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## Evodiamine induces tumor cell death through different pathways: apoptosis and necrosis

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**KEY WORDS** evodiamine; apoptosis; necrosis; caspases; MAP kinase signaling system

### ABSTRACT

**AIM:** To study the different death pathways in human cervical cancer HeLa and melanoma A375-S2 cells initiated by evodiamine. **METHODS:** Viability of evodiamine-induced HeLa and A375-S2 cells was measured by MTT assay. Apoptotic cells with condensed or fragmented nuclei were visualized by Hoechst 33258 staining. Nucleosomal DNA fragmentation was assayed by agarose gel electrophoresis. Proportion of cell death through apoptotic and necrotic pathways was determined by LDH activity-based cytotoxicity assays. Cell cycle distribution was observed by flow cytometry. **RESULTS:** Evodiamine induced HeLa and A375-S2 cell death dose- and time-dependently. Caspase-3 and -8 were activated in apoptosis induced by evodiamine 15  $\mu\text{mol/L}$ . However, over 24-h incubation of A375-S2 cells, evodiamine 15  $\mu\text{mol/L}$  initiated necrosis related to p38 and ERK (extracellular signal-regulated kinases) activities. Evodiamine-induced HeLa cell death was preceded by an accumulation of cells at the G2/M phase of the cell cycle, but there was no significant effect of evodiamine on A375-S2 cell cycle. **CONCLUSION:** Evodiamine induces caspase-3,8-dependent apoptosis in HeLa cells which is related to G2/M arrest of the cell cycle. On the other hand, in A375-S2 cells, evodiamine initiates caspase-3,8-mediated apoptosis at early stages and the induction of MAPK-mediated necrosis at later stages of cell culture.

### INTRODUCTION

The fruits of *Evodia rutaecarpa* Benth (Rutaceae) have been used as a traditional Chinese medicine ("Wu-zhu-yu") for the treatment of headache, abdominal pain, dysentery, postpartum hemorrhage, and menorrhagia. Phytochemical studies on the drug have shown the presence of numerous constituents including alkaloid such as evodiamine<sup>[1]</sup> which was reported to increase arterial pressure *in vivo*, and to possess

antitumor, antinociceptive, vasorelaxant, and catecholamine-seretory properties<sup>[2]</sup>. Evodiamine also had an inhibitory activity on tumor cell migration *in vitro*<sup>[3]</sup>.

Caspases appear to be involved in regulating activation of apoptotic signal transmission<sup>[4]</sup>, however, caspase participation in necrotic signaling mechanism has been still unclear. p38 and ERK are the members of mitogen-activated protein kinase (MAPK) family which were important mediators of signal transduction<sup>[4,6]</sup> and their activation is associated with apoptosis<sup>[7]</sup>.

The aim of this study was to investigate that evodiamine induced HeLa and A375-S2 cell death through distinct mechanisms and pathways.

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## MATERIALS AND METHODS

**Chemical reagents** Evodiamine was purchased from Beijing Institute of Biological Product (Beijing, China). The purity of evodiamine was measured by HPLC and determined to be about 98 %. Evodiamine was dissolved in dimethyl sulfoxide (Me<sub>2</sub>SO), and Me<sub>2</sub>SO concentration in all cell cultures was kept below 0.001 % which had no detectable effect on cell growth or apoptosis.

z-VAD-fmk (pan-caspase inhibitor), z-DEVD-fmk (caspase-3 inhibitor), and z-IETD-fmk (caspase-8 inhibitor) were purchased from Enzyme Systems (CA, USA) and Ac-YVAD-cmk (caspase-1 inhibitor) was obtained from Bachem (Bubendorf, Switzerland). p38 MAPK inhibitor (SB203580) and ERK MAPK inhibitor (PD 98059) were purchased from Calbiochem (CA, US).

**Cell culture** The human malignant A375-S2 and human cervical cancer HeLa cells obtained from American Type Culture Collection (ATCC, USA). were cultured in RPMI-1640 medium (Gibco, USA) supplemented with 5 % fetal bovine serum (FBS) (Dalian Biological Reagent Factory, Dalian, China) and 0.03 % *L*-glutamine (Gibco) at 37 °C in 5 % CO<sub>2</sub>. Cells in the exponential phase of growth were used in the experiments.

**Cytotoxicity assay** HeLa cells were cultured at 1×10<sup>4</sup> cells/well and A375-S2 at 5×10<sup>3</sup> cells/well in 96-well plates (NUNC, Denmark). After preincubation with caspase inhibitors, z-VAD-fmk, z-DEVD-fmk, z-IETD-fmk, Ac-YVAD-cmk, p38 MAPK inhibitor (SB 203580), or ERK inhibitor (PD 98059) at given concentrations for 1 h, the cells were incubated with evodiamine for different time periods. Cell growth was measured at different time points by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described with a plate reader (Bio-Rad, USA). The percentage of cell inhibition was calculated as follows:

$$\text{Cell death (\%)} = \frac{A_{570}(\text{control}) - A_{570}(\text{evodiamine})}{A_{570}(\text{control})} \times 100 \%$$

**Observation of morphological changes** HeLa and A375-S2 cells in RPMI-1640 containing 5 % FBS were seeded into 6-well culture plates (NUNC, Denmark) and cultured overnight. Evodiamine 30 μmol/L was added to the cell culture and the cellular morphology was observed using phase contrast microscopy (LEICA, Germany) at 12, 24, and 48 h.

**Nuclear damage observed by Hoechst 33258 staining** Apoptotic nuclear morphology was assessed using Hoechst 33258 (Sigma, USA) as described previ-

ously<sup>[8]</sup>. Cells were fixed with 3.7 % paraformaldehyde for 2 h at room temperature, then washed and stained with Hoechst 33258 167 μmol/L at 37 °C for 10 min. At the end of incubation, the cells were washed and resuspended in PBS for observation of nuclear morphology using fluorescence microscope (Nikon, Japan).

**DNA extraction and detection of DNA fragments<sup>[8]</sup>** HeLa and A375-S2 cells (1×10<sup>6</sup> cells) were harvested and centrifuged at 1000×g for 10 min. Cell pellets were suspended in Tris-HCl 10 mmol/L (pH 7.4), edetic acid 10 mmol/L, 0.5 % Triton X-100 and proteinase K 40 μg/L (Merck, USA) at 37 °C for 2 h. The lysate was extracted with 0.5 % NaCl 5 mol/L and 50 % 2-propanol and incubated overnight at -20 °C, and centrifuged at 7000×g for 20 min. The supernatant was washed with 70 % ethanol and centrifuged. The pellets were dried and suspended in Tris-HCl 10 mmol/L (pH 7.4) and edetic acid 1 mmol/L. DNA was incubated with RNase A 40 μg/L (Sigma, USA) at 37 °C for 60 min and separated by 2 % agarose gel electrophoresis at 100 V for 40 min and stained with ethidium bromide 0.1 mg/L.

**LDH activity-based cytotoxicity assays<sup>[9]</sup>** The cells were cultured with evodiamine for 8, 12, 24, and 36 h. Floating dead cells were collected from culture medium by centrifugation (240×g for 10 min at 4 °C), and the lactate dehydrogenase (LDH) content from the pellets lysed in 0.1 % NP-40 for 15 min was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (extracellular LDH or LDHe) was used as an index of necrotic cell death. The adherent and viable cells were lysed in 0.1 % NP-40 for 15 min to release LDH (intracellular LDH or LDHi). The substrate reaction buffer of LDH [L (+)-lactic acid 0.5 mmol/L, INT 0.66 mmol/L, PMS 0.28 mmol/L, NAD<sup>+</sup> 1.3 mmol/L in pH 8.2 Tris-HCl] was added. The absorbance value at 490 nm of reaction for 1 and 5 min were assayed. LDH activity=(A<sub>5 min</sub>-A<sub>1 min</sub>)/4. The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\% \text{ apoptosis} = \frac{\text{LDHp}}{\text{LDHp} + \text{LDHe} + \text{LDHi}} \times 100$$

$$\% \text{ necrosis} = \frac{\text{LDHe}}{\text{LDHp} + \text{LDHe} + \text{LDHi}} \times 100$$

**Caspase-3 and caspase-8 activity assay** HeLa and A375-S2 cells were cultured in 1×10<sup>6</sup> cells and treated with or without evodiamine 30 μmol/L for different time periods. After brief rinsing in phosphate buffered saline, cells were collected and lysed in 500 μL of the cell lysis buffer included in the caspase-3 apoptosis detection kit (Santa Cruz Biotechnology, USA).

The enzymatic reaction for caspase-3 activity was carried out with the DEVE-AFC substrate according to the manufacturer's instruction. The caspase-8 activity was measured using IETD-AFC substrate contained in the caspase-8 apoptosis detection kit (Santa Cruz Biotechnology, USA).

**Flow cytometric analysis of cell cycle** Flow cytometric analysis was conducted<sup>[10]</sup>. In brief, A375-S2 and HeLa cells, both adherent and floating, were collected and washed with PBS. The cells were fixed in 75 % ethanol at 4 °C overnight. After washing twice with PBS, the cells were stained with 1.0 mL of propidium iodide (PI) solution containing PI 50 mg/L (Sigma, USA), RNase A 1 g/L (Sigma, USA) and 0.1 % Triton X-100 in sodium citrate 3.8 mmol/L, followed by incubation on ice for 30 min in the dark condition. Samples were analyzed on a flow cytometer (Becton Dickinson FACScan, CA, USA) using the Cell Quest software (CA, USA) which was used to determine the percentage of cells at different phases of the cell cycle.

**Statistics** Data were expressed as mean±SD. Difference between groups was analyzed using Student's *t*-test. *P*<0.05 was considered statistically significant.

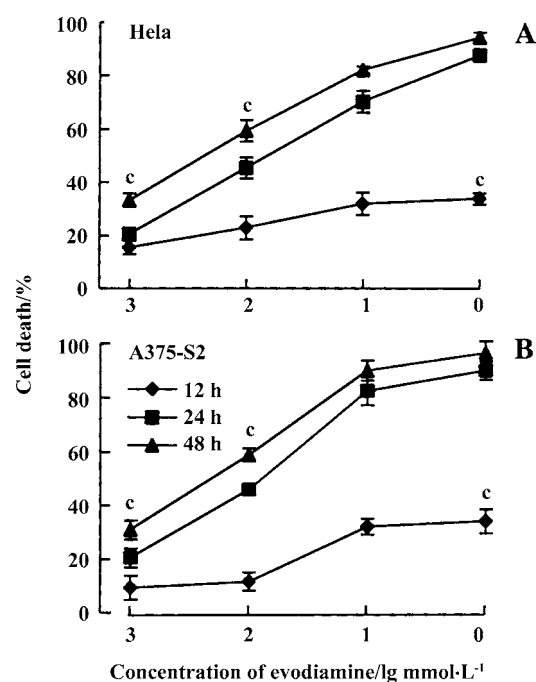
## RESULTS

### Growth inhibition of HeLa and A375-S2 cells

Evodiamine induced cell death in dose- and time-dependent manners in HeLa and A375-S2 cells. Evodiamine 3-300 μmol/L exerted potent inhibitory effects on HeLa and A375-S2 cell growth. By 48 h after evodiamine 30 μmol/L treatment, cell death rates of both cell types reached to 80 % (Fig 1).

### Evodiamine-induced morphological changes and DNA fragmentation of HeLa and A375-S2 cells

The nuclear morphological changes were observed by Hoechst 33258 staining. In control group, HeLa and A375-S2 nuclei were round and stained homogeneously with Hoechst 33258 (Fig 2A). Both in HeLa and A375-S2 cells, marked blebbing nuclei and granular apoptotic bodies were observed at 12 h after incubation. By 24 h, apoptotic characteristics in HeLa cells were more significant, but in A375-S2 cells, necrotic nuclei with weak fluorescence emission were observed. Typical DNA fragmentation was observed in evodiamine-treated HeLa cells (Fig 2B), however, in A375-S2 cells, smear-like DNA degradation characteristic to necrosis was observed (data not shown). Thus, evodiamine induced apoptosis in A375-S2 cells at the early stages, but at

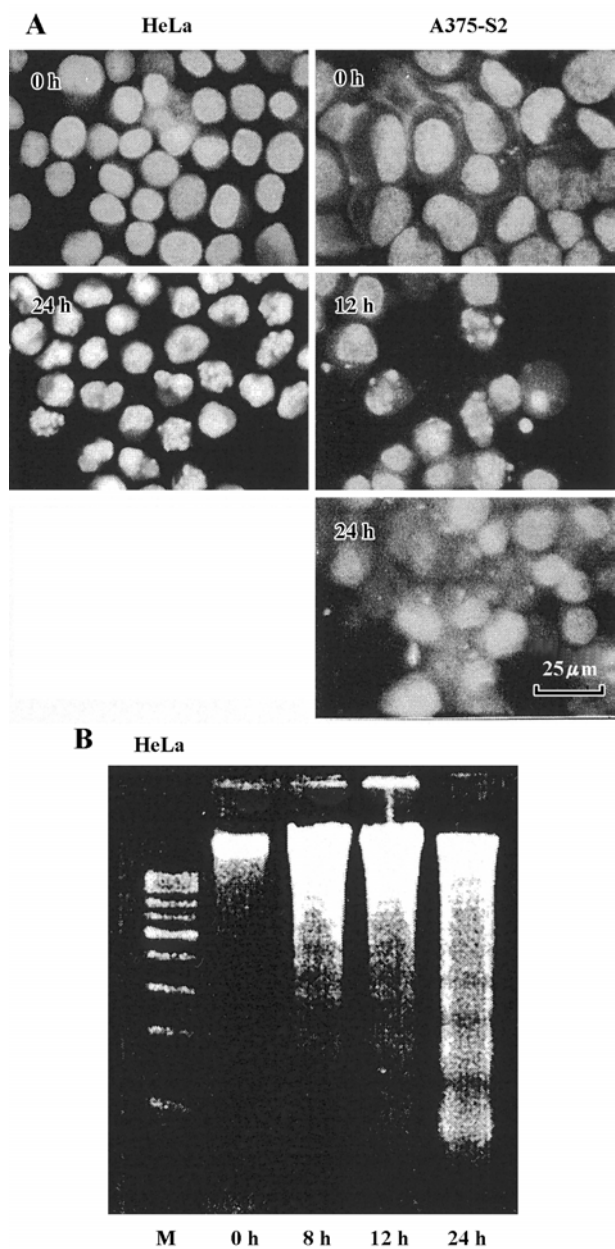


**Fig 1.** Inhibitory effects of evodiamine on (A) HeLa and (B) A375-S2 cell growth. The cells were cultured for 12, 24, and 48 h. *n*=3. Mean±SD. \**P*<0.01 vs negative control group.

later stages, it induced cell death through necrotic pathways.

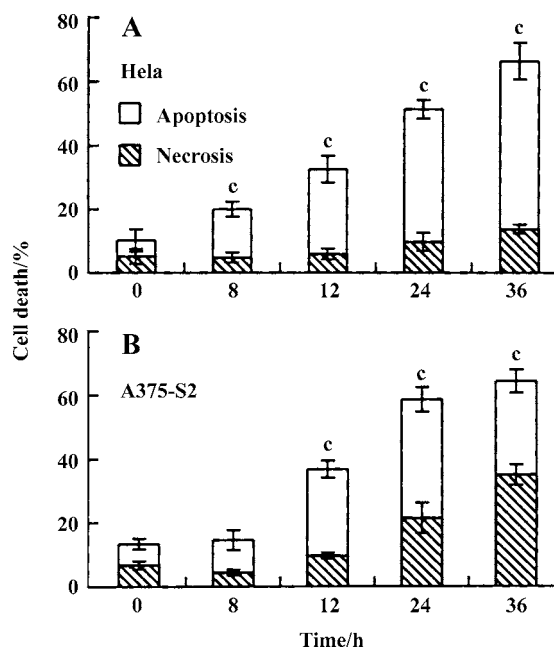
**Different types of cell death identified with LDH released assay** The rate of LDH released from viable cells, floating dead cells and the culture medium was used to distinguish the proportion of apoptotic and necrotic cells<sup>[9]</sup>. The number of apoptotic HeLa cells increased from 15.7 % at 8 h to 52.9 % at 36 h in the presence of evodiamine, however, that of necrotic cells was still negligible (Fig 3). In contrast, A375-S2 cell death before 24 h was mainly due to apoptosis, but at 24 h, the number of necrotic cells began to increase and was more than that of apoptotic cells at 36 h. These results were coincident with morphological changes and DNA fragmentation, suggesting that evodiamine induced not only apoptosis in HeLa and A375-S2, but also necrosis in A375-S2 at later stages.

**Effects of caspase inhibitors on evodiamine-induced cell death** Caspases were believed to mediate apoptotic pathway, therefore, the effects of caspase inhibitors on evodiamine-induced cell death were examined. In HeLa cells cultured for 12 h to 48 h, evodiamine-induced cell death was inhibited by z-VAD-fmk, z-DEVE-fmk, and z-IETD-fmk, but not by Ac-YVAD-fmk (Tab 1). In A375-S2 cells, Ac-YVAD-fmk also did not block evodiamine-induced cell death at 12 h,



**Fig 2.** Evodiamine-induced morphological changes of cell nuclei and DNA fragmentation in HeLa and A375-S2 cells. (A) HeLa and A375-S2 cells were incubated in the medium alone for 24 h or the medium containing 30  $\mu\text{mol/L}$  evodiamine for 12 h and 24 h. ( $\times 400$ , Hoechst 33258 stain). (B) HeLa cells were cultured with evodiamine 30  $\mu\text{mol/L}$  for 0, 8, 12, and 24 h. Hae-III-digested phage  $\phi\text{x-174}$  DNA fragments were used as molecular markers (M).

but z-VAD-fmk, z-DEVD-fmk, and z-IETD-fmk reduced the cell death. Following evodiamine treatment for 48 h, any caspase inhibitors' effects disappeared (Tab 1). These results were coincident with morphological changes of the A375-S2 cell nuclei. Interestingly, at 24 h, only z-VAD-fmk blocked A375-S2 cell death.



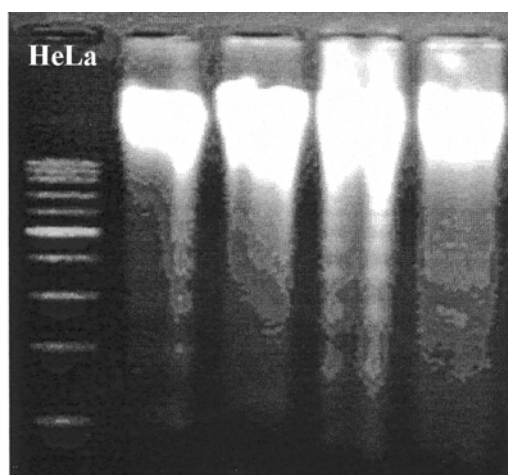
**Fig 3.** Characterization of cell death in (A) HeLa and (B) A375-S2 cells treated with evodiamine 15  $\mu\text{mol/L}$ . Cell death assessed by LDH activity-based assays was expressed as percentage of apoptosis and necrosis.  $n=3$ . Mean $\pm$ SD.  $^{\ast}P<0.01$  vs the necrosis percentage (at the same time point).

The inhibitory effects of z-DEVD-fmk and z-IETD-fmk disappeared. By 24 h, evodiamine activated other caspases except for caspase-3 and -8. Since typical apoptosis was associated with the activation of the caspases, the influences of caspase inhibitors on DNA fragmentation were examined. The results showed that z-DEVD-fmk prevented the DNA fragmentation, but z-IETD-fmk's inhibitory effect on HeLa DNA degradation was partial (Fig 4). z-IETD-fmk and z-DEVD-fmk failed to inhibit the DNA degradation in A375-S2 cells (data not shown).

**Activities of caspase-3 and caspase-8 in evodiamine-treated HeLa and A375-S2 cells** In HeLa cells, caspase-3 was significantly activated at 12 h and its activity was 6 times of negative control group at 24 h. But by 36 h, caspase-3 activity began to decline (Tab 2). Interestingly, caspase-8 was activated at 24 h and continued to increase. First, caspase-3 was activated, then caspase-8 activity began to increase. In A375-S2 cells, after administration of evodiamine, caspase-3 activity increased and reached 5 times units of control group at 24 h, while it again declined to the level of control group at 36 h. Caspase-8 was activated only at 12 h. The results were partially corresponded to the effects of caspase inhibitors on evodiamine-induced cell

**Tab 1. Effects of caspase inhibitors on evodiamine-induced cell death.** HeLa and A375-S2 cells were cultured in the presence or absence of the caspase inhibitors, z-VAD-fmk 20  $\mu\text{mol/L}$  (VAD), z-DEVD-fmk 20  $\mu\text{mol/L}$  (DEVD), z-IETD-fmk 20  $\mu\text{mol/L}$  (IETD) or Ac-YVAD-cmk 20  $\mu\text{mol/L}$  (YVAD), 1 h prior to the administration of evodiamine (EV) 15  $\mu\text{mol/L}$ , then incubated for 12, 24, and 48 h.  $n=3$ . Mean $\pm$ SD.  $^{\circ}P < 0.01$  vs evodiamine 15  $\mu\text{mol}\cdot\text{L}^{-1}$  group.

Group	12 h		Cell death/%		48 h	
	HeLa	A375-S2	HeLa	A375-S2	HeLa	A375-S2
EV	29.3 $\pm$ 0.3	37.6 $\pm$ 1.6	43.3 $\pm$ 1.1	56.8 $\pm$ 2.4	79.7 $\pm$ 3.6	84.0 $\pm$ 2.2
VAD	5.8 $\pm$ 1.0	-7.6 $\pm$ 0.8	-5.4 $\pm$ 0.6	-3.6 $\pm$ 0.2	12.1 $\pm$ 1.3	-3.3 $\pm$ 0.1
VAD+EV	18.6 $\pm$ 0.7 $^{\circ}$	0.5 $\pm$ 0.2 $^{\circ}$	16 $\pm$ 0.3 $^{\circ}$	32.3 $\pm$ 0.6 $^{\circ}$	53.2 $\pm$ 3.1 $^{\circ}$	74.3 $\pm$ 3.2
DEVD	5.6 $\pm$ 0.4	-4.8 $\pm$ 0.8	2.6 $\pm$ 0.4	7.9 $\pm$ 1.0	11.2 $\pm$ 0.9	-3.1 $\pm$ 0.4
DEVD+EV	14.9 $\pm$ 1.4 $^{\circ}$	-8.51 $\pm$ 0.6 $^{\circ}$	12.2 $\pm$ 1.7 $^{\circ}$	48.9 $\pm$ 1.3	32.8 $\pm$ 1.0 $^{\circ}$	74.4 $\pm$ 1.6
IETD	9.7 $\pm$ 1.3	6.09 $\pm$ 0.2	12 $\pm$ 0.8	16.8 $\pm$ 0.7	13.0 $\pm$ 0.5	17.8 $\pm$ 0.8
IETD+EV	26 $\pm$ 2.0	19.0 $\pm$ 1.2 $^{\circ}$	20.8 $\pm$ 1.8 $^{\circ}$	56.3 $\pm$ 2.0	50.4 $\pm$ 2.1 $^{\circ}$	83.4 $\pm$ 0.6
YVAD	3 $\pm$ 0.6	-9.5 $\pm$ 0.7	5.5 $\pm$ 0.6	13.7 $\pm$ 1.4	6.7 $\pm$ 0.3	15.9 $\pm$ 1.1
YVAD+EV	31 $\pm$ 1.1	27.1 $\pm$ 1.4	39.4 $\pm$ 2.1	51.7 $\pm$ 2.3	72.3 $\pm$ 2.3	76.1 $\pm$ 2.7



Marker	+	-	-	-	-
Evodiamine	-	+	+	+	-
z-DEVD-fmk	-	-	+	-	-
z-IETD-fmk	-	+	-	+	-
Control	-	-	-	-	+

**Fig 4. Effects of caspase inhibitors on evodiamine-induced DNA fragmentation of HeLa cells.** The cells were cultured in the absence or presence of caspase inhibitors, 1 h prior to the addition of evodiamine, then incubated for 24 h. Hae-III-digested DNA fragments were used as molecular marker. Evodiamine (30  $\mu\text{mol/L}$ ); z-DEVD-fmk (20  $\mu\text{mol/L}$ ); z-IETD-fmk (20  $\mu\text{mol/L}$ ).

death (Tab 1), except for that z-DEVD-fmk did not affect the evodiamine-induced A375-S2 cell death at 24 h, while the activity of caspase-3 was at the highest at this time point.

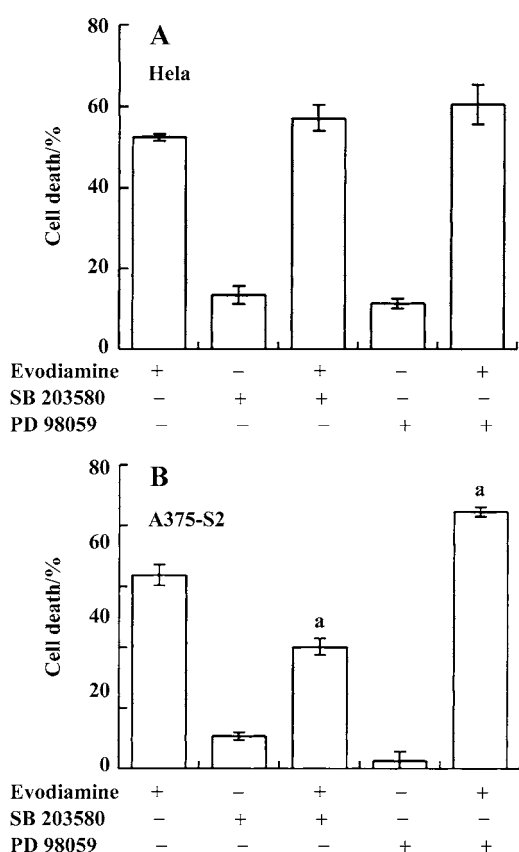
**Effects of MAPK inhibitors on evodiamine-induced HeLa and A375-S2 cell death** MAPK super-

**Tab 2. Activities of caspase-3 and caspase-8 in evodiamine (15  $\mu\text{mol/L}$ )-treated HeLa and A375-S2 cells.**  $n=3$ . Mean $\pm$ SD.  $^bP < 0.05$ ,  $^{\circ}P < 0.01$  vs 0 h group.

Time/h	Caspase-8 activity		Caspase-3 activity	
	HeLa	A375-S2	HeLa	A375-S2
0	1.5 $\pm$ 0.2	2.3 $\pm$ 0.1	2.2 $\pm$ 0.2	3.2 $\pm$ 0.1
12	1.8 $\pm$ 0.1	4.2 $\pm$ 0.3 $^{\circ}$	6.5 $\pm$ 0.5 $^{\circ}$	6.0 $\pm$ 0.2 $^{\circ}$
24	2.7 $\pm$ 0.2 $^{\circ}$	2.0 $\pm$ 0.2	12.4 $\pm$ 0.8 $^{\circ}$	10 $\pm$ 0.8 $^{\circ}$
36	4.0 $\pm$ 0.2 $^{\circ}$	2.2 $\pm$ 0.1	6.7 $\pm$ 0.8 $^b$	3.3 $\pm$ 0.5

family played an important role in apoptosis, but each MAPK had different influences on the complicate pathways of cell death. In the presence of evodiamine, selective p38 MAPK inhibitor (SB 203580) and ERK inhibitor (PD 98059) did not affect cell death at 12 h in HeLa and A375-S2 cells (data not shown). However, by 24 h, SB 203580 partially decreased A375-S2 cell death from 75.8 % to 40.2 %, but not HeLa cells (Fig 5). On the other hand, PD 98059 augmented the cytotoxicity of evodiamine against A375-S2 cells but not HeLa cells. These results suggested that at later stages, evodiamine activated p38 MAPK resulting in A375-S2 cell death but reduced ERK activity. However, in HeLa cells, evodiamine appeared to have no such effects on p38 and ERK MAPK.

**Effects of evodiamine on cell cycle arrest** The effects of evodiamine on HeLa and A375-S2 cell cycle were assayed by flow cytometry. In negative control



**Fig 5. Effect of MAPK inhibitors on evodiamine-induced (A) HeLa and (B) A375-S2 cell death.** The cells were pre-treated with SB203580 10  $\mu$ mol/L and PD 98059 10  $\mu$ mol/L for 1 h, then incubated with or without evodiamine 15  $\mu$ mol/L for another 24 h.  $n=3$ . Mean $\pm$ SD. <sup>a</sup> $P<0.01$  vs evodiamine 15  $\mu$ mol/L group.

group of HeLa, the cells at the G2/M phase was only 21.31 % at 24 h. However, Having been treated with evodiamine for 8 h, the cells arrested at G2/M phase reached to 44.84 % (Tab 3). Evodiamine induced significant G2/M arrest of HeLa cells and apoptosis induced by evodiamine mainly occurred at the G2/M phase. However, this compound did not affect any cell cycle distribution in A375-S2 cells (data not shown).

**DISCUSSION**

Majority of cells die in two distinct pathways: apoptosis and necrosis. They share some common pathways for death signal transduction<sup>[11]</sup>. Different drug doses or growing periods of cells might be a switch between apoptosis and necrosis<sup>[12]</sup>. In our present study, we also found that evodiamine not only caused typical apoptosis of HeLa cells, but also induced atypical apoptosis and necrosis of A375-S2 cells. Activated

**Tab 3. Flow cytometric analysis of the cell cycle distribution of HeLa cells treated with evodiamine 30  $\mu$ mol/L for 0, 8, 16, 24, and 36 h.**

Group	Treated time/h	Cell cycle/%			
		Sub-G1	G0/G1	S	G2/M
Control	0	4.37	51.23	25.64	19.76
	24	5.36	40.7	32.63	21.31
Evodiamine (30 $\mu$ mol/L)	8	7.25	25.75	22.16	44.84
	16	12.51	8.49	13.73	65.27
	24	32.28	8.22	20.52	39.98
	36	55.21	10.29	18.21	16.29

caspase-3 induced separation of inhibitor of caspase activated DNase (ICAD) from the DNase, resulting in degradation of DNA into fragmentation of 180 base pair units<sup>[13]</sup>. Although evodiamine induce apoptotic morphological changes in A375-S2 cells, DNA fragmentation was not observed, suggesting that A375-S2 cells may have some specific DNases, substrates of caspase, which did not induced typical apoptotic DNA ladder in the electrophoresis. Cytotoxicity assay also demonstrated that in A375-S2 cells, apoptotic pathway was dominant before 24 h, but necrosis became obvious at 36 h. Thus, it was probable that some as-yet unidentified proteases or protein kinases participate in this switching mechanism.

Unexpectedly, downstream caspase-3 was first activated by evodiamine, while upstream caspase-8 was later activated. This was different from previous reports<sup>[14]</sup> in which upstream caspase-8 was activated prior to the activation of downstream caspase-3. It was possible that before activation of upstream caspase-8, downstream caspase-3 was activated through other apoptotic pathways such as mitochondrial one by which the activation of caspase-9 initiated the activation of caspase-3. Then, activated caspase-3 in turn activated caspase-8, which amplified the caspase cascade<sup>[15]</sup>. In A375-S2 cells, caspase-3 was activated to high level by 24 h, but caspase-3 inhibitor failed to prevent evodiamine-induced A375-S2 cell death. Thus, at this time point, the induced cell death was not absolutely due to the activation of caspase-3.

ERK and p38 MAPK play important roles in cell survival and death. ERK MAPK was believed to protect cells from death, however, p38 MAPK promoted apoptosis<sup>[16]</sup>. On the other hand, in macrophages the activation of p38 MAPK led to NF- $\kappa$ B translocation to

the nuclei, resulting in inhibition of apoptosis<sup>[17]</sup>. Over 24 h incubation, caspase inhibitors failed to block A375-S2 cell death induced by evodiamine, suggesting that there must be other molecular mechanism to regulate the cell death. A375-S2 cell death induced by TNF or IL-1 was reported to be mediated by caspases and p38<sup>[18]</sup>. At 24 h, pretreatment with SB203580 reduced the number of apoptotic cells in A375-S2, but not in HeLa. On the contrary, pretreatment with PD98059 increased apoptosis in A375-S2 and had no significant effect on HeLa. These results suggest that at early stages, caspases contributed to evodiamine-induced A375-S2 cell death, but at later stages, the participation of p38 MAPK in the cell death pathway was more obvious.

Evodiamine induced cell cycle arrest at G2/M phase in HeLa cells, but failed to affect A375-S2 cells. Causal relationship between G2/M arrest and apoptotic signaling in evodiamine-treated HeLa cells remains to be elucidated.

In conclusion, evodiamine induced caspase-dependent apoptosis in HeLa cells which was related to the G2/M arrest. However, evodiamine initiated caspase-mediated apoptosis and necrosis in which the activation of p38 and ERK MAPK was involved.

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