Abstract. We assessed the effects of direct injection of docetaxel-loaded immuno-(trastuzumab)-liposomes (IDL) on a xenograft mouse tumor model to determine potential clinical applications of intratumoral tailored chemotherapy against Her2/neu-overexpressing gastric cancer. The NCI-N87 Her2/neu overexpressing gastric cancer cell line xenograft mouse model was treated with IDL or docetaxel-loaded liposomes (DL). The ratio of the tumor volume of the treatment:control was determined. In addition, docetaxel pharmacokinetics in tumors were measured using high-performance liquid chromatography, and the cell viability and cell cycle distribution of Her2/neu positive cells were determined by flow cytometric analysis. The IDL group showed a significantly higher distribution of docetaxel in the N87 xenograft tumor tissues and superior antitumor efficacy compared to crude administration of docetaxel and/or trastuzumab and DL. The number of viable Her2/neu positive cells decreased following treatment with either free trastuzumab or IDL. On day 7 after treatment, a decrease in the G0/G1 phase of the cell cycle was observed in the DL and IDL groups compared to the control group. No local adverse effects were observed. These results suggest that intratumoral administration of IDL maintains a high concentration of docetaxel within the tumors. These data suggest that immuno-liposome mediated delivery of drugs is a promising new therapeutic option for patients with advanced gastric cancer that over-express Her2/neu.

Introduction

In recent studies, Her2/neu overexpression and/or amplification has been observed, not only in breast cancers, but also in colon (1), bladder (2), ovarian (3), endometrial (4), lung (5), uterine cervix (6), head and neck (7), esophageal (8), and gastric carcinomas (9). The Her2/neu protein has also been reported to be overexpressed in 10-30% of patients with gastric cancer (9,10). These patients are known to be associated with poor outcome and distant metastasis (11,12). Treatment with trastuzumab has been shown to enhance survival rates in breast cancer patients. Nevertheless, the effectiveness of trastuzumab for HER2 positive gastric cancer patients is still controversial.

Docetaxel is one of the most important anticancer drugs used as a standard therapy for various types of solid tumors, such as breast, ovarian, non-small cell lung cancer and advanced gastric cancer. Preclinical studies of docetaxel in combination with trastuzumab demonstrate synergistic interactions of trastuzumab with docetaxel and additive interactions with paclitaxel in breast cancer patients (13).

Higher drug concentrations within the tumor and longer exposure to anticancer drugs will have a greater cytotoxic effect. Intratumoral chemotherapy is an ideal method for the generation of high drug concentrations within solid tumors and for limitation of total body exposure to the cytotoxic agent resulting in increased dose-related cell killing and a reduced systemic toxic effect.

To simultaneously administer both docetaxel and trastuzumab using a single drug delivery system, which may have advantages over their separate administration, we decided to deliver these drugs using liposomes.

Using liposome-mediated delivery of drugs that were selected based on recent advances in chemotherapy, we
devised a new therapeutic option for advanced gastric cancer using less-invasive and more effective local cancer control than direct administration of conventional anticancer drugs. For this purpose, we investigated the combination of safety, selectivity and anti-cancer effectivity of a monoclonal antibody-conjugated liposome in which an anti-cancer drug was incorporated. The results suggest that monoclonal antibody-liposome mediated tumor delivery of anti-cancer drugs may enhance their anti-tumor effectivity.

Materials and methods

Reagents. L-α-Phosphatidylethanolamine, Dipalmitoyl (DPPE) was obtained from Wako Pure Chemicals (Osaka, Japan). N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP) and cholesterol were purchased from Sigma (St. Louis, MO, USA). 1,2-Dipalmitoyl-sn-Glycero-3-Phosphocholine (DPPC) was from Alexis Biochemicals (Lausen, Switzerland), and Dithiothreitol (DTT) was from Nacalai Tesque (Kyoto, Japan). Docetaxel was provided as a gift by Sanofi-Aventis (Tokyo, Japan). Trastuzumab was a kind gift from Chugai Pharmaceutical (Tokyo, Japan). 3-(2-pyridyldithio)-propionyl-phosphatidylethanolamine (DTP-DPPE), used as a bridging agent between the lipids and trastuzumab, was prepared by the reaction of SPDP with DPPE as described previously (14).

Preparation of trastuzumab-conjugated docetaxel loaded liposomes. Small unilamellar vesicles were prepared by lipid film hydration and the Sobin extrusion method (15). First, the main ingredients, DPPC, cholesterol, and DTP-DPPE were dissolved in chloroform at a molar ratio of 2:1:0.03 and were mixed in a round-bottomed flask. The solvent was then evaporated using a vacuum in a rotary evaporator until a thin lipid film was formed. The thin lipid film obtained was further dried under vacuum overnight. The thin lipid film was then hydrated using a solution containing an aqueous docetaxel hydrate (0.5 mg/ml) and 2:1(2-hydroxy-1,1-bis[hydroxymethyl]ethyl)-amino]ethanesulfonic acid (TES) buffer (10 mM, pH 7.4) containing 140 mM NaCl, 0.1 mM EDTA and by rotating the flask at about 200 rpm at 40°C until the thin lipid film was completely hydrated and a homogeneous dispersion was formed (approximately 30 min). The lipid suspension was sonicated for 40 min in a bath-type sonicator (S-450D, Branson, Atsugi, Japan). The liposome suspension was then extruded under a nitrogen atmosphere through a 100-nm polycarbonate membrane filter (Whatman Inc., Piscataway, NJ, USA) using a high-pressure extruder (Lipex™ Extruder, Northern Lipids Inc., Vancouver, Canada).

The trastuzumab to be conjugated to liposomes (thiol-bearing trastuzumab) was prepared according to the SPDP method (14). SPDP was added to 1 mg/ml trastuzumab to a final concentration of 0.1 mM, and the mixture was incubated at room temperature for 30 min. DTT was then added to the mixture to a final concentration of 50 mM. The mixture was reduced by incubation at room temperature for 20 min. Non-bound SPDP and non-bound DTT were removed by passage through a MicroSpin G-25 column (Amersham Biosciences, Buckinghamshire, UK). Subsequently, thiol-bearing trastuzumab with an exposed SH radical was prepared in a similar manner.

Trastuzumab-conjugated, docetaxel-containing liposomes were prepared by addition of docetaxel (0.4 mg/ml) to the liposome suspension containing thiol-bearing trastuzumab, prior to incubation for 16 h at room temperature. Transmission electron microscopy (JEM-1230, Nihon Denshi, Tokyo, Japan) was also performed to examine the morphologic characteristics of the immunoliposomes.

Animals. Female 4-week-old BALB/c-nu/nu mice were obtained from Charles River Japan (Yokohama, Japan). The maintenance and care of all experimental animals used in this study were carried out according to the guidelines of the Animal Studies Committee of Ehime University, Ehime, Japan. The mice were kept for 1 week in our animal facility prior to tumor inoculation.

Cell culture and culture conditions. The NCI-N-87 (a well differentiated adenocarcinoma) cell line was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). This cell line was subcultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and an antibiotic-antimycotic agent containing 100 IU/ml penicillin and 0.1 mg/ml streptomycin under standard conditions.

Preparation of the murine subcutaneous tumor model. Log-phase NCI-N87 cancer cells were harvested using 1 mm EDTA in phosphate-buffered saline (PBS), then resuspended at a cell density of 5x10^6/ml PBS containing 300 μg/ml of Matrigel™ basement membrane matrix (BD Biosciences, San Jose, CA, USA). Each mouse was inoculated by subcutaneous injection of 0.1 ml cells (5x10^6 cells)/mouse or control PBS/ Matrigel into the left flank.

Her2 expression analysis. Tumor xenograft tissues were resected and were processed by formalin fixation and paraffin embedding followed by sectioning. These sections were examined for the expression of HER2 protein by immunohistochemistry (IHC) using the Hercep Test (Dako Corp., Carpinteria, CA, USA) and for HER2 gene amplification by fluorescence in situ hybridization (FISH) using the PathVysion HER-2 DNA Probe kit (Vysis, Downers Grove, IL, USA). The sections were then analyzed at SRL Medisearch, Inc. (Tokyo, Japan).

Antitumor efficacy. Intratumoral injection therapy of the anticancer agent was started when the tumor had grown to approximately 150-250 mm^3. The tumor volume was calculated using the following formula: (tumor volume mm^3) = 1/2 x (long diameter) x (short diameter)². Antitumor activity was quantified by calculation of the ratio of the tumor volume of the treatment/control (T/C) using tumor volumes measured on day 21. Mice were injected only once during the experiment. Tumors of the experimental and control groups were measured 3 times a week.

HPLC analysis. Docetaxel concentrations in the docetaxel loaded liposomes (DL), the trastuzumab-conjugated docetaxel loaded liposomes (IDL), and in tumor samples were quantified using the modified high-performance liquid chromatography...
(HPLC) method described by Marchettini et al (16). Briefly, samples were diluted using a sufficient amount of acetonitrile and were homogenized. After centrifugation, the extract was placed into another tube and was evaporated to dryness under nitrogen gas. The residue was resuspended in 250 μl of an isocratic mixture of acetonitrile and 0.1% phosphoric acid in deionized water. These suspensions were filtered, and 25 μl of the filtrate were injected into the HPLC system (665A-11, Hitachi, Tokyo, Japan) at a flow rate of 0.8 ml/min. A reverse phase ODS column (TSK GEL, ODS 80Ts, Tosoh, Tokyo, Japan), was used, and docetaxel concentration was determined by measurement of UV adsorption at 227 nm using a spectrophotometer (SPD6A, Shimadzu, Kyoto, Japan) connected to the HPLC system.

Flow cytometry. A FACSCalibur flow cytometer (BD Biosciences) was used for all of the flow cytometric analyses. The Neu 24.7-FITC antibody (BD Biosciences) was used to analyze cell surface Her2/neu expression. Cells (1x10^6) were blocked in Pharmingen Stain Buffer (BD Biosciences) for 10 min on ice and were subsequently incubated with the appropriate antibody for 20 min. After two washes, the cells were resuspended in Pharmingen Stain Buffer containing 7-Amino-actinomycin D (BD Biosciences), which was used to discriminate between live and dead cells.

Flow cytometric cell cycle analyses and detection of apoptotic cells were performed using the CycleTest™Plus DNA Reagent kit (BD Biosciences). In brief, 5x10^5 cells were prepared and trypsin buffer was then added to each sample and was reacted for 10 min at room temperature. Trypsin inhibitor and RNase buffer were then added to each sample and were reacted for 10 min at room temperature. Finally, propidium iodide stain buffer was added to each sample and was incubated for 10 min in the dark on ice.

Statistical analysis. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). The Mann-Whitney U test was used to obtain statistical differences. Statistical significance was established at p<0.05.

Results

HER2 levels in NCI-N87 cells. We first assayed the level of HER2 protein in the NCI-N87 model cell line by IHC using the HercepTest. Positively stained cells were scored for HER2 protein expression on a scale of 0 to 3+. The NCI-N87 cells had a score of 3+. The level of HER2 gene amplification in NCI-N87 cells was determined by FISH analysis. The FISH score was 3.8 signals/cell (Fig. 1).

Characterization of liposomes and immunoliposomes. Characterization of DL and IDL by dynamic light scattering (data not shown) indicated that the average diameter of both liposomes was about 100 nm. The amount of docetaxel incorporated into DL and IDL was 0.43±0.05 mg/ml, 0.43±0.03 mg/ml (n=3), respectively, as measured by HPLC. The amount of trastuzumab incorporated into IDL was 2.15±0.17 mg/ml (n=3) as measured using the Pierce 660 nm Protein Assay (data not shown). Based on transmission electron microscopy analysis, each IDL conjugated from two to four antibodies (Fig. 2).

Tumor response following intratumoral injection of docetaxel with PBS, docetaxel and trastuzumab, free liposomes, DL or IDL in the NCI-N87 xenograft model. For assay of the effectiveness of liposome-incorporated drugs against the NCI-N87 mouse xenograft tumor model, the mice were randomly assigned to one of 6 treatment groups (n=6 in each group).
The groups were injected intratumorally with 0.5 ml of either PBS (control group), free liposomes (group I), 1 mg/kg docetaxel (group II), 1 mg/kg docetaxel and 5 mg/kg trastuzumab (group III), 1 mg/kg DL (group IV, docetaxel-liposomes) or 1 mg/kg IDL (group V, docetaxel/trastuzumab-liposomes). Twenty-one days later, tumor volume was measured and the T/C ratio was calculated. The T/C ratio was 1.013 for group I, 0.668 for group II, 0.404 for group III, 0.383 for group IV and 0.169 for group V. Thus, the antitumor activity of IDL (group V) was significantly superior to that of the other groups (Fig. 3). No significant decrease in body weight was observed for any of the xenograft model groups (data not shown).

Concentration of docetaxel within tumors. The pharmaco-kinetic (PK) profile of docetaxel after intratumoral injection was followed by sampling the tumors in groups II, III, IV, and V at 24, 72 and 168 h, followed by measurement of the docetaxal level using HPLC. Tumor concentrations of docetaxel were measured using HPLC. Statistically significant data (P<0.05) vs. the result of the free docetaxel group are indicated by asterisk. Bars indicate the mean ± SEM.

Tumor cell viability. The effect of treatment on the viability of the Her2/neu positive NCI-N87 cells in the tumor xenograft was determined by flow cytometric analysis of single cell suspensions of the tumors of the 6 treatment groups. The groups treated with free docetaxel and trastuzumab or with IDL displayed a significant reduction in the percentage of viable Her2/neu positive cells compared to the control group 7 days after treatment. Treatment with a single agent, either
free docetaxel or DL, had no significant effect on Her2/neu positive cell viability. These data suggest that the combination of trastuzumab with docetaxel had a significant cytotoxic effect on the tumor of the NCI-N87 xenograft model (Fig. 5).

**Effect on cell cycle and cell cycle distribution.** To analyze the effect of incorporation of these drugs into liposomes on the cell cycle in tumor cells, the cell cycle distribution of NCI-N87 xenografted cells was analyzed on day 7 after each treatment, using FACS analysis (Fig. 6). Both DL and IDL treatment induced a decrease in the number of cells in the G0/G1 phase compared to the control group. The free-docetaxel and the docetaxel plus trastuzumab groups did not show any significant differences. Apoptosis was significantly induced in all treatment groups compared to control (Fig. 7). However, the percentage of apoptotic cells was not significantly different among the different treatment groups.

**Discussion**

There are several potential advantages in using direct intratumoral injection. These advantages include 1) assured precision in the local delivery of drugs, 2) complete perfusion of a drug within and around the lesion, 3) dramatically higher concentrations of the drug within the tumor tissue than is achievable by conventional systemic chemotherapy, and 4) little or no systemic toxic side effects (17).

Fujimoto-Ouchi *et al* (18) reported that intravenous administration of 60 mg/kg docetaxel combined with intraperitoneal injection of 20 mg/kg trastuzumab had effective anti-tumor activity in the NCI-N87 xenograft model. This concentration of docetaxel was considerably higher than that used in the present study. Nevertheless, our study showed effective anti-tumor activity of a lower docetaxel concentration in combination with trastuzumab.

Trastuzumab has been previously shown to have additive/synergistic interactions with docetaxel for breast cancer treatment (19). Our results are consistent with these previous findings in that trastuzumab interacted at least additively with docetaxel for anti-tumor activity in the NCI-N87 xenograft model.

In a review of intratumoral cancer treatment, Goldberg *et al* (20) concluded that it is reasonable to believe that preoperative intratumoral chemotherapy using drug carriers that prolong local drug activity is ready to achieve more widespread clinical use. A second approach to the administration of chemotherapeutic drugs for localized tumor treatment could be via a controlled-release implant.

There have been several studies aimed at achieving high intratumoral drug retention. Shikanov *et al* (21) evaluated the antitumoral activity of a polymeric formulation of paclitaxel administered via an intratumoral injection. They concluded that this polymer formulation of paclitaxel inhibited the growth of tumors. Almond *et al* (22) reported the *in vivo* evaluation of the efficacy of an intratumoral injection of mitoxantrone.
both as a free drug and as drug-loaded albumin microspheres. Finally, Bao et al. (23) reported the potential use of liposome drug carriers for cancer therapy via direct intratumoral injection. Higher intratumoral retention of the technetium-99m (99mTc)-liposomes was accompanied by improved intratumoral diffusion suggesting that intratumorally administered liposomal drugs are potentially promising agents for solid tumor local therapy.

Liposomes have gained considerable attention as carriers for a wide range of drugs and may have a number of advantages over administration of the free drug. Free docetaxel is solubilized in a polyoxyethylated surfactant prior to injection. A number of biological effects related to the use of this vehicle for the formulation of drugs for clinical use have been described, including acute hypersensitivity reactions and peripheral neuropathy (24). Furthermore, severe skin ulceration may occur during extravasation of docetaxel. Indeed, we found in the present study that percutaneous injection of docetaxel that was diluted in PBS into the tumors of 6 mice resulted in severe skin ulceration in 2 of the mice. However, treatment with liposome-incorporated docetaxel, either DL or IDL, did not induce any skin ulceration. Thus it appears that incorporation of docetaxel into liposomes may reduce the incidence and severity of side effects.

In our study, the liposomes used were approximately 100 nm in diameter. Wang et al. (25) reported that 99mTc-labeled liposomes of 100 nm diameter were retained for a longer period within the tumor than liposomes of larger size. They suggested that smaller liposomes may accelerate intracellular uptake due to greater surface-to-surface contact of the liposomes with the cell membrane. The liposomes used in this study were therefore considered to be of optimal size for tumor uptake and retention.

Active targeting of liposomes to tumor cells is generally attempted by conjugating ligands to the liposomal surface which allow a specific interaction with the tumor cells (26). In our study, the IDL group demonstrated much higher retention of docetaxel within the tumor. This result suggests that HER2 mediated endocytosis is involved in the tumor uptake of IDL and therefore that conjugation of trastuzumab to liposomes is a viable approach for the targeting of liposomes to HER2-expressing tumors. Thus, even in the absence of specific, liposome tumor targeting, liposome incorporation of docetaxel is more effective than treatment with free drugs.

The anti-tumor activity of trastuzumab has been reported to involve two mechanism of tumor growth inhibition: a direct anti-proliferative effect (27) and an indirect antitumor effect due to antibody-dependent cellular cytotoxicity (28). In our study, the docetaxel plus trastuzumab group showed much higher inhibition of tumor growth than the docetaxel group. Moreover, the IDL group showed much higher inhibition of tumor growth than the DL group. These data indicate that trastuzumab inhibits tumor proliferation in the NCI-N87 xenograft model. Thus incorporation of trastuzumab into liposomes not only targets the liposomes to the tumor but also has anti-tumor effects.

In our previous study we observed that docetaxel loaded magnetoliposomes injected locally into a tumor had gradually spread to almost the entire tumor and accumulated in the peripheral lymph nodes. Thus, many Berlin Blue-staining positive magnetic particles were observed in the regional lymph nodes within one day after injection (29). These data indicate that local injection of anticancer drug-containing liposomes of a small size may simultaneously treat regional metastatic lymph nodes and the primary tumor. We now plan to study the effectiveness of IDLs against lymph node metastases in the NCI-N87 xenograft model.

In conclusion, IDL showed good efficacy and limited toxicity as well as excellent anti-tumor properties in a Her2/neu-overexpressing gastric cancer xenograft model. Studies leading to greater understanding of the biological consequence of Her2/neu directed therapies should allow the integration of this molecular targeted approach with currently available cancer treatments.

Acknowledgements

We would like to thank Professor Maeyama, M.D., Ph.D., Ehime University Graduate School of Medicine, Department of Informational Biomedicine for his technical advice for HPLC.

References