Phospholipase D (PLD) as a candidate molecule to reduce the resistance to trastuzumab

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ABSTRACT

Trastuzumab is the crucial therapeutic agent both on adjuvant and advanced/recurrent settings in combination with cytotoxic agent for HER2-positive breast cancer. It is a powerful drug, however, the resistance develops during treatment. The crosstalk of signal transduction including PI3K/Akt pathway, the genesis of p95-HER2, and change in the HER2 status have been reported as the resistance mechanism to trastuzumab. It is the urgent necessity to avoid the resistance since recently the breast cancer patients have been increasing. The contribution of phospholipase D (PLD) to the resistance of trastuzumab was investigated in order to find the improvement of the efficacy of anti-HER2 therapy with trastuzumab in the current study. The breast cancer model cell lines, MDA-MB-231 (ER-PgR-HER2-) and MDA-MB-453 (ER+PgR+HER2-) were cultured with trastuzumab and the growth inhibition and PLD activity was estimated by the conventional method. Trastuzumab inhibited the cell growth dependent on HER2. The PLD activity was high in MDA-MB-231 cells but under detectable level in MDA-MB-453 cells. The overexpression of PLD reduced the sensitivity to trastuzumab of the MDA-MB-453 cells. It was suggested that PLD located between HER2 and intracellular signaling pathways and modulated the cell proliferation including cancer. In conclusion, the inhibition of PLD could be a promising method to reduce the resistance to trastuzumab.

INTRODUCTION

The breast cancer has become the most frequent malignant tumor and the most frequent cause of death for women in the prime of their life in the world. Recently the breast cancer has been categorized according to the gene expression profile[1),2)] and clinically four major groups of luminal A-like, luminal B-like, HER2-enriched, and triple negative, were important for decision-making because they reflect the sensitivity to anti-hormonal, anti-HER2, and cytotoxic agents.

Trastuzumab is the pioneering molecular target drug for HER2-overexpressing cancers and becomes the crucial therapeutic agent both on adjuvant and advanced/recurrent settings in combination with cytotoxic agent for breast cancer. Although it is powerful, the resistance develops during the treatment. It is the urgent necessity to avoid the resistance since recently the breast cancer patients have been increasing. The crosstalk of signal transduction, the genesis of p95-HER2, and change in the HER2 status have been reported

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as the mechanism for the development of the resistance to trastuzumab[3]. Phospholipase D (PLD) is one of the essential enzymes for cell functions such as cell proliferation and survival and its involvement in cancer development has been elucidated[4]. There are two isozymes with PLD activity, PLD1 and PLD2. PLD1 and PLD2 were reported the involvement in the angiogenesis and metastasis[5], and migration and invasion of the tumor[6], respectively, however the relationship between phospholipase D (PLD) and the resistance to trastuzumab has not been elucidated. Using breast cancer cell lines, the contribution of PLD to the sensitivity to trastuzumab was investigated.

MATERIALS AND METHODS

Cell cultures

MDA-MB-231 and MDA-MB-453 cells were obtained from ATCC and cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2 at 37℃.

Sensitivity to trastuzumab

The cells were inoculated to the 12-well plate by 3×10^4/well in duplicate with 0, 1, 10, or 100 μg/ml trastuzumab-containing culture medium and cultured for 4 days and harvested for viable cell count using 200 μl of trypsin-EDTA solution. Trastuzumab was given from the pharmaceutical company.

Viable cell count

The harvested cells were mixed with 600 μl of the culture medium and 200 μl of 1% trypan blue in phosphate buffered saline (PBS), and the number of viable cells was counted with a haemocytometer under a light microscope.

Quantification of PLD activity

The PLD activity of the cells was measured by the conventional method[7]. Briefly the procedures were described below. The cells were metabolically labelled with [^{14}C] lysophosphatidylcholine (0.5 μCi/1×10^7 cells; GE Healthcare, Buckinghamshire, UK) for 18 h in the absence of serum. After washing with PBS, PLD reaction was initiated by adding DMEM containing 0.3% 1 - butanol in the presence of FCS. After 120 min of incubation at 37℃, reactions were terminated by the addition of 1 ml of ice - cold chloroform–methanol–HCl (1:1:0.006, by volume). Lipids were extracted and analysed as described earlier[8]. PLD activity was expressed as a percentage of [^{14}C] phosphatidylbutanol (PBt) in the total radioactivity found in all spots in one lane.

Overexpression of PLD

The cells were cultured with adenovirus harboring PLD1 or PLD2 gene in the culture medium for 4 days. Both constructs as well as control vector were the generous gifts from Dr T Okada of Kobe University. The expression of PLD were evaluated by measuring the PLD activity of infected cells as mentioned above.

RESULTS

Growth inhibition of the breast cancer cell lines by trastuzumab

The viable cell number of the MDA-MB-231 and MDA-MB-453 cells after cultured with trastuzumab was shown in Figure 1. The proliferation of MDA-MB-453 cells was inhibited dose-dependently with trastuzumab. In contrast, the proliferation of MDA-MB-231 cells was not affected with trastuzumab. The result indicated that this assay method could evaluate trastuzumab activity properly because MDA-MB-453 cells express HER2[9),10)] and MDA-MB-231 cells do not express HER2[10)]. The concentration of the trastuzumab in the culture medium was fixed at 100 μg/ml thereafter since the concentration of 100 μg/ml

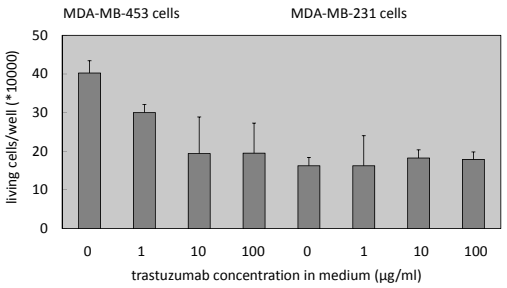

Figure 1　Growth inhibition of breast cancer cell lines by trastuzumab

The breast cancer cell lines, MDA-MB-453 cells and MDA-MB-231 cells were cultured in the DMEM-containing 10% FCS and various concentration of trastuzumab for four days. The cells were harvested, labelled with trypan-blue and the number of the viable cells was counted. The experiment was repeated several times in duplicate and the representative result was shown. The average number of the viable cells per well was indicated with SD.

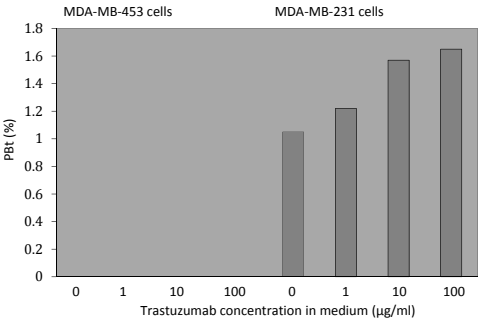

Figure 2　PLD activity in breast cancer cell lines cultured with trastuzumab

At the end of the culture in the DMEM containing 10% FCS as well as various concentration of trastuzumab, PLD activity of the breast cancer cell lines, MDA-MB-453 and MDA-MB-231 cells was quantified by the conventional method. The experiment was repeated several times and the representative result was shown. PLD activity was indicated as the percentage of phosphatidylbutanol.

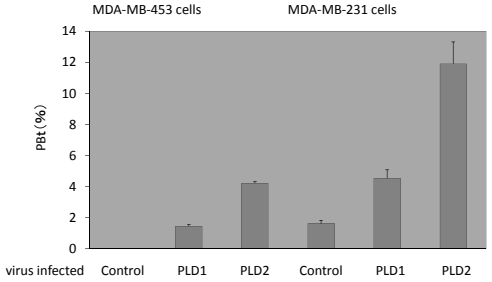

Figure 3　PLD activity in breast cancer cell lines infected with PLD-harboring adenovirus

The breast cancer cell lines, MDA-MB-453 and MDA-MB-231 cells were infected with PLD1 or PLD2-harboring adenovirus, and cultured for four days. Control in the figure means cells were infected with control virus without PLD gene. At the end of the culture the cells in the well were labelled to measure the PLD activity in duplicate. The experiment was repeated several times in duplicate and the representative result was shown. PLD activity was indicated as the percentage of phosphatidylbutanol. The average activity was indicated with SD.

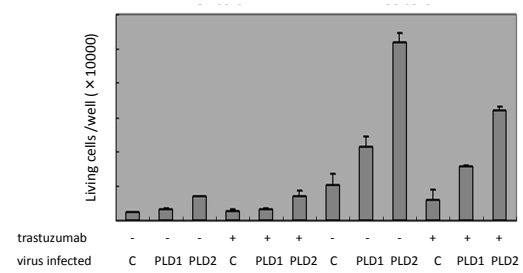

Figure 4　Effect of PLD overexpression on growth inhibition by trastuzumab in breast cancer cell lines

The breast cancer cell lines were infected as in the figure 3 and after culture with or without trastuzumab (100 μg/ml) the viable cells in the well were counted. The average number of the viable cells per well was indicated with SD. The experiment was repeated several times and the representative result was shown.

trastuzumab is almost equivalent to the blood concentration when the loading dose is infused.

PLD activity in breast cancer cell lines

PLD activity in the MDA-MB-231 and MDA-MB-453 cells was shown in Figure 2. PLD activity in the MDA-MB-453 cells was under detectable level with and without trastuzumab in the culture medium. MDA-MB-231 cells showed high PLD activity and the activity tended to increase in dose-dependent manner with trastuzumab.

Overexpression of PLD in breast cancer cell lines

The overexpression of PLD by adenovirus infection was confirmed by PLD activity of the cells. As indicated in Figure 3, PLD1 and PLD2 were successfully overexpressed in both cell lines. The remarkable increment in PLD activity was observed in MDA-MB-231 cells. This assay was performed in duplicate at the same time for growth inhibition assay by trastuzumab, i.e., the cells were inoculated in 12-well plate in quadruplicate and those in two wells were used for PLD assay and the viable cells in the remaining two wells were counted.

PLD activity and growth inhibition of the breast cancer cell lines by trastuzumab

The effect of PLD overexpression on growth inhibition by trastuzumab was indicated in Figure 4. The growth of the MDA-MB-231 cells was not affected with trastuzumab and with PLD overexpression. In contrast, the proliferation of the MDA-MB-453 cells was suppressed with trastuzumab and the inhibition was reduced with both PLD1 and PLD2 overexpression. Figure 5 shows the change in cell number with PLD overexpression indicated as percentage. PLD1 rather than PLD2 inhibited the trastuzumab activity for MDA-MB-453 cells.

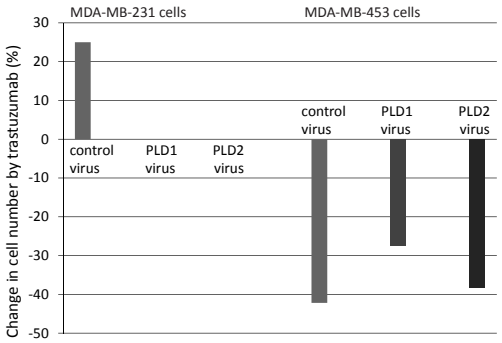

Figure 5　Effect of PLD overexpression on growth inhibition by trastuzumab
The effect of PLD overexpression on growth inhibition by trastuzumab was shown by the reduction percentage of viable cells by the drug. Each value was calculated using the data in fugure 4 that the viable cell number in the well with trasuzumab was divided by that without the drug and multiplied by hundred. The viable cell reduced with trastuzumab and the percentage was indicated negatively.

DISCUSSION

The relationship between the increment in PLD activity and the effectiveness of trastuzumab

HER2 is one of the ErbB receptor family, a receptor tyrosine kinase[11]. Ras/Raf/MAPK and PI3K/Akt pathways are downstream the ErbB receptor, the former and the latter play the significant role mainly on cell survival and proliferation, and invasion and migration, respectively. Phospholipase D (PLD) is an essential enzyme for cell function including growth, survival, proliferation[4)~6)], catalyzes phosphatidylcholine (PC), a phospholipid hydrolysis to generate phosphatidic acid (PA). PA regulates the Ras/Raf/MAPK and PI3K/Akt pathways by activation of CRAF(upstream of MAPK pathway) and mTOR (downstream of PI3K/Akt pathway)[12]. In addition, PA increased the epidermal growth factor receptor expression[13]. On the contrary, the ligand for ErbB receptor family such as EGF is a strong

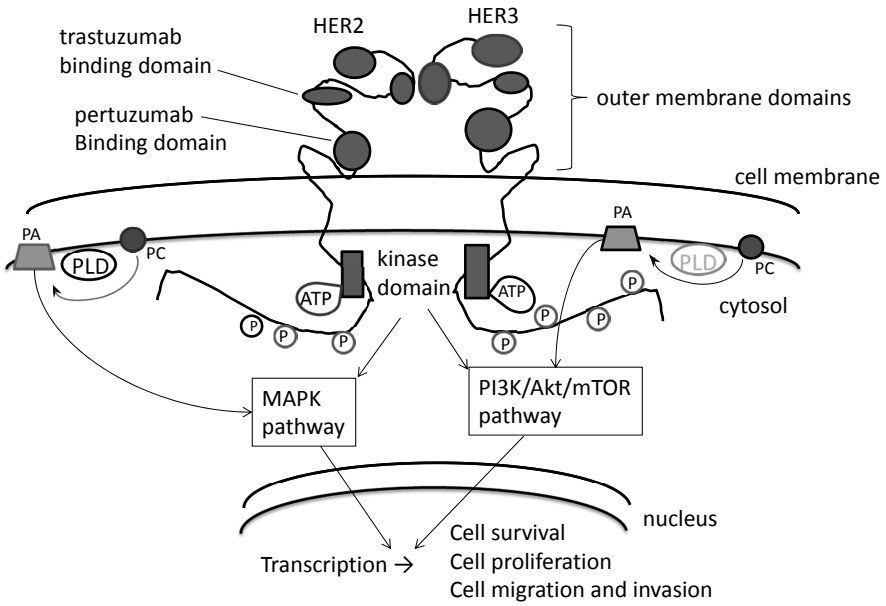

Figure 6　PLD and HER2 signaling pathway

The figure indicated that HER2 signaling pathway requires both MAPK and PI3K/Akt/mTOR systems to translate the outer signal like EGF into intracellular signal in order to execute cell functions, e. g., survival, proliferation, and metastasis. PLD locates between the cell surface receptors and intracellular systems and regulate the cell function to activate the enzymes involved in both systems by phosphatidic acid (PA), a product from phosphatidylcholine (PC) by PLD.

activator for PLD[14]. Namely, PLD locates between the cell surface receptors and signaling pathways and mediates the extracellular signals into cells. Once PLD is activated, the signal transduction proceeds for cell function including survival, proliferation, and carcinogenesis[4]. The PLD activity of the breast cancer was high, especially after development of resistance to chemotherapy and metastasis[15)~17)]. PLD is reported to be important for ErbB2 signaling through overexpression and stabilization of EGFR by PA[13] and the inhibition of the PLD by small inhibitors suppressed the ErbB2 signaling[18]. From these findings, it was suggested that overexpression of PLD may cause the activation of HER2 signaling and reduction in the sensitivity to trastuzumab. Therefore, the inhibitors for PLD might be effective for HER2+ breast cancer[14),19)].

PLD, a promising candidate for biomarker for the resistance to anti-HER2 therapy

The expression of HER2 is prerequisite but not sufficient for the effective anti-HER2 treatment and therefore the biomarker for the dependency of the tumor upon HER2 signaling pathway could be critical for effective treatment with anti-HER2 drugs such as trastuzumab. As mentioned above, PLD is a critical enzyme for ErbB2 signaling and the inhibition of PLD reduced the viability of the HER2-driven breast cancer. The several lines of evidence including this study suggested that PLD activity is promising candidate for biomarker for anti-HER2 therapy. The study on the relationship between PLD activity and the sensitivity to anti-HER2 therapy might be a significant contribution. Not only when to start the chemotherapy but also when to change

the regimen are crucial for longer OS, then the biomarker for the resistance shall be important. The current study suggested that PLD could be a biomarker for the resistance to anti-HER2 therapy.

Combination therapy with anti-HER2 drug and PLD inhibitor could restrain the resistance to anti-HER2 treatment

Current study suggested that PLD inhibitors might reinforce the anti-HER2 therapy. Frohman et al. reported the small molecule inhibitors against PLD[19] and Gomez-Cambronero described that the breast cancer cells with low PLD activity such as MCF-7 cells expressed microRNAs suppressing PLD expression which might be effective to the cancer treatment[14]. The mTOR inhibitors have been used for advanced breast cancer these days for reduction of the resistance to anti-hormonal therapy[20]. PA, the product of the PLD activity, is essential to mTOR activation[21]. Interestingly, MDA-MB-231 cells which had by 10-times higher endogeneous PLD activity than MCF-7 cells and the former and the latter cells were resistant and sensitive to rapamysin a mTOR inhibitor, respectively and the overexpression of PLD brought MCF-7 cells resistant to rapamysin whereas suppression of PLD resulted in MDA-MB-231 cells rapamysin-sensitive[22]. From these findings, PLD inhibitors may play a role to potentiate not only anti-HER2 treatment but also hormonal therapy.

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