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Adenovirus-mediated transfection of caspase-8 sensitizes hepatocellular carcinoma to TRAIL- and chemotherapeutic agent-induced cell death

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Abstract

Caspase-8 belongs to the cysteine protease family and is known to be activated at the initial step in the cascade of TRAIL-induced apoptosis. The activation of procaspase-8 can be blocked by a relatively large amount of c-FLIP, which renders resistance to death receptor-mediated apoptosis in many types of cancer cells. To ask if extrinsic over-expression of caspase-8 contributes to the induction of apoptosis, we introduced the caspase-8 gene into HCC cells using an adenoviral (Adv) vector (Adv-Casp8). We demonstrated that Adv-Casp8 increased expression of active forms of caspase-8 in MOI-dependent manner. A large amount of Adv-Casp8 (MOI of 50) induced apoptosis significantly in HCC cells and resulted in downregulation of c-FLIP (in SK-Hep1, HLE, and HepG2 cells), XIAP, survivin, and Bcl-xL (in HLE cells) and dynamic release of cytochrome *c* and Smac from the mitochondria into the cytosol. On the other hand, a small amount of Adv-Casp8 (MOI of 10) causes a slight but detectable increase in the level of apoptosis with only a small effect on anti-apoptotic proteins and mitochondrial activation. However, small amounts of Adv-Casp8 augmented TRAIL- or chemotherapeutic agent-induced cell death (with an MOI of 10 or 20, respectively). These results suggest both that exogenous over-expression of caspase-8 by Adv-Casp8 may be essential for induction of HCC cell death and that the combination of Adv-Casp8 and TRAIL or chemotherapeutic agents could provide a useful strategy for treatment of HCC.

1. Introduction

The process of programmed cell death, apoptosis, is fundamental in developmental and homeostatic maintenance of complex biological systems [1–4]. Mis-regulation or failure of normal apoptotic mechanisms can contribute to transformation of cells and provide a growth advantage to cancer cells [5].

Apoptosis can be triggered by the engagement of any one of several cell surface death receptors, each of which expresses extracellular cysteine-rich pseudo-repeats and contains homologous intracellular carboxyl-terminal death domains. Moreover, these cell surface receptors are members of the TNF-receptor (TNF-R) family and include Fas, TNF-receptor 1(TNF-R1); TNF-related apoptosis-induced ligand (TRAIL)-receptor 1(R1), TRAIL-R2, and TRAMP [4,6,7]. Stimulation of Fas, TRAIL-R1, or TRAIL -R2 results in clustering of the receptor, which in turn leads to recruitment of an adaptor molecule and the Fas-associated death domain (FADD) protein. Recruitment of these factors causes the receptor proximal to caspase-8 to become activated upon recruitment to the death-inducing signaling complex (DISC) via homophilic DED-DED interactions. Caspase-8 belongs to the cysteine protease family of proteins, whose members are activators of executioner caspases, and is activated at the initial step in the cascade of Fas- or TRAIL-induced apoptosis [6,8]. Activated caspase-8 then initiates the apoptosis executing caspase cascade [9].

Abbreviations: TRAIL, TNF-related apoptosis-induced ligand; TRAIL-R1, TRAIL-receptor 1; TRAIL-R2, TRAIL-receptor 2; DISC, death-inducing signaling complex; HCC, hepatocellular carcinoma; XIAP, X-linked inhibitor of apoptosis proteins; c-FLIP, cellular FLICE/caspase-8-inhibitory protein; NF-κB, nuclear factor-κB; AxCALNL-hCaspase-8, Adv-Casp8, Recombinant adenovirus expressing human caspase-8; AxCANCre, Adv-Cre, Recombinant adenovirus expressing Cre recombinase; MOI, multiplicity of infection; CPT, camptothecin; DOX, doxorubicin; CDDP, Cisplatin; 5-FU, 5-Fluorouracil; COX IV, Cytochrome oxidase subunit IV; IAPs, the inhibitor of apoptotic proteins; Smac, second mitochondrial-derived activator of caspase; DIABLO, the direct IAP binding protein with low pI

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Most hepatocellular carcinoma (HCC) cells show strong resistance to stimuli that induce apoptosis. Since this apoptotic sensitivity is closely related to resistance to conventional anticancer agents and radiotherapy, providing cells with a way to overcome apoptosis resistance may be critical for establishment of effective new strategies for treatment of HCC. Several cellular anti-apoptotic mechanisms exist in cells, including reduced expression of Fas [10,11] or caspases [12]; expression of anti-apoptotic proteins, Bcl-2 family proteins [13,14], and proteins in the various inhibitor of apoptosis families, such as survivin and X-linked inhibitor of apoptosis proteins (XIAP) [15,16]; or receptor-mediated survival signals [17,18]. These mechanisms contribute to resistance against immunologic cytotoxicity in human HCC cells.

Among caspase inhibitors, it may be cellular FLICE/caspase-8-inhibitory protein (c-FLIP) that is crucial for modulation of the cell death signal by inhibition of procaspase-8 processing at DISC. A recent study demonstrated that small amounts of c-FLIP allow for processing of procaspase-8, leading to the formation of the active caspase-8 heterotetramer, which is composed of the p18 and p10 subunits, but that the presence of large amounts of c-FLIP blocks cleavage of procaspase-8 [19]. In a previous study, we have demonstrated that c-FLIP is over-expressed in human HCC tissues and HCC cell lines and plays an important role in cell survival not simply by inhibiting death receptor-mediated apoptosis but also by regulating NF-κB activation [20].

Taken together, the results suggest that DISC formation by caspase-8 and c-FLIP is a critically important anti-apoptotic mechanism and thus, that is an ideal therapeutic gene target. In support of this idea, a recent study revealed that the relative levels of caspase-8 and c-FLIP are an important determinant of susceptibility to Fas-mediated apoptosis in malignant cells [19,21]. Although it is not clear what level or levels of c-FLIP function can increase apoptosis sensitivity, we tested transfer of the caspase-8 gene into HCC cells using adenovirus vector in an attempt to alter the c-FLIP, caspase 8 ratio and ask if apoptosis is affected. To do this, the expression level of caspase-8 must be lower than levels that results in self-oligomerization of caspase-8 precursor proteins in order to prevent a high level of non-specific apoptosis in non-transformed cells.

Previous studies demonstrated that TRAIL and chemotherapeutic drugs-induced cytotoxicity is closely related with apoptotic pathway. However, there is little report concerning the increased sensitivity of TRAIL and a panel of chemotherapeutic agent using caspase overexpression. Therefore, to explore the possibility that caspase-8 gene transfer could be useful in a gene therapy approach to the treatment of HCC cells, we asked if adv-mediated caspase-8 gene transfer could selectively affect cell growth and survival and augment TRAIL- or chemotherapeutic agent-induced apoptosis in HCC cells.

2. Materials and methods

2.1. Cell culture

The human HCC cell lines SK-Hep1 and HepG2 were purchased from the American Type Culture Collection (Rockville, MD). The HLE cell line (JCRB 0404) was purchased from the Health Science Research Resource Bank (Osaka, Japan). All HCC cells were cultured in Dulbecco's modified Eagle medium (Dainippon Pharmaceutical Co. Ltd., Osaka, Japan) supplemented with 1% penicillin/streptomycin (GIBCO BRL, Grand Island, NY) and 10% heat-inactivated fetal calf serum (GIBCO BRL) in 5% CO_2 at 37 °C.

2.2. Recombinant adenoviral vectors

Recombinant adenovirus expressing human caspase-8 (AxCALNL-hCaspase-8, referred to here as Adv-Casp8) was kindly provided by Dr. Hirofumi Hamada (Sapporo Medical University, Hokkaido, Japan). This recombinant adenovirus vector utilized the Cre/loxP system [22] in order to prevent damage to 293 virus producer cells induced by pro-apoptotic gene products such as caspase-8. In the vector, the human caspase-8 precursor can be expressed via the CA promoter but is silenced by a polyadenylate sequence flanked by a pair of loxP

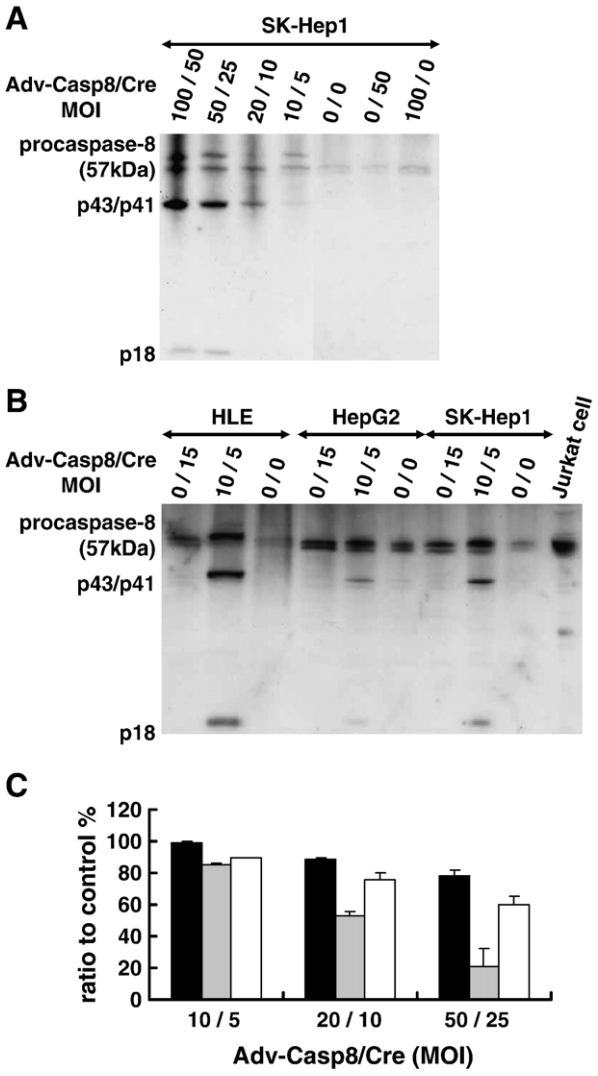

Fig. 1. Adv-mediated induction of caspase-8 in HCC cells. (A) Dose-dependent expression of caspase-8 by Western blot analysis in human HCC cell lines SK-Hep1 24 h after infection with Adv-Casp8 or Adv-Cre (control) at the indicate MOI. Co-infection ratio of Adv-Casp8 and Adv-Cre was 2:1. (B) Expression of caspase-8 in three cell lines, SK-Hep1, HLE, and HepG2. Cells were infected with Adv-Casp8 and Adv-Cre at MOI 10/5. For control experiments, cells were incubated with Adv-Cre alone at the same total MOI. Lysates were harvested at 48 h post-infection. Jurkat cell lysates were used as a positive control. (C) Effects of Adv-Casp8 expression on HCC cell viability. (SK-Hep1; closed columns, HLE; gray columns, and HepG2; open columns). Cells were infected with Adv-Casp8 or Adv-Cre (control) at the indicated MOIs for 48 h. Cell viability was assessed by MTT. The data are reported as a percent viability versus the control and represent the means±S.D. of six independent experiments.

sites. In the presence of Cre recombinase, loxP sites are excised and the CA promoter becomes juxtaposed to the caspase-8 gene, resulting in constitutive activation of the gene. Recombinant adenovirus expressing Cre recombinase (AxCANCre, referred to here as Adv-Cre) was purchased from Takara Shuzo Co., LTD. (Shiga, Japan). The viruses were propagated and purified, and viral titers were determined in 293 cells with the Adeno-X Rapid Titer Kit (BD Biosciences Clontech). Purified recombinant virus were kept at −80 °C until use.

Cells were seeded on the appropriate plates or dishes at a concentration of 2×10^5/ml 1 day prior to infection. Before infection, the culture medium was discarded and cells were washed in culture medium without antibiotics or fetal calf serum. For infection, the diluted viral solution was added to the target cells and after incubation for 1 h at 37 °C, growth medium was added and cells were cultured for 48 h. Adv-mediated caspase-8 gene transfection was performed by co-infection of Adv-Casp8 and Adv-Cre at a MOI of 2:1. In control experiments, cells were incubated with Adv-Cre alone at the same total MOI.

2.3. Assessment of viability of HCC cells

The HCC cells were plated at a density of 1×10^4 cell/well in 96-well microtiter plates (Corning Glass Works, Corning, NY) and each plate was incubated for 24 h, followed by addition of the diluted viral solution. After 24 h, the plates were incubated for 24 h with various concentrations of the following: recombinant human TRAIL (R&D Systems, Inc., Minneapolis, MN); (*S*)-(+)-camptothecin (camptothecin, Sigma); doxorubicin hydrochloride (doxorubicin, Sigma); cis diamminedichloroplatinum (Cisplatin, Sigma); Paclitaxel (Taxol, Sigma); or 5-Fluorouracil (5-FU; Sigma). The live-cell count was assayed by the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay using a Cell Titer 96 assay kit (Promega, Madison, WI) according to the manufacturer's instructions. The absorbance of each well was measured at 570/633 nm with a microtiter plate reader (Bio-Rad Laboratories, Hercules, CA).

2.4. Immunoblotting and antibodies

Expression of caspase-8, caspase-3, FLIP, XIAP, survivin, Bcl-xL, Smac, and cytochrome *c* was analyzed by immunoblotting. Cells were harvested an appropriate number of hours after transfection and lysed on ice in lysis buffer (50 mmol/L Tris–HCl pH 8, 150 mmol/L NaCl, 5 mmol/L ethylenediaminetetraacetic acid, 1% NP-40, 1 mmol/L phenylmethylsulfonyl fluoride). Total protein levels were measured using a Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein from each extract were separated by 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto PVDF membranes (Millipore Corporation, MA). Blots were blocked by incubation in 5% milk with Tris–HCl (pH 7.5) and 0.1% Tween 20 for 2 h at room temperature and probed overnight at 4 °C with primary antibodies. The following primary antibodies were used: mouse anti-human caspase-8 monoclonal (Cell Signaling Technology, Beverly, MA), mouse anti-caspase-3/CPP32 monoclonal, mouse anti-hILP/XIAP monoclonal, rabbit anti-Bcl-xL polyclonal (Transduction Laboratories, Lexington, KY), rabbit anti-c-FLIP polyclonal (Medical and Biological Laboratories Co., LDT, Nagoya, Japan), mouse anti-survivin monoclonal, goat anti-Smac polyclonal (Santa Cruz Biotechnology, Inc. Santa Cruz, CA), rabbit anti-cytochrome *c* polyclonal (CLONTCH Laboratories, Inc., CA), mouse anti-COX IV monoclonal (Abcam Ltd., Cambridge, UK), and mouse anti-α-Tubulin monoclonal (Oncogene Research Products, San Diego, CA). Antibodies were diluted with 5% milk in Tris–HCl (pH 7.5) and 0.1% Tween 20. The immunoblots were then probed with horseradish peroxidase-conjugated anti-mouse immunoglobulin G (IgG), horseradish peroxidase-conjugated anti-rabbit IgG (diluted 1:1000 with 1% milk in Tris–HCl pH 7.5) (Amersham Biosciences, Buckinghamshire, UK) or horseradish peroxidase-conjugated anti-goat IgG. (1:2000 dilution) (Zymed Laboratories, South San Francisco, CA). After the final wash, the signal was detected with an ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

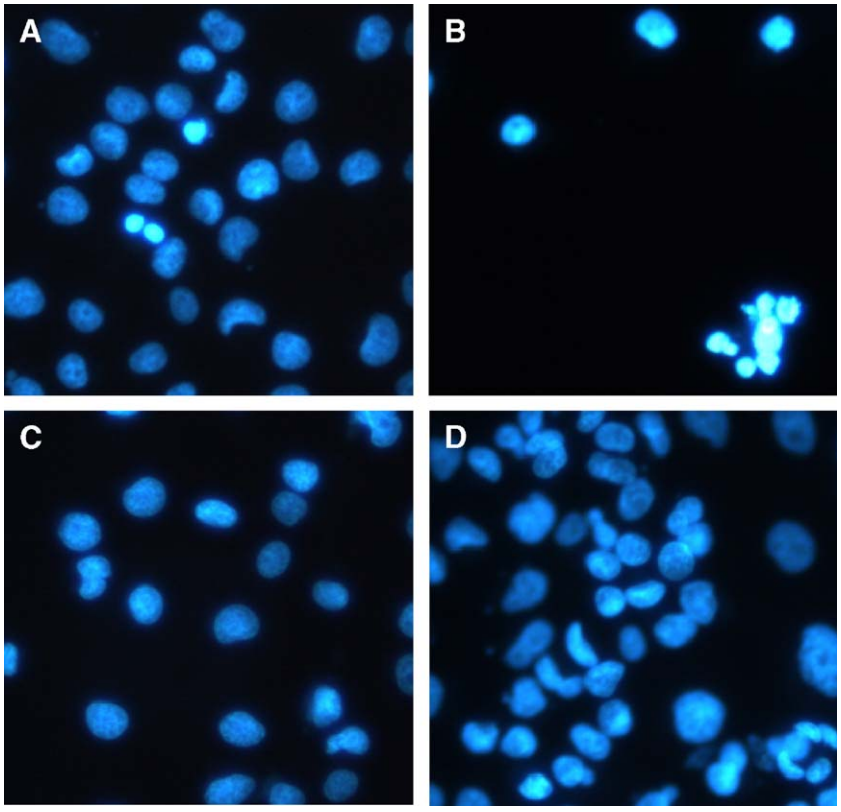

Fig. 2. Adv-Casp8-induced apoptosis in HLE cells. Cells were infected by either (A) Adv-Casp8/Cre at MOI of 10/5, (B) Adv-Casp8/Cre at MOI of 50/25, (C) Adv-Cre at MOI of 15 (control for A), or (D) Adv-Cre at MOI of 75 (control for B) for 48 h. Cell nuclei were visualized with DAPI. We observed five fields, respectively, and demonstrated the representative figure (×200). Note the typical apoptotic features, nuclear condensation and nuclear fragmentation.

2.5. Cellular fractionation

Two 10 cm culture dishes per sample were collected and washed twice in ice-cold Wash Buffer (CLONTECH Laboratories, Inc.). Whole-cell pellets were resuspended in ice-cold Fractionation Buffer Mix (0.2% Protease Inhibitor Cocktail, 0.1% DTT and Fractionation Buffer; CLONTECH Laboratories, Inc.) and subjected to 40 strokes in an ice cold Dounce tissue grinder. The homogenates were subjected to centrifugation at 700×*g* for 10 min at 4 °C. The supernatant was subsequently subjected to centrifugation at 10,000×*g* for 25 min at 4 °C to obtain a cytosolic fraction (supernatant) and a mitochondrial fraction (pellet).The pellets were suspended in Fractionation Buffer Mix. Cytochrome oxidase subunit IV (COX IV) and α-Tubulin were used as controls for the mitochondrial and cytosolic fractionations, respectively.

2.6. Detection of apoptosis

A total of 1×10^5 HLE cells were cultured in 35 mm culture dishes for 24 h, followed by addition of the diluted viral solution. After incubation for 48 h, cell nuclei were stained with 4'6,-diamidino-2-phenylindole (DAPI; Sigma, St. Louis, MO, USA) and observed under a fluorescence microscope (Zeiss, Göttingen, Germany).

3. Results

3.1. Adv-mediated caspase-8 increased expression of procaspase-8 and cleaved caspase-8 in HCC cells

We first determined the expression levels of caspase-8 from the adenovirus vector in SK-Hep1 cells. Infection of SK-Hep1 cells with AxCALNL-hCaspase-8 (Adv-Casp8) and AxCACre (Adv-Cre) at MOIs ranging from 10 to 100 for 24 h induced increased intracellular expression levels of procaspase-8 and cleaved caspase-8 in a MOI-dependent manner, whereas Adv-Casp8 or Adv-Cre alone could not (Fig. 1A). These results agreed with the report that over-expression of procaspase-8 leads to self-activation of caspase-8 [23]. We next investigated expression of caspase-8 in three HCC cell lines (SK-Hep1, HLE, and HepG2) 48 h after infection with the adenovirus vectors. An increase in the caspase-8 expression level was visible at MOI of 10 in three HCC cell lines (Fig. 1B).

3.2. Adv-Casp8 induced apoptosis in HCC cells

The effect of Adv-Casp8 expression on the viability of HCC cells was then assessed by the MTT assay. Infection of HCC cells with Adv-Casp8 at MOI of 10 did not markedly affect cell viability 48 h after infection. However, viability of cells infected with Adv-Casp8 did decrease in a MOI-dependent manner (Fig. 1C). Since we wished to explore whether a sublethal dose of Adv-Casp8 could augment apoptosis elicited by TRAIL or chemotherapeutic agents, we chose the sub-lethal dose of MOI of 10 or 20 for subsequent experiments.

In order to determine if Adv-Casp8 induces apoptosis in HCC cells, we assessed DAPI staining 48 h after infection of either Adv-Casp8 and Adv-Cre or Adv-Cre alone (at the same total MOI) in HLE cells. Apoptosis was not induced in cells infected with Adv-Cre alone. As expected, however, Adv-Casp8-infected cells did show typical apoptotic features, including nuclear condensation and nuclear fragmentation with DAPI staining (Fig. 2).

3.3. Adv-Casp8 augmented TRAIL- induced cell death

It is well known that HCC cell lines showed resistance to TRAIL-induced apoptosis caused by nuclear factor-κB (NF-κB) activation induction of genes whose products provide resistance to apoptosis. In order to assess the effects of Adv-Casp8 on TRAIL induced cell death, 24 h after of infection cells were incubated with TRAIL at various concentrations for 24 h and cell viability was assessed by the MTT assay (Fig. 3). Viability of Adv-Casp8 infected cells treated with TRAIL decreased synergistically or additively and in a dose-dependent manner; the effect was observed for all cell lines.

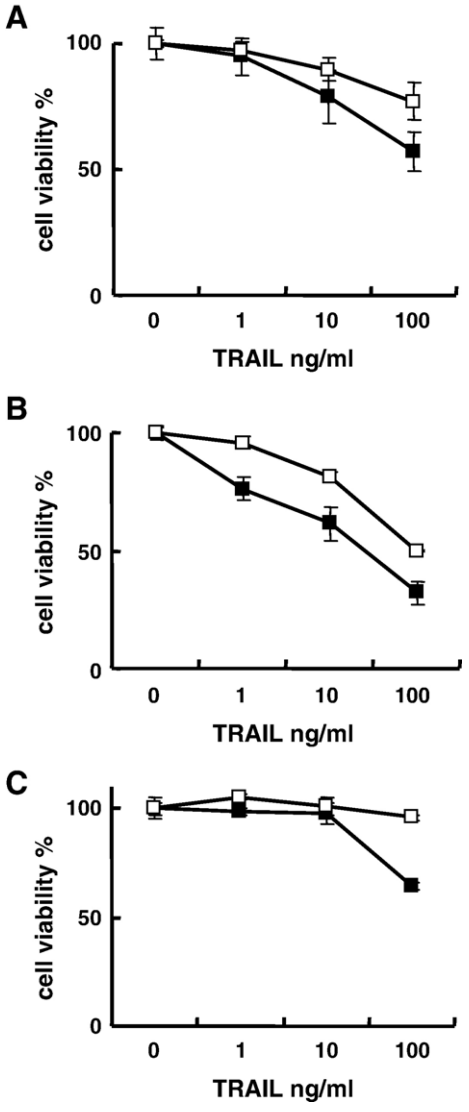

Fig. 3. Effect of Adv-Casp8 on TRAIL-induced apoptosis in HCC cell lines. HCC cells, (A) SK-Hep1, (B) HLE, and (C) HepG2, were infected by either Adv-Casp8/Cre at MOI of 10/5 (■), or Adv-Cre at MOI of 15 as control (□) for 24 h. And they were incubated with various concentrations of TRAIL for 24 h. Cell viability was assessed by MTT. The data are shown as the percent viability as compared to cells without TRAIL treatment, respectively, and represent the mean± S.D. of six independent experiments.

3.4. Adv-Casp8 augmented chemotherapeutic agent-induced cell death

In recent studies, it was shown that suppression of inhibitor of apoptosis proteins such as XIAP or survivin augmented chemotherapeutic agent-induced cell death on HCC cell lines [24]. To examine the effect of Adv-Casp8 on chemotherapeutic agent-induced cell death in HCC cell lines, 24 h after of infection of adenovirus vectors, infected cells were incubated with Cisplatin, Taxol, camptothecin, doxorubicin, or 5-FU for 24 h and cell viability was assessed using the MTT assay (Fig. 4A, B, C). The doses of the chemotherapeutic agents were determined by calculating the 50% growth suppression value (IC_{50}) in preliminary experiments (data not shown). At MOI of 20, Adv-Casp8 resulted in a decrease in cell viability in all cell lines when combined with chemotherapeutic agents, whereas at MOI of 10, Adv-Casp8 resulted in a decrease in cell viability only in the HLE cell line. In addition, we assessed DAPI staining with HLE cells, which were incubated with Cisplatin, Taxol, or 5-FU for 24 h after infection of adenovirus vectors (Fig. 4D). In infected cells with Adv-Casp8 at MOI of 20, we detected many apoptotic cells. And these apoptotic cell numbers were almost in proportion to the cell death ratio estimated by MTT assay.

3.5. Adv-Casp8 decreased expression level of c-FLIP and other Anti-apoptotic proteins

As shown by recent studies, over-expression of caspase-8 by Adv-Casp8 suppressed expression of FLIP but increased expression of FADD and caspase-3 activity in human gastric adenocarcinoma cells [25]. To investigate whether caspase-8 over-expressed using the transgenic construct Adv-Casp8 could alter the expression levels of anti-apoptotic proteins or activity of caspase-3 in HCC cells, immunoblot analyses of c-FLIP, XIAP, survivin, Bcl-xL, and procaspase-3 were performed. Expression of survivin and procaspase-3 decreased in two cells, c-FLIP was suppressed in SK-Hep1 cells, and XIAP was suppressed in HLE cells by Adv-Casp8 at MOI of 10. Moreover, expression of c-FLIP and procaspase-3 decreased in all cell lines at MOI of 50 and XIAP, survivin, Bcl-xL were suppressed by Adv-Casp8 at MOI of 50 in HLE cells (Fig. 5).

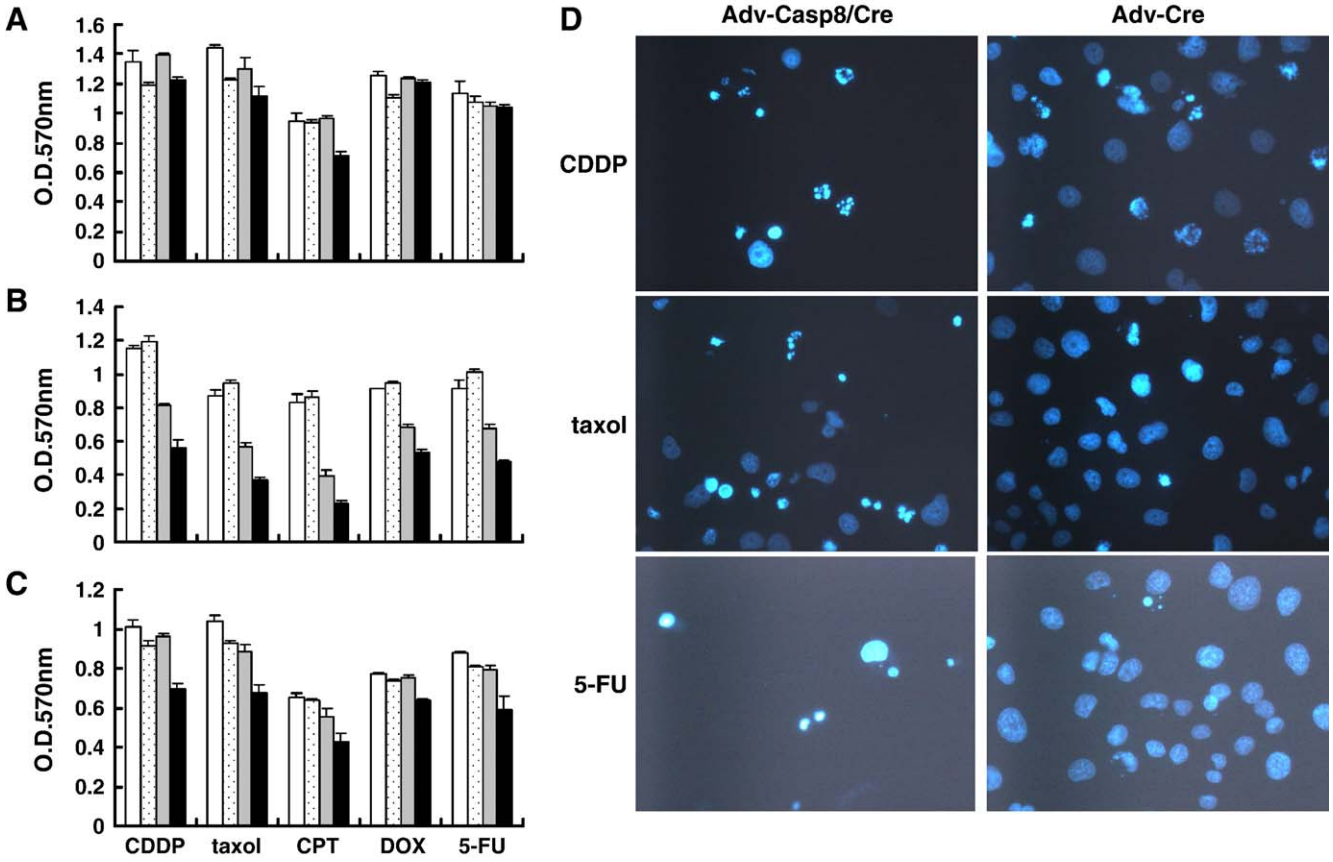

Fig. 4. Effect of Adv-Casp8 on Chemotherapeutic agent- induced cell death in HCC cell lines. HCC cells, (A) SK-Hep1, (B) HLE, and (C) HepG2, were infected by either Adv-Casp8/Cre at MOI of 10/5 (gray columns), Adv-Casp8/Cre at MOI of 20/10 (closed columns), Adv-Cre at MOI of 15 as control (open columns), or Adv-Cre at MOI of 30 as control (dot columns) for 24 h. And they were incubated with chemotherapeutic agents for 24 h at the IC_{50} concentration for (A) Cisplatin 500 μM, Taxol 50 nM, camptothecin 0.7 μg/ml, doxorubicin 0.5 μg/ml, and 5-FU 500 μM, (B) (C) Cisplatin 100 μM, Taxol 100 μM, camptothecin 0.5 μg/ml, doxorubicin 1.0 μg/ml, and 5-FU 80 μM. Cell viability was assessed by MTT. The data shown are the absorbance at 570 nm, which is directly proportional to the number of cells. Means ± S.D. of six independent experiments are shown. (D) HLE cells with DAPI staining after infection of either Adv-Casp8/Cre at MOI of 20/10 or Adv-Cre at MOI of 30 (control) and moreover incubation with Cisplatin 100 μM, Taxol 100 μM, or 5-FU 80 μM for 24 h (×200).

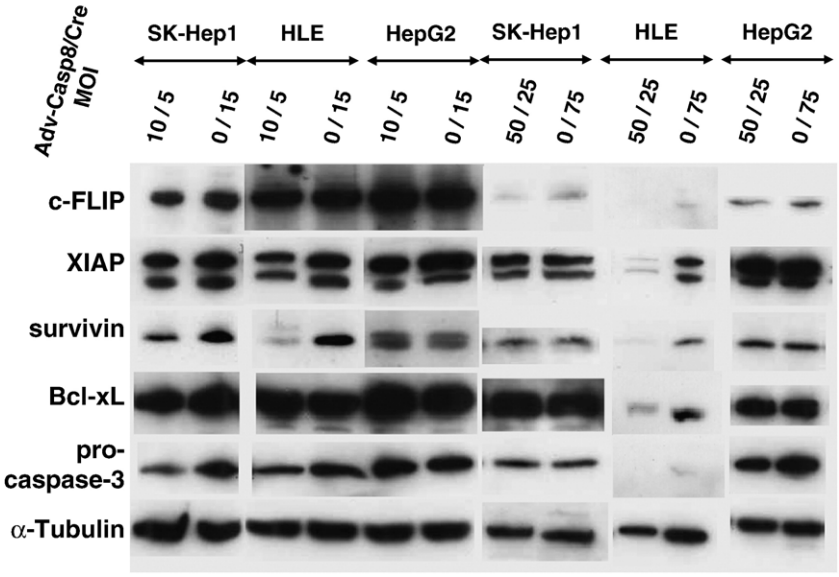

Fig. 5. Change in expression of anti-apoptotic proteins and procaspase-3 by Adv-Casp8 infection in HCC cell lines. Cells were harvested 48 h after infection, and cell lysates were separated by SDS-PAGE and transferred onto PVDF membranes. Protein levels were then detected by immunoblotting using antisera that recognize the indicated proteins.

3.6. Adv-Casp8 activated the mitochondrial apoptosis pathway

Mitochondrial release of Smac/DIABLO and cytochrome *c* into the cytosol has an important role in apoptosis, including TRAIL -induced apoptosis of malignant cells [26,27]. We next asked if over-expression of caspase-8 in HCC cells via Adv-Casp8 promotes release of Smac and cytochrome *c* from mitochondria. To do this, we used immunoblotting to detect the levels of Smac and cytochrome *c* in mitochondrial and cytosolic fractions from Adv-Casp8-infected cells.

In cells infected by Adv-Casp8 at MOI of 50, the levels of mitochondrial Smac and cytochrome *c* were lower than those in control cells and the cytosolic levels of both proteins were higher than those in control(Fig. 6B, C), while definite effect was determined in infected cells by Adv-Casp8 at MOI 10 (Fig. 6A, C). Over-expression of caspase-8 via Adv-Casp8 promoted release of Smac and cytochrome *c* from mitochondria to cytosol; thus, the mitochondrial pathway of apoptosis was activated by Adv-Casp8.

4. Discussion

The c-FLIP protein can modulate activation of procaspase-8 and thereby prevent induction of apoptosis mediated by death receptors. In this way, many cancer cell types are rendered resistant to death receptor-mediated apoptosis. Moreover, c-FLIP exerts other physiological functions related to cell proliferation and tumorigenesis. Therefore, c-FLIP is thought to be a critical target for therapeutic intervention [28]. We have demonstrated that there is a high level of expression of c-FLIP in human HCC tissues and cells. Conversely, c-FLIP is downregulated by chemotherapeutic agents [20] that normally act to augment TRAIL-induced apoptosis [29]. A large ratio of c-FLIP to procaspase-8 blocks cleavage of procaspase-8 [19]. Recent studies demonstrated that deletion or mutation of the caspase-8 gene is present in some cancer cells, including HCC, which presumably results in loss of the caspase-8 apoptotic function and contributes to pathogenesis [30–33]. These data suggest the importance of caspase-8 in hepatocarcinogenesis and that caspase-8 may be effective target for gene therapy. Indeed, efficient upregulation of procaspase-8 by adenovirus-mediated transfer of caspase-8 can induce apoptosis in several transformed cells [25,34–36].

In this study, we demonstrated that adenovirus-mediated transfer of the caspase-8 gene induces a significant level of apoptosis in an MOI-dependent manner. These results were in accordance with a report that showed that over-expression of procaspase-8 leads to self-activation of caspase-8 and subsequent induction of apoptosis [23]. Adenoviral vectors are considered a useful tool in cancer gene therapy since they can be used to efficiently introduce genes into many types of cancer cells. Recently, it was shown that Adv-Casp8-mediated gene transfer efficiently induced apoptosis in glioma cells [34]. However, over-expression of caspase-8 may induce apoptosis in all Adv-Casp8-infected cells rather than selective inhibition of detached cells. It is, therefore, necessary to reduce nonspecific cell death from over-expression of caspase-8 for anticancer therapy. For this purpose, the level of caspase-8 expression from the adenovirus vector must be lower than that which results in self-oligomerization of caspase-8 precursor proteins [25]. Thus, we considered that only relatively low levels of caspase-8 would be useful to augment cell death in HCC cells and accordingly, we used Adv-Casp8 at MOI of 10 or 20 to sensitize cytokine or chemotherapeutic agents in our study.

TRAIL, a member of the TNF family, selectively induces apoptosis in a variety of transformed cell lines. Interestingly, several tumor cell lines and most normal cells are, by contrast, resistant to TRAIL-mediated apoptosis [37]. This selective toxicity is the basis for the current enthusiasm for TRAIL as a potential target of novel anticancer therapeutics [38]. Previous

studies suggested that HCC cells are resistant to TRAIL-mediated apoptosis, despite the fact that they express TRAIL receptors. In the current study, whereas Adv-Casp8 infection at MOI of 10 induced minimal cell death in HCC cells, it augmented TRAIL-induced apoptosis synergistically in HCC cells.

Recently, we demonstrated that high levels of TRAIL-R1/DR4 and -R2/DR5 are expressed in human HCC tissues but that HCC cells are resistant to TRAIL-induced apoptosis. There are several molecular mechanisms that may account for this resistance. The first to consider is that c-FLIP is expressed at high levels in HCCs. C-FLIP blocks the cleavage of procaspase-8 at DISC and activates NF-κB pathways involved in cell proliferation or differentiation. In fact, TRAIL also activates NF-κB, which controls expression of genes whose products provide resistance to apoptosis, including c-FLIP, the inhibitor of apoptotic proteins (IAPs), Bcl-2, and Bcl-xL [39,40]. The IAPs regulate apoptosis by preventing the action of the central execution phase of apoptosis through direct inhibition of the effector caspase-3 and/or caspase-7 [41,42]. We have previously studied the expression and function of XIAP and survivin in human hepatocellular carcinomas (HCCs) [15,16] and demonstrated that downregulation of XIAP or survivin by siRNA sensitized TRAIL-induced apoptosis in HCC cells [24]. Bcl-xL, an anti-apoptotic member of the Bcl-2 family, is located mainly on the outer membrane of mitochondria and inhibits a common pathway of apoptosis by preventing the release of cytochrome *c* into the cytosol [43,44]. Bcl-xL was also over-expressed in HCC tissues and downregulation of Bcl-xL stimulated apoptosis in HCC cells in response to cellular stress [45].

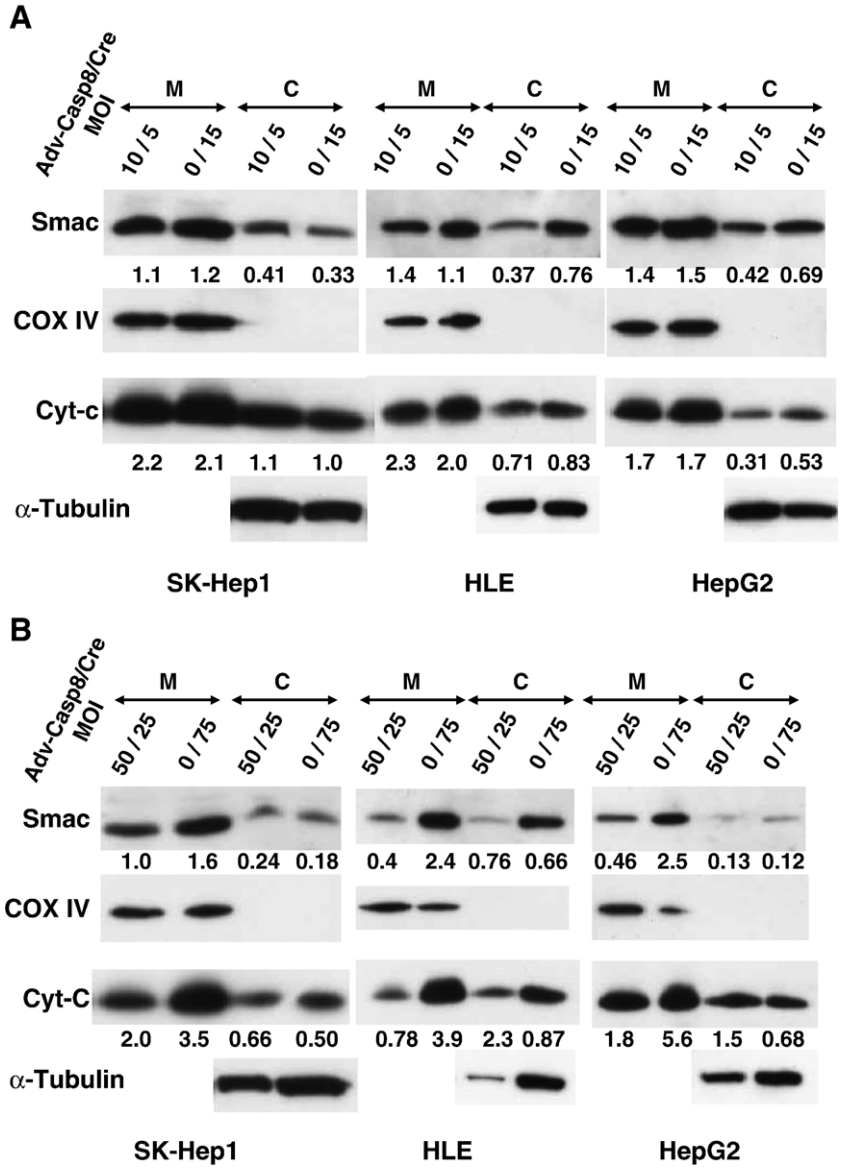

Fig. 6. Mitochondrial changes 48 h after infection of Adv-Casp8. After infection of (A) Adv-Casp8 at MOI of 10 or (B) MOI of 50, mitochondrial (M) and cytosolic (C) fractions were prepared and subjected to immunoblot analysis to detect Smac and cytochrome *c*. COX IV and α-Tubulin were used as controls for detection of the mitochondrial and cytosolic fractions, respectively. Protein signals were quantified by densitometry. Their ratios to either COX IV or α-Tubulin are expressed under the bands. (C) The relative ratios of Adv-Casp-8 to control are shown. (MOI of 10; open columns, MOI of 50; closed columns).

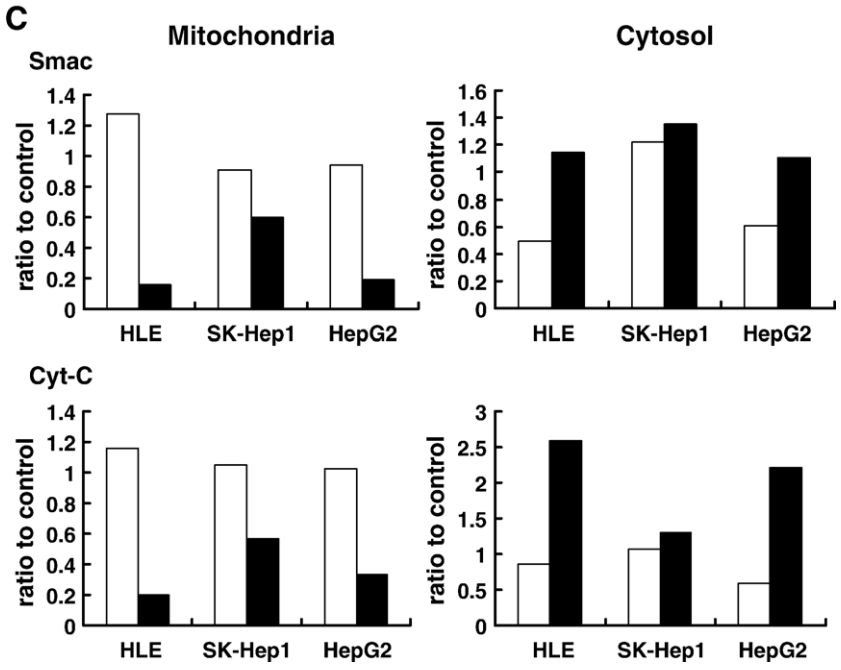

Fig. 6 (*continued*).

Here, we next looked at the molecular mechanism of sensitization of TRAIL by caspase-8 gene transfer. Immunoblot analysis revealed that at MOI of 10, Adv-Casp8 had a little effect on the level of c-FLIP, XIAP, survivin, or Bcl-xL, although it augmented TRAIL-induced apoptosis. These results suggested that a small amount of procaspase-8 over-expression cannot inhibit function of anti-apoptotic proteins. However, slight over-expression did have a significant affect on sensitization to TRAIL, presumably because introduction of the transgene resulted in an increase in the ratio of procaspase-8 to c-FLIP. These results also indicate that TRAIL-induced signaling was converted from acting on the proliferation pathway to acting on the apoptosis pathway and subsequently, TRAIL-induced apoptosis was augmented.

Moreover, we demonstrated that at MOI of 20, Adv-Casp8 augmented cell death induced by chemotherapeutic agents in all HCC cells examined. There are several reports that describe the mechanism of chemotherapeutic agent-induced cell death in HCC cells. Chemotherapeutic drug-induced apoptosis is mediated by the CD95 (Apo-1/Fas) receptor/ligand system in HCC cells and involves activation of wild-type p53 [46]. Moreover, mediation can act via Fas-dependent or Fas-independent pathways [47]. Analysis of the TRAIL DISC after treatment with chemotherapeutic drugs (for example, 5-FU) revealed upregulation of TRAIL-R2; enhanced caspase-8 recruitment to and activation at the DISC; and downregulation of c-FLIP in HCC cells with either wild-type or null p53 status [48]. Our current study indicates that sensitization to chemotherapeutic drugs by Adv-Casp8 did not depend on p53 status, suggesting the clinical utility of the approach in HCC cells independent of p53 status. We have recently demonstrated that chemotherapeutic agents sensitize HCC cells to TRAIL-induced apoptosis [29]. These reports indicated that chemotherapeutic agent-induced cell death is closely related to death receptor signaling. Together with the current study, the results suggested that exogenous over-expression of caspase-8 may be essentially critical for induction of HCC cell death by chemotherapeutic drugs.

The mitochondrial pathway is an important alternative pathway for apoptosis via death receptor signaling. In the mitochondrial pathway, death signals lead to changes in permeability of the mitochondrial membrane and a subsequent release of pro-apoptotic factors, such as cytochrome *c* [49] and a second mitochondrial-derived activator of caspase (Smac)/ the direct IAP binding protein with low pI (DIABLO) [50,51]. Cytosolic cytochrome *c* binds to Apaf-1 and the resulting complex activates procaspase-9, which in turn activate several downstream caspases, including caspase-3 [52]. Smac/DIABLO also functions to promote caspase activation by inhibiting IAP family proteins. In this study, activation of caspase-8 by Adv-Casp8 at MOI of 50 promoted the release of cytochrome *c* and Smac/DIABLO from the mitochondria into the cytosol. Also, Adv-Casp8 resulted in a decrease in the expression of procaspase-3. Thus, we can conclude that Adv-Casp8 processed procaspase-3 not only directly but also by release of cytochrome *c*, leading activation of caspase-9. In addition, downregulation of c-FLIP, XIAP and survivin by Adv-Casp8 may be caused by not only inhibition of NF-κB but also by Smac/DIABLO. That is, mitochondrial activation by Adv-Casp8 appears to play a role in elimination of factors that normally confer resistance to TRAIL in HCC cells and thus helps to augment TRAIL- or chemotherapeutic agent-induced cell death.

In summary, adenovirus vector-mediated gene transfer of caspase-8 induced apoptosis via downregulation anti-apoptotic proteins such as c-FLIP, XIAP, survivin, and Bcl-xL, critical factors for the normal resistance of HCC cells to several cancer therapies. Moreover, Adv-Casp-8 augmented TRAIL- and chemotherapeutic agent-induced cell death. Finally, Adv-Casp8

activated the mitochondria death pathway, releasing cytochrome *c* and Smac/DIABLO. We conclude that the combination of Adv-Casp8 treatment with TRAIL or chemotherapeutic agents may provide a useful strategy for treatment of HCC.

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