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AIRE-induced apoptosis is associated with nuclear translocation of stress sensor protein GAPDH

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ABSTRACT

AIRE (Autoimmune Regulator) has a central role in the transcriptional regulation of self-antigens in medullary thymic epithelial cells, which is necessary for negative selection of autoreactive T cells. Recent data have shown that AIRE can also induce apoptosis, which may be linked to cross-presentation of these self-antigens. Here we studied AIRE-induced apoptosis using AIRE over-expression in a thymic epithelial cell line as well as doxycycline-inducible HEK293 cells. We show that the HSR/CARD domain in AIRE together with a nuclear localization signal is sufficient to induce apoptosis. In the nuclei of AIRE-positive cells, we also found an increased accumulation of a glycolytic enzyme, glyceraldehyde-3-phosphate (GAPDH) reflecting cellular stress and apoptosis. Additionally, AIRE-induced apoptosis was inhibited with an anti-apoptotic agent deprenyl that blocks GAPDH nitrosylation and nuclear translocation. We propose that the AIRE-induced apoptosis pathway is associated with GAPDH nuclear translocation and induction of NO-induced cellular stress in AIRE-expressing cells.

1. Introduction

AIRE (Autoimmune Regulator) is expressed in medullary thymic epithelial cells [1], where it controls the ectopic expression of peripheral tissue-specific self-antigens [2]. The presentation of self-antigen proteins to developing T-cells is required for the negative selection of developing thymocytes [3]. Mutations in the AIRE gene cause autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy or APECED (OMIM 240300), a monogenic disease where patients develop multiple autoimmune disorders [4]. At the subcellular level, AIRE is located in nuclear structures that resemble but are distinct from PML (promyelocytic leukemia) bodies [1,5]. AIRE interaction with many proteins involved in transcriptional regulation, pre-mRNA processing, nuclear transport, and chromatin binding have been found [6,7]. For example, these include DNA-PK and topoisomerase 2, which are involved in reparation of DNA double strand breaks [6,8].

AIRE has been shown to act as a transcriptional regulator via binding of its PHD-type zinc finger to histone H3 [9,10], which is a mechanism currently thought to be central in AIRE-induced activation of tissue-specific antigens. The AIRE N-terminal region also contains a homogenously staining region or caspase recruitment domain (HSR/CARD). As this domain is characteristic of several pro-apoptotic proteins, such as APAF-1, caspase-1, -2, -4 and -9 [11], a number of recent studies have focused on the role of AIRE in apoptosis induction. Accordingly, AIRE has been shown to cause apoptosis in mouse thymic epithelial cell line 1C6 [6,12,13] as well as in mouse primary medullary epithelium [13]. The apoptotic activity of AIRE was also reported in a recent study that analyzed its effect on the cellular proteome using quantitative proteomics [14]. In addition, the transcriptional regulator DAXX has been shown to repress the transcriptional activity of AIRE [15], while DAXX itself is involved in Fas-induced apoptosis [16] and colocalizes with Sp100 protein in PML bodies [17]. AIRE shares a similar HSR/CARD domain with the Sp100 protein, which is involved in the DNA damage-induced apoptosis pathway [18]. Collectively, there is increasing evidence linking AIRE with thymic medullary epithelial cell apoptosis. As a result, it has been suggested that this mechanism may in fact promote cross-presentation of tissue-specific antigens to the developing thymocytes [12]. Thus, the pathways behind AIRE-induced apoptosis may be directly linked to the induction of central tolerance in thymus.

Glyceraldehyde-3-phosphate (GAPDH), a common housekeeping gene, is known for its role in glycolysis. Recently, GAPDH has also been implicated in cellular stress and apoptosis [19–23]. Apoptotic stimuli, such as oxidative or genotoxic stress, results in GAPDH translocation and accumulation into nuclei [23,24]. For example, cell stressors activate nitric oxide synthase (NOS) which increases generation of nitric oxide (NO), leading to *S*-nitrosylation of GAPDH [19]. This event abolishes GAPDH glycolytic activity and elicits nuclear translocation of GAPDH [19]. The nuclear translocation depends on interaction with E3-ubiquitin ligase Siah-1, which has a nuclear localization signal and specifically binds to GAPDH

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via the *S*-nitrosylation modification [19]. In the nucleus, GAPDH stabilizes the Siah-1 protein, facilitating its degradation of nuclear proteins and resulting in cell death [19]. It has been shown that the nuclear translocation of GAPDH and consecutive activation of death cascade can be blocked by deprenyl, a compound mainly known for its activity on monoamine oxidase-B inhibition [25,26]. Whether AIRE-induced apoptosis involves the nuclear translocation of GAPDH or if the inhibition of GAPDH translocation affects AIRE-induced cell death has remained unknown so far.

In this study, we focus on AIRE-mediated apoptosis in thymic epithelial and doxycycline-inducible AIRE (AIRE-HEK/Tet) cell lines. We demonstrate that the HSR/CARD region of the AIRE protein alone is sufficient to induce apoptosis in the transfected cells. Our data also show that GAPDH accumulates in nuclei after AIRE expression and that deprenyl inhibits the effect of AIRE on apoptosis induction.

These findings suggest that AIRE-induced cellular stress and apoptosis are associated with GAPDH translocation into the nuclei.

2. Materials and methods

2.1. Transfection of 1C6 cells and generation of doxycyclin-inducible AIRE-HEK/Tet cell line

An AIRE mutant expressing the N-terminal 1-143 amino acids was amplified from pGEX1λT-AIRE and cloned into the pcDNA3.1B plasmid between the NotI and EcoRI sites. Mouse thymic epithelial 1C6 cells were co-transfected with 1 μg pmaxGFP (Lonza, Germany) and 2 μg pcDNA3.1B (as a negative control), 2 μg pcAIRE or 2 μg pcAIRE 1-143 amino acids per 10^6 cells, using Basic Nucleofector Kit (Lonza) and Amaxa Nucleofector (T-023 program), and were analyzed 5–20 h post-transfection.

Human AIRE cDNA [1] was cloned into the pTRE vector. The resulting plasmid and hygromycin B selection vector pTK Hyg were co-transfected into a Tet-On Advanced HEK293 cell line (all from Clontech). Cells were grown in the presence of hygromycin B (75 μg/ml) for 2 weeks. The surviving clones were selected and expanded into monoclonal lineages. The established cell line was grown in the presence of G418 (0.15 mg/ml). AIRE expression was induced with 2.0 μg/ml doxycycline (DOX), and the cells were subsequently analyzed using Western blotting and immunofluorescence using an antibody to AIRE (clone 6.1, [1]).

2.2. Viability, apoptosis and proliferation studies

Cell number and viability was assessed in a Burker-Turk chamber using trypan blue staining and are shown as a percentage (mean ± SEM) of living cells relative to the number of living cells in the negative control group. *p* Values were calculated by paired *t*-test using GraphPad Prism5 software.

Apoptosis was detected with 7-amino-actinomycin D (7-AAD; Invitrogen) and/or with Annexin V-PE using a FACSCalibur (both from BD Biosciences) according to the manufacturer's protocol. Where indicated, 10.0 μM-(−) deprenyl (Sigma) was added 1.5 h before AIRE induction with DOX or immediately after co-transfection of 1C6 cells with pmaxGFP and AIRE plasmids. BrdU (final concentration 10 μM; BD Biosciences) was added into fresh medium for 1 h to doxycycline-induced (for 48 or 72 h) AIRE-HEK/Tet or control cells. The cells were fixed, permeabilized, and then stained with anti-BrdU-FITC (BD Biosciences) before analysis with a FACSCalibur.

2.3. RNA purification and real-time PCR

RNA was purified with Trizol Reagent (Invitrogen), and cDNA was then synthesized with Superscript III Reverse Transcriptase (Invitrogen). Oligonucleotides to detect human GAPDH (PPH00150-200; SuperArray, Qiagen) and human β-actin (Fwd 5′-CTGGAACGGTG AAAGGTGACA and Rev 5′-CGGCCACATTGTGAACTTTG) were used in real-time PCR (7900HT, Applied Biosystems).

2.4. GAPDH subcellular localization

Immunofluorescence was performed on AIRE-induced, etoposide-treated (2 μM for 24 h), or control cells using a polyclonal GAPDH antibody (ab9485, Abcam) and a confocal microscope (63× water-immersion objective, Zeiss LSM5 DUO).

For subcellular fractionation, the cells were lysed in ice-cold lysis buffer A (20 mM HEPES pH 7.5, 0.1% NP-40, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 0.4 mM Na_3VO_4, proteinase inhibitor cocktail (Sigma)) for 8 min. Nuclei were collected by centrifugation at 1000 rpm (200 g) for 5 min at 4 °C. The nuclear pellet was washed once with 1 ml lysis buffer A without NP-40 detergent, and half of the washed nuclear pellet was separately added into a fresh tube before dilution in 5 volumes of 2× SDS-loading buffer for histone H3 detection. The other half of the washed nuclei were lysed in 2 packed cell volumes of ice-cold buffer B (20 mM HEPES pH 7.5, 0.3% NP-40, 300 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM EGTA, 0.4 mM Na_3VO_4, proteinase inhibitor cocktail (Sigma)), incubated on ice for 45 min and were then used to detect GAPDH. The crude nuclear extracts were homogenized using a 23G syringe needle and vortexed for 10 s. Crude lysates were cleared by centrifugation at 16,600*g* for 15 min at 4 °C. Protein concentrations were measured using the Bradford method, and either 20 μg cytoplasmic or 30 μg nuclear proteins were loaded to SDS–PAGE. To detect total protein fraction, cells were lysed with 2xSDS sample buffer. Proteins were detected using Western blotting with antibodies against AIRE (6.1), GAPDH (ab8245, Abcam), β-actin (A2228; Sigma–Aldrich) and H3 (ab1791, Abcam).

3. Results

3.1. The AIRE CARD domain induces apoptosis in thymic epithelial 1C6 cells

Gray et al. [12] demonstrated that AIRE induces apoptosis in mouse thymic epithelial cell line 1C6. Therefore, we used these cells to further analyze the role of AIRE in apoptosis. We transfected pcDNA3.1B or pcAIRE plasmid together with pmaxGFP plasmid into 1C6 cells with Amaxa Nucleofector. Transfection efficiency was 25–35% according to the GFP signal detected via flow cytometry (data not shown). We analyzed apoptosis from the GFP-positive cell subset using Annexin V-PE as an apoptosis marker. As observed in Fig. 1, no significant apoptosis was detectable at 5 h post transfection ($p > 0.05$, $n = 3$). However, we detected increased apoptosis at 10 h and noted up to two times more apoptosis at 20 h when compared to negative controls (Fig. 1A; $p < 0.05$, $n = 3$).

We did not observe significantly cell death after 10 h, while approximately 50% (live cells compared to negative control: 49 ± 10%, $p < 0.05$, $n = 3$) of the cells died after 20 h transfection with pcAIRE, as determined by trypan blue staining. We also analyzed the level of AIRE protein by Western blotting, which showed a 40% decrease in AIRE protein after transfection at 20 h due to reduction of AIRE positive cells (Fig. 1B). These results confirm that AIRE induces increased cell death in thymic epithelial 1C6 cells.

We next examined whether the AIRE HSR/CARD domain is involved in cell death. 1C6 cells were co-transfected with pmaxGFP and a plasmid expressing the N-terminal 143 amino acids of AIRE (pcAIRE 1–143) that contains HSR/CARD region and a nuclear

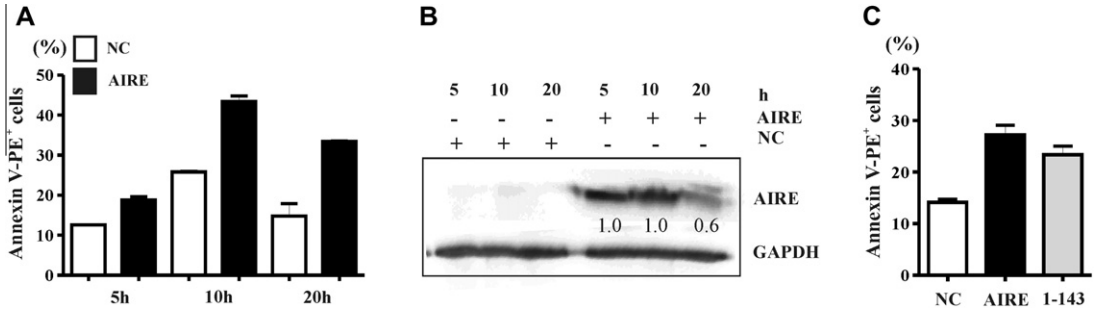

Fig. 1. Over-expression of AIRE or the AIRE CARD domain induces apoptosis in the thymic epithelial 1C6 cell line. (A) 1C6 cells were co-transfected with GFP and AIRE, and the Annexin V-PE positive cells were measured by flow cytometry in the GFP positive population at 5, 10 and 20 h. The data are shown as the mean ± SEM, determined from duplicate measurements of a representative sample of three independent experiments. (B) Cells were transfected with a negative control (NC) or AIRE plasmid, and the level of AIRE and GAPDH protein was measured using Western blotting. A representative sample of two independent experiments is shown. (C) 1C6 cells were co-transfected with GFP and full length AIRE or with GFP and a plasmid expressing the N-terminal 143 amino acids of AIRE (1–143). Apoptosis was detected 20 h later by flow cytometry of Annexin V-PE positive cells in (from) the GFP positive population. Data are the mean ± SEM of duplicate measurements of a representative sample of three independent experiments.

localization signal. The HSR/CARD region caused a similar extent of apoptosis as determined by Annexin V-PE staining compared to the full-length AIRE protein (Fig. 1C; $p < 0.05$ compared to negative control, $n = 3$). Additionally, a similar effect on cell death was detected by trypan blue staining when AIRE HSR/CARD domain-induced cell death was compared to the one induced by full-length AIRE (live cells after AIRE HSR/CARD plasmid transfection: 62 ± 9% compared to negative control, $p < 0.05$, $n = 3$). This result suggests that the HSR/CARD domain has an important role in the apoptotic effect caused by AIRE.

3.2. AIRE causes apoptosis in inducible AIRE-HEK/Tet cell line

To study the effect of AIRE on apoptosis and proliferation, we used a DOX-inducible AIRE-expressing HEK/Tet cell line. After induction with DOX for 24 h, all cells expressed AIRE, which was localized to the nuclear dots and cytoplasmic filaments (Fig. 2A). AIRE protein expression was confirmed by Western blotting (Fig. 3D). As observed in Fig. 2B, AIRE also induced apoptosis in DOX-induced AIRE-HEK/Tet cells compared to negative control cells (Fig. 2B, $p < 0.05$; $n = 3$) analyzed by flow cytometry. Like-

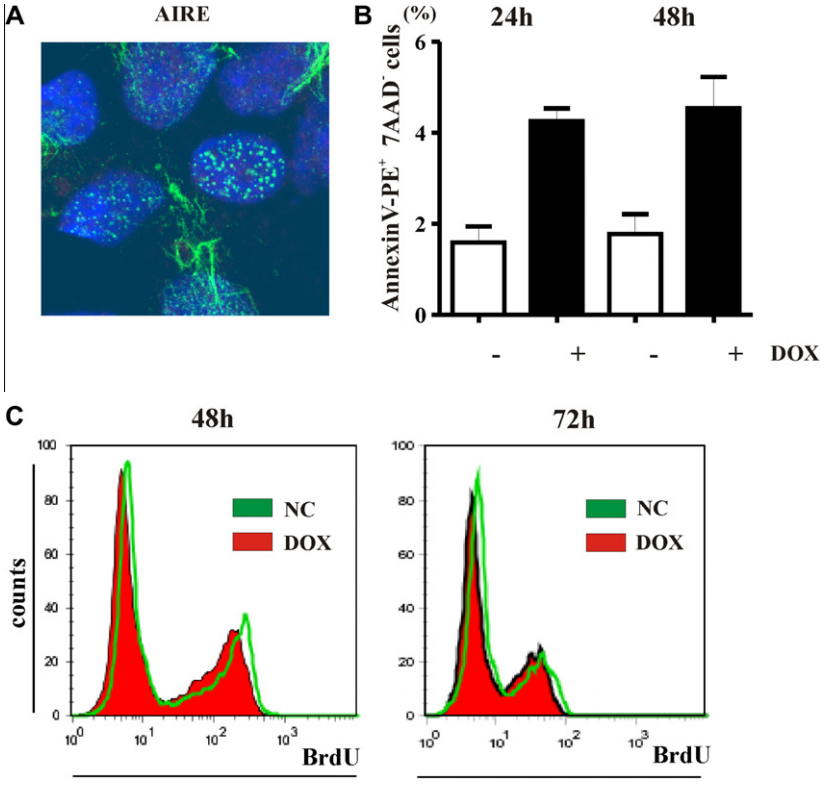

Fig. 2. Over-expression of AIRE induces apoptosis in the AIRE-HEK/Tet cell line but has no effect on cell proliferation. (A) AIRE expression was induced with DOX and visualized by immunofluorescence staining with AIRE antibodies (green) 24 h later. DAPI (blue) was used for nuclear counterstaining. (B) AIRE expression was induced with DOX, and apoptosis was measured by flow cytometry of Annexin V-PE positive and 7-AAD negative cells 24 and 48 h later. Data are the mean ± SEM of duplicate measurements of a representative sample of three independent experiments. (C) AIRE expression was induced with DOX, and cell proliferation was measured at 48 and 72 h based on BrdU incorporation. Representatives of two independent experiments are shown. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

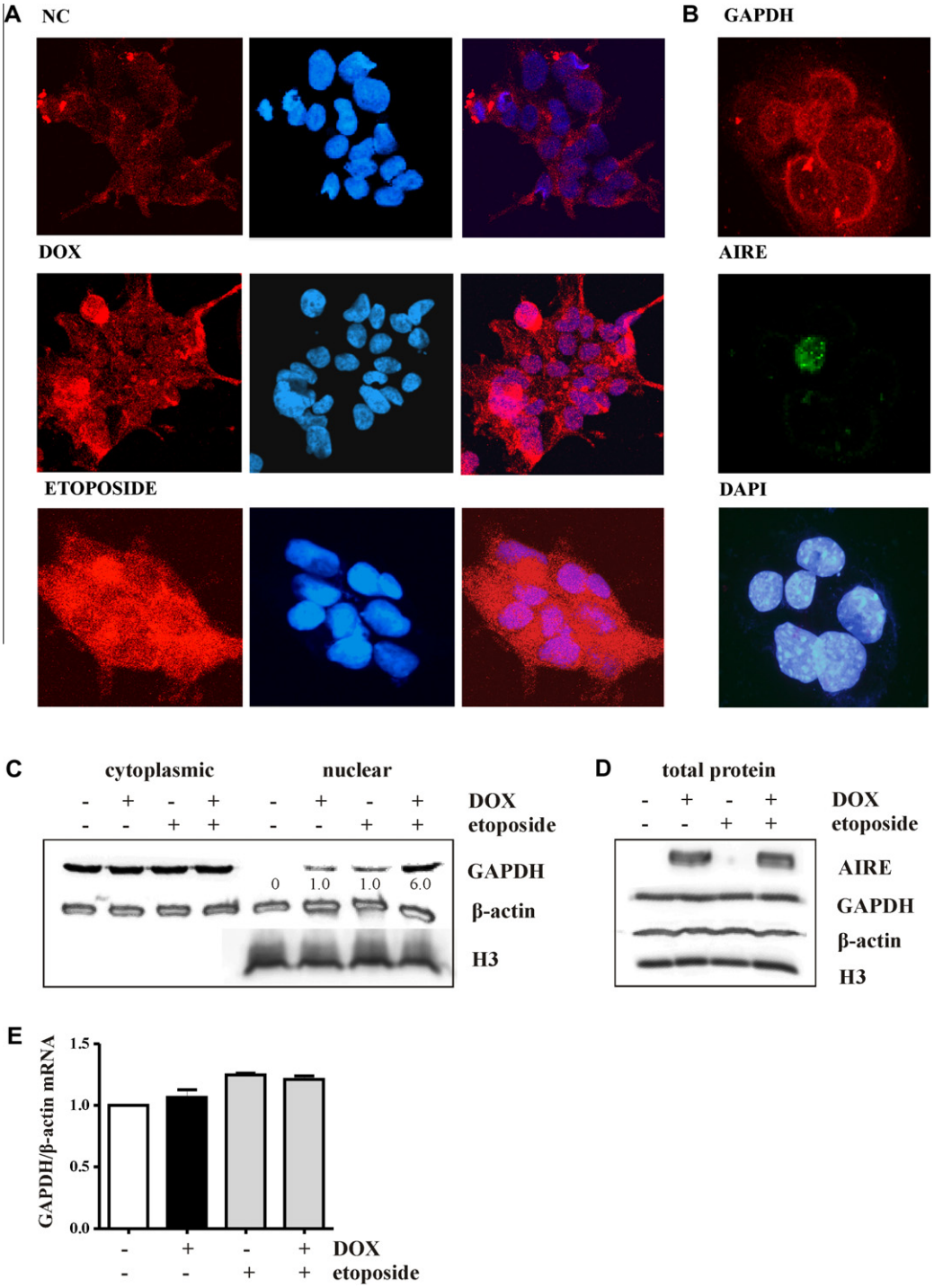

Fig. 3. AIRE-induced apoptosis is related to GAPDH accumulation in the nuclei. (A) DOX-inducible HEK-AIRE/Tet cells or negative controls (NC) were treated with DOX or etoposide and stained 24 h thereafter with GAPDH antibodies and DAPI. (B) The 1C6 mTEC cell line was transfected with AIRE for 20 h and stained for GAPDH, AIRE and DAPI. The AIRE-positive (green) cells were characterized by diffuse nuclear GAPDH staining (red). (C) DOX-inducible HEK-AIRE/Tet cells were treated for 24 h with DOX and/or etoposide followed by Western blotting of cell lysates for GAPDH and two housekeeping genes, β-actin and histone H3 (H3). DOX alone induced nuclear accumulation of GAPDH, which was further increased by etoposide, while the levels of cytoplasmic GAPDH remained unchanged. (D) An experiment was performed as in (C) except that the total protein levels of AIRE, GAPDH, β-actin and H3 were quantified. There was an increase in the levels of AIRE protein in the DOX and DOX plus etoposide cells, whereas all other protein levels remained unchanged. (E) An experiment was performed as in (C) except that after treatment, the mRNA levels for GAPDH and β-actin were quantified by real-time PCR. There was no major change in GAPDH mRNA levels after DOX and/or etoposide treatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

wise, the number of live cells was also reduced by AIRE induction at 24 as well as 48 h (percentage of live cells relative to negative control: 72 ± 5%, $p < 0.05$, $n = 3$ and 77 ± 1%, $p < 0.05$, $n = 3$, respectively).

3.3. AIRE expression does not change cell proliferation

To exclude the possibility that AIRE has an effect on cell proliferation in our cell model, we measured the extent of DNA synthesis

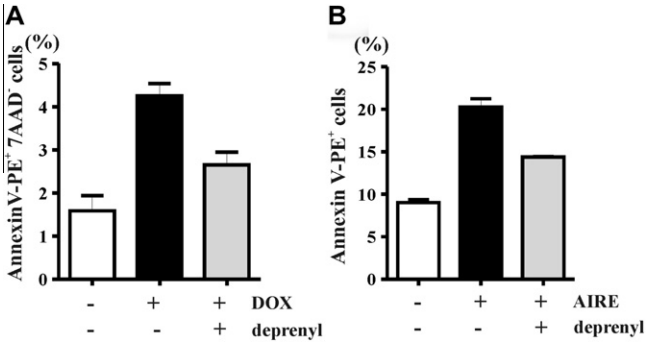

Fig. 4. (A) HEK-AIRE/Tet cells were pretreated with deprenyl or vehicle followed by treatment with DOX for 24 h, and were analyzed for apoptosis by Annexin V-PE and 7-AAD staining by flow cytometry. (B) 1C6 cells were co-transfected with GFP and AIRE or negative control plasmids. AIRE induction and deprenyl treatment were performed for 20 h. Data are the mean ± SEM of duplicate measurements of a representative sample of four (A) or three (B) independent experiments.

using a BrdU incorporation assay. Fig. 2C shows that AIRE-positive cells do proliferate with a speed similar to AIRE-negative control cells at 48 and 72 h after the induction of AIRE by DOX. This result is in concordance with previously described studies in 1C6 thymic epithelial cells, showing that AIRE has no effect on cell proliferation [12].

3.4. AIRE expression causes GAPDH translocation to the nucleus

Because GAPDH nuclear translocation is a marker of apoptosis after multiple stimuli, we studied the GAPDH translocation in DOX-induced AIRE-HEK/Tet cells by immunofluorescence and Western blotting. Treatment with etoposide, which has been shown to cause GAPDH accumulation to cell nuclei due to genotoxic stress [25], was used as a positive control. As shown in Fig. 3A, GAPDH staining was increased in DOX-inducible AIRE-HEK/Tet cell nuclei compared to AIRE-negative cells.

To further confirm the nuclear accumulation of GAPDH, we fractionated nuclei and cytoplasm from AIRE-HEK/Tet cells. Histone H3 and β-actin were used as cellular controls for nuclear and cytoplasmic lysates, respectively. The GAPDH protein was not observed in the nuclear lysate of negative control cells but was clearly present in AIRE-positive and etoposide-treated cells as determined by Western blotting and ECL quantification (Fig. 3C). Moreover, levels of GAPDH protein in the nucleus were further increased by approximately six times when etoposide was added to DOX-induced AIRE-expressing cells (Fig. 3C).

To rule out the possibility that GAPDH translocation to cell nucleus is due to DOX treatment, we transiently transfected HEK293 cells with AIRE and studied the subcellular location of GAPDH by immunofluorescence. We observed increased accumulation of GAPDH in the cell nucleus, indicating that the translocation is a result of AIRE expression, not DOX treatment (data not shown). Moreover, we also found nuclear accumulation of GAPDH in AIRE-transfected 1C6 cells by immunofluorescence analysis, which again indicates that this finding is not a result of DOX treatment itself (Fig. 3B).

3.5. GAPDH mRNA and total protein levels are constant in the control, AIRE-positive and etoposide-treated cells

We next wanted to confirm that the accumulation of GAPDH to cell nucleus is not simply due to the upregulation of GAPDH mRNA expression as a result of AIRE's function as a transcriptional activator. Thus, we performed real-time PCR analysis for mRNA and normalized the expression to β-actin levels as well as performed Western blot analysis for protein detection with β-actin and histone H3 as controls. The total GAPDH protein (Fig. 3D) and mRNA transcript levels (Fig. 3E; $p > 0.05$, $n = 3$) were similar in AIRE positive-(DOX), negative, etoposide- and etoposide-DOX-treated cells. These results confirmed previous studies showing that the total levels of GAPDH mRNA and protein do not change after the expression of AIRE [9] or etoposide treatment [19].

3.6. Deprenyl inhibits AIRE induced apoptosis

Because deprenyl has a inhibitory effect on GAPDH-associated apoptosis by preventing its nitrosylation and translocation to nuclei [25], we decided to study the effect of deprenyl on AIRE-induced apoptosis. Deprenyl inhibited AIRE-induced apoptosis both in HEK/Tet cells ($p < 0.05$, $n = 4$; Fig. 4A) and in the GFP-positive cell subset of AIRE-transfected 1C6 cells ($p < 0.05$, $n = 3$; Fig. 4B). This result demonstrates the association between AIRE-induced apoptosis and GAPDH nuclear translocation.

4. Discussion

In this report, we studied AIRE-induced apoptosis in thymic epithelial 1C6 cells, in which AIRE was overexpressed transiently by transfection and in doxycycline-inducible AIRE-HEK/Tet cells. The thymic 1C6 cells were more sensitive to AIRE-induced apoptosis than inducible AIRE-HEK/Tet cells. It is possible that the reduced apoptosis in HEK cells is due to a strong anti-apoptotic effect caused by adenovirus-5 immortalization described earlier [27].

We found that the N-terminal HSR/CARD region of AIRE is sufficient to cause apoptosis at the same level as full-length AIRE. Interestingly, the N-terminal region of 1-161 amino acids containing the HSR/CARD domain and the bipartite nuclear localization signal motif is also required for its interaction with DAXX protein [15]. Predominantly, DAXX localizes to PML containing nuclear bodies where it (together with PML) influences the nuclear pathway of apoptosis and transcription [17,28]. PML bodies, which contain a number of other proteins including p53, CBP, Sp100 and caspase-2 in addition to PML and DAXX, have been shown to be important in apoptosis and to have many other cellular functions, such as transcriptional regulation and antiviral responses. Thus, the apoptotic effect of AIRE HSR/CARD may suggest AIRE involvement in DAXX-mediated apoptosis.

AIRE-induced apoptotic signals may originate from genotoxic or oxidative stress. AIRE interacts with proteins involved in DNA double strand break repair, such as DNA-PK and topoisomerase 2 [6,8], which suggests that AIRE-induced gene activation might be associated with DNA damage and genotoxic stress. Alternatively, aberrant cytoplasmic folding of AIRE-induced proteins may cause oxidative stress. This would be in agreement with earlier reports showing AIRE-induced expression of heat shock proteins HSC70 and HSP27, tubulin-specific chaperone A and superoxide dismutase (Cu–Zn) [14]. HSC70 and HSP27 are chaperons responsible for the folding of nascent proteins, and SOD is an antioxidant that protects cells from superoxide radical (O_2^-) toxicity. SOD is also responsible for dismutation of superoxide or other biological radicals such as NO, which induces GAPDH translocation to the nucleus.

A wide range of apoptotic stimuli activate NO formation in inflammation [29]. In the thymus, epithelial and dendritic cells in medulla and corticomedullary junctions produce NO via inducible nitric oxide synthase during the negative selection of double-positive thymocytes [30,31]. Therefore, our results showing AIRE-induced nuclear accumulation of GAPDH in AIRE-HEK/Tet cells are in agreement with the described involvement of NO-induced cellular stress in thymic epithelial and dendritic cells and warrant further study.

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