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Research Article

MCF-7 and its Multidrug Resistant Variant MCF-7/ADR Overcome TNF Cytotoxicity through Prevention of Reactive Oxygen Species Accumulation

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ABSTRACT

Background: Signal transduction of numerous cytokines and growth factors are mediated by reactive oxygen species (ROS). Tumor necrosis factor-α (TNF-α) have stimulated accumulation of ROS in various *in vitro* studies. MCF-7 and its Adriamycin resistant variant MCF-7/ADR are resistant against TNF-α cytotoxicity. Role of ROS in the resistance of MCF-7 and MCF-7/ADR cells was investigated.

Methods: ROS accumulation and viability in MCF-7 and MCF-7/ADR after TNF-α exposure was evaluated using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as a fluorescent probe and 3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) cytotoxicity assay respectively.

Results: ROS level did not change significantly after TNF-α exposure. Induction of ROS accumulation along with TNF-α treatment sensitized these cells to TNF-α toxicity.

Conclusion: It can be concluded that lack of ROS accumulation following TNF-α exposure is involved at least by part in the resistance of MCF-7 and its drug resistant derivative MCF-7/ADR cells to TNF-α cytotoxicity.

Introduction

Multidrug resistance (MDR) hinders successful eradication of breast cancer, which is the most common cancer worldwide. In MDR, tumors resist against cytotoxic effects of different chemotherapy agents. Tumors are usually containing drug-resistant and drug-sensitive cells. Following initial chemotherapy regimens, resistant cells may propagate and lead to regrowth of tumors which are resistant to further therapy. Resistant tumor cells frequently fight against cytotoxic agent through decrement of intracellular drug concentration and substitution of signal transduction involved in cell death and survival.[1-2] Breast adenocarcinoma cell line MCF-7 and its Adriamycin resistant subline MCF-7/ADR have shown resistance against TNF-α cytotoxic effect. It has also been reported that overexpression of manganese superoxide dismutase (MnSOD) attenuates cytotoxic effects of TNF-α in MCF-7 cells.[3-4]

Reactive oxygen species (ROS) are mostly considered as harmful byproducts of cellular metabolism. However, accumulating evidence indicates that ROS are important and have essential role in cellular signaling. Free radical is a highly unstable and reactive atom, molecule or ion containing at least one unpaired electron in its outermost shell. ROS can be intricated in a cascade of pathologic events because they acquire electron from all nearby molecules and macromolecules.[5-6] ROS can modify some specific cysteine residues in proteins involved in signal transduction and alter their activity in various conditions.[7] Intracellular ROS are mainly produced by mitochondria but it has been demonstrated that production of ROS can also be induced by cytokines and growth factors through activation of specialized NADPH-dependent plasma membrane oxidases. Some intracellular enzymes including cyclooxygenases, cytochrome P_{450} enzymes, xanthine oxidase and lipoxygenases can also generate ROS as part of their normal enzymatic function. On the other hand, there are antioxidant enzymes including superoxide dismutase (SOD), catalase and glutathione peroxidase, converting ROS to stable molecules.[7] The level of antioxidant enzymes are influenced by level of ROS within the cells. Activity of transcription factors including Nrf2 and FoxO are dependent on the intracellular redox state and these transcription factors regulate the expression of antioxidant enzymes genes.[8-9]

Tumor necrosis factor-α (TNF-α) is a key cytokine in immune system and is involved in pathological states relevant to acute and chronic inflammation, autoimmune disease and cancer-related inflammation. Binding of TNF-α to its receptor activates different downstream

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mediators, which can affect redox state of cell.[10] TNF-α demonstrates pleiotropic nature, it leads to inflammation and cell survival as well as apoptosis or necrosis on various cells and tissues.[11-12] Different studies reported elevation of ROS level after TNF-α exposure, demonstrating role of ROS in TNF-α signaling.[13-15] TNF-α exposure induced ROS generation mainly in mitochondria while generation of ROS through NADPH oxidase was also reported.[13-15] Transcription factor activation, cellular proliferation and cell death can be mediated by ROS following to TNF exposure.[16-18] ROS mediate induction of sustaining JNK activation which is involved in the TNF-α toxicity.[19-20] On the other hand, ROS has been reported to be able to activate or repress NF-κB signaling. Effect of ROS on NF-κB is controversy. Cell specific behaviors as well as different methodology might be responsible for many diverse results investigating ROS and NF-κB crosstalk. Moreover, expression of antioxidant enzymes including MnSOD, glutathione S-transferase, glutathione peroxidase-1 and catalase are regulated by NF-κB, which influences ROS level.[17] Due to important role of ROS in TNF-α signaling in this investigation we examined role of ROS in response of MCF-7 and MCF-7/ADR cells to TNF-α.

Materials and Methods

Cell culture

MCF-7 and MCF-7/ADR cells were cultured in the RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO, Grand Island, NY) and penicillin (100 units/ml)/streptomycin (100 µg/ml) (GIBCO, Grand Island, NY, USA). Cells were incubated at 37˚C in the presence of 5% CO_2. To maintain the MDR phenotype ADR (Sigma-Aldrich, Taufkirchen, Germany) (250 nM) was add to the culture media of MCF-7/ADR cells but eliminated one week before the experiments.

Determination of intracellular ROS level

The intracellular level of ROS was determined using 2',7'-Dichlorofluorescein diacetate (DCFH-DA) (Sigma-Aldrich, St. Louis, MO, USA). The cellular esterases hydrolyze the nonfluorescent ester DCFH-DA to 2',7'-dichloro fluorescein (DCFH) following to the penetration, then DCFH is oxidized to the highly fluorescent compound 2,7-dichlorofluorescein (DCF) by intracellular ROS. Cells were incubated in a media containing 50 µM DCFH-DA for 30 min in the dark at 37 ° C following TNF-α (EMDMillipore, Billerica, MA, USA) treatment for indicated times and concentrations. Fresh medium was used to wash stained cells and fluorescence was evaluated with a microplate spectrofluorometer (Synergy 4, BioTek, VT, USA) where the excitation and emission were adjusted to 485 and 530nm, respectively. The percentage of measurement in control wells was employed to report ROS generation.

Cell viability assays

Cells were seeded in a density of 6000 cell/well and incubated overnight. MCF-7 and MCF-7/ADR cells were treated with different concentrations of TNF-α alone or in combination with ROS-generating system (RGS). After 5, 24 and 72 h cells were incubated with 0.5mg/ml dimethylthiazolyl-2,5-diphenyl tetrazolium bromide (MTT) for 3h, then 100 µL dimethyl sulfoxide was added to every well to dissolve Formosan crystals. Absorption at 570 nm of each well was determined using a microplate reader (Synergy 4, BioTek, VT, USA). Cell viability was calculated as a percentage of controls treated with vehicle. To investigate the role of ROS in TNF-α toxicity, RGS was employed along with TNF-α treatment. A composition of $CuSO_4$ (1 µM), 1, 10-phenanthroline (1 µM), and ascorbic acid (400 µM), H_2O_2 (20 µM) and paraquat (20 µM) (all from Sigma-Aldrich St. Louis, MO, USA) was utilized to generate ROS in the cells.[21] The concentrations causing the cell survival rate of at least 80% of untreated cultures after 72h were found using dose-response experiments.

Analysis of antioxidant enzymes activity

In addition to ROS production, intracellular redox homeostasis is also dependent on the activity of antioxidant enzymes. Therefore activity of enzymes including SOD, glutathione peroxidase (GP), glutathione reductase (GR) and catalase were analyzed using activity assay kites from Abcam (Abcam, Cambridge, MA, USA). The assays were done according to the manufacture's instruction. Briefly, treated cells were lysed using lysis buffers provided by the kites. All lysis buffers were supplemented by complete protease inhibitor cocktail (Roche, Welwyn, UK). Insoluble materials were sedimented using centrifugation in 14000×g at 4ºC for 15 min. Afterward, a standard Bio-Rad Bradford protein assay with bovine albumin as standard (Bio-Rad, Hemel Hempstead, UK) was employed to determine protein concentrations. Ten µg of the total protein extract was subjected to enzyme activity assay. Absorbance values were measured using a microplate spectrofluorometer (Synergy 4, BioTek, VT, USA) and data reported as percentage of vehicle treated controls.

Results

Determination of intracellular ROS level

Intercellular ROS level was evaluated by measuring the oxidation of nonfluorescent DCFH-DA to its highly fluorescent derivative DCF. MCF-7/ADR and MCF-7 cells were exposed to TNF-α (1, 10, 25 and 50 ng/ml) for 5, 24 and 72 h. Then DCF fluorescence was quantified and directly related to intracellular ROS level. As shown in Figure 1, TNF-α did not stimulate ROS generation in MCF-7 and MCF7/ADR cells.

Cell viability assays

Since MCF-7 and MCF-7/ADR resist against cytotoxic effects of TNF-α and ROS play important role in TNF-α induced cell death we hypothesized that resistance against TNF-α may be related to lack of ROS production after TNF-α exposure.[3-4]

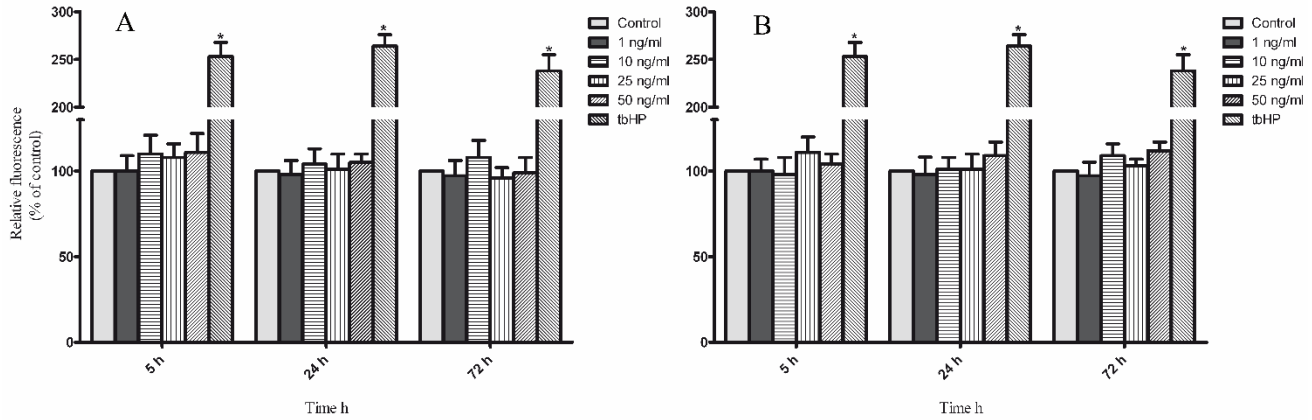

Figure 1. Analyzing ROS accumulation after TNF-α treatment. A fluorescent plate reader was employed to measure the fluorescent intensity of DCF which is directly related to ROS level. MCF-7(A) and MCF-7/ADR (B) cells where treated with TNF-α at various concentrations for 5, 24 and 72 h. Then ROS level was analyzed using DCFH-DA probe. tbHP was used as positive control. *Significantly different from control, P <0.05.

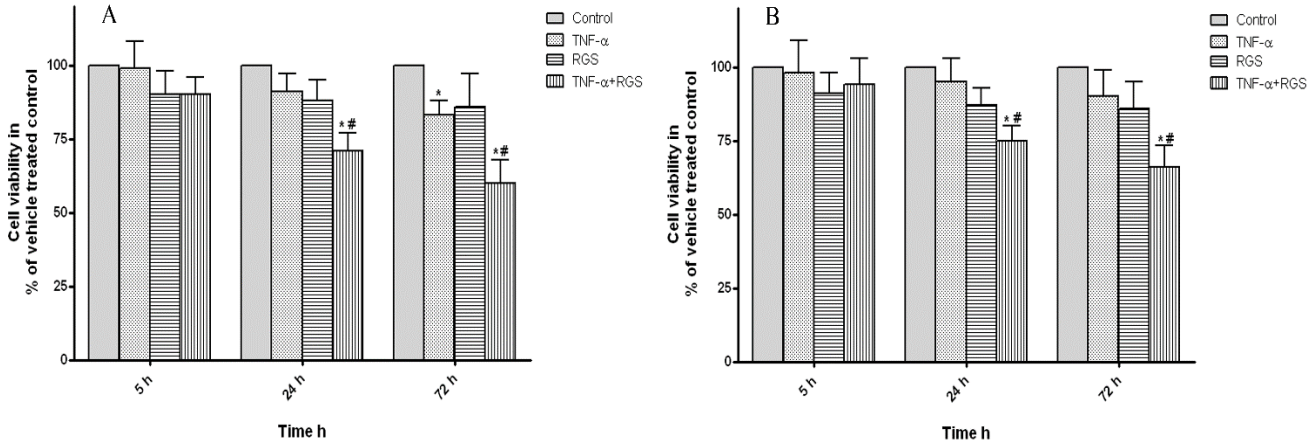

Figure 2. Role of ROS accumulation in the TNF-α cytotoxicity. MCF-7 (A) and MCF-7/ADR (B) cells where treated with TNF-α and/or a standard ROS generating system. As demonstrated, ROS generating system (RGS) sensitized MCF-7 and MCF-7/ADR cells to the cytotoxic effects of TNF-α. *Significantly different from control, P<0.05.

To examine the hypothesis TNF-α treatment was coincided with exposure to a standard ROS generating system. Accumulation of ROS following RGS treatment led to enhancement of cytotoxic effects of TNF-α in both MCF-7 and MCF-7/ADR cells (Figure 2).

Analysis of antioxidant enzymes activity

The balance between ROS generation and ROS detoxification is very important in the intracellular ROS accumulation therefore activity of ROS detoxifying enzymes including SOD, catalase, GR and GP were analyzed using commercial kits. Enzyme activity was analyzed based on manufacturer's instruction following exposure of both cell lines to 50 ng/ml TNF-α. All tested enzyme showed higher activity in MCF-7/ADR than MCF-7 cells (Figure 3) demonstrating better capacity of ROS detoxifying in MCF-7/ADR cells. TNF-α treatment enhanced activity of SOD and GP in both cell lines (P<0.05) (Figure 4) while catalase and GR activity were not demonstrated significant changes after TNF-α treatment (data not shown).

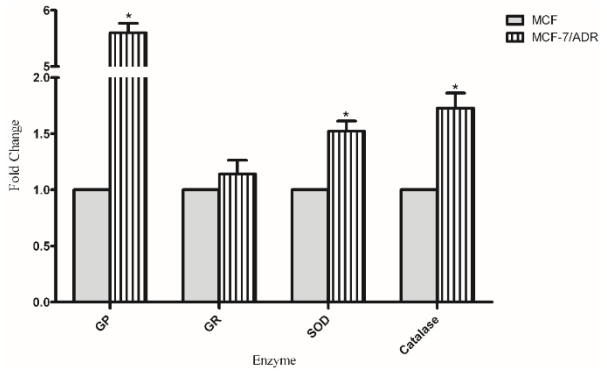

Figure 3. Comparison of antioxidant enzymes activity between MCF-7 and MCF-7/ADR cells. Enzymes activities were analyzed using commercial kites in MCF-7 and MCF-7/ADR cells. Data represented as mean±SD of three independent experiments. *Significantly different from MCF-7 enzyme activity, P<0.05.

Discussion

TNF-α activates various downstream signaling mediators leading cells to survival or death.[16] Despite their damaging effects, ROS also play important role in signal transduction.[7]

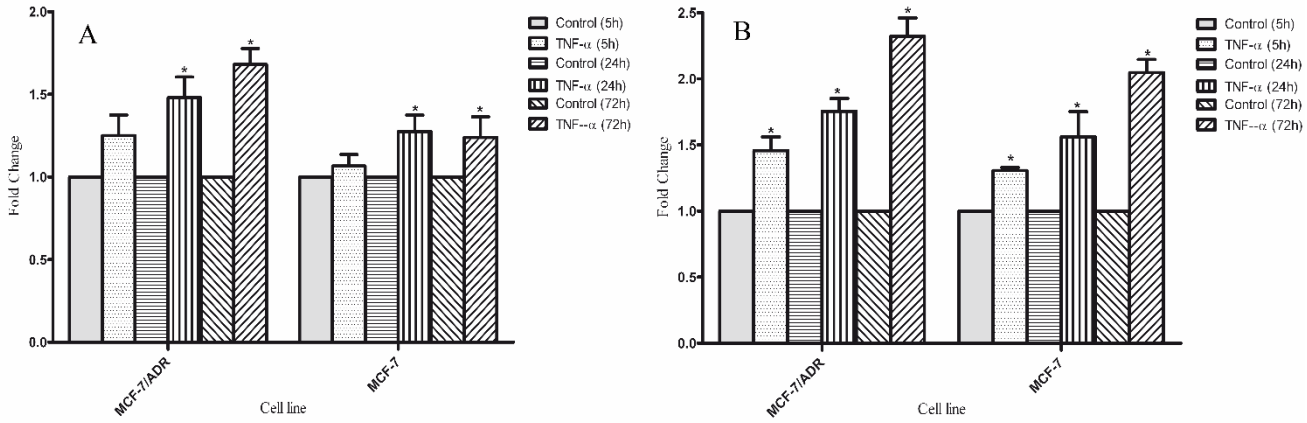

Figure 4. Analysis of GP and SOD activity. GP (A) and SOD (B) activity were assessed after TNF-α treatment in MCF-7 and MCF-7/ADR cell lines. As demonstrated, activity of GP and SOD were increased in MCF-7 and MCF-7/ADR cells after TNF-α treatment. Data represented as mean±SD of three independent experiments. *Significantly different from corresponding control, P<0.05.

To study mechanisms of resistance of breast cancer cell lines MCF-7 and MCF-7/ADR to cytotoxic effects of TNF-α, this study was focused on the role of ROS. Although enhancement of intracellular ROS level following TNF-α treatment has been reported in various studies, TNF-α exposure did not induce ROS accumulation in MCF7 and MCF-7/ADR cells. Further investigations using ROS generating system demonstrated that MCF-7 and MCF-7/ADR cells resistance against TNF-α toxicity at least in part is mediated by lack of ROS accumulation. On the other hand, TNF-α exposure increased activity of antioxidant enzymes which can be one of the mechanisms involved in the lack of ROS accumulation.

The cellular response (e.g., proliferation versus cell death) to TNF-α is determined by the balance between the cell death (apoptotic and non-apoptotic) and cell survivals signaling pathways.[22] TNF-α exposure has led to ROS accumulation in various murine and human cell lines including cultured human umbilical vein endothelial cells and L929 murine cell line.[13,23-24] Moreover, ROS accumulation and necrotic cell death was induced by TNF-α in RAW 264.7 monocytic and L929 fibroblasts cells.[25] TNF-α-induction of ROS can be involved in cell death as well as cell survival signaling pathways. Most of the pro-survival effects of TNF-α are mediated by NF-κB activation and ROS have various inhibitory or stimulatory roles in NF-κB signaling.[17] ROS are promoting TNF-α-induced cell death by activation of pro-death proteins including JNK as well as inhibition of pro-survival pathways including NF-κB.[26] Moreover, ROS represents direct anti-survival effects through oxidation of cellular macromoleculs and causes general damage and dysfunction.[27] IKK and thioredoxin-1 which are involved in the activation of NF- κB and JNK, can be regulated by ROS through oxidation of their redox sensitive cysteines.[28-29] Overall, moderate amounts of ROS usually induces cell proliferation while excessive rises of ROS triggers cell death.[30] This preliminary experiment demonstrates resistance of MCF-7 and its drug resistant derivative MCF-7/ADR cells against TNF-α cytotoxicity is mediated by lack of ROS accumulation at least by part.

Detoxifying of intercellular ROS to non-harmful products by antioxidant enzymes play role in the balance of intracellular redox homeostasis. Oxidized glutathione (GSSH) is converted to its reduced form (GSH) by GR. Organic hydroperoxides are detoxified by GP biological activity. Highly reactive O_2^- is converted to less toxic H_2O_2 by SOD activity and catalase reduces H_2O_2 to H_2O. Transfection of MCF-7 cells with Mn-SOD expressing vector blocked cytotoxicity of TNF-α in human breast and cervical carcinoma cell lines[31-32] and Mn-SOD expression and activity have been enhanced by TNF- α treatment in various cell lines including endometrial stromal (ESC) and MCF-7.[33-34]

In this study, in consistent with other reports, TNF-α treatment enhanced SOD and GP activity in MCF-7 and MCF-7/ADR cells. Moreover, basal activity of all tested enzymes in MCF-7/ADR cells was higher than MCF-7 cells. Expression of antioxidant enzymes including Mn-SOD are regulated by NF-κB activation, which is also involved in TNF-α signaling as prosurvival factor. Therefore, NF-κB may also plays a role in the resistance of MCF-7 and MCF-7/ADR cells to TNF-α toxicity.

Although DNA intercalating is the main mechanism of Adriamycin cytotoxicity but enhancement of ROS level following Adriamycin treatment have been reported in various studies and may involve in cytotoxic effects of Adriamycin.[35-37] Accordingly, improved capacity of ROS detoxifying in MCF-7/ADR cells can be one of the Adriamycin resistance mechanisms in this cell line. More studies on finding mechanisms underlying enhanced activity of antioxidant enzymes in MCF-7/ADR cells may help in clarifying molecular events causing MDR.

Conclusion

Overall, our data demonstrate that lack of ROS accumulation is involved in MCF-7 and MCF-7/ADR resistance against TNF-α cytotoxicity at least by part. Moreover, enhancement of antioxidant enzymes activity following to TNF-α exposure may be involved in this resistance. More investigation on TNF-α signaling mediators including NF-κB, JNK and Akt may help

clarifying the mechanisms contributing to MDR as well as development of new therapeutic strategies against it.

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Conflict of interests

The authors claim that there is no conflict of interest.

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