Antitumor effects by Wilfoside C3N treatment in ECA109 cells

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C21 steroidal glycoside (C21) is one of the most bioactive compounds of *Cynanchum auriculatum Royle*, known as Baishouwu, and possesses potent antitumor activity. Wilfoside C3N is one of the two most abundant and active C21 in it. The aim of this study was to further investigate the antitumor activity of C3N and to clarify its signaling pathway. The growth inhibition ofECA109 cells induced by C3N was assessed. Western blot analysis, reverse-transcription PCR, caspase-2 and Fas activity assay, and knockdown of caspase-2 with siRNA were used to study the apoptotic mechanisms. We showed that C3N inhibited the proliferation of ECA109 cells moderately in a dose and time-dependent manner and induced apoptosis in the ECA109 cell line through a mitochondrial pathway by triggered cytochrome *c* release from the mitochondria, with caspase-2 functioning upstream of caspase-9 rather than association with Fas and caspase-8. Furthermore, C3N-driven apoptotic events were associated with downregulation of Bcl-2. These results suggest that C3N-induced apoptosis of ECA109 cells *in vitro* was dependent on caspase-2 or mitochondria or caspase-9 and independent of the Fas-FasL or caspase-8 pathway. *Anti-Cancer Drugs* 21:625–631 © 2010 Wolters Kluwer Health | Lippincott Williams & Wilkins.

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Introduction

Planted in Jiangsu, Baishouwu, the tuber of *Cynanchum auriculatum Royle*, is a traditional Chinese herbal medicine that has multiple pharmacological actions. Contained within this tuber is 'C21 steroidal glycoside' (C21), which is one of its most bioactive compounds. Modern pharmacological studies have shown that it has antitumor, antimicrobial, immunological regulation, anti-inflammation, and antiaging functions and helps in the protection of liver tissues and in cholesterol lowering [1]. C21 was found to inhibit the growth of several human tumor cell lines and to induce apoptosis in human breast cancer (MCF-7) cells. It was evaluated for its in-vitro cytotoxicity against MCF-7, HO-8910, and Bel-7402 cells, and for its in-vivo antitumor effects on implanted sarcoma-180 (S180) tumors in mice [2]. Wilfoside C3N [Caudatin 3-*O*-*β*-D-cymarophranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-cymaropyranoside] and wilfoside C1N [Caudatin 3-*O*-α-L-cymarophranosyl-(1→4)-*β*-D-cymaropyranosyl-(1→4)-α-L-diginopyranosyl-(1→4)-*β*-D-cymaropyranoside] are the two most abundant and active C21 compounds in it. Wilfoside C3N showed a much better absorption than wilfoside C1N [3]. However, rarely has the antitumor effect of C3N been researched. Moreover, other than our recent report that C21 induced apoptosis in hepatocellular cancer cells (Heps) by downregulating Bcl-2 expression and inducing apoptosis [4], virtually no study has been conducted to elucidate its mechanisms. Through an understanding of the molecular mechanisms that mediate the proapoptotic activity of C3N, it will be possible to better understand its antitumor effects and to determine whether it is a candidate for clinical use. In addition, knowing the molecular targets of C3N and its mechanism of action will enable us to design rational combination therapies, which will eradicate tumor cells more efficiently.

Esophageal cancer (EC) is an aggressive malignancy of the alimentary system. Despite major efforts during the past years, limited progress has been made in the treatment of EC [5]. Although in patients with EC that can be completely removed with surgery, the use of chemotherapy and radiotherapy before or after surgery may be needed to achieve a cure. The standard of care in patients who are unable to tolerate surgery or who have disease that cannot be completely removed is chemoradiotherapy, which induces cell death mainly by apoptosis mediated by either the intrinsic mitochondrial or the extrinsic death receptor pathway, both of which lead to caspase activation and cell disintegration [6]. The development of innovative therapies and identification of more effective drugs, therefore, remain high priorities for EC research.

The effects of C3N on the esophageal carcinoma cell line (ECA109) are described and the mechanisms of C3N-induced apoptosis in these cells are investigated in this research.

Materials and methods

Materials

Esophageal squamous carcinoma cell line Eca109 was obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai Institute of

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Cell Biology, Chinese Academy of Sciences), cultured in RPMI 1640 medium (Invitrogen, Grand Island, New York, USA) supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin and penicillin and incubated at 37°C in a humidified atmosphere containing 5% CO_2. C3N was synthesized from the tuber of *Cynanchum auriculatum Royle*, planted in Jiangsu, and was extracted and identified as C3N with special purified-grade (98% C3N content) by the Chinese Medicine Preparing Center of Zhejiang University of Traditional Chinese Medicine. Antibodies for caspase-2, caspase-9, Bcl-2, Fas, and cytochrome *c* were purchased from BD Biosciences (San Diego, California, USA). Antibodies for β-actin, caspase-8, FasL, and Fas-associated death domain (FADD) were purchased from Santa Cruz Biotechnology, Inc. (Delaware Avenue Santa Cruz, California, USA). All other chemicals were of reagent grade.

Stimulation of cells

C3N was dissolved in incomplete RPMI 1640 at concentrations of 2, 4, 8, 16, and 32 μg/ml. Final concentrations of the solvents in the medium were 1, 2, 4, 8, and 16 μg/ml. Tumor cells were treated *in vitro* by different concentrations of C3N for the specified times (12, 24, 36, and 48 h). The same incomplete RPMI 1640 were used for the controls.

Cell proliferation assay

Cell proliferation was measured by the MTT assay. ECA109 cells were seeded into 96-well culture plates at 2×10^3 cells per well and each group consisted of six parallel wells. Absorption values were determined by MTT at 490 nm. The growth curve of each group was plotted based on absorption values. All experiments were performed three times.

Detection of DNA fragmentation

The total cellular DNA was extracted from the cells. The cells were washed and lysed overnight at 37°C in lysis buffer containing 10 mmol/l Tris-HCl (pH 8.0), 10 mmol/l edetic acid, 0.4% sodium dodecylsulfate, and 100 mg/l proteinase K. Subsequently, the cells were treated with 0.5 μg/ml RNase A for 2 h. Genomic DNA was extracted by phenolchloroform isoamyl alcohol extraction. The ECA109 cells treated with cisplatin that served as positive control and the ECA109 cells treated with the same incomplete RPMI 1640 were used as negative control. The quantities of genomic DNA were determined spectrophotometrically. Electrophoresis was performed on a 1% agarose gel. Images of gels stained with ethidium bromide were captured by the Gel Doc XR system (Bio-Rad, Hercules, California, USA).

Measurement of apoptosis using FACS

Cells were harvested through trypsinization after stimulation and washed twice with cold PBS. The cells were centrifuged at 500*g* for 5 min at 4°C. Thereafter, the supernatant was discarded and the pellet was resuspended in 500 μl of 1 × annexin binding buffer (BD Biosciences) with 5 μl of annexin V-fluorescein isothiocyanate (BD Biosciences) and 0.5 μl of propidium iodide (PI) (BD Biosciences) for 30 min at room temperature in the dark. The percentage of cells with annexin V^+/PI^- was measured using flow cytometry (FACSAria, BD Biosciences) within 1 h.

Western blot analysis

The cells that were stimulated as indicated were harvested in 5 ml of the medium, pelleted by centrifugation (1000*g* for 5 min at 4°C), then washed twice with ice-cold PBS and lysed in ice-cold HEPES buffer [HEPES (pH 7.5, 50 mmol/l), NaCl (10 mmol/l), $MgCl_2$ (5 mmol/l), EDTA (1 mmol/l), glycerol 110% (v/v), Triton X-100 1% (v/v), a cocktail of protease inhibitors, and 1 mg/l TSA on ice for 30 min]. The lysates were clarified by centrifugation (15 000*g* for 10 min at 4°C) and the supernatants were then either analyzed immediately or stored at −80°C.

The protein sample was loaded with 30 mg of protein per lane on the SDS–polyacrylamide gel electrophoresis gel. After electrophoresis, the proteins on the gel were transferred onto a nitrocellulose membrane, as described earlier. The nitrocellulose membrane was blocked with 50 g/l nonfat dried milk in TBS-T for 1 h at room temperature, incubated with the first antibody for 2 h and with the secondary antibody for 50 min at room temperature and finally washed three times after each incubation. ECL chemoluminescence reagent was used to show the positive bands on the membrane.

Reverse-transcription PCR

RNA was extracted with Trizol isolation reagent (Invitrogen). Sample RNA content was quantified by measuring the absorbance at 260 nm. Reverse-transcription (RT) was performed using PrimeScript One Step RT-PCR Kit (Takara, Japan) according to the manufacturer's instructions. Table 1 shows the synthetic oligonucleotide primer sequences used for RT-PCR and the product sizes. β-actin served as a control. A total of 20 μl of RT-PCR reaction mixture contained 4 μl RT product, 2.5 U Taq DNA polymerase, 20 μmol/l dNTP, 0.1 μmol/l primer, and 1X Taq DNA polymerase buffer (Takara, Japan). The reaction mixture was incubated in a thermocycler programmed to predenature at 94°C for 10 min, denature at 94°C for 30 s, anneal at 55°C for 30 s, and extend at 72°C for 1 min. The last cycle was followed by incubation at 72°C for 10 min and cooling to 4°C. The PCR products were analyzed in 1.5% (v/v) agarose gels. Densitometry was performed using Quantity One software (Hercules, California, USA). The data were recorded as the ratio of sample to internal standard.

Table 1 The synthetic primers used for reverse-transcription PCR

Apoptosis modulator	Sense primer (5′–3′) (forward)	Anti-sense primer (5′–3′) (reverse)	PCR conditions[a]	Size (bp)
Bcl-2	AGGATTGTGGCCTTCTTTGAGT	GCCAAACTGAGCAGAGTCTTCA	62 (30)	236
Caspase-2	CAGTTACCTGCACACCGAGTCACGG	AGCCGCATATCATGTCTGAGCGCG	69 (30)	375
Caspase-9	AACAGGCAAGCAGCAAAGTT	TCCATCTGTGCCGTAGACAG	69 (30)	497
β-actin	GGGAAATCGTGCGTGACA	TCAGGAGGAGCAATGATC	57 (30)	423

[a]Annealing temperature in degree centigrade (number of cycles).

Extraction of cytochrome *c* released into the cytosol

After treatment with the control or desired concentrations of C3N, the cells were harvested and resuspended in extraction buffer (20 mmol/l HEPES, pH 7.5, 10 mmol/l KCl, 1.5 mmol/l $MgCl_2$, 1 mmol/l EDTA, 1 mmol/l EGTA, 1 mmol/l DTT, 0.1 mmol/l phenylmethylsulfonyl fluoride, and 250 mmol/l sucrose) and homogenized. The homogenates were then centrifuged at 750*g* for 10 min at 4°C. The supernatants were centrifuged at 10 000*g* for 15 min at 4°C, and the remaining supernatant was designated as the cytosol fraction. Cytochrome *c* was identified by western blot analysis with a rabbit anticytochrome *c* polyclonal antibody from Santa Cruz. The subsequent western blot analysis was performed as described above.

Caspase-2 and Fas activity assay

The caspase-2-specific inhibitor z-Val-Ala-Asp(OMe)-Val-Ala-Asp(OMe)-CH2F (z-VDVAD-fmk) (BioVision, USA) and Fas antibody were used to measure caspase-2 and Fas activities. Briefly, cells were seeded at a density of 4×10^5 cells per well in a six-well plate, grown overnight, and then treated with 50 μmol/l z-VDVAD-fmk or Fas antibody for 3 h followed by C3N at 4, 8, and 16 μg/ml for 48 h of treatment. After the treatments, adherent and floating cells were collected by trypsinization and centrifuged at 500*g* for 5 min. Cell pellets were resuspended and incubated in complete medium for 10 min. After centrifugation, cell pellets were washed twice with PBS and resuspended. Cytochrome *c* was measured by western blot analysis as described above. Apoptosis was identified by incubation with fluorescein isothiocyanate (FITC)-conjugated annexin V and PI (BD Biosciences) for 30 min at room temperature in the dark, followed by analysis with flow cytometry as described above.

Knockdown of caspase-2

Cells were transfected in six-well plates with caspase-2 or nontarget control small interfering RNA (siRNA) constructs (Dhamacon, Chicago, Illinois, USA) for 24 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The construct used was the siRNA SMART pool caspase-2. After knockdown, cells with caspase-2 siRNA 50 nmol/l were treated for another 48 h with or without C3N at 4 μg/ml, and apoptotic cells were quantified by the PI method using flow cytometry. RNA interference was assessed by western blotting.

Statistical analysis

Statistical analyses were performed using Statistical Package for Social Survey (SPSS, Chicago, Illinois, USA) for Windows 13.0. Significant effects on multiple comparisons analysis of variance (ANOVA) were followed up by post-hoc *t*-tests with LSD correction. Differences were considered to be significant at a P value less than 0.05.

Results

The effect of C3N on cell proliferation

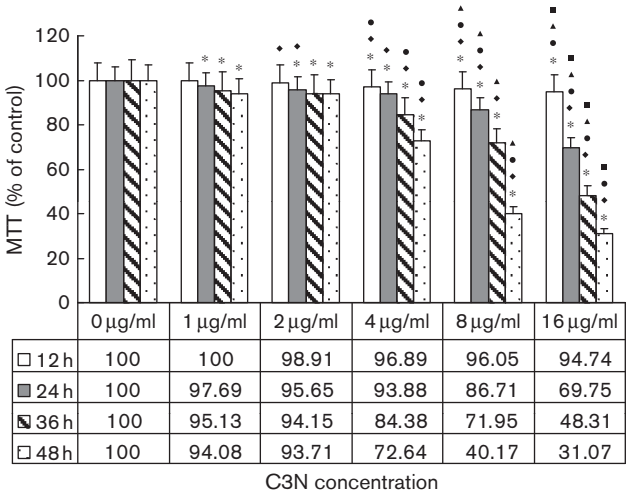
The absorption values of each group of cells were detected using MTT at 490 nm. The growth curve of each cell group showed that cell proliferation was slower in the C3N-treated cells than the control cells. There was no significant difference of cell proliferation between 2 μg/ml for 12-h group and the control group. C3N inhibited the growth of the ECA109 cells in a dose and time-dependent manner at concentrations of 1, 2, 4, 8, and 16 μg/ml at 12, 24, 36, and 48 h, respectively (Fig. 1). These results suggested that ECA109 cell proliferation was inhibited by treatment with C3N.

Induction of apoptosis by C3N

The cytotoxic effects of C3N on ECA109 cells were attributable to the induction of apoptosis, as shown by the following biochemical features. The induction of DNA fragmentation was shown by incubating ECA109 cells with C3N at different concentrations (4 and 16 μg/ml) for 48 h. A DNA fragmentation laddering assay was used to confirm the apoptotic effect of C3N on ECA109 cells. The occurrence of DNA fragmentation is a characteristic event of cell apoptosis with genomic DNA degrading into a ladder feature on agarose gel electrophoresis. Figure 2(a) notably shows that DNA fragmentations were observed in ECA109 cells after 48 h of treatment with C3N at 4 and 16 μg/ml.

To further characterize C3N-induced apoptosis, we performed cytofluorimetric analysis by FITC-PI double staining. Early apoptotic cells were annexin V-FITC positive and PI negative. The percentage of early apoptotic ECA109 cells treated with C3N at 2, 4, 8, and 16 μg/ml for 48 h were $3.6 \pm 0.5\%$, $10.2 \pm 0.9\%$, $18.9 \pm 1.2\%$, and $21.3 \pm 2.7\%$, respectively. Compared with $1.5 \pm 0.6\%$ in the negative control, the percentages were higher in the cells treated with C3N ($P < 0.05$). These results show that C3N induces apoptosis in ECA109 cells (Fig. 2b).

Fig. 1



	0 μg/ml	1 μg/ml	2 μg/ml	4 μg/ml	8 μg/ml	16 μg/ml
□ 12 h	100	100	98.91	96.89	96.05	94.74
▩ 24 h	100	97.69	95.65	93.88	86.71	69.75
▨ 36 h	100	95.13	94.15	84.38	71.95	48.31
□ 48 h	100	94.08	93.71	72.64	40.17	31.07

C3N induced a mean extent of inhibition of MTT conversion. C3N inhibited the growth of the ECA109 cells in a dose and time-dependent manner. Results are means and 95% confidence intervals of three independent experiments made in triplicate. Differences were considered statistically significant at $P<0.05$. *$P<0.05$ compared with controls; ◆$P<0.05$ compared with 1 μg/ml groups; ●$P<0.05$ compared with 2 μg/ml groups; ▲$P<0.05$ compared with 4 μg/ml groups; ■$P<0.05$ compared with 8 μg/ml groups.

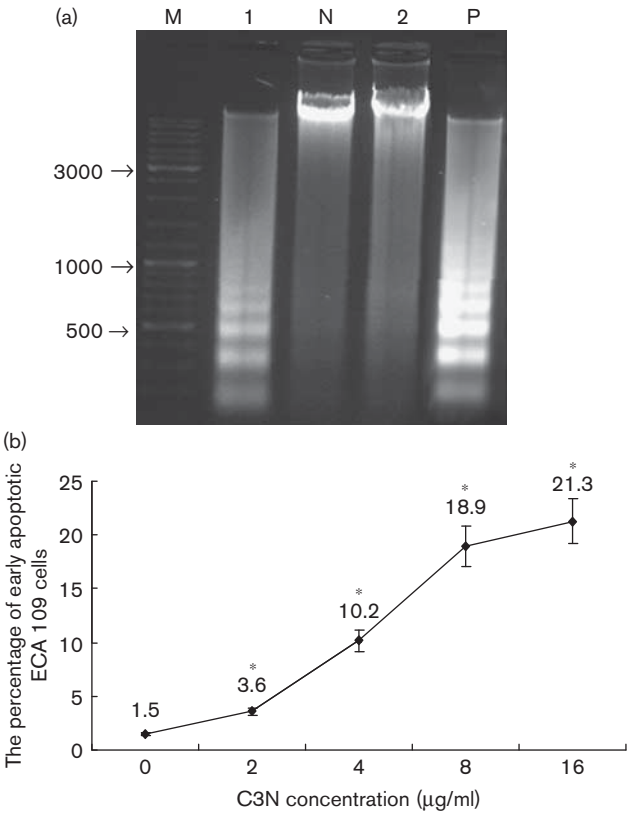
Regulation of proapoptotic and antiapoptotic proteins levels by C3N in ECA109 cells

To better understand the mechanism by which elevated expression of C3N induces apoptosis in ECA109 cells, lysates from the cells treated for 48 h with C3N and negative controls were prepared, and a western blot was carried out. Results of multiple western blot analyses showed that the proapoptotic factors, Fas, caspase-9, caspase-2, and cytochrome *c* levels, were remarkably higher (Fig. 3a–d), and the antiapoptotic factor, the Bcl-2 level, was lower than in the negative controls (Fig. 3e). However, there were not significant differences in the FasL, FADD, and caspase-8 levels between the treated groups and the controls (Fig. 3f–h).

The effects of C3N on Bcl-2, caspase-2, and caspase-9 on the transcriptional level

Cells were treated with 2, 4, 8, and 16 μg/ml concentrations of C3N for 48 h, then the total RNA of the cells was isolated and finally, RT-PCR was performed as described in the earlier section. PCR products visualized by UV transillumination showed an increase in the expression levels of caspase-2, 9 and a remarkable decrease in the expression of Bcl-2 (Fig. 4). These results suggest that C3N treatment (2, 4, 8, and 16 μg/ml for 48 h) upregulated caspase-2 and 9 and downregulated Bcl-2 at the transcriptional level in ECA109 cells.

Fig. 2



Induction of apoptosis by C3N treatment. (a) DNA was visualized by ethidium bromide staining and photographed under UV illumination. DNA fragmentations were observed in the ECA109 cells after 48 h of treatment with C3N at 4 and 16 μg/ml. Experiments were performed four times with similar results (M, marker; N, negative control; P, positive control; 1 = 4 μg/ml; 2 = 16 μg/ml). (b) The percentage of early apoptotic ECA109 cells treated with C3N at different concentrations by cytofluorimetric analysis. These results show that C3N induces apoptosis in ECA109 cells. *$P<0.05$ compared with negative control.

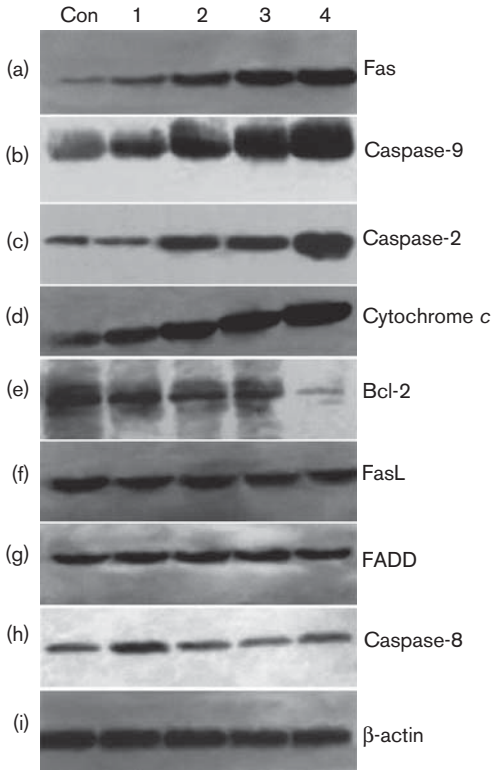
The role of caspase-2 and Fas in C3N-induced apoptosis

z-VDVAD-fmk and Fas antibody were used as specific inhibitors of caspase-2 and Fas. Figure 5 shows that inhibition of caspase-2 significantly decreased cytochrome *c* release (Fig. 5a–c) and C3N-induced apoptosis (Fig. 5d). However, there were no marked differences after the pretreatment with Fas antibody (Fig. 5d). Furthermore, inhibition of caspase-2 (Fig. 5e and f) using an siRNA specific for caspase-2 resulted in a marked inhibition of C3N-induced apoptosis (Fig. 5g).

Discussion

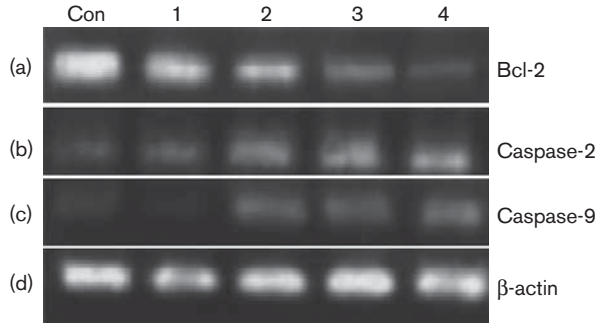
We have investigated the effect of C21 on inducing apoptosis of hepatocellular cancer cells and its potential mechanism [4]. Inhibiting the excessive expression of the Bcl-2 gene to promote apoptosis may be one of the antitumor mechanisms for the C21 sterols found in Baishouwu. The Bcl-2 (B-cell lymphoma-leukemia-2) gene becomes involved in chromosomal translocations in

Fig. 3



Detection of levels of Fas, caspase-9, caspase-2, cytochrome *c*, Bcl-2, FasL, FADD and caspase-8 protein expression in ECA109 cells by western blot analysis. The proapoptotic factors, Fas, caspase-9, caspase-2, and cytochrome *c* levels, were remarkably higher (a–d), and the antiapoptotic factor, the Bcl-2 level, was significantly lower than in the negative controls (e). There were no obvious differences in the FasL, FADD, and caspase-8 levels between treated groups and controls (f–h). Lane Con, control; lane 1, 2 μg/ml; lane 2, 4 μg/ml; lane 3, 8 μg/ml; lane 4, 16 μg/ml. (i) β-actin was used to control for loading differences.

Fig. 4



Effect of C3N treatment for 48 h on mRNA expression of Bcl-2 (a), caspase-2 (b), and caspase-9 (c) in ECA109 cells by reverse-transcription PCR analysis. C3N treatment upregulated caspase-2 and 9 and downregulated Bcl-2 at the transcriptional level in ECA109 cells. Lane Con, control; lane 1, 2 μg/ml; lane 2, 4 μg/ml; lane 3, 8 μg/ml; lane 4, 16 μg/ml. (d) β-actin was used to control for loading differences.

many human B-cell lymphomas. B cells and some other cells, such as tumor cells, which overexpress Bcl-2, exhibit enhanced in-vitro survival in the absence of growth factors [7]. The expressions of Fas/FasL are increased frequently whereas that of Bcl-2 is decreased. It is shown that Bcl-2 could act in suppression of apoptosis, which resulted in downregulating the expression of caspase-3 [8]. In addition, caspases and cysteine acid proteases are central regulators of apoptosis [9]. Once caspase-8 and caspase-10 are activated, these caspases cleave and the downstream caspases including caspase-3, 6, and 7 are activated, which in turn cleave cytoskeletal and nuclear proteins, further resulting in cell apoptosis through the Fas/FasL pathway [10].

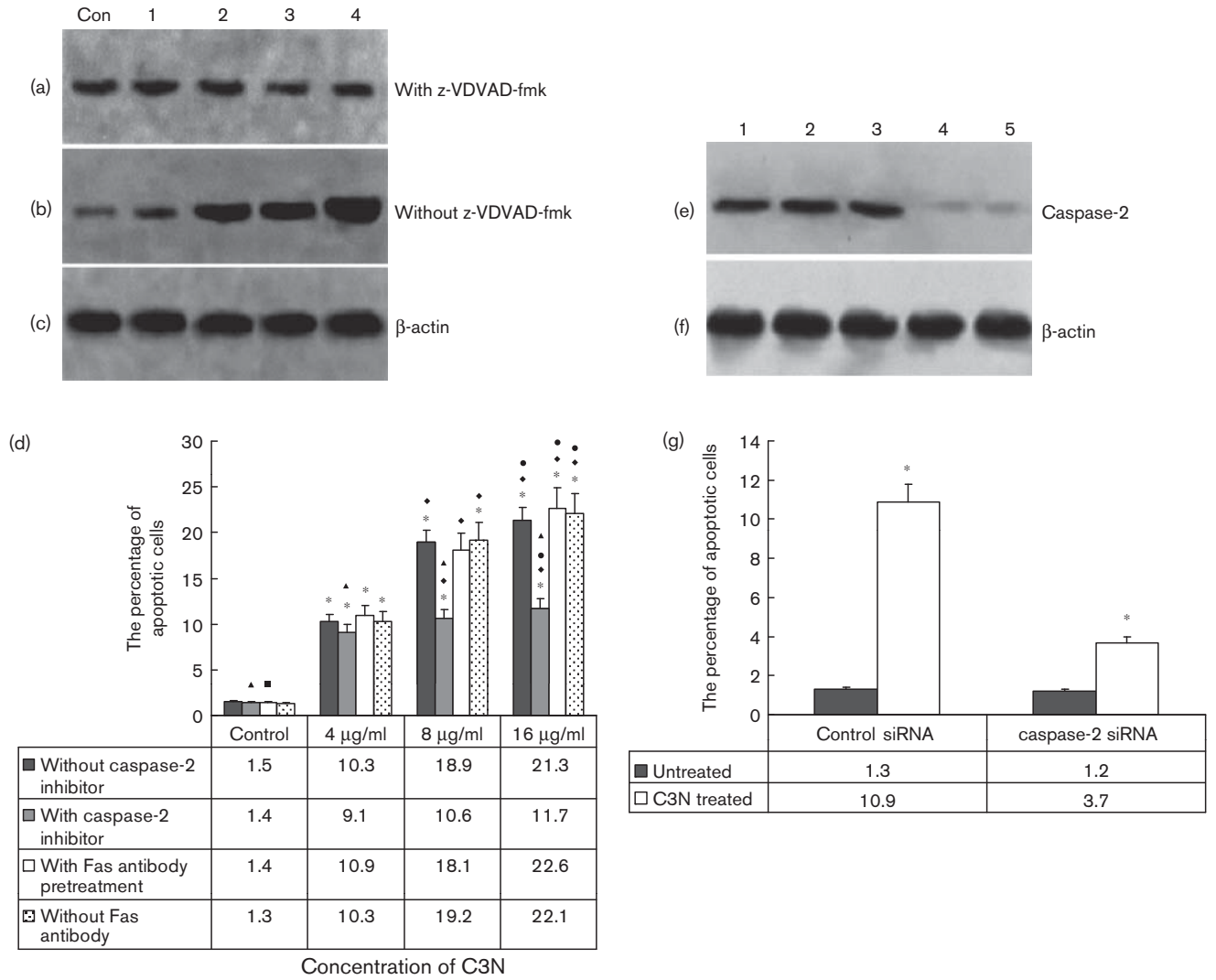
The death effector domain and the caspase recruitment domain (caspase activation and recruitment domain), which function as a domain for a homophilic protein interaction, play a role in death receptor-mediated apoptosis by binding to caspase-8 and/or the FADD [11]. Signaling in apoptosis is often mediated by proteins of the death domain superfamily within the Fas/FADD/caspase-8 group [12]. Caspase-8 is the first step in the cascade of apoptotic events induced by Fas. Cleaved caspase-8 then activates other downstream caspases including caspase-2, 3, and 9, thereby committing the cell to undergo apoptosis [13].

In contrast, caspase-2 is activated early in response to DNA-damaging antineoplastic agents and may be important for the engagement of the mitochondrial apoptotic pathway [14]. The apoptotic signaling cascades may proceed through the proteolytic activation of caspase-2, change in mitochondrial membrane potential, release of cytochrome *c* and second mitochondria-derived activator of caspase/direct IAP binding protein with low pI, activation of caspase-9 and 3, and cleavage of poly (ADP-ribose) polymerase [15].

Our findings showed that C3N, one of the two most abundant and active *C21*, moderately inhibited the proliferation of ECA109 cells in a dose and time-dependent manner by the MTT assay. The apoptosis morphology of the ECA109 cells was observed using an inverted microscope and it included the cells growing round and small and against the wall, the condensation of nuclear material, and the vacuolar degeneration of the cytoplasm. We showed that C3N induced extensive apoptosis in the ECA109 cell line by flow cytometry and DNA ladder analysis. Obviously, all these effects presented a significant influence on inhibiting the proliferation of ECA109 cells.

The aim of our study was to understand the mechanism whereby C3N produces this inhibition. Using western blotting, we found that the proapoptotic factors, Fas, caspase-9, caspase-2, and cytochrome *c* levels, were remarkably higher and the antiapoptotic factor, the Bcl-2 level, was significantly lower than in the negative

Fig. 5



(a–c) Comparison of cytochrome *c* release between caspase-2 inhibition (a) and none of the inhibitors (b). Lane 1, control; lane 2, 4 μg/ml; lane 3, 8 μg/ml; lane 4, 16 μg/ml. Inhibition of caspase-2 significantly decreased cytochrome *c* release. (d) Comparison of the percentage of early apoptotic cells between caspase-2 and Fas inhibition and none of the inhibitors. Inhibition of caspase-2 significantly decreased C3N-induced apoptosis. There were no marked differences after pretreatment with Fas antibody. *$P<0.05$ compared with controls; ◆$P<0.05$ compared with 4 μg/ml groups; ●$P<0.05$ compared with 8 μg/ml groups; ▲$P<0.05$ compared with without caspase-2 inhibitor groups; ■$P<0.05$ compared with without Fas antibody pretreatment groups. (e–g) Comparison of the percentage of 4 μg/ml 48 h C3N-induced apoptotic cells between with and without caspase-2 siRNA 50 nmol/l. (e and f) Knockdown of caspase-2 inhibits C3N-induced activation of caspase-2. Lane 1, control; lane 2, control with siRNA 50 nmol/l; lane 3, control with siRNA 100 nmol/l; lane 4, caspase-2 siRNA 50 nmol/l; lane 5, caspase-2 siRNA 100 nmol/l. (g) Inhibition of caspase-2 using an siRNA specific for caspase-2 resulted in a marked inhibition of C3N-induced apoptosis. *$P<0.05$ compared with controls.

controls. However, there were no remarkable differences in the FasL, FADD, and caspase-8 levels between the treated groups and controls. The results suggest that in ECA109 cells, C3N-induced apoptosis *in vitro* may not occur through an FADD-caspase-8 pathway. Recent findings have suggested that caspase-2 participates in the activation of the mitochondrial apoptotic pathway. Overexpressed procaspase-2 was shown to locate to the nucleus, in which it may be able to induce apoptosis by stimulating mitochondrial cytochrome *c* release by an unknown mechanism [16]. Purified caspase-2 is able to trigger cytochrome *c* release *in vitro*, possibly by cleaving Bid [17]. Although the expression of full-length caspase-2 did not show proteolytic activity, it was interesting to note that overexpression of caspase-2 was coincident with cellular apoptosis. This evidence suggests that activated caspase-2 induces death through two pathways. First, activated caspase-2 induces cell death independently of the mitochondrial pathway, in a manner similar to that of ced-3, a caspase in *Caenorhabditis elegans*. Second, activated caspase-2 also induces cell death upstream of the mitochondrial pathway. The choice of pathway may

depend on the type of death stimulus [18]. In this study, the caspase-2-specific inhibitor z-VDVAD-fmk may also exert a significant inhibitive effect on the apoptotic process. This evidence indicates that activated caspase-2 overexpression is in response to the apoptosis induced by C3N *in vitro*. Furthermore, silencing of caspase-2 by siRNA failed to induce apoptosis. These results suggest that the effect of C3N-induced apoptosis was activated caspase-2 dependent *in vitro*. Binding of Fas with FasL causes trimerization and recruitment of FADD proteins through homotypic death domain interactions. In turn, trimerized FADD recruits either procaspase 8 or 10, which undergoes a process of autoproteolysis to become an activated caspase. Effector T cells and NK cells express FasL, and target cells express Fas; thus, these cells are susceptible to apoptosis mediated by this pathway [10]. Although the Fas/FasL death pathway may play a role in cellular apoptosis, this observation that shows activated caspase-2 dependent apoptosis *in vitro* may explain why Fas antibody pretreatment does not prevent C3N-induced apoptosis or upregulates FADD and caspase-8 levels *in vitro* significantly.

Thus, this study shows that C3N-induced apoptosis of ECA109 cells *in vitro* seems to depend on the over-expression of activated caspase-2 and the decreased expression of Bcl-2, which induces cytochrome *c* release, upregulating caspase-9 expression, and initiating the subsequent events leading to apoptosis. This is the first step to study the important mediators of apoptosis induced by C3N *in vitro*. Whether Fas overexpression induced by C3N provides an important role *in vivo* is still to be determined, but these studies may present valuable insights. These and other ongoing studies promise to provide valuable evidence that may assist in a more effective use of C3N in the treatment of esophageal cancer.

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