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The apoptotic effect of intercalating agents on HPV-negative cervical cancer C-33A cells

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Summary. Cervical cancer is one of the leading causes of female cancer death worldwide with about 500,000 deaths per year. Both mitomycin C and cisplatin are alkylating agents, which bind and intercalate DNA, and thus used as anti-cancer drugs. In these studies, we focused on investigating the apoptotic effects of intercalating agents on HPV-negative cervical cancer C-33A cells. Accordingly, C-33A cells were treated with carboplatin, mitomycin C or cisplatin. Cell cycle analysis revealed that treatment with mitomycin C and cisplatin but not with carboplatin resulted in apoptosis. Both mitomycin C and cisplatin induced apoptosis in C-33A cells via caspase-8 and -3 processing in a Fas/FasL-dependent manner and also suppressed IL-18 expression, while they down-regulated IκB expression and up-regulated p65 expression. These results suggest that both mitomycin C and cisplatin induce apoptosis, not only via the caspase-8 and -3 dependent Fas/FasL pathway, but also via the regulation of NF-κB activity and IL-18 expression in HPV-negative cervical cancer C-33A cells.

Keywords: IL-18 – FasL – Mitomycin C – Cisplatin – Apoptosis – Cervical cancer

Abbreviations: IFN-γ, interferon-γ; FasL, Fas ligand; NF-κB, nuclear facor-κB; HPV, human papillomavirus; MMC, mitomycin C; CIS, cisplatin; CA, carboplatin

Introduction

Cisplatin has been shown to interact with a variety of small and large molecules. The major target of cisplatin with respect to cytotoxicity appears to be DNA. Cisplatin binds to DNA at pharmacologically relevant doses (Rosenberg, 1979), and selectively inhibits DNA synthesis over RNA and protein synthesis (Weiss and Christian, 1993). Particularly, the drug is more toxic to cells that are deficient in DNA repair. Cisplatin inhibits DNA synthesis by producing equal numbers of DNA lesions to a greater extent than the trans isomer, which has no anti-tumor activity (Johnson et al., 1980). Cisplatin is active against several human cancers including testicular, ovarian, small-cell lung, bladder, cervical, and head and neck cancers. Mitomycin C (MMC), a well known anti-tumor agent, exhibits a diverse range of biological effects: DNA alkylation, and the generation of reactive free radicals, thereby inducing DNA strand scission (Iyer and Szybalski, 1964; Gusev and Panchenko, 1982).

The cytokine interleukin-18 (IL-18), a member of the IL-1 superfamily (Gracie et al., 2003; Dinarello, 1999), elicits several biological activities that initiate and promote host defenses and inflammation following infection or injury. IL-18, initially described as IFN-γ inducing factor (Nakamura et al., 2000), is a pro-inflammatory cytokine (Puren et al., 1998). In addition, it possesses pleiotropic biological properties including activation of nuclear factor κB (NF-κB) (Matsumoto et al., 1997), Fas ligand expression (FasL) (Dao et al., 1996), induction of both CC and CXC chemokines (Balashov et al., 1999), enhancement of the production of IFN-γ and granulocyte macrophage-colony stimulating factor (GM-CSF) (Ushio et al., 1996), and induction of the CD^{4+} cytolytic T-cell response (Micallef et al., 1997). Moreover, IL-18 enhances the expression of FasL on NK cells, which specifically attack cells presenting Fas proteins on their membrane, but they do not attack

parental control cells (Tsutsui et al., 1997). Several pro-inflammatory cytokines negatively regulate endothelial cell survival (Madge and Pober, 2001; Robaye et al., 1991), and IL-18 regulates human cardiac endothelial cell death (Chandrasekar et al., 2004). However, the role of IL-18 in cervical cancer cell apoptosis is not known.

According to previous studies (Kang et al., 2005), MMC treatment induced a reduction in the expressions of E6 oncogene and IL-18 in HPV 16-positive cervical cancer cells in a p53-independent manner. In these studies, MMC and CIS were treated in C-33A cervical cell line, which is a p53-mutant and HPV-negative in order to compare the apoptotic mechanisms with those of HPV positive cervical cancer cells. As in the case of SiHa cell line which is HPV positive cervical cancer cell line, MMC and CIS induced apoptosis through similar pathways in p53 mutated and HPV-negative cervical cancer cell line C-33A. Our studies might be instrumental for understanding the role of endogenous IL-18 as it relates to drug-induced apoptosis in cervical cancer cells.

Materials and methods

Cervical cancer cell culture

The C-33A, which is a p53-mutant, HPV-negative, and human cervical cancer cell line, was obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). C-33A cells were grown in DMEM supplemented with 100 unit/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air.

Treatment of alkylating agents

C-33A cells were treated with 10 μg/ml of anti-cancer agents such as CA, MMC, and CIS (Sigma, St. Louis, MO, USA) for 24 h. Cell extracts were prepared with luciferase assay kit according to the manufacture's instructions (Promega, Madison, WI, USA) and were then subjected to Western blot analysis.

Cell proliferation assay

WST-1 (Boehringer Mannheim, Mannheim, Germany) was used to measure the viability of cervical carcinoma cell line C-33A. Cells were plated at a density of 1×10^4 cells/100 μl/well on a 96-well plate at a final concentration of 10 μg/ml of anticancer drugs. Then 10 μl of WST-1 cell proliferation assay reagent was added to the cells after 24, 48 or 72 h and incubated at 37 °C for 30 min, at which time the optical density was measured at 450–650 nm.

FACS analysis

Cell cycle was analyzed by flow cytometry. Cells were washed twice with cold PBS, and permeabilized in a cold saponin buffer (0.1% saponin in PBS). Subsequently, cells were treated with a propidium iodide (PI) buffer (100 μg/ml PI, 0.1 mM EDTA, 0.1% Triton X-100 in PBS, pH 7.4) containing 1 mg/ml RNase A for 30 min as previously described (Zou et al., 1989), and analyzed with the FACSCalibur system (Becton Dickinson, Mountain View, CA, USA). The distribution of the cells in different phases of the cell cycle was calculated using the Cell-FIT software (Becton Dickinson Instruments). For FasL and Fas analyses, cells (1×10^6) were suspended in 50 μl FACS buffer (0.1% FBS, 0.02% sodium azide), and incubated with the indicated antibodies on ice for 30 min. The cells were then washed twice with a FACS buffer. Data were collected with the FACSCalibur system (Becton Dickinson) and analyzed with CellQuest software. Except where noted, all antibodies were purchased from BD Biosciences Pharmingen (San Diego, CA, USA). For the analysis of apoptosis, cells were left untreated, or treated for 24 h with either MMC alone or in combination of 30 min pretreatment with anti-human Fas ligand (2.5 μg/one million cells), Z-IETD-FMK (a caspase-8 inhibitor; 20 μM), Z-DEVD-FMK (a caspase-3 inhibitor; 20 μM) or Z-FA-FMK (a negative control; 20 μM). Following incubation, cells were collected and resuspended in 100 μl of Annexin binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.4 mM CaCl) supplemented with 5 μl of Annexin V-FITC and 5 μl of PI (100 μg/ml) and incubated for 15 min at 4 °C. The cells were immediately analyzed using the FACSCalibur system.

Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from cervical carcinoma cells using the easy-BLUE™ Total RNA Extraction kit (iNtRON, Korea). RT-PCR was performed using a One-Step RT-PCR kit (iNtRON, Korea) according to the manufacturer's instructions with some minor modifications. The primers used for FasL: 5′-CAA GTC CAA CTC AAG GTC CAT GCC-3′ (sense) and 5′-CAG AGA GAG CTC AGA TAC GTT TGA C-3′ (antisense). The primers used for Fas: 5′-GAC CCT CCT ACC TCT GGT T-3′ (sense) and 5′-CTA GAC CAA GCT TTG GAT TTC-3′ (antisense). The primer sequences used for β-actin as an internal standard were: 5′-GTG GGG CGC CCC AGG CAC CA-3′ (sense) and 5′-CTC CTT AAT GTC ACG CAC GAT TTC-3′ (antisense).

Western blot analysis

Cellular proteins were separated on a 12% SDS-PAGE gel, and transferred onto nylon membranes (Immobilon-P, Millipore, Bedford, MA, USA) according to the established methods. The membranes were then probed with specific antibodies against IκB, p65, IL-18, FADD, caspase-8, caspase-3, and β-actin, and the immunoblots were developed using enhanced chemiluminescence (ECL) detection reagents (iNtRON, Korea) according to the manufacturer's instructions. The immunoblotted membranes were stripped with a stripping buffer (50 mM Tris–HCl, pH 6.8, 100 mM β-Mercaptoethanol, 2% SDS) and reprobed with specific other antibodies.

IL-18 ELISA

C-33A cervical carcinoma cells were treated with 10 μg/ml of anti-cancer agents such as CA, MMC, and CIS for 24 h. The IL-18 level in the supernatants was subsequently measured with an ELISA kit (MBL, Nagoya, Japan) according to the manufacturer's instructions.

Results

C-33A cell viability was decreased after treatment with intercalating agents, MMC and CIS

C-33A cells were used to monitor cell death after treatment with intercalating agents such as carboplatin, mitomycin C, and cisplatin. The cell viability was evaluated with WST-1

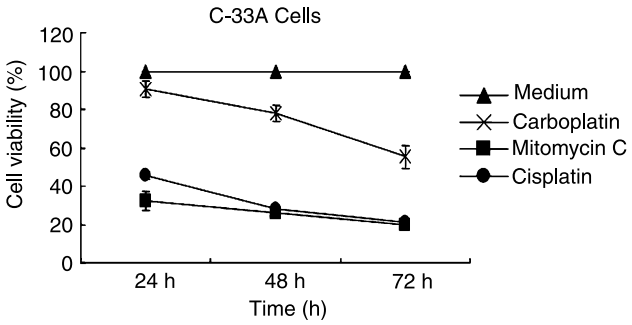

Fig. 1. Cytotoxic effects of both MMC and CIS on HPV-negative cervical cancer cell line, C-33A. Cell viability was evaluated using a cell proliferation reagent, WST-1 after treatment with carboplatin, cisplatin, or mitomycin C as described in Materials and methods

reagent. The viability of anti-cancer agent-treated cells was decreased in comparison with the untreated control cells (Fig. 1). At 24 h after MMC and CIS treatments, cell viabilities were reduced up to 32 and 45%, respectively, whereas most of cells (about 90%) were still viable at 24 hours after CA treatment (Fig. 1). Three days after CA, MMC, and CIS treatments, cell viabilities were reduced up to 55, 20, and 21%, respectively (Fig. 1). According to these results, both MMC and CIS exhibited more cytotoxic activity than CA at 24 h after treatment. Therefore, this 24 h time point was selected for all subsequent apoptosis experiments.

Treatment with either MMC or CIS increased apoptosis in C-33A cells

The changes in DNA content during the progression of cell cycle were evaluated by a flow cytometric assay. In the DNA content histogram (Fig. 2a), the presence of cells with fragmented DNA was indicated by a sub-G_1/G_0 peak (M1) of DNA content. The sub-G_1/G_0 population increased to 54.8 and 39.3% due to MMC and CIS treatment, respectively, in the C-33A cervical carcinoma cell line (Fig. 2a). The cell cycle was arrested at the G_1 phase by both MMC and CIS treatments in the same cell lines, as compared with that of non-treated control cells (Fig. 2a). These results suggest that MMC and CIS induced increased rates of cell death in the C-33A cervical carcinoma cell line.

In order to demonstrate that the cell death induced by MMC and CIS in C-33A cells was due to apoptosis, we performed Annexin V staining using FACSCalibur, which revealed the Annexin V-positive population in the MMC- or CIS-treated C-33A cells. The percentages of apoptotic cells were 21.4 and 47.9% after treatment with MMC and CIS, respectively (Fig. 2b). These results confirmed

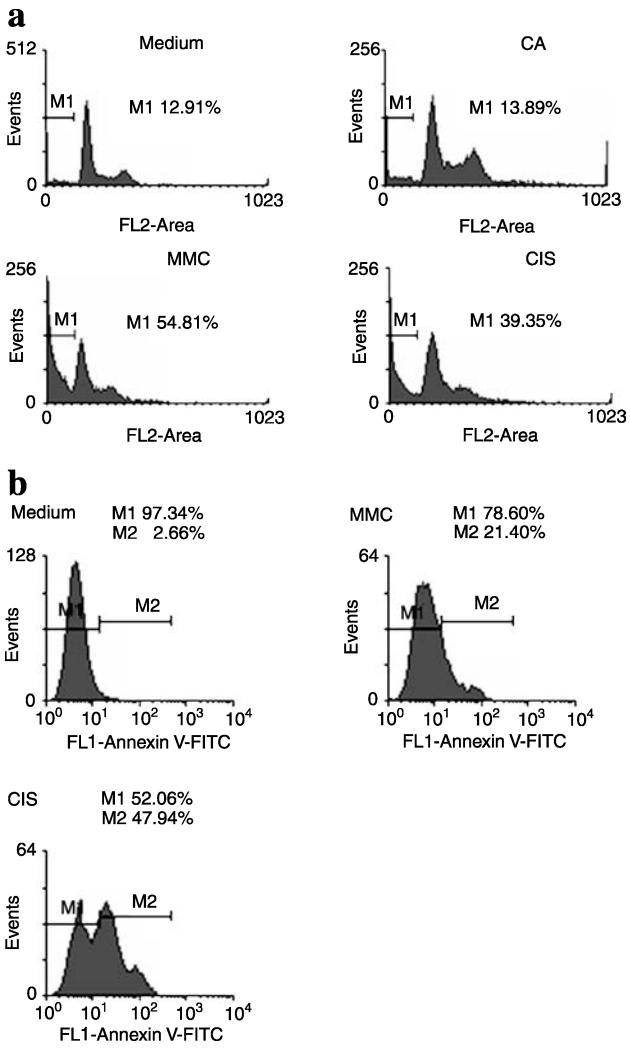

Fig. 2. The apoptotic effects of MMC and CIS on C-33A cervical cancer cells. **a** Profiling of DNA content using a FACS analysis. M1 indicates the percentage of a sub-G_1/G_0 peak of DNA content. **b** Annexin V staining at 24 h after drug treatment in the C-33A cervical carcinoma cell line. Cells were incubated with Annexin V-FITC in a buffer containing propidium iodide (PI), and were analyzed by a flow cytometry as described in Materials and methods. Medium-treated C-33A cells were primarily Annexin V-FITC and PI negative. There were two primary populations of cells observed at 24 h after drug treatment. Annexin V and PI-negative cells were viable and non-apoptotic (*M1*), whereas Annexin V-positive and PI-negative cells were apoptotic (*M2*)

that the cytotoxic activities of MMC and CIS were mainly due to the induction of apoptotic machinery in the C-33A cells.

MMC and CIS regulated expression of p65 and IL-18 proteins in C-33A cells

We elucidated whether anti-cancer agents would modulate the expression of IL-18, which is well-known for its

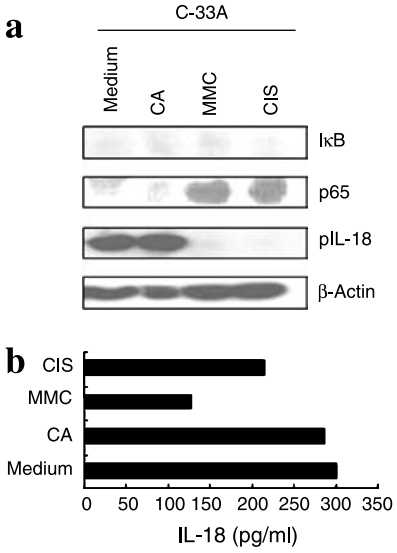

Fig. 3. The inhibitory effects of MMC and CIS on the expressions of IκB and IL-18 in the C-33A cervical cancer cell line. **a** Western blot analysis of IκB, p65, and IL-18. The cell lysates were subjected to 12% SDS-PAGE and electro-transferred onto PVDF membrane. They were then incubated with specific antibodies to IκB, p65, IL-18, and β-actin. **b** Detection of secreted IL-18 with an IL-18 ELISA kit in drug-treated cervical cancer cell lines. Cells were treated with 10 μg/ml of CA, MMC, or CIS for 24 h. The supernatants were then used for detecting IL-18 levels with an ELISA kit, according to the manufacturer's instructions. The optical density (OD) of each well was then measured at 450 nm using an ELISA reader. The concentration of human IL-18 was calibrated by a standard curve. The data represent one of two independent experiments

biological activities, notably immunomodulation and anti-cancer effects. The levels of IL-18 precursor in the C-33A cervical carcinoma cell line were decreased after treatments with MMC or CIS (Fig. 3a), and the secreted IL-18 levels were also found to be down-regulated by MMC and CIS (Fig. 3b). After MMC and CIS treatments, the secreted levels of IL-18 from the C-33A cervical carcinoma cell line were 127 and 214 pg/ml, respectively, whereas the amount of IL-18 from untreated control cells was 300 pg/ml. These results indicated that the levels of IL-18 protein were down-regulated by treatment with MMC or CIS.

Using immunoblotting, we then evaluated the expression levels of IκB and p65, which are members of the NF-κB subfamily. Decreased IκB and up-regulated p65 expression levels (Fig. 3a) suggested that NF-κB would be activated in both MMC- and CIS-treated C-33A cells. These results indicated that both MMC and CIS were able to induce C-33A cell death via NF-κB activation. Furthermore, our preliminary results suggested that MMC and CIS induced apoptosis in C-33A cells by down-regulating IL-18 via the IκB-dependent NF-κB pathway.

Apoptosis was dependent upon Fas/FasL activation in both MMC- and CIS-treated C-33A cells

It has been proposed that the Fas (CD95) signaling pathway is involved in chemotherapy-induced apoptosis (Muller et al., 1997; Fulda et al., 1997; Friesen et al., 1996). In the previous study (Kang et al., 2005), we confirmed that MMC treatment induced apoptosis through up-regulation of FasL expression in two HPV 16-positive cervical carcinoma cell lines. In further experiments, we determined that Fas and FasL expressions in HPV negative cervical carcinoma C-33A cells would be increased by both MMC and CIS treatment via NF-κB activation. FACS and RT-PCR analyses revealed that the expressions of Fas and FasL on the cell surfaces were higher in both MMC- and CIS-treated C-33A cells than in the control cells (Fig. 4a and b). These results suggested that the activation of Fas/FasL would induce cell death in a Fas/FasL-dependent pathway. We then detected FADD, Fas-associating with the death domain, by using the membrane that was used in Fig. 3b, stripped with a stripping buffer and reprobed with a specific antibody. According to the results obtained with Western blot analysis, FADD level also increased in C-33A cervical carcinoma cell line after treatment with either MMC or CIS (Fig. 4c). In order to confirm whether MMC would induce apoptosis by inducing FasL expression in C-33A cells, we treated the MMC-treated C-33A cells with a neutralizing anti-human FasL. As expected, the MMC-induced apoptosis was inhibited by treatment with a neutralizing anti-human FasL antibody (Fig. 5). Thus, these effects of the neutralization of FasL activity on Fas-mediated killing suggested that MMC-induced apoptosis in C-33A cells might be initiated via the Fas/FasL-dependent pathway.

MMC and CIS induced the cleavage of caspase-8 and caspase-3

The initiator caspases appear to display some degree of specificity according to the type of apoptotic signal (Sun et al., 1999; Earnshaw et al., 1999; Budihardjo et al., 1999). Fas receptor-ligand interactions utilize caspase-8 activation to trigger downstream executioner caspases. We tested the characteristics of both caspase-8 and -3 mediated cleavages by Western blot analyses. Both cleaved

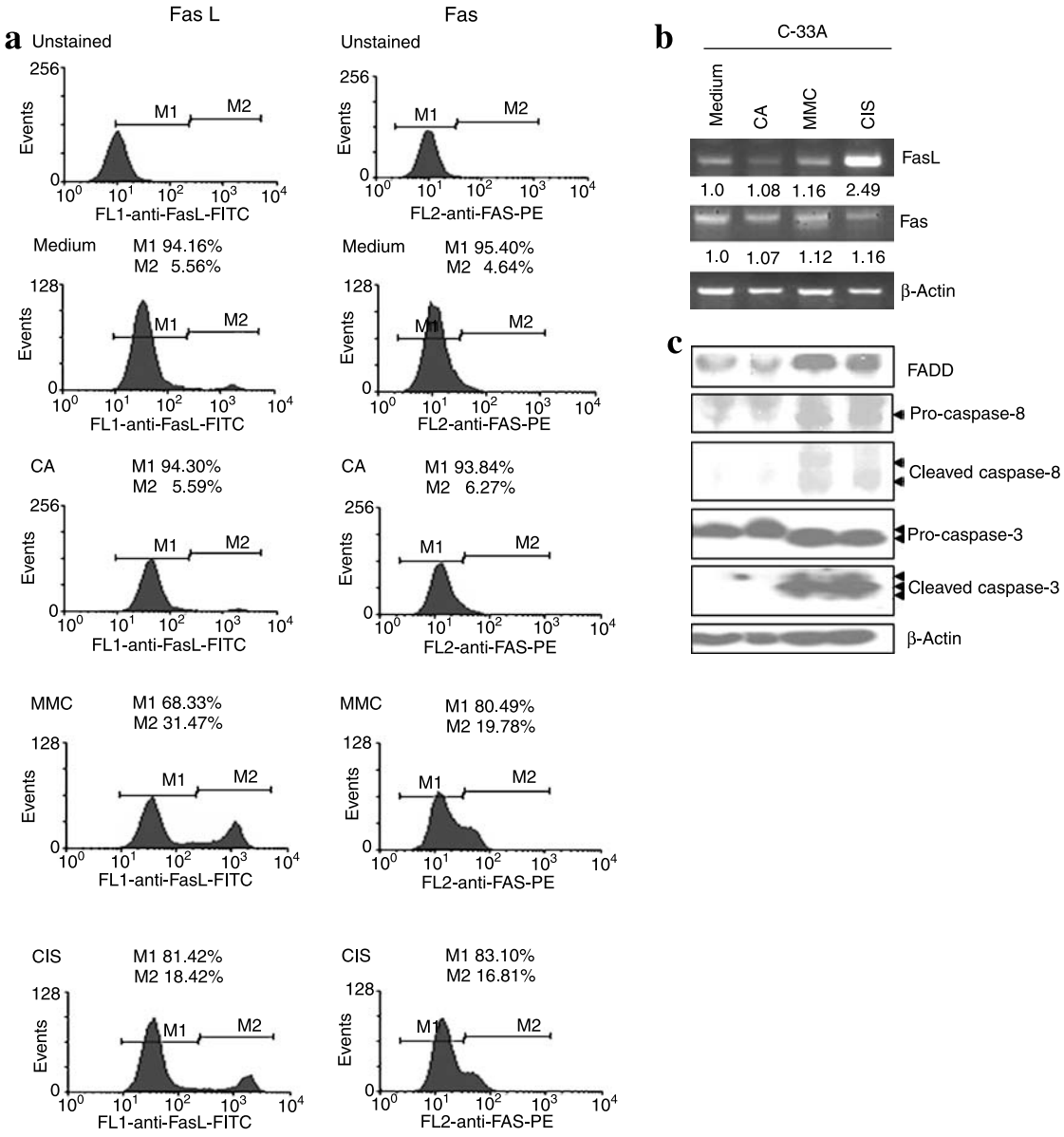

Fig. 4. The effects of MMC and CIS on the expressions of Fas/FasL in the C-33A cervical cancer cell line. **a** The effects of anti-cancer agents on Fas/FasL expression in C-33A cells. Fas/FasL expression was evaluated by a flow cytometry in C-33A cells, as described in Materials and methods. Results are representative of two independent experiments. **b** RT-PCR analysis of Fas and FasL. mRNAs were isolated from the C-33A cervical cancer cell line. RT-PCR was performed as described in Materials and methods. Numbers indicate relative band intensity relative to medium-treated C-33A cells after normalization to β-actin levels. **c** Western blot analysis of FADD, caspase-8, and caspase-3. The cell lysates were subjected to 12% SDS-PAGE and transferred onto PVDF membrane. They were then incubated with specific antibodies to FADD, caspase-8, caspase-3, and β-actin

caspase-8 (43/41 and 18 kDa) and cleaved caspase-3 (20, 18, and 16 kDa) were detected in the cells treated with MMC or CIS (Fig. 4c). In order to confirm these specific caspase activities, the C-33A cells were pre-incubated with caspase-8 inhibitor, capase-3 inhibitor, or negative control inhibitor, and treated with MMC. Treatment with either caspase-8 or caspase-3 inhibitor reduced the levels of apoptosis in the untreated controls (Fig. 5). These results suggested that treatment with MMC or CIS induced apoptosis via the processing of caspase-8 and -3 in a Fas/FasL-dependent manner.

Discussion

DNA damaging agents are now known to be an integral component of the treatment of many solid and hematological malignancies. Furthermore, it has been well known that DNA damage is a classic inducer of p53 function,

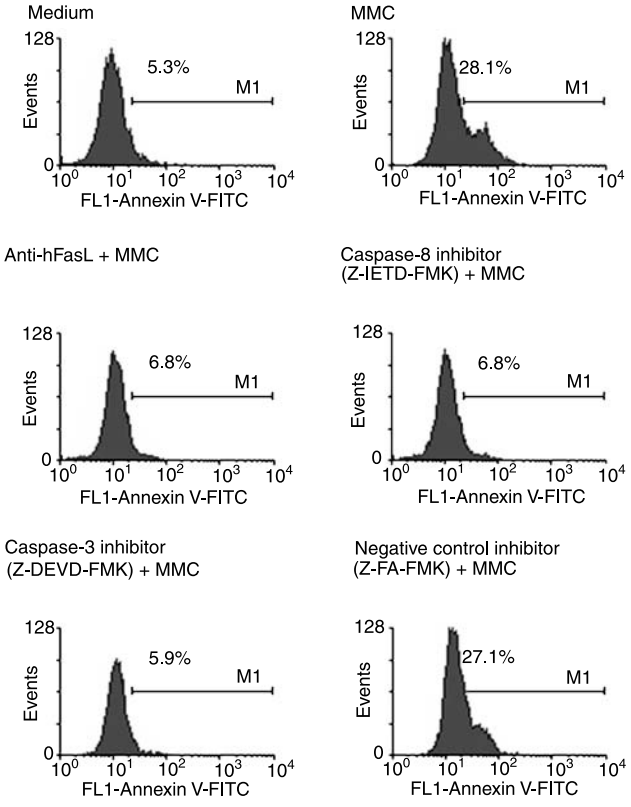

Fig. 5. The effect of FasL-neutralizing antibody and caspase inhibitors on apoptosis in C-33A cells treated with MMC. Cells were pretreated with a FasL-neutralizing antibody, the Z-IETD-FMK caspase-8 inhibitor, the Z-DEVD-FMK caspase-3 inhibitor, or the Z-FA-FMK negative control inhibitor for 30 min, and were then exposed to MMC for 24 h. The harvested cells were stained with Annexin V-FITC, followed by cytometric analysis described in Materials and methods. The percentages of cells (*M1*) represent Annexin V-positive and PI-negative cells

which orchestrates apoptosis or DNA repair, depending on the cellular background. However, it suggests that other p53-independent pathways of apoptosis induction are operational in the action of DNA damaging anti-cancer drugs (Ferrera et al., 1999; Borner et al., 1995; Strasser et al., 1994). In a recent report by other researchers (Hougardy et al., 2005), it was found that IFN-γ and cisplatin were able to increase sensitivity to anti-Fas in a subset of HPV-positive cervical cancer cell lines by up-regulation of Fas and caspase-8 expression without major changes in p53 levels. In our previous studies (Kang et al., 2003, 2005), p53 levels in both SiHa and SiHa/pRSV-luc cells remained stable after MMC treatment. Taken together these results, we suggested that both MMC- and CIS-induced apoptosis relied on p53-independent pathways in HPV-negative and p53-mutated C-33A cells. In general, apoptosis is characterized by a series of characteristic morphological features including chromatin condensation, nuclear fragmentation, and the appearance of membrane-enclosed apoptotic bodies. These morphological changes are executed by a family of aspartate-specific cysteine proteases (caspases), which can be activated by various apoptogenic signals. In the classical model (Thornberry and Lazebnik, 1998; Strasser et al., 1995), initiator caspases (such as caspase-8, -9) and executioner caspases (caspase-3, -6, -7) are differentiated according to their functions and activation sequences. The initiator caspases appear to display some degree of specificity according to the type of apoptotic signal transduced. Two main activation cascades have been implicated in the induction of apoptosis (Sun et al., 1999; Earnshaw et al., 1999; Budihardjo et al., 1999). Fas receptor-ligand interactions utilize caspase-8 activation to trigger downstream executioner caspases. Drug-induced apoptosis can be prevented by Fas neutralizing antibodies, and dysfunctions in the Fas pathway have been shown to correlate with a lack of sensitivity to anti-cancer agents in some cell lines. However, some studies have been unable to corroborate these findings (Newton and Strasser, 2000; Wesselborg et al., 1999), and the relevance of the Fas pathway with regard to chemotherapy-induced apoptosis remains controversial (Fulda et al., 1997; Villunger et al., 1997). An alternative mitochondrial pathway, which is triggered by various anti-cancer agents, involves the activation of caspase-9 upon recruitment to the mitochondria by cytochrome c, and also involves apoptosis protease activation factor-1 (APAF-1). The initiator caspases activate executioner caspases-3, -6, and -7, which then cleave specific proteins, finally resulting in the characteristic signs of apoptosis. Caspase-3 appears to play a central role in chemotherapy-induced apoptosis. It is specifically required for DNA fragmentation, which culminates in the typical apoptotic pattern of DNA laddering (Enari et al., 1998; Liu et al., 1998). Caspase-3 is also an active factor in other typical morphological phenomena associated with apoptotic cell death. In certain systems, including UV irradiation and osmotic shock to embryonic stem cells, or chemotherapy of mouse embryonic fibroblasts, caspase-3 activity clearly seems to be required for apoptosis to occur. Additionally, caspase-3 has been shown to be involved in the activation of the other effector capases, caspase-6 and -7. This finding was consistent with the results of previous experiments with caspase-3 reconstitution in caspase-3-deficient MCF-7 cells (Yang et al., 2001). In a recent report, MMC was observed to induce apoptosis and caspase-8 and -9 processing via caspase-3 and Fas-independent pathways (Pirnia et al., 2002). Our previous results (Kang et al., 2005), however, revealed that MMC induced apoptosis via the Fas/FasL-dependent pathway

and caspase-8 and -3 processing. Because of these results, we tested another cervical carcinoma cell line, C-33A, which is HPV-negative and p53 mutated, and observed the same result that MMC or CIS induced apoptosis via the Fas/FasL-dependent pathway (Figs. 4 and 5). IL-18 has also been reported to enhance FasL expression and to induce apoptosis in Fas-expressing human myelomonocytic KG-1 cells (Chandrasekar et al., 2004; Ohtsuki et al., 1997). In addition, IL-18 activated both intrinsic and extrinsic pro-apoptotic signaling pathways, thereby inducing endothelial cell death. Furthermore, in recent reports, IL-18 was found to induce apoptosis via a Fas/FasL-dependent pathway. However, Western blotting and IL-18 ELISA revealed the down-regulation of IL-18 in both MMC- and CIS-treated cells (Fig. 3a, b). Based on our previous and present results, we propose that the reduction of IL-18 levels via NF-kB activation might play critical roles in cervical carcinoma cell death (Fig. 6). Further study should be focused on determining the exact mechanisms that underlie the apoptosis induced by IL-18 down-regulation in both MMC- and CIS-treated cervical carcinoma cells.

In summary, cell cycle analysis revealed that treatment of C-33A cells with MMC or CIS resulted in apoptosis, whereas treatment of those with CA did not. In addition, MMC and CIS treatments induced reductions in the expressions of IL-18 in a p53-independent manner and activated NF-κB expression. Both MMC and CIS also increased the expression of Fas/FasL and induced the processing of caspases-8 and -3. Thus, we suggest that both MMC- and CIS-induced apoptosis in C-33A cells might involve NF-κB-mediated Fas/FasL pathways and IL-18 down-regulation.

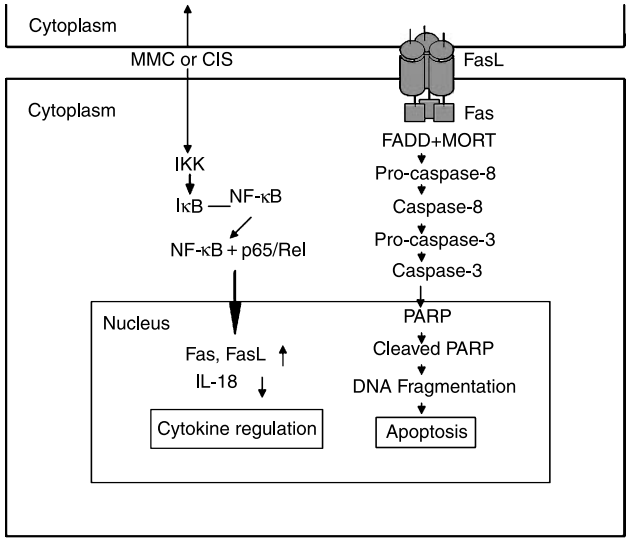

Fig. 6. Proposed mechanism for MMC- and CIS-induced apoptosis in HPV-negative cervical carcinoma C-33A cells. Both MMC- and CIS-induced apoptosis in C-33A cells progressed via the modulation of both NF-κB and IL-18 expression, and the Fas/FasL pathway

Acknowledgments

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