Biochemical and Biophysical Research Communications 424 (2012) 414–420

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

iASPPsv antagonizes apoptosis induced by chemotherapeutic agents in MCF-7 cells and mouse thymocytes

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ARTICLE INFO

Article history:
Received 13 June 2012
Available online 2 July 2012

Keywords:
iASPP
iASPPsv
p53
Apoptosis
Chemotherapeutic agents

ABSTRACT

iASPP was an inhibitory member of ASPP family and could specifically inhibit the apoptotic function of p53. iASPPsv was identified by our lab as the short isoform of iASPP, which encoded a 407 aa protein and highly matched the carboxyl terminus of iASPP. In this study, iASPPsv was stably transfected into the breast cancer cell line MCF-7 by means of lentivirus to explore the effects of iASPPsv on biological functions of MCF-7. Thymocytes from iASPP/iASPPsv transgenic mice were also used to explore the effects of iASPP/iASPPsv on cell biological function. The results demonstrated that iASPPsv antagonized the growth inhibition induced by etoposide (VP-16) in MCF-7 cells. iASPPsv also down-regulated proapoptotic genes (Bax, Puma and Noxa) expression to inhibit apoptosis caused by VP-16. Moreover, iASPP and iASPPsv could both help the thymocytes of transgenic mice to resist the growth inhibition and apoptosis caused by dexamethasone (Dex) or VP-16. At the same time, DNA double strand break damage accumulated in either iASPPsv MCF-7 cells or iASPP/iASPPsv thymocytes. These findings showed that iAPSS/iASPPsv reduced the growth inhibition and apoptosis induced by Dex or VP-16, with DNA damage accumulating which might promote the pathogenesis and/or progression of cancer.

1. Introduction

p53 is a transcription factor that has been most extensively studied in its capacity to mediate tumor suppression [1]. Genes transactivated by p53 extend across a diverse group of biological activities such as DNA metabolism [2], apoptosis [3], cell cycle regulating [4], senescence [5] and energy metabolism [6]. Above all, as a tumor suppressor, the major functions of p53 are to regulate growth arrest and apoptosis, and the balance of these two cellular events can determine the fate of individual cell [7].

Approximately 50% of the solid tumors have some mutation in p53 that alters transcriptional activity. Nonetheless, the mutation rate of p53 varies dramatically between different tumor types. For example, over 50% of head and neck, lung and colon tumors express mutant p53 whereas no more than 30% of breast tumors and only 5% of leukemias have mutant p53 [8]. How does wild-type p53 lose its tumor suppression function in these situations? Co-factors of p53 might give the answer [9]. Co-factors could interact with p53 and influence whether p53 induced apoptosis or cell-cycle arrest. For example, Hzf promoted p53 to transactivate cell cycle genes such as p21 and 14–3–3σ, while Brn-3b increased the transactivation of the proapoptotic gene Bax [10–12].

ASPP family has been identified as specific regulators of p53-mediated apoptosis. It was comprised of three members, which shared a highly conserved carboxyl terminus consisting of ankyrin repeats, SH3 (Src homology 3) domain and proline-rich region. ASPP1 and ASPP2 have similar sequences not only in the carboxyl terminus but also in the amino terminus. They could promote p53-mediated apoptosis but not cell cycle arrest [13–17]. iASPP was the inhibitory member of ASPP family, and there were two isoforms. iASPP, the 828 aa isoform, located mainly in cytoplasma inhibiting p53 induced apoptosis [18,19]. iASPPsv was a short isoform of 407 aa, with identification of 52–407 aa residues to the 473–828 aa residues of iASPP. In previous study [20], we found iASPPsv could reduce transactivity of p53 on the promoter of Bax, and we want to further discuss its biological function in this paper.

Abbreviations: iASPP, inhibitory member of the ASPP family; iASPPsv, a short isoform of iASPP; γ-H2AX, phosphorylated H2AX; DSB, double-strand breaking.

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http://dx.doi.org/10.1016/j.bbrc.2012.06.124

2. Materials and Methods

2.1. Cell culture

Human breast cancer MCF-7 cells and mouse thymocytes were cultured in 1640 medium supplemented with 10% fetal calf serum at 37 °C in a humidified environment of 5% CO_2. Mouse thymocytes were isolated from thymus of the wild type C57 BL/6 mouse and iASPP/iASPPsv C57 BL/6 transgenic mouse using lymphocyte separation medium (Dakewe Biotech Co., Beijing, China) following the protocol.

2.2. Construction of pCDH-iASPPsv expression vector

iASPPsv ORF was amplified from pcDNA3.0-iASPPsv expression vector constructed by our lab and cloned into the pCDH1-MCS1-EF1-copGFP (SBI, USA), which was an HIV-based lentiviral expression vector and was named pCDH-iASPPsv. The positive recombinant was identified and named pCDH-iASPPsv.

2.3. Stable transfection

pCDH-iASPPsv and the other three packing plasmid mixture pPACKH1-GAG, pPACKH1-REV and pVSV-G were transfected into 293T cells using calcium phosphate precipitation method. Viral supernatant was harvested after 48 h and infected MCF-7 cells. Both fluorescent microscopy and flow cytometric analysis of green fluorescent protein (GFP) were used to monitor lentivirus infection of MCF-7 cells. Individual clones with GFP expression were isolated by limited dilution, and stable transfected clones containing pCDH empty vector and pCDH-iASPPsv were established. iASPPsv expression was confirmed by Western Blot analyses.

2.4. Western Blot assays

Cell pellets were lysed in lysis buffer. Protein concentration of the supernatant was determined using BCA protein assay reagents (Pierce, USA). The equal amounts of protein were separated by 12% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE), electroblotted on nitrocellulose membranes, and immunostained with rabbit anti-iASPP (Abcam,) or mouse anti-γ-H2AX (Abcam) followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., USA). Finally, the proteins were detected using enhanced chemiluminescence (Pierce, USA) according to the manufacturer's instructions.

2.5. MTT assays

MTT assay was performed as previously described [21]. The optical density (OD) of the cells was immediately measured at 546 nm using a microplate reader (SLT-Lab, Salzburg, Austria). Growth inhibition ratio was calculated with the formula: growth inhibitory rate = $(1-T/C) \times 100\%$, where T is the absorbance rate of treatment group with VP-16 and C is the absorbance rate of control group. Triplet experiments were performed to obtain their average.

2.6. Assessment of cell apoptosis

Mitochondrial membrane potential was measured using DiOC6 test. Cells were washed in PBS and incubated at 37 °C for 30 min with 40 nM 3,3-dihexyloxacarbocyanine (DiOC6, Sigma, USA). The fluorescence was measured by flow cytometry.

Empty vector or iASPPsv-transfected MCF-7 cells were untreated or treated with VP-16, enzyme topoisomerase II inhibitor, and then the annexin V–PE/7-AAD apoptosis detection kit (BD Biosciences) was used to determine the apoptosis of MCF-7 according to the manufacturer's instructions. The cells were analyzed on the FACSscan (Becton Dickinson Immunocytometry Systems; San Jose, CA, USA). Early apoptotic cells were defined as those cells with Annexin V-PE^+/7-AAD^- in the GFP expression positive cells, while late apoptotic cells were those with Annexin V-PE^+/7-AAD^+ in the GFP expression positive cells. Apoptosis assays were run in triplicate.

2.7. Real-time quantitative RT-PCR

Total RNA was extracted using RNasio (TaKaRa, Japan) according to the protocol supplied with the reagent. The concentration and purity of isolated total RNA were determined by both spectrophotometry (NanoDrop Technologies, Thermo Scientific) at 260 nm/280 nm, and electrophoresis on 1% agarose gel. Complementary DNA (cDNA) was synthesized from 2 μg RNA with N6 primers using murine myeloleukemia virus reverse transcriptase (M-MLV, Promega, USA), following the procedure provided by the manufacturer.

Real-time quantitative RT-PCR (RQ-PCR) was performed using SYBR PremixEx Taq (Takara, Japan) on a 7500 Thermo cycler (Applied Biosystems, USA). Each sample was run in triplicate. Relative quantification of Bax, Puma and Noxa mRNA expression adopts comparative Ct method established by Livak et al. [22]. Using SDS 2.1 software (Applied Biosystems, USA), the output relative expression value was normalized to the endogenous reference (GAPDH) and calibrated to the untreated pCDH-empty vector-MCF-7 cell clone. The primer sequences are as follows: Bax forward 5′-GATGCGTCCACCAAGAAGCT-3′, reverse 5′-CGGCCCCAGTTGAAG TTG-3′; Puma forward 5′-ACGACCTCAACGCACAGTACG-3′, reverse 5′-TCCCA TGATGAGATTGTACAGGAC-3′; Noxa forward 5′-AGCTG-GAAGTCGAGTGTG CT-3′, reverse 5′-TCCTGAGCAGAAGAGTTTG GA-3′; GAPDH forward 5′-GAAG GTGAAGGTCGGAGTC-3′, reverse 5′-GAAGATGGTGATGGGATTTC-3′, with expected PCR products of 170 bp, 95 bp, 101 bp and 226 bp, respectively.

2.8. Statistical analysis

Three repetitions were performed for each experiment. The significance of differences in relative mRNA expression levels, the percentage of cell growth inhibition and rates of apoptosis between two groups was determined using paired T test. All analyses were done using the SPSS software package (SPSS, Chicago, IL, USA). $P < 0.05$ was deemed statistically significant.

3. Results

3.1. Cell strains that stably expressed iASPPsv

We stably transfected wild type p53-expressing MCF-7 cells with a lentiviral expression vector encoding iASPPsv. Individual clones with GFP expression were isolated by limited dilution and iASPPsv expression was confirmed by Western Blot analysis (Fig. 1A). The results revealed that the pCDH-iASPPsv-MCF-7 cell clones B2, D9 and F10 expressed a higher level of iASPPsv protein than that of the pCDH-empty vector-MCF-7 cell clone F6, and that these clones expressed endogenous iASPP at almost the same level.

3.2. Effects of iASPPsv on the growth inhibition of MCF-7 cells caused by VP-16

To investigate the effects of iASPPsv on the growth inhibition of MCF-7 cells, the pCDH-empty vector-MCF-7 cell clone F6 and the

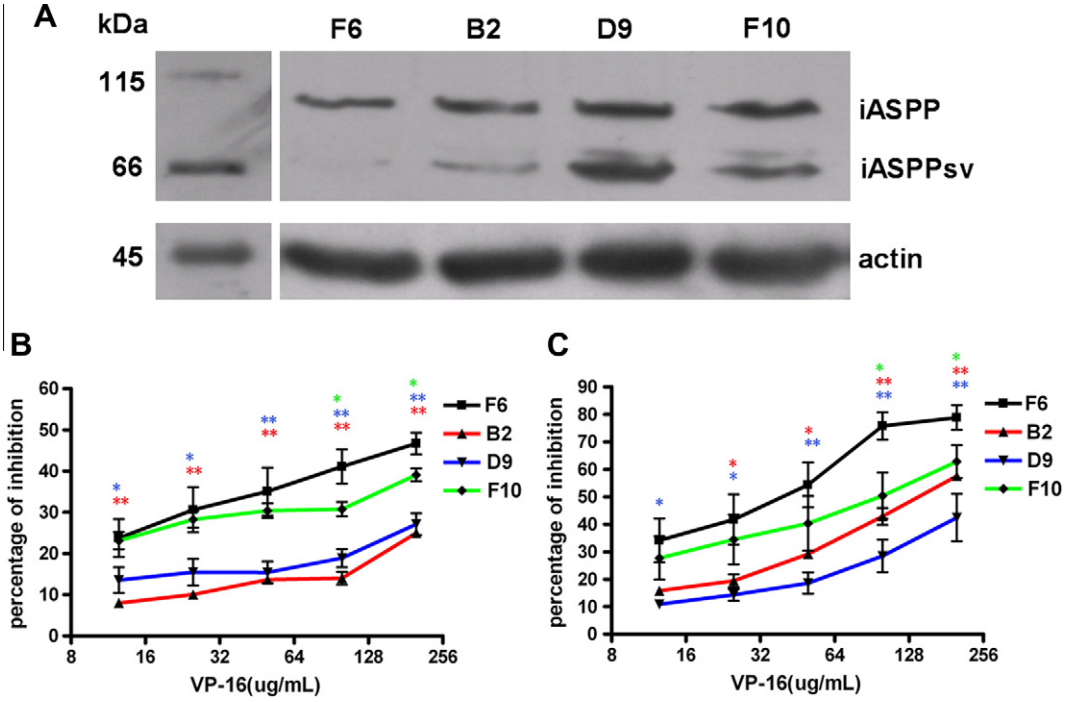

Fig. 1. The protein expression of iASPPsv in iASPPsv-transfected MCF-7 cell clones and MTT proliferation assays. (A) Western Blot analysis was performed with antibodies specific for iASPP. The 67 kDa band was iASPPsv and the 102 kDa band was endogenous iASPP. Actin was used as protein loading control. F6 was a clone of MCF-7 cells transfected with empty vector. B2, D9 and F10 were iASPPsv-transfected clones. (B and C) MCF-7 cells were treated with VP-16 of different concentration for 24 and 48 h, respectively. The wells containing untreated cells were defined as having 100% viability. The data are from three independent MTT experiments and represent as mean ± SD. *: $p < 0.05$, **: $p < 0.01$. Red, blue and green asterisk presents the significant difference of growth inhibition in B2, D9 and F10 cell clone compared to that of F6, respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

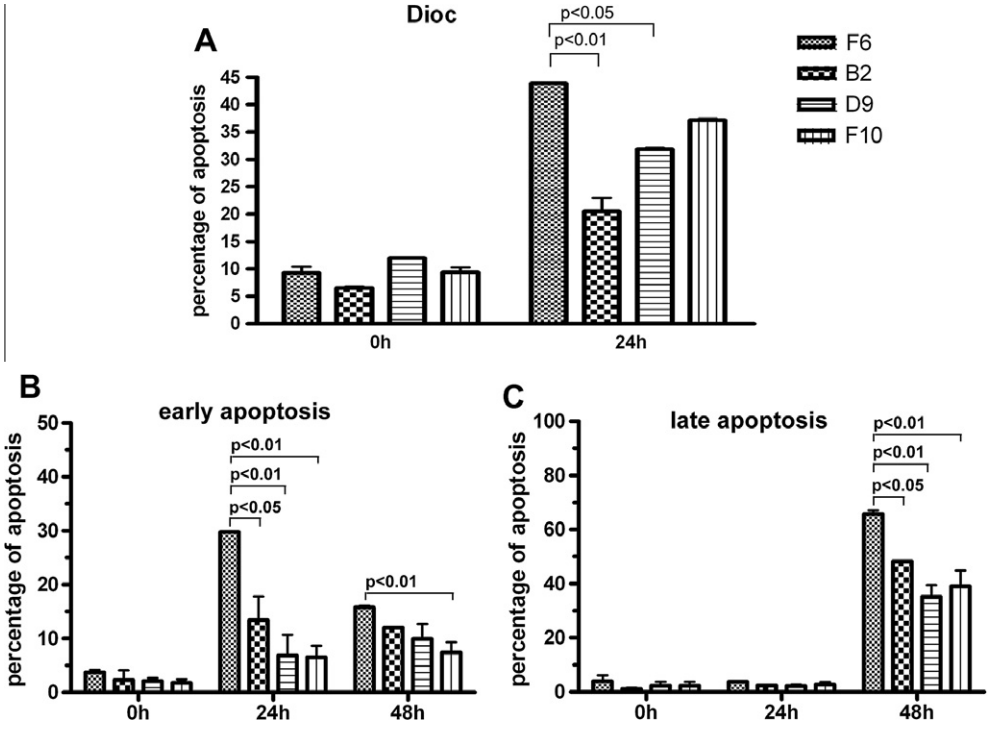

Fig. 2. iASPPsv antagonizes cell apoptosis induced by etoposide. (A) Mitochondria membrane potential was assessed by DiOC6 staining. Flow cytometric analysis of the effect of 200 μg/mL etoposide treatment for 24 h on mitochondrial transmembrane potential of empty vector-transfected (F6) or iASPPsv-transfected MCF-7 cells (B2, D9 and F10). (B and C) Early and late apoptosis assays with Annexin V/7-AAD analysis. Empty vector- (F6) or iASPPsv-transfected (B2, D9 and F10) MCF-7 cells were untreated or treated with 200 μg/mL VP-16 for 24 and 48 h. Data shown are representative of three independent experiments and represent as mean ± SD.

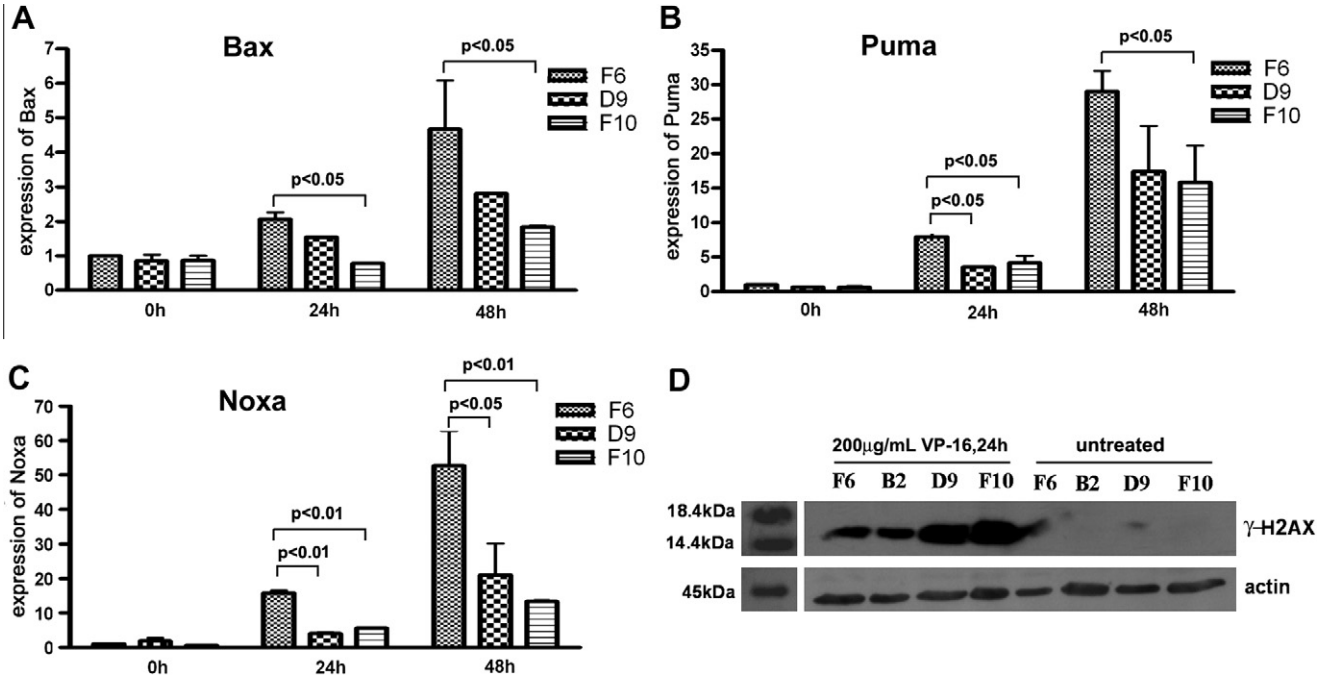

Fig. 3. The expression of proapoptotic genes and γ-H2AX protein in empty vector-(F6) or iASPPsv- transfected (B2, D9, F10) MCF-7 cells. Cells were treated with 200 μg/mL VP-16 for 24 and 48 h. Real time RT-PCR was used to measure the expression level of Bax (A), Puma (B) and Noxa (C). The value was presented as relative quantification (RQ) and shown as mean ± SD. (D), The 15 kDa γ-H2AX protein was identified by western blot. Actin was used as protein loading control.

pCDH-iASPPsv-MCF-7 cell clones B2, D9 and F10 were treated with VP-16 at different concentrations for 24 and 48 h, and then growth inhibition was measured by MTT assays. As a result, B2, D9 and F10 clones showed significantly decreased growth inhibition than that of F6 cell strain (Fig. 1B, C), indicating that iASPPsv could resist the growth inhibition caused by VP-16.

3.3. Effects of iASPPsv on apoptosis of MCF-7 cells caused by VP-16

As iASPP was identified as a negative regulator of p53-dependent apoptosis, effect of iASPPsv on apoptosis was examined in MCF-7 cells. The pCDH-empty vector-MCF-7 cell clone F6 and the pCDH-iASPPsv-MCF-7 cell clones B2, D9 and F10 were treated with 200 μg/mL VP-16 for 24 h and 48 h, and apoptosis was determined by flow cytometric analysis. As DiOC6 staining represents the mitochondria membrane potential, which occurs prior to plasma membrane translocation of phosphatidylserine, we used the DiOC6 staining method after 24 h treatment. pCDH-iASPPsv-MCF-7 cell clones showed significantly decreased apoptosis ($p < 0.01$ for B2, $p < 0.05$ for D9) compare to that of the pCDH-empty vector-MCF-7 cell clone (Fig. 2A).

We also detected early and late apoptosis by using AnnexinV/7-AAD staining. The results showed that iASPPsv expressing clones B2, D9 and F10 displayed lower level of early and late apoptosis than that of iASPPsv non-expressing clone F6 after VP-16 treatment for 24 and 48 h ($p < 0.01$), respectively, indicating that iASPPsv could antagonize the apoptosis induced by VP-16 (Fig. 2B, C).

3.4. Effects of iASPPsv on expression of proapoptotic genes in MCF-7 cells

In above study, we found that iASPPsv expressing cells displayed lower apoptosis rate after exposure to VP-16. In the following study, we further investigate the expression of apoptosis related genes. As Bax, Puma and Noxa were p53-induced proapoptotic genes, we examined whether iASPPsv had effects on the expression of these genes by using real time PCR. The results showed that after treated with 200 μg/mL VP-16 for 24 h and 48 h, the expression of Bax, Puma and Noxa were down-regulated in iASPPsv transfected clones D9 and F10 than that in the empty vector transfected clone F6, indicating that iASPPsv could down-regulate the proapoptotic genes expression, and therefore inhibited apoptosis (Fig. 3A–C).

3.5. Effects of iASPPsv on DNA damage repair in MCF-7 cells

MCF-7 cells were treated with 200 μg/mL VP-16 for 24 h and γ-H2AX (phosphorylated H2AX) protein was detected by Western blot analysis. pCDH-iASPPsv-MCF-7 cell clones B2, D9 and F10 showed higher level of γ-H2AX than that of pCDH-empty vector-MCF-7 cell clone F6, indicating that there was more DNA damage in pCDH-iASPPsv-MCF-7 cell clones than that in the pCDH-empty vector-MCF-7 cell clone (Fig. 3D).

3.6. Effects of iASPP/iASPPsv on the growth inhibition of mouse thymocytes caused by Dex or VP-16

As MCF-7 cells were malignant cells, tumor cell heterogeneity might be taken into account. Thymocytes from iASPP/iASPPsv transgenic mouse were used to explore the effects of iASPP/iASPPsv on biological function of normal cells, while the wild type C57 BL/6 mouse was used as control.

To determine whether the stages of thymocyte development from the iASPP/iASPPsv transgenic mice were similar to that of the wild type C57 BL/6 mice, the expression of a cluster of differentiation markers on thymocytes from above mice at 6–8 weeks of age was analyzed by flow cytometry. There were no significant differences on the expression of c-Kit, thy1.2, CD3, CD4 and CD8 between transgenic and wild type mice, indicating that iASPP/iASPPsv expression had no obvious effect on the thymocyte development (data not shown).

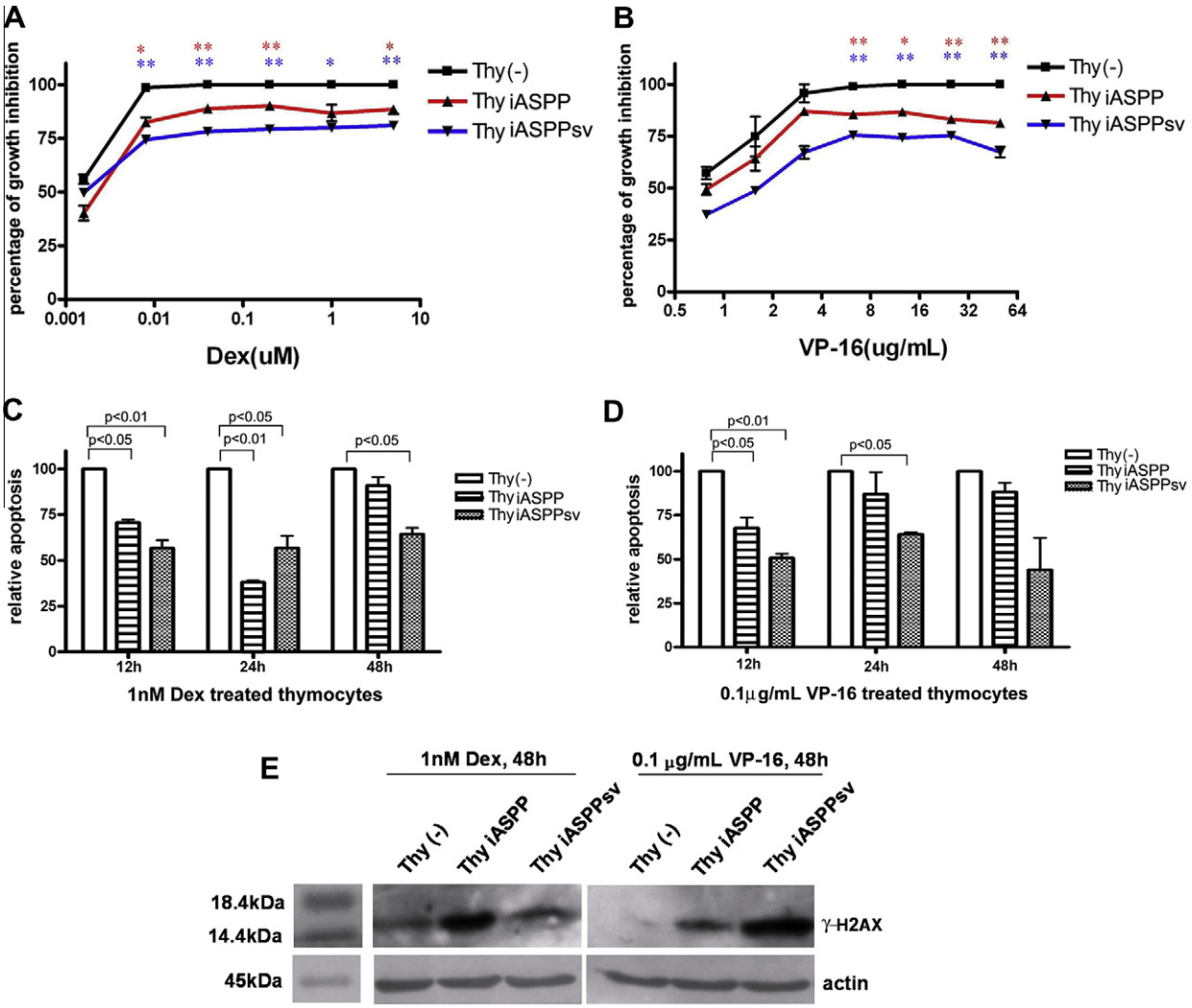

Fig. 4. iASPP/iASPPsv resists cell growth inhibition and apoptosis induced by Dex or VP-16. (A) Thymocytes from wild type and iASPP/iASPPsv transgenic C57 BL/6 mice were treated with Dex for 48 h. (B) The same thymocytes were treated with VP-16 for 48 h. The cells untreated were defined as having 100% viability. The data are from three independent MTT experiments and represent as mean ± SD. *: $p < 0.05$, **: $p < 0.01$. Red and blue asterisk presents the significant difference of thymocytes from iASPP and iASPPsv transgenic C57 BL/6 mice compared to that of wild type C57 BL/6 mice, respectively. The thymocytes were treated with Dex (C) or VP-16 (D) for 12, 24 and 48 h. Then the apoptosis was detected with Annexin V/ 7-AAD analysis. The relative apoptosis was in triplicate and represent as mean ± SD. (E) The γ-H2AX protein expression of thymocytes was identified by western blot. Actin was used as protein loading control. Thy(-), Thy iASPP and Thy iASPPsv represent the thymocytes from wild type, iASPP and iASPPsv transgenic C57 BL/6 mice, respectively (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

To investigate the effects of iASPP/iASPPsv on the growth inhibition of mouse thymocytes induced by Dex or VP-16, thymocytes from the three groups were treated with the above agents at various concentrations for 48 h, followed by the MTT assays to measure the proliferation capacity. The results showed that the inhibition rate of Dex and VP-16 on proliferation of the thymocytes from transgenic mice was lower than that of the wild type mice, suggesting that iASPP/iASPPsv could help the thymocytes to resist the growth inhibition caused by Dex or VP-16 (Fig. 4A, B).

3.7. Effects of iASPP/iASPPsv on apoptosis of mouse thymocytes induced by Dex or VP-16

As iASPP/iASPPsv was identified as a negative regulator of p53-dependent apoptosis, effect of iASPP/iASPPsv on apoptosis was examined in mouse thymocytes. The three groups of thymocytes were treated with 1 nM Dex or 0.1 μg/mL VP-16 for 12, 24 and 48 h, and apoptosis was determined with AnnexinV/PI staining by flow cytometric analysis. With either chemotherapeutic agent treatment, thymocytes from transgenic mice showed significantly decreased level of apoptosis compare to that of the wild type mouse (Fig. 4C, D), indicating that iASPP/iASPPsv can antagonize apoptosis induced by Dex or VP-16.

3.8. Effects of iASPP/iASPPsv on DNA damage repair in mouse thymocytes

Mouse thymocytes were treated with 1 nM Dex or 0.1 μg/mL VP-16 for 48 h, and γ -H2AX was detected by Western Blot analysis. Thymocytes from transgenic mice showed higher level of γ-H2AX than that of the wild type mice, indicating that there were more DNA damages accumulated in iASPP/iASPPsv expressing cells than in iASPP/iASPPsv non-expressing cells (Fig. 4E).

4. Discussion

iASPP is an inhibitory member of ASPP and can specifically inhibit the apoptotic function of p53. It has been found that iASPP was

overexpressed in various tumor cells [18,23–25]. Down-regulation of endogenous iASPP by RNA interfering could increased p53-dependent apoptosis of leukemia cells with wild type p53 [26], also lead to cell growth deceleration and slow colony formation in bladder cancer cell [27] or hepatocellular carcinoma cells [28]. The inhibition of iASPP binding to p53 could be a potential cancer therapy [18,29].

In our previous study, we identified a novel isoform of human iASPP—iASPPsv. It contains the conserved domains of ASPP family: ankyrin repeats domain, SH3 domain and proline-rich region and shares a high homology with other ASPP family members. It highly matches with the C terminus of iASPP but mainly located in nucleus. iASPPsv could inhibit the transcriptional activity of p53 on the promoter of Bax about threefold [20]. The effect of iASPPsv on apoptosis induced by p53 and its signaling pathway is a worthwhile challenge.

In this study, MCF-7 cells stably expressing iASPPsv are more resistant to growth inhibition and apoptosis induced by VP-16 compared to the parental cells. Apoptosis occurred through the extrinsic pathway or the intrinsic pathway. The former was induced by the ligand-induced activation of cell membrane-anchored death receptors, while the latter was induced by the endogenous stress such as DNA damage, growth factors withdrawal and oxidative stress. Most cell death in vertebrates proceeds via the intrinsic pathway of apoptosis [30]. Intrinsic pathway was regulated by the Bcl-2 family, which contains the pro-survival members Bcl-2 and Bcl-xL and the proapoptotic members Bax, Puma and Noxa. Briefly, Bax could be held inactively by Bcl-2, and be activated by Puma and Noxa. Activated Bax could translocate to the mitochondria and result in the permealization of outer membrane of mitochondria, followed by the release of cytochrome c [31]. As Bax, Puma and Noxa were transactivated by p53, and iASPPsv mainly located in nucleus, we examined whether iASPPsv inhibit the transcription of these proapoptotic genes by p53. As a result, pCDH-iASPPsv-MCF-7 cell clones expressed lower proapoptotic genes after VP-16 treatment compared to the control, suggesting iASPPsv inhibited apoptosis by reducing expression of proapoptotic genes.

In order to invest the effects of iASPPsv under different cell background, the thymocytes from iASPPsv transgenic mice were also used with those from iASPP transgenic mice. Chemotherapeutic agents such as Dex and VP-16 could induce DNA damage in thymocytes from iASPPsv and iASPP transgenic mice with p53 wild type, which could transduct to p53 network through the signaling pathway. As iASPP/iASPPsv could inhibit apoptosis induced by chemotherapeutic agent, the question, whether or not DNA damage in these cells had been repaired, deserved much attention. Double-strand breaking (DSB) was the most severe DNA damage, which could result in the loss of large pieces of DNA during mitosis. Immediately upon DSB formation, one or more of the PI3 K-like kinases, such as ataxia telangiectasia mutated (ATM), ataxia telangiectasia and Rad3-related (ATR) and DNA-dependent protein kinase (DNA-PK) are activated [32]. Thus H2AX was phosphorylated and γ-H2AX focused on the DSB sites, and acted as a scaffold recruiting DSB signaling and repair proteins such as MDC1, MCPH1, 53BP1, and BRCA1, and arresting these proteins until DSBs well repaired [33–36]. It is estimated that roughly 2,000 γ-H2AX molecules are formed per DSB [37], suggesting the number of γ-H2AX foci correlates well with the number of DSBs induced [32]. Therefore γ-H2AX could serve as an epigenetic mark for DNA damage. In our study, iASPP/iASPPsv expressing cells presented higher expression of γ-H2AX after VP-16 treatment, indicating there was more DNA damage accumulated in these cells than in control cells. This result corresponded to our assumption that iASPP/iASPPsv prevented apoptosis of damaged cells, and therefore resulted in increased proportion of damaged cells in cell population. These cells might be the source of malignant transformation. So, iASPPsv, like iASPP, plays an important role in tumorigenesis and needs further study.

Acknowledgments

We are grateful to kind assistance from the technicians in Flow Cytometry Core and Instrument Center, Institute of Hematology and Blood Diseases Hospital, Chinese Academy of Medical Sciences and Peking Union Medical College. This work was supported by the National Natural Science Foundation of China (No. 30971290 and No. 81070427), Tianjin Applied Fundamental Research Planning Key Project (10JCZDJC19600) and applied Basic Research Project of Tianjin (No. 08JCYBJC06300).

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