A combined treatment TNF-α/Doxorubicin alleviates the resistance of MCF-7/Adr cells to cytotoxic treatment

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Abstract

The efficiency of anticancer therapy is often restricted by the development of drug resistance. Here, we report that the doxorubicin (DOX)-resistant MCF-7/Adr cells were more resistant to DOX-treatment than MCF-7 cells. However, an alternative treatment of DOX/TNF-α enhanced the cytotoxic effect in multidrug resistant MCF-7/Adr cell line. Treatment of cells with TNF-α following doxorubicin (DOX) resulted in a decrease of the activated Rel A/p65 in nuclei. Histone deacetylase 1 (HDAC1) was found to interact with Rel A/p65 in the complex, suggesting that HDAC1 is involved in mediating nuclear export of Rel A/p65. The combined treatment of TNF-α/DOX also resulted in a significant decrease of mRNA levels of anti-apoptotic genes, such as the cellular inhibitor of apoptosis-1 (c-IAP1), and the long isoform of B cell leukemia/lymphoma x gene (Bcl-xL), leading to efficient induction of caspase-8 cleavage and cell death. In previous work, we demonstrated that TNF-α promotes DOX-induced cell death and anti-cancer effect through downregulation of p21 in p53-deficient tumor cells. Thus, we proposed that alternative administration of TNF-α and DOX may be a new and efficient therapeutic strategy for patients that develop resistance to cytotoxic treatment.

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1. Introduction

Tumor necrosis factor-α (TNF-α) is a glycoprotein that possesses antitumor activities against a wide range of tumor cell lines, whereas nonmalignant cells are relatively resistant [1]. But its clinical application is limited by its severe systemic side effects before therapeutic doses could be reached. Recently, the feasibility of TNF-α as a biological response modifier was demonstrated. Our clinical phase II and phase III trials showed that systemic administration of TNF-α in combination with a chemotherapeutic agent DOX for lung tumors remarkably enhanced the anti-tumor effect, as compared to the DOX treatment alone. In total, 44% of the 133 patients exhibited complete or partial remission of lung tumors in response to this combination therapy strategy [2–4]. Since it has been approved by the Chinese SDA, this innovative clinical application of TNF-α in combination with DOX may offer great opportunities for cancer patients. Furthermore, in the setting of hyperthermic isolated limb perfusion (HILP), TNF-α in combination with melphalan appeared to be very active against irresectable sarcomas and melanomas, tumors known to be resistant against chemotherapy [5,6]. The results of the combination TNF-α with other chemotherapeutic agents suggest the synergistic anti-tumor effects in chemotherapy-resistant tumors. However, the molecular basis for the combination treatment has not been well elucidated.

TNF-α binding to its receptors and the active TNF-α receptor complexes can interact with caspase-8 proteases via TRADD and FADD to induce cell death, whereas NFκB activation induced by TNF-α inhibits cell death [7]. The prototypical NFκB complex, which responds to heterodimer of p50 and Rel A/p65 subunits, exists as the inactive dimmer sequestered in the cytoplasm. The activated NFκB heterodimer rapidly translocates into the nucleus, where it engages the κB enhancer elements and alters gene expressions. In fact, NFκB regulates a set of anti-apoptotic genes including the members of the Bcl-2 family, cellular inhibitors of apoptosis protein C (c-IAP 1 and 2), A20 and superoxide dismutase 2 (SOD2). These anti-apoptotic
proteins inhibit the activation of caspases, which results in preventing cytotoxic actions induced by TNF-α stimulation [8].

The Doxorubicin (DOX)-resistant human breast cancer cell line MCF-7/Adr displays cross-resistance to many chemotherapeutic agents such as anthracyclines, taxol and other drugs [9]. Multidrug resistance in MCF-7/Adr cells is associated with several different mechanisms including overexpression of P-glycoprotein, glutathione s-transferase and glutathione peroxidase [10].

DOX, a widely used anti-tumor agent, targets DNA topoisomerase II enzyme activity, which involves sequential DNA binding, cleavage of DNA phosphodiester backbone and subsequently causes DNA breaks. Our previous data suggested that the abnormal progression of cell cycle is responsible for DOX-induced cell death [11,12].

In previous work, we demonstrated that TNF-α promotes the anti-cancer effect of DOX through suppressing the anti-apoptotic activity of p21, and that a combined treatment TNF-α/DOX is an effective chemotherapeutic strategy for p53-deficient tumor cells [13]. In this study, using MCF-7 and its multidrug resistant derivative cell line MCF-7/Adr, we demonstrated the treatment with TNF-α/DOX strongly increased MCF-7/Adr cells’ sensitivity to cytotoxic treatment, which was correlated with a down-regulation of NF-κB-mediated anti-apoptotic gene expressions. This effect is dependent, at least in part, on the cytoplasmic relocalization of NFκB/Rel A/p65 induced by HDAC1.

2. Materials and methods

2.1. Cells and cell culture

Human breast cancer cell line MCF-7 cells and the doxorubicin-resistant cell line MCF-7/Adr cells were cultured in Dulbecco’s modified Eagle’s minimum essential medium (Hyclone, Logan, UT) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT).

2.2. Measurement of cell death

Cells were seeded into 96-well microwell plate and treated either with 24 h of DOX (0.8 μg/ml) alone or with 24 h of DOX (0.8 μg/ml) and then 0.5 h of TNF-α (500 U/ml) treatment. Then, 10 μl of the CCK-8 solution is added to each well of the plate and it was incubated for 2 h in the incubator (37 °C and 5% CO2) according to the procedure of Cell Counting Kit-8 (Doshindo Laboratories, Tokyo, Japan). The absorbance was measured at 450 nm using a microplate reader (BioTek).

2.3. Western blotting and immunoprecipitation

The nuclear, whole-cell and cytoplasmic extracts from cells treated differently were prepared by the Nuclear Extract Kit (Active Motif, Carlsbad, CA). The protein content of the cell lysate was determined by using the Bradford colorimetric assay method (Bio-Rad, Richmond, CA). A 40-μg aliquot of the cell lysate was resolved by 12% polyacrylamide-sodium lauryl sulfate gel electrophoresis and transferred to a Hybond-C Super membrane (Amersham, Buckinghamshire, UK). P65 and caspase-8 were detected with an antibody against p65 (Upstate, USA) and an antibody against caspase-8 (Merck, USA), respectively. Then the blot was incubated with a secondary antibody, IRDye 800 conjugated affinity purified anti-mouse or anti-rabbit IgG (Rockland Immunoochemicals, Inc., Gilbertsville, PA) and detected with Odyssey Infrared Imaging System (LI-COR Biosciences Co., Nebraska, USA). For immunoprecipitation, a 500-μg aliquot of cell lysate was incubated at 4 °C with 2 μl of an antibody against p65 (Upstate, USA) for 1 h, then vortexed completely at 4 °C, centrifuged and collected the precipitation, and washed with NP-40 buffer and lysed with loading buffer to do Western Blotting analysis. HDAC1 was detected with an antibody against HDAC1 (Santa Cruz Biotechnology, USA).

2.4. RNA extraction and RT-PCR

Total RNA was extracted from MCF-7/Adr cells by using the RNeasy kit (Qiagen) following the manufacturer’s instructions. Five hundred to 100 ng of RNA was used to perform the semiquantitative PCR analysis by using the RT-PCR system (Fermentas). PCR products were resolved by agarose gel electrophoresis and visualized by staining with ethidium bromide. Primers used for Bcl-xL, c-IAP1, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as follows: Bcl-xL, sense, 5′-GGAGCTGG-TGGTTGACTTTCTC-3′; and antisense, 5′-CCGGAGAGTTCTTACCTACTA-C-3′; c-IAP1, sense, 5′-AGGGAAATTACGACAAACTGCTCCTCCA-3′, and antisense, 5′-CTCTCTCGAGGATGCCTTCAAGTGTTCAAC-3′; GAPDH, sense, 5′-GGTCCGATTGCGCCTGTTACC-3′, and antisense, 5′-CACACCCATGACGAAATGGG-3′.

2.5. Electrophoretic mobility shift assays

A 30-μl of annealing mixture containing 2 μl of each B site oligonucleotide (sense: 5′-agt tga ggg gac ttt ccc agg c-3′; antisense: 5′-gct gga gaa ggt ccc ctc aac t-3′) in 0.5 M NaCl was incubated 2 min at 80 °C then stopped the heating and maintained in water bath until 35 °C. The 8 μl of solution, 2 μl of 10× labeling Buffer and 2 μl IR Dye label solution (IRDye800 800 labeling kit, Odyssey, USA) were mixed, and the reaction proceeded for 5 min at 70 °C. MCF-7/Adr cells untreated or with treatment of DOX (0.8 μg/ml) for 0.5, 2, 6 or 12 h were washed with ice-cold PBS, and then collected. The nuclear extracts were prepared by the Nuclear Extract Kit (Active Motif, Carlsbad, CA), and 5 μg aliquots of the extracts were preincubated with 1.5 μl of above labeled oligonucleotide on ice for 30 min. The complexes were resolved on a 5% polyacrylamide gel. The gel was scanned by Odyssey infrared imaging system.

3. Results

3.1. A combined treatment TNF-α/DOX alleviates the resistance of MCF-7/Adr cells to cytotoxic treatment

The DOX-resistant human breast cancer cell line MCF-7/Adr displays resistance to many chemotherapeutic agents. To confirm this point we treated MCF-7 cells and its DOX resistant derivatives MCF-7/Adr cells with DOX for 24 h. Data from Fig. 1 showed that there were inherent differences between MCF-7 cells and MCF-7/Adr cells as displayed by cell survival curves. This result indicated that MCF-7/Adr cells were more resistant to DOX than MCF-7 cells.

To explore a condition for a killing effect on multidrug resistant MCF-7/Adr cells, we treated cells with DOX, and then incubated the cells with TNF-α for 0.5 h (i.e., 24 h DOX, 0.5 h TNF-α). Interestingly, MCF-7/Adr cells were more sensitive to the cytotoxic effect of this DOX-TNF-α treatment than to that of a simple 24 h DOX treatment. Its parental MCF-7 cells also showed more sensitive to this combined therapy (Fig. 1). Together, our data suggest that MCF-7/Adr cells displayed resistance to DOX-induced cell death, but the treatment TNF-α/DOX reduced its resistance to cytotoxicity to the level of the parental cell line MCF-7. This observation led us to suggest that the alternative administration of TNF-α with DOX may be a more effective protocol for the therapeutic treatment of chemotherapeutic agent-resistant tumors.
Consistent with other studies [14], we found that MCF-7 cell line that underwent cell death induced by various apoptotic stimuli did not display some of the distinct morphological features characteristic of apoptosis such as nuclear shrinkage and membrane blebbing. It was due to a partial deletion within exon 3 of caspase-3 gene in MCF-7 cells.

3.2. HDAC1 mediated Rel A/p65 translocation from nucleus to cytoplasm after treatment with DOX

Numerous reports indicate that NFκB plays an important anti-apoptotic role in TNF-α induced apoptosis by acting as a sequence-specific DNA binding transcription factor and inducing a variety of anti-apoptotic gene expressions. To examine the biochemical events of the activated Rel A/p65 in nuclei and the inactivated Rel A/p65 in cytoplasms in this study, MCF7 and MCF-7/Adr cells were either untreated or treated with TNF-α and with DOX alone or with TNF-α following DOX treatment. The levels of nuclear and cytoplasmic Rel A/p65 were assessed by Western blotting analysis with an antibody against Rel A/p65. As shown in Fig. 2A and B, the nuclear Rel A/p65 was elevated in both cell lines after TNF-α stimulation. In contrast, the nuclear activated Rel A/p65 was much lower in the treatment with DOX (24 h) alone or followed by a treatment with TNF-α. Moreover, the amount of cytoplasmic Rel A/p65 likely corresponded to its pattern in nuclei.

NFκB/Rel A is also activated by ionizing radiation and some chemotherapeutic compounds, such as DOX, to translocate to the nucleus. Study was next performed to examine the nuclear Rel A/p65 after treatment with DOX for 0, 0.5, 2, 6, and 12 h in MCF-7/Adr cells. The levels of nuclear Rel A/p65 were assessed by immunoblotting in DOX-treatment time course. As shown in Fig. 3A, DOX-induced nuclear Rel A/p65 was accumulated and sustained in the peak after 2 h–6 h treatment with DOX and then diminished.

Fig. 1. TNF-α/DOX alleviates the resistance of MCF-7/Adr cells to cytotoxic treatment. The fractions of survival cells after 24 h of DOX (0, 0.1, 0.2, 0.4, 0.6, 0.8 μg/ml) treatment in MCF-7 cells (○) and MCF-7/Adr cells (□), or 24 h of DOX (0.8 μg/ml) and then 0.5 h of TNF-α (500 U/ml) treatment in MCF-7 cells (●) and MCF-7/Adr cells (■). Results represent the mean±S.D. of the three determinations.

Fig. 2. DOX mediates to export Rel A/p65 from nucleus. (A) MCF-7/Adr cells or (B) MCF-7 cells were treated with DOX (0.8 μg/ml) alone for 24 h, with TNF-α (500 U/ml) alone for 0.5 h, or 24 h of DOX (0.8 μg/ml) followed by 0.5 h of TNF-α (500 U/ml) treatment. The nuclear and cytoplasmic p65 expressions were determined by Western blot analysis.

Fig. 3. HDAC1 mediates Rel A/p65 translocation from the nucleus to the cytoplasm after the treatment with DOX. (A) MCF-7/Adr cells treated with DOX (0.8 μg/ml) for 0, 0.5, 2, 6, and 12 h. The nuclear and cytoplasmic p65 expressions were determined by Western blotting analysis. (B) Rel A/p65 was immunoprecipitated from the nuclear extracts of MCF-7/Adr cells with DOX (0.8 μg/ml) treatment for 0, 0.5, 2, 6 and 12 h. HDAC1 protein was detected by Western blotting analysis in the complex. (C) Nuclear extracts from MCF-7/Adr cells treated with DOX (0.8 μg/ml) for 0, 0.5, 2, 6, and 12 h were subjected to electrophoretic mobility shift assay (EMSA) using IR Dye™ labeled αB oligonucleotide.
Recent studies have shown that the class 1 histone deacetylased, HDAC1,2 and 3 influence NF-κB activity through direct or indirect interaction with Rel A/p65. Those associations have been reported to regulate NF-κB’s DNA binding and nuclear export [15–17]. Consistent with this hypothesis, immunoprecipitation experiments were carried out as follows: MCF-7/Adr nuclear extracts were immunoprecipitated with an antibody against p65 and then HDAC1 protein was assessed by Western blotting analysis in the complex, after treated with DOX for the time indicated. The results in Fig. 3B demonstrated the treatment with DOX induced Rel A/p65 association with HDAC1. In contrast, TNF-α stimulation did not result in the increased association of Rel A/p65 with HDAC1 at this time point (data not shown). HADC2, HDAC3 proteins were also checked by Western blotting analysis in p65 immunoprecipitated complex, no apparent difference observed. Electrophoretic mobility shift assays suggested that DOX treatment resulted in the strong induction of NF-κB DNA-binding activity for the time when the nuclear p65 was accumulated in MCF-7/Adr cells (Fig. 3C).

Taken together, these results may indicate that the DOX-treatment mediated the association of Rel A/p65 with HDAC1 in the nucleus and then promoted Rel A/p65 export from the nucleus to the cytoplasm. In contrast, this cytoplasmic relocalization of Rel A/p65 did not occur in the treatment with TNF-α alone.

3.3. Treatment of TNF-α following DOX inhibited expression of anti-apoptotic genes

We were interested in confirming that the effects of the intracellular trafficking of Rel A/p65 induced by HDAC1 could be seen with NF-κB/Rel A/p65-regulated target genes. For these experiments, we analyzed c-IAP1 and Bcl-xL, which are anti-apoptotic genes regulated by NF-κB. The semiquantitative PCR analysis of RNA extracted from MCF-7/Adr cells stimulated with either DOX or TNF-α revealed increases in Bcl-xL and c-IAP1 mRNA levels. In contrast, c-IAP1 and Bcl-xL mRNA levels remarkably decreased in MCF-7/Adr cells with the treatment of DOX followed by TNF-α (Fig. 4A). In contrast to these observations, DOX treatment alone resulted in decrease in Bcl-xL and c-IAP1 mRNA levels in its parental MCF-7 cells (Fig. 4B). It led us to suggest that MCF-7/Adr cells were more resistant to DOX treatment may partly due to losing its ability of decreasing expression of these anti-apoptotic genes. As a specificity control, GAPDH mRNA expression was unaffected under these conditions. These results suggest that the combination of DOX and TNF-α treatment target the expression of some anti-apoptotic genes such as c-IAP1 and Bcl-xL in both cell lines.

3.4. Treatment of TNF-α following DOX potentiated the activation of caspase-8

TNF-α-induced cell death requires the activation of caspase-8. Studies were next performed to define the effects on apoptotic signal of caspase-8 for HDAC1-mediated nuclear export of Rel A/p65 and the inhibition of NF-κB-induced anti-apoptotic gene expressions. MCF-7/Adr cells were treated with TNF-α, alone, or the treatment with DOX followed by TNF-α. The activated caspase-8 protein amounts were measured by detecting its active 18-kDa subunit with an antibody specific against active (cleaved) caspase-8. Comparing to treatment of DOX or TNF-α alone, the treatment with DOX followed by TNF-α remarkably elevated the expression of the cleaved (active) subunit of caspase-8 (Fig. 5). There was also the correlation between the extent of cell death and the activation of caspase-8, when MCF-7/Adr cells were exposed to this treatment (Figs. 1 and 5).

Fig. 4. Treatment of TNF-α following DOX represses the expression of Bcl-xL and c-IAP1. (A) MCF-7/Adr cells and (B) MCF-7 cells were treated with DOX (0.8 μg/ml) alone for 24 h, with TNF-α (500 U/ml) alone for 0.5 h, or 24 h of DOX (0.8 μg/ml) followed by 0.5 h of TNF-α (500 U/ml) treatment. The semiquantitative PCR analysis was then performed in extracted RNA from above treated cells using primers specific to human Bcl-xL, c-IAP1 or a GAPDH control.

Fig. 5. Treatment of TNF-α following DOX potentiates the activation of caspase-8. MCF-7/Adr cells were treated with DOX (0.8 μg/ml) alone for 24 h, with TNF-α (500 U/ml) alone for 0.5 h, and 24 h of DOX (0.8 μg/ml) followed by 0.5 h of TNF-α (500 U/ml) treatment. The proteolysis of caspase-8 was analyzed by immunoblotting using a specific antibody against cleaved caspase-8.
4. Discussion

In this study, we reported that the treatment of multidrug-resistant MCF-7/Adr cells with TNF-α following DOX administrations remarkably increased the cytotoxic effect of chemotherapeutic agent DOX on tumor cells. This effect was associated with the inhibition of NF-κB-induced anti-apoptotic gene expressions and the increased activation of caspase-8. We also demonstrated HDAC1 was involved in mediating nuclear export of Rel A/p65.

MCF-7/Adr cell line originally came from human breast cancer cell line MCF-7, displaying cross-resistance to many chemotherapeutic agents. Several mechanisms including over-expression of P-gp, GST-Px contribute to the degree of drug resistance in MCF-7/Adr cells [18]. Moreover, Ogretmen’s group found that a 21-bp deletion beginning at exon 5 in the tumor suppression p53 gene alters its expression levels, stability and cellular localization of the protein in multidrug resistant MCF-7/Adr cells [19]. Recently, several groups revealed that MCF-7/Adr cells have different genotype and phenotypic characteristics from its parental MCF-7 cells [20].

The efficiency of anticancer therapy is limited by the development of drug resistance. Great efforts have been made to improve the efficacy of the cytotoxic response to chemotherapeutic agents on this drug-resistant human breast tumor [21]. TNF-α acts synergistically with cytotoxic drugs against a variety of tumor cells, and the observation that TNF-α is able to do so in drug-resistant tumor cell lines and thereby overcomes drug resistance is especially provocative [22–24]. In this study, we found the treatment of TNF-α following DOX reversed the resistance to cytotoxic treatment in MCF-7/Adr cells (Fig. 1). This might represent a new strategy to improve the efficiency of therapeutic response to the tumors known to be resistant to chemotherapy. However, it should be noted that this combined therapy was effective in overcoming the resistance to TNF-α rather than DOX treatment. Moreover, it had been shown by several studies that the possible synergistic effects of TNF-α with other drugs are strongly dependent on several conditions such as the sequence of administration. However, the molecular basis for that has to be well defined.

The activation of caspase-8 contributes to TNF-α-induced cytotoxic action [25,26]. However, the activation of NF-κB prevents TNF-α-induced cytotoxic action by blocking caspase-8 with anti-apoptotic gene expressions [27]. In this study, we found that treatment with TNF-α alone induced modest cytotoxic effect in MCF-7/Adr cells, i.e., about 83% survival rate after 500 U/ml TNF-α 0.5 h treatment (data not shown). The model for NF-κB’s anti-apoptotic action is that following TNF-α stimulation of cells, which results in degradation of IκBα, NF-κB is translocated to the nucleus and binds tightly to κB-DNA elements to induce anti-apoptotic gene expressions, such as c-IAP1, c-IAP2, TRAF1, TRAF2 and XIAP, whose protein products are caspase inhibitors [28]. In this report, we demonstrate that TNF-α/DOX-treatment eliminated NF-κB protection by HDAC1-mediating to export Rel A/p65 from the nucleus and subsequently turn off NF-κB-mEDIATE anti-apoptotic gene expressions. Then, cells could override the inhibition conveyed by the NF-κB pathway to carry out cell death due to expression of elevated activated caspase 8 (Fig. 5). Thus, translocation of Rel A/p65 from the nucleus may be a turning point in TNF-α-induced cell death. However, more intrinsic mechanistic studies between the two processes should be proved and addressed in our future work.

A growing number of reports suggest that HDACs not only deacetylate core histone leading to repressive changes in chromatin structure but also deacetylate various host transcription factors, altering transcriptional activity [15–17]. In combination with other data, we suggested that after the treatment with DOX, HDAC1 was identified as the histone deacetylase responsible for the deacetylation by it’s binding to Rel A/p65. We showed HDAC1 regulated the intracellular trafficking of Rel A/p65 in a time-dependent pathway. After 12 h treatment of DOX, the interaction between Rel A/p65 and HDAC1 led to the removal of Rel A/p65 from the nucleus to the cytoplasm and subsequently turn off NF-κB-mEDIATE anti-apoptotic gene expressions in MCF-7/Adr cells (Figs. 3 and 4). NH2-terminal regions of both HDAC1 and Rel A/p65 are reported to be required for the assembly of these two proteins in vivo.

Recently, several groups revealed that MCF-7/Adr cells have different genotype and phenotypic characteristics from its parental MCF-7 cells [20]. MCF-7/Adr cells contain full-length functional caspase-3 protein, while MCF-7 cells are caspase-3 deficient. It should be noted the activation of caspase-8 may be essential for this combined treatment TNF-α/DOX-induced cell death. As shown in Fig. 5, the combination of TNF-α and DOX was efficient in inducing caspase-8 cleavage in MCF-7/Adr cells. The activated caspase-8 played an important role in this study.

The ability of TNF-α to cause cytotoxic action in neoplastic cells is of great interest with respect to its potential roles in cancer therapy. The understanding of the combination of TNF-α treatment with chemotherapeutic agents and the underlying mechanisms might provide new strategies to overcome tumor drug resistance by synergistic interactions.

References


