Low Levels of Her2/neu Expressed by Ewing’s Family Tumor Cell Lines Can Redirect Cytokine-Induced Killer Cells

Michael R. Verneris,1 Arash Arshi,2 Matthias Edinger,2 Martin Kornacker,2 Yas0 Natkunam,3 Mobin Karami,2 Yu-an Cao,1 Neyssa Marina,4 Christopher H. Contag,4 and Robert S. Negrin2

Abstract

Purpose: To identify novel treatments for pediatric solid tumors and/or for malignancies with low-level Her2/neu expression.

Experimental Design: Using fluorescence-activated cell sorting and immunohistochemistry, Her2/neu expression was determined on cell lines derived from Ewing’s family tumors (EFT) and neuroblastoma. Sensitivity to trastuzumab treatment was investigated using an in vitro proliferation assay. Cytotoxicity against EFT cell lines was done with either freshly isolated or ex vivo activated and expanded T cells (cytokine-induced killer cells, CIK cells), with or without addition of a CD3xHer2/neu bispecific antibody. The effects of either trastuzumab, CIK cells alone, or CD3xHer2/neu bispecific antibody redirected CIK cells was determined using a SCID/hu model of EFTs and serial, noninvasive bioluminescent imaging.

Results: EFT cell lines express 5- to 10-fold lower levels of her2/neu than either breast (BT-474) or ovarian (SK-OV-3) cell lines. Treatment of EFT cell lines with trastuzumab did not induce growth inhibition either in vitro or in vivo. In contrast, Her2/neu could be used to redirect CIK cell to mediate cytotoxicity against EFTs both in vitro and in vivo (using two different treatment schemas).

Conclusions: CD3xHer2/neu bispecific antibody and CIK cells may be a suitable approach to treat malignancies with low-level Her2/neu expression not responsive to trastuzumab.

Her2/neu is one of four members of the epidermal growth factor receptor family. The expression of Her2/neu is found on a variety of adult epithelial malignancies, including breast, ovarian, colon, and bladder carcinomas (1–5). Overexpression of Her2/neu induces malignant transformation and tumorigenesis in NIH 3T3 cells, implicating it as an oncogene (6, 7). Her2/neu expression on breast carcinoma samples correlates with multiple adverse prognostic indicators, including advanced disease at presentation and shorter time to progression (8, 9). A variety of murine monoclonal antibodies have been generated against the Her2/neu molecule. One such antibody, 4D5, induces growth inhibition in some Her2/neu-expressing tumor cell lines, most notably those of breast origin (10). Humanized versions of this antibody (trastuzumab) have been generated (10). Clinical trials using trastuzumab treatment for women with Her2/neu-expressing breast malignancies have established that treatment delays the time to disease progression (9), thus leading to Food and Drug Administration approval in 1998.

Ewing’s sarcoma and peripheral primitive neuroectodermal tumor are malignancies of neuroectodermal origin that arise in bone and soft tissue, typically affecting children and young adults. These tumors span a histologic continuum, sharing both histoclonal markers (11), cytogenetic rearrangements (12), and respond similarly to therapy (13), thus leading to their grouping as Ewing’s family tumors (EFT). For patients with localized disease at presentation, the response to chemotherapy is considered aggressive chemoradiation therapy (14, 15). Given these results, novel therapies are necessary.

In this study, we examined EFT cell lines for Her2/neu expression and found low levels of cell surface Her2/neu on all of the four cell lines tested. Interestingly, none of these cell lines responded to treatment with trastuzumab either in vitro or in vivo. Because trastuzumab treatment was ineffective, we investigated whether low levels of Her2/neu expression could be used to redirect ex vivo activated and expanded T cells (also known as cytokine-induced killer cells, CIK cells) to tumor targets using a bispecific antibody. Our results show that CIK cells alone mediated relatively modest cytolysis against these tumor targets. In contrast, redirecting CIK cells to tumor targets using a CD3xHer2/neu bispecific antibody resulted in significantly higher cytotoxicity in vitro and a longer survival...
of tumor-bearing animals. Collectively, these results suggest that CD3xHer2/neu bspecific antibody and CIK cells may be a suitable form of therapy for trastuzumab nonresponsive malignancies.

Materials and Methods

Cell lines. The EFT cell lines CB-AGPN, SK-N-MC, and TC-32 were all grown in cRPMI and were a kind gift of Dr. Patrick Reynolds (Children’s Hospital Los Angeles, Los Angeles, CA). MC-IXC (EFT) was purchased from American Type Culture Collection (Manassas, VA) and cultured in a 1:1 mixture of Eagle’s MEM with nonessential acids and Ham’s F12 medium and 10% fetal bovine serum. The neuroblastoma cell lines NB-SD, NB-EB, and NB-1691 were all grown in cRPMI and were a kind gift of Peter Houghton (St. Jude Children’s Research Hospital, Memphis, TN). The cell line SK-N-DZ was cultured in RPMI 1640. SK-N-SH was cultured in MEM with 2 mmol/L l-glutamine and Earle’s balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, and 1.0 mmol/L sodium pyruvate. SN-N-FI (American Type Culture Collection) was cultured in DMEM with 4.5 g/L glucose and 0.1 mmol/L nonessential amino acids. SK-N-AS was cultured in DMEM with 4 mmol/L l-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose. BE(2)-M117 (American Type Culture Collection) were cultured in Eagle’s MEM with nonessential acids and Ham’s F12 medium.

Fluorescence-activated cell sorting analysis. Cells were collected after brief incubation with trypsin/EDTA and were rendered into a single cell suspension by gentle pipetting. 1 × 10⁶ cells were washed with fluorescence-activated cell sorting (FACS) staining buffer consisting of PBS supplemented with 2% FCS. Ten microliters of either Her2/neu or isotype control antibody (Becton Dickinson, San Jose, CA) were added and incubated for 20 minutes on ice. Excess antibody was washed off and cells were resuspended in FACS staining buffer containing 1 mg/mL of propidium iodine (Sigma, St. Louis, MO) to allow exclusion of dead cells. Cells were analyzed using a single laser FACScan that was modified and made available through the FACS shared-user group at Stanford University. Data analysis was done on >20,000 events using Flowjo software version 3.2 (Tree Star, Inc., San Carlos, CA).

Immunohistochemistry. Air-dried cytoplasm preparations were used for immunohistologic studies. The Food and Drug Administration–approved DAKO HercepTest kit (DAKO Corporation, Carpinteria, CA), using a primary rabbit antibody to human HER2 protein, was used. Pretreatment for antigen retrieval was done in a water bath at 95°C for 40 minutes, followed by cooling for 20 minutes. Detection was done using an automated staining machine (DAKO Autostainer, DAKO Corporation).

Proliferation assay. Proliferation assay was done by culturing either EFT or breast cancer cell lines in complete medium supplemented with increasing amounts of trastuzumab (0-12.5 µg/mL; kindly provided by Genentech, South San Francisco, CA). Either 5 × 10⁵ (MC-IXC, BT-474, and SK-N-DZ) or 1 × 10⁶ (CB-AGPN, TC-32, and SK-N-MC) cells were plated in 96-well flat-bottomed tissue culture plates and allowed to grow for a total of 4 days in a humidified incubator at 37°C with 5% CO₂. Cells were then pulsed with 1 µCi of [³H]thymidine (DuPont-NEN, Boston, MA) and further cultured for 18 hours. Samples were counted in a β-counter and % inhibition of growth was calculated by the following formula:

% Growth inhibition =

\[(⁴H incorporation in the presence of trastuzumab / baseline ⁴H incorporation) \times 100\]

Isolation of peripheral blood lymphocytes and generation of cytokine-induced killer cells. Briefly, mononuclear cells were isolated fromuffy coats obtained from healthy donors by Ficoll-Hypaque density centrifugation and washed thrice with PBS. The final product was either used in a ⁵¹Cr release assay (below) or to generate CIK cells as previously described (16). Briefly, isolated mononuclear cells were resuspended in cRPMI at a final concentration of 2 × 10⁶ cells/mL. On day 0, peripheral blood lymphocytes (PBL) were activated with IFN-γ (1,000 units/mL; Genentech) and the following day cells were stimulated with OKT-3 (25 ng/mL; OrthoBiotech, Raritan, NJ) and recombinant interleukin-2 (300 units/mL; Chiron, Emeryville, CA). Thereafter, cells received both recombinant interleukin-2 (300 units/mL) and fresh media to maintain a density of ~1 × 10⁶ to 3 × 10⁶ cells/mL every 3 to 5 days for a total of 21 days. We have previously shown that, at this time point, the majority of cells are T cells (CD3⁺), with ~50% to 80% being CD3⁺CD8⁺ and the remainder (20-50%) CD3⁻CD4⁻. Approximately 10% to 40% of T cells coexpress CD56 (i.e., CD3⁺CD56⁺). Most of these CD3⁺CD56⁺ cells coexpress CD8⁺ (16-18).

⁵¹Cr release assay. Target cells (MC-IXC, TC-32, CB-AGN, and SK-N-MC) were labeled with ⁵¹Cr (Dupont-NEN) by incubating 1 × 10⁶ to 2 × 10⁶ cells with 300 µCi ⁵¹Cr for 2 hours at 37°C. The labeled cells were washed with PBS thrice and distributed in 96-well plates at 2 × 10⁵ cells/well in triplicate. Effector cells (either freshly isolated PBLs or expanded T cells) were added at a 1:1 effector-to-target (E:T) ratio either alone or in the presence of a CD3xHer2/neu or a CD3xCD18 (both at 10 ng/mL, both kindly provided by Genentech). Targets and effectors were incubated for 4 hours at 37°C and cells were then pelleted by centrifugation and aliquots of supernatant were counted in a γ-counter. The percentage of specific ⁵¹Cr release was calculated according to the following formula:

\[
\text{test release} - \text{spontaneous release} \times 100
\]

\[
\text{maximal release} - \text{spontaneous release}
\]

Maximum ⁵¹Cr release was determined by incubation of target cells in 2% NP40.

RNase protection assay. On days 0, 7, 14, and 21 of culture, 2 × 10⁷ cells were collected and RNA was isolated using the RNeasy kit (Qiagen, Chatsworth, CA). RNase protection assays were done using 2.5 µg total RNA and the RiboQuant multiprobe assay kit (PharMingen, San Diego, CA). RNase protection assay was done according to the manufacturer’s specifications and the templates Apo-3 and Apo-4 were used.

SCID/hu model and bioluminescence imaging. Recipient CB.17 severe combined immunodeficient (SCID) mice (purchased from Stanford University, Stanford, CA) were prepared for tumor inoculation using sublethal irradiation to inhibit natural killer cell function. Animals received a single 2 Gy fraction using a 250/50 KV-X-ray machine at 100 rad/min. After irradiation, mice were maintained on antibiotic water containing trimethoprim/sulfamethoxazole. Recipient mice received 0.5 × 10⁶ MC-IXC-luc cells (0.5 mL/injection) via i.p. injection. Bioluminescent imaging was done after mice were sedated with a mixture of ketamine (100 mg/kg; Fort Dodge Animal Health, Wyeth, Madison, NJ), xylozone (10 mg/kg; Butler, Columbus, OH) and an aqueous solution of luciferin (150 mg/kg; Xenogen Corp., Alameda, CA), all injected i.p. After sedation, animals were placed into the light tight chamber of the charged coupled device camera system (IVIS, Xenogen) and a grayscale body surface reference image (digital photograph) was obtained under weak illumination. The light source was then extinguished and photons emitted from luciferase-expressing cells within the animal body and transmitted through the tissue were quantified using a specialized software program “Living Image” (Xenogen). For anatomic localization, a pseudocolor image representing light intensity (blue least intense and red most intense) was generated and superimposed over the grayscale reference image. Annotations were added using another graphics software package (Canvas 5.0, Deneba, Miami, FL).

Statistics. Statistical analysis of the proliferation and killing assays were done using the Student’s t test (unpaired). Survival analysis was done using the log-rank test.
Results

Cell surface expression of Her2/neu on Ewing's family tumor cell lines. In an attempt to identify novel therapies for the treatment of pediatric peripheral neural derived tumors, we evaluated the expression of the proto-oncogene Her2/neu on neuroblastoma and EFT cell lines. None of the neuroblastoma cell lines had appreciable cell surface expression of Her2/neu (Fig. 1A). In contrast, three EFT cell lines, TC-32, SK-N-MC, and CB-AGPN, and one established subclone of SK-N-MC, MC-IXC, had detectable cell surface expression of Her2/neu (Fig. 1A). When normalized for background staining, the surface density of Her2/neu was ~5- to 10-fold lower than is found on BT-474 (breast carcinoma) or SK-O-V-3 (ovarian carcinoma; Fig. 1A and B). All EFT cell lines had similar surface levels of Her2/neu, with MC-IXC staining slightly dimmer than the others (Fig. 1A and B). FACS histograms show that individual cells from each cell line had relatively homogenous levels of Her2/neu on the

![Image](https://www.aacrjournals.org/doi/fig/1)

**Fig. 1.** Expression of Her2/neu on EFT cell lines. A, columns (fluorescence) indicate mean fluorescence intensity minus background staining as determined by FACS analysis for the stated cell lines. Results are representative of more than three experiments. B, FACS histograms of cell lines stained with either isotype control (dotted line) or Her2/neu antibody (solid line). Shown are the positive control breast cancer cell line (BT-474) and the negative control cell line SK-N-DZ (neuroblastoma) in (1) and (2), respectively. The Ewing's/PNET cells MC-IXC, CB-AGPN, SK-N-MC, and TC-32 are shown in (3) to (6), respectively. Data is derived from >20,000 live events and results are representative of three experiments. C, immunohistochemistry staining of BT-474 and MC-IXC showing strong membrane and cytoplasmic staining for BT-474 and weak membrane staining for MC-IXC.
cell surface (Fig. 1B). Her2/neu expression on EFT cell lines was also investigated using immunohistochemistry. As shown in Fig. 1C, MC-IXC had fine membrane staining for Her2/neu; in contrast, BT-474 cells had strong membrane and cytoplasmic staining.

Given that EFT cell lines expressed Her2/neu, we investigated whether trastuzumab treatment would induce growth inhibition as has been shown in some breast cancer cell lines (10). Using a standard proliferation assay, EFT cell lines were cultured in the presence of increasing amounts of trastuzumab. As shown in Fig. 2, the breast cancer cell line BT-474 (positive control) displayed prompt growth inhibition when cultured with trastuzumab (0 versus 12.5 mg, P < 0.0001). In contrast, the Her2/neu negative neuroblastoma cell line SK-N-DZ (negative control) showed no growth inhibition (0 versus 12.5 mg, P < 0.74). Similarly, the EFT cell lines showed minimal growth inhibition when cultured with trastuzumab in this assay. In some cases, this reached statistical significance (0 versus 12.5 mg, MC-IXC P < 0.01, CB-AGPN P < 0.01, SK-N-MC P < 0.97, and TC-32 < 0.80), but the overall inhibition was quite modest.

Because the above in vitro proliferation assay was conducted over 96 hours and responses to trastuzumab may take longer than this in vivo (19), we tested the response to trastuzumab in tumor-bearing animals. To do this, we used a highly sensitive method of detecting tumor growth and response to therapy in living animals using the firefly gene luciferase (20–22). The dose of trastuzumab used in these studies was based on the recommended dose for humans (i.e., 4 mg/kg loading dose followed by 2 mg/kg). Using luciferase imaging, animals were monitored for response to therapy weekly until day 70. As shown in Fig. 3A, mice injected with tumor cells followed by treatment with PBS have a drop in signal from day 1 to day 7, representing death of nonengrafting tumor cells. After this first week, progressive tumor growth is noted in the PBS-treated animals. Essentially, the same pattern of growth inhibition of either the Her2/neu-negative SK-N-DZ (negative control) or the EFT cell lines (MC-IXC, CB-AGPN, SK-N-MC, TC-32).

CD3xHer2/neu bispecific antibody enhances cytotoxic activity of cytokine-induced killer cells. Our laboratory has characterized a population of ex vivo activated and expanded T cells that have potent antitumor activity without prior antigen priming.
Shown are the relative light emission detected from each individual animal over time.

These polyclonal T cells, called CIK cells, are generated by the timed addition of IFN-γ, cross-linking antibody to CD3 (OKT-3) and intermediate dose interleukin-2 (300 units/mL). After ~21 days of expansion and activation, these T cells mediate MHC-unrestricted antitumor activity against a variety of malignant target cells without prior priming (16–18). A CD3xHer2/neu bispecific antibody has previously been generated, which recognizes CD3 on one arm and Her2/neu on the other (23–25). This reagent enhances CIK cell cytotoxicity against Her2/neu-overexpressing breast and ovarian carcinoma cell lines, such as BT-474 and SKOV-3 (19, 26).

To test whether CIK cells mediate antitumor activity against EFT cell lines and whether cytotoxicity could be enhanced by the addition of a CD3xHer2/neu bispecific antibody, we obtained PBLs from healthy donors and did 51Cr release assays to determine the expression of these molecules over time in culture. As shown in Fig. 4C and D, there was a pronounced induction of granzyme A, granzyme B, perforin, FasL, and TRAIL over time in culture.

To test the antitumor activity of CIK cells against EFT cells in living animals, we engrafted MC-IXC-luc into SCID mice and treated the animals 1 day later (day 1) with PBS (Fig. 5A), CIK cells (Fig. 5B), or CIK cells and the CD3xHer2/neu bispecific antibody (Fig. 5C). Animals were followed over time using bioluminescent imaging to noninvasively determine response to therapy. Figure 5A to C shows the light emission patterns of individual animals over time when treated with effector to target ratios (E/T ratio) of 20:1. When these large numbers of effector cells were used (E/T = 20:1), the MC-IXC-luc cells were eradicated in four of five animals that received CIK cells (Fig. 5B) and in five of five animals treated with CD3xHer2/neu bispecific antibody redirected CIK cells (Fig. 5C). All animals treated at this dose of CIK cells (either with or without bispecific antibody) survived the 120-day observation period, except for one animal in the CIK cells + bispecific antibody group that unexpectedly died (possibly from anesthesia) and was tumor-free at the time of death (Fig. 5B-D). In contrast, mice that received mock treatment (PBS injection) all showed progressive tumor growth and death within 70 days (Fig. 5A and D).

In further experiments, the protection was less pronounced when fewer effector cells (E/T ratio = 1:1) were used. Animals treated with CIK cells alone had a slightly prolonged decrease in light emission (i.e., decreased tumor load) when compared with PBS controls (Fig. 5F versus E). Despite this, in both groups, all animals eventually succumbed to tumor although CIK cell–treated mice had a slightly prolonged survival compared with PBS-treated animals (Fig. 5H). Mice treated with CD3xHer2/neu bispecific antibody redirected CIK cells had a more prolonged decrease in light emission compared with either PBS control–treated animals or CIK cell–treated animals (Fig. 5G versus E or F). By the end of the 120-day observation period, three of seven CIK cell + bispecific antibody treated animals were alive, which resulted in a statistically significant survival advantage when compared with either CIK cells alone or PBS-treated animals (P < 0.0001 or P < 0.0001, respectively).

To better simulate established disease, we injected the MC-IXC-luc cells into mice and allowed the malignant cells to...
engraft for 7 days before therapy. As above, using this treatment schema, we tested the effect of either CIK cells alone or CD3xHer2/neu bispecific antibody redirected CIK cells. Both groups of mice were treated at an E/T ratio = 10:1. Figure 6A shows the Kaplan-Meier survival curves of the treated animals. Mice that received control treatment (PBS) all died within 80 days, whereas mice that received CIK cells had a statistically significant prolongation in survival ($P = 0.005$), but all animals eventually succumbed to tumor. Mice that received CD3xHer2/neu bispecific antibody redirected CIK cells had a significantly longer survival, compared with those that received CIK cells alone ($P = 0.001$; Fig. 6A). Some mice that were treated with bispecific antibody redirected CIK cells had progressive disease, whereas others were either cured or had only minimal residual disease (4 of 15 and 2 of 15, respectively) at the end of the 120-day experiment. Shown in Fig. 6B, are pseudocolor images from representative mice from each group. Thus, bispecific antibody–redirected CIK cells are superior to CIK cells alone in the treatment of established EFT tumors in SCID mice.

**Discussion**

Her2/neu is a 185 kDa transmembrane protein with tyrosine kinase activity and is one of the four members of the epidermal growth factor receptor family. Numerous studies have established that overexpression of this proto-oncogene has both prognostic and therapeutic implications for a variety of adult-derived malignancies, most notably breast carcinoma. Given that patients with EFT have a poor outcome despite aggressive multimodality therapy (14, 15, 29), we set out to determine whether EFT cell lines express Her2/neu and whether treatment with trastuzumab might be a useful adjunct in this setting. Using both FACS analysis and immunohistochemistry, we found low levels of cell surface Her2/neu expression on three separate EFT cell lines and one established subclone. The density of Her2/neu on the EFT cell lines was markedly lower than on breast and ovarian cancer cell lines. These results corroborate and extend two recent studies that show low-level expression of Her2/neu on EFT cell lines using Western blotting (30, 31) and immunohistochemistry (30). Like others (30), we found that trastuzumab did not inhibit EFT cell growth in vitro. Because it was possible that low-level expression of Her2/neu could result in growth inhibition not apparent in in vitro assays, we used trastuzumab in a SCID/hu model of EFT. Using cell lines transfected to express luciferase and bioluminescent imaging, we were unable to detect any effect of trastuzumab treatment on tumor progression. The reason for lack of response of EFTs to trastuzumab is not known, but is likely due to the low level of cell surface Her2/neu. In vitro studies support this notion because there is a direct correlation between Her2/neu expression levels on breast cancer cell lines and response to trastuzumab (10). Clinical studies also confirm the relationship between Her2/neu density and response to trastuzumab treatment, because patients with high levels of Her2/neu are...
more likely to respond to either trastuzumab alone or a combination of trastuzumab and chemotherapy than those with lower levels (reviewed in ref. 32).

Many adult malignancies express Her2/neu, including breast, ovarian, intestinal, uroepithelial, and lung cancers (1–5, 33). Less is known about the Her2/neu status of pediatric-derived malignancies and the available data has yielded conflicting results. For example, two studies showed that ~40% of patients with high-grade osteosarcoma expressed Her2/neu and that these patients were more likely to have a poor response to therapy and a worse overall treatment outcome (34, 35). In contrast, other investigators have found little to no Her2/neu expression on samples from a clinically similar cohort of osteosarcoma patients (36, 37). The reasons for these differences are not entirely clear, but may be due to the differences in the fixation process, the antibody used for immunohistochemistry, methods of antigen retrieval, or the interpretation of the staining (36). Other pediatric malignancies that express Her2/neu include medulloblastomas and this subset of patients also has a poorer clinical outcome when compared with medulloblastoma patients not expressing Her2/neu (38–40). Immunohistochemistry has failed to detect Her2/neu expression on hepatoblastoma, rhabdomyosarcoma, Wilms tumor, and liposarcoma (37, 41). Like osteosarcoma, controversy exists as to whether Her2/neu is expressed on neuroblastoma tissue because Layfield et al. (42) found expression in 4 of 31

![Figure 5. Bispecific antibody redirected CIK cells are more effective in eradicating EFT tumors engrafted into SCID mice. Mice were engrafted with 5 x 10^5 MC-IXC-luc on day 0 and treated on day 1 with (A) PBS, (B) 1 x 10^7 CIK cells alone (E/T = 20:1), or (C) 1 x 10^7 CIK cells + CD3xHer2/neu bispecific antibody (E/T = 20:1). Four of five animals treated with CIK cells alone showed prompt eradication of the tumor, whereas five of five animals in the bispecific antibody redirected group showed eradication (B versus C). Survival analysis shows that all but one animal survived the 120-day observation period. The one animal that did not survive was in the CIK + CD3xHer2/neu bispecific antibody group who unexpectedly died but was disease-free at the time of death (D). In a second set of experiments, animals were engrafted and treated as above, but instead received small numbers of effector cells [5 x 10^5 (E/T = 1:1)]. Under these conditions, the protection was less complete. Mice that received CIK cells had a slightly prolonged decrease in light emission when compared with PBS-treated controls (E versus F), but mice in both groups eventually succumbed to disease (H). Mice that received low numbers of CIK cells that were redirected to tumor targets using the CD3xHer2/neu bispecific antibody (10 µg) had a more prolonged decrease in light emission when compared with either CIK cells treated animals or PBS controls (G versus F or E). These results translated into a survival advantage for mice that received bispecific antibody redirected T cells (P < 0.0001; H).]
patients, but George et al. (37) were unable to show tissue staining in 15 patients. Using 10 neuroblastoma cell lines, we were able to detect a slight increase above background staining in only one cell line using FACS analysis. Like other investigators (30, 43–45), our preliminary studies evaluating paraffin-embedded EFT patient samples showed no detectable staining in 18 patient samples with both nonmetastatic and metastatic disease. Given the low level of Her2/neu seen in the EFT cell lines, it is possible that the technique used (immunohistochemistry) was not sufficiently sensitive to detect low level of Her2/neu expression on patient samples or that Her2/neu expression in EFT cell lines is an artifact of tissue culture. Studies on freshly isolated EFT tissue using both immunohistochemistry and FACS analysis are needed to clarify this issue.

Whereas the above in vitro and in vivo results cast doubt on the utility of trastuzumab as a single agent in the treatment of EFT, other immunologic approaches toward malignancies with low level Her2/neu expression (including EFT) may still be possible. The development of such approaches are important because it is

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Fig. 6. Established tumor model. A, SCID mice are injected with $5 \times 10^5$ MC-IXC-luc on day 0 and were treated on day 7 with either PBS (dashed line, $n = 11$), $5 \times 10^6$ CIK cells (dotted line, $n = 14$), or $5 \times 10^6$ CIK cells + $10 \mu g$ CD3/Her2/neu bispecific antibody on (E/T ratio = 10:1; solid line, $n = 15$). Whereas treatment with CIK cells significantly prolonged the survival of the mice compared with PBS controls ($P < 0.0001$), all animals died of tumor. Treatment with bispecific antibody redirected CIK cells lead to a significant improvement in survival compared with the CIK cells treatment group ($P < 0.0001$) and 6 of 15 mice survived >120 days when the experiment was terminated. B, pseudocolor images of representative animals in each treatment group on the day of treatment (established disease), 1 and 6 weeks after therapy.
likely that large numbers of patients with malignancies have low level Her2/neu expression, which will not respond to trastuzumab treatment. Here, we show that ex vivo activated T cells (CIK cells) redirected to tumor targets using a CD3xHer2/neu bispecific antibody efficiently induce cytolysis of EFT cells both in vitro and in vivo. Our results also show that redirecting CIK cells (with a CD3xHer2/neu bispecific antibody) significantly enhances antitumor cytotoxicity when compared with bispecific antibody redirected unactivated PBLs. These findings are in agreement with other investigators who have used either the same or different bispecific antibodies to target T cells to malignant cells (46–48). These improvements do not seem to be through nonspecific activation of the CD3 (and the triggering of cytosis) because CD3 × CD18 bispecific antibody did not increase cytocytotoxicity in vitro. Due to limiting amounts of the CD3 × CD18 reagent, we did not test this reagent in our in vivo studies, but we have previously shown that the CD3 × CD18 bispecific antibody did not enhance antitumor activity against ovarian carcinoma cell lines (SKOV-3) in a similar SCID/hu model (19). Using RNase protection assays, we show an up-regulation in the expression of perforin, granzyme A and B, Fas ligand, and TRAIL in these T cells (before redirection with the CD3xHer2/neu bispecific antibody). The mechanism of tumor cytosis in EFT targets may involve multiple mechanisms. Recent studies have established that EFT cell lines express both the Fas (CD95) and the TRAIL receptors (DR4 and DR5). Treatment of EFT cell lines with agonist antibodies against the Fas receptor–induced apoptosis in three of seven cell lines tested. In contrast, treatment of EFT cells with recombinant TRAIL-induced apoptosis in 9 of 10 cell lines tested, including some that were resistant to Fas ligation, suggesting that EFT cells may be more sensitive to the effects of TRAIL than Fas (49). Given these findings and the results from the RNase protection assay, it is reasonable to assume that both FasL or TRAIL signaling may contribute to cytosis, but previous work from our laboratory suggests that the perforin/granzyme pathway is also operative. In murine studies, we have shown that CIK cells generated from FasL-deficient mice (gld) had full antitumor activity against lymphoma cells both in vitro and in vivo, whereas CIK cells derived from perforin-/- mice (pfp) were devoid of antitumor activity (50). It should be noted that in the above studies, no bispecific antibody was used and it is possible that different effector molecules may be operative with bispecific antibody redirection. Despite this, we have recently showed that bispecific antibody redirected CIK cells mainly function through perforin/granzyme because incubation with either concanamycin A or EGTA markedly attenuates the cytotoxicity against either breast or ovarian tumor cell lines (19).

In two different treatment schemes, we show that the addition of a CD3xHer2/neu bispecific antibody to CIK cells leads to improved treatment of EFTs in a SCID/hu model. In the first scheme, animals were treated 1 day after tumor inoculation. Using this approach, we found low numbers of CIK cells were ineffective in rescuing the animals but therapeutic efficacy could be dramatically improved with the addition of the bispecific antibody. In contrast, large numbers of these cells with and without bispecific antibody could rescue freshly inoculated tumors. In the second treatment scheme, tumors were established for 7 days before treatment. These animals had minimal response to CIK cell therapy, but again this resistance could in part be overcome by redirecting T cells to the tumor targets with the CD3xHer2/neu bispecific antibody. Using bioluminescent imaging, we were able to serially observe the response to therapy without the need for sacrifice. Using this technology, we found that the kinetics of response to cellular therapy are rapid, usually occurring within 48 hours. This is considerably different than the response to herceptin (in herceptin responsive tumors), which takes up to a month (19).

In summary, our results show that EFT cell lines express low levels of Her2/neu and are resistant to the effects of trastuzumab treatment either in vitro or in vivo. Whereas low level Her2/neu may not be sufficient for effective trastuzumab treatment, it is sufficient to redirect CIK cells using a CD3xHer2/neu bispecific antibody. Here, we have used bioluminescent imaging to noninvasively monitor the response to therapy with either ex vivo expanded T cells alone or the same population of cells redirected to tumor targets using a CD3xHer2/neu bispecific antibody. Our results show that bispecific antibody redirected T cells are highly effective in treating mice engrafted with human EFT cell lines and these studies provide the rationale for such an approach in patients with low level expression of Her2/neu that do not respond to trastuzumab treatment.

References


Correction: Low Levels of Her2/neu Expressed by Ewing’s Family Tumor Cell Lines Can Redirect Cytokine-Induced Killer Cells

In this article (Clin Cancer Res 2005;11:4561–70), which was published in the June 15, 2005, issue of Clinical Cancer Research (1), one of the authors’ names was misprinted. The corrected name should read as follows: "Mobin Karimi." The publisher regrets this error.

Reference

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