Therapeutic Effects of Autologous Lymphocytes Activated with Trastuzumab for Xenograft Mouse Models of Human Breast Cancer

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Trastuzumab (TTZ) is molecular targeted drug used for metastatic breast cancer patients overexpressing human epidermal growth factor receptor 2 (HER2). Therapeutic effects of lymphocytes activated with TTZ (TTZ-LAK) using xenograft mouse models of human breast cancer (MDA-MB-453) cells were examined in vivo. Remarkable reduction of tumor volume in a xenograft mouse models intravenously treated with TTZ-LAK cells after the subcutaneously inoculated of MDA-MB-453 cells was verified in vivo. The migration of TTZ-LAK cells in tumor of mouse models subcutaneously inoculated MDA-MB-453 cells was observed on the basis of histological analysis using immunostaining with CD-3. Induction of apoptosis in tumor of xenograft mice treated with TTZ-LAK cells was observed in micrographs using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) method. It was noteworthy that the therapeutic effects of TTZ-LAK cells along with apoptosis were obtained for xenograft mouse models of human breast tumor in vivo.

Key words trastuzumab; immunotherapy; human epidermal growth factor receptor 2; breast cancer; xenograft mouse model.

Various anticancer drugs which target specific molecules involved in growth and progression of tumor have been developed. The human epidermal growth factor receptor (HER) 2 is overexpressed in 20% of patients with breast cancers, increasing the aggressiveness of the tumor. HER2 gene, also known as neu and c-erbB-2, encodes a 185-kd transmembrane glycoprotein receptor (p185HER2), p185HER2 has partial homology with the epidermal growth factor receptor and shares intrinsic tyrosine kinase activity with that receptor. HER2 overexpressed in human breast cancers correlated with poor clinical outcome in women with breast cancers.1–3) HER2 is known as epidermal growth factor receptor (ErbB) 2 of the tyrosine kinase (TK) receptor, belonging to ErbB growth factor receptor family. There are four members of HER family receptors; epidermal growth factor receptor (EGFR)/ErbB1/HER1, HER2/ErbB2, HER3/ErbB3, and HER/ErbB4.4) HER family receptors are activated by ligand-induced dimerization, or receptor pairing. Dimerization is a critical step in HER family-mediated signaling, and HER receptors are able to homodimerize or heterodimerize with other HER family members. HER2 is the preferred dimerization partner for all HER family receptors. However, there are no known endogenous ligands that bind to HER2.5)

The extracellular domain of HER2 is in a conformation that is open and ready for dimerization. So, molecules such as monoclonal antibodies would bind to the extracellular domain of HER2 to suppress that activity. Several monoclonal antibodies (mAbs) directed against HER2 ectodomain that significantly inhibit the growth of tumor cell lines overexpressing HER2 have been developed.5) Trastuzumab (TTZ) is a humanized immunoglobulin G1 kappa (IgG1k) light chain mAb in which the complementary-determining regions (CDR) of a HER2-specific mouse mAb were joined to in human immunoglobulin variable regions.5) TTZ binds to the domain IV of the extracellular segment of the HER2/neu receptor. Because TTZ belong to IgG1, its effects may be related to Fab (antigen binding fragment) or Fc (crystallizable fragment) regions.6) It has been reported that antibody-dependent cellular cytotoxicity (ADCC) plays an important role in the antitumor activity of TTZ.7) Although the mechanisms underlying the action of TTZ are still not fully understood, inhibitory effects of TTZ by cell cycle arrest in G1 and induction of apoptosis on the growth of HER2-positive breast cancer cells in vitro have been reported.8) The combination chemotherapy with TTZ has been shown to improve survival for women with HER2-overexpressing metastatic breast cancer patients.9–11) However, there were severe side-effects in combination chemotherapy.12,13) On the other hand, immunotherapy using lymphocyte stimulated with immunomodulators such as cytokines was noted in cancer therapy as attractive approach, since there is no serious side effects in immunotherapy. Significant prolongation in survival and disease free period in group of immunotherapy compared with no-treatment group have been reported.14)

Yoshida et al. have studied metastasis mechanism of cancer cells15,16) and for clinical applications of immunotherapy using CD3- and lymphokine interleukin (IL)-2-activated killer (CD3-LAK) cells. On the other hand, Ueoka et al. have investigated inhibitory effects of hybrid liposomes17,18) composed of phosphatidylcholine and polyoxyethylenealkyl ether on the growth of tumor cells in vitro, in vivo, and for clinical applications.19–21) On the basis of these studies for cancer, we have tried to develop new immunotherapy using TTZ which is molecular target drug. We have already reported on inhibitory effects of lymphocytes activated with TTZ (TTZ-LAK) on the growth of human breast cancer cells in vitro.22) However, anticancer effects of TTZ-LAK in vivo have not been elucidated.

In this study, we investigated the therapeutic effects of in-
travenous injection of TTZ-LAK using xenograft mouse models subcutaneously inoculated human breast cancer (MDA-MB-453) cells were examined in vivo.

MATERIALS AND METHODS

Cell Culture Human breast cancer (MDA-MB-453) cell lines were obtained from Riken Cell Bank. Cells were cultured in RPMI-1640 medium (GIBCO, U.S.A.) and 10% fetal bovine serum (FBS, HyClone Laboratories Inc., U.S.A.) in humidified atmosphere containing 5% CO2 at 37°C.

Preparation of TTZ-LAK Cells We collected peripheral blood mononuclear cells (PBMCs) samples from a healthy donor. This protocol was reviewed and approved by the Institutional Review Board of Omote Sando Yoshida Hospital for use in these studies. To prepare TTZ-LAK cells, TTZ (5 µg/mL) dissolved in phosphate buffered saline (PBS)(-) and human anti-CD3 antibodies (Janssen Pharmaceutical KK, Japan) were added to flasks and immobilized at room temperature for 4 h. In CD3-LAK, human anti-CD3 antibodies only dissolved in PBS(-) and were added to flasks and immobilized at room temperature for 4 h. After discarding these solutions, flasks were washed with PBS, and then PBMCs (1×10^6 cells/mL) were cultured for a humidified 5% CO2 incubator at 37°C in culture media including IL-2 1000 IU/mL. Proliferated lymphocytes were cultured in a CO2 permeability culture bag for scale-up for two weeks.

Animal The mice were handled in accordance with the guidelines for animal experimentation set out in Japanese law. The animal studies were approved by the committee on animal research of the Sojo University. BALB/c-R/J mice were kindly provided by Prof. Okada (Kumamoto University, Japan). Animals were housed with a 12 h dark/light cycle with food and free access to food and water.

Assessment of Antitumor Effects in Vivo MDA-MB-453 cells (5.0×10^6 cells) suspended into matrigel (BD Co., U.S.A.) were subcutaneously inoculated to dorsal of mice. The mice were randomly grouped on the basis of the tumor volume using the stratified randomization method after confirming increase of tumor volume in mice. Number of mice was five in each group. The tumor volume was measured using Vernier caliper and calculated using the equation of \( V = 0.5 \times a^2 \times b \), where \( a \) and \( b \) denote the smallest and longest superficial diameter, respectively. The tumor volume reached 100–150 mm^3 at day 6 after the inoculation of MDA-MB-453 cells, and then TTZ-LAK and CD3-LAK cells were intravenously administered twice each week for 14 d from day 6. The solid tumors were weighed after anatomizing the mice after 21 d of inoculation of MDA-MB-453 cells.

Immunohistochemical Staining MDA-MB-453 cells (5.0×10^6 cells) suspended into matrigel (BD Co., U.S.A.) were subcutaneously inoculated to dorsal of mice. The mice were randomly grouped on the basis of the tumor volume using the stratified randomization method after confirming increase of tumor volume in mice. Number of mice was five in each group. TTZ-LAK and CD3-LAK cells were intravenously administered twice each week for 14 d from day 6. The solid tumor was removed from anaesthetized mice after the treatment with TTZ-LAK and CD3-LAK cells and fixed in 10% formalin solution. Paraffin-embedded sections were cut, de-waxed in xylene and rehydrated through a series of ethanol to water. Tumor sections were heated at 120°C for 10 min for antigen activation and were blocked with a solution PBS and 1% H2O2 for 5 min. The sections were washed with PBS(-) and incubated with anti-human primary antibody against CD3 (anti-CD3 mAbs) (N1580, Dako, Denmark) in a humidified box at 4°C for overnight. The sections were washed twice with PBS, immunostained with histofine simple stain MAX-PO (Rabbit, Nichirei, Japan) in a humidified box at 4°C for overnight. The sections were washed twice with PBS, immunostained with histofine simple stain MAX-PO (Rabbit, Nichirei, Japan) in a humidified box at 4°C for overnight. Finally, the detection of the antigen-antibody link was made through immunoperoxidase followed by 3,3'-diaminobenzidine (DAB) chromogen. The tumor sections were observed by an optical microscope (Nikon TS-100, Tokyo, Japan).

Fig. 1. Reduction of Tumor Volume in Xenograft Mouse Models of Human Breast Cancer Intravenously Treated with TTZ-LAK Cells after the Subcutaneous Inoculation of the MDA-MB-453 Cells Overexpressing HER2 in Vivo
Arrows indicate treatment days.

Fig. 2. Therapeutic Effects of TTZ-LAK Cells for Xenograft Mouse Models of Human Breast Cancer in Vivo
(A) Tumor of xenograft mouse models of breast cancer treated with TTZ-LAK cells. Scale bar, 5 mm. (B) Tumor weight of xenograft mouse models of breast cancer treated with TTZ-LAK cells.
Terminal Deoxynucleotidyl Transferase-Mediated Deoxyuridine Triphosphate Nick-End Labeling (TUNEL) Methods Detection of apoptotic cells was performed on the basis of the TUNEL method using apoptag peroxidase in situ apoptosis detection kit (S7100, Merck, Millipore, U.S.A.) according to the manufacturer’s directions. MDA-MB-453 cells (5.0×10⁶ cells) suspended into matrigel (BD Co., U.S.A.) were subcutaneously inoculated to dorsal of mice. TTZ-LAK and CD3-LAK cells were intravenously administered twice each week for 14 d from day 6. Tumor was removed from anesthetized mice after the treatment with TTZ-LAK and CD3-LAK cells and fixed in 10% formalin solution. Paraffin-embedded sections were made, and the detection of apoptosis of a solid tumor was performed on the basis of TUNEL assay according to the conventional method. The tumor sections were stained with 3,3′-DAB chromogen and observed by an optical microscope.

Statistical Analysis Results are presented as mean±S.D. Data were statistically analyzed using Student’s t-test. A p value less than 0.05 was considered to represent a statistically significant difference.

RESULTS AND DISCUSSION

Therapeutic Effects of TTZ-LAK for Xenograft Mouse Models of Breast Cancer in Vivo We have already reported on inhibitory effects of TTZ-LAK cells on the growth of SK-BR-3 and MDA-MB-453 cells over-expressing HER2 in vitro.²²) It has been reported that HER-2 is overexpressed in MDA-MB-453 cells on the basis of enzyme-linked immunosorbent assay (ELIZA), Western blotting and immunostaining in vitro and in vivo.²⁶–²⁹) We examined inhibitory effects of intravenous treatment with TTZ-LAK cells on the growth of tumor in subcutaneous xenograft mouse models of human breast cancer overexpressing HER-2. The tumor volume reached 100–150 mm³ at day 6 after the inoculation of MDA-MB-453 cells, and then TTZ-LAK and CD3-LAK cells were intravenously administered twice each week for 14 d from day 6. First, we examined the therapeutic effects of TTZ-LAK cells on the xenograft mouse models of breast cancer using subcutaneous tumor volume. The results are shown in Fig. 1. The mean of tumor volume was 242±97 mm³ in the control group and 211.8±64.5 mm³ and 138±35 mm³ in the group treated with CD3-LAK and TTZ-LAK cells, respectively. The more remarkable reduction rate of 45% (p<0.05) in tumor volume was obtained in mice treated in TTZ-LAK cells, in contrast with that of 5% (p>0.1) in mice treated with CD3-LAK cells. Statistical significance between CD3-LAK and TTZ-LAK cells was obtained (p<0.05).

Next, we examined the therapeutic effects of TTZ-LAK on the xenograft mouse models of human breast cancer in autopsy and tumor weight. The results are shown in Fig. 2. The tumor enlargement of the group treated with TTZ-LAK cells remarkably was inhibited, although the enlargement of tumor with angiogenesis in the untreated control group and group treated with CD3-LAK cells were confirmed (Fig. 2A). Furthermore, the tumor weight was 0.14±0.03 g in the control group and 0.13±0.04 g in the group treated with CD3-LAK, respectively (Fig. 2B). On the other hand, that was 0.07±0.02 g in the group treated with TTZ-LAK cells (Fig. 2B). There was a significant difference (p<0.01) in the tumor weight between the control group and the group treated with TTZ-LAK cells.

Fig. 3. Micrographs of Solid Tumor in Xenograft Mouse Models of Human Breast Cancer Treated with TTZ-LAK Cells after the Subcutaneous Inoculation of MDA-MB-453 Cells Using Immunostaining with Anti-CD3 mAbs Arrows indicate CD3-positive cells. Scale bar: 100 µm.

Fig. 4. Micrographs of Solid Tumor in Xenograft Mouse Models of Human Breast Cancer Treated with TTZ-LAK Cells after the Subcutaneous Inoculation of MDA-MB-453 Cells Using TUNEL Method Arrows indicated apoptotic cells. Scale bar: 100 µm.
It was attractive that statistical significance between CD3-LAK and TTZ-LAK cells was obtained ($p<0.05$).

It is noteworthy that a remarkable reduction of tumor volume was observed in xenograft mouse models of human breast cancer intravenously treated with TTZ-LAK cells after subcutaneously inoculating MDA-MB-453 cells.

**Immunohistochemical Staining with Anti-CD3 mAbs**

To elucidate the direct attack (lymphocyte migration) of LAK cells against tumor ones, we have carried out immunohistochemical staining with anti-CD3 mAbs. The results are shown in Fig. 3. Many CD3 positive lymphocytes (brown color) were observed in tumor of mice treated in TTZ-LAK cells, although few lymphocytes were observed in the group treated with CD3-LAK cells. These results indicate that TTZ-LAK cells (lymphocyte) migration into the tumor could be implicated in therapeutic effects of TTZ-LAK cells on the xenograft mouse models of human breast cancer.

**Induction of Apoptosis by TTZ-LAK Cells**

We examined the induction of apoptosis by TTZ-LAK cells for solid tumor in xenograft mouse models of human breast cancer using the TUNEL method. The results are shown in Fig. 4. Fairly more numerous apoptotic cells (brown color) in the tumor cells of the group treated with TTZ-LAK cells were observed compared with case of the group treated with CD3-LAK cells. On the other hand, apoptotic cells were not observed in the control group. With regard to the group treated with TTZ-LAK cells, it is noteworthy that the positive area of lymphocyte (Fig. 3) was the same as that of apoptotic cells (Fig. 4). These results indicate that TTZ-LAK cells should have remarkable inhibitory effects along with apoptosis due to lymphocyte migration on the growth of MDA-MB-453 cells.

We have previously reported that remarkable increases in the number of natural killer and γδT cells in TTZ-LAK cells were observed.\(^{22}\)

Activation of the lymphocytes having Fc receptor (FcR) in TTZ-LAK cells could enhance cellular cytotoxicity. On the other hand, T cells which recognized a specific antigen (HER2) produced by human breast cancer cells in TTZ-LAK cells could also induce cytotoxic T-lymphocyte (CTL) by antigen presentation to lymphocyte.

In conclusion, our study demonstrates the remarkable therapeutic effects of TTZ-LAK cells on the xenograft mouse models of human breast cancer in vivo for the first time. The noteworthy aspects are as follows. (1) Remarkably high therapeutic effects of TTZ-LAK cells were obtained in the xenograft mouse models of human breast cancer. (2) TTZ-LAK cells (lymphocyte) migration was observed in the tumor of xenograft mouse models of human breast cancer. The results in this study should be advantageous in the cancer immunotherapy for patients with breast cancer overexpressing HER2 in the near future.

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