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Initiation of autophagy and apoptosis by sonodynamic therapy in murine leukemia L1210 cells

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ABSTRACT

Sonodynamic therapy (SDT) has shown great potential in target cancer therapy, but it induced cell death modes has not been fully investigated. This study was to examine autophagic and apoptotic responses to protoporphyrin IX (PpIX) mediated SDT in murine leukemia L1210 cells. After SDT, the occurrence of autophagy was identified by morphological observation and biochemical analysis. Meanwhile, the mitochondria dependent apoptosis pathway was examined to participate in SDT induced cell death. The relationship between autophagy and apoptosis was further investigated by applying pharmacological inhibition studies, which indicated that impairment of autophagy enhanced the anti-tumor effect of SDT through induction of apoptosis and necrosis, while caspase inhibition did not affect autophagic vacuoles formation or protect SDT induced cytotoxicity. The findings supported that autophagic vacuoles formed upstream and independently from caspase-dependent cell death. Additionally, the possible mechanism of SDT-induced autophagy was evaluated by measurement of ROS (reactive oxygen species) formation. Result suggested ROS play important role in initiating autophagy, possibly through the sono-damaged mitochondria being enclosed by autophagic vacuoles. All together, these data indicate that autophagy may be cytoprotective in our experimental system, and point to an important insight into how autophagy inhibitors, in combination with SDT may contribute a regimen for cancer therapy.

1. Introduction

Sonodynamic therapy (SDT) involves the irradiation of sono-sensitized cells with ultrasound (Tachibana et al., 2008; Rosenthal et al., 2004). It is based on the ability of certain sono-sensitizer to localize in malignant cells. Subsequent irradiation with ultrasound can cause lethal sono-damage including cell apoptosis and necrosis by both direct mechanical stress and indirect chemical reactions. Therefore, SDT has great advantage as a target cancer therapy.

The ultimate goal of anti-cancer therapy is to kill cancer cells quickly and effectively. Up to now, three different types of cell death have been distinguished: apoptosis, autophagy, and necrosis (Edinger and Thompson, 2004). Autophagy is a newly described cellular response to various cancer therapies (Kondo and Kondo, 2006). Briefly, the autophagy process comprises four stages. In mammalian cells, the process begins with an isolation membrane formation. Second, the isolation membrane expands to encompass cytosolic proteins and organelles, which involves conjugation of phosphatidylethanolamine to LC3 (microtubule associated protein light chain 3), leading to the conversation of soluble LC3-I to membrane-bounded LC3-II. Atg5-Atg12 system also play key role in this stage. Thirdly, the expanded membranes fuse to form a double-membrane vesicle called an autophagosome or autophagic vacuoles (AVOs). The last step is the fusion of autophagosome and lysosome, followed by the degradation of its contents (Wang and Klionsky, 2003; Levine and Klionsky, 2004; Klionsky and Emr, 2000).

Autophagy was originally characterized as a survival response to nutrient deprivation, but recent studies clearly indicate that the induction of autophagy can sometimes lead to cell death, which is considered to be type II programmed cell death (Kuma et al., 2004; Gozuacik and Kimchi, 2004). It is also suggested that there is significant cross-talk between autophagy and apoptosis (type I programmed cell death) (Maiuri et al., 2007; Boya et al., 2005). Apoptosis, as the best described form of programmed cell death, irreversibly leads to cell death, while autophagy can be both a survival and a death pathway. Autophagy can either delay cell apoptosis or act as part of apoptotic program, or sometimes being a parallel pathway with apoptosis to contribute to cell death (Yang and Klionsky, 2009). Therefore, the understanding of either pro-survival or pro-death function of autophagy might shed lights on enhancing efficacy of anticancer therapy by the modulation of autophagy.

Few studies reported the role of autophagy in the efficacy of SDT. Previously, we used sarcoma 180 cells as the study mode,

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and primarily found autophagy linked with apoptosis after SDT treatment (Wang et al., 2010). Because the ultrasonically induced cytotoxicity is greatly cell line dependent (Wang et al., 2009a), So, in this study, it is very important to investigate the autophagy occurrence and its function in SDT caused cellular fate of murine leukemia L1210 cells, and further to evaluate the possible mechanism for SDT induced autophagy. Protoporphyrin IX (PpIX) was employed as a sono-sensitizer, known to have high affinity for peripheral benzodiazepine receptor on the outer mitochondrial membrane, and mainly mediate mitochondria stress during ultrasound irradiation (Mi et al., 2009; Wang et al., 2010). Following PpIX-SDT, hallmarks of apoptosis and autophagy were detected by morphological observation, biochemical analysis and molecular measurements. The relationship between autophagy and apoptosis was further illustrated by pharmacological inhibition studies with apoptosis suppressor z-VAD-fmk and autophagy inhibitor 3-methyladenine (3-MA) or bafilomycin A1 (Ba A1). The potential mechanisms of SDT induced cellular responses were also evaluated by measuring ROS (reactive oxygen species) generation and mitochondria damage. The available findings may provide new insights into the mechanism of SDT-induced cell death, suggesting potential therapeutic intervention for SDT mediated cancer therapy by manipulating autophagy.

2. Materials and methods

2.1. Cell culture and reagents

Murine L1210 cells were purchased from the American type culture collection (ATCC, CCL-219™). Cells were cultivated in DMEM (Gibco) supplemented with 10% horse serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 1 mM L-glutamine. Cells were maintained at 37 °C in a humidified 5% CO_2 atmosphere. Each experiment utilized cells in the exponential phase.

PpIX, 3-MA, 4′-6-diamidino-2-phenylindole (DAPI), 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltertrazolium bromide tetrazolium (MTT), acridine orange (AO) were purchased from Sigma company. Mito Tracker Red (MTR), Mito Tracker Green (MTG) and 2′,7′- dichlorodihydrofluorescein-diacetate (DCFH-DA) were supplied by Molecular Probes Inc. z-VAD-fmk (z-VAD) was purchased from Biovision. Bafilomycin A1 was obtained from Millipore.

Antibodies raised against Bax and PARP were from Cell signaling technology; anti-Bak, cytochrome c (Cyto c), LC3 and β-actin were from Santa Cruz; anti-Atg5 were obtained from Sigma.

2.2. Intracellular PpIX localization

L1210 cells were incubated with 1 μg/ml PpIX for 60 min at 37 °C. Mito Tracker Green (20 nm) was added to incubation medium together with PpIX, images were captured by a laser scanning confocal microscope (TCS SP5, Leica, Germany). In multi-channel imaging, photomultiplier sensitivities and offsets were set to a level at which bleed through effects from one channel to another were negligible.

2.3. SDT protocols

L1210 cells (1×10^6 cells/ml) were harvested and incubated in DMEM medium with 25 mM HEPES buffer (pH 7.2) replacing $NaHCO_3$ to promote pH maintenance at high cell densities. Sono-sensitization with PpIX involved a 60 min drug-loading incubation at 37 °C, allowing sufficient time for cell uptake of the sensitizer to reach the maximum level. The cells were then exposed to ultrasound. In the inhibitory studies, 3-MA (1 mM), Ba A1 (0.1 μM), z-VAD (5 μM), and NAC (5 mM) were added to culture medium prior to loading PpIX by 1 h. The inhibitors used at the selected concentrations did not yield any significant cell damage to cultured cells.

The experiment set-up for insonation was the same as previously described (Wang et al., 2010). After SDT treatment, cell suspensions were cultured for an additional period as specified in the text and subjected to different analysis.

2.4. Cell viability assay

MTT assay provides a rapid and simple method to evaluate the cell viability following SDT (Wang et al., 2010). This assay was performed as a regular procedure and the absorbance at 570 nm was recorded using a microplate reader (BIO-TEK ELX800, USA) against the reference value at 690 nm. Results were expressed as percentage of control.

2.5. Western blot analysis

After treatment, cells were lysed in RIPA buffer (containing 50 mM Tris HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 1 μM leupeptin and 0.01 μM aprotinin). Similar amounts of protein were analyzed in each lane. Electrophoresis was carried out on 12% to 15% acrylamide gels and the proteins were transferred to PVDF membranes (Millipore, 0.22 μm pore size). Membrane blocking, washing, primary and secondary antibody incubations and chemiluminescence reactions were carried out according to the manufacture's ECL protocol (Amersham biosciences, ECL Plus). Anti-actin was used to ensure equal loadings. Antibodies dilutions were carried out as per the data sheet provided by the manufacture.

2.6. Immunofluorescence assay

At the indicated times after SDT, cells were fixed with 4% paraformaldehyde for immunofluorescence assay. Cells pre-incubated with 20 nM MTG were stained to detect the Bax, Bak translocation and the Cyto c release. Cells pre-incubated with 100 nM MTR were stained to detect the co-localization of damaged mitochondria and Atg5. The corresponding secondary antibodies were performed by immunoglobulin FITC or TRITC conjugates. Cells were imaged with a confocal microscope.

2.7. Fluorescence microscopy

DAPI (4 μg/ml), was used to assess the nuclear morphology of the sonicated cells. After labeling, cells were washed with PBS and viewed under a fluorescence microscope (Nikon E600, Japan). Phase-contrast and fluorescence images were acquired using a CCD camera with the same exposure settings. The percentage of apoptotic nuclei were calculated, all cells from 10 random microscopic fields at 40× magnification were scored.

2.8. TEM and SEM observations

For TEM (transmission electron microscopy) observation, cells were harvested and then fixed in 2.5% glutaraldehyde in 0.1 M PBS (pH 7.2) for 1 h at 4 °C, followed by post-fixation for 1 h at 4 °C in 1% osmium tetroxide. After washing with PBS (0.01 M, pH 7.2), the samples were dehydrated by graded alcohol (30%, 50%, 70%, 80%, 90%, 95%, 100%, 30 min each time), embedded with Epon812 and cut into ultrathin sections. The sections were stained with uranium acetate and lead citrate, and examined under a TEM (JEM-2100, Japan).