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Restoration of ASC expression sensitizes colorectal cancer cells to genotoxic stress-induced caspase-independent cell death

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ABSTRACT

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), an essential component of the inflammasome complex, is frequently silenced by epigenetic methylation in many tumor cells. Here, we demonstrate that restoration of ASC expression in human colorectal cancer DLD-1 cells, in which ASC is silenced by aberrant methylation, potentiated cell death mediated by DNA damaging agent. Contrarily, ASC knockdown in HT-29 cells rendered cells less susceptible to etoposide toxicity. The increased susceptibility of ASC-expressing DLD-1 cells to genotoxic stress was independent of inflammasome or caspase activation, but partially dependent on mitochondrial ROS production and JNK activation. Thus, our data suggest that ASC expression in cancer cells is an important factor in determining their susceptibility to chemotherapy.

1. Introduction

Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) is a bipartite protein consisting of two death domains, an N-terminal pyrin domain (PYD) and a C-terminal caspase recruitment domain (CARD) [1]. A number of proteins containing the death-fold domain have been widely shown to play a pivotal role in apoptotic cell death and inflammation [2]. In particular, accumulating reports have recently demonstrated that ASC is an essential adaptor molecule of the inflammasome complex, which mediates the conversion of procaspase-1 to active caspase-1 and the subsequent maturation of interleukin-1 beta (IL-1β) and IL-18, the key proinflammatory cytokines [3].

Upon stimulation of the macrophages or dendritic cells by pathogen-associated molecular pattern (PAMP) or danger-associated molecular pattern (DAMP), PYD-containing pattern-recognition receptors such as the NOD-like receptor family, pyrin domain containing-3 protein (NLRP3) or absent in melanoma 2 (AIM2) associate with ASC through PYD homotypic interactions [4]. ASC then recruits procaspase-1 through CARD–CARD interaction to form an inflammasome complex leading to the activation of caspase-1. The PAMP- or DAMP-triggered assembly of the inflammasome induces not only the maturation of the inflammation-initiating proinflammatory cytokines IL-1β and IL-18 but also caspase-1-dependent pyroptotic cell death depending on the cell type [5,6].

ASC was originally identified in human leukemia cells based on its ability to form speck-like aggregates and trigger apoptosis in response to retinoic acid or anti-tumor drugs [7]. Interestingly, ASC was also identified as a downstream target of methylation-induced gene silencing by DNA methyltransferase and thus initially named as 'target of methylation-induced silencing 1 (TMS1)' [8]. ASC is indeed epigenetically silenced by aberrant methylation of its CpG islands in many tumor cells; particularly, it has been reported to be silenced in 40% of primary breast tumors [8–10]. This observation suggests that ASC might be a potential tumor suppressor gene, and its silencing might contribute to carcinogenesis in some tumors.

The cell death-promoting role of ASC has been suggested in a number of reports; however, the underlying mechanism of apoptosis induction is quite controversial depending on the cell type. The most intriguing involvement of ASC in cell death is the mediation of caspase-1-dependent pyroptosis through inflammasome formation [5]; however, pyroptosis is known to mainly occur in innate immune cells to clear intracellular pathogens [11]. In addition to pyroptosis, overexpression of ASC was initially shown to induce

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mitochondria- or caspase-9-mediated apoptosis in 293T cells [12]. By contrast, many other reports have also demonstrated that ASC triggers caspase-8-dependent apoptotic cell death [13–15]. In human mammary epithelial cells, DNA damage or loss of extracellular matrix contact induced ASC expression leading to apoptosis [15,16], indicating that ASC is definitively associated with the suppression of cancer progression.

Here, we demonstrated that ASC expression in colorectal cancer cells clearly augments the cell death caused by DNA damaging agents. Contrary to previous speculations, we found that the increased susceptibility of ASC-expressing cells to genotoxic stress is not caused by caspase activation, but can be partially attributed to the production of mitochondrial reactive oxygen species (ROS) and the activation of c-jun kinase (JNK) mitogen-activated protein kinase (MAPK) signaling pathway.

2. Materials and methods

2.1. Cell culture

DLD-1 cells were purchased from Korean Cell Line Bank and maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and streptomycin. A549, NCI-1650, HCC827, PC-3, Du145, LNCaP, MDA-MB231, SK-BR3, MCF-7, HCT116, HT-29, and THP-1 cells were purchased from the American Type Culture Collection (ATCC). THP-1 cells were grown in RPMI 1640 medium supplemented with 10% FBS, 2 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 0.05 mM 2-mercaptoethanol, and 100 U/ml penicillin and streptomycin. Wild-type or ASC-deficient immortalized mouse bone marrow-derived macrophages were kindly gifted by Dr. Fitzgerald (University of Massachusetts) and cultured in DMEM supplemented with 10% FBS, 5% L929 cell supplements, and 100 U/ml penicillin and streptomycin. To generate ASC stably-expressing cells, DLD-1 cells were transfected with pMSCV-puromycin with or without the pcDNA3-T7-ASC construct using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Puromycin-resistant clones were then selected during 3–4 weeks of culture and screened for ASC expression.

2.2. Reagents

Etoposide, camptothecin, doxorubicin, 5-aza-2′-deoxycytidine (5-AD), lipopolysaccharide (LPS), nigericin, poly (dA:dT), cycloheximide (CHX), and N-acetyl-L-cysteine (NAC) were purchased from Sigma. Z-VAD(OMe)-fluoromethylketone (zVAD-fmk) and acetyl-YVAD-chloromethylketone (YVAD-cmk) were obtained from Bachem. The p38 MAPK inhibitor SB203580, JNK inhibitor SP600125, and necrostatin-1 were from Calbiochem. Recombinant human tumor necrosis factor α (TNF-α) was purchased from R&D Systems. ASC-specific small interfering RNA (siRNA) and negative control siRNA were from Invitrogen (Stealth RNAi).

2.3. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total cellular RNA was isolated from DLD-1 cells using the PureLink™ RNA Mini Kit (Invitrogen) and reverse transcribed using the SuperScript cDNA Synthesis Kit (Invitrogen). Template DNA was then amplified by PCR using the following primers: 5′-TGG GCC TGC AGG AGA TG-3′ and 5′-ATT TGG TGG GAT TGC CAG-3′ for ASC, and 5′-CCT TCC TGG GCA TGG AGT CCT G-3′ and 5′-GGA GCA ATG ATC TTG ATC TTC-3′ for β-actin.

2.4. Immunoblot assay

Cells were lysed in 20 mM HEPES (pH 7.5) buffer containing 0.5% Nonidet P40, 50 mM KCl, 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, and protease inhibitors. Soluble lysates were subjected to SDS–PAGE, transferred onto PVDF membranes, and then immunoblotted with the appropriate antibodies. To determine inflammasome activity, culture supernatants were precipitated as described previously [17]. Anti-ASC, anti-human caspase-1 (p10), and anti-β-actin antibodies were from Santa Cruz Biotechnology. Anti-human IL-1β, anti-caspase-3, anti-poly(ADP-ribose) polymerase (PARP), anti-phospho JNK, and anti-phospho-c-Jun antibodies were from Cell Signaling Technology. Anti-JNK antibody was from BD Biosciences. Anti-NLRP3 antibody was purchased from Alexis Biochemicals. Anti-HMGB1 antibody was from Abcam.

2.5. Cell death assay

Cell death was determined by the extracellular release of lactate dehydrogenase (LDH) using a CytoTox® 96 Non-Radioactive Cytotoxicity Assay Kit (Promega). LDH release was calculated as $[A_e/(A_e + A_i)] \times 100$, where A_e and A_i indicate the levels of extracellular and intracellular LDH, respectively. Cell survival was determined by measuring the conversion of methyl-thiazolyldiphenyl tetrazolium bromide (MTT; Sigma) into formazan at 570 nm. The type of cell death was determined using a Fluorescein Isothiocyanate (FITC) Annexin V Apoptosis Detection Kit (BD Biosciences) as described previously [18]. In brief, attached cells, after appropriate treatment, were washed with phosphate-buffered saline (PBS) and stained with Annexin V-FITC and propidium iodide (PI) in binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) for 10 min. Following trypsinization, the cells were washed two times with PBS and resuspended in binding buffer (5×10^5 cells/ml). Simultaneously, floating cells, after appropriate treatment, were resuspended in binding buffer and stained with Annexin V-FITC for 10 min. After washing with binding buffer, the floating cells were stained with PI and resuspended in binding buffer. Fluorescence of the mixture of attached and floating cells was then analyzed by flow cytometry (BD, FACSCalibur™). Extracellular releases of ATP were measured by ATP determination kit (Invitrogen) according to the manufacturer's protocol.

2.6. Immunofluorescence assay

Cells were grown on a cover slip and treated with etoposide for 24 h. After washing with PBS, the cells were fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton X-100. Subsequently, cover slips were blocked with 4% normal goat serum and incubated with an anti-ASC antibody (Santa Cruz Biotechnology), followed by incubation with the Alexa Fluor 488 anti-rabbit IgG antibody (Invitrogen). The cell samples were mounted using the ProLong Gold reagent (Invitrogen) containing 4′, 6-diamidino-2-phenylindole (DAPI) as the nuclei stain and examined under a confocal microscope (Olympus, FluoView FV1000).

2.7. Mitochondrial ROS production

Cells, after appropriate treatment, were trypsinized and resuspended in Hank's balanced salt solution containing calcium and magnesium. The MitoSox (Invitrogen) stock solution was then added to the cells at a final concentration of 2.5 μM and incubated at 37 °C for 10 min. Cells were washed three times, and the fluorescence was monitored by flow cytometry.

2.8. Knockdown of ASC by siRNA

HT-29 cells were transfected with a control non-targeting siRNA or with an ASC-targeting siRNA (50 nM) using Lipofectamine 2000 according to the manufacturer's instructions. After 48 h of transfection, cells were washed and treated with chemotherapeutic agents.

2.9. Statistical analysis

All values were expressed as mean ± standard deviation (SD) of n observations. Data were statistically analyzed by using an unpaired Student's t-test. p-values of 0.05 or less were considered significant.

3. Results

3.1. ASC is epigenetically silenced by aberrant methylation in human colorectal cancer DLD-1 cells

To explore the molecular function of ASC in cancer cells, we first determined ASC expression in twelve cancer cell lines derived from various tissues. Two-thirds of the tumor cell lines tested did not express ASC, indicating that ASC is frequently silenced in tumor cells (Fig. 1A). It has indeed been reported that many tumor cells, such as Du145, LNCaP, MDA-MB231, HCT 116, and DLD-1, showed complete methylation of the ASC promoter region [19–21]. By contrast, both MCF-7 and HT-29 cells, in which the ASC promoter is rarely methylated according to previous reports [20,21], exhibited robust expression of ASC (Fig. 1A), suggesting that ASC expression is primarily regulated by epigenetic methylation-induced silencing in cancer cells.

In innate immune cells, ASC plays a crucial role in assembling the inflammasome complex, leading to secretion of proinflammatory cytokines [22]. Because chronic inflammation is closely linked to the initiation of colorectal cancer, we decided to investigate the role of ASC in colorectal cancer DLD-1 cells, in which ASC is epigenetically silenced by methylation [9,21]. To examine whether ASC expression could be restored by demethylation, we treated DLD-1 cells with 5-AD, a DNA methyltransferase inhibitor. ASC expression was not observed in the resting state of DLD-1 cells, but was

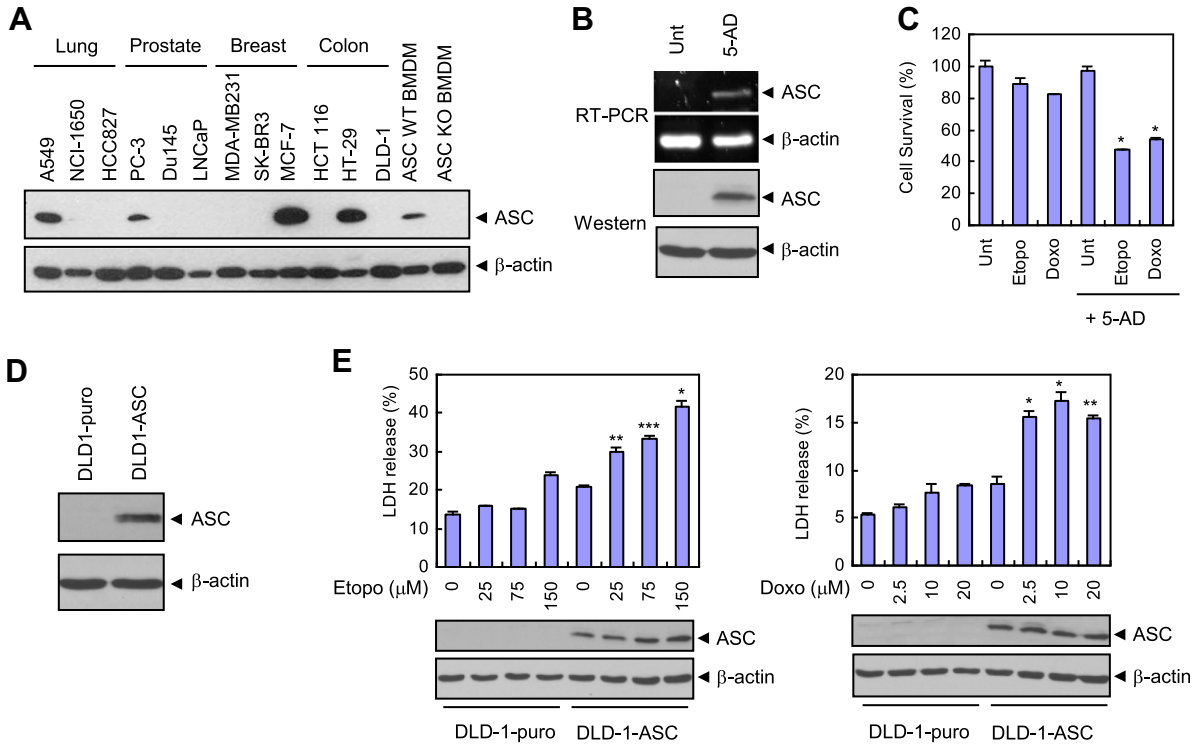

Fig. 1. ASC expression sensitizes human colorectal cancer DLD-1 cells to DNA damaging agents. (A) Cellular lysates from various cancer cell lines or mouse bone marrow-derived macrophages (BMDMs) were immunoblotted with anti-ASC or anti-β-actin antibodies. (B) ASC expression in DLD-1 cells with or without 5-AD priming (2 μM, 4 days) was determined by RT-PCR or western blot analysis. (C) DLD-1 cells with or without 5-AD-priming (2 μM, 48 h) were treated with etoposide (50 μM) or doxorubicin (1 μM) for additional 38 h. Cell survival rate was determined by MTT assay. Asterisks indicate significant differences between unprimed and 5-AD-primed cells ($n = 3$, $^{*}p < 0.05$). (D) Western blotting of the lysates of DLD-1-puro or DLD-1-ASC stable cells. (E) DLD-1-puro or DLD-1-ASC cells were treated with etoposide or doxorubicin at the indicated concentration for 30 h. Culture supernatants and cellular lysates were assayed to determine extracellular LDH release. Asterisks indicate significant differences compared to DLD-1-puro cells (left panel, $n = 3$, $^{*}p < 0.01$; $^{**}p < 0.005$; $^{***}p < 0.0005$; right panel, $n = 3$, $^{*}p < 0.005$; $^{**}p < 0.001$).

restored after 4 days of 5-AD treatment, as determined by RT-PCR and Western blot analysis (Fig. 1B). These results suggest that ASC expression in DLD-1 cells is mainly suppressed by epigenetic methylation of its promoter region.

3.2. ASC expression sensitizes DLD-1 cells to DNA damaging agents

To examine the tumor-suppressive function of ASC in DLD-1 cells, we first treated unprimed or 5-AD-primed DLD-1 cells with the chemotherapeutic drugs, etoposide and doxorubicin, which are known to induce DNA damage leading to cell death [23]. 5-AD-primed DLD-1 cells showed a significant decrease in cell survival upon treatment with DNA damaging agents, compared with unprimed DLD-1 cells, as revealed by the MTT assay (Fig. 1C). In addition, the DNA damaging agents caused more severe cytotoxicity to 5-AD-primed DLD-1 cells than to unprimed DLD-1 cells, as determined by extracellular LDH release (Supplementary Fig. 1A). These results indicate that demethylation-induced ASC expression renders DLD-1 cells more susceptible to genotoxic stress.

In addition to ASC, demethylation by 5-AD treatment is able to induce the expression of other aberrantly methylated genes at their CpG islands in DLD-1 cells. Indeed, it has been previously reported that several genes such as *LKB1* and *DKK-1* are hypermethylated in DLD-1 cells [24,25]. To exclude the possibility that expression of other epigenetically silenced genes via demethylation can affect the susceptibility of DLD-1 cells to genotoxic stress, we generated DLD-1 cells stably expressing ASC (Fig. 1D). We then determined the susceptibility of both the control DLD-1-puro and the DLD-1-ASC cells to DNA damaging agents. Cell death was significantly higher in the DLD-1-ASC cells than that in the control DLD-1-puro cells when treated with etoposide, doxorubicin, or camptothecin (Fig. 1E and Supplementary Fig. 1B).

3.3. Decreased ASC expression attenuates cell death caused by DNA damaging agents

To verify the above-mentioned observation that ASC expression sensitizes colorectal cancer cells to DNA damaging agents, we knockdowned ASC expression in human colorectal cancer HT-29 cells, which express ASC due to unmethylation of the ASC promoter [21]. ASC expression in HT-29 cells was markedly decreased by transfection with a siRNA specific to ASC (Fig. 2A). Etoposide induced a dose-dependent cell death in ASC-expressing HT-29 cells, but not in ASC-knockdowned cells (Fig. 2A). In addition, both caspase-3 and PARP cleavage were attenuated in ASC-knockdowned HT29 cells (Fig. 2A).

To further examine whether ASC is critical for genotoxic stress-induced cell death, cells other than cancer cells (wild-type and ASC-deficient mouse bone marrow-derived macrophages) were treated with DNA damaging agents. As observed in DLD-1 cells, etoposide-, camptothecin-, and doxorubicin-induced cell death was significantly suppressed in ASC-deficient macrophages compared with that in ASC-expressing wild-type cells (Fig. 2B and Supplementary Fig. 1C). These results collectively demonstrate that ASC expression is an important factor for genotoxic stress-induced cell death of colorectal cancer cells and macrophages.

3.4. Inflammasome signaling is not required for the genotoxic stress-induced cell death of ASC-expressing DLD-1 cells

One recent report proposed that chemotherapeutic agents such as doxorubicin and daunorubicin induce NLRP3 inflammasome activation in bone marrow-derived macrophages [26]. To understand whether inflammasome signaling, which is responsible for caspase-1-dependent pyroptotic cell death, is involved in DNA

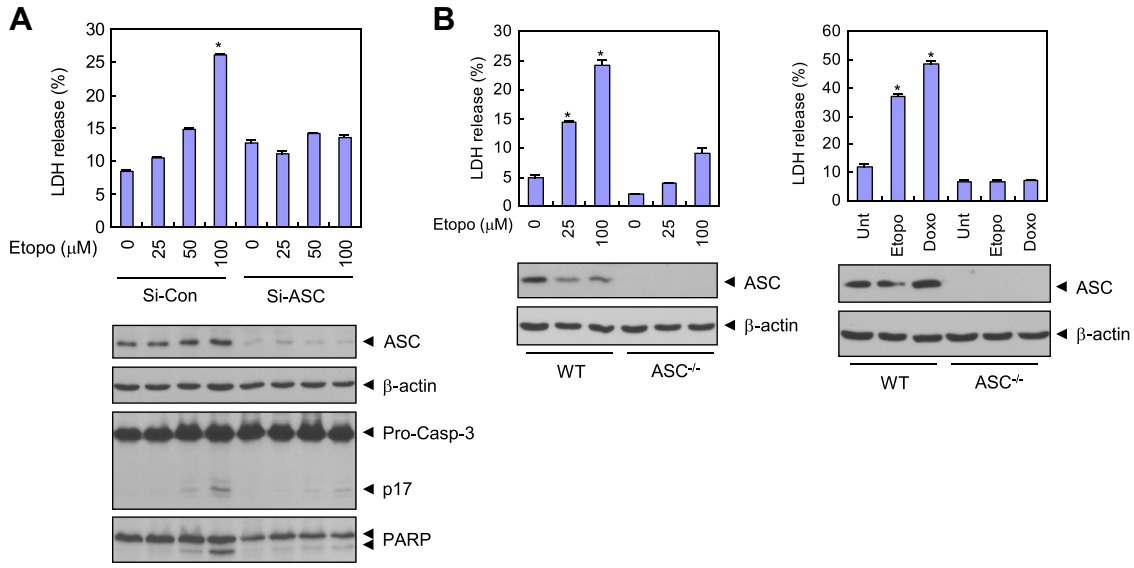

Fig. 2. Decreased ASC expression attenuates DNA damage-induced HT-29 or macrophage cell death. (A) HT-29 cells were transfected with si-control or si-ASC RNA (50 nM), and after 48 h of transfection, cells were incubated with etoposide as indicated for 30 h and assayed for LDH release. Cellular lysates were immunoblotted with the indicated antibodies. Asterisk indicates significant difference compared to si-ASC-transfected group ($n = 3$, $^*p < 0.001$). (B) Wild-type or ASC-deficient BMDMs were incubated with etoposide for 5 h (left panel), and with etoposide (2 μM) or doxorubicin (0.5 μM) for 24 h (right panel). Asterisks indicate significant differences compared to ASC-deficient cells (left panel, $n = 3$, $^*p < 0.001$; right panel, $n = 3$, $^*p < 0.0005$).

damage-induced cytotoxicity, the inflammasome activity was examined in DLD-1 cells after treatment of DNA damaging agents. Although DLD-1 cells express procaspase-1, none of the DNA damaging agents tested induced caspase-1 activation in DLD-1-puro and DLD-1-ASC cells, as determined by extracellular secretion of active caspase-1 (Fig. 3A). However, considering that these DNA damaging agents definitely induced cell death in the present study, particularly in DLD-1-ASC cells, we speculate that caspase-1 or inflammasome activity may not be required for ASC-mediated cell death induced by genotoxic stress.

To further examine whether the activation of the inflammasome could promote colorectal cancer cell death, ASC-deficient or ASC-expressing DLD-1 cells were treated with known inflammasome stimulators. As shown in Fig. 3B, LPS plus nigericin, an activator of the NLRP3 inflammasome, had no effect on the activation of the inflammasome in DLD-1 cells, as determined by the secretion of active caspase-1 and IL-1β. Similarly, transfection of poly (dA:dT), which activates AIM2 inflammasome [17], failed to induce caspase-1 or IL-1β activation in DLD-1 cells. In contrast, LPS plus nigericin as well as poly (dA:dT) promoted robust caspase-1 activation and subsequent secretion of active IL-1β in phorbol 12-myristate 13-acetate (PMA)-primed THP-1 cells, which is a monocytic leukemia cell line (Fig. 3B). Moreover, the activation of the NLRP3 or AIM2 inflammasome triggered substantial cell death in THP-1 cells, but not in DLD-1 cells (Fig. 3B).

ASC oligomerization is a unique feature of inflammasome activation and pyroptotic cell death [5,27]. ASC is also reported to form speck-like structures in human leukemia cell lines upon stimulation with chemotherapeutic agents [7]. However, ASC oligomerization was not obvious in DLD-1-ASC cells treated with etoposide (Fig. 3C). These results collectively indicate that ASC-mediated inflammasome formation is not required for colorectal cancer cell death that occurs in response to genotoxic stress.

3.5. Etoposide-induced cell death of ASC-expressing DLD-1 cells is caspase-independent

To gain a molecular insight into the type of cell death induced by genotoxic stress, we performed annexin V and PI staining of the etoposide-treated and control DLD-1 cells, followed by flow cytometric analysis of the cells. Etoposide treatment increased annexin V-positive and PI-positive cell populations, which represent both apoptotic and necrotic cell death, more prominently in DLD-1-ASC cells (48.7%) than in DLD-1-puro cells (15.67%, Fig. 4A). However, the population of annexin V-positive and PI-negative cells, a characteristic of apoptotic cell death, was relatively very scarce in DLD-1-ASC cells. We thus inferred that etoposide-induced cell death in DLD-1-ASC cells might be necrotic rather than apoptotic (Fig. 4A).

Next, to examine whether caspase signaling pathway, a crucial player in apoptotic cell death, is implicated in etoposide-induced cell death of ASC-expressing DLD-1 cells, the effect of caspase inhibition by zVAD-fmk, a cell permeable pan-caspase inhibitor, and Ac-YVAD-cmk, a selective inhibitor of caspase-1, was examined. Remarkably, etoposide cytotoxicity in 5-AD-primed DLD-1 cells was unaffected by zVAD or YVAD pretreatment (Fig. 4B, left panel), indicating that caspase signaling pathways are dispensable for etoposide-induced cell death of ASC-expressing DLD-1 cells. In support of these results, both caspase inhibitors failed to prevent cell death from etoposide cytotoxicity in DLD-1-ASC stable cells (Fig. 4B, right panel).

To confirm the caspase-independent cell death by etoposide, the effect of caspase inhibition was also tested in ASC-expressing HT-29 cells, well-studied as a caspase-independent cell death model [28]. Interestingly, inhibition of caspase activity by zVAD completely blocked caspase-3 and PARP processing (Fig. 4C, lower panel), but did not prevent etoposide-promoted cell death (Fig. 4C, upper panel). These results verified our speculation that etoposide could trigger caspase-independent cell death in ASC-expressing cells. We, then, stimulated HT-29 cells with TNF-α plus cycloheximide (TC), which is known to induce caspase-8-dependent apoptosis. The presence of zVAD completely suppressed caspase-3 and PARP cleavage in HT-29 cells upon stimulation with TC (Fig. 4C, lower panel, 6th lane), but zVAD did not attenuate TC-induced cell death (Fig. 4C, upper panel, 6th lane). It has been already demonstrated that the inhibition of caspase activity by zVAD in the presence of TC treatment can trigger receptor interacting protein 3 (RIP3)-dependent necrotic cell death in RIP3-expressing cells, such as HT-29 [29]. In this regard, our results demonstrate that

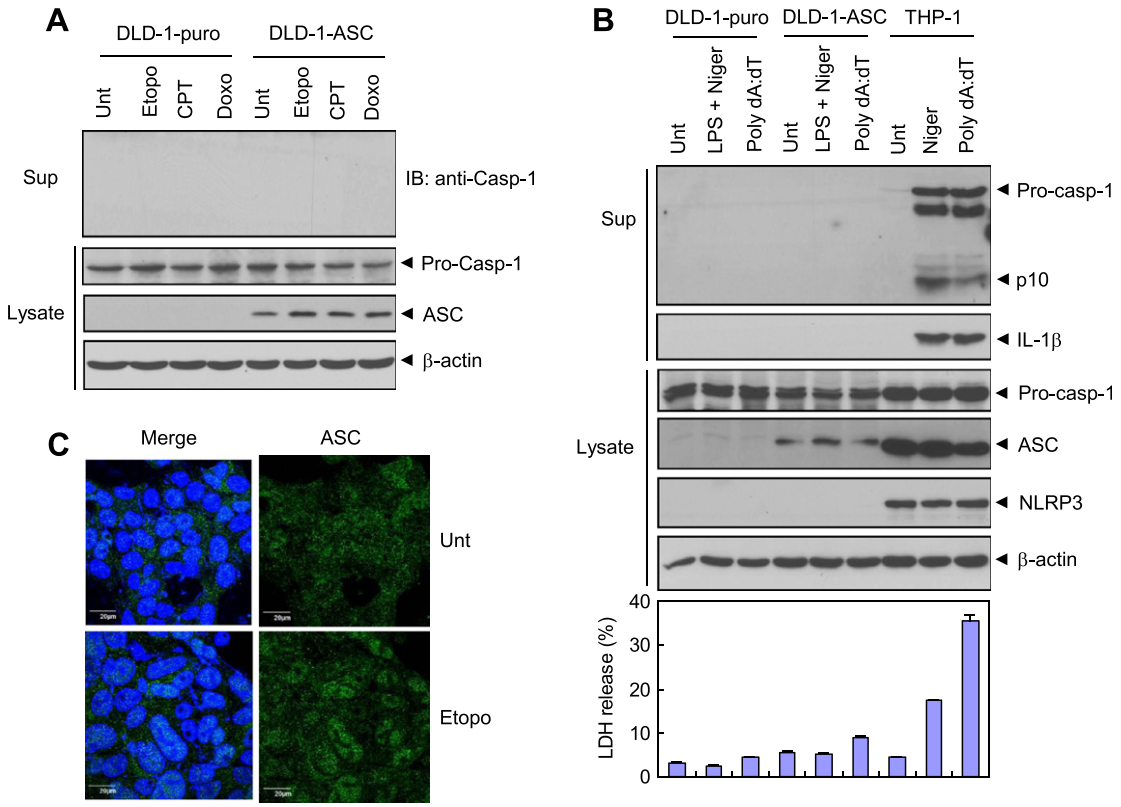

Fig. 3. Inflammasome signaling is not required for DNA damage-induced cell death of DLD-1 cells. (A) ASC-deficient or ASC-expressing DLD-1 cells were treated with etoposide (75 μM), camptothecin (CPT, 2 μM), or doxorubicin (1.5 μM) for 28 h, and the culture supernatants or cellular lysates were immunoblotted with the indicated antibodies. (B) DLD-1 cells or PMA-primed THP-1 cells were treated with LPS (0.5 μg/ml, 4 h) and nigericin (10 μM, 1 h), or transfected with poly (dA:dT) (1 μg, 6 h). Culture supernatants or lysates were immunoblotted with the indicated antibodies, or assayed for LDH release. (C) DLD-1-ASC cells were treated with etoposide (100 μM, 24 h) and stained with an anti-ASC antibody. Immunofluorescence was then detected as described in Section 2.

etoposide could promote caspase-independent necrotic cell death in DLD-1-ASC or HT-29 cells.

3.6. Etoposide promotes necrotic cell death in ASC-expressing colorectal cancer cells

Then, to examine whether etoposide could trigger necrotic cell death in DLD-1-ASC cells, we employed necrostatin-1 (Nec-1), a selective inhibitor of RIP1 kinase to prevent RIP1-dependent necrosis. Unlike zVAD, Nec-1 significantly attenuated etoposide-mediated cell death in DLD-1-ASC cells (Fig. 4D, left panel). In HT-29 cells, Nec-1 also showed a significant inhibition of etoposide-induced cell death, whereas zVAD failed to protect cell death from etoposide toxicity (Fig. 4D, right panel). As expected, Nec-1 effectively prevented TCZ-induced necrotic cell death of HT-29 cells (Fig. 4D, right panel). These observations collectively indicate that RIP1-dependent pathway possibly contributes to the etoposide-mediated cell death of ASC-expressing tumor cells.

We, then, examined the other features of necrotic cell death in ASC-expressing colorectal cancer cells under DNA damage toxicity. Cells undergoing necrosis or secondary necrosis from apoptosis release several endogenous molecules, called danger signals, such as ATP, heat shock proteins and high mobility group box 1 protein (HMGB1), leading to local inflammatory responses [30]. Etoposide, indeed, promoted the release of ATP in DLD-1-ASC cells, and the ATP release was significantly prevented by Nec-1, but not by zVAD (Fig. 4E), indicating that etoposide-mediated ATP release is caspase-independent. Interestingly, DLD-1-puro cells also secreted substantial amount of ATP responding to etoposide, but this ATP release was less than DLD-1-ASC cells and was not significantly inhibited by Nec-1 (Supplementary Fig. 2A). In addition to ATP, HMGB1 release was also detected by etoposide or TCZ stimulation, but not by TC treatment in HT-29 cells (Fig. 4F and Supplementary Fig. 2B), indicating that HMGB1 release is necrosis-specific in our experiment. Indeed, etoposide-mediated HMGB1 release was partially inhibited by Nec-1, but not by zVAD (Fig. 4F). Taken together, our data suggest that etoposide could potentiate caspase-independent necrotic cell death in ASC-expressing colorectal cancer cells.

3.7. Mitochondrial ROS and JNK is implicated in the increased susceptibility of ASC-expressing DLD-1 cells to DNA damaging agent

Based on the above-mentioned results, caspase-independent necrotic pathway is likely to contribute to the etoposide-induced cell death of ASC-expressing DLD-1 or HT-29 cells. Necrotic cell death is often characterized by the following features: plasma membrane rupture, mitochondrial dysfunction and swelling, enhanced mitochondrial ROS generation, and ATP depletion [31]. To compare the etoposide-mediated production of mitochondrial ROS between DLD-1-puro and DLD-1-ASC cells, we measured the mitochondrial ROS by MitoSox staining. Compared to DLD-1-puro cells, DLD-1-ASC cells produced higher amounts of mitochondrial ROS after etoposide stimulation (Fig. 5A and Supplementary Fig. 3A).

Next, to assess the sensitivity of DLD-1-puro and DLD-1-ASC cells to cellular ROS, we added H_2O_2 to ASC-null or ASC-expressing DLD-1 cell cultures. Remarkably, H_2O_2 had a higher cytotoxic effect on DLD-1-ASC cells than on DLD-1-puro cells (Supplementary Fig. 3B). These observations provide biochemical evidence that

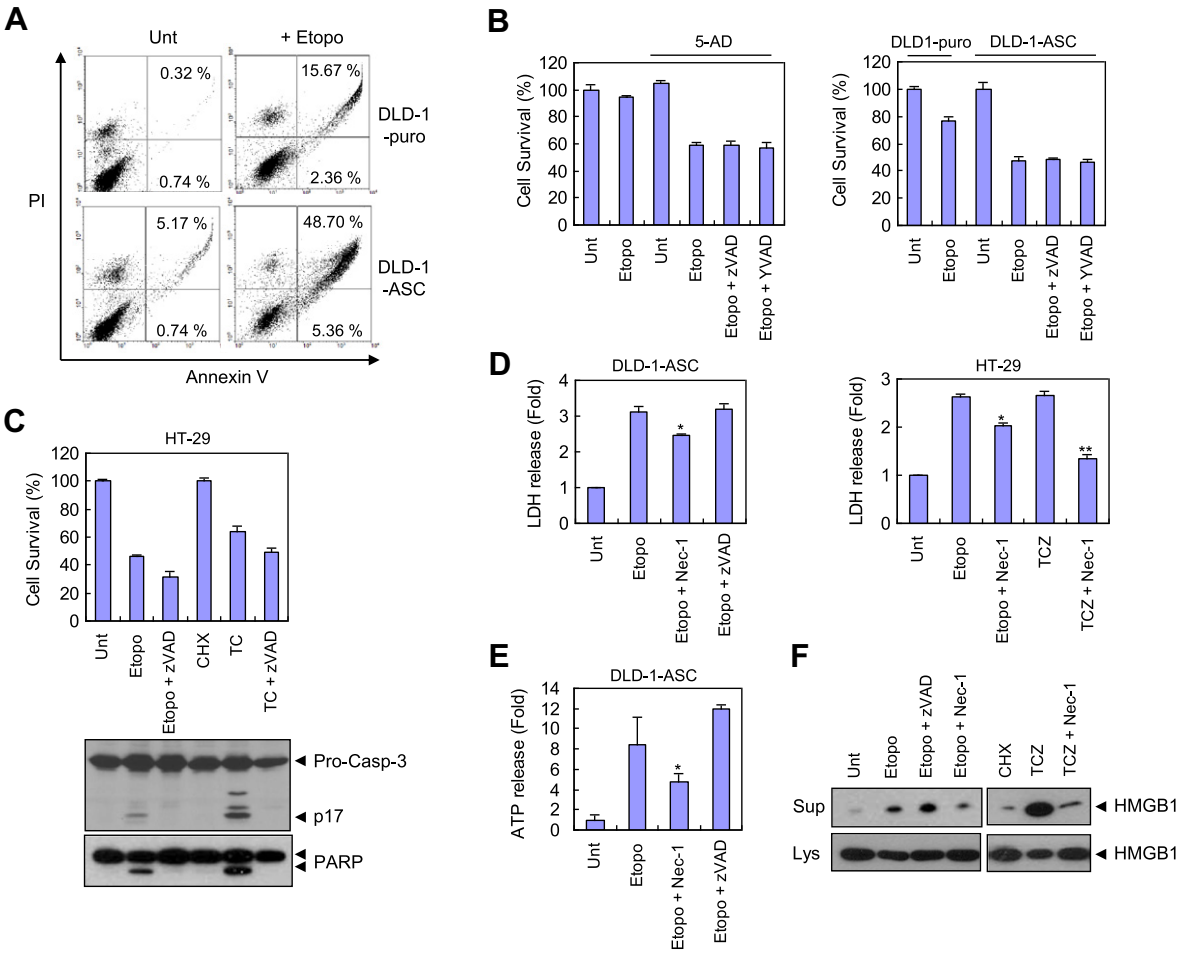

Fig. 4. Etoposide-induced cell death of ASC-expressing colorectal cancer cells is caspase-independent. (A) DLD-1-puro or DLD-1-ASC cells were treated with etoposide (75 μM, 24 h) and assayed for annexin V/PI staining by flow cytometry. (B) DLD-1 cells with or without 5-AD priming, DLD-1-puro, and DLD-1-ASC cells were treated with zVAD or YVAD (20 μM, 30 min), followed by treatment with etoposide (50 μM) for 38 h, as indicated. Cell survival was measured by MTT assay. (C) To determine cell survival rate, HT-29 cells were treated with etoposide (100 μM, 36 h) in the presence of zVAD (20 μM, 30 min pretreat), or treated with TNF-α (30 ng/ml, 16 h) after pretreatment with CHX (10 μg/ml) or zVAD (20 μM) for 30 min, as indicated. TC indicates TNF-α plus CHX. For immunoblotting, HT-29 cells were treated with etoposide (100 μM, 24 h) or TNF-α (30 ng/ml, 16 h) after pretreatment with zVAD (20 μM) or cycloheximide (10 μg/ml) for 30 min, as indicated. (D) DLD-1-ASC cells were pretreated with Nec-1 (40 μM) or zVAD (20 μM) for 30 min, and treated with etoposide (100 μM) for 31 h. LDH release was then assayed for determining cell death. Asterisk indicates the significant difference compared to only etoposide-treated cells ($n = 4$, $^{*}p < 0.005$, left panel). HT-29 cells were pretreated with Nec-1 (40 μM, 30 min) and treated with etoposide (100 μM, 36 h), or pretreated with zVAD (20 μM) or cycloheximide (10 μg/ml) for 30 min, and then treated with TNF-α (30 ng/ml, 16 h). Asterisks indicate the significant differences compared to etoposide- and TCZ-treated cells, respectively ($n = 3$, $^{*}p < 0.05$; $^{**}p < 0.001$, right panel). (E) DLD-1-ASC cells were treated as described in (D), and cultural supernatants were assayed for ATP release. Asterisk indicates the significant difference from etoposide-treated cells ($n = 4$, $^{*}p < 0.05$). (F) HT-29 cells were treated as similar as described in (D), and cultural supernatants or lysates were immunoblotted as indicated.

ASC expression in DLD-1 cells augments the chemotherapeutic agent-induced production of mitochondrial ROS and sensitizes the cells to ROS toxicity. Then, we examined whether ROS scavenger could prevent etoposide-mediated cell death in ASC-expressing tumor cells. NAC, a well-studied antioxidant, effectively protected DLD-1-ASC cells against H_2O_2-induced cytotoxicity (Supplementary Fig. 3C). Similarly, NAC significantly reduced the etoposide-mediated cell death in DLD-1-ASC and HT-29 cells (Fig. 5B).

Chemotherapeutic agents, including etoposide, have been known to activate MAPKs that contribute to cell death [32]. To examine whether MAPKs such as p38 MAPK and JNK are implicated in etoposide-mediated cell death of ASC-expressing colorectal cancer cells, we first observed the activation of both kinases by DNA damaging agents. DNA damaging agents including etoposide or camptothecin promoted stronger phosphorylation of JNK in 5-AD-primed DLD-1 cells expressing ASC than in unprimed DLD-1 cells (Fig. 5C, upper panel and Supplementary Fig. 4A, left panel). In addition, ASC-deficient macrophages showed attenuated JNK phosphorylation upon treatment with etoposide (Supplementary Fig. 4A, right panel), demonstrating that ASC facilitates etoposide-mediated JNK activation by unknown mechanism. To support these findings, knockdown of ASC decreased etoposide-mediated JNK activity as determined by the phosphorylation of c-Jun in HT-29 cells (Fig. 5C, middle panel). On the contrary, etoposide did not activate p38 MAPK in HT-29 cells (Fig. 5C, lower panel) and in ASC-null or ASC-expressing DLD-1 cells (Supplementary Fig. 4B).

We, then, tested specific MAPK inhibitors for their ability to suppress etoposide-promoted cell death. SP600125, an inhibitor of JNK, showed a partial but significant protection against etoposide cytotoxicity in ASC-expressing DLD-1 cells, whereas SB203580, a selective p38 MAPK inhibitor, failed to prevent etoposide-induced cell death of ASC-expressing DLD-1 cells (Fig. 5D, left panel and Supplementary Fig. 4C). Consistently, SP600125 significantly increased cell survival against etoposide stimulation to HT-29 cells (Fig. 5D, right panel). These results collectively demonstrate that JNK activation might be involved in the etoposide-induced cell death of ASC-expressing colorectal cancer cells.

Then, we asked whether JNK activity is required for the etoposide-induced production of mitochondrial ROS. Mitochondrial ROS

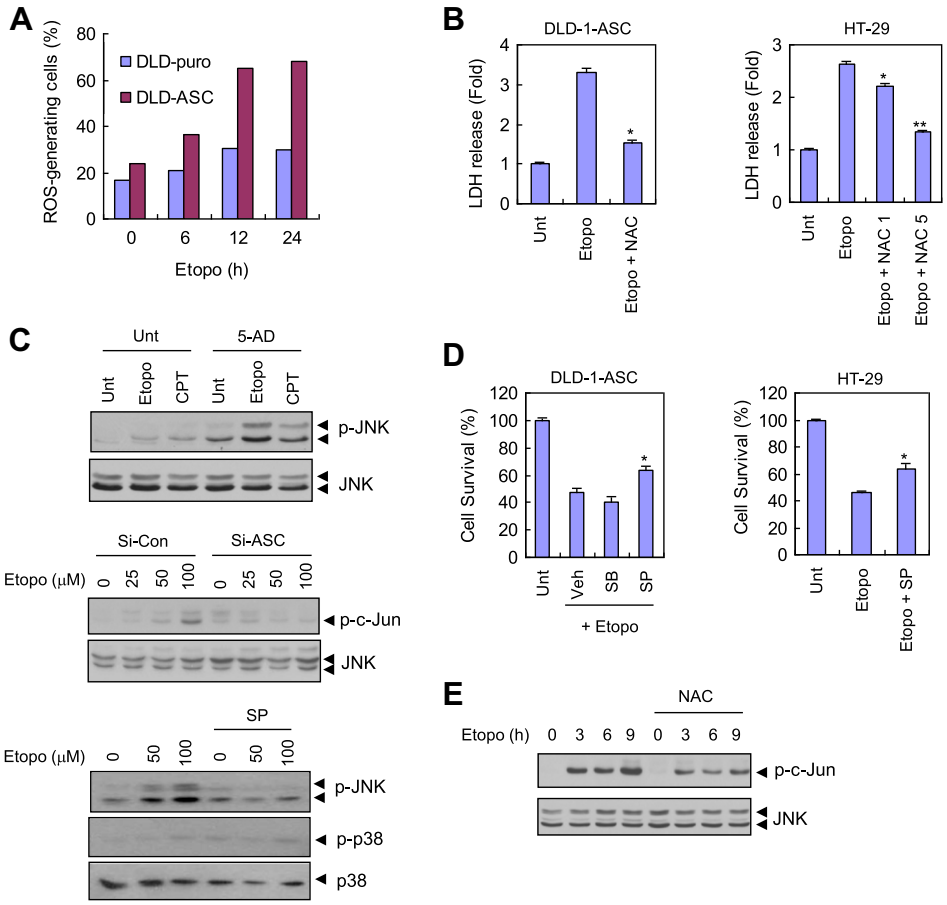

Fig. 5. Mitochondrial ROS and JNK activation is required for etoposide-induced cell death of ASC-expressing colorectal cancer cells. (A) DLD-1-puro or DLD-1-ASC cells were treated with etoposide (100 μM) for the indicated times. Cells were stained with MitoSox and analyzed for mitochondrial ROS production by flow cytometry. (B) DLD-1-ASC cells were treated with etoposide (100 μM, 30 h) in the presence of NAC (2 mM, 30 min pretreat), and assayed for LDH release. Asterisk indicates the significant difference from etoposide-treated cells ($n = 4$, $^{*}p < 0.001$, left panel). HT-29 cells were treated with etoposide (100 μM, 36 h) in the presence of NAC as indicated, and assayed for LDH release. Asterisks indicate the significant difference from etoposide-treated cells ($n = 3$, $^{*}p < 0.005$; $^{**}p < 0.001$, right panel). (C) DLD-1 cells with or without 5-AD priming (upper panel), HT-29 cells transfected with si-control or si-ASC (middle panel), and HT-29 cells pretreated with SP600125 (lower panel) were treated with etoposide or camptothecin, as indicated. Cellular lysates were immunoblotted with the indicated antibodies. (D) DLD-1-ASC and and HT-29 cells were treated with etoposide (75 μM, 38 h) with or without the 30 min pretreatment of SB203580 (10 μM) or SP600125 (10 μM), as indicated. Cell survival was determined by MTT assay. Asterisks indicate significant differences in cell survival compared to only etoposide-treated cells ($n = 3$, $^{*}p < 0.05$). (E) DLD-1-ASC cells were treated with etoposide (75 μM) in the presence of NAC (2 mM, 30 min pretreat) for the indicated times. Cellular lysates were immunoblotted with the indicated antibodies.

production in etoposide-treated DLD-1-ASC cells was not impaired by the pretreatment of the JNK inhibitor, SP600125 (Supplementary Fig. 4D), suggesting that mitochondrial ROS production is an independent or upstream process of the JNK signaling pathway. Then, we checked the effect of NAC on the activation of JNK. Scavenging of ROS by NAC showed a partial inhibitory effect on the phosphorylation of c-Jun (Fig. 5E). This observation suggests that ROS, more produced in ASC-expressing cells, might positively modulate etoposide-mediated JNK activation leading to cell death. Taken together, our data demonstrate that ASC potentiates genotoxic stress-induced cell death of colorectal cancer cells via mitochondrial ROS production and JNK activation.

4. Discussion

Inflammasome is a cytoplasmic multiprotein complex, which is assembled and activated upon sensing of a microbial infection or a danger signal derived from tissue injury [3]. To our knowledge, inflammasome has been mainly studied in myeloid cells, because the major function of inflammasome signaling is the maturation and secretion of proinflammatory cytokines IL-1β and IL-18, which results in triggering local inflammation. However, emerging evidences have demonstrated that inflammasome is implicated in the progression of several chronic or metabolic diseases including carcinogenesis, type II diabetes, and atherosclerosis [33,34]. It is thus of interest to investigate the function of the inflammasome in nonmyeloid cells.

The physiological role of inflammasome signaling in tumorigenesis is still controversial. Activation of the inflammasome induces caspase-1-dependent pyroptotic cell death, resulting in the removal of tumorigenic cells, particularly in the colon [6]. Indeed, *caspase-1*-deficient mice exhibit enhanced tumor formation in a colorectal cancer model and decreased apoptosis in colonic epithelial cells [35,36]. This tumor-suppressive role of the inflammasome is supported by the finding that *Nlrp3*$^{-/-}$, *ASC*$^{-/-}$, and *caspase-1*$^{-/-}$ mice are more susceptible to colitis-associated colorectal cancer than wild-type mice [35–37]. In contrast, it is also reported that *Nlrp3*$^{-/-}$ and *caspase-1*$^{-/-}$ mice showed less severe dextran sulfate sodium-induced colitis [38,39]. Therefore, the contribution of the inflammasome to tumor suppression or progression is debatable based on the context and remains to be clarified.

The inflammasome adaptor molecule, ASC, seems more directly implicated in the regulation of tumorigenesis, because it is found to be epigenetically silenced with a high frequency in many cancer cells [8–10]. Overexpression or ligand-induced oligomerization of

ASC clearly makes nontumorigenic cells susceptible to apoptotic stimuli [12]. Interestingly, in MCF-7 cells upon genotoxic stress, ASC is upregulated and localized to the mitochondria in a p53-dependent manner, leading to mitochondria-mediated apoptosis [16]. However, it has not been examined in detail whether restoration of ASC expression in epigenetically ASC-silenced cancer cells facilitates cell death in response to chemotherapeutic agents. In the present study, we demonstrated the putative tumor-suppressive effect of ASC expression in human colorectal cancer cells, DLD-1. Given that DLD-1 cells lack wild-type p53 [40], DNA damaging agents might trigger p53-independent cell death in DLD-1 cells. Moreover, we found no difference in the DNA damage response between ASC-deficient and ASC-expressing DLD-1 cells, as determined by the phosphorylation of H2AX (Supplementary Fig. 5A).

According to a recent report, ASC was able to induce not only apoptotic, but also necrotic cell death, depending on the cellular context [41]. After being activated by the upstream recombinant Nod-like receptor family protein, ASC induces apoptosis in caspase-1-deficient cells, but necrotic cell death in caspase-1-expressing cells, regardless of caspase-1 activity [41]. ASC is thus likely to promote necrotic cell death in DLD-1-ASC or HT-29 cells due to an expression of caspase-1 in both cells (Supplementary Fig. 5B), although the precise mechanism as to how caspase-1 controls the type of cell death is not clear. Further study will be needed to clarify how ASC contributes to the progression of apoptotic or necrotic cell death under the different cellular context.

In conclusion, ASC expression clearly promotes colorectal cancer cell death in response to genotoxic stress. This finding suggests that ASC possesses a tumor-suppressive function, and that its expression might be epigenetically silenced during carcinogenesis in several ASC-deficient cancer cells, such as DLD-1. ASC is able to trigger apoptotic cell death; however, in our study, it also induced necrotic cell death through the production of mitochondrial ROS and through the activation of JNK signaling upon stimulation with a chemotherapeutic agent. As the epigenetic regulation of ASC expression is definitively correlated with the colorectal carcinogenesis, methylation status of ASC gene could be used for cancer prognosis. Furthermore, the restoration of ASC expression by demethylating agents could be a potent strategy to enhance the efficacy of chemotherapeutic drugs in ASC-silenced cancer patients.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.canlet.2012.12.020.

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