the gastric cell line N87, which overexpresses gp185erbB-2 at high levels. An immunoblot of the N87 cell line and a tumor formed by implantation of N87 cells into a nude mouse is shown in Fig. 2 compared to the breast cell lines SK-Br-3 (high level of gp185erbB-2 overexpression) and MDA-MB-231 (low level of gp185erbB-2 overexpression). The levels of erbB-2 gene amplification in N87, as shown in Fig. 3, surpassed those found in the well-characterized SK-Br-3 and SK-OV-3 cell lines (26).

The effect of these antibodies on growth was first studied in vitro using a semiautomated colorimetric MTT assay. A dose-
response analysis of the effects of the antibodies on N87 cell proliferation is shown in Fig. 4. Either e21 or e23 alone had no effect on the monolayer growth of cells up to a concentration of 10 μg/ml (6 μM). A 1:1 combination of the two, however, markedly affected cell proliferation at doses as low as 1 μg/ml. Fab fragments prepared from both antibodies also had no effect on cell growth alone or in combination (data not shown). The Fab fragments were found to inhibit >80% of the binding of 125I-e23 at the concentrations used. This was comparable to the complete antibody. In analogous experiments with three other gastric cell lines displaying little or no overexpression by immunoblot analysis, no activity was observed with the antibody combination or the antibodies alone (data not shown). The same results were obtained using crystal violet staining.

The efficacy of combination antibody therapy was subsequently tested on the growth of N87 cells growing as tumor xenografts. N87 cells injected s.c. into nude mice produce rapidly growing tumors with a short latency. Tumor growth at the injection site was easily quantitated. As shown in Fig. 5, the N87 cells did not form tumors in the animals treated with the combination of e21 and e23. In sharp contrast they were potently tumorigenic in animals treated with the single antibodies or PBS and grew substantially over the period measured. In contrast to in vitro experiments, each monoclonal antibody alone may have limited activity, thus partially restricting the rate of tumor growth. To determine if the combined therapy with e21 and e23 was able to eradicate established tumors, an experiment was performed in which tumors were allowed to grow to measurable sizes prior to antibody treatment. The results are illustrated in Fig. 6. In animal groups randomized so that the starting size of the tumors was approximately the same volume, the tumors continued to grow when the animals were given single-antibody treatment of e21 or e23. In contrast, in the animals given combination treatment with e21 and e23, tumors completely regressed after 11 days (4 treatments of 200 μg of total antibody) and continued to be absent for another 2 weeks. After another month, the tumors reappeared, demonstrating that some of the cells had escaped the treatment.

To investigate the molecular basis for the antiproliferative effects of e21 and e23, we measured the rate of gp185erb2 turnover in the presence or absence of antibodies. N87 cells were pulse-labeled with [35S]Cys and then chased for various times in the presence of single antibody or the e21/e23 combi-
e23 combination mimics the ligand, then treatment should result in increased gp185erbB-2 autophosphorylation upon e21/e23 combined treatment. We used antiphosphotyrosine immunoblots to test this hypothesis. As shown in Fig. 8, increases in the tyrosine phosphorylation of gp185erbB-2 from N87 cells were observed 15 min and 1 h after the addition of the single antibodies or the antibodies in combination. (The same results were observed at 30 min and 2 h; data not shown.) This suggests that activation of tyrosine kinase activity may be necessary but is probably not sufficient for growth inhibition.

A possible explanation for the decreased half-life of gp185erbB-2 is that the combined treatment with e21 and e23 acts by constraining gp185erbB-2 into an activated conformation, thus mimicking an agonist ligand. This kind of mechanism has been postulated for other agonistic antibodies (20). If the e21/e23 combination mimics the ligand, then treatment should result in increased gp185erbB-2 autophosphorylation upon e21/e23 combined treatment. We used antiphosphotyrosine immunoblots to test this hypothesis. As shown in Fig. 8, increases in the tyrosine phosphorylation of gp185erbB-2 from N87 cells were observed 15 min and 1 h after the addition of the single antibodies or the antibodies in combination. (The same results were observed at 30 min and 2 h; data not shown.) This suggests that activation of tyrosine kinase activity may be necessary but is probably not sufficient for growth inhibition.

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\[ \text{erbB-2 \ antibody has been proposed as a therapeutic. Following} \\\	ext{kinase activation, another sterically distinct receptor-receptor} \\\	ext{interaction may then facilitate internalization and down-regu-} \\\	ext{lation. This alignment may be induced when the \text{erbB-2} \text{ protein} \\\	ext{molecule is constrained by binding a combination of anti-erbB-2} \\\	ext{e21 and e23. This model is supported by the finding that the} \\\	ext{Fab fragments do not show antigrowth activity in combination,} \\\	ext{nor do other combinations of anti-erbB-2 monoclonal antibo-} \\\	ext{dies that we have tested. Alternatively, the combined e21/e23} \\\	ext{treatment may induce a nonphysiological pattern of receptor} \\\	ext{oligomerization that shunts gp185*erbB-2 to the down-regulation} \\\	ext{pathway.} \\\	ext{Regardless of the mechanism, our results demonstrate that a} \\\	ext{combination of anti-receptor antibodies leads to different and} \\\	ext{more potent antitumor activities than single antibodies, and} \\\	ext{therefore, combination antibody therapy may be a useful ap-} \\\	ext{proach to the treatment of human malignancies overexpressing} \\\	ext{gp185*erbB-2. This approach may be particularly important in} \\\	ext{the treatment of gastric cancer, a disease which responds poorly} \\\	ext{to current systemic chemotherapies.} \\

\text{ACKNOWLEDGMENTS} \\
\text{We are grateful to Adi Gazdar for providing the N87 human gastric} \\
\text{cell line. We thank Teresa Clayton, John Henry, and Laura Porter} \\
\text{for excellent technical assistance and Michael Berman and Nancy Turner} \\
\text{for critical reading of the manuscript.} \\

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\text{Discussion} \\
\text{Our results are the first reported observation of tumor regres-} \\
\text{sion induced by anti-erbB-2 monoclonal antibodies. While complete} \\
\text{regression was observed, the treatment was not curative in that tumors grew back in 6 weeks. Other investigators have} \\
\text{focused their studies on the use of single anti-erbB-2 (or HER-} \\
2) antibodies against the growth of human breast cancer cells \text{in vitro} and against the formation of tumors by fibroblasts \\
\text{transformed by the overexpression of human \text{erbB-2} (or HER-} \\
2). Previous studies have shown that two anti-neu antibodies can inhibit the growth of tumors by murine cells transformed by the mutationally activated \text{neu} oncogene (27). This effect is also seen with the inhibition of leukemic 
\text{tumor cell growth using anti-transferrin monoclonal antibodies (28). In a recent publication by Hancock et al. (29), the authors studied the} \\
\text{synergistic effect of an antibody against c-erbB-2 plus cis-} 
\text{diaminedichloroplatinum.} \\

\text{Our results suggest that each antibody singly can stimulate gp185*erbB-2 autophosphorylation; the combined antibody treat-} 
\text{ment also stimulates gp185*erbB-2 autophosphorylation but acts additionally by altering the turnover rate of gp185*erbB-2. Since} 
\text{combination antibody treatment probably acts primarily to spatially constrain the erbB-2 molecule, our results raise the intriguing possibility that two different receptor-receptor inter-} 
\text{actions might take place during the response of gp185*erbB-2 to} 
\text{natural ligand. One receptor-receptor interaction is required for} 
\text{tyrosine kinase activation, which might be mimicked by certain monoclonal antibodies that activate erbB-2 autophosphory-} 
\text{lation (20, 30) or e21 and e23. Such a monoclonal anti-}


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