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Enhanced Anti-tumor Effect of Trastuzumab in Combination with Cisplatin

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The oncogenenic transmembrane tyrosine kinase receptor HER-2/neu is a promising target for treatment of HER-2-overexpressing cancers. The humanized anti-HER-2/neu antibody Trastuzumab is under clinical evaluation in combination with chemotherapy against breast cancer. The combination of Trastuzumab and cisplatin is expected to be active against HER-2/neu-expressing tumors. We examined the mechanisms of this combination effect against human solid tumor cells in the presence of human peripheral blood mononuclear cells (PBMCs) using an *in vitro* MTT assay. The growth-inhibitory effects of cisplatin (CDDP) on the tumor cells were not significantly affected by Trastuzumab in the absence of effector cells. CDDP alone at a dose of less than 12.5 μM did not affect the viability of PBMCs, as determined by MTT assay, suggesting that PBMCs could exert antibody-dependent cell-mediated cytotoxicity (ADCC) at this CDDP concentration. The combination of Trastuzumab and CDDP showed higher cytotoxic effects against the tumor cells in the presence of PBMCs. The CDDP concentration required to inhibit tumor cell growth by 50% was reduced to ~20% by Trastuzumab in the presence of PBMCs at an effector/target ratio of 10. It may be important to select combined chemotherapeutic agents which do not diminish the ADCC activity of Trastuzumab via PBMCs. Both the expression of HER-2/neu and the ADCC activity may be important determinants of the therapeutic benefit of the Trastuzumab/CDDP combination.

Key words: Trastuzumab (Herceptin) — Anti-HER-2/neu antibody — Antibody-dependent cell-mediated cytotoxicity — Cisplatin — Combination therapy

HER-2/neu (*c-erbB-2*) proto-oncogene encodes a transmembrane tyrosine/kinase receptor with homology to the epidermal growth factor receptor family,[1, 2] and is overexpressed on breast and ovary cancers, as well as uterine cervical carcinomas.[3–6] HER-2/neu overexpression induces transformation and tumorigenesis of NIH3T3[7] and was correlated with histological grade and disease stage as an indicator of poor prognosis.[8–10] The anti-HER-2/neu humanized antibody Trastuzumab recognizes a carbohydrate epitope on the extracellular domain of HER-2/neu and anti-HER-2/neu antibody suppresses *in vitro* proliferation of several human solid tumor cell lines.[11–13] Anti-HER-2/neu antibody action is partially mediated by an antagonistic effect on signal transduction[11, 14] and partially by an anti-tumor effect via antibody-dependent (effector) cell-mediated cytotoxicity (ADCC).[15, 16] Therefore, it can be speculated that the anti-HER-2/neu antibody Trastuzumab also exerts its antitumor effect through these mechanisms. Trastuzumab has been used to treat HER-2/neu-overexpressing tumors in clinical trials,[16–19] and showed an overall response of 11.6%.[20]

Combination therapy with conventional chemotherapeutic agents is now being evaluated clinically in order to increase the anti-tumor activity of anti-HER-2/neu antibodies.[21] It is important to select an appropriate partner for combination therapy. Trastuzumab reportedly enhances the anti-tumor effect of paclitaxel and doxorubicin by affecting the cell cycle distribution.[21, 22] Cisplatin (CDDP) is also considered as a possible partner for combination therapy with Trastuzumab because anti-HER-2/neu antibody interferes with DNA repair following cisplatin-mediated DNA damage[23–27] and CDDP has actually been used in combination therapy with Trastuzumab clinically.[18, 19, 27] However, it remains unclear whether ADCC and an antagonistic effect initiated by Trastuzumab are responsible for the combination effect and whether the chemotherapeutic agents inhibit the ADCC activity of Trastuzumab. In this study, we examined the combination effect of Trastuzumab and CDDP in the presence of effector cells for ADCC and demonstrated that CDDP alone did not affect the viability of human peripheral blood mononuclear cells (PBMCs), suggesting that ADCC by PBMCs is not affected by CDDP. On the other hand, Trastuzumab, in the presence of PBMCs (10 effector: target ratio), reduced the CDDP IC_{50} values in HER-2/neu-overexpressing tumor cells to less than 0.20-fold as compared with the IC_{50} value of CDDP alone. This suggests that, in combination therapy, the ADCC activity of Trastuzumab is not affected by any influence of CDDP on PBMCs.

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MATERIALS AND METHODS

Cell lines and cell culture The cell lines used were human solid-cancer cell lines including PC-14 (human non-small cell lung cancer), JRST (gastric cancer), BT-474 (breast cancer), SK-Br-3 (breast cancer), and SKOV-3 (ovarian carcinoma). PC-14 was donated by Dr. Y. Hayata (Tokyo Medical University, Tokyo). JRST, BT-474, and SK-Br-3 were obtained from the ATCC (Rockville, MD). SKOV-3 was kindly donated by Dr. K. H. Cowan (National Cancer Institute, Bethesda, MD). PC-14 was cultured in RPMI-1640 medium (Gibco, Grand Island, NY), others were cultured in MEM (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 atmosphere.

Antibodies and anti-tumor compounds Trastuzumab was donated by Nippon Roche, Ltd. (Tokyo). Cisplatin (CDDP) was purchased from Nippon Kayaku Co. (Tokyo).

Analysis of HER-2/neu-expression For indirect immunofluorescence, 1×10^6 tumor cells were incubated with 10 mg/ml Trastuzumab diluted with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) (w/v) at 4°C for 1 h. After the first reaction, cells were washed with PBS including 1 m*M* ethylenediaminetetraacetic acid and incubated for 1 h at 4°C with 10 μg/ml fluorescein-conjugated anti-human IgG (Vector, Burlingame, CA) diluted with 1% BSA-PBS. After the second antibody reaction, cells were washed with PBS twice and resuspended in 2% formaldehyde PBS (v/v). Human IgG (Sigma, St. Louis, MO) was used as a control. The reactivity in 2×10^4 cells was measured using FACScan (Becton Dickinson, Mansfield, MA) and analyzed by Cell Quest (Becton Dickinson). Negative, low, and high expression levels of HER-2/neu were defined as ratios of flow-cytometric intensity less than 2, 2–10, and over 10, respectively.

Chromium (^{51}Cr) release assay for analysis of ADCC and complement-dependent cytolysis (CDC) One million tumor cells were labeled with 3.7 Mbq of ^{51}Cr for 1 h at 37°C, washed with medium and then seeded in 96-well plates (1×10^4 cells/well). For the ADCC assay, PBMCs were obtained from a healthy volunteer, separated using Lymphocyte Separation Medium (Organ Teknika-Capple, Durham, NC) and used as the effector cells. The labeled tumor cells were incubated for 6 h in the presence of Trastuzumab (final concentration: ~5 μg/ml) and 2×10^5 PBMCs. The effector/target (E/T) ratio was 20. For the CDC assay, human serum was obtained from a healthy volunteer as a source of complement. The labeled tumor cells were incubated for 4 h in the presence of 25% human serum (v/v). After incubation, ^{51}Cr released into the supernatant was counted with a γ-scintillation counter, ARC-300 (Aloka, Tokyo). The percentage of specific cytolysis was calculated from the experimental release (*A*) cpm, the release by natural killer (NK) or complement alone (without antibody) (*B*) cpm, total release (*C*) cpm, and spontaneous release (*D*) cpm as follows; $[(A-D)-(B-D)]/(C-D)\times100$.[28]

Growth-inhibitory effect of Trastuzumab and CDDP Fifteen hundred tumor cells/well were cultured in 96-well microtiter plates in the presence of 0, 0.25, 2.5 μg/ml Trastuzumab and 0–50 μM CDDP for 72 h. After exposure to Trastuzumab and/or CDDP, MTT reagent was added, followed by a 4-h incubation.[29] The absorbance at 630–562 nm was measured with an EL340, 96-well microtiter plate reader ("Bio-Tek" Instruments, Winooski, VT) and % cell growth inhibition was calculated from the absorbance value of untreated cells (*A*), the absorbance value of treated cells (*B*) and the absorbance value of medium alone (*C*) as follows; $(B-C)/(A-C)\times100$.

Cell cycle analysis One million cells were cultured in the medium including 0–2.5 μg/ml Trastuzumab for 24–72 h. Cells were collected, fixed in 70% ethanol and stocked at −20°C until analysis. Samples were treated with an ethanol gradient to remove ethanol. RNA was removed by treatment with 0.1 mg/ml RNase in PBS (w/v) for 1 h at 37°C. The cells were stained with 10 mg/ml propidium iodide in PBS (w/v) for 1 h at 4°C. A total of 2×10^4 cells were measured by flow cytometry and analyzed using Modefit (Becton Dickinson).

Viability of cultured PBMCs PBMCs were cultured in medium for 7 days, and dead PBMCs were detected microscopically using trypan-blue dye. PBMCs (3.75×10^3, 7.5×10^3, 15×10^3 or 30×10^3) were cultured in a 96-well microtiter plate for 3 days in the presence of 0–50 μM CDDP. The E/T ratio was 2.5, 5, 10, or 20 at 1500 tumor cells. After incubation, the absorbance value at 630–562 nm was measured using EL340.

MTT assay for Trastuzumab-mediated ADCC For analysis of the combination effect with ADCC and CDDP we analyzed the ADCC activity of Trastuzumab using MTT assay.[30–32] Fifteen hundred tumor cells per well were exposed to 0–5 μg/ml Trastuzumab and 0–50 μM CDDP in the presence of PBMCs at an E/T ratio of 20 for 72 h. The absorbance value at 630–562 nm was measured using an EL340 96-well microtiter plate reader. NK activity was calculated from the absorbance value of the cells cultured without Trastuzumab, CDDP, and PBMCs (*A*), the absorbance value of cells cultured with PBMCs (*B*), the absorbance value of medium alone (*C*), and the absorbance value of PBMCs (*D*) as follows; $\{1-[(B-C)-(D-C)]/[(A-C)-(D-C)]\}\times100$. ADCC was calculated from the values of *A*, *D*, the NK activity (*E*), and absorbance value of the cells treated with Trastuzumab, CDDP, and PBMCs (*F*); $[1-(F-D)/(A-D)]\times100-E$. The combination effect of Trastuzumab and CDDP in the presence of PBMCs was evaluated as a percentage from *A*, *D*, and the absorbance

value of cells treated with Trastuzumab and CDDP in the presence of PBMCs (G); $(G-D)/(A-D)\times100$. The correlation coefficient between MTT assay and ^{51}Cr release assay for ADCC was analyzed using Pearson's correlation coefficient (StatView).

RESULTS

Expression of HER-2/neu on human cancer cell lines The expression of HER-2/neu was evaluated in terms of the ratio of average intensity to the control intensity using human IgG in flow-cytometric analysis. PC-14 cells expressed low levels of reactive HER-2/neu on the cell surface. JRST and BT-474 cells expressed more than 10-fold higher levels, and SK-Br-3 and SKOV-3 cells expressed more than 20-fold higher levels than PC-14 (Fig. 1, A–E).

Viability of PBMCs exposed to CDDP The viability of PBMCs detected by trypan-blue staining did not change significantly until 4 days of culture (Fig. 2A). In the MTT assay, 7500 and 3750 PBMCs had undetectable absorbance (less than 0.01) (Fig. 2B). Totals of 30 000 and

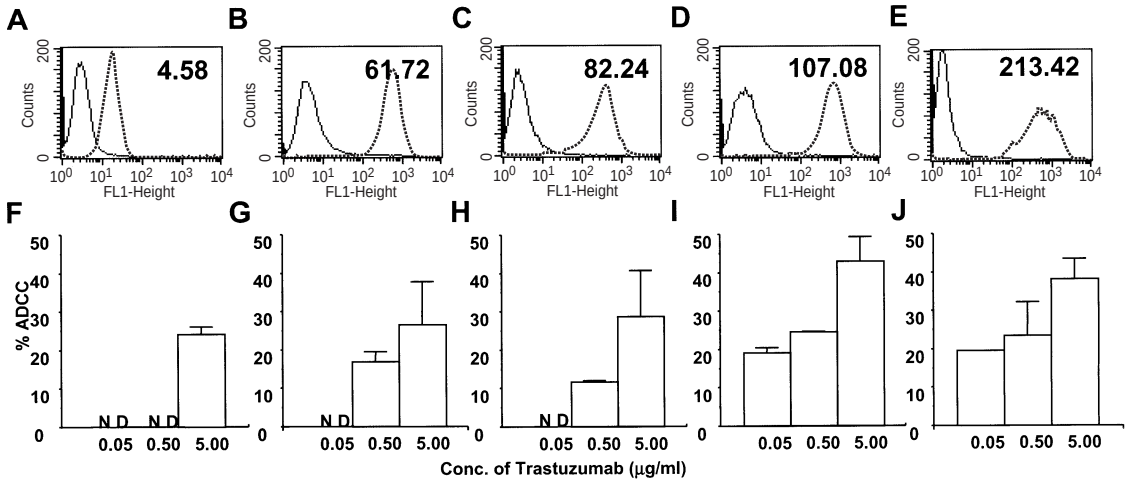

Fig. 1. HER2/neu-expression. A, PC-14; B, JRST; C, BT-474; D, SK-Br-3; E, SKOV-3. Indicated numbers are ratios of mean intensity as compared with using control human IgG. Gray lines, HER2/neu antibody; solid lines, control (human IgG). ADCC activity of Trastuzumab detected by ^{51}Cr release assay at an E/T ratio of 20 (n=3); F, PC-14; G, JRST; H, BT-474; I, SK-Br-3; J, SKOV-3. CDC activity was analyzed by ^{51}Cr release assay using 25% human serum. CDC activity was not detected in the concentration range of 0.05–5.00 μg/ml Trastuzumab (n=3). ND, not determined.

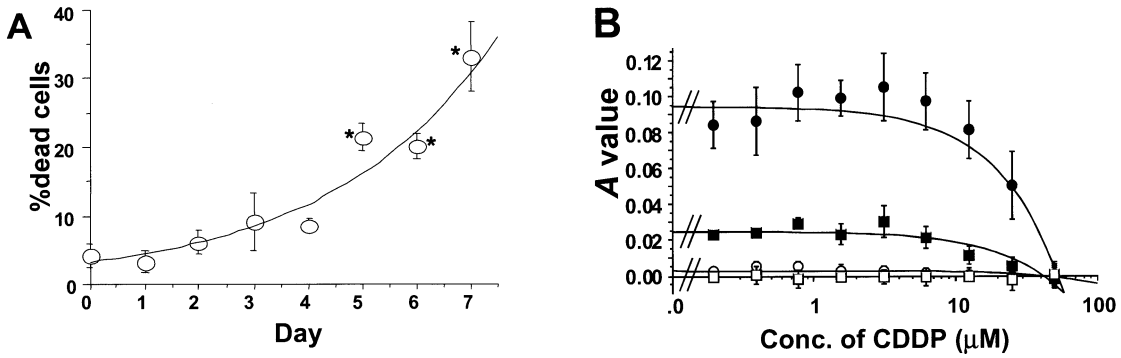

Fig. 2. The viability and A value of PBMCs. A, Percentage of dead PBMCs found by trypan-blue dye assay. Dead PBMCs increased time-dependently (R^2=0.924). Bars means SD (n=3). * Significant increase (P<0.01; Student's t test). B, A value of PBMCs cultured for 72 h in MTT assay. ● 30 000, ■ 15 000, ○ 7500, □ 3750 PBMCs, bars are SD (n=3). 7500 and 3750 PBMCs did not show measurable absorbance (<0.01), 30 000 and 15 000 PBMCs showed A 0.095 and 0.025 at 0.0 μM CDDP, respectively. The A value of PBMCs treated with less than 12.5 μM CDDP was not changed. The correlation between A and concentration of CDDP was R^2=0.848 for 30 000, and R^2=0.851 for 15 000 PBMCs.

15 000 PBMCs gave values of 0.095 and 0.025, respectively. Exposure to CDDP at less than 12.5 μM did not affect the absorbance value of PBMCs (Fig. 2B).

Trastuzumab-mediated ADCC and CDC activity IgG-type antibodies containing the Fc portion are usually clinically active through immunological cytotoxicity via ADCC or CDC. Therefore we examined ADCC activity against ^{51}Cr-labeled cancer cells as targets using Trastuzumab and human PBMCs containing NK cells and monocytes with Fc receptor. We also examined CDC activity against ^{51}Cr-labeled cancer cells using Trastuzumab and human serum containing human complement. Percent ADCC and %CDC were calculated as described in "Materials and Methods." A cytotoxic effect of Trastuzumab via ADCC by human PBMCs and Trastuzumab was detected against all HER-2/neu-overexpressing cells at 0.5 μg/ml of Trastuzumab. On the other hand, no CDC-mediated cytolytic effect of human complement and Trastuzumab against any of the cells was observed even at 5 μg/ml of Trastuzumab (Fig. 1).

Table I. Growth-inhibitory Effect of Trastuzumab

Cell line	Survival ratio±SD[a)]		
	Control	Conc. of Trastuzumab (μg/ml)	
		0.25	2.5
PC-14	1.00±0.03	0.99±0.07	0.98±0.02
JRST	1.00±0.04	0.92±0.07	0.85±0.09[b)]
BT-474	1.00±0.06	0.71±0.02[b)]	0.57±0.07[b)]
SK-Br-3	1.00±0.07	0.76±0.08[b)]	0.71±0.11[b)]
SKOV-3	1.00±0.05	1.01±0.17	1.01±0.14

a) Survival ratio±SD of cells determined as compared with the control (0 μg/ml) Trastuzumab using 72 h exposure to 0–2.5 μg/ml Trastuzumab as described in "Materials and Methods."
b) Cell growth was significantly inhibited ($P<0.05$, $n=3$, Student's t test).

Growth-inhibitory effect of Trastuzumab alone Trastuzumab (2.5 μg/ml for 72 h) showed more than 10% growth-inhibitory effect as compared with the control (without Trastuzumab) against JRST, BT-474, and SK-Br-3 cells, as determined by MTT assay. BT-474 cells were most sensitive to the Trastuzumab-induced growth-inhibitory effect. Survival ratios of these cells at 0.25 and 2.5 μg/ml Trastuzumab were 0.71 and 0.57, respectively. On the other hand, SKOV-3 cells were the least sensitive to the growth-inhibitory effect of Trastuzumab (Table I). Cell lines sensitive to Trastuzumab showed dose-dependent growth inhibition, but no correlation was detected between HER-2/neu expression and growth inhibition. The values of Pearson's correlation coefficient at 2.5 and 0.25 μg/ml Trastuzumab were 0.181 and 0.141, respectively.

Evaluation of ADCC by MTT assay We evaluated the ADCC activity using MTT assay in advance of analysis of the combination effect with ADCC and CDDP. When 15 000 PBMCs and 1500 target tumor cells were incubated in the medium containing 1.0 μg/ml Trastuzumab, the %ADCC values against PC-14, JRST, BT-474, SK-Br-3, and SKOV-3 determined by MTT assay were 9.9±0.2, 28.1±1.2, 33.4±1.5, 34.1±1.4, and 32.4±0.8, respectively. Percent ADCC values against PC-14, JRST, BT-474, SK-Br-3, and SKOV-3 determined by ^{51}Cr release assay were 7.1±0.7, 15.2±4.1, 19.7±6.2, 24.5±5.6, and 22.9±18.3, respectively (Fig. 3). The correlation coefficient between MTT assay and ^{51}Cr release assay for ADCC, at 1.0 μg/ml Trastuzumab, was 0.939 at an E/T ratio of 10.

Effects of Trastuzumab on CDDP-induced growth inhibition To clarify whether Trastuzumab increases the

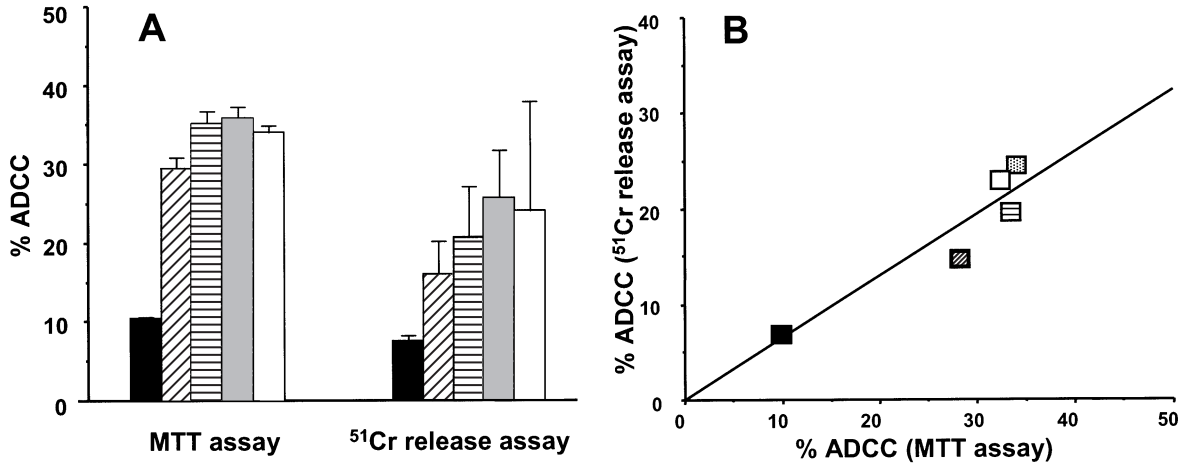

Fig. 3. A, Percent ADCC detected by MTT and ^{51}Cr release assay under the condition of E/T=10, 1.0 μg/ml Trastuzumab; ■ PC-14, ▨ JRST, ▤ BT-474, ▩ SK-Br-3, □ SKOV-3, bars are SD ($n=3$). B, Correlation between these assays.

growth-inhibitory effect of CDDP, the sensitivity of HER-2/neu-overexpressing cells to CDDP was analyzed by MTT assay with or without 0.25 or 2.5 μg/ml Trastuzumab. We evaluated relative resistance to CDDP as the change of sensitivity because the cancer cell lines had different sensitivity to CDDP alone (4.91–22.67 μM IC_{50}, 1.42–10.45 μM IC_{30}). Relative resistance was calculated as follows; relative resistance=CDDP IC_{50} or IC_{30} in combination with Trastuzumab/CDDP IC_{50} or IC_{30} alone (Table II). The IC_{30} of CDDP for BT-474 cells decreased markedly from 10.45 to 5.19 μM and less than 0.2 μM with 0.25 and 2.5 μg/ml Trastuzumab, respectively. Relative resistance to CDDP was 0.50 at 0.25 μg/ml and <0.02 at 2.5 μg/ml. That for SK-Br-3 cells was also decreased from 1.42 to 0.36 μM and 0.27 μM with 0.25 and 2.5 μg/ml Trastuzumab, with relative resistance values of 0.25 and 0.19, respectively. However, relative resistance values based on the IC_{50} values of CDDP for these cells were 0.90 and 0.22, and 0.38 and 0.33, respectively. These values were decreased as compared with those based on the IC_{30}. The direct growth-inhibitory effect of Trastuzumab seems to be masked by the strong cytotoxic effect of a high concentration of CDDP (Table II).

Combination effect of Trastuzumab and CDDP in the presence of PBMCs The existence of PBMCs adds the ADCC activity of Trastuzumab to the antagonistic effect on signal transduction of Trastuzumab and the cytotoxic effect of CDDP. Relative resistance was defined as follows; Relative resistance=CDDP IC_{50} or IC_{30} in combination with Trastuzumab/CDDP IC_{50} or IC_{30} alone. The presence of PBMCs (E/T=2.5) and Trastuzumab (1.0 μg/ml) required only 0.06–0.28-fold lower concentration of CDDP in terms of IC_{50} in HER-2/neu-overexpressing cells to give an effect equivalent to that of CDDP alone (Table III). Under the condition of E/T=5 and E/T=10, the IC_{50} relative resistance values in the HER-2/neu-overexpressing cells were less than 0.25 and less than 0.20, respectively. The IC_{30} relative resistance values at the E/T ratio of 10 were <0.2 in all cell lines (Table III). Exposure to CDDP at less than 12.5 μM did not affect the viability of PBMCs *in vitro* (Fig. 2B). These results indicate that the immunological cytotoxicity of Trastuzumab via PBMCs

Table II. Growth-inhibitory Effect of Trastuzumab in the Presence of CDDP

Cell line	Conc. of CDDP (μM) at IC_{50} [Relative resistance][a]			Conc. of CDDP (μM) at IC_{30} [Relative resistance][a]		
	CDDP alone	Conc. of Trastuzumab (μg/ml)		CDDP alone	Conc. of Trastuzumab (μg/ml)	
		0.25	2.50		0.25	2.50
PC-14	4.91 [1.00]	4.75 [0.97]	3.99 [0.81]	2.38 [1.00]	2.17 [0.91]	1.67 [0.70]
JRST	6.81 [1.00]	4.88 [0.72]	4.68 [0.69]	3.54 [1.00]	2.25 [0.64]	2.07 [0.58]
BT-474	22.67 [1.00]	20.29 [0.90]	5.08 [0.22][b]	10.45 [1.00]	5.19 [0.50][b]	<0.2 [ND][b]
SK-Br-3	5.20 [1.00]	1.96 [0.38][b]	1.69 [0.33][b]	1.42 [1.00]	0.36 [0.25][b]	0.27 [0.19][b]
SKOV-3	8.27 [1.00]	8.26 [1.00]	8.69 [1.05]	3.96 [1.00]	3.60 [0.91]	3.69 [0.93]

Concentration of CDDP and relative resistance were indicated at IC_{50} and IC_{30} in the presence of 0, 0.25, and 2.5 μg/ml Trastuzumab. Cells were exposed to 0–50 μM CDDP for 72 h. ND: not determined.
a) Relative resistance=CDDP IC_{50} or IC_{30} in combination with Trastuzumab/CDDP IC_{50} or IC_{30} alone.
b) CDDP IC_{50} or IC_{30} value was 0.5-fold lower than for CDDP alone.

Table III. Combination Effect of Trastuzumab and CDDP in the Presence of PBMCs

Cell line	Conc. of CDDP (μM) at IC_{50} [Relative resistance][a]				Conc. of CDDP (μM) at IC_{30} [Relative resistance][a]			
	CDDP alone	Effector/target ratio			CDDP alone	Effector/target ratio		
		E/T=2.5	E/T=5	E/T=10		E/T=2.5	E/T=5	E/T=10
PC-14	4.91 [1.00]	4.92 [1.00]	2.18 [0.44]	1.17 [0.24]	2.38 [1.00]	0.72 [0.30]	0.29 [0.12]	<0.2 [ND][b]
JRST	6.81 [1.00]	1.45 [0.21]	1.69 [0.25]	1.38 [0.20]	3.54 [1.00]	0.81 [0.23]	0.56 [0.16]	<0.2 [ND][b]
BT-474	22.67 [1.00]	5.99 [0.26]	5.11 [0.24]	1.01 [0.04]	10.45 [1.00]	1.84 [0.18]	<0.2 [ND][b]	<0.2 [ND][b]
SK-Br-3	5.20 [1.00]	0.31 [0.06]	<0.2 [ND][b]	<0.2 [ND][b]	1.42 [1.00]	<0.2 [ND][b]	<0.2 [ND][b]	<0.2 [ND][b]
SKOV-3	8.27 [1.00]	2.32 [0.28]	1.44 [0.17]	0.29 [0.04]	3.96 [1.00]	1.33 [0.34]	0.49 [ND][b]	<0.2 [ND][b]

Concentration of CDDP and relative resistance were indicated at IC_{50} and IC_{30}. Cells were exposed to 0–50 μM CDDP with Trastuzumab (1.0 μg/ml) and effectors for 72 h. ND: not determined.
a) Relative resistance=CDDP IC_{50} or IC_{30} in combination with Trastuzumab and PBMCs/CDDP IC_{50} or IC_{30} alone.
b) Less than 0.2 μM CDDP showed IC_{50} or IC_{30}.

produces a strong anti-tumor effect in combination with CDDP *in vitro*.

DISCUSSION

It is difficult to evaluate the anti-tumor activity of the antibody itself *in vitro* because the ADCC is mediated by effector cells. Trastuzumab showed high ADCC activity during a 6-h period in HER-2/neu-expressing cell lines (Fig. 1, F–J). When the direct growth-inhibitory effect of Trastuzumab against adhesive cells was examined by MTT assay after a 72-h incubation, Trastuzumab alone did not inhibit the growth by more than 30% and IC_{30} could not be determined, except for BT-474 (Table I). When Trastuzumab was combined with other cytotoxic agents including CDDP, it was difficult to evaluate the combined effect by using conventional *in vitro* techniques such as the isobologram and the combination index.[33, 34] It was also difficult to analyze the ADCC of Trastuzumab *in vivo* because the ADCC activity of a humanized antibody is less in mice.[28, 35] Therefore, *in vitro* analysis of the anti-tumor effect of the antibody in the presence of human effector cells for ADCC is useful as a pre-clinical test for a humanized antibody combined with chemotherapeutic agents. The *in vitro* MTT assay for ADCC was validated by the good correlation with the ^{51}Cr release assay results (Fig. 3).

We demonstrated that the cytotoxic effect of Trastuzumab is mediated by ADCC, which in turn is influenced by the status of HER-2/neu expression on the tumor cell surface. ADCC activity was correlated with HER-2/neu expression measured by flow cytometry (Fig. 1). In combination with CDDP, Trastuzumab-induced ADCC was not inhibited by a concentration of CDDP sufficient to inhibit tumor cell growth. This might be due to the lower sensitivity of PBMCs to CDDP. The results suggest that the efficacy of ADCC would not be diminished if Trastuzumab is combined with CDDP.

A supra-additive effect of combination therapy with CDDP regimens is often explained by the inhibition of repair of drug-induced DNA damage.[36–38] It was reported that modulation of anti-HER-2/neu antibody TAb-250 was associated with inhibition of DNA repair,[23, 24, 36] and it has been shown that Trastuzumab interferes with repair of CDDP-induced damage.[27] In addition, Baselga *et al.* reported a synergistic anti-tumor effect using Trastuzumab and chemotherapeutic agents *in vivo*.[21] Our results with BT-474 and JRST (Table II) support increased sensitivity of these cell lines to CDDP. However, an HER-2/neu-overexpressing cell line, SKOV-3, did not show a remarkable increase in sensitivity to CDDP (Table II) and the cell cycle distribution of SKOV-3 exposed to Trastuzumab was not greatly changed (data not shown).

CDDP at 12 μM showed no cytotoxicity towards human PBMCs. Therefore, the combination effect of Trastuzumab and CDDP was considered to have been due to the additive effects of ADCC induced by Trastuzumab and cytotoxicity induced by CDDP (Figs. 1, 3 and Table III). We considered that Trastuzumab-mediated ADCC is mainly involved in the synergistic mechanism, because the synergistic effect was observed only in the presence of PBMC.

The influence of CDDP on the ADCC activity remains unclear. However, the synergistic effect with Trastuzumab was observed only with CDDP when preliminarily experiments were done using several cytotoxic agents (data not shown). The optimum selection of anticancer agents should be further studied.

In the clinical situation, important determinants of the therapeutic benefit of combination therapy with Trastuzumab and chemotherapeutic agents may include not only the expression of HER-2/neu and cell-cycle regulatory molecules, but also the patient's ADCC effector activity.

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