Cell Surface Density of p185c-erbB-2 Determines Susceptibility to Anti-P185c-erbB-2-Ricin A Chain (RTA) Immunotoxin Therapy Alone and in Combination with Anti-P170EGFR-RTA in Ovarian Cancer Cells

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ABSTRACT

Approximately 30% of ovarian and breast cancers overexpress p185c-erbB-2 with as many as 10⁶ receptors/cell. Normal cells have as few as 10⁴ receptors/cell. We have examined the susceptibility of SKOV3 human ovarian cancer cells to anti-c-erbB2 antibodies and immunotoxins as a function of c-erbB-2 density on the cell surface. A panel of SKOV3 clones that expressed different densities of p185c-erbB-2 receptor were generated through transfection with the c-erbB-2 gene. A significant correlation was found between p185c-erbB-2 density and susceptibility to killing by anti-p185c-erbB-2-ricin A chain (anti-p185c-erbB-2, RTA) immunotoxins. With 10⁵ copies/cell of p185c-erbB-2, <10% of clonogenic ovarian cancer cells could be eliminated, whereas in clones that expressed 10⁶ copies/cell of p185c-erbB-2, 99.9% of clonogenic tumor cells were killed. In cell lines that overexpressed p185c-erbB-2 and also expressed p170EGFR, anti-p185c-erbB-2, RTA and anti-p170EGFR, RTA immunotoxins exerted synergistic cytotoxicity. Treatment with the two immunotoxins could eliminate 99.9% of clonogenic cells. Importantly, tumor cells that had survived first treatment with anti-p185c-erbB-2-RTA alone still retained sensitivity to repeat treatment with the same immunotoxin and also proved susceptible to the synergistic cytotoxicity of anti-p185c-erbB-2, RTA in combination with anti-p170EGFR, RTA. Growth characteristics of the clones expressing various levels of p185c-erbB-2 were also studied. No correlation was found between p185c-erbB-2 expression levels and the rate of anchorage-dependent growth, anchorage-independent growth, or in vivo growth in nude mice.

INTRODUCTION

c-erbB-2/HER-2 is a M, 185,000 receptor tyrosine kinase with a high degree of homology to the EGFR. It has been suggested that c-erbB-2 is important in the pathogenesis of a variety of human malignancies, including breast and ovarian cancers. Overexpression of p185c-erbB-2 in ovarian cancers has been associated with a poor prognosis in several (1–3), but not all (4, 5), studies. Poor prognosis may relate to increased invasiveness or decreased response to chemotherapy rather than to increased proliferative capacity (6, 7). The protein encoded by the c-erbB-2 gene is an attractive target for serotherapy because 30% of breast and ovarian cancers overexpress this receptor. Binding of specific monoclonal antibodies to the extracellular domain of c-erbB-2 can inhibit tumor growth both in vitro and in vivo (8–16). When various cancer cell lines were compared, the most marked growth inhibition in vitro was seen in those cells that overexpressed c-erbB-2 (16, 17). However, native anti-c-erbB-2 antibody produces only modest inhibition of human breast and ovarian cancer cells (9). More potent growth inhibition can be achieved when antibodies are conjugated with radionuclides (18) or toxins (19–23). Other cell surface growth factor receptors also lend themselves as potential targets for immunotoxins. EGFR is also frequently expressed on the surface of ovarian cancer cells both in vitro and in vivo (24). Monoclonal antibodies against EGFR are available that can inhibit growth of cells that bear the receptor (25). Again, greater cytotoxicity in vitro and augmented antitumor activity against heterografts in vivo has been obtained when anti-EGFR antibodies were conjugated with toxins (26–28). When two distinct immunotoxins are used in combination, synergistic cytotoxicity is often observed (20).

In the present study, we examined the relationship between c-erbB-2 receptor density and susceptibility to anti-c-erbB-2 immunotoxin alone and in combination with anti-EGFR. Selection of cell clones with different levels of p185c-erbB-2 expression allowed us to correlate the number of receptors expressed with the inhibitory potency of native as well as toxin-conjugated anti-erbB-2 antibodies. We also examined spontaneous growth rate of the SKOV3 ovarian cancer cells expressing different...
levels of p185-c-erbB-2 under various tissue culture conditions, as well as in nude mice.

MATERIALS AND METHODS

Cell Lines and Plasmids. The SKOv3 human ovarian cancer cell line was obtained from American Type Culture Collection (Manassas, VA) and was maintained in monolayer culture in McCoy's medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Transfected cells were grown in the same media, but also supplemented with 400 μg/ml G418.

Plasmid pABT 9002 containing a cytomegalovirus promoter and the coding sequence for the full-length human c-erbB-2 gene and a selection marker, G418, was obtained from Applied Biotechnology/Oncogene Sciences (Cambridge, MA), and antibody 520C9 was obtained from Chiron Corporation (Emeryville, CA). Anti-EGFR monoclonal antibody 225 was generated against the extracellular domain of EGFR; the production and characterization of 225 have been described (32). Murine monoclonal antibody MOPC21 was used as an isotype-matched control that did not bind to p185 or EGFR and was obtained from American Type Culture Collection.

To prepare immunotoxins, antibodies were conjugated to recombinant RTA, as described previously (33). Radioconjugation with Na125I (Amersham, Arlington Heights, IL) was performed using the iodogen method (34).

Scatchard Analysis. The number of receptors/cell was estimated using different concentrations of antibodies to saturate binding sites. SKOv3 cells were seeded in 48-well plates (4 x 10^6/well) and incubated overnight until monolayers were confluent. Subsequently, cells were incubated with 10% BSA in McCoy's medium for 1.5 h at 37°C to block nonspecific binding of the antibody. The 125I-labeled antibody was then added to final concentrations of 2.5, 0.5, 0.25, 0.05, and 0.005 μg/ml. After a 2-h incubation at 37°C, cells were washed 3 times in McCoy's medium and lysed with 2N NaOH, and radioactivity was counted with a Packard gamma counter. Aliquots of the 125I-labeled antibody were also counted as a control. The number of cells in each microtiter well was estimated by detaching monolayers from six unlabeled wells using trypsin and counting the number of cells in each microtiter well. SKOv3 parental and transfected cells were seeded into tissue culture flasks (10^6 cells/flask) and incubated for 2, 4, 6, and 8 days, respectively. Cells were counted in a hemocytometer at the end of each incubation interval.

Assays of Cell Growth. To test anchorage-dependent growth, SKOv3 parental and transfected cells were seeded into T75 tissue culture flasks (10^6 cells/flask) and incubated for 2, 4, 6, and 8 days, respectively. Cells were counted in a hemocytometer at the end of each incubation interval.

Anchorage-independent growth was measured in 35-mm tissue culture dishes (Nunc, Naperville, IL) coated with 1 ml of 0.6% agar (DIFCO Laboratories, Detroit, MI). SKOv3 cells (5 x 10^4 cells/dish) were resuspended in 1 ml of 0.3% agar in McCoy's medium, and different monoclonal antibodies were added to the suspension. Colonies containing 30 or more cells were counted at regular time intervals after further incubation.

To test growth in immunosuppressed mice, nu/nu mice were bred and maintained in a pathogen-free environment. Portions (10^5) of the SKOv3 parental cells and individual transfected clones, respectively, were injected intradermally into nu/nu mice. Antitumor activity was measured as the increase in tumor diameter, as described previously (33). Radioconjugation with Na125I (Amersham, Arlington Heights, IL) was performed using the iodogen method (34).

Scatchard Analysis. The number of receptors/cell was estimated using different concentrations of antibodies to saturate binding sites. SKOv3 cells were seeded in 48-well plates (4 x 10^6/well) and incubated overnight until monolayers were confluent. Subsequently, cells were incubated with 10% BSA in McCoy's medium for 1.5 h at 37°C to block nonspecific binding of the antibody. The 125I-labeled antibody was then added to final concentrations of 2.5, 0.5, 0.25, 0.05, and 0.005 μg/ml. After a 2-h incubation at 37°C, cells were washed 3 times in McCoy's medium and lysed with 2N NaOH, and radioactivity was counted with a Packard gamma counter. Aliquots of the 125I-labeled antibody were also counted as a control. The number of cells in each microtiter well was estimated by detaching monolayers from six unlabeled wells using trypsin and counting the cells in the presence of 0.1% trypsin blue using a hemocytometer.
groups of six mice. Three-dimensional tumor measurements were taken every 3–4 days for up to 5 months. Mean tumor diameter was calculated from the cube roots for the products of the three measurements averaged over six mice for each time point in an experiment.

Cell proliferation was also assayed in 96-well flat-bottomed plates by [3H]thymidine incorporation (Costar Corp., Cambridge, MA). Cells were seeded in wells (2.5 × 10^4 cell/well) and incubated for 42 h, and 1 μCi [3H]thymidine was added to each well. After 6 h of additional incubation, cells were washed three times with 50 mM PBS (pH 7.4), and harvested by adding 2N sodium hydroxide to each well. Incorporated radioactivity was measured using a Packard scintillation counter.

**Determination of the Effects of Immunotoxins on Clonogenic Growth.** A limiting dilution assay was used to determine inhibitory effects of immunotoxins on clonogenic growth (20). SKOV3 cells (10^6) were incubated with 5 μg/ml immunotoxin for 3 h at 37°C on a rocking platform in a humidified atmosphere containing 5% CO2 and 95% air. At the end of the incubation period, cells were washed twice with medium, and 1:10 serial dilutions in fresh medium were performed 5 times. Aliquots (100 μl) of each dilution were plated onto 96-well microtiter plates and incubated for 14 days. The clonogenic growth of surviving cells was evaluated by phase-contrast microscopy, scoring the number of wells with at least one colony containing 30 or more cells. An estimate of the number of surviving clonogenic units was calculated using a modification of the method of Spearman and Karber (35).

**Isobolographic Analysis.** Isobolographic analysis examines the dose of individual agents required to produce a given antitumor effect and its interaction with other agents (36). For agents with linear dose-response curves, a concave isobole indicates synergy. For agents with nonlinear dose-response curves, a concave isobole may indicate either synergy or additivity. An “envelope of additivity” as described by Steel and Peckham (36) was used to determine the nature of interaction between immunotoxins that exhibit nonlinear dose-response curves. The envelope consisted of two components, mode I and mode II, which represented the theoretical limits of the additive effects of two immunotoxins. Mode I was calculated from the dose-response curves of each agent based on the assumption that the two agents operated independently. Mode II was calculated assuming that the two agents interacted. Immunotoxin combinations were considered synergistic if the isobole fell outside the envelope created by mode I and mode II, and combinations were considered additive if the isobole fell within the envelope.

**Statistical Analysis.** Differences in c-erbB-2 expression were evaluated with the Student’s t test. To compare trends in cell counts and inhibitory efficacy of immunotoxins as functions of c-erbB-2 receptor density, E001 and E12 statistics for the simple order were used (37). This method provides a test for statistical comparison of trends without assumptions about the underlying nature of a trend (e.g., linear). The Pts for the tests comparing the averages in one set of groups to the average in a separate set of groups were based on the Scheffe method of multiple comparisons (38).

**RESULTS**

### p185c-erbB-2 Expression on SKOV3 Cells and c-erbB-2 Transfected Clones.

The expression of p185c-erbB-2 was evaluated in parental SKOV3 cells and in eight different c-erbB-2 transfected clones using 125I-labeled TA1 antibody and Scatchard analysis. The parental SKOV3 cell line expressed 2.6 × 10^5 p185c-erbB-2 sites/cell, whereas the various transfecants expressed 2–10-fold higher levels ranging from 5.6 × 10^5 to 3.3 × 10^6 sites/cell (Table 1). The expression of p170EGFR was also measured. In the parental SKOV3 cells 2.8 × 10^5 EGFR sites/cell were detected, compared with 4.8–7.9 × 10^5 in the different c-erbB-2 transfected clones.

**Growth Rate of SKOV3 Cells Is Not Related to p185c-erbB-2 Receptor Density.** There was no clear association between p185c-erbB-2 receptor density on the cell surface and growth of SKOV3 cells (Table 1). Neither anchorage-dependent nor anchorage-independent growth correlated with levels of p185c-erbB-2 expression (data not shown). Six of eight clones formed colonies in soft agar, as did the parental SKOV3 cells. Interestingly, the two clones with the highest receptor density (3.3 × 10^5 and 1.8 × 10^6 sites/cell, respectively) failed to form colonies in soft agar. Cell proliferation was also assayed with [3H]thymidine incorporation in short-term cultures of SKOV3 cells expressing various levels of erbB-2 receptor and, again, there was no clear correlation between expression of

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**Fig. 2** Anti-p185c-erbB-2 antibody-mediated inhibition of anchorage-independent growth of parental SKOV3 cells (■) and transfectants (●) that express different levels of p185c-erbB-2.

**Fig. 3** Anti-p185c-erbB-2, RTA immunotoxin-mediated inhibition of anchorage-independent growth of parental SKOV3 cells (■) and transfectants (●) that express different levels of p185c-erbB-2.
p185\textsuperscript{\textit{c-erbB-2}} receptor and proliferative activity. Consistent with the above findings, the parental SKOv3 ovarian cancer cell line as well as the four sublines tested, each expressing various levels of p185\textsuperscript{\textit{c-erbB-2}} receptors, grew progressively in nude mice but there was no correlation between \textit{in vivo} growth and p185\textsuperscript{\textit{c-erbB-2}} expression (Fig. 1).

\textbf{Anti-p185\textsuperscript{\textit{c-erbB-2}} Antibody Has Modest Growth Inhibitory Activity on Cells that Overexpress \textit{erbB-2}.} Parental SKOv3 cells and six transfected clones expressing different levels of p185\textsuperscript{\textit{c-erbB-2}} were tested for clonogenic growth inhibition by ID5 antibody in concentrations ranging up to 5 \textmu g/ml. The ID5 antibody was used because it has previously demonstrated significant growth inhibitory activity on other cell lines grown in soft agar, whereas TA1 and 520C9, which bind different epitopes of the extracellular domain of p185\textsuperscript{\textit{c-erbB-2}}, had no such growth inhibitory effect (16). ID5 inhibited clonogenic growth ranging from 10–90%, depending on the clone studied (Fig. 2). There was a trend toward greater inhibition in cells that exhibited the highest density of receptors, but this trend was not linear and has not reached statistical significance.

\textbf{Cytotoxicity of Anti-p185\textsuperscript{\textit{c-erbB-2}}-Ricin A Immunotoxin Correlates with p185\textsuperscript{\textit{c-erbB-2}} Expression.} Cytotoxicity of anti-p185\textsuperscript{\textit{c-erbB-2}} ricin A immunotoxins (520C9-RTA and TA1-RTA) was assessed by limiting dilution clonogenic assay. A positive correlation \((P < 0.01)\) was observed between receptor density and susceptibility to immunotoxins. Cells that expressed \(>10^6\) receptors/cell exhibited 100–1,000 times greater sensitivity to cytotoxic effects of anti-p185\textsuperscript{\textit{c-erbB-2}}-RTA immunotoxin than did parental cells (Fig. 3). The sensitivity curve indicated a plateau for cell kill above \(10^6\) receptors/cell.

\textbf{Anti-p185\textsuperscript{\textit{c-erbB-2}}, RTA and Anti-p170\textit{EGFR}-RTA Immunotoxins Exhibit Synergistic Cytotoxicity.} Possible synergy between anti-c-erbB2 (520C9-RTA, TA1-RTA) and anti-EGFR (225-RTA) immunotoxins were evaluated on parental SKOv3 and SKOv3 clone 9002-18 cells. SKOv3 cells express \(2.5 \times 10^5\) copies of p185\textsuperscript{\textit{c-erbB-2}}/cell, combination of 225-RTA and either 520C9-RTA or TA1-RTA exerted synergistic cytotoxicity, but eliminated only approximately 90% (1 log) of clonogenic cells (Table 2). In experiments with SKOv3-9002-18 cells that express approximately five times higher a number of p185\textsuperscript{\textit{c-erbB-2}} receptors than parental cells, the combination of 520C9-RTA and 225-RTA could eliminate 99.97% (3.86 logs) of clonogenic tumor cells (Table 2). Isobolographic analysis of the interaction between the distinct immunotoxins indicated a synergistic effect (Fig. 4A). Similar synergistic cytotoxicity was obtained when a combination of TA1-RTA and 225-RTA were used (Fig. 4B). This suggests that the synergistic interactions were related to the antigenic targets and not to the particular immunotoxins used.

Cells that had survived previous treatment with single agent 520C9-RTA, TA1-RTA, or 225-RTA immunotoxins still demonstrated sensitivity to cytotoxicity at a second exposure to the same agent. Also, synergistic cytotoxicity of combination therapy was obtained when a combination of TA1-RTA and 225-RTA was used.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|c|c|}
\hline
\hline
SKOv3 & 0.82 & 0.35 & 0.70 & 1.17 & 0.93 & Synergistic \\
SKOv3-9002-18 & 2.91 & 1.55 & -- & 3.86 & 4.0 & Synergistic \\
\hline
\end{tabular}
\caption{Additive and synergistic cytotoxicity (log kill) of anti-p185\textsuperscript{\textit{c-erbB-2}}-RTA and anti-p170\textit{EGFR}-RTA immunotoxins}
\end{table}

The table illustrates synergistic interaction between distinct immunotoxins. The numbers indicate mean log kill observed in clonogenic assays after treatment with either individual or combination immunotoxins. SDs of means were within 15%.
immunotoxin therapy was still observed with cells that had survived previous treatment with individual immunotoxins (Fig. 4C).

**DISCUSSION**

It has been suggested that overexpression of p185<sup>erbB-2</sup> alters growth characteristics in cell lines (39). Considering that p185<sup>erbB-2</sup> is a tyrosine kinase growth factor receptor, it may be assumed that increased expression of this receptor will result in an increased rate of proliferation. Our results do not demonstrate a consistent positive correlation between the level of p185<sup>erbB-2</sup> expression and growth rate under either anchorage-dependent or anchorage-independent tissue culture conditions or in nude mice. In general, there was a trend toward faster growth in cells that expressed more receptors, but this was not statistically significant. Of interest is that the two SKOv3 clones that expressed the highest density of the receptor (3.3 × 10<sup>6</sup> and 1.8 × 10<sup>6</sup> receptors/cell) were unable to grow in soft agar. Thus, the marked overexpression of p185<sup>erbB-2</sup> might inhibit, rather than favor, clonogenic growth of malignant cells. This is consistent with recent observations that have demonstrated growth inhibitory effect of heregulin and agonistic anti-p185<sup>erbB-2</sup> antibodies in breast cancer cells that overexpress p185<sup>erbB-2</sup> (10<sup>6</sup> receptors/cell; Ref. 17). A similar growth inhibitory experience was reported with EGF in a breast cancer cell line that expresses a very high number of EGF-Rs (40).

It has been shown that antibodies raised against the p185<sup>erbB-2</sup> receptor are able to inhibit growth of various tumor cell lines (8–16). In studies conducted with different cell lines, susceptibility to antibody-mediated growth inhibition seemed to correlate with the number of antigenic targets expressed by a given cell line (17). In this study, we tested the hypothesis that the degree of susceptibility to anti-p185<sup>erbB-2</sup> immunotoxin is a function of antigen density. A series of clones differing in levels of p185<sup>erbB-2</sup> expression were derived from SKOv3 cells by transfecting them with the c-erbB2 gene. A positive correlation has emerged between c-erbB2 expression on the cell surface and susceptibility to anti-c-erbB2 immunotoxins. A 5-fold increase in expression of p185<sup>erbB-2</sup> was associated with a 3 log (1000 time) increase in the efficiency of immunotoxin-mediated elimination of clonogenic cells. The sensitivity curve indicated a plateau for cell kill by immunotoxins above 10<sup>6</sup> antigenic sites/cell. These findings are consistent with an earlier report in which anti-p185<sup>erbB-2</sup> immunotoxin inhibited 99.9% of the clonogenic growth of different cell lines that expressed 10<sup>4</sup>-10<sup>5</sup> copies of the receptor, but exhibited little cytotoxicity for cell lines with lower receptor density (17). The marked dependence of cytotoxicity on overexpression of p185<sup>erbB-2</sup> is not readily explained since, in theory, the translocation of a few RTAs to the cytosol could kill a cell. The binding of many immunotoxin molecules to the surface of the tumor cell may be required to assure adequate internalization and translocation of ricin toxin. Indeed, internalization is not very efficient. Only 20% of the antibodies bound to p185<sup>erbB-2</sup> were internalized within 1 h (41).

In previous studies, immunotoxins reactive with distinct cell surface antigens have exerted either additive or synergistic antitumor activity (19, 20, 23, 26, 33). In this study, we also demonstrated synergistic cytotoxicity of anti-p185<sup>erbB-2</sup>-RTA and anti-p17<sup>EGFR</sup>-RTA immunotoxins. By formal isobolographic analysis, the two immunotoxins exerted synergistic toxicity against tumor cell lines that expressed from 2.6 × 10<sup>5</sup> to 1.1 × 10<sup>6</sup> p185<sup>erbB-2</sup> sites/cell. Interestingly, cells that survived the first treatment with one or more immunotoxins often remained susceptible to retreatment with the same immunotoxin(s) and to combination therapy with anti-p185<sup>erbB-2</sup>-RTA and anti-p17<sup>EGFR</sup>-RTA.

Several features of immunotoxin-mediated cell kill observed in vitro could have relevance to designing clinical studies for serotherapy of cancer. The marked dependence of cytotoxicity on the level of p185<sup>erbB-2</sup> expression may provide a favorable therapeutic index for immunotoxin therapy. On the basis of immunohistochemical analysis, most normal tissue exhibit no more than 10<sup>4</sup> sites/cell of p185<sup>erbB-2</sup>, whereas approximately 30% of ovarian and breast cancers overexpress p185<sup>erbB-2</sup> (41, 42). Also, in immunohistochemical studies some 60% of ovarian cancers coexpressed p185<sup>erbB-2</sup> and p17<sup>EGFR</sup>, whereas similar coexpression was found in relatively few normal tissues (24). These findings together with the observation that distinct immunotoxins often produce synergistic cytotoxicity provide a further rational for exploring combination immunotoxin therapy.

**REFERENCES**


Cell surface density of p185(c-erbB-2) determines susceptibility to anti-p185(c-erbB-2)-ricin A chain (RTA) immunotoxin therapy alone and in combination with anti-p170(EGFR)-RTA in ovarian cancer cells.

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