CSE1L/CAS, a microtubule-associated protein, inhibits taxol (paclitaxel)-induced apoptosis but enhances cancer cell apoptosis induced by various chemotherapeutic drugs

Ching-Fong Liao1, Shue-Fen Luo2, Tzu-Yun Shen1, Chin-Huang Lin1, Jung-Tsun Chien1, Shin-Yi Du1 & Ming-Chung Jiang1,∗

1Institute of Cellular and Organismic Biology, Academia Sinica, Taipei, 2Department of Medicine, Division of Allergy, Immunology and the Rheumatology, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Tao-yuan, Taiwan, ROC

CSE1L/CAS, a microtubule-associated, cellular apoptosis susceptibility protein, is highly expressed in various cancers. Microtubules are the target of paclitaxel-induced apoptosis. We studied the effects of increased or reduced CAS expression on cancer cell apoptosis induced by chemotherapeutic drugs including paclitaxel. Our results showed that CAS overexpression enhanced apoptosis induced by doxorubicin, 5-fluorouracil, cisplatin, and tamoxifen, but inhibited paclitaxel-induced apoptosis of cancer cells. Reductions in CAS produced opposite results. CAS overexpression enhanced p53 accumulation induced by doxorubicin, 5-fluorouracil, cisplatin, tamoxifen, and etoposide. CAS was associated with α-tubulin and β-tubulin and enhanced the association between α-tubulin and β-tubulin. Paclitaxel can induce G2/M phase cell cycle arrest and microtubule aster formation during apoptosis induction, but CAS overexpression reduced paclitaxel-induced G2/M phase cell cycle arrest and microtubule aster formation. Our results indicate that CAS may play an important role in regulating the cytotoxicities of chemotherapeutic drugs used in cancer chemotherapy against cancer cells. [BMB reports 2008; 41(3): 210-216]

INTRODUCTION

Paclitaxel, a kind of complex diterpenoid from the bark of yew, has antitumor activities against a broad range of cancers (1, 2). Most chemotherapeutic drugs exert their antitumor activities through a p53-dependent mechanism (3, 4). Experimental and clinical studies have shown that the cytotoxicities of paclitaxel against cancer cell lines and cancers are not related to p53 (5-7). Microtubules are mainly constituted of α- and β-tubulin heterodimers and are the target of paclitaxel-induced apoptosis (8). In paclitaxel-induced apoptosis, paclitaxel binds β-tubulin subunits of microtubules, disrupts normal microtubule dynamics of cell division, and arrests cells in the G2/M phase of the cell cycle (9-11). The cytotoxicity of paclitaxel against cancer cells can be affected by the status of tubulin expression in cancer cells (12). The dynamics of microtubules can be regulated by microtubule-associated proteins (13, 14) and thus the status of microtubule-associated proteins in cancer cells may also affect the sensitivity of cancer cells to paclitaxel (15, 16).

The cellular apoptosis susceptibility (CAS) protein was identified in a study of an antisense DNA fragment that is capable of causing cell resistance to apoptosis induced by Pseudomonas exotoxin, diphtheria toxin, and tumor necrosis factors, but not by ricin or cycloheximide (17). CAS also regulates apoptosis induced by cypermethrin (18) and interferon-γ (19). CAS is highly expressed in various cancers (20, 21). CAS is an apoptosis susceptibility protein and is also a microtubule-associated protein (22), thus CAS may play an important role in regulating the cytotoxicities of chemotherapeutic drugs (particularly paclitaxel) against cancer cells. We report here that CAS differentially regulates apoptosis of cancer cells induced by paclitaxel and other chemotherapeutic drugs.

RESULTS

Doxorubicin-induced apoptosis is enhanced by CAS overexpression and reduced by CAS reduction

HT-29 cells and MCF-7 cells were separately transfected with the pcDNA3.1 empty vector (EV), pcDNA-CAS vector (CAS), and pcDNA-anti-CAS vector (anti-CAS) to obtain HT-EV, HT-29-CAS, HT-anti-CAS, MCF-EV, MCF-CAS, and MCF-anti-CAS cells, respectively (Fig. 1A). Cells were changed to fresh media containing the vehicle solvent or doxorubicin (5 μg/ml for transfected HT-29 cells and 3 μg/ml for transfected MCF-7 cells) and incubated for 72 h. Percentages of apoptotic cells were determined by TUNEL assay. The results showed that

*Corresponding author. Tel: 886-2-27899532; Fax: 886-2-27858059; E-mail: jiangmcedu@yahoo.com.tw

Received 16 July 2007, Accepted 20 September 2007

Keywords: Apoptosis, Cancers, Chemotherapeutic drugs, Paclitaxel, Taxol
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Fig. 1. Doxorubicin-induced apoptosis is enhanced by CAS overexpression and reduced by CAS reduction. (A) Immunoblot analyses of CAS expressions in HT-EV, HT-CAS, HT-anti-CAS, MCF-EV, MCF-CAS, and MCF-anti-CAS cells with anti-CAS antibodies. The β-actin levels were assayed to control for variations in protein loading. (B) Doxorubicin-induced apoptosis was enhanced by CAS overexpression and reduced by CAS reduction. Cells were treated with the vehicle solvent (control) or doxorubicin for 72 h and apoptosis were analyzed by TUNEL assay. Data are the mean ± SD of three independent assays. (C) CAS enhanced doxorubicin-induced p53 accumulation. Cells were treated with the vehicle solvent (control) or 3 μg/ml doxorubicin for 16 h. The p53 levels were determined by immunoblotting with anti-p53 antibodies. The assays were repeated three times with similar results; a representative immunoblot is shown here.

doxorubicin-induced apoptosis was enhanced by CAS overexpression and reduced by CAS reduction in both HT-29 and MCF-7 cells (Fig. 1B). The percentages of apoptotic cells were 44.7 ± 2.9%, 87.2 ± 4.3%, 23.6 ± 2.6%, 34.0 ± 1.1%, 64.5 ± 3.1%, and 20.4 ± 2.7% for doxorubicin-treated HT-29 cells and 40 nM for transfected MCF-7 cells for 12 h. DAPI staining showed that caspase-induced chromatin condensation or fragmentation were reduced by CAS overexpression and enhanced by CAS reduction in HT-29 and MCF-7 cells (Fig. 2A). Paclitaxel-induced apoptosis was determined by TUNEL assay. The percentages of apoptotic cells after being treated with 10 nM paclitaxel for 24 h were 65.7 ± 3.6%, 34.7 ± 3.7%, and 88.6 ± 2.8% for HT-EV, HT-CAS, and HT-anti-CAS cells, respectively (Fig. 2B). The percentages of apoptotic cells after being treated with 40 nM paclitaxel for 24 h were 40.8 ± 1.7%, 25.1 ± 2.7%, and 67.3 ± 4.0% for MCF-EV, MCF-CAS, and MCF-anti-CAS cells, respectively (Fig. 2B). These results indicate that CAS differently regulates apoptosis of cancer cells induced by doxorubicin and paclitaxel.

Paclitaxel-induced apoptosis is reduced by CAS overexpression and enhanced by CAS reduction
Because CAS is a microtubule-associated protein and microtubules are the target of paclitaxel-induced apoptosis, we tested the effect of CAS expression on apoptosis induced by paclitaxel. Cells were changed to fresh media containing the vehicle solvent or paclitaxel (10 nM for transfected HT-29 cells and 40 nM for transfected MCF-7 cells) for 12 h. DAPI staining showed that paclitaxel-induced chromatin condensation or fragmentation were reduced by CAS overexpression and enhanced by CAS reduction in HT-29 and MCF-7 cells (Fig. 2A). Paclitaxel-induced apoptosis was determined by TUNEL assay. The percentages of apoptotic cells after being treated with 10 nM paclitaxel for 24 h were 65.7 ± 3.6%, 34.7 ± 3.7%, and 88.6 ± 2.8% for HT-EV, HT-CAS, and HT-anti-CAS cells, respectively (Fig. 2B). The percentages of apoptotic cells after being treated with 40 nM paclitaxel for 24 h were 40.8 ± 1.7%, 25.1 ± 2.7%, and 67.3 ± 4.0% for MCF-EV, MCF-CAS, and MCF-anti-CAS cells, respectively (Fig. 2B). These results indicate that CAS differently regulates apoptosis of cancer cells induced by doxorubicin and paclitaxel.

CAS affects paclitaxel-induced G2/M-phase cell cycle arrest
Apoptosis and cell cycle control are intimately coupled (23, 24). Paclitaxel treatment can prolong cells at the G2/M-phase cell cycle during apoptosis induction (9-11). Also, G2/M phase arrest was reported to occur prior to apoptosis induced by paclitaxel (25). Thus we analyzed the effect of CAS overexpression on the cell cycle phase distribution of paclitaxel-treated cells. MCF-EV and MCF-CAS cells were changed to fresh media containing the vehicle solvent or 40 nM paclitaxel and incubated for 24 h. The sub-G1 fraction in a DNA histogram determined by flow cytometry is considered to be the apoptotic cells. Flow cytometry analyses showed that CAS overexpression decreased the percentage of the paclitaxel-induced sub-G1 fraction of cells (Supplementary Fig. 1). The results also showed that the percentage of G2/M phase in the paclitaxel-treated MCF-CAS cells was increased by 36.2 % (from 28.3 % to 64.5 %), while the percentage of G2/M phase in the paclitaxel-treated MCF-CAS cells was only increased by 25.3 % (from 31.3 % to 56.6 %) (Supplementary Fig. 1). Thus, CAS expression affects paclitaxel-induced G2/M-phase cell cycle arrest.

CAS overexpression enhances the association between α-tubulin and β-tubulin
CAS is a microtubule-associated protein. Our immunoprecipitation studies showed that both α-tubulin and β-tubulin immunoprecipitated with CAS, and CAS overexpression en-
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Fig. 2. Paclitaxel-induced apoptosis is reduced by CAS overexpression and enhanced by CAS reduction. (A) Paclitaxel-induced chromatin condensation or fragmentation was reduced by CAS overexpression and enhanced by CAS reduction in HT-29 and MCF-7 cells. Cells were treated with the vehicle solvent (control) or paclitaxel for 12 h. Cells were stained with DAPI, and chromatins were analyzed with a fluorescence microscope. Arrows indicate condensed or fragmented chromatin in some of the cells. (B) Paclitaxel-induced apoptosis was reduced by CAS overexpression and enhanced by CAS reduction. Cells were treated with the vehicle solvent (control) or paclitaxel and apoptosis were analyzed by TUNEL assay. Data are the mean ± SD of three independent assays.

Enhanced the immunoprecipitation of CAS with α-tubulin and β-tubulin (Supplementary Fig. 2A). The cellular α-tubulin and β-tubulin levels were not affected by CAS overexpression (Supplementary Fig. 2A). The results of immunoprecipitation studies also showed that the association between α-tubulin and β-tubulin was enhanced in MCF-CAS cells (Supplementary Fig. 2B). Thus, CAS overexpression enhances the association between α-tubulin and β-tubulin.

CAS regulates paclitaxel-induced microtubule aster formation of cells
Paclitaxel is known to induce microtubule aster formation of cells during apoptosis induction (26, 27). CAS associates with microtubules and CAS reduces paclitaxel-induced apoptosis, thus CAS may regulate paclitaxel-induced microtubule aster formation. In the control assay, vehicle solvent-treated HT-29 cells displayed organized microtubule networks that excluded the nucleus and extended throughout the cytoplasm, while paclitaxel treatment induced chromatin condensation or fragmentation of cells, and these cells also showed microtubule aster formation (Supplementary Fig. 3A). Cells were changed to fresh media containing the vehicle solvent, paclitaxel, or 4-OH-tamoxifen. Paclitaxel treatment induced chromatin condensation or fragmentation were reduced by CAS overexpression and enhanced by CAS reduction, and paclitaxel-induced microtubule aster formations were also reduced by CAS overexpression and enhanced by CAS reduction in MCF-7 cells (Fig. 3) and in HT-29 cells (Supplementary Fig. 3B). Thus CAS regulates paclitaxel-induced microtubule aster formation of cells.

CAS regulates apoptosis of cancer cells induced by various chemotherapeutic drugs
Various chemotherapeutic drugs including 5-fluorouracil, cisplatin, etoposide, and tamoxifen were tested to study whether CAS also regulates the susceptibility of cancer cells to apoptosis induced by these drugs. Cells were changed to fresh media containing the vehicle solvent, 5-fluorouracil, cisplatin, etoposide, or 4-OH-tamoxifen. Apoptosis was analyzed by TUNEL assay 72 h after chemotherapeutic drug treatment. The results showed that etoposide-induced apoptosis was not obviously affected by CAS expression (Fig. 4A). Etoposide is special compared with the other chemotherapeutic drugs that we tested, as it also induced microtubule aster formation during apoptosis induction (Supplementary Fig. 4). The ability of etoposide to induce microtubule aster formation during apoptosis induction may be involved the invalidation of CAS in enhancing apoptosis.
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Fig. 3. CAS regulates paclitaxel-induced microtubule aster formation in MCF-7 cells. Cells were treated with the vehicle solvent or paclitaxel for 12 h. Cells were labeled with anti-β-tubulin antibodies and stained with DAPI. The chromatin and the microtubules were analyzed with a fluorescence microscope. Arrows indicate some of the microtubule asters. Bars, 50 μm.

Thus, it is possible that a conflict between the p53-mediated apoptosis pathway enhanced by CAS and the microtubule-related apoptosis pathway reduced by CAS invalidates the CAS' ability to enhance or reduce apoptosis induced by etoposide.

Our studies show that CAS inhibited paclitaxel-induced apoptosis of cancer cells. CAS might enhance cancer cell apoptosis induced by chemotherapeutic drugs (except paclitaxel and etoposide), and p53 may be involved in this regulation (Fig. 1 and 4). However, our data also show that the extents of chemotherapeutic drugs-induced apoptosis enhanced by CAS were not the same for the various chemotherapeutic drugs we tested (Fig. 4). Also, CAS enhanced apoptosis induced by 4-OH-tamoxifen (Fig. 4), and tamoxifen has been reported to be able to induce apoptosis of p53-null breast cancer cells (28).

DISCUSSION
Our results show that CAS can enhance apoptosis of cancer cells induced by chemotherapeutic drugs (except paclitaxel and etoposide), and p53 may be involved in this regulation (Fig. 1 and 4). However, our data also show that the extents of chemotherapeutic drugs-induced apoptosis enhanced by CAS were not the same for the various chemotherapeutic drugs we tested (Fig. 4). Also, CAS enhanced apoptosis induced by 4-OH-tamoxifen (Fig. 4), and tamoxifen has been reported to be able to induce apoptosis of p53-null breast cancer cells (28).

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Thus, it is possible that a conflict between the p53-mediated apoptosis pathway enhanced by CAS and the microtubule-related apoptosis pathway reduced by CAS invalidates the CAS' ability to enhance or reduce apoptosis induced by etoposide.

Our studies show that CAS inhibited paclitaxel-induced apoptosis of cancer cells. CAS might enhance cancer cell apoptosis induced by chemotherapeutic drugs (except paclitaxel) by increasing the p53 accumulation induced by chemotherapeutic drugs. But the mechanism on which CAS inhibits paclitaxel-induced apoptosis is not known. Paclitaxel binds β-tubulin subunits of microtubules, and CAS is also able to bind with β-tubulin (Supplementary Fig. 2A). The molecular weight of CAS is 100 kDa, and the molecular weight of β-tubulin is 55 kDa. Thus, the association of CAS with β-tubulin may be able to occupy the space of paclitaxel binding site and thus inhibits apoptosis induced by paclitaxel. In addition, the increased association between α-tubulin and β-tubulin induced by CAS (Supplementary Fig. 2B) may also be able to affect the binding of paclitaxel with β-tubulin and thus affects apoptosis induced by paclitaxel.

Microtubules are the target of paclitaxel-induced apoptosis of cancer cells. The association of the microtubule-associated protein with microtubules in tumor cells may affect the sensitivity of cancer cells to paclitaxel-induced apoptosis. Caveolin-1 has been reported to be associated with microtubules and to enhance paclitaxel-mediated apoptosis of MCF-7 cells (29). Low expression of the microtubule-binding protein, tau, was reported to enhance the sensitivity of human breast cancer to...
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Fig. 4. CAS regulates apoptosis induced by various chemotherapeutic drugs. (A) CAS regulated apoptosis of MCF-7 and HT-29 cancer cells induced by various chemotherapeutic drugs. Cells were treated with the vehicle solvent (control), 200 μg/ml 5-fluorouracil (5-Fu), 15 μg/ml cisplatin, 150 μM etoposide, or 1 μM 4-OH-tamoxifen (for transfected MCF-7 cells), or treated with the vehicle solvent, 100 μg/ml 5-fluorouracil, 5 μg/ml cisplatin, 100 μM etoposide, or 2 μM 4-OH-tamoxifen (for transfected HT-29 cells). Apoptosis was analyzed by TUNEL assay 72 h after drug treatment. Data are the mean ± SD of three independent assays. (B) CAS overexpression enhanced p53 accumulation induced by 5-fluorouracil, etoposide, 4-OH-tamoxifen, and cisplatin. Cells were treated with the vehicle solvent (control), 200 μg/ml 5-fluorouracil (5-Fu), 1 μM 4-OH-tamoxifen, 150 μM etoposide, or 15 μg/ml cisplatin for 16 h. The p53 levels were determined by immunoblotting with anti-p53 antibodies. The assays were repeated two times and showed similar results, shown here is a representative immunoblot.

paclitaxel treatment (30). As a microtubule-associated protein, CAS is particularly interesting as it is an apoptosis susceptibility protein and is highly expressed in various cancers; thus CAS may play an important role in regulating the cytotoxicity of paclitaxel in cancer chemotherapy.

The genetic profiles of cancer cells can affect the efficacies of chemotherapeutic drugs in cancer chemotherapies. CAS is highly expressed in various cancers, and our studies showed that CAS plays important roles in regulating apoptosis of cancer cells induced by chemotherapeutic drugs. Tumor necrosis factors and interferon-γ also possess anticancer activities against cancers (19, 31), and CAS also regulates apoptosis induced by tumor necrosis factors and interferon-γ (17, 19). Thus, CAS may regulate the cytotoxicities of a variety of anticancer substances against cancer cells. It is worthwhile further studying CAS-mediated apoptosis to improve the efficacies of cancer therapies.

MATERIALS AND METHODS

Drugs and antibodies
Paclitaxel, doxorubicin, etoposide, and 4-OH-tamoxifen were from Sigma (Sigma Chemical Co.). Cisplatin and 5-fluorouracil were from Merck (Merck KGaA). Primary antibodies used in the experiment were anti-CAS (clone 24) and anti-COX-2 (clone 33) (BD Pharmingen); anti-p53 (Ab-2) (Oncogene Research Products); anti-α-tubulin (clone TU-01) (Zymed); anti-β-tubulin (clone D66) (Sigma); and anti-β-actin (Ab-5) (Lab Vision Corp.).

Vectors, cells, and DNA transfections
The pcDNA-CAS vector was constructed previously (32). The pcDNA-CAS vector was cut with Apa I and Hind III, and the 516-bp CAS fragment (bp 1 to 516) was cloned into pcDNA3.1 vector in an antisense direction to obtain pcDNA-anti-CAS vector. Cultures of cells were as previously described (33). Cells were transfected with vectors using the Lipofectamine plus reagent (Invitrogen). Transfected cells were selected with G418 for 3 weeks. Multiple drug-resistant colonies (> 100) were pooled together and amplified in mass culture. The transfected cells were maintained in media containing G418. For the experiments, cells were cultured in media without G418.

Immunofluorescence
Cells were grown on glass coverslips (12 x 12 mm). Cells were cytospun and fixed with 4% paraformaldehyde, treated with 0.1% Triton X-100 in 4% paraformaldehyde, and rinsed with PBS containing 0.1% BSA and 0.5% Tween-20. Cells were incubated with primary antibodies, washed with PBS, incubated with 10 μg/ml DAPI and goat anti-mouse IgG secondary antibodies coupled to Alexa Fluor 568 (Molecular Probes), washed with PBS, and examined with a fluorescence microscope. Experiments were carried out on duplicate coverslips of three independent experiments, and five to ten random fields were imaged per coverslip.

TUNEL assay
Cell death was measured by TUNEL method with an Apoptosis Detection System according to the manufacturer’s instructions (Promega). Adherent cells were harvested by 0.1 % trypsin-EDTA digestion. Non-adherent cells and adherent cells were combined and applied to glass slides by centrifugation.
Samples were then fixed in 4 % paraformaldehyde and permeabilized with 0.1 % Triton X-100. Samples were incubated with Nucleotide Mix and TdT enzyme, and stained with propidium iodide (PI). Fluorescence from apoptotic cells was counted on the propidium iodide background fluorescence under a fluorescence microscope. Percentages of apoptosis were determined as the number of the TUNEL-positive cells of the total number of cells and were expressed as mean ± SD. Three independent experiments were conducted for each assay, and five random fields of each field of approximately 100 cells were counted.

**Immunoblotting**

Cells were washed with PBS and lysed in RIPA buffer (25 mM Tris-HCl [pH 7.2], 0.1 % SDS, 0.1 % Triton X-100, 1 % sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin). Fifty micrograms of each protein sample was loaded onto a SDS-polyacrylamide gel and immunoblotting was done as previously described (32).

**Immunoprecipitation**

Cells were washed with PBS and incubated in lysis buffer (10 mM Tris-HCl [pH 7.4], 10 mM EDTA, 0.4 % deoxycholic acid, 1 % NP40 [or Triton X-100], 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 5 μg/ml leupeptin) at 4°C for 20 min and then disrupted by pipetting. The cell lysate was cleared of insoluble materials by centrifugation at 12,000 g for 10 min at 4°C, and the protein concentration was determined. Immunoprecipitation was carried out as previously described (32).

**Flow cytometry analysis**

Cells were harvested by 0.1 % trypsin-EDTA digestion, washed with PBS containing 0.1 % glucose, and fixed in 70 % ethanol at 4°C for 16 h. Cells was stained with a propidium iodide staining solution containing 100 μg/ml PI, 100 μg/ml RNase A, and 0.1 % glucose for 30 min. The PI fluorescence was measured with a BD FACS Canto flow cytometer (BD Biosciences). A minimum of 10,000 cells in each treatment was analyzed in the flow cytometry analysis.

**Acknowledgments**

We thank the Core Facility of the Institute of Cellular and Organismic Biology, Academia Sinica for assistance in fluorescence microscopy.

**REFERENCES**


