Antihuman Epidermal Growth Factor Receptor 2 (HER2) Monoclonal Antibody Trastuzumab Enhances Cytolytic Activity of Class I-restricted HER2-specific T Lymphocytes Against HER2-overexpressing Tumor Cells1

Christian Meyer zum Büschenfelde, Christine Hermann, Burkhard Schmidt, Christian Peschel, and Helga Bernhard2

Department of Hematology/Oncology, Klinikum rechts der Isar, Technical University of Munich, D-81675 Munich, Germany

Abstract

The monoclonal antibody trastuzumab (Herceptin) directed against the human epidermal growth factor receptor 2 (HER2) results in tumor regressions when administered to patients with HER2-overexpressing breast cancer. One of the underlying mechanisms of this antibody-induced tumor regression is based on the internalization and degradation of HER2 by tumor cells on interaction with trastuzumab, subsequently inhibiting signal transduction pathways. As antibody-induced degradation of HER2 is likely to be accompanied with increased numbers of HER2 peptides presented with MHC, we asked whether trastuzumab-treated tumor cells were more susceptible to CTL-mediated lysis. Here we show that the cytolytic activity of human, HER2-specific CD8+ CTLs is augmented by anti-HER2 antibody trastuzumab. HER2-reactive CTL clones lyse class I-matched, HER2-overexpressing tumor cells more efficiently after treatment with trastuzumab. The potentially synergistic activity of HER2-specific antibody and CTL encourages the development of an HER2-targeted immunotherapy using a combination of inhibitory antibodies and CTLs for patients with HER2-overexpressing tumors.

Introduction

HER21 represents an appealing target for humoral and cellular immunotherapy because HER2 is expressed at high levels in a variety of human cancers, such as breast cancer (1). Overexpression of antigenic peptides, such as HER2, is coupled with a high protein turnover, leading subsequently to a high number of MHC class I peptide complexes on the cell surface (Ref. 2; for review, see Ref. 3). Therefore, HER2++ tumor cells are potentially good target cells for tumor-reactive CTLs recognizing HER2-derived peptides in context with MHC class I molecules. Indeed, HER2-specific CTLs have been isolated from normal donors and patients with HER2++ tumors (4–7). However, lytic activity of separated CTLs has been low because HER2 is a self-antigen attributable to induction of tolerance, and, therefore, HER2-specific T cells display a low avidity T-cell receptor. On this basis, we investigated whether the lytic potential of HER2-specific CTLs could be improved with the help of trastuzumab, an inhibitory antibody against HER2. On binding of anti-HER2 mAb trastuzumab, the receptor is internalized and degraded, subsequently inhibiting HER2-mediated signal transduction and tumor cell growth (8–10). Theoretically, degradation of the internalized HER2 might increase the amount of HER2 protein available for loading into MHC class I molecules, subsequently leading to an enhanced tumor lysis by HER2-specific CTL.

Here we show that anti-HER2 mAb trastuzumab sensitizes HER2++ tumor cells to lysis by HER2-specific CTLs in vitro. HER2-reactive T-cell clones generated from healthy donors and breast cancer patients lyse class I-matched, HER2-overexpressing tumor cells more efficiently after incubation with trastuzumab. These findings encourage the further development of a combined immunotherapy with trastuzumab and autologous HER2-specific CTLs for patients with HER2-overexpressing tumors.

Materials and Methods

Cells. The HER2++ ovarian cancer cell line SKOV3 (HLA phenotype: A3, A28, B18, and B35) and the HER2++ breast cancer cell line SKBR3 (HLA phenotype: A11, B40, and B18) were obtained from American Type Culture Collection (Manassas, VA). The HLA-A2+-transfectant cell line SKOV3A2 was a gift from M. L. Disis (University of Washington, Seattle, WA). SKOV3A2 express the TIR on the cell surface as determined by fluorescence-activated cell sorting analysis (data not shown). The HLA-A2+, transporter associated with antigen processing (TAP)-deficient T2 cell line was provided by P. Cresswell (Yale University, New Haven, CT). Tumor cell lines and T2 cells were cultured in RPMI 1640 (Life Technologies, Inc., Paisley, Scotland) supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 μM glutamine. For down-regulation experiments, confluent SKOV3A2 cells were cultured in the presence of 100 μg/ml anti-HER2 mAb trastuzumab (Herceptin; Roche, Grenzach-Wyhlen, Germany) or 100 μg/ml anti-CD71 mAb (clone 32001A; BD Pharmingen, San Diego, CA) for 2 days.

HER2-specific, class I-restricted CTL clones were generated from different donors by repetitive stimulation with autologous DCs as described recently (6–7). In brief, HLA-A3-restricted CTLs FS-32 and HLA-A2-restricted CTLs FS-D10 and HR-8 were generated from different donors by repetitive stimulation with autologous stem cell-derived DCs (11) retrovirally transduced with HER2. HLA-A2-restricted CTLs NK-1 and IR-1 were established by stimulation with monocyte-derived autologous DCs pulsed with peptides known to be naturally presented with HLA-A2. HER2-specific CTLs NK-1 were generated against HER2369–377 and melan-A-reactive CTLs IR-1 against melan-A27–35. Antigen-specific T-cell lines were cloned by limiting dilution and expanded according to the method described previously by Yee et al. (12).

Western Blot Analysis. Cell lysates were made from SKOV3A2 cells that were cultured in the presence of anti-HER2 mAb trastuzumab or anti-CD71 mAb. Lysates from equal amounts of cells were separated by SDS-PAGE and transferred to nitrocellulose. The HER2 protein was identified using the mAb c-neu-Ab3 recognizing the intracellular domain of HER2 (Oncogene Science, Uniondale, NY) as primary Ab and rabbit antimouse peroxidase-conjugated Fab2 fragment (Amerham Pharmacia Biotech, Freiburg, Germany) as secondary Ab. The blots were developed using a chemiluminescent reaction (enhanced chemiluminescence; Amerham Pharmacia Biotech).

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2 To whom requests for reprints should be addressed, at Medizinische Klinik, Klinikum rechts der Isar, Technical University of Munich, D-81675 Munich, Germany. Phone: 89-4140-6223; Fax: 89-4140-4824; E-mail: helga.bernhardlrz.tum.de.

3 The abbreviations used are: HER2, human epidermal growth factor receptor 2; HER2++, HER2-overexpressing; DC, dendritic cell; mAb, monoclonal antibody; TIR, transferrin receptor.
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Cytotoxicity Assay. Cytolytic activity was determined as described previously (6). Briefly, 10^6 tumor cells were labeled in 100 μl of FCS with 100 μCi/ml ^51^Cr for 1 h at 37°C. T2 cells were labeled in 100 μl of FCS with 200 μCi/ml for 1.5 h at 37°C and then loaded with 10 μg/ml peptide and 3 μg/ml B2-microglobulin (Sigma, Taufkirchen, Germany) for 1 h at room temperature. 31Cr-labeled target cells and graded doses of T cells were incubated with anti-CD71 mAb (Sigma, St. Louis, MO) to 100 μl of supernatant were collected, and radioactivity was measured in a gamma counter. The percentage of specific ^51^Cr release was calculated as follows: % specific ^51^Cr release = (experimental ^51^Cr release – spontaneous ^51^Cr release) / (maximum ^51^Cr release – spontaneous ^51^Cr release). Maximum ^51^Cr release was obtained by adding 100 μl of 1% NP40 (Sigma, St. Louis, MO) to 100 μl of labeled target cells. Spontaneous ^51^Cr release ranged from 5 to 10% of the total counts incorporated. The data in the figures refer to the mean of two replicates. SDs were <5% of the mean. Similar results were obtained with at least three independent experiments.

Results and Discussion

Drebin et al. (8) have demonstrated that exposure of HER2-transformed cells to inhibitory anti-HER2 antibodies results in the rapid loss of both cell surface and total cellular HER2. This antibody-induced down-regulation of HER2 is a result of accelerated endocytosis and correlates with the tumor-inhibitory potential of the antibody (9). The anti-HER2 mAb trastuzumab was engineered from a murine mAb that inhibits growth of HER2^+^ tumor cells via receptor internalization (13). In our experimental in vitro model, antibody trastuzumab, on ligation to its receptor HER2, leads to HER2 down-regulation of total cellular HER2 protein expression in HER2^+^ ovarian cancer cells SKOV3tA2 (Fig. 1. An anti-CD71 mAb, known to induce internalization of the TIR (CD71), was used as an irrelevant antibody control. This anti-CD71 mAb did not influence the HER2 protein level of HER2^+^ ovarian cancer cells expressing the TIR.

It has been shown recently by Klapper et al. (10) that tumor-inhibitory antibodies to HER2 enhances ubiquitination of HER2. Protein tagged to ubiquitin is known to be targeted to the proteasome, which cleaves the protein into small peptides. These peptides are then transported into the endoplasmic reticulum, loaded on MHC class I molecules, and transferred to the cell surface for recognizing CTLs. On the basis of these findings, we next examined whether tumor cells that had down-regulated the HER2 receptor on antibody ligation could become sensitized to lysis by HER2-specific CTLs. We cultured the HER2^+^ ovarian cancer cell line SKOV3tA2 in the presence of trastuzumab. The trastuzumab-treated SKOV3tA2 cells were then used as target cells for class I-restricted HER2-reactive CTL clones in a cytotoxicity assay. The HER2-specific CTL clones (6) were derived from different donors by in vitro stimulation with autologous DCs transduced retrovirally with HER2. The HER2-specific CTL clone HR-4, generated from an HLA-A2^+^ patient with HER2^+^ breast cancer, lysed the HER2^+^ SKOV3tA2 cells in an HLA-A2-restricted manner (Fig. 2a). The amount of specific tumor cell lysis correlated with the number of HR-4 CTLs present during the cytotoxicity assay. Pretreatment of SKOV3tA2 cells with trastuzumab resulted in a remarkable increase of tumor cell lysis by CTL clone HR-4. In contrast to nontreated tumor cells, lysis of trastuzumab-treated tumor cells was already detectable at a very low E:T ratio of 1:1. In general, much fewer effector CTLs were needed to lyse equal numbers of antibody-treated tumor cells compared with nontreated tumor cells (e.g., 30% of tumor cell lysis at E:T of 10:1 versus 90:1). Moreover, maximum tumor cell lysis by CTL clone HR-4 was higher (47 versus 26%) and reached at a lower E:T ratio (30:1 versus 90:1), when tumor cells had been incubated with the anti-HER2 antibody. The anti-CD71 mAb capable of inducing TIR internalization did not enhance HR-4-mediated lysis of CD71^+^ SKOV3tA2. Therefore, it can be excluded that enhanced lysis of tumor cells is a general phenomenon observed after receptor internalization.

We next asked whether trastuzumab-treated HER2^+^ tumor cells were also more efficiently lysed by HER2-specific CTLs using a restriction element other than HLA-A2. CTL clone FS-32 recognizing HER2 in context with HLA-A3 was used for lysing HLA-A3^+^ SKOV3tA2, which had been cultured in the presence or absence of anti-HER2 and anti-TIR antibody, respectively (Fig. 2b). Similar to HLA-A2-restricted CTL clone HR-4, the lytic activity of HLA-A3-restricted CTL clone FS-32 was enhanced markedly when tumor cells were preincubated with anti-HER2 mAb trastuzumab. Again, the tumor lysis by CTLs remained unchanged after the incubation of the tumor cells with anti-TIR mAb.

To validate the HER2-specific interaction of trastuzumab and CTLs, we next used CTLs with known peptide specificity as effector cells. CTL line NK-1 (6) recognizing HLA-A2-bound peptide HER2^35_377 (4) lysed SKOV3tA2 more efficiently after preincubation with trastuzumab (Fig. 3a). HLA-A2-restricted CTL clone IR-1 directed against the melanoma-associated peptide melan-A^35_55 did not lyse melan-A-negative SKOV3tA2 cells (Fig. 3b). Culturing SKOV3tA2 cells with trastuzumab did not induce lysis by melan-A-specific CTLs IR-1, indicating that both

Fig. 1. HER2-overexpressing tumor cells down-regulate total cellular HER2 protein expression after ligation with anti-HER2 mAb trastuzumab. Ovarian cancer cell line SKOV3tA2 expressing the HER2 molecule and the TIR (CD71) was cultured in the absence or presence of either mAb trastuzumab directed against the extracellular domain of HER2 or anti-CD71 mAb. After 2 days, Western blot analysis of SKOV3tA2 cells was performed using mAb c-neu-A3 directed against the intracellular domain of HER2. In comparison to untreated SKOV3tA2 cells (Lane a), trastuzumab-treated SKOV3tA2 cells exhibited reduced total cellular HER2 protein expression (Lane b), whereas SKOV3tA2 cells incubated with anti-CD71 mAb did not down-regulate the HER2 molecule (Lane c).

Fig. 2. HER2-specific CTL clones using different restriction elements lyse HER2-overexpressing tumor cells after incubation with anti-HER2 mAb more efficiently. HLA-A2-restricted CTL clone HR-4 (a) and HLA-A3-restricted CTL clone FS-32 (b), both specific for HER2, were generated by repetitive stimulation with autologous DCs retrovirally transduced with HER2. HER2^+^ SKOV3tA2 cells expressing the class I molecules HLA-A2 and HLA-A3 were lysed by HLA-A2-restricted CTL clone HR-4 and by HLA-A3-restricted CTL clone FS-32. Lysis of SKOV3tA2 cells were enhanced when tumor cells were preincubated with anti-HER2 mAb trastuzumab. Lytic activity of CTL clones was not altered when SKOV3tA2 cells were treated with an irrelevant mAb recognizing TIR also expressed by SKOV3tA2 (a). HER2^+^ SKBR3 (c) being negative for HLA-A2 and HLA-A3 served as negative control.
provided evidence that the infusion of antigen-specific T lymphocytes represents an effective modality for the treatment of cancer (12). Up until now, attempts to treat HER2+ tumors by adoptive transfer of HER2-reactive T cells have not been initiated because of the difficulty of generating autologous CTLs directed against the HER2 antigen. We have succeeded in generating and expanding class I-restricted HER2-specific CTL clones (6–7) from patients with HER2-overexpressing breast cancer to numbers that allow adoptive T-cell transfer. The synergistic in vitro antitumor activity of trastuzumab and anti-HER2 CTLs described here encourages the transfer of humoral and cellular immunity to patients who fail to completely eradicate cancer by conventional treatment. Braun et al. (19) observed a correlation between HER2 expression on HER2+ micrometastatic tumor cells in bone marrow and poor prognosis. In this context, the relative resistance of HER2+ micrometastatic cells to adjuvant chemotherapy might be successfully overcome by HER2-targeted immunotherapy that is independent of the proliferative status of the target cells.

Because of the expression of HER2 in some normal tissues, it cannot be ruled out that a combination therapy of trastuzumab and anti-HER2 CTLs might induce autoimmune disease. However, because of low-receptor density on normal cells, the binding of trastuzumab could potentially selectively promote class I-restricted presentation of HER2 peptides on HER2+ tumor cells only. Therefore, the combination of trastuzumab and HER2-specific CTLs with low avidity might lead to tumor rejection without damaging normal tissues in vivo (20). Ongoing studies focus on the toxicity and efficacy of adoptively transferred HER2-specific CTLs with and without trastuzumab in patients with HER2+ breast cancer.

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References


Several mechanisms have been proposed to account for the antitumor activity of antibodies against HER2. The capacity of a HER2-directed antibody to inhibit the growth of HER2+ tumor cells depends on the ability to down-regulate HER2, subsequently leading to the interruption of signaling pathways (8–10). Therefore, one of the main mechanisms responsible for the therapeutic effect of anti-HER2 mAb trastuzumab is thought to be mediated through the inhibition of signal transduction (for review, see Ref. 14). However, Stankovski et al. (15) have postulated that inhibition of tumor growth by anti-HER2 mAb is not only because of the blockade of HER2 receptor function but also involves host-tumor interactions. Indeed, Clynes et al. (16) have shown that antibody-dependent cell-mediated cytotoxicity contributes substantially to the antitumor activity of trastuzumab in vivo. In this murine model, growth inhibition of HER2+ tumors was dependent on the engagement of trastuzumab with Fc receptors on myeloid cells, such as monocytes and macrophages. We propose a novel immune mechanism of trastuzumab-mediated tumor killing. Trastuzumab-induced degradation of the HER2 molecule is accompanied probably by the enhanced generation of class I-bound HER2 peptides, and this might lead to a higher susceptibility of HER2-overexpressing tumor cells to CTL-mediated lysis. The presence of both antibodies and CTLs directed against HER2 in patients with HER2+ tumors (17) suggests that the antitumor activity of HER2-specific antibodies and CTLs might be intertwined. To date, attempts to enhance the preexistent cellular and humoral immunity to HER2 in patients with HER2+ tumors have focused on peptide-based vaccination strategies. In initial clinical studies, the vaccination of women with HER2-overexpressing breast cancer elicited HER2-specific cytotoxic T-cell responses in vivo but have thus far failed to induce tumor regressions (18). Recent studies have


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