Impaired Antibody-dependent Cellular Cytotoxicity Mediated by Herceptin in Patients with Gastric Cancer

Koji Kono, Akihiro Takahashi, Fumiko Ichihara, Hidemitsu Sugai, Hideki Fujii, and Yoshirou Matsumoto

First Department of Surgery, Yamanashi Medical University, Yamanashi 409-3898, Japan

ABSTRACT

The humanized monoclonal antibody Herceptin, which specifically targets HER-2/neu, exhibits growth inhibitory activity against HER-2/neu-overexpressing tumors and is approved for therapeutic use with proved survival benefit in patients with HER-2/neu-positive breast cancer. In the present study, we investigated whether Herceptin could affect the HER-2/neu-overexpressing gastric cancer cells based on antibody-dependent cell-mediated cytotoxicity (ADCC) and compared immune effector cells from gastric cancer patients with normal individuals on ADCC. HER-2/neu-expressing gastric cancer cells could be killed by Herceptin-mediated ADCC and the Herceptin-induced ADCC correlated with the degree of HER-2/neu expression on the gastric cancer cells. However, the Herceptin-mediated ADCC was significantly impaired in peripheral blood mononuclear cells from advanced disease patients (n = 10) compared with that in early disease (n = 12; P = 0.04) or healthy individuals (n = 10, P = 0.02). Moreover, natural killer (NK) cells purified from patients with advanced disease indicated less Herceptin-mediated ADCC in comparison with that from healthy donors (P = 0.04), whereas monocytes purified from the patients showed an almost equal amount of Herceptin-mediated ADCC in comparison with that from healthy individuals, indicating that NK cell dysfunction contributed to the impaired Herceptin-mediated ADCC in gastric cancer patients. Furthermore, the NK-cell dysfunction on Herceptin-mediated ADCC correlated with the down-regulation of CD16 expression in the patients, and interleukin 2 ex vivo treatment of NK cells could restore the impairment of Herceptin-mediated ADCC, concomitant to the normalization of the expression of CD16 molecules. Thus, some modalities such as interleukin 2 treatment aimed at reversing NK dysfunction may be necessary for successful Herceptin treatment of gastric cancer.

INTRODUCTION

Gastric cancer is one of the most common cancers in Japan today. Despite the efforts to introduce new treatment modalities such as surgery combined with chemotherapy (1), hyperthermia (2), or chemoradiotherapy (3), control of gastric cancer at the advanced stage remains difficult. Therefore, the utilization of antitumor T cells or antibody against tumor antigens as immunoadjuvant therapy for gastric cancer is extremely appealing. We and others have reported that MHC class I-restricted CTLs from gastric-cancer patients can react specifically against autologous tumor cells (4–7). In fact, we have reported recently that adoptive transfer of ex vivo cultured T cells derived from tumor-associated lymphocytes resulted in a survival effect in a randomized control study in patients with gastric cancer (8).

The HER-2/neu proto-oncogene encodes a M 185,000 transmembrane glycoprotein that contains an extracellular domain and intracellular domain with tyrosine-specific kinase activity (9). We reported that amplification and overexpression of the HER-2/neu proto-onogene were seen in ~20% of gastric cancers and correlated with stage progression of gastric cancer (10, 11). Abundant examples from experimental models and clinical trials suggest that HER-2/neu can be immunogenic and generate antibody, CTL-, and helper T-cell-specific responses in individuals with HER-2/neu-overexpressing tumors (12, 13). On the basis of the above reports, anti-HER-2/neu immune targeting may be used as an attractive approach to treat gastric cancer. The humanized mAb Herceptin, which specifically targets HER-2/neu, exhibits potent growth inhibitory activity against HER-2/neu-overexpressing tumors (14). The underlying mechanisms of the action of Herceptin include the inhibition of tumor cell growth such as the down-regulation of the HER-2/neu receptor and the enhancement of the immune system such as ADCC (14). Herceptin has boosted the interest of clinicians in immunotherapy based on this molecule, because it represents the first mAb approved for therapeutic use with proven survival benefit in patients with HER-2/neu-positive breast cancer with metastasis (15, 16). Thus, it is desirable to apply the treatment with Herceptin to other HER-2/neu-expressing tumors. However, there has been no previous report describing the possibility of applying Herceptin to gastric cancer patients.

In the present study, we: (a) investigated the biological activity of Herceptin against HER-2/neu-overexpressing gastric cancer cells with respect to ADCC; and (b) compared the degree of ADCC associated with immune effector cells obtained from gastric cancer patients to those from normal individuals.

MATERIALS AND METHODS

Patients. PBMCs were isolated from gastric cancer patients with early disease corresponding to stage I (n = 12, 65 ± 14 years old), with advanced disease (n = 10, 68 ± 16 years old), corresponding to stage II (n = 2), III (n = 2), IV (n = 6), and healthy individuals (n = 10, 63 ± 18 years old). The stage of the disease was defined according to the Unio Internationale Contra Cancrum classification. All of the patients were treated at Yamanashi Medical University Hospital and pathologically diagnosed with gastric cancer. None of the patients received radiotherapy, chemotherapy, or other medical interventions during this study. This study was approved by the ethical committee of Yamanashi Medical University, and informed consent for blood donations was obtained for all of the individuals.

Preparation of Cells. PBMCs were isolated from heparinized peripheral blood by Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient centrifugation.

To prepare monocytes with negative selection, monocytes were isolated from PBMCs by Ficoll-Paque (Pharmacia) density gradient centrifugation after being incubated with RosetteSep antibody mixture for monocytes (StemCell Technologies Inc., Vancouver, British Columbia, Canada). RosetteSep antibody was bound in bispecific antibody complexes, which are directed against cell surface antigens on human hematopoietic cells (CD2, CD3, CD8, CD19, CD56, and C66b) and glycophorin A on RBCs. Unwanted cells, which adhered to RBCs, and desired cells were separated on a Ficoll-Paque density gradient.

To prepare NK cells with negative selection, NK cells were isolated by Ficoll-Paque density gradient after being incubated with RosetteSep antibody.
mixture (CD3, CD4, CD36, CD19, and CD66b) for NK cells as described above. NK cells resulted in >88% purity confirmed by flow cytometric analysis using anti-CD16 mAb (Becton Dickinson).

**Cell Lines.** MKN-7 (a well-differentiated gastric adenocarcinoma) and KATOIII (a signet ring cell gastric carcinoma) cell lines were obtained from the Immuno-Biological Laboratories cell bank (Gunma, Japan). MKN-28 (a well-differentiated gastric adenocarcinoma) and MKN-45 (a poorly differentiated gastric adenocarcinoma) cell lines were provided by Chugai (Tokyo, Japan). AME-1 gastric cancer cell line (a poorly differentiated gastric adenocarcinoma) was established by our laboratory. All of the gastric cancer cell lines were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 7.5% of FCS (Life Technologies, Inc.) and penicillin (100 units/ml; Sigma Chemical Co., St. Louis, MO), and streptomycin (100 μg/ml; Sigma).

**ADCC Assay.** After the target cells were labeled with 100 μCi 51Cr for 60 min, target cells (5 × 10^4/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 6 h at 37°C with Herceptin (2 μg/well; Roche) or control antibody, Rituxan (2 μg/well; Roche). 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 = (experimental cpm − spontaneous cpm)/(maximum cpm − spontaneous cpm). Controls included the incubation of target cells alone with Herceptin. Rituxan was used as a control mAb in some experiments.

**Flow Cytometric Analysis.** For CD16 ζ expression on NK cells, intracellular staining was performed as described previously by us (17). Briefly, the isolated PBMCs were fixed with 0.5% formaldehyde in PBS for 20 min on ice after permeabilization by digitonin (10 μg/ml; Sigma) for 15 min on ice. The intracellular component of ζ molecules in the CD16 complex was stained by anti-ζ mAb conjugated with PE (TIA-2, IgG1; Coulter) or by IgG1 isotype control mAb at a saturating concentration, followed by counterstaining with FITC-CD16 (Becton Dickinson). The stained cells were assessed by flow cytometric analysis.

**Biochemical Analysis for ζ Chain.** Negatively selected NK cells (2 × 10^7/ml) were lysed in NP40 lysis buffer [1% NP40, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris (pH 8.0)]. Cellular lysates were electrophoresed in 10% SDS gels, transferred to nitrocellulose membrane, and blotted with anti-ζ mAb (TIA-2) or isotype-matched control mAb. The membrane was incubated with antipolypeptide immunoglobulin-horseradish peroxidase (Amersham, Arlington Heights, IL) followed by the enhanced chemiluminescence reagent (enhanced chemiluminescence; Amersham).

**IL-2 Treatment of NK Cells.** Negatively selected NK cells (1 × 10^6 cell/ml) from gastric cancer patients were incubated with AIM-V medium (Life Technologies, Inc.) in 48-well culture plates in the presence or absence of IL-2 (500 IU/ml; Shionogi, Tokyo, Japan) for 5 days.

**Antibodies.** Clinically marketed anti-HER-2/neu mAb Herceptin and anti-CD20 mAb Rituxan, as an isotype-matched control mAb for Herceptin, purchased from Roche were used in the ADCC experiment. For the evaluation of HER-2/neu expression on the tumor cells, a PE-labeled anti-HER-2/neu mAb (Becton Dickinson, San Jose, CA) and PE-labeled mouse IgG1 mAb (Beckman-Coulter, Miami, FL) as a negative control were used for immunostaining by flow cytometric analysis.

**Statistics.** To determine statistical differences between groups, nonpaired Student’s t test was used. Statistical significance was determined when Ps were <0.05.

**RESULTS**

**Herceptin Mediates ADCC against HER-2/neu-overexpressing Gastric Cancers.** To test whether Herceptin induces ADCC against gastric cancer cells, we performed an ADCC assay by PBMCs from

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Fig. 1. Herceptin mediates ADCC against HER-2/neu-overexpressing gastric cancers. PBMCs from healthy donors were tested for ADCC in the presence of Herceptin against several different gastric cancer cell lines with various levels of HER-2/neu. HER-2/neu expression on the tumor cells was evaluated by flow cytometric analysis (A). Representative data of ADCC against MKN-7 with HER-2/neu-overexpression (B), MKN-45 with moderate levels of HER-2/neu (C), or AME-1 with low levels of HER-2/neu (D) are shown.
healthy donors against several different gastric cancer cell lines with various levels of HER-2/neu. Representative data showed that Herceptin induced much higher cytotoxicity against MKN-7 with HER-2/neu overexpression in comparison to that against MKN-45 with moderate levels of HER-2/neu or AME-1 with low levels of HER-2/neu (Fig. 1). There were no direct cytotoxicities for the three targets by Herceptin (Fig. 1). Summarized data from healthy individuals (n = 10) showed that Herceptin significantly mediated the lysis of HER-2/neu-overexpressing MKN-7 and the Herceptin-induced cytotoxicity positively correlated with the degree of HER-2/neu expression on the gastric cancer cells (Fig. 2). There was no significant ADCC by control antibody Rituxan against any targets (Fig. 2).

**Herceptin-mediated ADCC Is Impaired in PBMCs from the Patients with Gastric Cancer.** To evaluate Herceptin-mediated ADCC in gastric cancer patients, we compared the ADCC in PBMCs from patients with that from healthy individuals. The Herceptin-mediated cytotoxicity against HER-2/neu-overexpressing MKN-7 was significantly impaired in advanced disease patients in comparison with that in early disease (P = 0.04) or healthy individuals (P = 0.02) as indicated in Fig. 3. These results indicated that Herceptin-mediated ADCC was impaired in PBMCs from the patients with advanced gastric cancer.

**Impaired Herceptin-mediated ADCC Was Dependent on NK Cell Dysfunction.** Monocytes and NK cells are known to be important effectors in the role of ADCC (18). To additionally investigate the impaired ADCC by Herceptin in the patients, monocytes and NK cells were purified from PBMCs with negative selection from the patients and healthy individuals. NK cells purified from the patients with advanced disease (n = 8) indicated less Herceptin-mediated ADCC against HER-2/neu-overexpressing MKN-7 in comparison with that from healthy donors (n = 10; P = 0.04), as shown in Fig. 4A. On the other hand, monocytes purified from the patients showed an almost equal amount of Herceptin-mediated ADCC against HER-2/neu-overexpressing MKN-7 in comparison with that from healthy individuals (Fig. 4B). These results indicated that impaired Herceptin-mediated ADCC in PBMCs from the patients contributes to NK cell dysfunction.

Because Herceptin exhibits ADCC depending on FCγRIII (CD16) molecules (19, 20) and the phosphorylation of CD16ζ molecules is one of the major signal transductions in NK cell-mediated ADCC through CD16 molecules (21, 22), we additionally studied the expression of signal-transducing CD16ζ molecules on NK cells using an intracellular staining method and Western blotting. As expected, the expression of signal-transducing CD16ζ molecules on NK cells from the gastric cancer patients was significantly down-regulated compared with that from healthy donors (Fig. 5, A–C; P = 0.03). Thus, NK cell dysfunction on ADCC mediated by Herceptin in gastric cancer patients correlated to the down-regulation of CD16ζ molecules.
Restoration of Herceptin-mediated ADCC by ex vivo IL-2 Treatment of NK Cells. We next investigated the effect of ex vivo IL-2 treatment of patient-derived NK cells. Negatively selected NK cells from gastric cancer patients ($n = 5$) were incubated with AIM-V medium in the presence or absence of IL-2 (500 IU/ml) for 5 days. The amount of Herceptin-mediated ADCC by IL-2-treated NK cells was increased compared with that by nontreated NK cells (Fig. 6) and restored to the levels of Herceptin-mediated ADCC by healthy individuals. The expressions of signal-transducing CD16ξ molecules on IL-2-treated and untreated NK cells were $94.5 \pm 10.7$ MFI and $61.5 \pm 12.1$ MFI, respectively. Thus, the IL-2 treatment of patient-derived NK cells resulted in the restoration of Herceptin-mediated ADCC, concomitant to the recovery of the expression of CD16ξ molecules.

**DISCUSSION**

The present report contains several novel findings relevant to clinical therapeutic application of Herceptin against gastric cancer. First, HER-2/neu-overexpressing gastric cancer cells could be killed by Herceptin-mediated ADCC; however, the Herceptin-mediated ADCC was impaired in PBMCs from the gastric cancer patients compared with that from healthy individuals, mainly because of the NK-cell dysfunction. Second, IL-2 ex vivo treatment of NK cells could restore the impairment of Herceptin-mediated ADCC in the patients with gastric cancer.

The fully humanized anti-HER-2/neu mAb, Herceptin, has been designed to specifically antagonize the HER-2/neu function by directing against the extracellular domain of the HER-2/neu protein (14). Herceptin was clinically proven to have survival effects for HER-2/neu-overexpressing breast cancer (15, 16). Many mechanisms have been proposed to account for the therapeutic effect of Herceptin, including the blockade of signaling pathways (14), down-modulation of the HER-2/neu receptor (14), activation of apoptotic signals of the tumor cells (23), and the interaction with the immune system via its immunoglobulin G, Fc domain, such as ADCC (14). In the present study, we clearly showed that HER-2/neu-expressing gastric cancer cells, as well as breast cancer cells with HER-2/neu expression, could be killed by Herceptin-mediated ADCC. This result encourages us to apply the therapeutic approach with Herceptin to gastric cancer.

In addition, we have indicated an important issue in the present study that this Herceptin-mediated ADCC was impaired in PBMCs from gastric cancer patients with advanced disease. NK cells, a principal cell type involved in ADCC, express the activation Fcγ receptor, FCγRIII (CD16), but do not express the inhibitory counterpart, FCγRIIB, whereas monocytes express both FCγRIII and FCγRIIB (20). Clynes et al. (20) have reported that both NK cells and monocytes play a role in Herceptin-mediated ADCC by demonstrating that mice deficient in the FCγRIII receptor were unable to inhibit tumor growth by treatment of Herceptin, and Herceptin exhibit ADCC depending on FCγRIII (CD16) molecules. In addition, it has also been reported that the inhibitory FCγRIIB receptor is a potent regulator of ADCC mediated by Herceptin in vivo, modulating the activity of FCγRIII on NK cells and monocytes (20). It was also shown in human PBMCs that Herceptin-mediated ADCC was CD16 molecule-dependent (19). In the present study, we showed that NK cells from gastric cancer patients were dysfunctional in Herceptin-mediated ADCC, whereas monocytes were not. These results indicated that NK cell-dysfunction contributed to the impaired Herceptin-mediated ADCC in gastric cancer patients. Similar to the present study, it has been shown that there was impairment of ADCC activity in colon cancer patients in comparison with healthy donors (24).

The signaling pathways leading to the activation of NK-cell killing...
have only been partly elucidated (22). Both the CD16 receptor complex and activatory counterpart of MHC-I inhibitory receptors or other NK triggering receptors are known to activate protein tyrosine kinase-dependent signaling pathways in NK cells (25). The protein tyrosine kinase activity has been shown to constitute a mandatory step in the initiation of the CD16-mediated target cell killing (25). The α subunit of CD16 associated noncovalently with the signal-transducing CD16ζ and FcγRI-γ molecules (26). We and others showed previously that the expression of signal-transducing CD16ζ molecules on NK cells was down-regulated in cancer patients compared with that in healthy donors, and the alteration of signal transducing molecules was associated with NK-cell dysfunction (17, 27, 28). Here, we emphasized these observations again, in the case of Herceptin-induced ADC. For the gastric cancer patients. The present study clearly showed that NK-cell dysfunction in Herceptin-mediated ADC in the gastric cancer patients was concomitant to down-regulated expression of CD16ζ molecules on NK cells, and the restoration of CD16ζ molecules by ex vivo IL-2 treatment resulted in the normalization of NK function in Herceptin-mediated ADC. Considering these results, some modalities such as IL-2 treatment aiming at reversing NK dysfunction may be necessary for successful Herceptin treatment of gastric cancer.

There are several problems to be resolved in Herceptin treatment. The safety data collected in different studies indicate that Herceptin achieves selective therapeutic effects without severe side effects (29), except for heart failure, which was observed in 15% of the patients in except for heart failure, which was observed in 15% of the patients in a large clinical trial of Herceptin (30). It was also reported that both the antiproliferative activity and the ability of anti HER-2/neu mAb to induce ADC were blocked by soluble HER-2/neu (31). Here, as a new finding, we have shown that Herceptin-mediated ADC was impaired in PBMCs from gastric cancer patients with advanced disease.

In conclusion, HER-2/neu-overexpressing gastric cancer cells could be killed by Herceptin-mediated ADC; however, the Herceptin-mediated ADC was impaired in PBMCs from the gastric cancer patients compared with that from healthy individuals, mainly because of the NK-cell dysfunction. IL-2 ex vivo treatment of NK cells could restore the impairment of Herceptin-mediated ADC in the patients with gastric cancer. Several combinations of anti-HER-2/neu immune targeting are desirable, such as anti-HER-2/neu antibodies and cytokines.

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