Detection of ROS and translocation of ERP-57 in apoptotic induced human neuroblastoma (SH-SY5Y) cells

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Abstract: Several toxic compounds are known to induce apoptosis in mammalian cell lines. The human neuroblastoma cells (SH-SY5Y) were exposed to the phosphatase inhibiting toxin okadaic acid (OA) or hydrogen peroxide (H2O2) to induce apoptosis as well as generate reactive oxygen species (ROS). Mitoxantrone (MXT) was used as a positive control for apoptosis. The SH-SY5Y cells were transfected with eukaryotic expression plasmid pHyPer-dMito encoding mitochondrial-targeted fluorescent or pHyPer-dCito encoding cytoplasmic-targeted fluorescent sensor for hydrogen peroxide (HyPer). The ERP57, also called GRP58 (Glucose-regulated protein 58), is a stress protein induced in conditions like glucose starvation and viral infection. Recently ERP57 was shown to translocate from the endoplasmatic reticulum to the cell surface in anthracycline-induced apoptotic cells. ERP57 co-translocation together with calreticulin has been suggested to be crucial for recognizing tumor cells to induce immunogenic cell death. ERP57 translocation after exposure to okadaic acid was studied using immunofluorescence and confocal microscopy. These studies indicated that okadaic acid has induced the translocation of ERP57 to the cellular membrane.

Introduction

Apoptosis a cell death process characterized by a series of specific morphological changes, such as cell shrinkage, rounding-up of the cell, pseudopodia retraction, budding of the cell membrane, chromatin condensation, nuclear as well as cellular fragmentation, and eventually, in vivo, engulfment of the apoptotic cell (Kerr et al., 1972; Anita et al., 2014). Apoptosis is genetically controlled, natural process playing crucial role in clean and safe removal of dead, damaged and old cells. Thereby apoptosis is a crucial process for the proper biological functions as well as development of the organisms. Thus, apoptosis prevents the inflammatory responses, cancer, and autoimmune diseases, however accelerated apoptosis could lead to acute and chronic degenerative disorder, infertility and immunodeficiency situations (Rosenberg et al., 2004; Elmore, 2007). Apoptosis can be induced by many different independent signalling pathways i.e., extracellular (the extrinsic or death receptor pathway) or intracellular (the intrinsic or mitochondrial pathway) pathways. These pathways converge on a common mechanism for causing cell death, which is the proteins degradation by caspases (Wilk, 2005). The third pathway causes the accumulation of unfolded proteins due to the stress in the endoplasmic reticulum (ER), leading the cells to apoptotic death.

Endoplasmic reticulum (ER), the important organelle in eukaryotic cells has multiple functions such as protein secretion, glycosylation as well as folding, intracellular calcium homeostasis and lipid synthesis. The disturbance in the homeostasis of ER called ER stress leads to the activation of a series of signal transduction cascades which are collectively called unfolded protein response (UPR). The UPR could reduce the translocation of proteins into the ER and ultimately result in the increased number of unfolded or misfolded proteins leading to apoptosis (Szegedi et al., 2006; Ron et al., 2007; Scull et al., 2011; Glab et al., 2017). The ER stress induces apoptosis by activating Caspase-3 and thereafter Caspase-12 (Groenendyk et al., 2005). The ER stress agents cause mitochondrial release of cytochrome c and loss of mitochondrial trans-membrane potential. Mitochondria plays significant role in intracellular bioenergetics, ion homeostasis, fatty acid and carbohydrate metabolism. Mutations and malfunction of mitochondria

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have been implicated in several human diseases, like cancer, neurodegeneration, diabetes, cardiomyopathy, aging and obesity (Wallace, 1999; Bravo et al., 2012). It has been reported that mitochondria initiate the process of apoptosis by releasing many mediators like cytochrome C and apoptosis-inducing factors. These mediators activate the caspase family proteases which results in apoptosis (Suen et al., 2008). Mitochondria are the most reactive oxygen species (ROS) producing organelles in the cell, due to its operative electron-transport chain which is the main source of ROS during normal mitochondrial metabolism (Willems et al., 2015). ROS are reactive molecules and free radicals derived from molecular oxygen. Oxygen is an important element of life, but it can cause the production of different kinds of ROS such as hydrogen peroxide (H_2O_2), peroxynitrite (ONOO-), superoxide (O_2-) and hydroxyl radicals (OH-). In human body, ROS are produced as part of the usual metabolism and can be eliminated by metal chelators, enzymatic reduction and antioxidants (endogenous and exogenous) such as 4-hydroxy-2,2,6,6-tetramethylpiperidin-N-oxyl (TEMPO) (Hancock et al., 2001; Zhang et al., 2004). In small quantity ROS plays role in extracellular signalling, but under oxidative stress conditions such as radiation and cytotoxic drugs, ROS induces apoptosis (Hancock et al., 2001). In intracellular apoptotic signalling cascades, H_2O_2 has been shown to act as a second messenger. Thereby causes lipid peroxidation as well as DNA damage leading to apoptosis induction in different cell lines (Yoshikawa et al., 2006; Ježek et al., 2018).

Endoplasmic Reticulum Protein 57 (ERp57), 54 kDa protein, is a close homologue of protein disulide isomerase (PDI) (Koivunen et al., 1996). ERp57 is found abundantly in those tissues, which are actively involved in the synthesis of ERp57 in ER and mitochondrial stress-induced apoptosis (H Hancock et al., 2004). In small quantity ERp57 plays important role in the formation of disulide bond, proper protein folding and oxidoreductase activity within the cytosol as well as nucleus (Turano et al., 2002). It is also involved in the cytoplasmic signal transduction as well as regulation of transcription by activation of STAT313 in the nucleus (Eufemi et al., 2004). ERp57 interacts with the membrane bound lectin Cnx and Crt to catalyze disulide bond formation in glycoproteins (Ellgaard & Ruddock, 2005).

Further roles of ERp57 include effectiveness in the fertility of rodent, biomarker in the determination of ovarian and some other type of cancer and crucial role in chemoresistance mechanisms after paclitaxel exposure (Cicchillitti et al., 2009). The ERp57 is involved in mitochondrial import of proteins. Recently ERp57 associated with mitochondrial μ-calpain and m-calpain was found to be involved in the converting of apoptosis-inducing factor (AIF) to truncated form (tAIF). Thereby leading to the subsequent release of tAIF, from the mitochondrial inner membrane (IM) into the intermembrane space (IMS). Thereby ERp57 is involved in causing cell death by the AIF-induced signalling pathway (Joshi et al., 2009). Thus, ERp57 might undergo some modifications that may cause its release from ER and participates in apoptosis induction (Panaretakis et al., 2008; Wiersma et al., 2015). But the actual physiological function of ERp57 in ER and mitochondrial stress-induced apoptosis still needs further exploration.

This study was an effort to understand the time increase ROS generation in apoptotic induced cells (mitochondrial and cytoplasmic) and its possible effect on the translocation of ERp57 from ER to the cell membrane. The translocation of the ER might prove a suitable biomarker for apoptotic cells, however the key role of ERp57 in apoptotic cells needs further exploration.

Material and Methods

General maintenance of cell line

General maintenance of SH-SY5Y cell line was done in sterile environment inside a laminar flow hood bench. The cells were grown in OPTI-MEM media with 10% fetal bovine serum and 1% penicillin/streptomycin antibiotics under incubation conditions of 5% CO_2 level at 37°C. At 80-100% confluency the cells were washed with 1x PBS pH 7.4, and trypsinated (1:10 dilution of 10 x Trypsin-EDTA in 1x PBS) for 3 min under same conditions. Fresh media was added to stop trypsination and cell suspension was centrifuge at 900x g for 5 min. The supernatant was discarded and the pellet re-suspended in fresh media to get 1:3 dilution, and grown in a new flask under same conditions.

Transfection

SH-SY5Y cells were seeded in 24-well plates with 22000 cells per well. When cells were approximately 35% confluent they were shifted from OPTI-MEM media containing ampicillin/ streptomycin and 10% serum to the same media without antibiotics and serum. Cells were transfected with plasmid using Lipofectamine® 2000 according to manufactures protocol. After six h medium was change to OPTI-MEM containing ampicillin/streptomycin and serum and was incubated further 48 h.

ROS detection

The SH-SY5Y cells were transfected with eukaryotic expression plasmid pHPer-dMito or pHPer-dCito encoding mitochondrial and cytoplasmic-targeted fluorescent sensor for hydrogen peroxide (HyPer). In the presence of hydrogen peroxide These HyPer sensors emit a green fluorescence signal. ROS-induced fluorescence was detected firstly in by a fluorescence (LICA) Microscope using 10 × 0.75 objective and then by a Zeiss LMS Meta Confocal Scanning Microscope by 63 × 1.40 Plan-Apochromat Oil objective lens.

Immunolabelling

SH-SY5Y cells were seeded on cover slips in a 24-well plate. When the cells were 80% confluent they were treated with 400 nM okadaic acid, 300 μM H_2O_2 or left untreated. Thereafter the cells were fixed on cover slips by incubating for 20 min at RT in 2% formaldehyde in 1x PBS. Then the cells were washed with 1x PBS for 5 min. For permeabilization the cells were incubated in 500 μl of 0.2% Triton X-100 in 1x PBS for 10 min. Thereafter, the cells were washed three times with 1x PBS for 5 min at RT. Then the cells were blocked by incubating overnight at 4°C in blocking solution (500 μl of 1 mg/ml BSA in 1x PBS with 0.5% NP-40). Cells were immunostained overnight at 4°C using primary antibody anti-ERp57 (1:100) in blocking solution. Then the cells were
washed three times with 1x PBS at RT. Thereafter the cells were incubated with appropriate secondary antibody with appropriate dilutions in blocking solution, for one h in dark at RT. The cells were again washed with 1x PBS three times for 5 min at RT. Then the cells were incubated with 1:4000 diluted DAPI staining solution for 7 min in dark at RT. The cells were washed with 1X PBS four times for 5 min on rocker at RT. Each cover slip was washed with a droplet of dH2O and was mounted with 5 µl mounting solution on an object glass.

Confocal microscopy
Oil objective 63 × 1.40 Plan-Apochromat of Zeiss LMS510 Meta Confocal Scanning Microscope was used for imaging of the cell sections. The FITC anti-rabbit secondary antibodies against ERP57 were excited with 488 nm laser beam, while the emission filter LP505 was used.

Results

Apoptosis induction
Okadaic acid (OA), hydrogen peroxide (H2O2) and mitoxantrone dihydrochloride (MTX) were used throughout the study as apoptosis inducers for SH-SY5Y cells. To determine the optimal time for harvesting and fixing of cells the time course of apoptosis development for the individual inducers was determined. The cells were grown in 48-well plates to approximately 80% confluency and either left untreated or treated with 10 µM MTX, 300 nM OA, or 300 µM H2O2 for different time periods. The cells were fixed, and the development of apoptosis was determined by light microscopy. As been evident from Fig. 1, the monolayer cell cultures have adopted a rounded shape and have detached from solid support forming beads on a string that are consistent with apoptosis after exposure to OA, H2O2, or MTX (Figs. 1(B)-1(D)) whereas the untreated control cells (Fig. 1(A)) do not show such morphological changes. The time dependent development of apoptosis induced by OA, H2O2 and MTX is shown in Fig. 2. As can been seen approximately 100% apoptosis was reached after 1.5 h with OA, 1 h with H2O2, and 6 h with MTX.

ROS development in apoptotic cells

The eukaryotic expression vectors pHyPer-dMito and pHyPer-Cyto were used to study the time-dependent ROS release in SH-SY5Y cells exposed to apoptotic inducers. The pHyPer vectors encode fluorescent sensors, which are capable of detecting cytoplasmic and mitochondrial hydrogen peroxide (H2O2). For this purpose, pHyPer-dMito and pHyPer-Cyto vectors were transfected into SH-SY5Y cells and intracellular ROS, detected as H2O2, was evaluated using a fluorescent microscope. Fig. 3 show the examples of mitochondrial ROS (Fig. 3(A)) and cytoplasmatic ROS (Fig. 3(B)), after exposure to ROS inducing agents. The mitochondrial and cytoplasmatic ROS detecting probes were further used to determine the time scale ROS development after OA and H2O2 exposure. SH-SH5Y cells were co-transfected with either pHyPer-dMito or pHyPer-Cyto together with a transfection control plasmid (red). ROS development was visualized by using fluorescence microscopy. Fig. 4 shows the cytoplasmic ROS development, while Fig. 5 shows mitochondrial ROS after OA and H2O2 exposure. Increased levels of cytoplasmic as well as mitochondrial ROS (green) can be observed after OA and H2O2 exposure of transfected cells (red). Time dependent ROS generation in cytoplasm (A-B) and mitochondria (C-D) is shown in Fig. 6. It is clear from the plotted lines in the figure that H2O2 induces more ROS than OA additionally more ROS can be observed in the mitochondria compared to the cytoplasm after OA and H2O2 exposure.

FIGURE 1. OA, H2O2 and MTX induced apoptosis. The figure shows the morphological appearance of SH-SY5Y cells left untreated (A) or treated with either 300 nM OA for 1 h (B), 300 µM H2O2 for 1 h (C) or 10 µM MTX for 6 h (D). Cells were grown in 48-wells plates to approximately 80% confluency before addition of apoptosis inducers, fixed in formaldehyde and viewed in a light microscope with 10x magnification.
FIGURE 2. Time-dependent development of apoptosis. SH-SY5Y cells were left untreated or treated with either 300 nM OA (blue line) or 300 µM H₂O₂ (red line) or 10 µM MTX (green line) for different time durations. The cells were fixed, and the number of apoptotic cells was counted based on morphological features of apoptosis. The number of apoptotic cells is expressed as percentage of the total number of cells. One-way ANOVA was applied on triplicate data at $p \leq 0.5$. The mean values with common letter/s are significantly not different.

FIGURE 3. Detection of mitochondrial and cytoplasmic ROS development. SH-SY5Y cells were transfected with the eukaryotic expression vectors pHyPer-Mito and pHyPer-Cyto. 48 hr after transfection the cells were exposed to the ROS inducing agent H₂O₂. ROS development was imaged using confocal microscopy. The figure shows examples of mitochondrial ROS (A) and cytoplasmic ROS (B).

FIGURE 4. Cytoplasmic ROS development after okadaic acid and H₂O₂ exposure. SH-SY5Y cells were co-transfected with pHyPer-Cyto (green) and M Cherry C1 (red). 48 h after transfection the cells were exposed to 400 nM OA (35 min) or 300 µM H₂O₂ (25 min). Cytoplasmic ROS development was imaged after fixation using a fluorescence microscope with 10x magnification.
FIGURE 5. Mitochondrial ROS development after okadaic acid and H₂O₂ exposure. SH-SY5Y cells were co-transfected with pHyPer-Mito (green) and M Cherry C1 (red). 48 h after transfection the cells were exposed to 400 nM OA (40 min) or 300 µM H₂O₂ (15 min). Mitochondrial ROS development was imaged after fixation using a fluorescence microscope with 10x magnification.

FIGURE 6. Time dependent cytoplasmic and mitochondrial ROS development after okadaic acid and H₂O₂ exposure. SH-SY5Y cells were co-transfected with either pHyPer-Cyto (blue and red lines) or pHyPer-Mito (green and purple lines). 48 h post-transfection the cells were exposed to 400 nM OA (blue and green) or 300 µM H₂O₂ (red and purple) for different time durations. The cells were fixed, and the number of ROS-positive cells was calculated to determine the number of transfected (red) cells which showed ROS (green) development (Figs. 4 and 5). One-way ANOVA was applied on triplicate data at p ≤ 0.5. The mean values with common letter/s are significantly not different.
Localization of ERp57 in apoptotic cells

SH-SY5Y cells were grown on coverslips with approximately 80% confluency and either left untreated or treated with 10µM MTX or 300 nM OA for different time periods. ERp57 localization was studied using immunolabeling and confocal microscopy (Fig. 7). In Fig. 7 the DAPI stained nuclei are shown in panel A, C, and E, while ERp57 staining are shown in panel B, D, and F. As can be seen in the figure, ERp57 is translocated to the cell surface membrane by okadaic acid (D) and mitoxantrone (F). This is indicated by arrows. ERp57 translocation is absent in control cells (B). The mitoxantrone treatment was used as a positive control in this study as recently ERp57 translocation was confirmed by mitoxantrone treatment of CT 26 cells (Obeid, 2008; Wiersma et al., 2015). These results indicated that ERp57 is translocated to the cell surface in OA-induced apoptotic SH-SY5Y cells.

Discussion

The light microscopy images revealed that SH-SY5Y cells exposed to OA, H₂O₂ and MTX undergo morphological changes characteristic to apoptosis i.e., decrease in cellular volume, chromatin condensation and plasma membrane bud formation (Fig. 1). In the case of SH-SY5Y monolayer cell cultures, cells adopt a round shape and detach from solid support forming beads on a string. Different chemicals induce apoptosis with different speed. The time duration for apoptosis induction was different for both of these toxins (OA and MTX) and OA (H₂O₂). As can be seen in Fig. 1, in the case of OA (Fig. 1(B)), many of the cells had shrunk and rounded up after 90 min. Such a reduction of cell size is characteristic for apoptosis, while necrotic cells show swelling and lyses (Raff, 1992). On the other hand, H₂O₂ (Fig. 1(C)) was found to induce apoptosis faster, showing the same morphological changes after 60 min, while MTX (Fig. 1(D)) was the slowest apoptosis-inducing agent among these three and showed morphological apoptosis after approximately 6 h. Furthermore, we observed that the morphology of the nuclei changes during apoptosis process with OA, H₂O₂, and MTX in SH-SY5Y cell line. Some of the cells exhibit nuclear fragmentation observed in confocal images DAPI stained nuclei (data not shown). All these findings indicate that OA, H₂O₂, and MTX induce apoptosis in SH-SY5Y cells with different time speed. Among these three H₂O₂ is the fastest and MTX is the slowest. In many cell lines the induction of apoptotic pathways is initiated by ROS development. Thus, the apoptotic cells were further studied for ROS generation.

ROS is the major, well known, reason for numerous pathological processes including aging, apoptosis, and cellular injury. Mitochondria are the most common site for ROS generation. It has been reported that OA induces rapid increase in ROS in the early stages of apoptosis induction and remain persisted until 4 h of exposure. This ROS burst occurs in the initial stages after OA exposure and acts as an early mediator in apoptotic pathway (Jayaraj et al., 2009). Also it has been reported that H₂O₂ in the form of ROS, results in tissue damaged of numerous cell lines by necrotic and apoptotic pathways (Mittal et al., 2014) Additionally, ROS are produced in cells as a result of the partial reduction of oxygen by the electron transport chain in mitochondria and in the cytoplasm by lipoxygenases (Rhee, 2006). The specific mitochondrial and cytoplasmatic ROS-detection probes have been successfully implemented in our study (Fig. 3). To monitor the changes of H₂O₂ level in the cytoplasm and mitochondria, cells transfected with plasmids expressing the two different ROS detecting probes were exposed to the apoptosis inducing agents for different time durations. As can be seen in Fig. 6, over the time H₂O₂ (red and purple lines) generated more ROS than OA (blue and green lines), also it is clear that more ROS is produced in the mitochondria (green...
and purple lines) than cytoplasm (blue and red lines). The reason may be that electron-transport chain is the main source of ROS during normal mitochondrial metabolism. These findings showed that in SH-SY5Y cells ROS are produced in cytoplasm and mitochondria after OA and H₂O₂ exposure and increases with increasing the time exposure till an hour. Thereby it conforms that ROS is one of the major initiators of apoptosis induced by OA and H₂O₂ in SH-SY5Y cells. 

ERp57 was shown to co-translocate together with calreticulin from the endoplasmic reticulum to the cell surface in anthracycline-induced apoptotic cells. Translocation of ERp57 has been suggested to be crucial for recognizing tumor cells to induce immunogenic cell death (Obeid, 2008; Wiersma et al., 2015). In this study we used mitoxantrone as a positive control to study the ERp57 delocalisation from ER to the cell surface membrane in OA induced apoptotic SH-SY5Y cells. As can be seen in the Fig. 7 D, ERp57 has been translocated to the cell surface membrane in the OA induced apoptotic cells. This indicated that OA causes translocation of ERp57 from ER to cell surface membrane in SH-SY5Y cells. The confocal microscopy image in Fig. 7 is not much clear to show the translocated ERp57 by surface staining, due to the intracellular staining of the cell. The 20 min fixation with 2% formaldehyde is hard for the SH-SY5Y cells making the cell membrane permeable for the antibodies. As a result, we get intracellular staining in the non-permeabilized cells. This hurdle might be minimized by soft fixation of the cells with more diluted formaldehyde for short duration. Similar ERp57 translocation was not observed in H₂O₂ treated cells (data not shown).

Conclusions

OA, H₂O₂ and MTX were found to induce apoptosis in SH-SY5Y cells. Additionally, both OA and H₂O₂ were found to produce cytoplasmic and mitochondrial ROS in the cytoplasm and mitochondria. OA seems to cause translocation of ERp57 to the cell surface membrane.

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