Trastuzumab (Herceptin) Enhances Class I-Restricted Antigen Presentation Recognized by HER-2/neu-Specific T Cytotoxic Lymphocytes

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ABSTRACT

Purpose: Numerous examples from animal models and clinical trials showed that HER-2-derived peptides are naturally processed as a CTL epitope and can be recognized by tumor-specific CTLs in several tumors with HER-2 overexpression. The humanized anti-HER-2 monoclonal antibody, Herceptin, has been designed to specifically antagonize the HER-2 function by directing against the extracellular domain of the HER-2 protein. One of the actions of Herceptin includes the internalization and degradation of HER-2, which might increase the amount of HER-2-derived peptides available for loading to MHC class I.

Experimental Design: In the present study, we investigated how Herceptin treatment of HER-2-overexpressing targets affects lysis by HER-2-specific CTLs.

Results: We showed that Herceptin sensitized HER-2-overexpressing tumors to lysis by HLA-A2-restricted or HLA-A24-restricted CTLs, without any effect of the expression of MHC class I, costimulatory molecules, adhesion molecules, or TAP-1 on the targets. Furthermore, the enhancement of cytolytic activity with Herceptin was inhibited by addition of a specific proteasome inhibitor, lactacystin.

Conclusions: These results suggested that Herceptin treatment might enhance the class I-restricted presentation of endogenous HER-2 antigen via the proteasome step, resulting in higher susceptibility of HER-2-overexpressing tumors to lysis by the HER-2-specific CTLs.

INTRODUCTION

HER-2/neu (designated as HER-2) is a receptor that contains an extracellular domain and intracellular domain with tyrosine-specific kinase activity and is a member of the epidermal growth factor receptor family (1). HER-2 is overexpressed in 10–35% of human breast cancer, ovarian cancer (2), or gastric cancer (3), in contrast to the low expression in normal cells. Numerous examples from experimental models and clinical trials suggest that HER-2 can be immunogenic and generate antibody, cytolytic T cell (CTL), and helper T-cell-specific responses in individuals with HER-2-overexpressing tumors (4, 5). In fact, it has been shown that HER-2-derived peptides are naturally processed as tumor-associated antigens and can be recognized by tumor-specific CTLs in several tumors with HER-2 overexpression (6, 7). On the basis of the above reports, anti-HER-2 immunotargeting may be used as an attractive approach for HER-2-overexpressing tumors, because HER-2 represents a good target for humoral and cellular immunotherapy.

The fully humanized anti-HER-2 monoclonal antibody (mAb), Herceptin, has been designed to specifically antagonize the HER-2 function by directing against the extracellular domain of the HER-2 protein (8). Herceptin was clinically demonstrated to have survival effects for HER-2-overexpressing breast cancer (9, 10). Many mechanisms have been proposed to account for the therapeutic effect of Herceptin, including the blockade of signaling pathways (8), down-modulation of the HER-2 receptor (8), activation of apoptotic signals of the tumor cells (11), and the interaction with the immune system via its immunoglobulin G1 Fc domain, such as antibody-dependent cellular cytotoxicity (8, 12). Also, it has been shown recently that Herceptin enhances cytotoxic activity of CTLs against HER-2-overexpressing tumors, although the underlying mechanisms involved remain unclear (13). It is important to clarify how Herceptin affects MHC class I-restricted antigen presentation to design an effective modality for anti-HER-2 immunotargeting for patients with HER-2-overexpressing tumors.

In general, to generate antigenic peptides, endogenously synthesized proteins are mainly cleaved by the multicytolytic proteasome complex (14). Most of the peptides are then translocated from the cytosol to the endoplasmic reticulum by the ATP-dependent heterodimeric transporter-associated with antigen processing complex TAP1 and TAP2 (15). TAP selectively transports peptides of specific sequence and length, thereby acting as a chaperone to support the correct loading of peptides onto MHC class I molecules.

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Received 10/17/03; revised 12/24/03; accepted 12/31/03.


The authors declare that they have no conflicting interests.

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MATERIALS AND METHODS

Cell Lines. SW626 (HER-2+, HLA A2−) and HCT-15 (HER-2+, HLA A24+), was obtained from American Type...
Culture Collection (Rockville, MD). MKN-7 (HER-2+, HLA A2601), PC-9 (HER-2+, HLA A24+), and KATOIII (HER-2+, HLA A24+) were obtained from the IBL cell bank (Gunma, Japan). SW626 was transfected with an HLA-A2 expression vector containing the full-length of HLA-A2.1 cDNA with Lipofectin (Life Technologies, Inc., New York, NY) according to the manufacturer’s protocol (6). The C1R/A2 is a MHC class I-defective LCL cell line, which expresses HLA-A2.1 (B35 low and Cw low), and C1R/A2HER-2 is a HER-2 transfectant cell line of C1R/A2 (6). SKOV3 and its HLA-A2 transfectant, SKOV3A24, were obtained from Dr. Hiroshi Shiku (Mie University, Tsu, Japan). HER-2 expression on these cell lines was confirmed by flow cytometric analysis as described previously (6). TISI cells are a human B-lymphoblastoid cell line expressing HLA-A24. The lymphoblastoid cell line (LCL) LCL-MTB was obtained by in vitro transfection of B cells from healthy donors with EBV supernatant. All of the cell lines were maintained in RPMI 1640 (Life Technologies, Inc.) supplemented with 5–10% FCS (Life Technologies, Inc.), penicillin (100 units/ml; Sigma, St. Louis, MO), and streptomycin (100 µg/ml; Sigma).

Induction of HER-2-Specific CTLs. The HER-2-specific, HLA-A2-restricted CTL line (A2CTL) was generated from tumor-associated lymphocytes in ascites of a gastric cancer patient with HER-2-overexpressing tumors by repetitive stimulation of lymphocytes with autologous tumor as described previously (6). In brief, tumor-associated lymphocytes were kept in AIM-V medium (Life Technologies, Inc.) supplemented with 50 IU/ml recombinant interleukin 2 (Shionogi, Tokyo, Japan) and were restimulated with mitomycin-C (50 mg/ml; Kyowa Hakko, Tokyo, Japan)-treated autologous tumor cells four times, at 2-week intervals, at a 10–20:1 (lymphocyte:tumor) ratio.

To generate the HER-2-specific, HLA-A24-restricted CTL line (A24CTL), peripheral blood mononuclear cells in a HLA-A24+ gastric cancer patient with HER-2-overexpressing tumors were stimulated with antigen loaded, autologous mature dendritic cells (DCs). Briefly, peripheral blood mononuclear cells were separated from peripheral blood by centrifugation over Ficoll-Paque (Pharmacia, Uppsala, Sweden), and monocytes were enriched by adherence to a plastic tissue culture flask (Corning, Corning, NY) for 90 min at 37°C. Adherent cells were cultured with 1000 units/ml of granulocyte macrophage colony-stimulating factor (Peprotech EC Ltd., London, United Kingdom) and 1,000 units/ml of interleukin 4 (Peprotech EC Ltd.) in X-VIVO15 (Life Technologies, Inc., Gathersburg, MD) with 1% autologous serum. On day 5, DCs were harvested with vigorous washing and coincubated with irradiated (40 Gy) PC-9 cell line (HER-2+, HLA A24+) at DC/PC-9 ratio of 1:1 for 12 h, and then matured with tumor necrosis factor α (50 units/ml; Peprotech EC Ltd.). Antigen-loaded, mature DCs were coincubated with autologous peripheral blood mononuclear cells at 1:20 in a 96-well round-bottomed plate (Corning) in X-VIVO with 1% autologous serum, and 50 IU/ml of interleukin 2 (Shionogi). Then, the cultured cells were restimulated with antigen-loaded, mature DCs every 14 days.

Cytotoxicity Assay. After the target cells were labeled with 100 µCi 51Cr for 60 min, target cells (5 × 105/well) and effector cells at various E:T ratios were coincubated in 200 µl of RPMI 1640 in a 96-well U-bottomed plate in triplicate for 4 h at 37°C. Then, the radioactivity of the supernatant (100 µl) was measured with a gamma counter. The percentage of specific lysis was calculated according to the formula: % specific lysis = 100 × (experimental cpm – spontaneous cpm)/(maximum cpm – spontaneous cpm).

In some experiments, the target cells were pretreated with Herceptin (2 µg/well) or control antibody, Rituxan (2 µg/well), for 10 h before the cytotoxicity assay. Clinically marketed anti-HER-2 mAb Herceptin and anti-CD20 mAb Rituxan, as an isotype-matched control mAb for Herceptin, were purchased from Roche (Basel, Switzerland).

Enzyme-Linked Immunospot (ELISPOT) Assay. HER-2-specific response was determined by IFN-γ ELISPOT assay. The ELISPOT assay was performed with a Mabtech assay system (Mabtech, Nacka, Sweden). After the 96-well plates with a nitrocellulose membrane (Millipore, LA) were precoated with a primary anti-IFN-γ antibody (1D1K) for 24 h, the plates were blocked with AIM-V containing 1% human serum albumin. Target cells (2 × 104/well) and CTLs (2 × 103/well) were incubated in 200 µl of AIM-V for 24 h. Thereafter, a biotinylated secondary anti-IFN-γ antibody (7-B6–1) was added for 2 h, and then the plates were incubated with streptavidin-alkaline phosphatase reagent and stained with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Life Technologies, Inc.).

Apoptosis. Apoptosis in SW626A2 or PC-9 cell lines after treatment with Herceptin (10 µg/ml) or control antibody, Rituxan (10 µg/ml), for 10 h, or irradiation (40 Gy) was measured by staining with FITC-conjugated annexin-V and PI using a MEBCYTO Apoptosis kit (MBL, Nagoya, Japan) following the manufacturer’s recommendations.

Blocking of the Antigen-Processing Pathway. To inhibit the antigen presentation pathway on target cells, SW626A2 or PC-9 cells were incubated with the specific proteasome inhibitor lactacystin (Alexis, San Diego, CA) at nontoxic doses (0.5 and 1.0 µM; Ref. 16) when treated with Herceptin.

Antibodies and Flow Cytometry. mAb HB54 recognizing HLA-A2.1 and mAb HB-164 recognizing HLA-A24 were derived from culture supernatants of American Type Culture Collection HB54 or HB-164 hybridomas. mAbs against CD80 (FITC conjugated; BD PharMingen), MHC class I (HLA-ABC; Dako, Glostrup, Denmark) and ICAM-1 (Dako) were used. Cells were incubated with either primary mAb or murine IgG of the appropriate class as a control, and unlabelled mAbs were visualized using FITC-conjugated rabbit antimouse immunoglobulins (Dako).

Western Blot Analysis for TAP-1 and LMP2. After SW626A2 or PC-9 cells were treated with Herceptin (10 µg/ml) or control antibody, Rituxan (10 µg/ml), for 10 h, cells were lysed in lysis buffer (CelllyticTM-MT; Sigma) and a mixture of protease inhibitor mixture (Sigma). Cellular lysates (30 µg) were electrophoresed in 10% SDS gels, transferred to a polyvinylidene difluoride membrane (Immobilon-P) and blotted with rabbit anti-TAP1 antibody (StressGen, Victoria, British Columbia, Canada) or rabbit anti-LMP2 antibody (Affinity, Manhead, United Kingdom). The membrane was incubated with swine antirabbit immunoglobulin-AP (Dako) and developed using CDP-star (Roche, Mannheim, Germany). For the positive con-
trols for the TAP-1 and LMP2 expression, the lymphoblastoid cell line LCL-MTB was used.

RESULTS

Generation of HER-2-Reactive CTLs. To evaluate how Herceptin affects CTL-mediated lysis, two sets of HER-2 specific, MHC-class I-restricted CTLs were generated. To confirm the HER-2 specificity of the CTLs, they were tested against various targets in a cytotoxic assay or ELISPOT assay. In Fig. 1, the A2CTL lysed HER-2-positive, HLA-A2-positive SW626A2, or C1RA2HER2 target, but not C1RA2, and this lytic activity for SW626A2 was inhibited with anti-HLA-A2 monoclonal antibody, indicating that the A2CTL was HLA-A2-restricted and HER-2-reactive.

Herceptin Sensitized HER-2-Overexpressing Tumors to Lysis by HER-2-Specific CTLs. By using the HER-2-specific CTLs (A2CTL and A24CTL), we analyzed the effect of Herceptin treatment on the target cells on the HER-2-specific CTL activity. Pretreatment of SW626A2 cells with Herceptin resulted in an increase in cytolysis by the HLA-A2-restricted CTLs (A2CTL) in comparison with that treated with control mAb, as show in Fig. 3A. Similarly, pretreatment of PC-9 cells with Herceptin enhanced the cytolytic activity of HER-2 weak-positive KATOIII target as well as HER-2 strong-positive PC-9 target (Fig. 3C).

These results indicated that Herceptin treatment sensitized HER-2-overexpressing tumors to lysis by HER-2-specific CTLs.

Enhancement of Cytolytic Activity with Herceptin Was Inhibited by the Proteasome Inhibitor Lactacystin. To analyze the mechanisms by which Herceptin sensitized HER-2-overexpressing target to lysis by HER-2-specific CTLs, we examined the proteolysis step mediated by the proteasome in the class I-restricted antigen-processing pathway. Endogenously synthesized proteins are degraded by proteasome, which generates antigenic peptides 9–12 amino acids long that are subsequently transported into the endoplasmic reticulum and loaded on MHC class I molecules, resulting in the antigenic target being recognized by CTLs (17, 18). The proteolysis step was...
inhibited by treatment with a selective proteasome inhibitor, lactacystin (15).

Then, SW626A2 cells were treated with Herceptin in the presence of a nontoxic dose of lactacystin (16), and then subjected to the cytotoxic assay induced by HER-2-specific A2CTL (Fig. 3A). As a result, the enhancement of the CTL activity induced by Herceptin was inhibited by the addition of lactacystin in a dose-dependent manner (Fig. 3A). Similarly, the enhancement of CTL activity (A24CTL) by Herceptin was inhibited by the addition of lactacystin in a dose-dependent manner (Fig. 3B).

Furthermore, the CTL activity against control mAb-treated SW626A2 or PC-9 induced by HER-2-specific CTLs (A2CTL and A24CTL) were also inhibited by the addition of the selective proteasome inhibitor, lactacystin (Fig. 3A and B), indicating that class I-restricted antigen presentation of HER-2 molecule was proteasome dependent.

Taken together, these results suggested that Herceptin treatment might enhance the class I-restricted presentation of endogenous HER-2 antigen via the proteasome step.

Herceptin Treatment Did Not Alter MHC Class I Expression. To additionally evaluate the mechanisms by which Herceptin sensitized targets to lysis by the HER-2-specific CTLs, the expression of MHC class I, costimulatory molecules, and adhesion molecules on SW626A2 or PC-9 after treatment with Herceptin was analyzed. The expression of MHC class I was not altered with Herceptin treatment (Table 1). The expression of CD80, CD86, or ICAM-1 was not enhanced by Herceptin (Table 1).

Lactacystin treatment did not affect the surface expression of class I molecules, CD80, CD86, or ICAM-1 (Table 1).

Herceptin Treatment Did Not Alter the Expression of TAP1 Molecules. Because MHC class I antigen-processing machinery is involved in several antigen-processing components such as transporter associated with antigen processing (TAP)1 or low molecular weight protein (LMP)2 (17, 18), we next analyzed the expression of TAP1 on SW626A2 and PC-9 cells after treatment with Herceptin or control mAb by Western blot analysis. As shown in Fig. 4, Herceptin treatment did not up-regulate the TAP1 expression on SW626A2 and PC-9 cells in comparison to those treated with control mAb. Positive control for the expression of TAP1 was included with LCL-MTB cells.

Herceptin Treatment Did Not Alter the Expression of LMP2 Molecules. We additionally analyzed the expression of LMP2 as one of the proteasome subunits (17, 18) by Western blot analysis. The expression of LMP2 in SW626A2 was originally faint (Fig. 5). Herceptin treatment of SW626A2 and PC-9 cells did not up-regulate the expression of LMP2 in comparison to those treated with control mAb (Fig. 5). Positive control for the expression of LMP2 was included with the LCL-MTB cells.

Fig. 3  Herceptin sensitized HER-2-overexpressing tumors to lysis by HER-2-specific CTLs. The A2CTL was tested for cytotoxicity against SW626A2 target pretreated with trastuzumab (Herceptin) or control monoclonal antibody in the presence of the specific proteasome inhibitor, lactacystin (A). The A24CTL was tested for cytotoxicity against PC-9 target pretreated with trastuzumab (Herceptin) or control monoclonal antibody in the presence of specific proteasome inhibitor, lactacystin (B). The A24CTL was tested for cytotoxicity against HER-2 strong-positive PC-9 or HER-2 weak-positive KATOIII targets, when pretreated with trastuzumab (C); bars, ±SD.
Herceptin Treatment Did Not Induce Apoptosis of HER-2-Overexpressing Tumors. We next investigated whether Herceptin induced apoptosis in SW626A2 or PC-9 cells. Annexin-V/PI staining showed that Herceptin treatment of SW626A2 or PC-9 cells did not result in apoptosis, whereas irradiation as a positive control could induce apoptosis in both cell lines as shown in Fig. 6.

**DISCUSSION**

The present report contains several important findings relevant to the underlying mechanisms of the action of Herceptin against HER-2-overexpressing tumors. First, Herceptin sensitized HER-2-overexpressing tumors to lysis by MHC class I-restricted, HER-2-specific CTLs. Second, Herceptin treatment might enhance the class I-restricted presentation of endogenous HER-2 antigen via the proteasome step.

Herceptin has been designed to specifically antagonize the HER-2 function and was clinically demonstrated to be a humanized mAb with survival effects for HER-2-overexpressing breast cancer (9, 10). In addition to the mechanisms including the blockade of signaling pathways (8), down-modulation of the HER-2 receptor (8), activation of apoptotic signals of the tumor cells (11), and interaction with the immune system via its immunoglobulin G1 Fc domain, such as antibody-dependent cellular cytotoxicity (8, 12), it has been shown recently that Herceptin enhances cytolytic activity of class I-restricted, HER-2-specific CTLs against HER-2 targets (13). In the present study, we confirmed this phenomenon and additionally proposed an immune mechanism that Herceptin enhances MHC class I-restricted antigen presentation in HER-2-overexpressing tumors, resulting in a higher susceptibility of HER-2-overexpressing tumors to lysis by the HER-2-specific CTL.

Effective CTL response requires both an antigen presentation delivered by antigenic peptides in the context of MHC class I molecules and costimulatory signals (19). In the present study, Herceptin treatment did not affect the expression of MHC class I molecules, costimulatory molecules, or adhesion molecules on HER-2-overexpressing targets. Because it has been reported that Herceptin can induce the internalization and degradation of the HER-2 receptor (20–22), degradation of internalized HER-2 might increase the amount of HER-2-derived peptides available for loading to MHC class I. Then, to clarify the mechanisms behind Herceptin enhancement of the activity of HER-2-specific CTLs against HER-2 targets, we analyzed how Herceptin affects the MHC class I-antigen presentation.

In the antigen presentation machinery, several molecules are involved, including the subunits of the multicatalytic proteasome complex such as LMP-2 and LMP-7, the heterodimeric peptide transporter consisting of TAP1 and TAP2, and chaperones such as calnexin and tapasin (17, 18, 23, 24). In general, to generate antigenic peptides, endogenously synthesized proteins are mainly cleaved by the multicatalytic proteasome complex (17, 18), although some nonproteasomal cytosolic or endoplasmic reticulum-resident protease(s) play a significant role in the generation of cytosolic MHC class I binding peptides (15). Most of the peptides are then translocated from the cytosol to the endoplasmic reticulum by the ATP-dependent heterodimeric transporter associated with antigen processing complex TAP1 and TAP2. TAP selectively transports peptides of specific sequence and length, thereby acting as a chaperone to support the correct loading of peptides onto MHC class I molecules.

Because the cytolysis by HER-2-specific CTLs was inhibited by treatment of target cells with the proteasome...
inhibitor lactacystin, it is likely that the generation of anti-
genic peptides derived from HER-2 molecules is proteasome
dependent. This observation is consistent with a previous
report that the proteasomal pathway is functional and partic-
ipates in the formation of E75 epitope, which is one of the
immunodominant HER-2 epitopes, in SKOV3.A2 cells (25).
Furthermore, as an important finding in the present study,
lactacystin inhibited the phenomenon by which Herceptin
sensitized HER-2-overexpressing tumors to lysis by HER-2-
specific CTLs without any effect of the expression of class-I
molecules, adhesion molecules, or costimulatory molecules.
Also, we showed that the expression of TAP1 molecules in
the target cells was not enhanced by the treatment with
Herceptin. These results suggest that the enhancement of
CTL lysis by Herceptin is mainly due to up-regulation of the
proteasome step in the class I-restricted antigen presentation
pathway. This was additionally supported by a previous
report that anti-HER-2 mAb enhances ubiquitination of
HER-2 (22). Protein tagged to ubiquitin is known to be
targeted to the proteasome, which cleaves the protein into
small peptides. It has also been reported that the accelerated
HER-2 degradation due to treatment with the tyrosine kinase
inhibitor geldanamycin significantly increased the presenta-
tion of the HER-2-specific epitope E75, which enhanced the
sensitivity of tumor cells to CTL lysis (25). Furthermore, it
has been shown recently that treatment with neu-specific
mAb enhances antigen-specific CD8+ T-cell function in
HER-2/neu-transgenic mice model (26). Thus, these observa-
tions suggest that Herceptin treatment might enhance the
class I-restricted presentation of endogenous HER-2 antigen
via the proteasome step.

One of the subunits of the proteasome, LMP-2, is encoded
within the MHC region, close to the TAP-1/2 genes (17, 18).
LMP-2 is not absolutely required for the production of MHC
class I-bound peptide epitopes (17, 18, 27), but it increases the
efficiency by which the proteasome produces peptides that can
bind to MHC-class I molecules (17, 18, 28). In the present
study, the expression of LMP-2 in HER-2-overexpressing tu-
mors was not enhanced by Herceptin treatment. Further study
will be needed to determine how Herceptin affects the quality
and quantity of the proteasome subunits.

These findings described here, showing that Herceptin
enhanced MHC class I-restricted antigen presentation in HER-
2-positive tumors, encourage us to initiate a combination ther-
apy including both antibody and CTLs directly against HER-2.
Because only animals receiving the combination of HER-2-
specific CTLs and HER-2-specific IgG were fully protected
from tumor challenge (29), it is suggested that both cellular
and humoral immunity should be activated for the induction of
a more potent antitumor immunity directed against HER-2.
Also, the combination of neu-specific mAbs with whole cell
tumor vaccination enhances tumor-free survival in HER-2/neu-
transgenic mice model (26). In contrast to the findings, several
reports argue against the requirement of antibodies for the
rejection of HER-2-positive tumors and rather support the in-
volvement of HER-2-specific T cells in this process (30). The
present study indicates that the combination of Herceptin and
anti-HER-2-specific CTLs results in the synergic antitumor ef-
effect of CTL-mediated lysis.

To enhance the immunity to HER-2, peptide-based vacci-
nation trials have been reported recently. We reported a Phase I
vaccination trial in gastric cancer patients using DCs pulsed
with immunodominant HER-2 (p369) peptides (31). Others
confirmed the immunodominance of HER-2 peptides in a
clinical vaccination trial in patients with ovarian and breast
cancer (32). These observations indicate that vaccination with
HER-2 peptides is immunogenic and HER-2 could be a good
target for immunotherapy. To develop an effective immunother-
apy targeted for HER-2, the combination of Herceptin and
anti-HER-2 CTLs is desirable.

**Fig. 6** Apoptosis of SW626A2 or PC-9 cells. SW626A2 or PC-9
cells treated with trastuzumab (Herceptin), control monoclonal
antibody, or irradiation (40 Gy) were analyzed for apoptosis with
Annexin-V/propidium iodide staining.
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