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Caspase independent cleavages of TDP-43 generates 35kD fragment that cause apoptosis of breast cancer cells

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ABSTRACT

Transactive response DNA-binding protein 43kD (TDP-43) is a major component of tau-negative and ubiquitin-positive inclusions that characterize ALS (amyotrophic lateral sclerosis) and FTLD (frontotemporal lobar degeneration). Due to its central role in neurodegenerative disease pathogenesis, most research recently has focused on its role associated with neurodegeneration disease, research on neuron and glial cell showed that pathological TDP-43 is associated with cell apoptosis which lead to loss of functional neurons and glial cells. However, little is known about its role on cancer cells, here we report a 35kD fragment of TDP-43 also plays a key role in apoptosis of breast cancer cells, and may be served as a potential therapeutic target to cure cancer.

1. Introduction

Transactive response DNA-binding protein 43 (TDP-43) is a highly conservative RNA and DNA binding protein encoded by the TARDBP gene on chromosome 1. Its physiological function is RNA transport and stabilization, DNA silencing [1]. Accumulating evidence suggested that pathological TDP-43 is the main hallmark of many neurodegenerative disease, including amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), they share a common pathogenesis which is known as TDP-43 proteinopathies. Tau-negative and ubiquitin-positive inclusions was abundantly observed in neurons and glial cells of these patients, TDP-43 is the major component [2]. Many research showed that mutation of TARDBP is the cause of inclusion formation rather than consequence [3,4].

TDP-43 can be cleaved by caspase3 and generate 25/35 kDa C-terminal fragments (CTF), CTF plays a key role in redistribution of TDP-43 from nucleus to cytoplasm in neuroglioma cells [5], the 25 kDa CTF generated by caspase3 can lead to the formation of pathological inclusions and induce apoptosis through a toxic gain-of-function [6].

Since TDP-43 is such a crucial protein involved in many key process of cells, and its pathological form can damage neurons and glial cells drastically which leads to neurodegenerative disease, TDP-43 should also play a key role in anti-apoptotic cancer cells. This study is mainly about the role of TDP-43 35 kDa fragment in apoptosis of breast cancer cells, hoping could shed some light on future TDP-43 targeted therapy for cancer.

2. Materials and methods

2.1. Cell cultures and treatments

Human breast cancer cells (MCF7-vec, MCF7-C3, MDA-435-wt) were purchased from the American Type Culture Collection (ATCC) and were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO) supplemented with 10% fetal bovine serum (FBS, GIBCO BRL, Grand Island, NY), penicillin (100 units/mL) and streptomycin (100 μg/mL) were added into the medium (Gibco-BRL, Grand Island, NY). All cell lines were cultured in incubator with humidified atmosphere and 5% CO2 at 37 °C. Cells were passaged every 3–4 days at 80% confluency. Cell passages before 40th generation were used for experiment. Cells were seeded into 6/12/24/96-well plates at least 12 h prior to treatment. STS, Tamoxifen, Z-VAD-FMK, MG132, Wor were all dissolved in DMSO to make a stock solution and stored at −20 °C. Before treatment, drug was thawed and diluted in DMEM with 10% FBS to indicated final

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concentration. Old medium was removed and new medium was added for desired period of time.

2.2. Cell viability assay

Cells were seeded into 24/96-well plate at a concentration of 2.5×10^5/ml 24 h ahead of treatment. After adding drug for intended time, culture medium was replaced with MTT staining solution (0.05 mg/ml in serum-free medium). Then incubated at 37 °C with 5% CO2 for 4 h, followed by adding same volume (100μl for 96-well plate, 500ul for 24-well plate) of MTT lysis buffer (50% v/v *N*,*N*-dimethylformamide (Sigma), 20% SDS (BIO-RAD), and 0.4% (v/v) glacial acetic acid in distilled water (pH 4.8)). The plate was incubated in incubator overnight, OD value of each well was measured with a microplate reader (BIO-TEK, Winooski, VT, USA) at 595 nm. MTS assay (CellTilter 96 Aqueous One Solution, Promega) was also performed to investigate cell viability according to manufacturer's instruction.

2.3. Protein extraction and immunoblotting

After indicated treatment, cells in the plates were washed with ice-cold PBS, then 1× sample buffer (62.5 mM Tris-HCl at pH 6.8 and 25 °C, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% (w/v) bromophenol blue) was added into plate to lyse cells. Cell lysate was collected and transferred into 1.5 ml tubes followed by heating at 95–100 °C for 5 min. Before loading the samples, protein concentration was measured by a modified DC protein assay (Bio- Rad), equal amount of protein was used for immunoblotting. SDS-PAGE was performed to separate different size of protein, then protein was transferred to nitrocellulose membranes using a wet transfer system (BIO-RAD). Block the membrane with 5% nofat milk (BIO-RAD) for 1 h at room temperature before incubating with primary antibody at 4 °C overnight. Wash the membranes with TBST (Tris-buffered saline with 0.05% Tween 20) for 3 times, 15min each, followed by incubation of second antibody (HRP-conjugated anti-rabbit or mouse IgG) for 1 h at room temperature. After 3 times wash with TBST, protein signals were detected by ECL kit (Perkin-Elmer Life Sciences, Boston, MA) using a Kodak image system (Kodak, Rochester, NY). Images were analysed by Image J (NIH, Bethesda, MD, USA).

2.4. Plasmid and siRNA transfection

Cells were inoculated into 6/12/96-well plate at a proper seeding concentration 24 h before transfection. For plasmid transfection, we used Lipofectamine 2000 Transfection Reagent (Invitrogen); for siRNA transfection, we used Lipofectamine RNAiMAX Transfection Reagent (Invitrogen). Both are according to manufacturer's instruction. Other treatment was applied at least 36 h after transfection.

2.5. Statistical analysis

At least 2 independent experiments were performed for data analysis. Charted data are presented in terms of means with SD. Unpaired, two-tailed Student's t-test was used to compare means of 2 groups, one way ANOVA was used to compare means of 3 or more groups, differences were regarded as statistically significant when $P < 0.05$ and were marked with asterisk (*) in figures.

3. Results

3.1. Staurosporine (STS) caused reduction of full-length TDP-43 and activation of caspase-3, generating a 35kD fragment in MCF7-C3 cells

Staurosporine (STS) is a classic agent used for inducing apoptosis [7]. MCF7 is a human breast cancer line that is null in caspase-3 and MCF7-C3 is an engineered daughter cell line of MCF7 which is able of expressing caspase-3 via permanently transfection [8] It has been well known that TDP-43 can be cleaved by caspase-3, resulting 25kD or 35kD fragments [6] Indeed, 4 h treatment with STS at 0.5uM caused significant reduction of full-length TDP-43 and production of 35kD fragment shown by western blots (Fig. 1 A,C,D). STS also caused activation of caspase-3 indicated by reduction of pro-caspase-3 (Fig. 1 B).

3.2. TDP-43 35kD fragment is degraded through ubiquitin-proteasome pathway

Cleavage of TDP-43 generate a 35kD fragment [6] which is seen in MCF7-C3 cells treated with STS (Fig. 1 A). To investigate the degradation pathway of TDP-43, we introduced two protein degradation inhibitors, Wortmannin and MG132. Wortmannin (Wor) can block the autophagy-lysosomal pathway [9] while MG132 can block ubiquitin-proteasome pathway [10]. The results showed that MG132 but not Wor significantly increased the amount of 35kD TDP-43 fragment in STS treated MCF7-C3 cells (Fig. 2A and B). Therefore, the 35kD fragment is mainly degraded by ubiquitin-proteasome pathway.

3.3. Increased 35kD TDP-43 cleavage product enhances STS cytotoxicity

Since TDP-43 35kD has been shown to cause cytotoxicity in various types of cells [11], we speculated that blocking degradation of 35kD fragment may enhance STS induced cytotoxicity in MCF7-C3 cells. Indeed, in the presence of MG132 which prevents proteasome degradation of TDP-43 35kD significantly increased STS caused cell death in MCF7-C3 cells while blocking lysosomal pathway by Wor that does not involved in 35kD fragment degradation had no effect (Fig. 2C). Similarly, in the presence of Z-VAD-FMK (20μM) that reduced cleavage of TDP-43 in caspase-3 positive MCF7-C3 cells (Fig. 3A and B), significantly less cell death was seen in STS treated cells (Fig. 3C). Thus, the above results suggested that intracellular levels of 35kD TDP-43 cleavage product are closely associated with STS induced cytotoxicity.

3.4. Tamoxifen induced cytotoxicity in breast cancer cells is also dependent on generation of 35kD TDP-43 cleavage product

Tamoxifen (TAM) is the first-line agent used to treat estrogen positive breast cancer which can lead to apoptosis of cancer cells [12]. To explore the role of TDP-43 in Tamoxifen-induced apoptosis of breast cancer cells, MCF7-C3 cells were subjected to various concentrations of TAM for 24 h, followed by western blot for TDP-43. Similar to STS, TAM at 40uM can cause decrease of full-length TDP-43 and 35kD fragment was also seen in the presence of ubiquitin-proteasome pathway blocker MG132 (Supplementary Fig.1 A). The same concentration of TAM that caused TDP-43 cleavage is associated with significant cell death. Furthermore, blocking 35kD fragment degradation by MG132 also caused significantly increased cytotoxicity of TAM (Supplementary Fig.1 B).

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