Anti-estrogen-resistant breast cancer cells are sensitive to cisplatin plus TRAIL treatment

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Abstract. Breast cancer patients who are positive for estrogen receptor (ER) are usually treated with anti-estrogen drugs, such as tamoxifen (Tam). However, a great majority of such patients eventually develop resistance to Tam. In this study, MCF-7 cells (with de novo and/or acquired resistance to Tam) as well as T47D cells (acquired resistance to Tam) models were used to investigate the effect of treatment with cisplatin plus tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). The results in the two cell types treated with cisplatin plus TRAIL showed significantly increased cell death compared to that in the untreated control cells. A similar treatment had a minimal effect on normal breast cells, the increased cell death appeared to be caused by the activation of caspases and, inhibition of the activity of caspases (using relatively specific inhibitors) reduced the cell death caused by cisplatin plus TRAIL treatment. Taken together, the results suggested that cisplatin plus TRAIL treatment has the potential to provide a novel treatment strategy to improve the treatment outcome in anti-estrogen-resistant breast cancer patients.

Introduction

Hormonal influence on breast tissue and in breast cancer development has long been recognized. Examinations of ~70% of breast cancer patients have been found to be positive for estrogen receptor (ER)/progesterone (PR) receptor (1,2). Additionally, the receptor status has been used as a biomarker for therapy and prognosis. Currently, ER/PR+ breast cancer patients are treated with anti-estrogen drugs, such as tamoxifen (Tam) or aromatase inhibitors (AIs) (3,4). Tam binds to ER, thereby preventing the binding of estrogen and subsequent activation of ER. Aromatase inhibitors (AI) suppress the activity of aromatase enzyme and thus, the production of estrogen. Although these inhibitors were shown to be effective, some patients develop resistance and do not respond to Tam therapy or other targeted endocrine therapies. The de novo resistance appears to be due to the up-regulation/overexpression of the HER2 receptor and/or Akt protein. Clinical and in vitro studies have also suggested that overexpression of the HER2 receptor is associated with resistance to hormone therapy (5-7). In addition, the majority of breast cancer patients with ER expression initially respond to endocrine therapy but, develop ‘acquired’ resistance over a period of time (8). MCF-7 and T47D breast cancer cell lines have been used as experimental models to investigate the mechanism(s) involved in acquired resistance to Tam (5,9,10). The results from such studies reported by our and other research groups have shown that drug resistance in these cells can be acquired through various mechanisms, including: i) loss of ERα expression, ii) altered activity of co-regulators, iii) cross-talk between the ERα and growth factor signaling pathways and, iv) overexpression of PKCα or PKCδ (3,5,11). These anti-estrogen-resistant models are also useful for the identification of other ‘potential’ therapeutic agents that can inhibit or eliminate anti-estrogen-resistant breast tumors.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) induced apoptosis through the death receptors, DR4 and DR5, which are present at the surface of target cells (12,13). Recombinant human TRAIL was shown to induce apoptosis preferentially in cancer over normal cells, and appear to have little or no overt toxicity when systemically administered to animals (12,14). Consequently, numerous clinical trials targeting TRAIL receptors, including TRAIL, as well as agonistic antibodies to DR4 and DR5 have been conducted. However, a significant portion of tumor cells, including breast cancer cells, were reported to be resistant to these agents (13,15-17). In this context, the use of platinum complexes for breast cancer therapy has emerged as a new treatment modality. Cisplatin, either independently or in combination with other chemotherapeutic drugs, was shown to be effective in the treatment of breast cancer (18,19). Cisplatin induces intra- and inter-strand cross-links in DNA leading to
cell death. The aim of the present study was to investigate whether cisplatin plus TRAIL treatment induced cell death and apoptosis in Tam-resistant cells and whether such a combinatorial treatment provides a new strategy for future therapeutic intervention of patients with anti-estrogen-resistant breast cancer.

Materials and methods

Cell lines and reagents. The MCF-7 human breast carcinoma cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 5% FCS and insulin, and penicillin and streptomycin (Life Technologies, Gaithersburg, MD, USA). Stably transfected MCF-7 cells with full-length HER2 cDNA (MCF-7/HER2-18) or control vector (MCF-7/neo) have been previously described (9). Tam/MCF-7 cells were grown in the presence of 4-OH Tam (10^-6 M) (20). The cells obtained from ATCC were immediately expanded and frozen, and restarted every 3-4 months from a frozen vial of the same batch of cells and no additional authentication was done on these cells. 4-OH Tam was purchased from Sigma Chemical Co. (St. Louis, MO, USA). The anti-glyceraldehyde-3-phosphate dehydrogenase (G3PDH) rabbit polyclonal was purchased from Trevigen (Gaithersburg, MD, USA). Monoclonal antibodies of PARP and Bid were purchased from BD Biosciences (San Diego, CA, USA). Caspase-9 antibodies were purchased from Cell Signaling Technology, Inc. (Boston, MA, USA). Caspase-3 inhibitor Z-DEVD-FMK, Caspase-8 inhibitor Z-IETD-FMK, Caspase-9 inhibitor Z-LEHD-FMK, and pan-caspase inhibitor Z-VAD-FMK were purchased from R&D System (Minneapolis, MN, USA). Unless otherwise specified, any other chemicals were obtained from Sigma Chemical Co. at highest suitable purities.

MTT assay. Briefly, 5x10^4 cells were added in 96-well tissue culture plates. After 24 h, the cells were treated with TRAIL (10 ng/ml), cisplatin (10 µg/ml), or a combination of TRAIL plus cisplatin for another 24 or 48 h. Then, 100 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (1 mg/ml) was added into each sample and incubated for 3 h under 5% CO_2 and 37°C. The cell viability was measured by MTT, which was converted by succinate dehydrogenase in the mitochondria of viable cells to yield a purple formazan dye. The formazan dye was dissolved in dimethyl sulfoxide (DMSO) and measured by absorption at a wavelength of 550 nm using Benchmark® microplate reader from Bio-Rad (Hercules, CA, USA).

Apoptosis assay. Apoptosis was assessed using the Cell Death Detection ELISAPLUS kit (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's instructions. This kit quantitatively detects cytosolic histone-associated DNA fragments. Briefly, the cells were treated with cisplatin (10 µg/ml) and TRAIL (10 ng/ml) or a combination of cisplatin (10 µg/ml) plus TRAIL (10 ng/ml) for 16 h of treatment. Apoptosis was quantified by ELISA and normalized to values measured in untreated cells. Data were presented as the mean ± SEM of triplicate determinants.

Western immunoblot analysis. The cells were grown in 6-well plates, to near confluence in the presence or absence of various treatments. The cells were lysed and western blotting was performed as previously described (5) using a standard protocol. Briefly, the cell extracts were obtained by lysing the cells in RIPA buffer (20 mM Hepes, 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, 1 mM Na_3VO_4, 1 mM EGTA, 50 mM NaF, 1% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor mixture). The samples containing 100 µg of total protein were electrophoresed on 8% or 15% SDS-polyacrylamide gels and transferred on to PVDF membranes by electroblotting. The membranes were probed with antibodies as indicated, followed by HRP-conjugated mouse or rabbit secondary antibodies (Amersham Amersham Biosciences, Piscataway, NJ, USA). Anti-G3PDH was used for loading controls.

Statistical analysis. Data are presented as mean ± SEM, and statistically analyzed using the unpaired Student's t-test. Differences were considered statistically significant when P<0.05.

Results

Cisplatin plus TRAIL enhances cell death in anti-estrogen-resistant and -sensitive cells without significantly effecting normal breast cells. To develop new strategies to eliminate anti-estrogen-resistant and -sensitive breast cells, we examined the effect of cisplatin on Tam/MCF-7 and Tam/T47D cells. Cell survival is shown in Fig. 1. Treatment of the cells with Cisplatin induced a significant decrease in cell survival in the
Tam/MCF-7 and Tam/T47D cells. The efficacy of cisplatin was dose-dependent; however, a significant risk of nephrotoxicity frequently hindered the use of higher doses to maximize the antitumor effects of cisplatin (21). Our data showed that 10 μg/ml of cisplatin exhibited optimum inhibition in MCF-7- and T47D-derived anti-estrogen-resistant cells (Fig. 1). In addition, the results showed that cisplatin was more effective in killing anti-estrogen-resistant cells when compared to sensitive cells in the MCF-7 cell line. However, in the T47D-derived cell lines Tam-resistant and -sensitive cells were killed (Fig. 1). The reasons for this discrepancy in ER-positive breast cell lines remains to be determined.

We evaluated whether a combination of cisplatin plus TRAIL enhanced killing in anti-estrogen-resistant cells. Fig. 2 shows that cisplatin or TRAIL treatment decreased cell survival in Tam/MCF-7 (acquired resistance to Tam), HER2/MCF-7 (de novo resistance) and Tam/T47D (acquired resistance to Tam) cells to varying degrees. However, cisplatin plus TRAIL had a maximum effect on anti-estrogen-resistant cells by 48 h. A similar treatment of CRL8799 normal breast cells resulted in a moderate increase in cell death. Data are the mean ± SE of triplicate determinants (**P<0.01, *P<0.05).

Cisplatin plus TRAIL enhances apoptosis in Tam-resistant cells. Most chemotherapy agents and irradiation trigger apoptosis through the cell-intrinsic pathway, as an indirect consequence of cell damage. The intrinsic pathway usually requires functional p53. However, inactivation of p53, either directly through mutation(s) or indirectly through p53 modulation through the MDM2 protein, is common in many human tumors. The extrinsic pathway induces apoptosis in response to the engagement of death receptors by their ligands such as TRAIL. This pathway stimulates the apoptotic caspase mechanism independent of p53 (22). The effect of cisplatin, TRAIL, and cisplatin plus TRAIL treatment on apoptosis in Tam/MCF-7 and Tam/T47D cells are shown in Fig. 3. Although treatment with cisplatin or TRAIL increased apoptosis to varying degrees, a combination of cisplatin plus TRAIL had a maximum effect on apoptosis in Tam/MCF-7 (Fig. 3A), and Tam/T47D (Fig. 3B) cells (p<0.05). These data suggested that cisplatin plus TRAIL increased apoptosis in anti-estrogen-resistant cells compared with the untreated controls.

Caspase inhibition reduces cisplatin plus TRAIL-induced cell death. Caspases have been previously shown to play an important role in TRAIL-induced apoptosis (23). It was previously shown that 75% of human breast tumors lack caspase-3 transcripts, whereas other caspases, such as caspase-8 and -9 were shown to be normal (24). MCF-7 cells lack caspase-3 expression as a
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result of a functional deletion mutation in the caspase-3 gene, while the expression of other caspase-8 and -9 remains normal. We used MCF-7 and T47D anti-estrogen cells to determine the possible mechanism by which cisplatin plus TRAIL increase cell death. The results from the western blot analyses revealed an increased caspase-9 activation in Tam/MCF-7 and Her-2/MCF-7 anti-estrogen-resistant cells compared to -sensitive cells and this led to increased PARP and Bid cleavage (Fig. 4A) and apoptosis (Fig. 3A). By contrast, T47D and Tam/T47D (express caspase-3) showed a higher activation of caspase-3 and -9 in resistant and sensitive cells in the presence of cisplatin plus TRAIL, leading to increased PARP and Bid cleavage (Fig. 4B).

To determine whether caspase activation is essential for cisplatin plus TRAIL-induced cell death, we blocked caspase-3, -8, -9 or pan-caspase using relatively specific caspase inhibitor(s). The effect of caspase inhibitors on cisplatin plus TRAIL treatment in Tam/MCF-7 HER2/MCF-7 and Tam/T47D cells are shown in Fig. 5. However, the addition of caspase inhibitors alone to anti-estrogen-resistant cells had a minimal or no effect on these cells. These data suggested that cisplatin plus TRAIL-induced apoptosis is predominantly mediated through the activation of caspases in Tam-resistant cells.

Discussion

Anti-estrogenic drugs such as Tam provide an effective therapy for women with hormone-dependent breast cancer in the neo-adjuvant, adjuvant and advanced disease settings (25). However, a major clinical problem with the use of anti-estrogens is that the majority of patients with advanced disease eventually develop resistance to the compounds and even stimulate tumor growth in some cases (26,27). Thus, Tam resistance is a daunting challenge for the successful treatment of ER-positive and hormone-dependent breast cancer. Preclinical and clinical investigations conducted to understand this resistance have led to the identification of multiple pathways that contribute to anti-estrogen resistance, most of which arise from alterations in ER and/or growth factor receptor signaling (28).

In this study, instead of managing or preventing anti-estrogen resistance, we attempted the strategy of eliminating endocrine-resistant cells by inducing enhanced cell death. The results from the present investigations have shown that Tam/MCF-7 and Tam/T47D cells, when treated with cisplatin plus TRAIL, exhibited significantly enhanced cell death as compared with the untreated cells. Furthermore, a similar
treatment had a minimal effect on CRL8799 normal breast cells. In addition, results from the present study provided evidence that cisplatin enhances TRAIL-induced cell death by activation of caspases. Inhibition of the activity of caspases decreased cell death in cisplatin alone and cisplatin plus TRAIL-treated cells.

The results also showed that cisplatin plus TRAIL significantly induces cell death in the de novo Tam-resistant (HER2/MCF-7) and acquired Tam-resistant (Tam/MCF-7) cells when compared to the MCF-7 parental cells. These observations were similar to those reported by Yde et al (29): MCF-7-derived anti-estrogen-resistant cells were more sensitive to cisplatin-induced cell death than the parental cells. The results from this study suggest that treatment with cisplatin or TRAIL alone induced cell death in 30-50% of Tam-resistant cells, however, the maximum effect is evident when these cells are treated with cisplatin plus TRAIL. There were differences in the response between Tam-resistant and parental T47D cells when compared to MCF-7 cells. Cisplatin plus TRAIL treatment significantly inhibited cell survival in Tam/T47D and T47D cells. However, the extent of cell death was different between Tam/MCF-7 and MCF-7 cells, the latter being relatively resistant to cisplatin plus TRAIL treatment. The reason behind these discrepancies has yet to be determined. One possibility is that MCF-7 cells lack caspase-3 expression as a result of a functional deletion mutation in caspase-3 gene, while expression of caspase-8 and -9 remains normal (30). By contrast, T47D expresses all caspases.

We have previously shown that re-introduction of caspase-3 in MCF-7 cells increases TRAIL-induced apoptosis in PKC-δ-induced anti-estrogen resistance (31). Caspase-3 mRNA expression levels in breast cancer were shown to be ≥10- to 50-fold lower than those in normal breast tissues (24) which is a significant clinical observation. It is possible that Tam/MCF-7 models can be used for more detailed mechanistic studies to identify additional targets that can be used to treat anti-estrogen-resistant breast tumors.

The results from the present investigations suggest that treatment of Tam-resistant cells, i.e., Tam/MCF-7 and Tam/T47D, with cisplatin can enhance TRAIL-induced apoptosis by activating caspases while similar treatment of immortalized CRL8799 normal breast cells had a minimal effect. Individually, platinum compounds, carboplatin or cisplatin and TRAIL have been approved by the FDA to treat different forms of cancer. The results of the present investigation suggest that cisplatin plus TRAIL treatment can be used as a potential and novel treatment strategy for anti-estrogen-resistant breast tumors.

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References


