HER2 signaling downregulation by trastuzumab and suppression of the PI3K/Akt pathway: An unexpected effect on TRAIL-induced apoptosis

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Abstract **We investigated whether HER2 downregulation by trastuzumab modulates the responsiveness of breast cancer cells to TNF-related apoptosis-inducing ligand (TRAIL). Interestingly, in contrast to increased response to TRAIL in SKBr3 cells, trastuzumab decreased the susceptibility of BT474 cells to TRAIL. This decrease was also observed after exogenous inhibition of PI3-K/Akt kinase, but not MAPK/ERK kinase (MEK)/mitogen-activated protein kinase (MAPK). In BT474 cells, but not SKBr3 cells, inhibition of the HER2/phosphatidylinositol 3′ kinase (PI3K)/Akt pathway resulted in downregulation of the pro-apoptotic receptors TRAIL-receptor 1 (TRAIL-R1) and TRAIL-R2. TRAIL-induced caspase-8 activation, Bid processing, drop of $\Delta\Psi_m$, and poly ADP-ribose polymerase (PARP) cleavage but not in caspase-9 activation, and these events were inhibited in HER2/PI3K/Akt-suppressed BT474 cells, which on the other hand exhibited downregulation of Bcl-x_L and increased response to mitomycin C. We show that HER2/PI3K/Akt pathway may play a specific pro-apoptotic role in certain cell type by inducing TRAIL-R1 and -R2 expression and thereby enhancing responsiveness to TRAIL.**

Keywords: TRAIL; Apoptosis; HER2/*neu*; Trastuzumab; PI3K; Breast cancer

1. Introduction

Aberrations in the apoptotic signaling promote tumorigenesis and underlie resistance of human tumors to anticancer agents. At the cell surface, signals for cell death can be transmitted through TNFR1, CD95/Fas, TNF-related apoptosis-inducing ligand-receptor 1 (TRAIL-R1)/DR4 and TRAIL-R2/DR5 which are members of the tumor necrosis factor (TNF) receptor family known as death receptors. Apoptosis via TRAIL-R1 and TRAIL-R2 is induced by their interaction with TRAIL, a death ligand that is interesting for cancer therapy for several reasons. First, unlike other members of the TNF ligand family, TRAIL preferentially induces apoptosis in transformed cells, whereas it is relatively benign to normal cells [1]. Second, the TRAIL effector pathway appears a physiological component of the immunosurveillance of tumor cells by natural killer and T cells [2–5].

Binding of TRAIL to its receptors TRAIL-R1 and TRAIL-R2 triggers formation of a death-inducing signaling complex (DISC) leading to activation/self-processing of proximal procaspase-8 and -10 [6]. Subsequently, in type I cells, the apoptotic cascade might proceed, in a mitochondria-independent manner, directly to the processing of effector caspases and downstream apoptotic events; or alternatively, in type II cells, it involves insertion of caspase-8-processed Bid into the mitochondrial membrane followed by release of apoptogenic molecules and the activation of effector caspases. Thus, starting at the cell membrane, TRAIL-induced cell death can be modulated at any of these several stages. The transcriptional activities of p53 [7] and NF-κB [8,9] are mostly responsible for regulating the surface levels of TRAIL-R1 and TRAIL-R2, and are frequently modulated in cancer cells. Furthermore, susceptibility to TRAIL might be negatively regulated by abundant expression of the decoy receptors TRAIL-R3 and TRAIL-R4 [10,11]. At the DISC, a high level of a competitive caspase-8 inhibitor Flice-inhibitory protein $(FLIP)_{L/S}$ has been shown to diminish TRAIL-induced apoptosis [12]. As apoptotic signaling from TRAIL-Rs often requires mitochondria-mediated amplification, a high level of mitochondrial anti-apoptotic Bcl-2 family proteins, such as Bcl-2 and Bcl-x_L, can suppress TRAIL-induced apoptosis. Similarly, overexpression of inhibitor of apoptosis protein (IAP) family proteins that inhibit caspase activity, particularly XIAP and survivin negatively regulates responsiveness to TRAIL [13]. Likewise, resistance to TRAIL has been correlated with impeded release of mitochondrial Smac/DIABLO [14] and Omi/HtrA2 [15], proteins that promote caspase activity by inhibiting the IAP proteins.

In various cancer types including breast carcinomas, overexpression of the HER2 (neu/ErbB-2) receptor tyrosine kinase, a member of the ErbB (epidermal growth factor receptor) family, underlies enhancement in proliferative, prosurvival and metastatic signaling [16,17]. Thus, patients with HER2-overexpressing carcinomas exhibit poor clinical outcome, exemplified by a lower overall survival rate and shorter time to relapse, compared to those with HER2 negative tumors [18,19].

The humanized anti-HER2 antibody trastuzumab (Herceptin®) was developed to abrogate the growth of

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Abbreviations: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TRAIL-R, TRAIL-receptor; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI3K, phosphatidylinositol 3′ kinase; FLIP, Flice-inhibitory protein; IAP, inhibitor of apoptosis protein; $\Delta\Psi_m$, mitochondrial membrane potential; PARP, poly ADP-ribose polymerase

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HER2-overexpressing cancer cells [20,21]. To date, trastuzumab has been approved for use in the treatment of metastatic breast cancer when combined with chemotherapy [22,23] and as a single agent in the first-line therapy of HER2-overexpressing advanced breast cancer [24,25]. Nevertheless, HER2 expression levels might only partially determine the therapeutic efficacy of trastuzumab; in clinical settings of trastuzumab monotherapy of metastatic breast cancer, close to two-thirds of patients with HER2 overexpression do not experience tumor regression, and the majority of patients initially responding to this drug subsequently develop resistance [26]. Although the biological effects of trastuzumab alone or its combination with chemotherapy are not completely understood, both extracellular and intracellular mechanisms seem to play a role in the antitumor activity of trastuzumab. In vitro studies demonstrated that the major part of antitumor action of trastuzumab results from upregulation of cyclin-dependent kinase inhibitor $p27^{Kip1}$ and subsequent cell cycle arrest in breast cancer cell lines [27–29]. However, in primary breast tumors [30] and in a model of cellular-dependent cytotoxicity of glioblastoma cell lines [31], trastuzumab has been shown to increase apoptosis rate but not cell cycle inhibition. In the whole-body context, binding of trastuzumab on the surface of Her-2-overexpressing cell stimulates NK cell-mediated antibody-dependent cellular cytotoxicity [32–34] and complement-dependent toxicity [35]. Nevertheless, both pro-apoptotic and antiproliferative actions of trastuzumab on the intracellular level are attributed to inhibition of transforming signals downstream of HER2, including constitutively activated Raf/MEK/mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3′ kinase (PI3K)/Akt kinase pathways [32,36]. Particularly, the prosurvival activity of PI3K/Akt has been frequently described resulting from regulation of number of proteins involved in the cell cycle, apoptosis, protein synthesis and glycogen metabolism [37], including $p27^{Kip1}$ [28] and Bad whose Akt-mediated phosphorylation on Ser136 prevents its pro-apoptotic action on the mitochondrial membrane [38].

Here, we investigated whether HER2-overexpressing breast carcinoma cells responded to trastuzumab by enhanced susceptibility to TRAIL which was reported for other cell lines [39]. Finding unexpected suppressive effect of trastuzumab on TRAIL-induced apoptosis in BT474 cells, we further analyzed signaling pathways downstream of the HER2 receptor and apoptosis-related proteins and events regulated by trastuzumab.

2. Materials and methods

2.1. Cell culture and treatment

Breast adenocarcinoma-derived cell lines were cultured in medium supplemented with 10% fetal bovine serum (Gibco, NY, USA), glutamine, and antibiotics (Sigma, St. Louis, MO, USA) at 37 °C in 5% CO_2; specifically BT474 and MCF-7 cells in Dulbecco's modified Eagle's medium (Sigma) and SKBr-3 cells in McCoy's medium (Sigma). In experiments determining the effect of pretreatments on TRAIL-induced apoptosis, subconfluent cultures were incubated with trastuzumab (Herceptin® Genentech, CA, USA), LY-294002 (Alexis, Switzerland), or PD-98059 (Calbiochem, CA, USA). Apoptosis was induced by addition of human recombinant TRAIL, prepared as in [40], at a final concentration of 20 ng/ml.

2.2. Apoptotic features

Cells were examined for apoptotic morphology using phase-contrast microscopy and photographed. Apoptotic cells exhibiting fragmentation of intracellular cytokeratin-18 were analyzed using the M30 Cyto-death assay (Roche Diagnostics, Germany). Briefly, cells were trypsinized, washed in Hank's balanced salt solution (HBSS) containing 0.1% Tween 20 (HBSS-Tween), fixed for 30 min in pure methanol at −20 °C, washed twice in HBSS-Tween, and incubated for 1 h at room temperature in the dark with fluorescein isothiocyanate (FITC)-conjugated M30 antibody diluted 1:250 in HBSS-Tween containing 1% bovine serum albumin (BSA). After two washes in HBSS-Tween, the percentage of M30-positive cells was analyzed on Epics XL flow cytometer (Coulter, FL, USA) using the manufacturer's analysis software. For DNA content analysis, floating cells were pooled together with trypsinized adherent cells, washed twice with HBSS, and incubated for at least 4 h at 4 °C in DNA staining solution [50 μg/ml propidium iodide (PI), 10 mM Tris, 0.1% TRITON X-100, 0.07% RNase, 0.1% sodium chloride] and analyzed by flow cytometry. The percentage of sub-G_1 gated signals reflects the proportion of apoptotic cells.

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were assessed according to accumulation of tetramethylrhodamine ethyl ester (TMRE; Molecular Probes, OR, USA) in the negatively charged mitochondrial matrix of intact living cells. Cells were incubated with 400 nM TMRE for the last 30 min in the cell culture medium at 37 °C. Then, attached cells were harvested by trypsinization, washed once in PBS (phosphate buffered saline) supplemented with 100 nM TMRE (PBS–TMRE), and pooled with floating cells. Cells were resuspended in 1 ml of PBS–TMRE, stained with PI at a final concentration of 0.5 μg/ml and analyzed by flow cytometry. Dead cells were excluded according to their high PI fluorescence (FL-3 channel). TMRE fluorescence was measured at 575 nm (FL-2 channel).

Expression of mitochondrial apoptosis-related protein 7A6 was detected using PECy5-conjugated Apo2.7 mAb (Immunotech, France). Adherent cells were trypsinized, pooled with floating cells, washed once in PBS containing 0.2% BSA (PBS-BSA) and permeabilized by incubation with 100 μl of 100 μg/ml digitonin (Sigma) solution for 20 min on ice. Cells were washed in PBS-BSA and stained with 10 μl of either Apo2.7-PECy5 Ab or PECy5 isotype control for 15 min at room temperature. After one wash in PBS-BSA, Apo2.7 staining was detected at 675 nm using flow cytometry.

2.3. Immunofluorescent staining

The cell surface expression of HER2 receptor was measured as the amount of selective binding sites for trastuzumab, a humanized antibody containing the murine antigen binding loops and human variable region framework residues plus human IgG_1 constant domains [41], followed by secondary staining with an antibody reacting specifically with the Fc portion of the heavy chain of human IgG_1 (Molecular Probes). First, breast cancer cells were trypsinized and washed twice with ice-cold HBSS containing 0.2% BSA (HBSS-BSA). Resuspended cells were incubated with trastuzumab for 30 min at 4 °C. Next, cells were washed twice, incubated with Alexa Fluor® 488-conjugated mouse anti-human IgG_1 secondary mAb, washed twice in HBSS-BSA, stained with PI at a final concentration 0.5 μg/ml and analyzed. Dead cells and debris were excluded according to their increased PI fluorescence and forward scatter properties. Alexa Fluor® 488-conjugated mouse anti-human IgG_1 mAb alone was used as an isotype-matched control.

To determine surface expression of TRAIL-Rs, adherent cells were trypsinized and washed twice with ice-cold HBSS-BSA. Resuspended cells were incubated with FITC-conjugated anti-human TRAIL-R1 (clone HS101), anti-human TRAIL-R2 (clone HS201) mAbs (Alexis), or FITC-IgG1 isotype control Ab (Immunotech, ME, USA) for 1 h at 4 °C, washed twice, stained with PI and analyzed as described above.

To determine the expression of intracellular proteins, adherent cells were harvested by trypsinization, washed in PBS, and fixed by resuspending in PBS containing 0.4% formaldehyde for 10 min at 37 °C. After washing, cells were permeabilized by incubation with pure methanol for 30 min at −20 °C. After one wash in PBS-BSA, cells were stained according to the manufacturer's recommendations with FITC-conjugated anti-human Bcl-x_L mAb (Chemicon, CA, USA) or with FITC-conjugated anti-human Bcl-2 mAb (DAKO A/S, Denmark) and with isotype-matched Abs, washed twice in PBS-BSA and analyzed by flow cytometry.

2.4. Western blot

Whole-cell lysates were separated by SDS–PAGE gels and transferred onto nitrocellulose membranes (BioRad, CA, USA). The membranes were blocked with 5% non-fat milk in PBS containing