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ORIGINAL ARTICLE

Antitumor activity of trastuzumab in combination with chemotherapy in human gastric cancer xenograft models

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Abstract
Purpose To clarify the antitumor activity of trastuzumab and its potential as an effective treatment for gastric cancer patients.
Methods Levels of HER2 expression in tumor tissues of gastric cancer cell lines were examined using immunohistochemistry (IHC), fluorescence in situ hybridization (FISH), and mRNA quantification. Efficacy of trastuzumab was examined as a single agent or in combination with chemotherapeutic agents widely used clinically for gastric cancers in HER2-overexpressing human gastric cancer xenograft models.
Results Two of nine human gastric cancer xenograft models, NCI-N87 and 4-1ST, showed overexpression of HER2 mRNA and protein by IHC (HercepTest®) and HER2 gene amplification by FISH (Pathvysion®). HER2 protein showed potent staining in peripheral membranes, similar to the staining pattern of breast cancer. FISH scores were also comparable to those of breast cancer models. Trastuzumab as a single agent inhibited the tumor growth in both of the HER2-overexpressing models but not in the HER2-negative models, GXF97 and MKN-45. In any combination with capecitabine, cisplatin, irinotecan, docetaxel, or paclitaxel, trastuzumab showed more potent antitumor activity than the anticancer agents alone. A three-drug combination of capecitabine, cisplatin, and trastuzumab showed remarkable tumor growth inhibition. In NCI-N87 in vitro, trastuzumab showed direct antiproliferative activity according to cell count or crystal violet dying, and showed indirect antitumor activity such as antibody-dependent cellular cytotoxicity.
Conclusion The antitumor activity of trastuzumab observed in human gastric cancer models warrants consideration of its use in clinical treatment regimens for human gastric cancer as a single agent or a combination drug with various chemotherapeutic agents.

Keywords Trastuzumab · Antitumor activity · Human xenograft models · Gastric cancer · Chemotherapy

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Introduction

The incidence of gastric cancer worldwide is reported to be especially high in Asia, South America, and Eastern Europe. In 2005, the number of gastric cancer patients in Japan was presumed to be about 106,000 [10]. Clinical gastric cancer patients were treated with various therapies such as chemotherapy and radiation, though further improvement and progress would be required. The overexpression of HER2 protein has been reported in gastric cancer as well as in various other cancers. Numerous reports have noted the expression of HER2 in gastric cancer, but the frequency remains controversial. Recently, Yano et al. [22] reported that up to 23% of patients with differentiated-type gastric cancer in Japan were HER2-positive according to HercepTest® results; the concordance rate between immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) was 87%. These diagnostic methods are being clinically used for the selection of breast cancer patients for treatment

with trastuzumab, and the resulting data are considered to be reliable. The expression of HER2 in breast cancer is known to be a factor for poor prognosis [18]. This relationship has also been reported for gastric cancer [4, 19]; thus, therapy targeted to HER2 overexpression in gastric cancer patients is thought to be beneficial.

Trastuzumab is widely used as a standard therapy for patients with HER2-overexpressing metastatic breast cancer with clear evidence of clinical efficacy as a single agent or in combination with chemotherapeutic agents such as taxanes [2, 17], and is being applied as adjuvant therapy for breast cancer [13]. Therefore, it is reasonable to consider the treatment of HER2-overexpressing gastric cancer with trastuzumab as a single agent or in combination.

In in vitro preclinical studies, HER2-overexpressing gastric cancer cell lines were reported with HER2 overexpression levels determined by IHC, CISH, RT-PCR, or Western blot analysis [5, 9, 19]. For in vitro studies, however, results of the antitumor activity of trastuzumab were controversial [5, 9, 19]. Furthermore, there have been no results reported with using xenograft models for combinations of trastuzumab with chemotherapeutic agents used in treating clinical gastric cancer. In the current study, we investigated whether trastuzumab would be useful for the treatment of HER2-overexpressing gastric cancer in cell culture and xenograft models as an indication of its usefulness for treating clinical gastric cancer.

Materials and methods

Test agents

Trastuzumab was provided by F. Hoffman-La Roche (Nutley, NJ) as a freeze-dried powder and was dissolved in water and diluted with saline for in vivo experiments or with a culture medium for in vitro experiments. Human immunoglobulin G (HuIgG) was purchased from MP Biomedicals, Inc. (Aurora, OH, USA) and was reconstituted with water and diluted with saline. Capecitabine was provided by F. Hoffman-La Roche as a bulk powder and dissolved in 40 mM citrate buffer (pH 6.0) containing 5% (w/v) gum arabic. Docetaxel was synthesized by Kanto Chemical Co., Inc. (Tokyo, Japan) as a fine powder and dissolved in saline containing 2.5% (v/v) polysorbate 80 (Sigma-Aldrich, Inc., St. Louise, MO, USA) and 2.5% (v/v) ethanol. Paclitaxel was purchased from Sigma-Aldrich and was dissolved in saline containing 5% (v/v) cremophol EL (Sigma-Aldrich) and 5% (v/v) ethanol. Cisplatin and irinotecan were purchased from Nihon Kayaku Co. (Tokyo, Japan) and Daiichi Pharmaceutical Co., Ltd. (Tokyo, Japan), respectively, and diluted with saline.

Animals

Male 5-week-old BALB/c-nu/nu mice (CAnN.Cg-Foxn1⟨nu⟩/CrlCrlj nu/nu) were obtained from Charles River Japan (Yokohama, Japan). All animals were allowed to acclimatize and recover from shipping-related stress for 1 week prior to the study. The health of the mice was monitored by daily observation. Chlorinated water and irradiated food were provided ad libitum, and the animals were kept in a controlled light–dark cycle (12 h–12 h). All animal experiments were performed in accordance with the Guidelines for the Accommodation and Care of Laboratory Animals in Chugai Pharmaceutical Research Center.

Cell lines and culture conditions

Nine human gastric cancer cell lines were used in the present study. NCI-N87, SNU-1, and SNU-16 human gastric cancer cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA) and were maintained in RPMI-1640 supplemented with 10% (v/v) fetal bovine serum (FBS). SNU-5 human gastric cancer cells were purchased from the ATCC and were maintained in IMDM supplemented with 4 mM L-glutamine and 20% (v/v) FBS. MKN-28 and MKN-45 were purchased from Immuno-Biochemical Laboratories Co., Ltd. (Fujioka, Japan) and were maintained in RPMI-1640 supplemented with 10% (v/v) FBS. AGS was purchased from ATCC and an in vivo line was established in our laboratory. 4-1ST was purchased from the Central Institute for Experimental Animals (Yokohama, Japan), and GXF97 was kindly provided by Prof. H. H. Fiebig (University of Freiberg, Freiberg, Germany). AGS, 4-1ST, and GXF97 cell lines were maintained in BALB/c-nu/nu mice by the subcutaneous (sc) inoculation of tumor pieces. The KPL-4 HER2-positive human breast cancer cell line was kindly provided by Dr. J. Kurebayashi (Kawasaki Medical School, Okayama, Japan) and was maintained in D-MEM supplemented with 5% (v/v) FBS [6]. MDA-MBA-361 and MDA-MB-231 were purchased from the ATCC and in vivo lines were established in our laboratory.

mRNA quantification, IHC, and gene amplification of HER2

To test the HER2 status of tumor tissues, each mouse was inoculated sc into the right flank with either 5×10^6 cells/mouse of human gastric cancer cell lines

NCI-N87, SNU-1, SNU-16, SNU-5, MKN-28, or MKN-45, or an 8 mm^3 piece of AGS, 4-1ST, or GXF97 tumor tissue. As a positive control for HER2 testing, the human breast cancer cell line KPL-4 (5×10^6 cells/mouse) was inoculated into the secondary mammary fat pad of one mouse. 8 mm^3 piece of MDA-MB-361 or MDA-MB-231 tumor tissue was inoculated into the right flank of the mouse. Tumor xenograft tissues were resected and processed as formalin-fixed, paraffin-embedded specimen sections and as fresh frozen tissues for HER2 mRNA quantification. These were examined for the expression of HER2 protein by IHC using HercepTest®, for HER2 gene amplification by FISH using Pathvysion®, and then diagnosed by blind testing at SRL Medisearch, Inc. (Tokyo, Japan), a central laboratory that conducts tests on clinical specimens. Total RNA was isolated from xenograft tumor tissues with Sepazol reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's protocol and reverse transcribed with Superscript II (Invitrogen, CA, USA). Quantitative PCR of HER2 was performed in a LightCycler™ (Roche Diagnostics, Mannheim, Germany) using a SYBR Green-based real-time assay. Standard curves for absolute quantification were obtained with plasmids containing PCR-amplicons. The PCR-amplification primers for HER2 were as follows: forward primer, 5′-TGT GTG GAC CTG GAT GAC AA-3′, and reverse primer, 5′-CCA GCT CCG TTT CCT GC-3′, generating a 190 bp amplicon. The housekeeping gene GAPDH was quantified with the following primers: 5′-CTC CTG CAC CAC CAA CTG-3′ and antisense 5′-TCA CGC CAC AGT TTC CCG-3′. The absolute copy number of HER2 mRNA was normalized to the GAPDH mRNA expression level.

In vivo tumor growth inhibition studies

The methods for tumor inoculation were the same as for the HER2 testing described above. Several weeks after tumor inoculation, mice were randomly allocated to control and treatment groups. Administration of anticancer agents was started when tumor volumes reached approximately 0.2–0.3 cm^3. To evaluate the antitumor activity and tolerability of the test agents, tumor volume and body weight were measured twice a week. The tumor volumes (V) were estimated from the equation $V = ab^2/2$, where a and b are tumor length and width, respectively. The percentage of tumor growth inhibition (TGI%) was calculated as follows: TGI% = {1 − (tumor volume of treatment group on evaluation day − tumor volume of treatment group on day 1)/(tumor volume of control group at evaluation day − tumor volume of control group on day 1)} × 100. For the criteria of antitumor activity in Table 1, * was marked when the tumor volume of the 80 mg/kg trastuzumab-treated group was significantly smaller than that of the control group.

Treatment of animals

Mice bearing tumors were treated with the test agents (5–8 mice/group). Trastuzumab was administered intraperitoneally (ip) twice a week (biw), once a week (qw), or once in 3 weeks (q3w) for 3 weeks. Capecitabine was administered orally (po) once a day for 14 consecutive days. The dose of trastuzumab was determined in reference to the doses used in breast cancer models [3]. The doses of chemotherapeutic agents in

Table 1 HER2 status of xenograft tumor tissues of human gastric and breast cancer

	mRNA HER2/GAPDH copies/100 copies	IHC score	FISH gene amplification of HER2	Antitumor activity of trastuzumab
Gastric cancer cell line				
4-1ST	70.4	3+	5.3	*
NCI-N87	56.7	2+	8.4	*
GXF97	1.21	0	0.9	–
SNU-5	1.12	0	1.7	–
SNU-16	0.46	1+	1.4	–
MKN-28	0.37	0	1.0	–
AGS	0.24	0	1.0	–
SNU-1	0.19	0	1.2	–
MKN-45	0.068	0	1.1	–
Breast cancer cell line				
KPL-4	22.0	3+	7.0	*
MDA-MB-361	16.2	2+	9.0	*
MDA-MB-231	0.081	0	1.1	–

mRNA was purified from fresh frozen tissues and mRNA of HER2 by Light Cycler™ was qualified. Immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH) were performed on the formalin-fixed, paraffin-embedded specimens

*Antitumor activity was significantly observed by 80 mg/kg of trastuzumab treatment; –antitumor activity was not observed

combination with trastuzumab were selected as two-third of the maximum tolerated dose (MTD) in the xenograft models. The administration of chemotherapeutic agents followed the schedules of clinical regimens. Cisplatin was administered ip qw for 3 weeks. Irinotecan was administered intravenously (iv) qw for 3 weeks. Docetaxel or irinotecan was administered iv q3w. Paclitaxel was administered iv qw. The MTD was defined as half of the minimum toxic dose causing death in more than one mouse out of six mice or resulting in more than 20% of body weight loss in a separate experiment.

In vitro antiproliferative assay

MKN-45 cells or NCI-N87 cells were seeded at 1×10^4 cells/well in 96-well flat bottom plates and precultured for 16 h. The cells were then treated with serially diluted trastuzumab or HuIgG and cultured for 72 h at 37°C in 5% CO_2. For comparison of methods in antiproliferative activity experiments, actual cell count or crystal violet methods were examined. For actual cell count, cells were harvested by trypsin treatment. Cell suspensions were diluted with equal volume of trypan blue, and the living cell number were counted under the microscope. For the crystal violet method, the cells were fixed with 10% formalin in 150 mM NaCl, stained with 0.5% (w/v) crystal violet in 6% methanol, washed three times with distilled water, and dried. Finally, the cells were dissolved with 1% (w/v) citric acid in 50% (w/w) ethylene glycol and 35% (w/w) ethanol, and their absorbance was measured at 595 nm. To estimate the effect of trastuzumab on cell proliferation, the percentage of cell proliferation inhibition (%Inhibition) was calculated as follows: %Inhibition by actual cell count = {1 − (cell number of treatment well − initial cell number)/(cell number of nontreatment well − cell number of initial cell number)} × 100; %Inhibition by crystal violet = {1 − (absorbance of treatment well − absorbance of blank well)/(absorbance of nontreatment well − absorbance of blank well)} × 100. IC50 or IC30 indicates the concentration of trastuzumab causing a rate of 50 or 30% of %Inhibition, respectively. For the comparison of cell lines in Fig. 5b, cells were dyed with crystal violet.

Antibody-dependent cellular cytotoxicity (ADCC)

Peripheral blood mononuclear cells (PBMC) obtained from healthy volunteers were isolated from heparinized whole blood by density gradient sedimentation using Ficoll-Paque PLUS (Amersham Biosciences AB, Sweden). The cells were washed three times with RPMI-1640 medium, resuspended at 5×10^6 cells/ml in RPMI-1640 medium containing 10% (v/v) FBS, and diluted into 96-well round bottom microtiter plates (Costar #3799, Corning Incorporated, NY, USA) to give effector to target (E:T) ratios of 25:1, 12.5:1, and 6.25:1. Various dilutions of trastuzumab or medium were then added. Antibody concentrations used were 1, 10, 100, 1,000, and 10,000 ng/ml. The target cell line, NCI-N87 or MKN-45, was labeled with 5.55MBq $Na^{51}CrO4$ (Amersham Biosciences, Japan) for 60 min at 37°C, then washed three times with RPMI-1640 medium, and 1×10^4 cells/well was added to the plates pre-seeded with PBMC. After incubation for 4 h at 37°C, an aliquot of culture supernatant (0.1 ml) was removed and measured for radioactivity in an automatic gamma counter. The percentage of specific lysis was calculated as follows: specific lysis (%) = $(A - B)/(C - B) \times 100$, where A represents ^{51}Cr release (cpm) from test supernatants, B represents spontaneous release (^{51}Cr release from untreated target cells), and C represents maximum release (^{51}Cr release from target cells lysed with 0.4% NP-40). Net percent cytotoxicity (%) = the percentage of specific lysis with antibody − the specific lysis without antibody. Each treatment was performed in triplicate. Spontaneous release from target cells alone was less than 20% of the maximum for all experiments.

Statistical analysis

For in vivo experiments, the Mann–Whitney U test was used to detect the statistical differences in tumor volume ($P < 0.05$). For in vitro experiments, Student's t test was used ($P < 0.05$). The statistical analysis was carried out with the SAS preclinical package (SAS Institute, Inc., Tokyo, Japan).

Results

HER2 levels in human gastric cancer cell lines

Nine gastric cancer cell lines—4-1ST, NCI-N87, GXF97, SNU-5, SNU-16, MKN-28, AGS, SNU-1, and MKN-45—were examined for their expression of HER2 protein by HercepTest®. Among the gastric tumor tissues examined, two tumor models, NCI-N87 and 4-1ST, showed overexpression of HER2 protein. Both NCI-N87 and 4-1ST models showed positive staining from the HercepTest® but negative staining for HuIgG as a negative control (Table 1, Fig. 1a, b). The expression scores for HER2 protein in the NCI-N87 and 4-1ST models were 2+ and 3+, respectively, from

Herceptest® on a scale of 0 to 3+. All others showed negative staining (0 or 1+) for HER2-overexpression (Fig. 1c). 4-1ST and NCI-N87 showed potent and typical peripheral membrane staining, similar to that seen in the KPL-4 breast cancer model used as a positive control (Fig. 1d). The levels of HER2 gene amplification determined by FISH were 8.4 and 5.3 for NCI-N87 and 4-1ST, respectively, whereas the levels for all other gastric tumor tissues were less than 2.0 or 7.0 and 9.0 for the HER2-positive KPL-4 and MDA-MB-361, respectively (Table 1). The HER2 expression status of NCI-N87 and 4-1ST determined by mRNA, IHC, and FISH was comparable to that of KPL-4 in which trastuzumab demonstrated antitumor activity [4]. Nine gastric cancer cell lines were examined for their expression of HER2 mRNA by RT-PCR. The mRNA ratios of HER2/GAPDH (copies/copies $\times 10^{-2}$) for the nine cell lines were 70.4, 56.7, 1.21, 1.12, 0.46, 0.37, 0.24, 0.19, and 0.068, respectively (Table 1). Both 4-1ST and NCI-N87 showed high levels of HER2 mRNA.

Effects of trastuzumab as a single agent on HER2-overexpressing human gastric cancer xenograft models

We investigated the antitumor activity of trastuzumab against NCI-N87 and 4-1ST xenograft models. Trastuzumab demonstrated significant antitumor activity against NCI-N87 tumors at doses ranging from 20 mg/kg as the loading dose followed by 10 mg/kg as the maintenance dose (20/10) to 80 mg/kg followed by 40 mg/kg (80/40) (Fig. 2a). On day 22 (21 days after starting treatment), tumor growth inhibition rates (TGI%) were 51, 61, 81, and 89% at doses of 10/5, 20/10, 40/20, and 80/40 mg/kg, respectively. Trastuzumab also showed significant antitumor activity at the doses

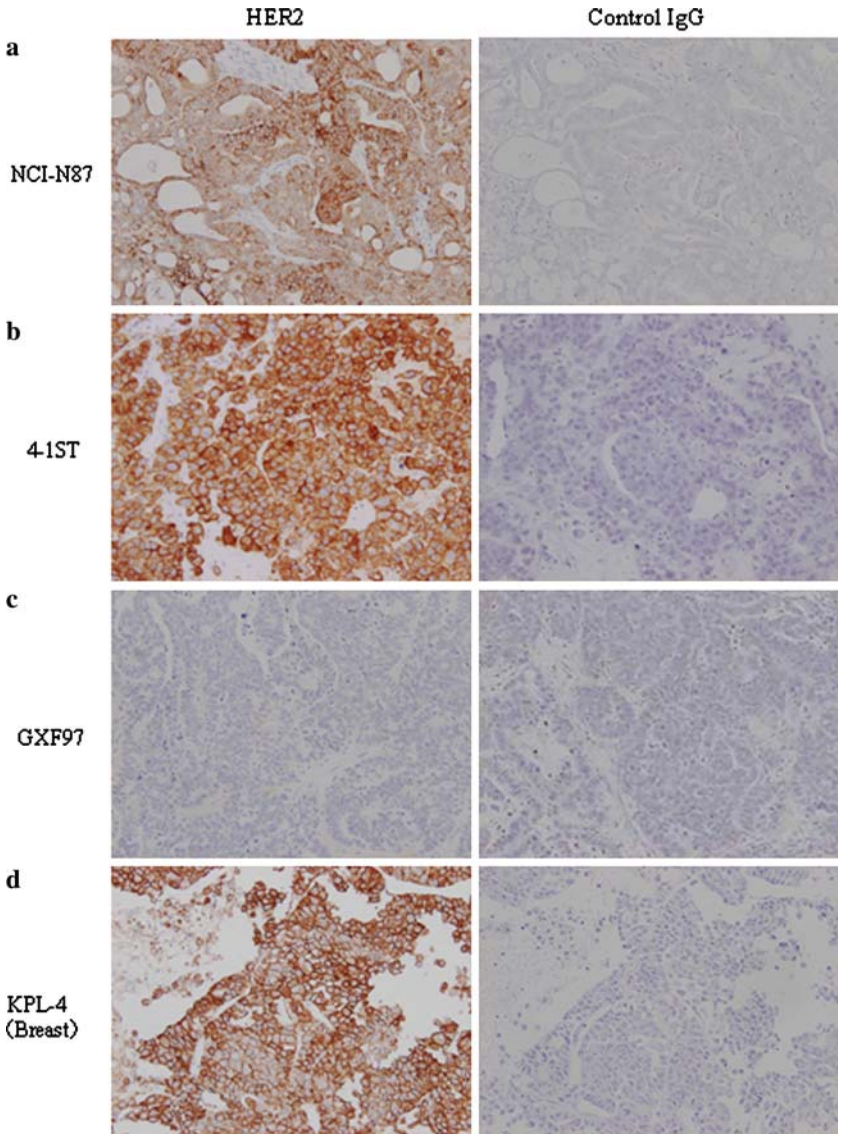

Fig. 1 HercepTest® (HER2 IHC) of the tumor tissues of gastric cancer cell lines. The method for immunohistochemistry (IHC) is described in the Materials and methods. **a** NCI-N87, **b** 4-1ST, **c** GXF97, **d** MKN-45

of 40/20 and 80/40 mg/kg against 4-1ST (Fig. 2b). No weight loss (≥20%) was observed for any of the doses tested in either model (data not shown). In contrast, trastuzumab showed no antitumor activity in the GXF97 (Fig. 2c) or MKN-45 (Fig. 2d) HER2-negative xenograft models at 80/40 mg/kg (TGI% on day 22: 14 and −6%, respectively).

For the NCI-N87 model, the antitumor activity was examined in three different treatment schedules for 3 weeks, with 80 mg/kg as the total administered amount of trastuzumab. . The TGI% results for the regimens of q3w, qw, and biw were 61, 57, and 59%, respectively, on day 29. These results showed that the antitumor activity of trastuzumab was nearly the same for all three schedules.

Combination therapy of trastuzumab with chemotherapeutic agents

The antitumor activities of trastuzumab in combination with chemotherapeutic agents were examined in HER2-overexpressing human gastric xenograft models. The combination of trastuzumab with capecitabine, irinotecan, cisplatin, docetaxel, or paclitaxel was examined. Two-third MTD of each chemotherapeutic agent was used for the combination, and 20 mg/kg of trastuzumab was administered qw for 3 weeks.

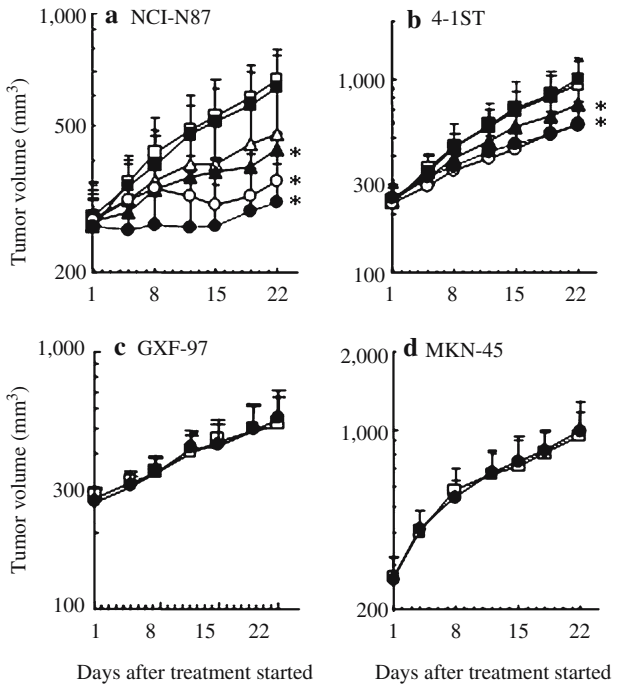

Fig. 2 Antitumor activity of trastuzumab in NCI-N87 (**a**), 4-1ST (**b**), GXF-97 (**c**), and MKN-45 (**d**) human gastric cancer xenograft models. Mice were randomly assorted into groups of six each. Hu-IgG or trastuzumab was administered ip twice a week for 3 weeks. Data points: mean values ± SD of tumor volume (mm^3). *Open square* Nontreatment, *filled square* HuIgG 80/40 mg/kg, *open triangle* trastuzumab 10/5 mg/kg, *filled triangle* trastuzumab 20/10 mg/kg, *open circle* trastuzumab 40/20 mg/kg, *filled circle* trastuzumab 80/40 mg/kg. Statistically significant differences between the nontreatment group and each trastuzumab-treated group are shown with an *asterisk* (*) ($P < 0.05$)

Figure 3 shows the results for the combinations of trastuzumab (20 mg/kg, qw for 3 weeks) with capecitabine (359 mg/kg, daily for 14 days), irinotecan (67 mg/kg, qw for 3 weeks), cisplatin (5 mg/kg, qw for 3 weeks), docetaxel (60 mg/kg, q3w on the first day of treatment), and paclitaxel (80 mg/kg, qw for 3 weeks) in the NCI-N87 model. The TGI% for trastuzumab in combination with capecitabine was 114%, and that for trastuzumab or capecitabine was of 71 and 97%, respectively (Fig. 3a). The TGI% for trastuzumab in combination with irinotecan was 110%, and that for trastuzumab or irinotecan 71 and 68%, respectively (Fig. 3b). The TGI% for trastuzumab in combination with cisplatin was 95%, and that for trastuzumab or cisplatin 71 and 65%, respectively (Fig. 3c). The TGI% for trastuzumab in combination with docetaxel was 133%, and that for trastuzumab or docetaxel 57 and 110%, respectively (Fig. 3d). The TGI% for trastuzumab in combination with paclitaxel was 125%, and that for trastuzumab or paclitaxel 57 and 107%, respectively (Fig. 3e). Antitumor activity of cisplatin in combination with trastuzumab was also examined in the 4-1ST model. The TGI% of the combination with 5 mg/kg of cisplatin in once weekly administration for 3 weeks was 78% on day 22. The TGI% of trastuzumab was 21% and that of cisplatin was 26% (Fig. 3f). In contrast to the NCI-N87 and 4-1ST models, the combination with trastuzumab provided no potentiation of the antitumor activity of paclitaxel in the HER2-negative GXF97 model (data not shown). In the HER2-overexpressing gastric cancer xenograft models, however, all of the chemotherapeutic agents examined showed more potent antitumor activities in combination with trastuzumab than did trastuzumab or the chemotherapeutic agents as single treatments.

Furthermore, we examined the three-drug combination therapy with trastuzumab, capecitabine, and cisplatin in the NCI-N87 model: 20 mg/kg of trastuzumab and 5 mg/kg of cisplatin (two-third of MTD) were administered once a week for 3 weeks, and 180 mg/kg of capecitabine (one-third of MTD) was given once a day for 14 days. This three-drug combined treatment achieved a significant increase in tumor growth inhibition (TGI% on day 22: 106%) as compared with trastuzumab (63%) as a single agent or cisplatin–capecitabine (82%) and trastuzumab–cisplatin (85%) as two-drug combinations (Fig. 4). Trastuzumab in

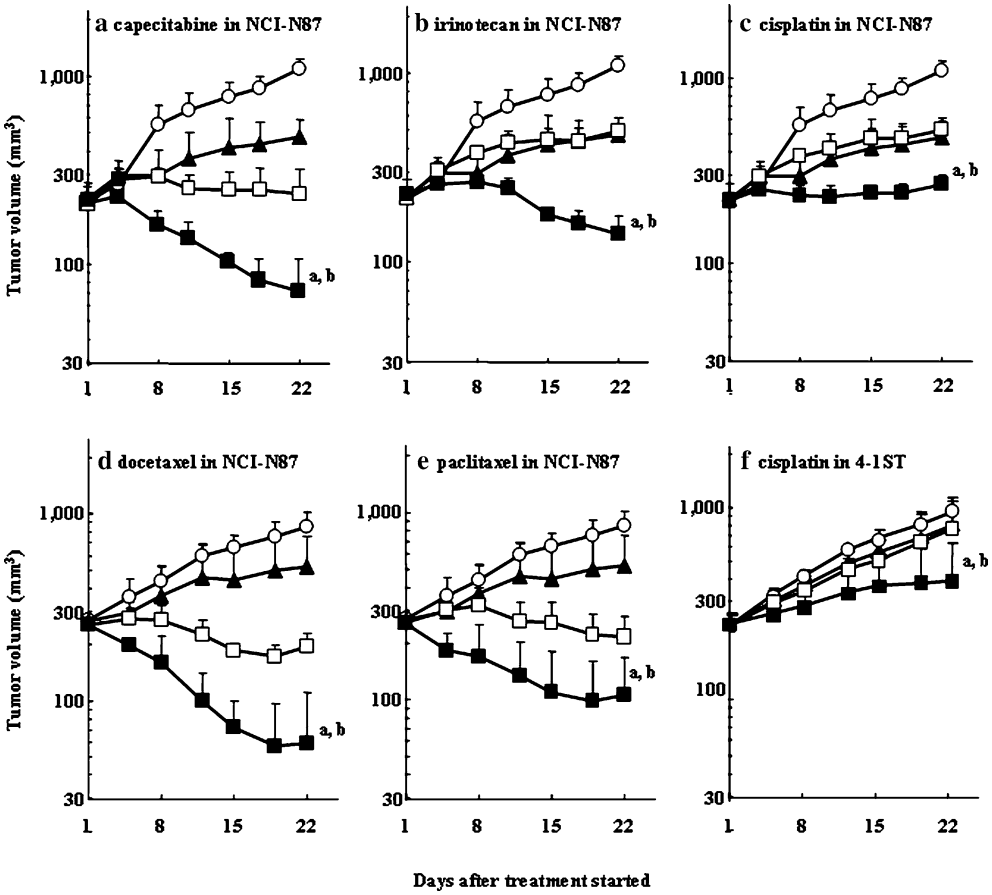

Fig. 3 Effect of trastuzumab in combination with chemotherapeutic agents on tumor growth in HER2-positive human tumor xenograft models. Mice were randomly assorted into groups of six (**a**–**e**) or eight (**f**) each. Statistically significant differences between the combination group and trastuzumab (**a**) or chemotherapeutic agent (**b**) as a single agent group ($P < 0.05$). Data points: mean values ± SD of tumor volume (mm^3). *Open circle* HuIgG and vehicle of chemotherapeutic agent, *filled triangle* trastuzumab and vehicle of chemotherapeutic agent, *open square* chemotherapeutic agent and HuIgG, and *filled square* combination with chemotherapeutic agent and trastuzumab. A dose of 20 mg/kg of HuIgG or trastuzumab was administered ip once a week for 3 weeks; **a** 359 mg/kg of capecitabine and trastuzumab in combination in NCI-N87. Capecitabine was administered po once a day for 14 days; **b** 67 mg/kg of irinotecan and trastuzumab in combination in NCI-N87—irinotecan was administered iv once weekly for 3 weeks; **c** 5 mg/kg of cisplatin and trastuzumab in combination in NCI-N87—cisplatin was administered iv once weekly for 3 weeks; **d** docetaxel and trastuzumab in combination in NCI-N87—60 mg/kg of docetaxel was administered iv once on day 1; **e** 80 mg/kg of paclitaxel and trastuzumab in combination in NCI-N87—paclitaxel was administered iv once weekly for 3 weeks; **f** 5 mg/kg of cisplatin and trastuzumab in combination in 4-1ST—cisplatin was administered iv once weekly for 3 weeks

combination with cisplatin and capecitabine showed more potent antitumor activity compared with the two-drug combination of cisplatin and capecitabine (Fig. 4a). Capecitabine in combination with cisplatin and trastuzumab also showed more potent antitumor activity than did the two-drug combination of cisplatin and trastuzumab (Fig. 4b).

ADCC activity and direct anti-proliferative effect of trastuzumab on HER2-overexpressing NCI-N87 cells in culture

To investigate the antitumor mechanisms of trastuzumab in a gastric cancer cell line, ADCC activity and direct anti-proliferative effects on NCI-N87 and MKN-45 were examined. At the ratio of E:T of 25:1, 12.5:1, and 6.25:1, 100 ng/ml of trastuzumab showed cytotoxicity rates of 96, 81, and 65, respectively, on NCI-N87 cells, whereas on MKN-45 cells, cytotoxicity was 27, 16, and 9.4%, respectively (Fig. 5a). At the E:T ratio of 25:1, even 10 ng/ml of trastuzumab generated ADCC activity on NCI-N87 cells (27%, data not shown).

Anti-proliferative activity on NCI-N87 from trastuzumab was examined using actual cell count or the crystal violet method (Table 2). The antiproliferative activity was observed using crystal violet method as well as actual cell count. The anti-proliferative activities were observed by crystal violet method with an

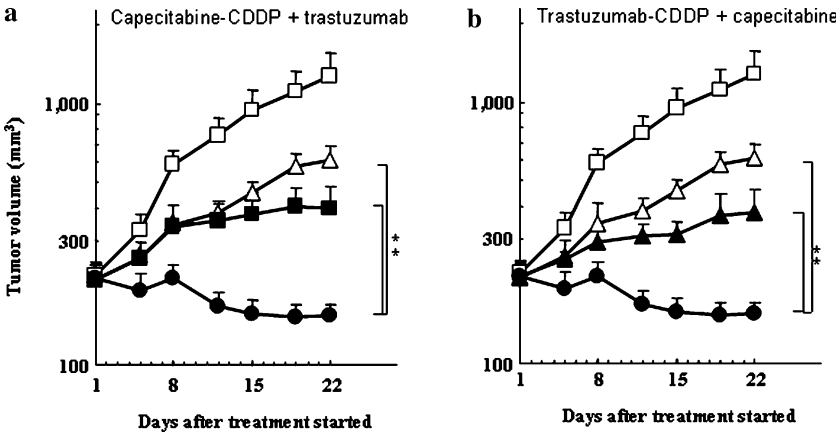

Fig. 4 Effect of trastuzumab in combination with cisplatin and capecitabine on tumor growth in NCI-N87 HER2-positive human tumor xenograft model. Mice were randomly assorted into groups of eight each. A dose of 20 mg/kg of HuIgG or trastuzumab was administered ip once a week for 3 weeks; 180 mg/kg of capecitabine or vehicle was administered po for 14 consecutive days; 5 mg/kg of cisplatin or saline was administered once a week for 3 weeks. *Open square* HuIgG, vehicle of capecitabine, and saline, *open triangle* trastuzumab and vehicle of capecitabine, and saline, *filled square* HuIgG, capecitabine, and cisplatin (**a**), *filled triangle* trastuzumab, vehicle of capecitabine, and cisplatin (**b**), *filled circle* three-drug combination with trastuzumab, capecitabine, and cisplatin. Mean values ± SD of tumor volume (mm^3). Statistically significant differences between the combination group and trastuzumab as a single agent group, trastuzumab and cisplatin or capecitabine and cisplatin as a two-drug combination group: $*P < 0.05$

IC50 of 27.3 μg/ml, or by actual cell count with an IC30 of 0.79 μg/mL. The anti-proliferative activity of trastuzumab for NCI-N87 showed moderate but significant and dose-dependent activity, whereas neither human IgG for NCI-N87 nor trastuzumab for MKN-45 generated activity at 1,000 μg/mL by the crystal violet dying method (Fig. 5b). Trastuzumab showed both ADCC and anti-proliferative activity on NCI-N87 cells in the in vitro experiments.

Discussion

Trastuzumab (Herceptin®) is a humanized antibody against human epidermal growth factor receptor 2 (HER2). It is widely used clinically as a standard therapy for patients with metastatic breast cancers or as adjuvant therapy for post-operative breast cancers that over express HER2 [2, 17]. The overexpression of HER2 protein in gastric cancer has been reported, though the frequency of overexpression in gastric cancer has been controversial. Recently, Yano et al. [22] reported that up to 23% of patients with differentiated-type gastric cancer in Japan were HER2-positive according to HercepTest® results. They also reported that the concordance rate between IHC and FISH was 87%. These diagnostic methods are being clinically used to select breast cancer patients for treatment with trastuzumab, and the resulting data are considered to be reliable. The expression of HER2 in breast cancer is known to be a factor for poor prognosis [18]. This relationship has also been reported for gastric cancer [4, 19]; thus, therapy targeted to HER2 overexpression in gastric cancer patients is thought to be beneficial. Recently, the case study of a HER2-overexpressing gastric cancer patient treated with trastuzumab was reported [15]. The patient was treated with trastuzumab, docetaxel, and capecitabine and survived for 4 years with metastatic disease controlled for 2 years by immuno-chemotherapy. Considering the high incidence of gastric cancer and an admitted lack of progress in its treatment, it is reasonable to consider the treatment of HER2-overexpressing gastric cancer with trastuzumab.

To conduct clinical studies, research demonstrating the scientific rationale is required. In the present study, we investigated whether trastuzumab would be useful for the treatment of HER2-overexpressing gastric cancer. The levels of HER2 expression in tumor tissues of gastric cancer cell lines were examined by using clinical diagnostic methods, IHC and FISH, and performing the diagnosis by blind testing in a clinical laboratory; these are the most appropriate methods in actual clinical application for selecting candidates for treatment with trastuzumab. HER2 staining in 4-1ST and NCI-N87 showed potent and typical peripheral membrane staining, similar to that seen in the KPL-4 breast cancer tissue used as a positive control. The antitumor activity of trastuzumab as a single agent was examined for the HER2-overexpressing gastric cancer models and

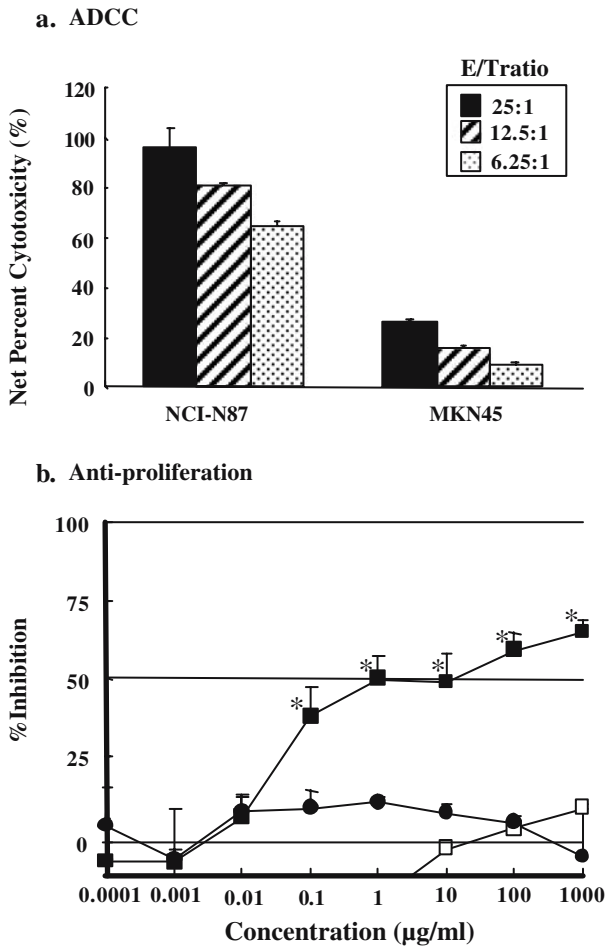

Fig. 5 Antitumor mechanisms of trastuzumab for NCI-N87 and MKN-45 gastric cancer cell lines in vitro. **a** Antibody-dependent cellular cytotoxicity (ADCC) activity of trastuzumab (100 ng/ml). The target cell was NCI-N87 or MKN-45. The effector cell was healthy human PBMC. Data points: mean values ± SE ($n = 3$) of net percent toxicity (%). ET ratios of 25:1 (*black*), 12.5:1 (*cross-hatched*), and 6.25:1 (*white*) are indicated. **b** Anti-proliferative activity of trastuzumab. Data points: mean values ± SD ($n = 4$) of percent cell proliferative inhibition (%Inhibition). *Filled square* trastuzumab on NCI-N87, *open square* HuIgG on NCI-N87, and *filled circle* trastuzumab on MKN-45. Statistically significant differences between the treatment well and control (trastuzumab 0 μg/ml) are shown with an *asterisk* (*) ($P < 0.05$)

compared with HER2-overexpression results in vivo and in vitro. Trastuzumab showed significant antitumor activity as a single agent in these two HER2-overexpressing human gastric cell line xenograft models, but did not do so in HER2-negative gastric cell lines. Tokuda et al. and Matsui et al. also reported that the humanized anti-HER2 antibody showed antitumor activity as a single agent in the 4-1ST model [20] or NCI-N87 model [13]. From the result of the concordance of efficacy as classified by HercepTest® or Pathvysion® in gastric cancer with those results for breast cancer, these two diagnostic methods would be also expected to be useful for diagnosing the HER2 status of gastric cancer.

In clinical regimens, trastuzumab has been used once in a week for metastatic breast cancer patients, though once in 3 weeks will be planned for use in an adjuvant setting. Also in a clinical phase III study for metastatic gastric cancer, a q3w regimen for trastuzumab is planned. In this study, we confirmed that the efficacy of trastuzumab was equivalent in the present preclinical model for biw, qw, and q3w treatment regimens.

Trastuzumab has been reported to show antitumor activity in in vitro breast cancer models through two mechanisms of tumor growth inhibition: a direct antiproliferative effect [20] and an indirect antitumor effect from ADCC [7]. In the present in vitro experiments, trastuzumab showed remarkable inhibition of cell proliferation and exerted ADCC activity in the HER2-overexpressing gastric cancer NCI-N87 cell line, similarly as in the breast cancer model. Although contradictory results for antiproliferative activity in gastric cancer cells have been reported using the dsDNA method and the WST-1 method [5, 19], we concluded that trastuzumab showed antiproliferative activity on NCI-N87 according to the results of our actual cell counts.

In clinical [8, 12, 17, 21] and preclinical [1, 3, 11, 14] studies on breast cancers, trastuzumab used in combination with various chemotherapeutic agents—capecitabine, cisplatin, docetaxel, and paclitaxel—showed potent efficacy and longer survival than chemotherapeutic agents alone. Recently, the new fluorinated pyrimidine—capecitabine—was approved for use in Korea for gastric cancer. And docetaxel has been reported to show a 54% response rate in combination with cisplatin and 5-fluorouracil (5-FU) in a European study [16]. In Japan, a standard treatment for gastric cancer has not been clearly defined, although fluorinated pyrimidines, cisplatin, mitomycin C, irinotecan, docetaxel, and paclitaxel are widely used. Because no chemotherapeutic agents have shown survival prolongation yet in gastric cancer patients, a new drug that would prolong patient survival, such as trastuzumab has shown in breast cancer, is desired. Thus, it is expected that trastuzumab could be clinically used in combination with other chemotherapeutic agents for HER2-overexpressing gastric cancer. In the present study, trastuzumab in combination with chemotherapeutic agents clinically used for gastric cancers, such as capecitabine, cisplatin, docetaxel, paclitaxel, and irinotecan, showed more potent antitumor activity than each anticancer agent alone. Furthermore, a three-drug combination study of trastuzumab, capecitabine, and cisplatin, in planning as a global clinical PIII study, showed remarkable tumor growth inhibition in the

Table 2 Anti-proliferative activity of trastuzumab for NCI-N87 in vitro

Method	Trastuzumab (μg/ml)			
	0	0.01	1	100
Cell count ($\times 10^4$ cells/well)	6.17 ± 0.32	5.97 ± 1.03	3.80 ± 1.45*	2.95 ± 1.16*
%Inhibition	–	3	38	52
Crystal violet (Abs at 595 nm)	0.41 ± 0.05	0.39 ± 0.05	0.21 ± 0.03*	0.17 ± 0.02*
%Inhibition	–	6	50	59

Details are described in Materials and methods. %Inhibition was calculated as follows: %Inhibition by actual cell count = {1 − (cell number of treatment well − initial cell number)/(cell number of nontreatment well − cell number of initial cell number)} × 100; %Inhibition by crystal violet = {1 − (absorbance of treatment well − absorbance of blank well)/(absorbance of nontreatment well − absorbance of blank well)} × 100. $n = 4$

*Statistically significant differences for 0 μg/ml of trastuzumab group

NCI-N87 model. In HER2-negative GXF97 models, however, trastuzumab showed no potentiation of the efficacy of paclitaxel. Therefore, it is clear that trastuzumab, even in combination therapy, should only be used for HER2-positive gastric cancers.

The present study showing remarkable tumor growth inhibition from trastuzumab in gastric cancer xenograft models clearly indicates that clinical evaluation of trastuzumab for gastric cancer is warranted.

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