Cisplatin and TRAIL enhance breast cancer stem cell death

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Abstract. Triple negative breast cancer (TNBC) has increased recurrence and poor survival, despite a high response rate to neoadjuvant chemotherapy. The aim of this study was to determine whether current drug treatment(s) eliminates bulk of tumor cells, but it has a minimal effect on cancer stem cells (CSCs) leading to tumor recurrence. We studied the effects of PARP inhibitors (AZD2281 and BSI-201), paclitaxel, docetaxel, cisplatin and cisplatin plus TRAIL on CSCs derived from CRL-2335 and MDA-MB-468 TNBC cells in vitro. The in vitro data indicate that cisplatin plus TRIAL treatment was most effective in eliminating CSCs compared to PARP inhibitors, cisplatin, paclitaxel and docetaxel. Treatment with cisplatin plus TRAIL also inhibits Wnt-1 signaling and its downstream target, β-catenin, phospho β-catenin, cyclin D1, increased apoptosis, reduced proliferation and mammosphere formation. Inhibition of Wnt-1 by siRNA significantly reduced the ability of CSCs to form mammospheres compared to control. However, maximum effect was seen in cisplatin plus TRIAL-treated cells. Taken together the data suggest that cisplatin plus TRAIL treatment has the potential of providing a new strategy for improving the therapeutic outcome in TNBC patients.

Introduction

Breast cancer is a common malignancy diagnosed among women in the United States, and is the second leading cause of mortality. Triple-negative breast cancer (TNBC) is the most aggressive and difficult to treat form of cancer compared to other breast cancers. Approximately, 12-15% of women with breast cancer have TNBC. They are negative for estrogen receptor (ER) and progesterone receptor (PR) expression and HER-2/neu amplification (1). They do not benefit from hormonal- or herceptin-based targeted therapies due to loss of target receptors. The disease is diagnosed more frequently in younger and pre-menopausal women (2-6). Current therapeutic approaches such as chemo- and/or radiation therapy is able to shrink the malignant mass and achieve an objective clinical response; however, too often these responses are followed by recurrence of the tumor (7). Unlike most cells within the tumor, cancer stem cells (CSC) were shown to be resistant to conventional chemo- and/or radiation therapy. After treatment, they can regenerate all the cell types in the tumor through their stem cell-like behavior (8,9). There is a critical need for the development of more effective therapies to eliminate both tumor and CSCs in TNBC.

The existence of CSCs has been described in a variety of hematologic and solid tumors, including those of the breast, brain, pancreas, and lung (10). The in vitro and animal models have demonstrated CSCs are relatively resistant to both radiation and chemotherapy (8,9). There is growing evidence to support that cancers originate in tissue stem cells through the dysregulation of self-renewal processes. A number of self-renewal pathways such as Wnt (11,12), Notch (13) and Hedgehog (14) have been shown to be involved in the regulation of stem cells. Furthermore, these pathways are shown to be frequently dysregulated during carcinogenesis (11,12,15). Recent studies have reported a pivotal role of Wnt/β-catenin signaling pathway in the regulation of stem cell self renewal (16). In Wnt/β-catenin pathway secreted proteins of the Wnt family bind to specific Frizzler (FZD) receptors on the surface of target cells to activate intracellular pathways, resulting in the accumulation and nuclear localization of the β-catenin protein. The nuclear β-catenin, binds to T-cell factor 4 (Tcf4) to drive activation of specific target genes, including cyclin D1, c-Myc and survivin, which have been characterized to be critical for cancer development. The strategies aimed at interfering with these interactions represent a rational approach to target breast CSCs. CSCs have distinct properties such as self renewal, potential to proliferate, and eventually leading to tumor recurrence and metastasis (17,18). We investigated the effect of cisplatin plus TRAIL on self renewal pathways such as Wnt/β-catenin signaling, mammosphere formation, cell proliferation and apoptosis in CSCs derived from TNBC cells in vitro.

Materials and methods

Cell lines and reagents. The human breast triple-negative breast cell line CRL2335 and MDA-MB-468 cells were
obtained from the American Type Culture Collection (ATCC). Cells were maintained in ATCC-recommended culture media. All cells obtained from ATCC were immediately expanded and frozen down such that all cell lines could be restarted every 3–4 months from a frozen vial of the same batch of cells, and no additional authentication was done in our laboratory. DR4/TRA1R1 monoclonal antibody and DR5/TRA1R2 polyclonal antibody was purchased from Imegenx (San Diego, CA). Monoclonal antibody actin was purchased from BD Biosciences (San Diego, CA). Phospho-β-catenin was purchased from Cell Signaling Technology (Danvers, MA). TRAIL was purchased from R&D systems (Minneapolis, MN). Wnt-1 (H-89), cyclin D1 (CD1), β-catenin (10H8) antibodies were purchased from Santa Cruz Biotechnology. PARP inhibitors AZD2281 (Olaparib) and BSI-201 (Iniparib) were purchased from Chemietek (Indianapolis, IN), docetaxel was purchased from LC Laboratories (Wobum, MA). Cisplatin, paclitaxel was purchased from Sigma (St. Louis, MO). Reagents for protein concentration analysis and protein gel electrophoresis were obtained from Bio-Rad (Hercules, CA). All other chemicals, unless otherwise specified, were purchased from Sigma in the highest suitable purities.

**ALDEfluor assay and separation of the ALDH-positive population by fluorescence-activation cell sorter.** The ALDEfluor assay was performed as described by the manufacturer using the ALDEfluor kit from StemCell Technologies. Briefly, cells were incubated in an ALDEfluor assay buffer containing ALDH substrate (1 µmol/l per 1x10^6 cells). In each experiment, a sample of cells was stained under identical conditions with 50 nmol/l of diethylaminobenzaldehyde, a specific ALDH inhibitor, as a negative control. The sorting gates were established using propidium iodide-stained cells for viability. Flow cytometry work was done at Imaging and cytometry resources core at The Karmanos Cancer Institute, WSU.

**Mammosphere culture.** Mammosphere culture was performed as described by Dontu et al (19) with minor modification. In brief, CRL2335 mammospheres were cultured in suspension (1,000 cells/ml) in serum-free RPMI-1640 media, supplemented with B27 (1:100; Invitrogen). MDA-MB-468 mammosphere were cultured in suspension (1,000 cells/ml) in serum-free DMEM media, supplemented with B27 (1:100; Invitrogen), N2 (1:50; Invitrogen) and 10 ng/ml of EGF. For mammosphere formation assay, cultured mammospheres were enzymatically dissociated by incubation in a trypsin-EDTA solution (Invitrogen) at 37°C. Cells were plated at 4000 cells per well of six-well ultra low-attachment plate (Corning, MA). Mammospheres were counted after 5–7 days. Mammosphere counting; mammospheres were centrifuged and transferred to a 96-well flat bottomed plate in 100 µl of the media and counted using a microscope under low magnification. Experiments were done in triplicates.

**MTT assay.** In brief, 5x10^4 cells were added in 96-well ultra low-attachment plate. After 24 h, cells were treated with TRAIL (10 ng/ml), cisplatin (10 µg/ml), or combination of TRAIL plus cisplatin or other drugs for another 24 h. Following treatments, 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% CO₂ and 37°C. The cell viability was measured by MTT, which is converted by succinate dehydrogenase in 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.

**RNA interference assay.** Cultured mammospheres were enzymatically dissociated by treating them with trypsin-EDTA. Cells (viable) were plated in 6-well ultra low-attachment plate in Opti MEM I medium (Invitrogen) at a density of 2x10^4 per well. After 16 h, cells were transfected with 75 nmol of Wnt-1 siRNA, random siRNA with scrambled sequence was used as control. Lipofectamine 2000 (Invitrogen) transfection reagent was used to transfect sequence according to the manufacturer’s instructions. After 72 h of transfection, cells were dissociated and used to determine the mammosphere formation and remaining cells were harvested for Western blot analysis.

**Apoptosis assay.** Apoptosis was assessed using the cell death detection ELISA<sup>PLUS</sup> kit (Roche) according to the manufacturer's instructions. This kit quantitatively detected cytosolic histone-associate DNA fragments. In brief, mammospheres were dissociated and 2x10^4 cells were plated in 96-well ultra low attachment plate. Cells were treated with cisplatin, TRAIL and cisplatin plus TRAIL for 16 h. Apoptosis was quantified by ELISA and normalized to values measured in untreated cells. Data are mean ± SE of triplicate determinations.

**Western blot analysis.** Cells were grown in 6-well plates, to near confluence in the presence or absence of various treatments. Cells were lysed and Western blotting was performed as described previously (20) using a standard protocol. In brief, cell extracts were obtained by lysing the cells in RIPA buffer (20 mM Heps, 100 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, 1 mM Na<sub>2</sub>VO<sub>3</sub>, 1 mM EGTA, 50 mM NaF, 10% glycerol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 1X protease inhibitor mixture). Samples containing 100 µg of total protein were electrophoresed on 8 or 15% SDS-polyacrylamide gels and transferred on to PVDF membrane by electroblotting. Membranes were probed with antibodies as indicated, followed by HRP-conjugated mouse or rabbit secondary antibodies (Amersham). Anti-actin was used for loading controls.

**Immunohistochemical analysis (IHC).** Immunohistochemistry was performed on tumor tissue sections placed on glass slides using the standard laboratory protocols as previously described (21,22). Briefly, after deparaffinizing and hydrating with phosphate-buffered saline (PBS) buffer (pH 7.4), tissue sections were pretreated with hydrogen peroxide (3%) for 10 min to remove endogenous peroxidase, followed by antigen retrieval via steam bath for 20 min in EDTA. A primary antibody was applied, followed by washing and incubation with the biotinylated secondary antibody for 30 min at room temperature. Detection was performed with diaminobenzidine (DAB) and counter stained with Mayer hematoxylin followed by dehydration and mounting. Cancer stem cells (CSCs) in human breast tumors were identified by CD44<sup>+</sup> and CD24<sup>low</sup> staining. The
staining intensity of each specimen was judged relative to the intensity of a control slide containing known positive tissue section. A score of zero indicates no staining relative to background; 1+, weak staining; 2+, moderate staining; and 3+, strong staining. For comparison of staining among tissues, the results were quantified by calculation of a complete H-score which considers both staining intensity and the percentage of cells stained at a specific range of intensities. A complete H-score was calculated by summing the product of the percentage cells stained (0-100%) and staining intensity (0-3) according to Kerfoot et al (23). Statistical analysis of the complete H-scores obtained for the TNBC tissue and ER\(^++\), PR\(^++\) and HER2\(^-\) breast tumors were carried out by using the one-tailed Student’s t-test with unpaired data of equal variance.

Results

**TNBC tumors express relatively higher levels of cancer stem cells compared to ER-positive, PR-positive and HER2-negative breast tumors.** The existence of CSCs has been described in a variety of hematologic and solid tumors, including those of the breast (10). Unlike most cells within the tumor, CSC was shown to be resistant to conventional chemo- and/or radiation therapy. After treatment, they can regenerate all the cell types in the tumor through their stem cell-like behavior (8,9). As tumor recurrence is a major problem in TNBC, we postulated that TNBC tumors may have higher CSC levels compared to other breast cancer tumors. We determine the CSC levels in nine TNBC tumors and eight ER\(^++\), PR\(^++\) and HER2\(^-\) human breast tumors by immunohistochemical staining. Interpretation of the slides was performed by microscopic examination by a board certified pathologist. Representative staining pattern of the tumor is shown in Fig. 1. The data indicate that TNBC tumors have CD44\(^+/\)CD24\(^{low/-}\) staining in \(\approx 20-70\%\) of the tumor cells compared to 2-10% of the cells in ER\(^++\), PR\(^++\) and HER2\(^-\) breast tumors. However, staining intensity and percentage of stained cells varied in different tumor samples. In order to compare the differences between TNBC and ER\(^++\), PR\(^++\) and HER2\(^-\) tumors, we calculated the H-score. H-score takes into account the intensity of staining and percentage of stained cells in each tumor (22). Our data indicate a significant high H-score in TNBC \(\approx 1090\) compared to an H-score of \(\approx 200\) in ER\(^++\), PR\(^++\) and HER2\(^-\) breast tumors (p<0.01) (Table I). Consistent with our postulation data indicate that human TNBC cancers express relatively higher levels of CSCs compared to ER\(^++\), PR\(^++\) and HER2\(^-\) breast tumors.

Cisplatin plus TRAIL had a synergistic effect on cancer stem cells. Clinical trials indicate that PARP-inhibitors AZD2281 (Olaparib) and BSI-201 (Iniparib) have emerged as a promising new class of antineoplastic agents (24,25). However, significant numbers of TNBC tumors reoccur (26,27). We postulated that above drugs eliminates bulk of tumor cells, but they have limited ability to eliminate CSCs and this could lead to tumor recurrence. To test the above concept, we treated the CSCs derived from TNBC cell lines CRL2335 and MDA-MB-468 with cisplatin, TRAIL, cisplatin plus TRAIL, two PARP inhibitors (AZD2281 and BSI-201), paclitaxel and docetaxel. CSCs levels were determined by ALDEfluor kit (StemCell Technologies) using flow cytometric analyses in the presence or absence of...
the drug(s) after 24 h treatment. The data indicate that untreated CRL2335 cells had ~36% CSCs (ALDEfluor-positive cells) and treatment with AZD2281 (50 µM) reduced CSCs from 36 to 25%, BSI-201 (50 µM) to ~20%, docetaxel (10 nM) to ~24%, paclitaxel (20 nM) had no effect on CSCs, cisplatin (10 µg/ml) to ~21%, TRAIL (10 ng/ml) to ~30%, and combination of cisplatin (10 µg/ml) plus TRAIL (10 ng/ml) to ~1% (Fig. 2A). Similarly, untreated MDA-MB-468 TNBC cells had
~19% cancer stem cells (ALDEfluor-positive cells), AZD2281 treatment reduced CSCs from ~19% to 16%, BSI-201 to ~15%, docetaxel marginally increased CSC to ~24%, paclitaxel had no effect on CSCs, cisplatin (10 µg/ml) to ~14%, TRAIL (10 ng/ml) to ~17%, and combination of cisplatin (10 µg/ml) and TRAIL(10 ng/ml) to ~2% (Fig. 2B). As cisplatin plus TRAIL had a maximum effect on CSCs compared to other drugs, we also determine the effect of cisplatin plus TRAIL on CSCs and total cells after 24 h treatment. Our data indicate that cisplatin plus TRAIL significantly inhibits both CSCs and total cells compared to cisplatin or TRAIL alone (Fig. 2C). Thus, taken together, the results suggest that cisplatin plus TRAIL treatment increased cell death in both CSCs and total cells in TNBC cells.

Therefore, we determine whether CSCs derived from TNBC cell lines express DR4 and DR5 receptors. Our data show that DR4 and DR5 receptors are expressed by CSCs (Fig. 3B) and cisplatin sensitizes the CSCs to TRAIL-induced apoptosis (Fig. 3C and D). Our data also indicate that there are differences in susceptibility to cisplatin treatment among different cell lines; however combination of cisplatin plus TRAIL had maximum effect in both the TNBC cell lines. Taken together, the data suggest that cisplatin plus TRAIL significantly reduce Wnt1/β-catenin signaling, which could lead to reduced self renewal, and cell proliferation in CSCs.

To further determine if inhibition of Wnt-1 is sufficient to achieve similar results as seen in cisplatin plus TRAIL treatment, we inhibited Wnt-1 by Wnt-1 siRNA in CRL2335-derived cancer stem cells. Our data indicate that inhibition of Wnt-1 by siRNA significantly reduced phospho-β-catenin, cyclin D1 levels (Fig. 4A) compared to random siRNA-treated CSCs. However, maximum inhibition was seen in cisplatin plus TRAIL-treated CSCs (Fig. 4A). Consistent with these observations Wnt-1 inhibition by siRNA reduced mammosphere formation by ~25% compared to random siRNA-treated CRL2335 cells, maximum inhibition ~95% was seen in cisplatin plus TRAIL-treated CSCs (Fig. 4B).

Cisplatin plus TRAIL inhibited mammosphere forming ability of CSCs cells. One of the major characteristic features of CSCs is its ability to form mammospheres in suspension culture (19). We investigated the effect of cisplatin plus TRAIL on apoptosis
and mammosphere formation in CSCs derived from TNBC cell lines CRL2335 and MDA-MB-468 cells. The results presented in Fig. 5A and B suggested that cisplatin treatment reduced mammosphere formation ability of CSCs by ~75% compared to an untreated control group, TRAIL treatment reduced mammosphere formation ability by ~42% and combination of cisplatin plus TRAIL reduced mammosphere formation ability by ~96% (Fig. 5B). Similarly, in MDA-MB-468 derived CSCs, cisplatin treatment reduced mammosphere formation ability by ~62%, TRAIL by ~21% and combination of cisplatin plus TRAIL reduced mammosphere formation ability by ~87% (Fig. 5B). Thus, taken together, the results suggest that cisplatin plus TRAIL treatment significantly inhibits Wnt-1 signaling, and decreases mammosphere formation in CSCs.

**Discussion**

TNBC is the most aggressive and difficult to treat form of cancer compared to other forms of breast cancer. TNBC patients do not benefit from hormonal or herceptin-based therapies due to loss of target receptors such as ER, PR and HER-2 (1). Chemotherapy is currently the main stay of systemic treatment, although patients with TNBC, when considered as a group, have a worse outcome after chemotherapy than patients with other subtypes of breast cancer (30,31). One of the reasons may be the inability of the chemotherapeutic drugs to kill CSCs. Some studies suggest that 75% of the tumors arising in women carrying a mutation in BRCA1 gene have a triple-negative phenotype (27,32,33). The PARPs constitute a family of enzymes involved in base-excision repair, a key pathway in the repair of DNA single-strand breaks. Despite exciting developments in the treatment options such as poly(ADP-ribose)polymerase (PARP) inhibitors to target BRCA1-deficient TNBC cancers (24), many women still experience a relapse, and metastatic breast cancer remains a largely incurable disease. Our data show 20-70% of cancer cells from TNBC tumors display cell surface markers for cancer stem cells, such as CD44+/CD24low and expression of aldehyde dehydrogenase 1...
Our data show that PARP inhibitor AZD2281 (olaparib) reduced CSCs from ~36% to 25% and BSI-201 (iniparib) reduced CSCs from ~36% to 20% in CRL2335 cells. However, cisplatin plus TRAIL has reduced CSCs from ~36% to 1% in CRL-2335 cells. Previous studies from preclinical and phase I trials, show a response rate of ~40% in patients with BRCA1 or BRCA2 mutations (24,25). Our data show that cisplatin plus TRAIL is much more effective in eliminating CSCs compared to cisplatin alone or PARP inhibitors. These observations suggest that cisplatin plus TRAIL is highly effective against TNBC by eliminating both CSCs and bulk of tumor cells. It is also becoming evident that cancer treatment that fails to eliminate CSCs allows relapse of the tumor (34,35).

One of the defining characteristics of CSCs is their ability to undergo self-renewal. The self-renewal pathways are regulated by Akt, Notch and Hedgehog signaling. Interestingly, dysregulation of each of these pathways in transgenic mice leads to breast cancer (36-38). Therapies that inhibit a self-renewal pathway(s) could lead to a significant reduction of CSCs in tumor. Previous studies have shown that Akt activation leads to β-catenin translocation into the nucleus, and cause induction of TCF family of transcription factors leading to increased synthesis of several proteins that are critically involved in cell growth (39). Our data indicate that cisplatin plus TRAIL inhibit Akt-1 signaling leading to reduced phospho-β-catenin and cyclin D1 levels, which could lead to significant inhibition of self-renewal and proliferation in CSCs. The therapeutic strategies that target CSCs are effective in reducing the risk of relapse and metastasis.

The commonly accepted criteria for clinical efficacy in phase II trials are tumor shrinkage using criteria defined by RECIST (40). The premise is that tumor regression equates with a clinical benefit. Literature suggests that there is only a modest overall survival advantage for pancreatic, prostate and metastatic breast cancer patients even with the tumor regression (41-43). This discrepancy between response and survival may be partially explained by an inability of chemotherapeutic drugs to effectively target the CSCs. Cisplatin plus TRAIL targets both tumor and CSCs in vitro and this could provide new treatment options for TNBC tumors.

We demonstrate for the first time that cisplatin plus TRAIL is highly effective in reducing CSCs compared to PARP inhibitors, docetaxel or paclitaxel in TNBC cells in vitro. Cisplatin plus TRAIL significantly inhibits Wnt-1 signaling pathways, reduces cell proliferation and increases apoptosis in CSCs and tumor cells. Individually, cisplatin and TRIAL are approved by FDA to treat other forms of cancer. Since TNBC patients do not benefit from hormonal or herceptin-based therapies or chemotherapeutic drugs to effectively target the CSCs. Cisplatin plus TRAIL treatment is suggested as a potential new cancer treatment strategy in TNBC patients.

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