Abstract. The cytotoxic effect of trastuzumab in combination with oral fluoropyrimidine S-1 on human epidermal growth factor receptor 2 (HER2)-overexpressing human pancreatic cancer cell line TRG in vitro and in vivo was investigated. HER2 expression in TRG was analyzed by RT-PCR and flow cytometry. For in vitro experiments, 5-fluorouracil (5-FU) was used instead of S-1. In vivo studies were conducted with TRG xenografts in athymic mice. Trastuzumab (10 mg/kg) was administered intraperitoneally once a week for 4 weeks. S-1 (10 mg/kg) was administered orally 5 days a week for 4 weeks. The results showed that TRG cells were positive for HER2 mRNA and overexpressed HER2 protein. Either trastuzumab or 5-FU concentration-dependently inhibited the growth of TRG cells. The combination of trastuzumab and 5-FU resulted in a significant inhibition of growth of TRG cells compared to either agent alone (P<0.001). Incubation of TRG cells with peripheral blood mononuclear cells after treatment with trastuzumab enhanced the antiproliferative effect of trastuzumab, which could be the result of antibody-dependent cellular cytotoxicity. The combination of trastuzumab and S-1 resulted in a significant reduction in xenograft volume compared to each agent alone (P<0.0001). In conclusion, this study showed that combination therapy with trastuzumab and S-1 may be effective for HER2-overexpressing pancreatic cancer patients.

Introduction

HER2 (human epidermal growth factor receptor 2) is a transmembrane tyrosine kinase receptor, which regulates cell growth and differentiation. A ligand binds to HER2 receptor, and HER2 forms a heterodimer with other receptors of the HER family (HER1, HER3, HER4), causing it to mediate signaling to cancer cells to proliferate. HER2 is overexpressed in 25-30% of breast cancers and is associated with poor prognosis for breast cancer patients (1-3). The anti-HER2 antibody trastuzumab (Herceptin) inhibits tumor growth in HER2-overexpressing breast cancer (4), and the response rate of trastuzumab alone for HER2-overexpressing breast cancer patients is 15-26% (5,6). Since the mechanism of action of trastuzumab is different from that of traditional chemotherapeutic agents, trastuzumab is expected to enhance the antitumor effect of chemotherapeutic agents without increasing their toxicity. Therefore, various combinations of trastuzumab and chemotherapies have been tried for breast cancer patients (7,8). In pancreatic cancer, HER2 is overexpressed in 17-45% of the patients (9-13). However, the efficacy of trastuzumab as a single agent or in combination with chemotherapy for pancreatic cancer patients has not been fully evaluated. In a phase II study of gemcitabine and trastuzumab for pancreatic cancer patients, the response rate of trastuzumab and gemcitabine was similar to gemcitabine alone (14).

S-1 is an oral preparation of 5-fluorouracil (5-FU) consisting of tegafur (a prodrug of 5-fluorouracil), 5-chloro-2,4-dihydroxypyridine, which inhibits the degradation of 5-fluorouracil, and potassium oxonate, which reduces gastrointestinal toxicity. Hayashi et al (15) reported that the response rates of S-1 alone and the combination of S-1 and cisplatin for pancreatic cancer patients were 20.0 and 57.1%, respectively.

In this study, we investigated the antitumor activity of trastuzumab and S-1 in HER2-overexpressing human pancreatic cancer in vitro and in vivo.

Materials and methods

Compounds. Trastuzumab (Genentech, Inc., San Francisco, CA) was provided by Chugai Pharmaceutical (Tokyo, Japan).
S-1 was from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan) and 5-fluorouracil was from Kyowa Hakko Co. (Tokyo, Japan).

**Cell line.** The human pancreatic cancer cell line TRG was established from pancreatic ductal adenocarcinoma resected from a pancreatic cancer patient at Kanagawa Cancer Center. Cells were cultured at 37°C with 5% CO₂ in RPMI-1640 complete medium, consisting of RPMI-1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 1% glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Sigma Chemical Co., St. Louis, MO).

**Peripheral blood mononuclear cells (PBMCs).** PBMCs were isolated from the blood of a healthy volunteer on Ficoll-Hypaque density gradients using LSM (ICN/Cappel, Aurora, OH). The cells were cultured at 37°C with 5% CO₂ in the RPMI-1640 complete medium described above.

**Animals.** Five-week-old male nude mice (BALB/c) were purchased from Charles River Japan, Inc. (Yokohama, Japan). These mice were bred and maintained in a 12-h dark-light cycle animal facility with controlled temperature (22-26°C) and humidity (40-70%) at Kanagawa Cancer Center. All animal experiments were conducted in accordance with the guidelines of the ethics committee at the Kanagawa Cancer Center.

**HER1 (EGFR), HER2, HER3, and HER4 RNA expression in TRG cells analyzed by RT-PCR.** Total RNA was extracted from TRG cells using TRIzol reagent (Invitrogen Corp., Carlsbad, CA). cDNA was synthesized with oligo(dT) primer and Superscript II reverse transcriptase (Invitrogen Corp.). PCR amplifications were carried out in a T-Personal thermal cycler (Biometra, Göttingen, Germany) with Platinum Taq DNA polymerase (Invitrogen Corp.). The following primers were used: HER1, forward (5’ CCT GAG CTC TCT GAG TGC AAC C 3’) and reverse (5’ AGA TAC TCG GGG TTG CCC ACT G 3’); HER2, forward (5’ CGG GAG ATC CCT GAC CTG CTA GGA 3’) and reverse (5’ CTG CGT GGG TAC CAG GTA CTC TCT C 3’); HER3, forward (5’ CGG GAG GTC TTT CCA GGA GTC 3’) and reverse (5’ GAG GAG GGG GTA CTT TTG AG 3’); and HER4, forward (5’ CCT CTT CTT GGG GTG TTT AG 3’) and reverse (5’ AAG TCT GGC AAT CAG CTT GGG G 3’) (16). Thermal cycling conditions for HER1, HER2 and HER3 were as follows: denaturation at 94°C for 30 sec; annealing at 60°C for 30 sec; and extension at 72°C for 1 min for 28 cycles. For HER4, the same conditions were used except that the PCR annealing temperature was 55°C. An additional PCR amplification directed at the glucose-6-phosphate dehydrogenase (G6PDH) gene was performed as a control using primers forward (5’ CTG ACC TAC GGC AAC AGA TA3) and reverse (5’ AAC CCA CTC TCT TCA TCA GGC 3’) under the following conditions: denaturation at 94°C for 15 sec; annealing at 55°C for 15 sec; and extension at 72°C for 30 sec for 30 cycles. PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with ethidium bromide and visualized under UV light. The expected amplicon sizes were 315, 300, 320, 404 and 238 bp for HER1, HER2, HER3, HER4 and G6PDH respectively.

**Expression level of HER2 protein in TRG cells and the effect of trastuzumab and 5-fluorouracil on the HER2 expression level analyzed by flow cytometry.** HER2 protein expression in TRG cells and the change in the HER2 expression level following exposure to trastuzumab alone (1 and 10 μg/ml), 5-fluorouracil alone (1 and 10 ng/ml) and the combination of trastuzumab and 5-fluorouracil were analyzed by flow cytometry. Cells (1x10⁶) were suspended in 20 μl of Hank’s balanced solution (HBS) containing 10% FBS. Anti-erbB2/HER-2 mouse monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY) which was used as the primary antibody was added, and the cells were incubated for 30 min on ice. After washing three times with HBS containing 10% FBS, the cells were suspended in 20 μl of HBS containing 10% FBS. Fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG monoclonal antibody (Becton Dickinson Labware, Lincoln Park, NJ) as a secondary antibody was added, and the cells were incubated for 30 min. After washing with HBS containing 10% FBS twice, the cells were re-suspended in 1 ml of HBS containing 10% FBS. The expression level of HER2 was analyzed by flow cytometry using FACS (Becton Dickinson Labware). Unstained (autofluorescence control) cells and cells stained with the secondary antibody alone (secondary control) were analyzed by flow cytometry.

**Effect of trastuzumab and 5-fluorouracil on proliferation of human pancreatic cancer cells in vitro.** TRG cells (1x10⁴) were seeded in each well of a BD Falcon 96-well microplate (Becton Dickinson Labware). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum. After a 1-day incubation at 37°C in a 5% CO₂ incubator, various concentrations of trastuzumab (0, 0.1, 1, 10 and 100 μg/ml) or 5-fluorouracil (0, 1, 10, 100 and 1000 ng/ml) were added. After 3 days of incubation at 37°C in a 5% CO₂ incubator, the proliferation of the cells was measured by MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay.

**Effect of combinations of trastuzumab and 5-fluorouracil on human pancreatic cancer cells in vitro.** After a 1-day incubation of 5x10⁶ TRG cells seeded in a 10-cm dish with 5 ml 10% fetal bovine serum at 37°C in a 5% CO₂ incubator, trastuzumab and 5-fluorouracil were added as single agents and in combination. No agent was added to the control. After 3 days of incubation at 37°C in a 5% CO₂ incubator, cells were harvested by trypsinization and were counted. Experiments were performed in triplicate. Percentages of control cell proliferation (cell number/mean cell number in controls x 100) were compared in each group.

**Antibody-dependent cellular cytotoxicity (ADCC) of trastuzumab on human pancreatic cancer cells in vitro.** After a 1-day incubation of 1x10⁶ TRG cells in 5 ml 10% fetal bovine serum at 37°C in a 5% CO₂ incubator, trastuzumab and 5-fluorouracil were added as single agents and in combination. No agent was added to the control. After 2 days of incubation at 37°C in a 5% CO₂ incubator, cells were washed to remove the added agent. In order to analyze antibody-dependent
cellular cytotoxicity (ADCC) with trastuzumab, 1x10^7 peripheral blood mononuclear cells (PBMCs) were added to each group. After 12 h of incubation at 37°C in a 5% CO₂ incubator, cells were counted. Experiments were performed in triplicate. Percentages of control cell proliferation were compared in each group.

Effect on human pancreatic cancer xenografts of a combination of trastuzumab and S-1. The in vivo effects of trastuzumab and S-1 as single agents and in combination were tested in a human pancreatic cancer xenograft model in athymic mice. The viable TRG cells (5x10^6) were injected subcutaneously into the mediodorsal regions of athymic mice. After a week of subcutaneous tumor growth, the mice were randomized into three treatment groups and a control group of 10 mice each, and the treatment was started. Trastuzumab was given intraperitoneally once a week for 4 weeks at a dose of 10 mg/kg. S-1 was given orally 5 days a week for 4 weeks at a dose of 10 mg/kg. The control group received no treatment. Tumor size and body weight were measured once a week, and tumor volume was calculated by the formula 1/2 x large diameter x (small diameter)^2.

Statistical analysis. Results were expressed as the means ± SE. Statistical significance was determined by the Student’s t-test in comparing treatment groups. A P value <0.05 was accepted as statistically significant.

Results

HER1 (EGFR), HER2, HER3, and HER4 RNA expression in TRG cells analyzed by RT-PCR. The mRNA expression of HER1, HER2, HER3, and HER4 was analyzed by RT-PCR. TRG cells were positive for HER1, HER2, and HER3, but not for HER4 (Fig. 1).

Expression level of HER2 protein in TRG cells and the effect of trastuzumab and 5-fluorouracil on the HER2 expression...
level analyzed by flow cytometry. TRG cells overexpressed HER2 protein in flow cytometric analysis (Fig. 2). Although the HER2 level of TRG cells did not change after treatment with trastuzumab alone, the HER2 level was increased after treatment with 5-fluorouracil alone and with a combination of trastuzumab and 5-fluorouracil (Fig. 3).

**Effect of trastuzumab and 5-fluorouracil on proliferation of human pancreatic cancer cells in vitro.** TRG cells were cultured in the presence of 0.1, 1, 10, and 100 μg/ml trastuzumab or 1, 10, 100, and 1000 ng/ml 5-fluorouracil. After 4 days of incubation, MTT assay was performed. Either trastuzumab or 5-fluorouracil concentration-dependently inhibited the growth of TRG cells (Fig. 4).

**Effect of a combination of trastuzumab and 5-fluorouracil on proliferation of human pancreatic cancer cells in vitro.** TRG cells were cultured in the presence of 1 μg/ml trastuzumab and 10 ng/ml 5-fluorouracil alone and in combination, and the growth inhibition effects were compared. The combination of trastuzumab and 5-fluorouracil resulted in significant inhibition of the growth of TRG cells compared to either agent alone (P=0.0004 vs. 5-fluorouracil alone, P=0.0006 vs. trastuzumab alone) (Fig. 5).

**Antibody-dependent cellular cytotoxicity (ADCC) of trastuzumab on human pancreatic cancer cells in vitro.** After incubation of TRG cells with trastuzumab and 5-fluorouracil, PBMCs were added to the TRG cells and the growth inhibition effects were compared. The inhibition effect of the trastuzumab group (P=0.001) and 5-fluorouracil group (P=0.009) in the presence of PBMCs was enhanced compared to that without PBMCs (Fig. 5). Although the inhibition effect in the trastuzumab group and the 5-fluorouracil group was not significantly different when PBMCs were not added, the inhibition effect in the trastuzumab group was greater than in the 5-fluorouracil group (P=0.06) in the presence of PBMCs. In the combination group, the enhancing effect on growth inhibition induced by PBMCs was not observed.

**Effect of the combination of trastuzumab and S-1 on human pancreatic cancer xenografts.** The in vivo effects of trastuzumab and S-1 alone and in combination were tested in a TRG subcutaneous xenograft model in athymic mice. Trastuzumab was given intraperitoneally once a week for 4 weeks at a dose of 10 mg/kg. S-1 was given orally 5 times a week for 4 weeks at a dose of 10 mg/kg. Two mice in the control group that received no treatment died within 4 weeks. The tumor volumes at 4 weeks in the three treatment groups...
were significantly reduced as compared with the control group (P<0.01) (Fig. 6). The combination of trastuzumab and S-1 resulted in a significant reduction (P<0.0001) in tumor volume compared to either agent alone. Weight loss in the combination group was significantly less than in the control group (P=0.0004) and the S-1 group (P<0.0001), which demonstrated that the combination of trastuzumab and S-1 probably did not increase toxicity (Fig. 7).

Discussion

Although chemotherapy for pancreatic cancer patients has not been established, Hayashi et al (15) recently reported that the response rates of S-1 alone and the combination of S-1 and cisplatin for pancreatic cancer patients were 20.0 and 57.1%, respectively. Therefore S-1 is one of the hopeful drugs for pancreatic cancer patients. S-1 is an oral preparation of 5-fluorouracil consisting of tegafur, 5-chloro-2,4-dihydroxy-pyridine and potassium oxonate. Tegafur is metabolized into 5-fluorouracil in the liver in vivo. 5-chloro-2,4-dihydroxy-pyridine competitively inhibits the 5-fluorouracil degradative enzyme dihydropyrimidine dehydrogenase (DPD), resulting in the retention of a prolonged concentration of 5-fluorouracil in the blood. For in vitro experiments, we used 5-fluorouracil, its metabolite, instead of tegafur itself. 5-fluorouracil concentration-dependently inhibited the proliferation of TRG cells in vitro. In vivo experiments, S-1 inhibited the growth of TRG xenografts in athymic mice. There are two possible mechanisms of action for 5-fluorouracil; interference with RNA function and inhibition of DNA synthesis. After intracellular conversion to 5-fluorouridine triphosphate (FUTP), 5-fluorouracil is incorporated into RNA and interferes with RNA processing. 5-fluorouracil is also converted to 5-fluoro-2'-deoxyuridine monophosphate (FdUMP), whereby a complex is formed with thymidylate synthase (TS), inhibiting the function of TS and impairing DNA synthesis. The high activity of tumoral TS and DPD (an enzyme of 5-fluorouracil catabolism) reduces the antitumor effect of 5-fluorouracil (17). However, the activity of TS and DPD was not evaluated in this study.

HER2 is overexpressed in 17-45% of pancreatic cancer patients (9-13). TRG cells expressed HER1, HER2, and HER3 mRNA in RT-PCR and overexpressed HER2 protein in flow cytometry. HER2 forms heterodimers with other members of the HER family to stimulate intercellular signaling pathways. In pancreatic cancer tissue, HER-2 co-expression with HER-3 and HER-4 is frequently seen (18). Treatment of TRG cells with trastuzumab resulted in concentration-dependent growth inhibition in vitro. An antitumor effect of trastuzumab for TRG xenografts was also observed. HER2 down-regulation is one possible mechanism of the antitumor effect of trastuzumab, which has not yet been completely elucidated (19,20). The binding of trastuzumab to HER2 proteins decreases expression of cell surface HER2, thereby weakening growth signaling and inhibiting cell proliferation. In this study, we analyzed the change in HER2 expression level after treatment of TRG cells with trastuzumab, but HER2 down-regulation was not observed. Therefore the action of trastuzumab against TRG cells was likely due to the prevention of dimer formation, initiation of G1 arrest and...
induction of p27, or prevention of HER2 cleavage (21), although further research is needed to explore these possibilities. Trastuzumab possesses the Fc domain of human IgG1, indicating that trastuzumab induces antibody-dependent cellular cytotoxicity (ADCC), and inhibits cell growth (4), in addition to direct antitumor activity as previously described. In order to determine the ADCC induced by trastuzumab against TRG cells in vitro, we added peripheral blood mononuclear cells (PBMCs) to TRG cells after treatment with trastuzumab and 5-fluorouracil and analyzed cell growth inhibition. The growth inhibition in 5-fluorouracil was increased by PBMCs as well as in trastuzumab. The enhanced growth inhibition in 5-fluorouracil, which is not an antibody-induced agent, seemed to be due to a direct cytotoxic effect of PBMCs. The enhanced effect by PBMCs in trastuzumab was greater than in 5-fluorouracil suggesting that ADCC could be operative in trastuzumab. In the combination group, the enhanced effect by PBMCs was not observed, since the cytotoxic effect of the combination of trastuzumab and 5-fluorouracil was too strong.

Previous studies have demonstrated synergistic or additive interactions between trastuzumab and a variety of chemotherapeutic drugs, such as paclitaxel, doxorubicin, and cisplatin (22-25), but few studies have reported the effect of chemotherapeutic drugs, such as paclitaxel, doxorubicin, and cisplatin. Pegram et al (25) reported that following exposure to doxorubicin, HER2 expression levels in SK-BR-3 breast carcinoma cells were unaltered. Treatment of TRG cells with trastuzumab resulted in an increase in HER2 expression. These data suggest that the enhanced effect with the combination of trastuzumab and 5-fluorouracil could be due to increased sensitivity for trastuzumab in TRG cells resulting from HER2 up-regulation. However, the interaction with trastuzumab and chemotherapeutic agents remains to be elucidated, and further research is needed to explore these effects.

In conclusion, we demonstrated the additive interaction with the combination of trastuzumab and 5-fluorouracil in vitro and the additive interaction with the combination of trastuzumab and S-1 in vivo using a HER2-overexpressing human pancreatic cell line. These data indicate that the clinical efficacy of the combination of trastuzumab and S-1 against HER2-overexpressing pancreatic cancer is worth investigating.

References


