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Diethylstilbestrol (DES) induces autophagy in thymocytes by regulating Beclin-1 expression through epigenetic modulation

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ABSTRACT

Diethylstilbestrol (DES) is an endocrine disruptor that was used to prevent adverse effects of pregnancy in women in late 1940s until early 1970s. Its use was banned following significant toxicity and negative effects not only in the mothers but also transgenerationally. Previous studies from our laboratory showed that DES induces thymic atrophy and immunosuppression in mice. In this study, we investigated the molecular mechanisms through which DES triggers thymic atrophy, specifically autophagy. To that end, we treated C57BL/6 mice with DES, and determined expression of two autophagy-related proteins, microtubule-associated protein-1 light chain 3 (LC3) and Beclin-1 (Becn1). We observed that DES-induced thymic atrophy was associated with increased autophagy in thymocytes and significant upregulation in the expression of both Becn1 and LC3. DES also caused downregulation in the expression of miR-30a in thymocytes, and transfection studies revealed that miR-30a targeted Becn1. Upon examination of methylation status of Becn1, we noted hypomethylation of Becn1 in thymocytes of mice exposed to DES. Together, these data demonstrate for the first time that DES induces autophagy in thymocytes potentially through epigenetic changes involving hypomethylation of Becn1 and downregulation of miR-30a expression.

1. Introduction

Diethylstilbestrol (DES) is a nonsteroidal estrogen that was first synthesized in 1938 and classified as an endocrine disruptor (Alves and Oliveira, 2013; Gibson and Saunders, 2014; Nohynek et al., 2013). DES exposure to adult mice has been shown to cause various abnormalities including thymic atrophy, skeletal tissue damage, female reproductive organs, and muscles (Maier et al., 1985; Okada et al., 2001). In the thymus, several changes such as apoptosis in thymic cells, T cell differentiation, immunotoxicity, and immunosuppression have been reported post-DES exposure (Badewa et al., 2002; Brown et al., 2006a). Previous studies have demonstrated that DES caused a decrease in prothymocyte stem cells (Holladay et al., 1993), decrease in double positive $CD4^+CD8^+$ cells (Smith and Holladay, 1997; Brown et al., 2006a), as well as cell death in thymocyte subsets $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$ (Calemine et al., 2002; Brown et al., 2006b). DES has also been shown to induce apoptosis in double- negative ($CD4^-CD8^-$) cells in fetal thymic organ culture system (Lai et al., 2000). The studies from our laboratory have also demonstrated DES-mediated thymic atrophy as well as upregulation of Fas and FasL expression leading to apoptosis occurs in both mothers and neonatal mice (Brown et al., 2006b; Shamran et al., 2017).

Autophagy has been in the forefront of regulating cellular physiological processing and is a major degradation system responsible for removing cellular constituents. It is a lysosome-dependent physiological mechanism that degrades and recycles cellular proteins and organelles (Xie and Klionsky, 2007). It has been reported that basal level of autophagy contributes to the maintenance of intracellular homeostasis and is required for cell cleansing and remodeling (Dikic et al., 2010). The thymus is an important organ in which pre-T cells differentiate into mature T cells following positive and negative selection. In addition to apoptosis, autophagy in the thymus has also been shown to shape the T cell repertoire (Nedjic et al., 2008). Moreover, there is cross-talk between autophagy and apoptosis (Kemp, 2017). Beclin-1 (Becn1) and microtubule-associated protein light chain 3 (LC3) are two important players in autophagy.

It is estimated that ~5–10 million Americans received DES during pregnancy or were exposed to DES in utero. Such an exposure to DES is associated with an increased risk for breast cancer in DES mothers and cervicovaginal cancers in DES daughters (Giusti et al., 1995). Based on

Abbreviations: DES, diethylstilbestrol; ER, estrogen receptor; miR, microRNA; miR-30a, microRNA-30a; Becn1, Beclin 1; LC3, microtubuleassociated protein light chain 3

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the transgenerational toxic effects of DES, it has been suggested that DES may trigger epigenetic events (Newbold et al., 2006). However, the precise mechanisms of epigenetic pathways are still unclear. Because DES is well-established to trigger thymic atrophy, in the current study, we decided to test if epigenetic changes can be seen following DES exposure in thymocytes and whether such changes can induce autophagy in thymocytes.

The current study demonstrates that DES induces autophagy in thymocytes, which correlates with increased expression of Becn1 and LC3. Furthermore, we demonstrate that these changes are related to the ability of DES to cause hypomethylation of Becn1 and down-regulation of miR-30a which targets Becn1. To our knowledge, this study is the first to demonstrate DES-induced autophagy in the thymus and it highlights the potential role of epigenetic pathways in the regulation the autophagy.

2. Materials and methods

2.1. Chemicals and reagents

We purchased Diethylstilbestrol (DES) powder and Acridine Orange (AO) solution from Sigma-Aldrich (Sigma-Aldrich, St. Louis, MO). DES suspended in corn oil was used for in vivo studies and DES suspended in Dimethyl sulfoxide (DMSO) was used for in vitro studies, as described (Singh et al., 2012, 2015a,b). The culture medium (RPMI 1640, Penicillin/ Streptomycin, HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), L-glutamine, Fetal Bovine Serum (FBS), and PBS (Phosphate-buffered saline) were purchased from Invitrogen Life Technologies (Carlsbad, CA). Polymerase chain reaction (PCR) reagents, Epicentre's PCR premix F, and Platinum *Taq* Polymerase, were purchased form Invitrogen Life Technologies (Carlsbad, CA). Anti-LC3 (PA5-22990) polyclonal antibody was purchased from Thermo-Fisher Scientific (Rockford, IL), and anti-Becn1 (H-300) polyclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The following reagents including miRNeasy kit, miScript cDNA synthesis kit, miScript primer assays kit, miScript SYBR Green PCR kit, and EpiTect Bisulfite Kit were purchased from QIAGEN (QIAGEN INC., Valencia, CA).

2.2. Mice

Female C57BL/6 mice (8 weeks old) were purchased from National Cancer Institute (NCI). The mice were housed in the animal facility at the University of South Carolina School of Medicine. All the mice were given ad libitum access to water and normal chow diet and were housed at 23–24 °C with a 12-h/12-h light/dark cycle. Mice were used for the experiments when they were 9 weeks old. The mice were sacrificed at the end of experiments and were ~9.4 weeks old. This AAALAC-accredited animal facility is equipped with a light- and temperature-maintained system. The mice were maintained and cared according to the guidelines for the care and use of laboratory animals as adopted by Institutional IACUC and NIH guidelines.

2.3. Cell line

EL4, a mouse T cell lymphoma cell line, purchased from American Type Culture Collection (ATCC), was maintained in complete RPMI 1640 medium containing 10 mM HEPES, 10 mM L-Glutamine, 100 µg/ml penicillin/ streptomycin), and 10% heat-inactivated fetal bovine serum (FBS) at 37 °C and 5% CO_2. The medium was changed every other day to maintain healthy growth of EL4 cells. Before changing the medium, EL4 cells in flask were observed to determine the confluency of the cells as well as for viability by observing the shape of EL4 cells under inverted phase contrast microscope. Dead EL4 cells were removed by washing the cells repeatedly using complete medium. After washing, the cells were suspended in fresh complete RPMI medium (1×10^6/ml) to maintain healthy growth of EL4 cells. EL4 cells were analyzed for STR profile (DDC Medical, Fairfield, OH) and for Mycoplasma as follows: El4 cells, cultured for two-three days and when the cells were 80–85% confluence, were tested for the presence of Mycoplasma species. PCR using Mycoplasma Detection kit from Applied Biological Materials, Inc (abm, Inc, Richmond BC, Canada) and primers for over 200 Mycoplasma species was performed at the following amplification conditions (Enzyme activation at 95 °C for 5 min, PCR amplification (40 cycles) was performed with denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 60 s, and final extension at 72 °C for 10 min. There was no Mycoplasma contamination present in the culture medium of EL4 cells as both EL4 cultures (1 and 2) showed no PCR amplification (500 bp) for Mycoplasma species, whereas, positive control for Mycoplasma showed the amplified band (500 bp) (Fig S1), Also, negative control for Mycoplasma did not show the amplified band.

2.4. Treatment of mice with DES

Mice (C57BL/6) were administered (ip) with a single dose of DES (5 µg/kg), as described previously (Singh et al., 2015a, 2015b). Mice, treated with corn oil, were used as control for vehicle.

2.5. Determination of thymic cellularity and autophagy

On day 2 and 4 following DES treatment, mice were euthanized, thymi from DES- and VEH-treated mice were harvested, thymic cellularity and autophagy was determined. In brief, single cell suspensions of thymi were prepared as described earlier (Camacho et al., 2004a,b). Thymic cell number and viability were determined by staining the cells with trypan blue dye and using Hematocytometer and an inverted phase contrast microscope. The dead and live thymic cells were counted in all the 64 squares of the Hematocytometer. Cells were counted at least 4–5 times with a minimum five mice for each group. The dead and live cells of thymi of both groups were also verified using an automated cell counter TC20 from Bio-Rad (Bio-Rad). Thymic cellularity was expressed as total number of thymocytes/mice. To determine autophagy in thymic cells post-DES exposure, thymic cells were stained with Acridine orange (AO) as it has been used to determine autophagy (Thome et al., 2016). AO crosses into lysosomes (and other acidic compartments) and becomes protonated. The protonated dye stacks and stacked AO emits in the red range. In brief, thymic cells freshly isolated from thymi of mice treated with VEH or DES were stained for 20 min with AO (1 µg/ml) at room temperature as described previously by Thome et al. (2016). AO emission was captured by red laser using FC500 Flow cytometer (Beckman Coulter). At least five mice were used for each treatment group and the experiments were repeated at least three times.

2.6. Histopathology of thymi exposed to DES

The thymi of VEH- and DES-treated mice were fixed by immersion in 4% paraformaldehyde in PBS. Paraffin blocks of fixed thymi were prepared, and microtome sections (5-µm-thick) were generated. The tissues sections were stained using hematoxylin and eosin (H&E) as described by Singh et al. (2009). In brief, the sildes were washed in deionized water and stained with Mayer's hematoxylin and eosin (Sigma-Aldrich) for 1–2 minutes. Next, the tissue sections were washed with deionized water and then de-hydrated in 70%, 95%, and absolute alcohol for 5 min each. The slides were then passed three times through xylene for one minute each. Finally, the sections were mounted with histomount mounting solution (ThermoFisher Scientific). The thymi sections post H&E staining were examined for architecture of cortex and medulla of the thymi using a Cytation-5 Imaging Reader (BioTek Instruments, Winooski, VT, USA). Five sections per mouse were analyzed, and a minimum of five mice were included in the study.