Apoptin Causes Apoptosis in HepG-2 Cells by Inducing Stress Injury in the Endoplasmic Reticulum

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Abstract

Apoptin is derived from the chicken anemia virus and exhibits specific cytotoxic effects against tumor cells. In our previous study, we demonstrated that Apoptin induced significant changes in the expression levels of endoplasmic reticulum stress (ERS) related proteins and caused a strong and lasting ERS response. The aim of this study was to explore the effects of ERS injury induced by Apoptin on the endoplasmic reticulum (ER) and the apoptotic pathway in mitochondria. ERS injury induced the intracellular levels of calcium (Ca^{2+}) were determined by electron microscopy, flow cytometry and fluorescence staining. Mitochondrial injury was determined by mitochondrial membrane potential and electron microscopy. The relationship between Ca^{2+} level and mitochondrial injury on Apoptin-treating cells was analyzed using Ca^{2+} chelator, flow cytometry and fluorescence staining. Western blotting was used to investigate the levels of key proteins in the ER and the apoptotic pathway in mitochondria. We also investigated the *in vivo* effects of ERS injury on the ER and the mitochondrial apoptotic pathway via the immunohistochemical analysis of tumor tissues from HepG-2 cells acquired from nude mice undergoing xenografts. *In vitro* and *in vivo* experiments showed that Apoptin caused ERS injury and an imbalance in Ca^{2+} , damaged the structure of the mitochondria, and increased the expression levels of Caspase-12, CHOP, AIF, HtrA2, Smac/Diablo, and Cyto-C. In summary, Apoptin induced apoptosis in HepG-2 cells via ERS and the mitochondrial apoptotic pathway. This study showed that Apoptin induced apoptosis in HepG-2 cells via ERS injury.

Introduction

Apoptin (GenBank: AY171617.1) is derived from chicken anemia virus, which exhibits cytotoxicity that is specific to tumor cells [1, 2]. In most of the cell lines investigated thus far, Apoptin has been shown to induce apoptosis in tumor cells via the intrinsic mitochondrial pathway. Apoptin reduces the mitochondrial membrane potential in tumor cells and induces the mitochondria to release cytochrome c (Cyto-C) and apoptosis inducing factor (AIF) [3–5], thus resulting in apoptosis. Apoptin-induced apoptosis is known to occur independently of p53 [6]. In our research, we found that Apoptin induced a strong and long-lasting endoplasmic reticulum stress (ERS) injury. However, as yet, the specific mechanism that underlying the association between ERS injury and Apoptin has yet to be elucidated.

The endoplasmic reticulum (ER) is an important intracellular organelle and participates in a number of cellular processes *in vivo*. The ER is also the main site of calcium (Ca^{2+}) storage. Ca^{2+} can be stored in the ER in concentrations of 10–100 mmol/L, but is present at concentrations of 100–300 nmol/L in the cytoplasm. A strong and lasting ERS response triggers the apoptotic pathway in the ER and creates an imbalance in Ca^{2+} . ERS-induced ER apoptotic pathways are known to involve the CCAAT/enhancer-binding protein homologous protein (CHOP) pathway [7, 8], the Inositol-Requiring Enzyme1 α (IRE1 α) - Apoptosis Signal Regulating Kinase 1 (ASK1) - C-Jun N-terminal Kinase (JNK) pathway [9, 10], and the Caspase-12 pathway [11]. However, the specific effect of Apoptin on apoptotic pathways in the ER have yet to be elucidated.

As the key site of energy transformation, the mitochondria also represents the main regulatory site for apoptosis and also plays a key role in most of the regulatory processes related to apoptosis. An abnormal change in the mitochondrial membrane potential triggers the release of apoptosis-related proteins from the mitochondria, including AIF, High-Temperature Requirement Protein A2 (HtrA2), Endonuclease G (Endo G), Apoptosis-Related Proteins (ARTs), Second Mitochondria-Derived Activator of Caspase/Direct IAP Binding Protein With Low pI (Smac/Diablo), and Cyto-C. A Ca^{2+} imbalance caused by ERS injury may affect the structure and function of the mitochondria. However, some research has indicated that Apoptin induces mitochondria to release Cyto-C and AIF. However, no previous study has investigated how Apoptin-induced ERS injury can cause Ca^{2+} imbalance and influence the structure and function of the mitochondria. Moreover, previous studies have not investigated the specific effect of Apoptin on the expression of key apoptosis-related proteins in the mitochondria, such as HtrA2, Smac/Diablo, Endo G, and ATRS.

Therefore, it is of great significance to investigate the specific effect of ERS injury on the ER and apoptosis-related pathways in the mitochondria with regards to the induction of apoptosis. Our previous studies have found that Apoptin can cause a strong and lasting ER stress response and damage ER functional structure[12]. In the present study, we demonstrated that Apoptin can induce ERS injury, cause Ca^{2+} imbalance, affect the structure and function of the mitochondria, and can induce significant changes in the expression of key proteins related to the ER and the mitochondria. Our research involved the assessment of dynamic changes in the ER and mitochondria, the determination of Ca^{2+} levels, and the immunohistochemical analysis of tumor tissue from a tumor-bearing nude mouse model.

Materials And Methods

Cells, virus, reagents and animals

Human hepatoma cell line HepG-2 was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). We also used a recombinant type 5 adenovirus (Ad-Apoptin; hereafter referred to as Ad-Vp3) that contained a CMV promoter and the Apoptin gene from chicken anemia virus. We used the recombinant type 5 adenovirus (hereafter referred to as Ad-Mock) without the Apoptin gene as a blank control. Ad-Vp3 and Ad-Mock were constructed previously in our laboratory. Dihydrorhodamine 123 (DHR) (No. D632), JC-1 (No. T3168), MitoTracker™ Red CMXRos (TMRM) (No. M7512), Oregon Green™ 488 BAPTA-1, AM, and Cell-permeant Special Packaging (No. O6807) were purchased from ThermoFisher Scientific (Shanghai, China). We also purchased an FITC Annexin V Apoptosis Detection Kit I (No. 556547) from BD Biosciences (Beijing, China). We also acquired a Minute™ Total Protein Extraction Kit (No. SD-001/SN-002) and a Mitochondria Isolation Kit For Mammalian Cells And Tissues (No. MP-007) from Invent (Beijing, China). We purchased a range of reagents from Cell Signaling Technology (Shanghai, China), including a PARP (46D11) Rabbit mAb (No. 9532), Caspase-3 (D3R6Y) Rabbit mAb (No. 14220), Cytochrome c (D18C7) Rabbit mAb (No. 11940), Smac/Diablo (D5S3R) Rabbit mAb (No. 15108), Endonuclease G Antibody (No. 4969), ASK1 (D11C9) Rabbit mAb (No. 8662),

SAPK/JNK Antibody (No. 9252S), and a Phospho-SAPK/JNK (Thr183/Tyr185) (81E11) Rabbit mAb (No. 4668S). We also purchased a monoclonal anti-ARTS antibody mAb (No. 106M4840) from Sigma-Aldrich (Shanghai, China). We purchased an anti-AIF antibody (No. ab1998), anti-HtrA2/Omi antibody (No. 75982), anti-Caspase-12 antibody (No. ab62484) from Abcam (Shanghai, China). Six-week-old male BALB/c nude mice were housed in light, comfortable temperature and humidity room ($22 \pm 2^{\circ}\text{C}$, $50 \pm 5\%$ relative humidity), given solid diet (Changchun billion Adams Laboratory Animal Technology Co., Ltd.) and sterilized tap water. All mice were obtained from the Experiment Animal Center of the Chinese Military Medical Academy. The animal experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the Chinese Academy of Military Medical Science, Changchun, China (Reference: 10ZDGG007). All mice surgery was performed under 40 mg/kg 1% sodium pentobarbital via intraperitoneal injection.

Annexin V analysis

HepG-2 cells were seeded at a density of 6×10^5 cells per well in a 6-well plate and incubated for 24 h at 37°C in 5 % CO_2 . Cells were then infected with Ad-Vp3 (MOI: 50) for 12, 24, 36, 48, and 72 h. Ad-Vp3-infected HepG-2 cells were incubated in the dark for 15 min at room temperature in the presence of 5 μL Annexin V-FITC and 5 μL of PI in 100 μL of $1 \times$ binding buffer. We then quantified the extent of apoptosis in HepG-2 cells by flow cytometry. Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

ROS levels analysis

HepG-2 cells were seeded at 6×10^6 cells per well in a 6-well plate and incubated for 24 h at 37°C with 5 % CO_2 . The cells were then infected with 50 MOI Ad-Vp3 for 12, 24, 36, 48, and 72 h. HepG-2 cells infected with Ad-Vp3 were incubated in the dark for 15 min at room temperature in the presence of 1 μL DHR in 1 mL of DMEM. Flow cytometry was used to detect the level of ROS in HepG-2 cells infected with Ad-Vp3. Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

Electron microscopy

HepG-2 cells were seeded at a density of 6×10^5 cells/well into a 6-well plate, cultured for 24 h at 37°C in 5% CO_2 , and infected with Ad-Vp3 (MOI: 50) for 48 h. Cells were then harvested, washed with PBS, and sections prepared using ultramicrotomy. Cells were analyzed by electron microscopy. Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

Ca^{2+} levels analysis

HepG-2 cells were seeded at a density of 6×10^5 cells/well into a 6-well plate, cultured for 24 h at 37°C in 5% CO_2 , and infected with Ad-Vp3 (MOI: 50) for 12, 24, 36, 48, and 72 h. Cells were then incubated in the dark for 20 min at 37°C , in the presence of 1 μg Oregon Green™ 488 BAPTA-1. We then quantified Ca^{2+} levels in the HepG-2 cells by fluorescence microscopy and flow cytometry, in accordance with the

manufacturer's instructions. Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

Mitochondrial analysis: structure and function

HepG-2 cells were seeded at a density of 6×10^5 cells/well into a 6-well plate, cultured for 24 h at 37°C in 5% CO₂ and infected with Ad-Vp3 (MOI: 50) for 12, 24, 36, 48, and 72 h. Cells were then incubated in the dark for 20 min at 37°C in the presence of 1 µL JC-1 in 1 mL of DMEM. We then determined the mitochondrial membrane potential of HepG-2 cells by fluorescence microscopy and flow cytometry, in accordance with the manufacturer's instructions. Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

For structural analysis, HepG-2 cells were seeded at a density of 1×10^7 cells into a 100 mm cell culture dish, cultured for 24 h at 37°C in 5% CO₂ and infected with Ad-Vp3 (MOI: 50) for 48 and 72 h. We then collected mitochondria by use of a Mitochondria Isolation Kit for Mammalian Cells and Tissues, in accordance with the manufacturer's guidelines. We then prepared sections by ultramicrotomy and investigated the structure of the mitochondria by electron microscopy. Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

Western blotting

HepG-2 cells were seeded at a density of 6×10^5 cells/well into a 6-well plate, cultured for 24 h at 37°C in 5% CO₂ and infected with Ad-Vp3 (MOI: 50) for 12, 24, 36, 48, and 72 h. Ad-Mock-infected cells were used as negative controls. Total protein was extracted from cells using the Minute™ Total Protein Extraction Kit, separated by SDS-PAGE, and transferred to membranes for Western blotting so that we could determine the expression levels of proteins related to apoptotic proteins and apoptotic pathways in the ER and mitochondria.

Immunohistochemical analysis

Tumor tissues were isolated from HepG-2 cells nude mice xenograft models xenografted [12]. We then used immunohistochemical staining to investigate the ER apoptotic pathway, and a range of proteins involved in the apoptotic pathway in mitochondria, including Caspase-12, CHOP, AIF, HtrA2, Smac/Diablo, and Cyto-C. All immunohistochemical detection was performed by Servicebio (Wuhan, China). Mice were sacrificed by injecting with 100 mg/kg 1% sodium pentobarbital when tumor volume exceeded 3000 mm³.

Ca²⁺ chelator analysis

HepG-2 cells were seeded at a density of 6×10^5 cells/well into a 6-well plate, cultured for 24 h at 37°C in 5% CO₂ and treated with Ad-Vp3 (MOI: 50). In addition, 5 µM Ca²⁺ chelator (BAPTA-AM) was administered to the Ad-Vp3-infected cells. The Cells were incubated in the dark for 20 min at 37°C in the presence of 1 µL TMRM in 1 mL of DMEM at 12, 24, 36, 48, and 72 h post-infection. Then, we determined the

mitochondrial membrane potential of HepG-2 cells by fluorescence microscopy and flow cytometry according to the manufacturer's instructions. Ad-Vp3-infected cells, Ad-Mock-infected cells and non-infected HepG-2 cells were used as negative controls.

For Western blot, HepG-2 cells were seeded at a density of 6×10^5 cells/well into a 6-well plate, cultured for 24 h at 37°C in 5% CO₂ and treated with Ad-Vp3 (MOI: 50) and 5 µM Ca²⁺ chelator (BAPTA-AM) for 12, 24, 36, 48, and 72 h. The total protein was extracted and probe for expression of mitochondrial apoptosis related proteins was detected. Ad-Vp3-infected cells were used as negative controls.

Statistics

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) statistical software package (version 15.0; SPSS Inc., Chicago, IL, USA). Data were presented using GraphPad Prism version 7.0 (GraphPad Software Inc., La Jolla, CA, USA). For analysis, we used the Student's t-test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test; $p < 0.05$, $p < 0.01$, or $P < 0.001$, were considered to be statistically significant. Data are presented as the mean $\pm$ standard deviation (SD).

Immunohistochemical analyses were performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA). At least 3 fields of vision were randomly selected for each slice (and for each group). When acquiring images, we organized the entire field of view to ensure that the background illumination was consistent. The method was used to define positive staining in cells for other types of proteins; a brown/yellow stain indicated positive immunostaining. We used Image-Pro Plus 6.0 software to determine the integrated optical density (IOD) and the area occupied by the entire tissue (AREA). The Areal density was then defined by IOD/AREA. A larger areal density indicated a higher level of positive expression.

Results

Apoptosis of HepG-2 cell induced by Apoptin

The apoptosis of HepG-2 cell can be significantly induced by Apoptin (Fig. 1a). The apoptotic rate in Ad-Vp3-infected cells was significantly higher than that in Ad-Mock-infected cells and HepG-2 cells at 48 and 72 h post-infection ($p < 0.05$). The results of apoptosis-related proteins in Ad-Vp3-infected HepG-2 cells are shown in Fig. 1b. The expression levels of Cleaved-PARP and Cleaved-Caspase-3 in Ad-Vp3-infected HepG-2 cells were significantly higher at 48 and 72 h post-infection, when compared with HepG-2 cells infected with Ad-Mock ($p < 0.01$). The ROS levels of HepG-2 cells can be significantly induced by Apoptin (Fig. 1c). ROS levels in Ad-Vp3-infected cells was significantly higher than that in Ad-Mock-infected cells and HepG-2 cells at all time points ($p < 0.05$).

Analysis of ERS injury

Our previous study demonstrated that Apoptin induced significant changes in the expression levels of ERS-related proteins and caused a strong and lasting ERS response [12]. The effect of Apoptin on the structure of the ER was evaluated by electron microscopy. Electron microscopy revealed clear structural damage in the ER and mitochondria of infected cells at 48 h (Fig. 2a). The structure of ER and the crest of the mitochondria in Ad-Vp3-infected cells had incurred significant damage by 48 h post-infection. This result indicated that a strong and lasting ERS response can result in ERS injury.

The intensity of green fluorescence (as an indicator of Ca^{2+} level) in HepG-2 cells infected with Ad-Vp3 was significantly higher than cells infected with Ad-Mock at 24, 36, and 48 h, post-infection. At 72 h post-infection, the level of green fluorescence in the HepG-2 cells infected with Ad-Vp3 was significantly lower than that in the control group (Figs. 2b, c) ($p < 0.05$). The trend for green fluorescence over time was similar to that shown by flow cytometry (Fig. 2d). These results showed that ERS injury increased the levels of Ca^{2+} and led to an imbalance in Ca^{2+} .

Our analysis showed that some of the proteins related to the apoptotic pathway in the ER were significantly affected by Apoptin (Fig. 2e). For example, the expression levels of ASK1 in Ad-Vp3-infected HepG-2 cells were significantly higher than those in Ad-Mock-infected cells and non-infected cells at 24 and 48 h post-infection ($p < 0.05$); there was also a significant difference in the expression of JNK and p-JNK in HepG-2 cells infected with Ad-Vp3 and Ad-Mock ($p > 0.05$). The expression levels of Caspase-12 in Ad-Vp3-infected cells were significantly higher than those in Ad-Mock-infected cells and non-infected cells at 24, 36, 48 and 72 h ($p < 0.05$). Furthermore, the expression of CHOP in Ad-Vp3-infected cells was significantly higher at 12 and 24 h than cells infected with Ad-Mock or non-infected cells, but were significantly lower at 36 h post-infection ($p < 0.05$). Consequently, these results showed that Apoptin triggered the apoptotic pathway in the ER and induced the expression of Caspase-12 and CHOP. Therefore, Apoptin can induce a strong and lasting ERS that ultimately leads to ERS injury; this leads to Ca^{2+} imbalance and triggers the apoptotic pathway in the ER of HepG-2 cells.

Effect of Apoptin on mitochondria

The mitochondrial membrane potential of HepG-2 cells was significantly reduced by Apoptin. The relative fluorescence (R2/R3; Red/Green) associated with mitochondrial membrane potential in Ad-Vp3-infected and HepG-2 cells was significantly lower than that in Ad-Mock-infected cells and non-infected cells at 24, 36, 48 and 72 h post-infection (Fig. 3a) ($p < 0.05$).

It was also evident that Apoptin caused damage to the mitochondria of HepG-2 cells (Fig. 3b); the crest of the mitochondria in Ad-Vp3-infected cells appeared to have been broken and dissolved by 48 h post-infection. By 72 h post-infection, it was evident that the mitochondria had been completely destroyed.

The expression levels of AIF, HtrA2, Smac/Diablo, and Cyto-C, in Ad-Vp3-infected cells were significantly higher than those in Ad-Mock-infected cells at all time points (Figs. 3c, d) ($p < 0.05$); however, there were no significant differences with regards to the expression of ARTs and Endo G in HepG-2 cells infected

with Ad-Vp3 and Ad-Mock ($p > 0.05$). It was evident that Apoptin can trigger the apoptotic pathway in mitochondria by increasing the expression levels of AIF, HtrA2, Smac/Diablo, and Cyto-C.

Collectively, these results indicated that Apoptin can significantly reduce mitochondrial membrane potential, cause damage to the mitochondrial structure and trigger the apoptotic pathway in the mitochondria of HepG-2 cells.

Immunohistochemical detection of key proteins in tumor tissues

Immunohistochemical studies of an *in vivo* model showed that Apoptin caused significant changes in proteins related to apoptotic pathways in both the ER and the mitochondria. Immunohistochemical data relating to ER proteins are shown in Figs. 4a, b. The areal densities of Caspase-12 and CHOP, in the tumors grown by the Ad-Vp3 group were significantly higher than those in the Ad-Mock and non-infected HepG-2 groups ($p < 0.01$). Immunohistochemical data relating to proteins of the apoptotic pathway in mitochondria are shown in Figs. 4c-f. The areal densities of AIF, HtrA2, Smac/Diablo, and Cyto-C, in tumors grown by the Ad-Vp3 group were significantly higher than those in the Ad-Mock and non-infected HepG-2 groups ($p < 0.01$). Collectively, these *in vivo* results showed that Apoptin could significantly induce the expression of proteins related to apoptotic pathways in the ER and mitochondria. We did not detect any significant differences between groups in relation to the expression of IRE1 α or ASK1 (data not shown).

Effect of Ca^{2+} chelator on mitochondria

In order to verify whether Ca^{2+} chelator has an effect on mitochondria in HepG-2 cells, we performed TMRM staining and Western-blotting on Ad-Vp3-infected HepG-2 cells (Fig. 5A-D). The mitochondrial membrane potential of HepG-2 cells in Ad-Vp3 + BAPTA-AM group was significantly higher than that in Ad-Vp3 group at 24, 36 and 48 h post-infection (Fig. 5a) ($p < 0.05$). However, there were no significant differences with regards to the mitochondrial membrane potential in HepG-2 cells treated with Ad-Vp3 and Ad-Vp3 + BAPTA-AM ($p > 0.05$). The trend for red fluorescence over time was similar to that of flow cytometry (Fig. 5b). The mitochondrial membrane potential in Ad-Vp3-infected HepG-2 cells was significantly increased by Ca^{2+} chelator. The expression levels of the mitochondrial apoptotic-related proteins were also analyzed after treatment with the Ca^{2+} chelator (BAPTA-AM). The expression level of Cyto-C in Ad-Vp3 group was significantly higher than that in Ad-Vp3 + BAPTA-AM group at 36 and 48 h post-infection (Figs. 5c, d) ($p < 0.05$); however, there were no significant differences with regards to the expression of AIF, HtrA2 and Smac/Diablo in HepG-2 cells treated with Ad-Vp3 and Ad-Vp3 + BAPTA-AM ($p > 0.05$). It was evident that the expression level of Cyto-C in Ad-Vp3-infected HepG-2 cells was significantly reduced by Ca^{2+} chelator.

Discussion

The endoplasmic reticulum is a highly dynamic organelle that plays a key role in homeostasis. The homeostatic balance created by the ER can, however, be disrupted by genetic and environmental damage, thus resulting in ERS. In order to restore dynamic balance, the ER produces increased levels of several stress-related proteins, including PERK [13], Calnexin [14–16], Ero1-L α [17–19], PDI [20], IRE1 α [21–23], and BIP [24]. Collectively, these proteins lighten the load on the ER by reducing misfolding and helping to avoid the accumulation of unfolded proteins. In our previous study [12], we found that the expression levels of several stress-related proteins in the ER, including PERK, Calnexin, Ero1-L α , and BIP, were significantly higher in the HepG-2 cells infected with Ad-Vp3 than cells infected with Ad-Mock when tested at 12 h and 24 h post-infection. These changes were caused by ER stress. However, the expression levels of these proteins in Ad-Vp3-infected cells were significantly lower than those in the Ad-Mock-infected cells, at 36 h, 48 h and 72 h post-infection. These changes might be caused by ERS injury. Electron microscopy also revealed that the ER in HepG-2 cells had incurred significant structural damage when assessed 48 h post-infection. Collectively, these results indicated that the structure and function of the ER were both damaged by strong and persistent ER stress, thus resulting in the increased expression of ER-related proteins; subsequently, these ER-related proteins showed a significant decrease in expression. In conclusion, these data indicate that Apoptin can cause ERS injury in HepG-2 cells by inducing strong and persistent ER stress.

From one perspective, ERS and ERS injury can both cause an increase in intracellular Ca²⁺ levels, thus resulting in Ca²⁺ imbalance. However, from another perspective, these processes can also trigger the apoptotic pathway in the ER to induce apoptosis. The levels of Ca²⁺ in Ad-Vp3-infected cells was significantly higher than those in Ad-Mock-infected cells and non-infected HepG-2 cells at 24, 36, and 48 h post-infection. This result may have been caused by a strong and lasting ERS, and consequential ERS injury. However, the Ca²⁺ levels in the Ad-Vp3-infected cells were significantly lower than that in the control groups at 72 h post-infection. We believe that this result was due to the fact that the Ad-Vp3-infected cells underwent a significant extent of apoptosis. *In vitro* and *in vivo* experiments indicated that Apoptin was able to trigger the apoptotic pathway in the ER; within this pathway, the CHOP protein was able to inhibit the expression of BCL-2, an anti-apoptotic protein [8] and increase the expression of BIM and BAX/BAK, which are pro-apoptotic proteins [7]. The expression of these proteins can disrupt the integrity of the ER membrane and cause an imbalance in Ca²⁺, thus inducing cell apoptosis via the apoptotic pathway in the mitochondria [25, 26]. Previous research has shown that the IRE1 α -ASK1-JNK pathway can activate the pro-apoptotic protein BIM to inhibit the activity of the anti-apoptotic protein BCL-2 and thus induce apoptosis. Furthermore, the IRE1 α -ASK1-JNK pathway can also participate in the CHOP pathway to induce apoptosis. Previous research has also shown that the Caspase-12 pathway can activate both Caspase-9 and Caspase-3 to induce caspase-mediated apoptosis [27–29]. In the present study, Apoptin triggered the apoptotic pathway in the ER, thus increasing the expression levels of Caspase-12 and CHOP. Consequently, we believe that a strong and lasting ERS leads to the increased expression of CHOP and that this may be one of the key determinants that can induce ERS injury and Ca²⁺ imbalance.

An imbalance in the intracellular concentration of Ca^{2+} can affect the mitochondrial membrane potential [30] and subsequently induce the release of pro-apoptotic proteins from the mitochondrial membrane mesenchyme [31]. Several pro-apoptotic proteins can be released from the mitochondria, including AIF, HtrA2, Endo G, ARTS, Smac/Diablo, and Cyto-C. AIF is a flavoprotein and represents the main protein responsible for Caspase-independent apoptosis. HtrA2 and Caspase are known to competitively bind Inhibitors of Apoptosis Proteins (IAP) [32, 33]. The interaction between these pro-apoptotic proteins and IAP can relieve the inhibition of caspase by IAP, thus allowing caspase to become activated and then induce apoptosis. Endo G is known to migrate to the nucleus and trigger cell apoptosis by causing DNA fragmentation [34, 19]. ARTs are known to mediate various pro-apoptotic stimuli in order to induce apoptosis. The function of Smac/Diablo is the same as HtrA2 [33, 35]. In the present study, we found that the mitochondrial membrane potential of Ad-Vp3-infected cells was significantly lower than that in Ad-Mock-infected cells and non-infected HepG-2 cells at 24 post-infection. Furthermore, compared with all other timepoints, the mitochondrial membrane potential at 36 h post-infection, was also significantly reduced in the cells infected with Ad-Vp3. We speculate that this result might be caused by an abnormal change in intracellular Ca^{2+} level. In order to analyze whether Ca^{2+} imbalance affects the abnormal changes of mitochondrial membrane potential mediated by Apoptin in HepG-2 cells, Ad-Vp3 infected cells were further treated with Ca^{2+} chelator. These results showed that the imbalance of Ca^{2+} induced by Apoptin could reduce the mitochondrial membrane potential and induce the expression of Cyto-C. Ca^{2+} chelator could antagonize this phenomenon, but could not completely suppress it.

In this study, we found that Apoptin induced a strong and lasting ERS and thus cause ERS injury. These events subsequently led to an imbalance of intracellular Ca^{2+} , an increase in the expression levels of CHOP and Caspase-12 by triggering the apoptotic pathway in the ER, a reduction in mitochondrial membrane potential, a significant extent of damage in the mitochondrial structure, and an increase in the expression levels of AIF, HtrA, Smac/Diablo, and Cyto-C, by triggering the mitochondrial apoptotic pathway in HepG-2 cells. Ca^{2+} imbalance played a role in apoptosis of HepG-2 cells induced by Apoptin. Apoptin-triggered mitochondrial apoptotic pathway was regulated by multiple factors. However, it is not clear how Apoptin induces ERS. We speculate that Apoptin-induced ERS may be related to Apoptin-induced G2/M phase arrest [36, 37]. G2 phase involves the synthesis of a large number of RNAs and proteins. Therefore, G2/M phase arrest may cause an accumulation of unfolded or misfolding proteins, thus triggering the Unfolded Protein Response (UPR), thus leading to ERS. Previous studies have not reported the specific relationship between G2/M phase arrest and UPR/ERS. It is clearly evident that we now need to be able to elucidate the specific relationships between these three factors. By investigating the relationships between G2/M phase arrest, UPR, and ERS, we may be able to enhance our understanding of the role of Apoptin-induced ERS injury in Apoptin-induced apoptosis.

Declarations

Ethics approval and consent to participate The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Military Medical Science

(10ZDGG007). All surgeries were performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering.

Consent for publication Not applicable.

Availability of data and materials The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests Not applicable.

Funding Not applicable.

Authors' contributions Yi-Long Zhu and Yi-Quan Li conceived and performed the experiments and analysed the data. Bing Bai, Chao Shang, Jia-Nan Cong, Jin-Bo Fang, Wen-Jie Li, Shan-Zhi Li, Zhi-Ru Xiu, Gao-Jie Song, Zi-Rui Liu, Xia Yang and Guang-Ze Zhu conducted the mouse experiments. Li Xiao and Ning-Yi Jin overall supervised the experiments and revised the manuscript. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Disclosure of potential conflicts of interest The authors declare that they have no competing interests.

Research involving Human Participants and/or Animals The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of the Chinese Academy of Military Medical Science (10ZDGG007). The manuscript does not contain clinical studies or patient data.

Informed consent For this type of study, informed consent is not required.

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Figures

Figure 1

The apoptotic effects of Apoptin on HepG-2 cells. Apoptin significantly induced apoptosis in HepG-2 cells. a The detection of Annexin V-FITC/PI by flow cytometry in Ad-Vp3-infected HepG-2 cells. The apoptosis level of HepG-2 cells infected with Ad-Vp3 was significantly higher than that of other groups at 48 h and 72 h. b Western blot analysis of apoptosis-related proteins PARP and Caspase-3. The levels of Cleaved-PARP and Cleaved-caspase-3 in Ad-Vp3 group were higher than those in Ad-Mock at 48 h and 72 h. c The intracellular ROS was detected by flow cytometry. The level of ROS in Ad-Vp3 group were significantly higher than that in Ad-Mock-infected cells and non-infected HepG-2 cells starting from 12 h to 72 h post-infection. Data are presented as mean $\pm$ SD, * p < 0.05, ** p < 0.01, *** p < 0.001.

Figure 2

The effect of ERS injury on HepG-2 cells. **a** The structure of the ER in Ad-Vp3-infected HepG-2 cells was determined by electron microscopy at 48 h post-infection (5000 $\times$). The arrows represent ER and the mitochondria. The structure of the ER and the crest of the mitochondria in Ad-Vp3-infected cells had incurred damage at 48 h post-infection. The detection of Ca^{2+} levels and proteins related to the apoptotic pathway in ER. Apoptin induces the imbalance of Ca^{2+} in HepG-2 cells infected with Ad-Vp3. **b, c** Flow cytometry detection of ER using Oregon Green[™] 488 BAPTA-1, AM, cell permeant-Special Packaging. **d** Endoplasmic reticulum Oregon Green[™] 488 BAPTA-1, AM, cell permeant-Special Packaging staining (200 $\times$). **e** Western blot analysis of the expression levels of proteins related to the apoptotic pathway in ER. Apoptin triggered the apoptotic pathway in the ER, which could increase the expression of CHOP and Caspase-12. Data are presented as mean $\pm$ SD, * p < 0.05, ** p < 0.01.

Figure 3

Apoptin significantly reduced mitochondrial membrane potential and caused damage to the mitochondrial structure. **a** Mitochondrial membrane potential, as determined by JC-1 and flow cytometry. **b** The structure of the mitochondria, as observed by electron microscopy at 48 and 72 h post-infection (30000 $\times$). The arrows represent mitochondria. The crest of the mitochondria in Ad-Vp3-infected cells had been broken and dissolved at 48 h post-infection. The mitochondria had been completely destroyed at 72 h post-infection. **c, d** Western blot analysis of the expression of proteins related to the apoptotic pathway in mitochondria. Apoptin triggered the mitochondrial apoptotic pathway to increase the expression levels of AIF, HtrA2, Smac/Diablo, and Cyto-C. Data are presented as mean $\pm$ SD, * p < 0.05, ** p < 0.01.

Figure 4

The immunohistochemical detection of apoptotic proteins related to the ER and the mitochondria. a-f The expression of proteins related to apoptotic pathways in the ER and mitochondria (Caspase-12, CHOP, AIF, HtrA2, Smac/Diablo, Cyto-C), as detected by immunocytochemistry (400×). Apoptin could significantly induce the expression levels of aspase-12, CHOP, AIF, HtrA2, Smac/Diablo, and Cyto-C in tumoral tissue. Data are presented as mean ± SD, *p < 0.05, **p < 0.01.

Figure 5

Relationship between Ca^{2+} level and mitochondria in HepG-2 cells infected with Ad-Vp3. **a** Flow cytometry detection of mitochondrial membrane potential using TMRM. **b** TMRM staining (200 $\times$). The mitochondrial membrane potential of HepG-2 cells infected with Ad-Vp3 was significantly higher than that of other groups; the addition of Ca^{2+} chelator (BAPTA-AM) could significantly increase in the mitochondrial membrane potential of HepG-2 cells infected with Ad-Vp3. **c, d** Western blot analysis of the expression of proteins related to the apoptotic pathway in mitochondria after treatment with Ca^{2+} chelator (BAPTA-AM). After adding BAPTA-AM, the expression level of Cyto-C in Ad-Vp3+BAPTA-AM was significantly higher than that in the Ad-Vp3 group at 36 h and 48 h post-infection. Data are presented as mean $\pm$ SD, * $p < 0.05$, ** $p < 0.01$.