

Trastuzumab-Mediated Antibody-Dependent Cellular Cytotoxicity against Esophageal Squamous Cell Carcinoma

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Abstract Purpose: In the present study, we investigated the degree of protein expression and gene amplification of HER-2 in esophageal squamous cell carcinoma (SCC) cell lines and freshly isolated tumors, and trastuzumab-mediated biological activity, in particular antibody-dependent cellular cytotoxicity (ADCC) against HER-2-expressing esophageal SCC cell lines.

Experimental Design: Ten different SCC cell lines with various levels of HER-2 status evaluated by flow cytometry, immunocytochemistry (HerceptTest), and fluorescence *in situ* hybridization were evaluated for ADCC, growth inhibitory, or apoptosis-inducing activities mediated by trastuzumab.

Results: Trastuzumab induced ADCC against HER-2-expressing esophageal SCC and the activities reflected the degree of HER-2 expression analyzed by flow cytometric analysis, but not by HerceptTest nor fluorescence *in situ* hybridization analysis. Furthermore, trastuzumab-mediated ADCC against transforming growth factor- β -producing SCC was enhanced by the treatment with SB-431542, which is a selective inhibitor of the phosphorylation induced by transforming growth factor- β . There were very marginal effects of anti-proliferative or apoptosis-inducing activities mediated by trastuzumab for HER-2-expressing esophageal SCC.

Conclusion: HER-2-expressing esophageal SCC cells could be killed by trastuzumab-mediated ADCC and the activity reflected the degree of HER-2 expression detected by flow cytometry.

Most patients with esophageal cancer in Japan have squamous cell carcinoma (SCC), whereas most of those in Western countries have adenocarcinoma. Despite aggressive treatment modalities such as surgical resection with extensive lymphadenectomy (1, 2) and surgery combined with chemotherapy (3) and/or radiotherapy (4, 5), the control of esophageal SCC at the advanced stage remains difficult. Therefore, for esophageal SCC patients, immunoadjuvant therapy such as the utilization of anti-tumor specific T cells or antibody against tumor antigens is very much needed.

The HER-2 proto-oncogene encodes a 185-kDa transmembrane glycoprotein with tyrosine-specific kinase activity (6). According to numerous examples from experimental models and clinical trials, HER-2 can be immunogenic and generate antibodies and cytotoxic T cell- and helper T cell-specific responses in individuals with HER-2-overexpressing tumors (7, 8). Moreover, the fully humanized monoclonal antibody

(mAb) trastuzumab (Herceptin), which specifically targets the extracellular domain of the HER-2 protein, exhibits potent growth inhibitory activity against HER-2-overexpressing tumors (9). In fact, trastuzumab was clinically shown to have survival benefit in patients with HER-2-overexpressing breast cancer with metastasis (10, 11). Based on the above reports, anti-HER-2 immune targeting is as an attractive approach to treat esophageal SCC.

Many mechanisms are linked to the therapeutic effect of trastuzumab, including the blockade of signaling pathways (9), inhibition of tumor cell growth such as the down-regulation of the HER-2 receptor (9), activation of apoptotic signals of tumor cells (12), and enhancement of the immune system such as antibody-dependent cellular cytotoxicity (ADCC; refs. 9, 13). In addition, we recently reported that trastuzumab enhances class I-restricted antigen presentation recognized by HER-2-specific CTLs (14). These observations prompted us to use trastuzumab against esophageal SCC. However, there is little information about the possibility of applying trastuzumab to esophageal SCC patients.

In the present study, we investigated (a) the degree of protein expression and gene amplification of HER-2 in esophageal SCC cell lines and freshly isolated tumors evaluated by flow cytometry, immunocytochemistry, and fluorescence *in situ* hybridization (FISH); (b) the biological activity of trastuzumab against HER-2-expressing esophageal SCC cell lines; and (c) the synergic effect of trastuzumab and SB-431542, which is a selective inhibitor of the phosphorylation induced by transforming growth factor- β (TGF- β ; ref. 15), in an SCC cell line producing TGF- β .

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Materials and Methods

Chemicals and antibodies. Anti-HER-2 monoclonal antibody trastuzumab (Herceptin) and anti-CD20 mAb Rituxan, which is an isotype-matched control mAb for trastuzumab, were purchased from Roche (Basel, Switzerland). Phycoerythrin-labeled anti-HER-2 mAb (Becton Dickinson, San Jose, CA) and phycoerythrin-labeled mouse immunoglobulin G1 mAb (Beckman-Coulter, Miami, FL) as a negative control were used for immunostaining by flow cytometric analysis. Human recombinant TGF- β 2 was purchased from AUSTRAL Biologicals (San Ramon, CA). Anti-TGF- β 2 neutralizing antibody was purchased from R&D Systems (Minneapolis, MN). SB-431542, which is a selective inhibitor of TGF- β /activin receptor-like kinase (ALK) type 1 activity, was purchased from TOCRIS (Ellisville, MO), and dissolved at concentration of 10 mmol/L in DMSO and stored at -20°C .

Cell lines. Esophageal SCC cell lines TE1, TE2, TE3, TE4, and TE5 were a gift from Dr. Nishihara (Institute of Development, Aging and Cancer, University of Tohoku, Sendai, Japan). Esophageal SCC cell lines KYSE30, KYSE50, KYSE70, KYSE110, and TT were purchased from Health Science Research Resources Bank (Osaka, Japan). Ovarian cancer cell line SKOV3 was obtained from American Type Culture Collection (Rockville, MD). All cell lines were kept in RPMI 1640 with 5% FCS, 50 units/mL penicillin, and 2 mmol/L L-glutamine.

Cell preparation. Primary solid tumor from esophageal SCC patients was isolated during surgery and was homogenized by mechanical mincing. Then, cell mixtures were passed through a cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) and suspended as a single-cell suspension. A single-cell suspension derived from solid tumors and malignant pleural effusion was purified by centrifugation with Ficoll-Paque (Pharmacia, Uppsala, Sweden) and subjected to flow cytometry or immunocytochemistry.

Peripheral blood mononuclear cells (PBMC) were separated from peripheral blood obtained from healthy donors and esophageal SCC patients by centrifugation with Ficoll-Paque (Pharmacia).

Immunocytochemistry analysis. Immunocytochemical staining was done using the HercepTest (DakoCytomation, Glostrup, Denmark) according to the recommendations of the manufacturer. After each cell line was centrifuged into a cell pellet, pelleted cells were formalin fixed and paraffin-embedded. Archival, formalin-fixed, paraffin-embedded material was used to obtain 4- μm -thick sections. Briefly, deparaffinized and rehydrated tissue sections were incubated with the Epitope Retrieval Solution in hot water bath for 40 minutes at 95°C to 99°C . Then, the sections were cooled at room temperature for 20 minutes, and the sections were washed with TRIS-buffer for 5 minutes, and endogenous peroxidase was blocked with 3% hydrogen peroxide for 5 minutes. The primary antibody was rabbit polyclonal antibody to human HER-2, which recognizes an intracytoplasmic part of HER-2, and the primary negative control antibody was an immunoglobulin fraction of normal rabbit serum at an equivalent protein concentration as the antibody to HER-2. The sections were washed with TRIS-buffer for 5 minutes and incubated with the primary antibody or the primary negative control antibody at room temperature for 30 minutes. After rewashing with TRIS-buffer for 5 minutes twice, the primary antibody was detected using visualization reagents, which were dextran polymer conjugated with horseradish peroxidase and affinity-isolated goat anti-rabbit immunoglobulins, for 30 minutes of incubation at room temperature. Subsequently, following the rewashing with TRIS-buffer for 5 minutes twice, diaminobenzidine was added as a visualization reagent for 10 minutes and the section was counterstained with Hematoxylin. The control slides provided with the HercepTest kit, which contained three human breast cancer cell lines with staining intensity scores of 0, 1+, and 3+, were used in the present study.

Immunocytochemistry analysis was done by two observers (K.M. and K.K.) according to the staining intensity scores provided by the HercepTest kit. Each section was classified into four categories (0, 1+, 2+, and 3+), in which tumor cells with complete absence of staining were scored as 0; those with incomplete membranous staining were classified as 1+; those with moderate, complete membranous staining were classified as 2+; and those with strong, complete membranous staining were classified as 3+.

Fluorescence in situ hybridization analysis. FISH analysis was done using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL). The HER-2/neu-SpectrumOrange probe is specific for the HER-2 gene locus (17q11.2-q12). The CEP 17 (chromosome enumeration probe)/SpectrumGreen probe is specific for the α -satellite DNA sequence (centromere region of chromosome 17). To determine the copy number for chromosome 17, we used CEP 17 as the control. FISH procedures were conducted according to the guidelines of the manufacturer, except the removal of the protein from the sections where we used our own protocol as previously described (16). The serial sections in each cell lines were analyzed by immunocytochemistry and FISH analysis. Briefly, sections were deparaffinized, dehydrated, and incubated in 20% sodium bisulfate/ $2\times$ SSC at 43°C for 20 minutes. Sections were washed with SCC and treated with proteinase K (Boehringer-Mannheim, Mannheim, Germany) at 37°C for 25 minutes. Subsequently, denaturation, hybridization, and post-hybridization washing were done according to the guidelines of the manufacturer, and after hybridization and post-hybridization washing, the sections were counterstained with 4',6-diamidino-2'-phenylindole dihydrochloride. FISH analysis was done using a fluorescence microscope (Olympus, Tokyo, Japan) equipped with Triple Bandpass Filter sets (Vysis). Signals were counted for at least 40 cancer nuclei per tumor. In accordance with our previous studies with FISH, a cell was considered to show amplification when a definite cluster or more than 10 orange signals of HER-2 were observed (16).

A positive control, a breast tumor with previously identified HER-2 amplification and overexpression, was used as a positive control for HER-2 FISH.

Antibody-dependent cell-mediated cytotoxicity assay. After the target cells were labeled with 50 μCi of ^{51}Cr for 60 minutes, target cells (5×10^3 /well) and effector cells at various effector/target ratios were co-incubated in 200 μL of X-VIVO medium in a 96-well U-bottomed plate in triplicate for 8 hours at 37°C with trastuzumab (2 μg /well; Roche) or a control antibody, Rituxan (2 μg /well; Roche). After 8 hours of incubation, the radioactivity of the supernatant (100 μL) was measured with a γ counter. The percentage of specific lysis was calculated according to the following formula: % specific lysis = $100 \times (\text{experimental cpm} - \text{spontaneous cpm}) / (\text{maximum cpm} - \text{spontaneous cpm})$. Controls included the incubation of target cells alone with trastuzumab.

Immunoblotting for phosphorylated Smad2. PBMCs (7×10^6) from healthy donors were pre-treated with SB-431542 or DMSO (vehicle) at indicated concentrations for 30 minutes in X-VIVO (1 mL) medium in a six-well plate and then pre-treated PBMCs were cultured in the presence of TGF- β 2 (5 ng/mL) for 60 minutes. After incubation, each sample of PBMCs was washed twice with sterile PBS and then suspended in Laemmli sample buffer [62.5 mmol/L Tris-HCl (pH 6.8), 25% glycerol, 2% SDS, 0.01% bromophenol blue; Bio-Rad Laboratories, Inc., Hercules, CA] supplemented with 5% β -mercaptoethanol. Extracts were cleared by centrifugation. Whole cell extracts (10 μg) were fractionated on 10% SDS-polyacrylamide gels and transferred onto Immobilon-P membrane (Millipore, Billerica, MA). Immunoblotting was done using antibodies against Smad2/3 (Upstate Biotechnology, Lake Placid, NY) and phosphorylated Smad2 (Upstate Biotechnology). Immunoreactive bands were detected by enhanced chemiluminescence (ECL-plus, Amersham Life Science, Piscataway, NJ) using horseradish peroxidase-linked anti-rabbit or anti-mouse immunoglobulin G (Amersham Life Science).

Treatment with anti-transforming growth factor- β neutralizing monoclonal antibody or SB-431542 of trastuzumab-mediated antibody-dependent cellular cytotoxicity. In experiment with anti-TGF- β 2 neutralizing mAb, after the target cells were labeled with 50 μ Ci of 51 Cr for 60 minutes, target cells (5×10^3 /well) and PBMCs (1×10^5 /well) were co-incubated in 200 μ L of X-VIVO medium in a 96-well U-bottomed plate in triplicate for 8 hours at 37°C with or without anti-TGF- β 2 mAb (5 ng/mL; R&D Systems) in the presence of trastuzumab (2 μ g/well; Roche).

In experiment with SB-431542, after PBMCs from healthy donors were pretreated with SB-431542 or DMSO (vehicle) at indicated concentrations for 30 minutes in X-VIVO medium, 51 Cr-radiolabeled target cells (5×10^3 /well) and the pre-treated PBMCs (1×10^5 /well) were co-incubated in X-VIVO (200 μ L) in a 96-well U-bottomed plate for 8 hours at 37°C with trastuzumab (2 μ g/well) in the presence of SB-431542 or DMSO.

Flow cytometric analysis. To evaluate HER-2 expression, a phycoerythrin-labeled anti-HER-2/neu mAb (Becton Dickinson) and phycoerythrin-labeled mouse immunoglobulin G1 mAb (Beckman-Coulter, Miami, FL) as a negative control were used for immunostaining by flow cytometric analysis.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell proliferation assay. Each cell line (2,500/well) was cultured in 200 μ L of X-VIVO with trastuzumab (2 μ g/well) in 96-well flat-bottomed plates (Corning, Corning, NY) in triplicate. After 96 hours of incubation in a humidified incubator at 37°C, 5% CO₂, 50 μ L of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (2 mg/mL, Sigma, St. Louis, MO) were added to each well and incubation was done for 4 hours. Then, the supernatant was discarded and the crystal products were eluted with DMSO (50 μ L/well, Sigma). The colorimetric evaluation was tested using a spectrophotometer at 570 nm. The inhibition of proliferation was shown as percent cell growth inhibition induced by trastuzumab in comparison with that induced by control mAb.

Apoptosis. Each cell line (2×10^5 cells) was cultured in 2 mL of X-VIVO with or without trastuzumab (10 μ g/mL) at 37°C for 24 hours in a six-well plate. After incubation, apoptosis in each cell line was measured by staining with FITC-conjugated Annexin-V and propidium iodide using a MEBCYTO Apoptosis kit (MBL, Nagoya, Japan) following the recommendations of the manufacturer.

Quantitative determination of interleukin-10 and transforming growth factor- β . Each cell line (2×10^5 cells) was cultured in 2 mL of X-VIVO at 37°C for 24 hours in a six-well plate. After incubation, the culture supernatants in each cell line were collected and tested for cytokine

using a human interleukin 10 (IL-10) Enzyme Immunometric Assay Kit (Assay Designs, Ann Arbor, MI), Human TGF- β 1 Immunoassay Kit (R&D Systems), and Human TGF- β 2 Immunoassay kit (R&D Systems) following the recommendations of the manufacturers.

Statistics. To evaluate statistical differences between groups, a non-paired Student's *t* test was done. Statistically significant difference was considered at $P < 0.05$.

Results

The status of HER-2 expression and gene amplification of esophageal squamous cell carcinoma. HER-2 expression in esophageal SCC cell lines ($n = 10$) was assessed by flow cytometric analysis, immunocytochemistry, and FISH. By flow cytometric analysis, there were variable levels of HER-2 expression (Table 1; Fig. 1). Furthermore, by immunocytochemistry analysis using the HercepTest, only the TE4 cell line was strong positive (3+) with the HercepTest, whereas the remaining nine cell lines showed no staining (Table 1). The HER-2 level of TE4 analyzed by flow cytometry was almost equal to that of SKOV3, which is a well-known HER-2-overexpressing ovarian cancer cell line (Fig. 1). In FISH analysis, HER-2 gene amplification was only found in TE4 (Table 1), and polysomy, in which cancer nuclei showed more than three HER-2 signals accompanied with the same number of centromere 17 signals, was found in cell lines (Table 1). These results indicated that there was a difference in the detection of HER-2 status among flow cytometry, immunocytochemistry analysis, and FISH.

Next, we examined HER-2 expression in freshly isolated tumors (primary tumor and malignant pleural effusion) derived from two different esophageal SCC patients. Both samples revealed the moderate levels of HER-2 expression in comparison with esophageal SCC cell lines analyzed by flow cytometry (Fig. 1). Furthermore, we did immunocytochemistry (HercepTest) analysis for the same samples. In spite of the HER-2 expression in flow cytometric analysis, both samples showed no staining in HercepTest (data not shown). These results indicated that there was a difference in the detection of HER-2 status between flow cytometry and immunocytochemistry analysis, and flow cytometric analysis might be more sensitive in the detection of HER-2 status.

Table 1. Anti-proliferative activity and apoptosis-inducing activity of trastuzumab

Cell lines	HER-2 status			% Inhibition of growth (MTT)	Apoptosis (%)	
	FACS (MFI)	HercepTest	FISH		Medium	Medium + trastuzumab
TE4	666	3+	Cluster	16.3 \pm 3.9	3.7	4.9
KYSE30	123	—	Polysomy	0	5.9	6.6
KYSE50	114	—	Polysomy	5.1 \pm 2.0	11.7	10.7
TE5	113	—	Polysomy	12.0 \pm 4.8	3.4	2.3
TE1	107	—	Polysomy	10.0 \pm 2.2	3.9	3.7
TE2	71	—	Polysomy	N.D.*	N.D.	N.D.
TT	56	—	Polysomy	5.3 \pm 2.1	9.3	9.9
TE3	47	—	Polysomy	N.D.	N.D.	N.D.
KYSE70	34	—	No amplification	N.D.	N.D.	N.D.
KYSE110	25	—	Polysomy	0	4.6	4.6

Abbreviations: MFI, mean fluorescence intensity; FACS, fluorescence-activated cell sorting; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.
*N.D., not determined.

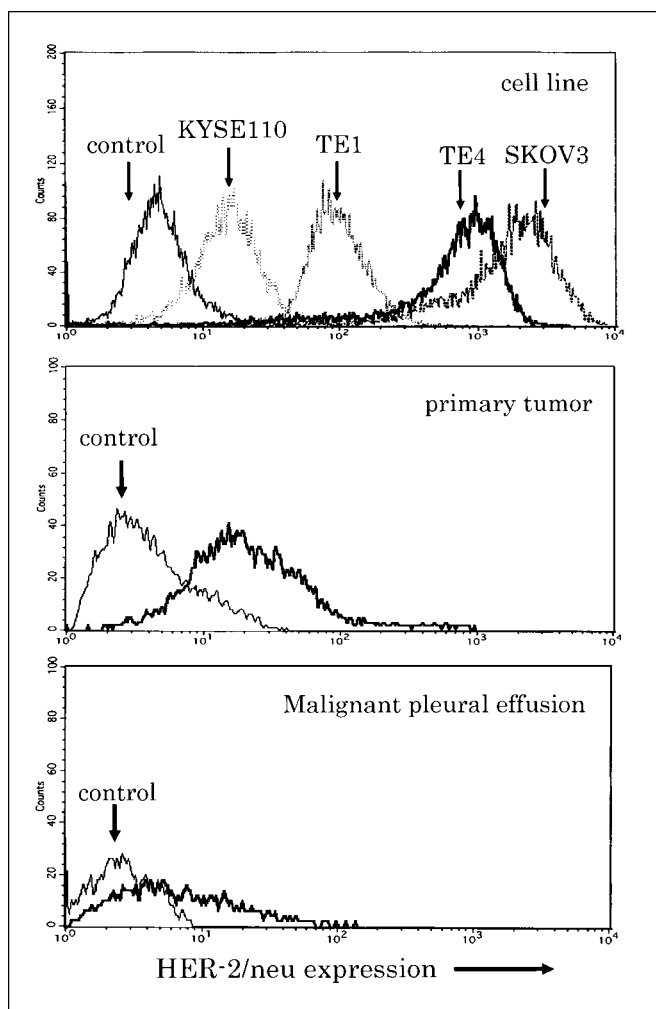


Fig. 1. The degree of HER-2 expression on esophageal SCC cell lines and freshly isolated SCC tumors. HER-2 expression was evaluated by flow cytometric analysis on esophageal SCC cell lines and freshly isolated SCC tumors derived from two different patients. Esophageal SCC cell lines KYSE110, TE1, and TE4 and ovarian cancer cell line SKOV3, which is a standard cell line with strong HER-2 expression, were used.

The biological activity of trastuzumab against HER-2-expressing esophageal squamous cell carcinoma cell lines. To evaluate the anti-proliferative activity and apoptosis-inducing activity of trastuzumab, we did 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, and Annexin V and propidium iodide staining for esophageal SCC cell lines. Inhibition of tumor cell growth induced by trastuzumab was found in TE4, TE1, and TE5 with very marginal but significant levels (Table 1). In addition, significant levels of apoptosis induced by trastuzumab were not found in all tested cell lines (Table 1).

Trastuzumab mediates antibody-dependent cellular cytotoxicity against HER-2-expressing esophageal squamous cell carcinoma. To investigate whether trastuzumab induces ADCC against esophageal SCC, we did an ADCC assay of PBMCs from healthy donors and esophageal SCC patients against several different esophageal SCC cell lines with various levels of HER-2 (Table 1; Fig. 1). Summarized data from healthy donors ($n = 7$) showed that trastuzumab could induce ADCC activities against esophageal SCC cells and the activities

reflected the degree of HER-2 expression by flow cytometric analysis on the esophageal SCC cell lines (Fig. 2). However, even when the HER-2 levels were almost the same among TE5, TE1, KYSE30, and KYSE50, there were significant differences in the levels of trastuzumab-induced ADCC (Fig. 2). There was no direct cytotoxicity for the targets using trastuzumab alone (Fig. 2).

Furthermore, we did an ADCC assay of PBMCs from esophageal SCC patients ($n = 5$) against KYSE110, TE1, and TE4 (Fig. 3). Trastuzumab could also induce ADCC activities against esophageal SCC cells and the activities reflected the degree of HER-2 expression (Fig. 3), although the levels of ADCC in esophageal SCC patients were weaker in comparison with those of healthy donors (Figs. 2 and 3).

Modification of trastuzumab-mediated antibody-dependent cellular cytotoxicity by SB-431542 in transforming growth factor- β -producing esophageal squamous cell carcinoma. As indicated in Fig. 2, the levels of trastuzumab-induced ADCCs were different among TE5, TE1, KYSY30, and KYSE50, although the HER-2 levels in these four cell lines were almost equal. Therefore, we next investigated the quantity of immunosuppressive cytokines, IL-10 and TGF- β , in the culture supernatants in KYSE30, KYSE50, TE5, and TE1, as it was well known that both IL-10 and TGF- β suppress natural killer (NK) activity (17–20). As a result, there were detectable TGF- β 2 in the supernatants, in which TE1 produced much higher TGF- β 2 (Fig. 4A), whereas no cell lines produced detectable levels of TGF- β 1 or IL-10 (data not shown).

Next, we investigated whether anti-TGF- β 2 neutralizing mAb enhanced trastuzumab-mediated ADCC against TGF- β 2-producing TE1. Representative ADCC assays from three independent experiments from different healthy donors ($n = 3$) were shown (Fig. 4B). We found that the treatment with anti-TGF- β 2 mAb resulted in the enhancement of trastuzumab-mediated ADCC against TE1, whereas there were no effects for TE5 and KYSE30 (Fig. 4B). These results indicated that TGF- β 2 produced by TE1 might inhibit the activity of trastuzumab-mediated ADCC against TE1.

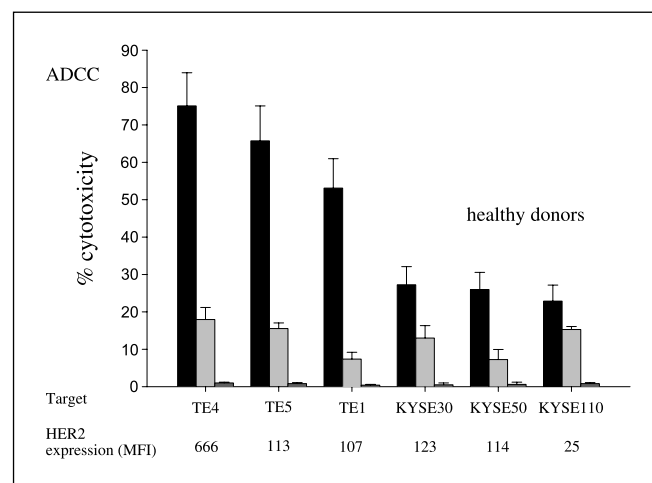


Fig. 2. Trastuzumab-mediated ADCC against HER-2-expressing esophageal SCC in healthy donors. Trastuzumab-mediated ADCC assays of PBMCs from healthy donors ($n = 7$) against esophageal SCC cell lines. The HER-2 expression on esophageal SCC cell lines was evaluated by flow cytometric analysis. *MFI*, mean fluorescence intensity; ■, Target + PBMC; ▒, Target + PBMC + Trastuzumab; □, Target + Trastuzumab (PBMC/target = 20:1).

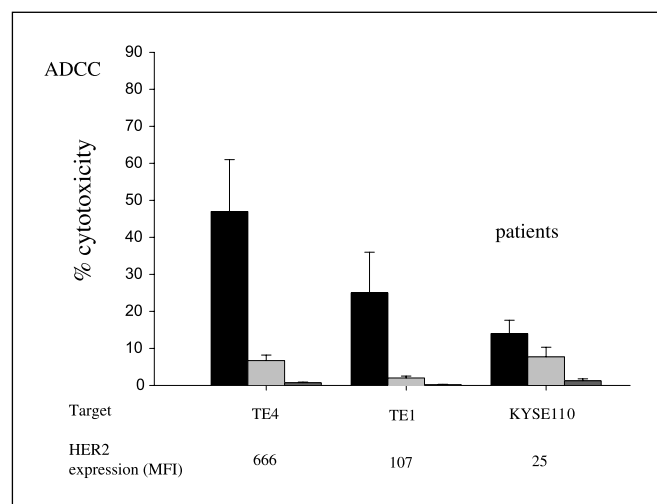


Fig. 3. Trastuzumab-mediated ADCC against HER-2-expressing esophageal SCC in esophageal SCC patients. Trastuzumab-mediated ADCC assays of PBMCs from esophageal SCC patients ($n = 5$) against esophageal SCC cell lines. The HER-2 expression on esophageal SCC cell lines was evaluated by flow cytometric analysis. ■, Target + PBMC + Trastuzumab; □, Target + PBMC + Control mAb; ▨, Target + Trastuzumab (PBMC/target = 20:1).

Furthermore, we investigated whether SB-431542 blocks TGF- β -induced phosphorylation in PBMCs and whether inhibition of TGF- β signaling results in enhancement of trastuzumab-mediated ADCC against esophageal SCC. It has been shown that SB-431542 is a specific inhibitor of ALK-4, ALK-5, and ALK-7, the type I receptors for TGF- β , and inhibits the phosphorylation of Smad2/3 induced by TGF- β (15). A representative immunoblotting assay for phosphorylated Smad2 is shown in Fig. 5A, indicating that SB-431542 blocks TGF- β -induced phosphorylation of Smad2 in PBMCs in a dose-dependent manner. Representative ADCC assays from six independent experiments from different healthy donors ($n = 6$) were shown in Fig. 5B. We found that SB-431542 significantly enhanced trastuzumab-mediated ADCC for TGF- β -producing TE1 in comparison with that in the DMSO control (Fig. 5B). However, SB-431542 did not enhance trastuzumab-mediated ADCC against TE5 and KYSE30 (Fig. 5B). Furthermore, these observations were confirmed in another five different healthy donors and the average synergic effect of SB-431542 was $12.2 \pm 5.5\%$ increase of trastuzumab-mediated ADCC, in which the maximal response was observed at around 0.1 $\mu\text{mol/L}$ of SB-431542 (Fig. 5B).

Discussion

The present study contains several important findings relevant to the action of trastuzumab against HER-2-expressing esophageal SCC. First, trastuzumab was able to induce ADCC against HER-2-expressing esophageal SCC and the activities reflected the degree of HER-2 expression analyzed by flow cytometric analysis, but not by HercepTest or FISH analysis. Second, trastuzumab-mediated ADCC against TGF- β -producing SCC was enhanced by treatment with SB-431542, which is a selective inhibitor of the phosphorylation induced by TGF- β .

There are many mechanisms linked to the therapeutic effect of trastuzumab, including the blockade of signaling pathways (9), inhibition of tumor cell growth (9), activation of apoptotic

signals of tumor cells (12), and enhancement of ADCC (9, 13). In the present study, we clearly showed that HER-2-expressing esophageal SCC cells were killed by trastuzumab-mediated ADCC, as well as breast cancer cells and gastric cancer cells with HER-2 overexpression (9, 13). However, the anti-proliferative activity of trastuzumab for HER-2-expressing esophageal SCC was marginal and there was no effect on the activation of apoptosis.

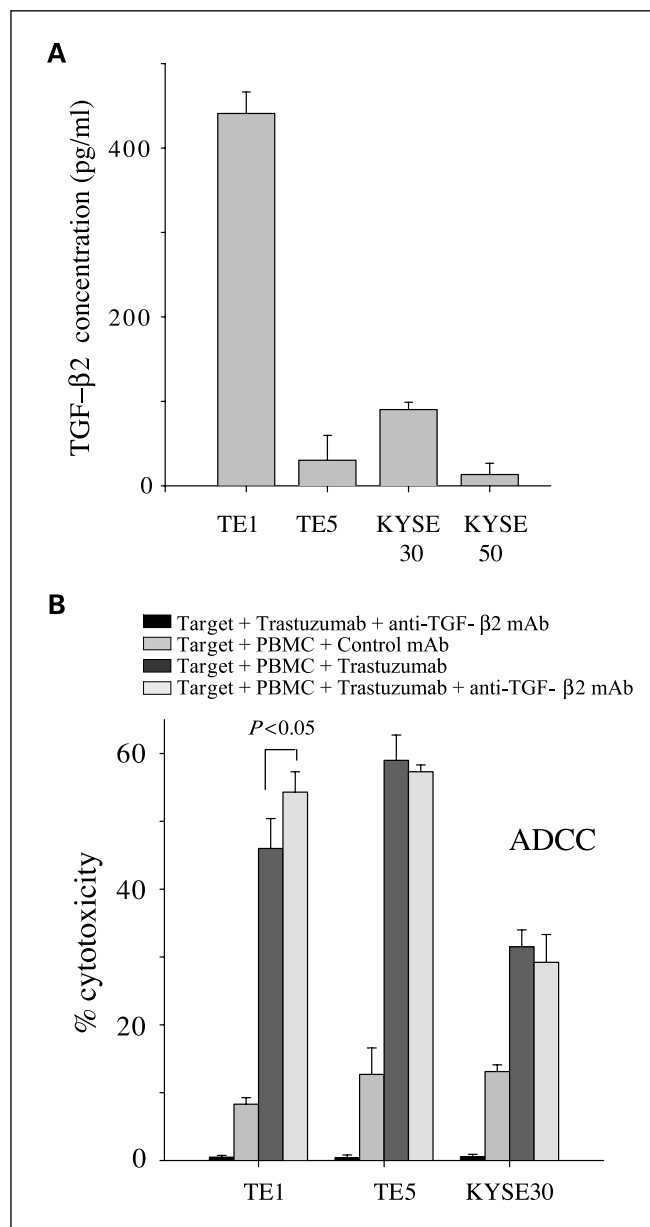
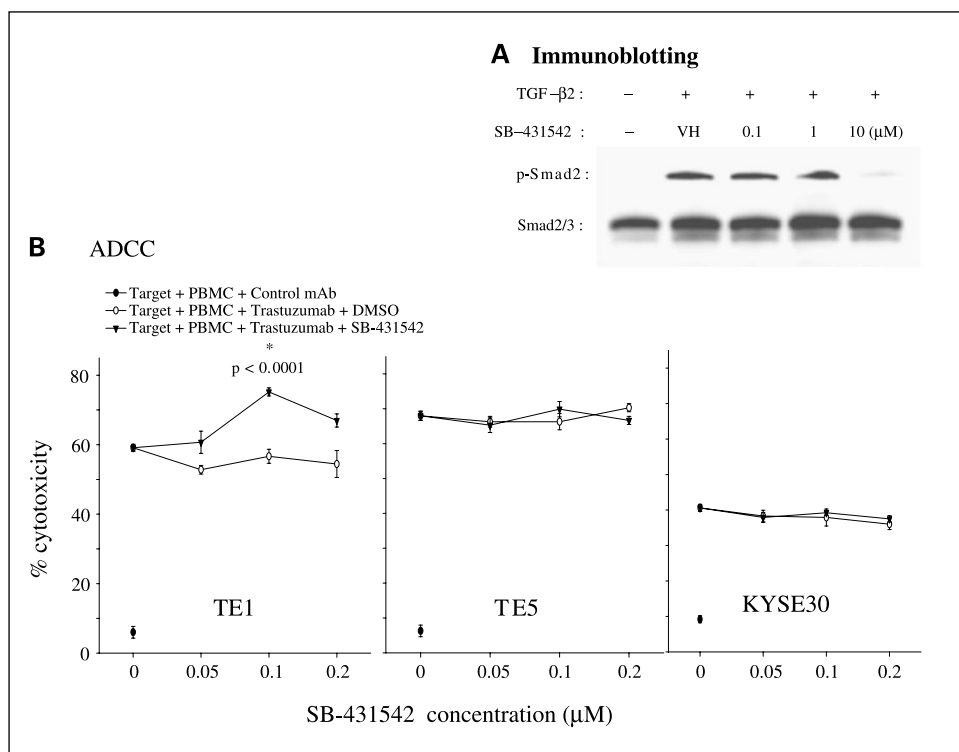


Fig. 4. Quantification of TGF- β 2 in the culture supernatants in esophageal SCC (A) and treatment with anti-TGF- β 2 neutralizing mAb in trastuzumab-mediated ADCC (B). A, each cell line (2×10^5 cells) was cultured in 2 mL of X-VIVO at 37°C for 24 hours in a six-well plate. After incubation, the culture supernatants were measured with a Human TGF- β 2 Immunoassay Kit. B, representative ADCC assays from three independent experiments from different healthy donors ($n = 3$). After the target cells were labeled with 50 μCi of ^{51}Cr for 60 minutes, target cells (5×10^3 /well) and PBMCs (1×10^6 /well) were co-incubated in 200 μL of X-VIVO medium in a 96-well U-bottomed plate in triplicate for 8 hours at 37°C with or without anti-TGF- β 2 mAb (5 ng/mL) in the presence of trastuzumab (10 $\mu\text{g/mL}$). Statistical analysis was done with a Student's t test.

Fig. 5. SB-431542 significantly blocks TGF- β 2-induced phosphorylation of Smad2 in PBMCs (A) and enhances trastuzumab-mediated ADCC for a TGF- β -producing esophageal SCC TE1 cell line (B). A, representative immunoblotting assay for phosphorylation of Smad2 in PBMCs. PBMCs (7×10^6) were pre-treated with SB-431542 or DMSO (vehicle, VH) for 30 minutes in X-VIVO medium and then pre-treated PBMCs were cultured in the presence of TGF- β 2 (5 ng/mL) for 60 minutes. After incubation, whole cell extracts (10 μ g) were fractionated on 10% SDS-polyacrylamide gels and immunoblotting was done using antibodies against Smad2/3 and phosphorylated Smad2 (p-Smad2). B, representative ADCC assays from six independent experiments from different healthy donors ($n = 6$). The TE1 cell line produced much higher TGF- β 2, whereas the TE5 and KYSE30 cell lines did not produce significant amounts of TGF- β 2 (Fig. 4A). After PBMCs from healthy donors were pre-treated with SB-431542 or DMSO for 30 minutes, 51 Cr-radiolabeled target cells (5×10^3 /well) and the pre-treated PBMCs (1×10^5 /well) were co-incubated for 8 hours with trastuzumab (10 μ g/mL) in the presence of SB-431542 or DMSO. Statistical analysis was done with a Student's t test.



In esophageal SCC, TE4 was shown to have high HER-2 expression analyzed by both flow cytometric analysis and HercepTest, and to possess *HER-2* gene amplification detected by FISH in the present study. Thus, it is reasonable to assume that TE4 was efficiently killed by trastuzumab-mediated ADCC. However, another six cell lines which had moderate levels of HER-2 expression analyzed by flow cytometry, but negative staining with the HercepTest, were also killed by trastuzumab-mediated ADCC, and the activity reflected the degree of HER-2 expression by flow cytometric analysis. In a previous study, Bunn et al. (21) investigated the relationship among flow cytometric analysis, HercepTest, and FISH (PathVysion test) in human lung cancer cell lines and human breast cancer cell lines. They reported that there was a correlation between these three assays in the detection of HER-2 status. However, our present study indicated that the HercepTest may not be enough, or may underestimate, in the sensitivity of screening for trastuzumab-mediated ADCC. Furthermore, FISH analysis detected *HER-2* gene amplification or polysomy in the tested SCC cell lines. However, there was a discrepancy between the HER-2 status analyzed by FISH and the levels of trastuzumab-mediated ADCC. Also, freshly isolated tumor samples in the present study revealed moderate levels of HER-2 expression detected by flow cytometry, whereas these samples showed no staining in HercepTest. Thus, these results indicated that flow cytometric analysis may be a better method for screening the candidates when targeting the action of ADCC in trastuzumab therapy for esophageal SCC.

The trastuzumab-mediated ADCC of PBMCs from patients with esophageal SCC was impaired in comparison with that in healthy donors, in line with our previous report for gastric cancer patients (13). We showed that NK cell dysfunction contributed to the impaired trastuzumab-mediated ADCC in

gastric cancer patients (13). It is likely that esophageal SCC patients might also be in the immunosuppressive state with NK cell dysfunction.

Clyens et al. and ourselves reported that NK cells and monocytes play a role in trastuzumab-mediated ADCC (13, 22). It is well known that immunosuppressive cytokines such as IL-10 and TGF- β are produced within the tumor microenvironment and suppressed the activity of NK cells, monocytes, or T cells (17–20). Therefore, to enhance the effect of trastuzumab-mediated ADCC, we next investigated the involvement of IL-10 and TGF- β in the trastuzumab-mediated ADCC for esophageal SCC. As a result, trastuzumab-mediated ADCC against TGF- β -producing TE1 cells was enhanced by treatment with anti-TGF- β 2 neutralizing mAb and by blocking the TGF- β signaling with SB-431542. SB-431542 is a specific inhibitor of ALK-4, ALK-5, and ALK-7, the type I receptors for TGF- β , which affected the ALK-1- and ALK-5-mediated signaling induced by TGF- β and inhibited the phosphorylation of Smad2/3 induced by TGF- β (15). In the present study, we confirmed that SB-431542 could block TGF- β 2-induced phosphorylation of Smad2 in PBMCs. It is likely that the inhibition of TGF- β signaling by SB-431542 on the effector cells (PBMCs) may result in the enhancement of trastuzumab-mediated ADCC. Considering these results, some modalities, such as the inhibition of TGF- β signaling aimed at enhancing trastuzumab-mediated ADCC, may be useful for successful trastuzumab treatment of TGF- β -producing esophageal SCC. Also, these observations suggest that if, in the setting of adoptive immunotherapy, *ex vivo* activated T cells and NK cells are pre-treated with SB-431542, adoptively transferred T cells and NK cells may overcome the immunosuppression induced by TGF- β *in vivo*. However, it is impossible to explain the inhibition of trastuzumab-mediated ADCC with immunosuppression by TGF- β alone. In fact,

ADCC activity for KYSE30 and KYSE50 cell lines was relatively low in comparison with that for TE5 or TE1 with almost the same amount of HER-2 levels, although the KYSE30 and KYSE50 cell lines did not produce significant amounts of TGF- β 1 and TGF- β 2. Further study will be needed to clarify the involvement of other immunosuppressive factors.

In conclusion, HER-2-expressing esophageal SCCs were killed by trastuzumab-mediated ADCC and the activity reflected the degree of HER-2 expression detected by flow cytometry. Furthermore, SB-431542 significantly enhanced the trastuzumab-mediated ADCC against a TGF- β -producing cell line.

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