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Apoptosis and necrosis induced by sulfur mustard in Hela cells

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KEY WORDS apoptosis; cell cycle; necrosis; mustard gas; Hela cells; cultured tumor cells

ABSTRACT

AIM: To study the apoptotic effect of sulfur mustard (SM) on Hela cells. **METHODS**: Exponentially growing Hela cells were treated with SM at various concentrations for 3 h, then apoptosis was examined by electron-microscope, DNA gel electrophoresis, and flow cytometry. **RESULTS**: SM 1 μmol · L^{-1} arrested cell growth. After treatment with SM 10 - 100 μmol·L^{-1}, cells were mainly blocked at G_1-phase with apoptosis. Agarose gel electrophoresis of DNA from cells treated with SM revealed "DNA Ladder." About 33 % of the Hela cells showed apoptosis 12 h after 3-h treatment with SM 100 μmol · L^{-1} as determined by flow cytometry and the S-phase cells were more susceptible. However, SM 1000 μmol · L^{-1} caused marked necrosis in Hela cells. **CONCLUSION**: SM caused 2 distinct forms of cell death, apoptosis or necrosis, in Hela cells in a concentration-dependent manner.

INTRODUCTION

Sulfur mustard [bis-(2-chloroethyl)sulphide, SM] is the first chemical compound shown to possess a mutagenic activity[1]. It is a radiomimetic alkylating agent that has mutagenic, carcinogenic, and cytotoxic properties. It is also a powerful vesicant that produces incapacitating injuries at the site of exposure. SM alkylates a range of biomolecules and DNA is the most sensitive molecular target of SM. Despite decades of years of research on SM, the mechanism of toxic action of SM is not very clear[2-5].

In recent years, apoptosis induced by alkylating agents such as nitrogen mustard[6], ethylmethane-sulfonate[7], and ethyl nitrosourea[1] have been reported. SM-induced apoptosis was also observed in the bovine pulmonary artery endothelial cells[8]. In this study, the apoptotic as well as necrotic effects of SM on Hela cells were investigated.

MATERIALS AND METHODS

SM (97 % purity) from our institute was dissolved in 1.2-propanediol and stored at 4 ℃. DMEM was purchased from Gibco. DNA molecular markers and RNase A were from Sino-American Biotech Co. Propidium iodide (PI) was from Sigma Co.

Cell culture and drug treatment Hela cells, a gift from Institute of Microbiology and Epidemiology were cultivated in DMEM supplemented with 10 % heat-inactived calf serum, benzylpenicillin 100 kU · L^{-1}, streptomycin 100 kU·L^{-1} in a humid atmosphere containing 5 % CO_2 at 37 ℃. Exponentially growing cells (2×10^6 cells·L^{-1}) were exposed to SM for 3 h, then maintained in fresh drug-free medium for 12 - 24 h for assessment.

Morphological assessment Morphological changes of the cells was examined by electron microscopy. Control and SM-exposed cells were collected with a cell scraper, followed by three 10-min washes in phosphate buffer saline (PBS, NaCl 8.0, KCl 0.2, $Na_2HPO_4 \cdot H_2O$ 1.56, and KH_2PO_4 0.2 g· L^{-1}, pH 7.4) at 20 ℃. The cell pellet was fixed with gentle agitation at 20 ℃ for 1 h in PBS containing 3.0 % glutaraldehyde, rinsed with PBS thrice, postfixed at 20 ℃ in PBS containing 1 % osmium tetroxide for 1 h, dehydrated in grading ethanols, and embedded in epoxy resin. Sections (100 nm thick) were counterstained with uranyl acetate and lead citrate, and examined under transmission electron microscope

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(Philips 400T).

DNA content and cell cycle Cells were harvested with trypsin, washed twice in ice-cold PBS by centrifugation, fixed in 70 % ethanol, and incubated with 10 μL RNase A (5 g·L^{-1}) at 37 ℃ for 30 min. The cellular DNA was stained with PI 0.05 g·L^{-1}) for 30 min. The DNA contents of cells at distinct phases in the cell cycle were determined by FACS 420 flow cytometer[9]. The DNA content less than that in the G_1-phase indicated the occurrence of apoptosis. The data were processed by DNA cell-cycle analysis software (Becton Dickson, USA).

DNA gel eletrophoresis The fragmentation of Hela cell DNA was assessed according to the method[10] with minor modifications. After drug treatment, aliquots of 500 μL lysis solution (Tris-HCl 100 mmol·L^{-1}, pH 8.5, disodium edetate 5 mmol·L^{-1}, NaCl 0.2 mol·L^{-1}, 0.1 % sodium dodecyl sulfate, and proteinase K 0.2 g·L^{-1}) were added and incubated at 37 ℃ overnight. NaCl was then added at a final concentration of 1.5 mol·L^{-1} and the nuclear debris was spun down in a microcentrifuge at about 18 ℃ for 15 min. The DNA in the supernatant was precipitated with 70 % ethanol and dissolved in 40 μL of TE (Tris-HCl 10 mmol·L^{-1}, pH 7.5, disodium edetate 1 mmol·L^{-1}) containing DNase-free RNase A 0.2 g·L^{-1}. After incubation at 37 ℃ for 2 h, the DNA was loaded onto a 1.5 % agarose gel containing ethidium bromide 5 mg·L^{-1} in TBE (Tris 45 mmol·L^{-1}-borate buffer, disodium edetate 1 mmol·L^{-1}, pH 8.0) and electrophoresed at 50 V for 2 h. DNA in gel was visualized under UV light.

Statistical analysis Data were analyzed by *t*-test.

RESULTS AND DISCUSSION

Cell growth The growth of Hela cells exposed to SM 1 – 100 μmol·L^{-1} for 3 h and in fresh medium for 12 – 24 h was retarded within 12 h and profoundly inhibited at 24 h in a concentration-dependent fashion (Fig 1).

Cell morphology The control cells had large nuclei and nucleoli, evenly distributed chromatin and intact membrane. Treatment of Hela cells with SM 100 μmol·L^{-1} for 3 h and in fresh medium for 24 h caused condensation of the chromatin, membrane-enclosed nuclear fragments, whereas ribosomes were well preserved. It implied that lower concentration (100 μmol·L^{-1}) of SM induced apoptosis of Hela cells.

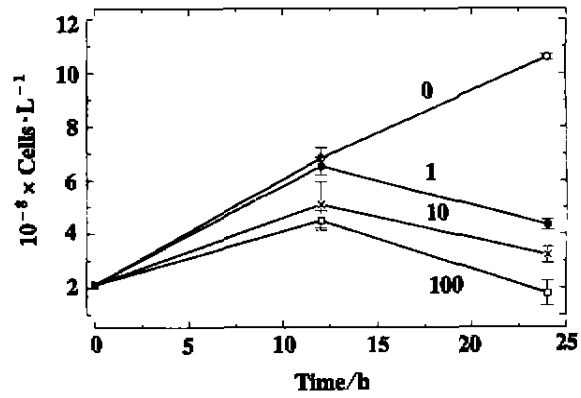

Fig 1. Inhibition of growth of Hela cells by sulfur mustard (1, 10, 100 μmol·L^{-1}). $n = 3$ experiments (3 samples in each experiment, 2 × 10^6 cells·L^{-1}, 10 mL in each sample). $\bar{x} \pm s$.

After the Hela cells were treated with SM 1000 μmol·L^{-1} in the same way, the cell membrane lost its structural integrity, showing a typical pattern of cell necrosis (Fig 2).

DNA fragmentation Agarose gel electrophoresis of DNA extracted from cells treated with SM 10 or 100 μmol·L^{-1} revealed patterns of "DNA ladder" (Fig 3), suggesting the preferential degradation of DNA molecules at the internucleosomal linker regions (apoptosis).

DNA from the Hela cells exposed to SM 1 or 10 mmol·L^{-1} showed smear patterns (Fig 4), suggesting the occurrence of necrosis. These results were in accordance with the above-mentioned morphology observations.

Cell cycle After treatment of Hela cells with SM 100 μmol·L^{-1} for 3 h, the G_1-phase cells varied from 41 % to 50 %, and G_2/M phase cells from 8 % to 15 %, whereas S-phase cells were markedly decreased from 51 % to 35 %. It suggested that SM mainly arrested Hela cells at G_1-phase, S-phase cells preferentially underwent apoptosis. The percentages of apoptotic cells concurrently increased from 1.9 % to 33 % in a concentration-dependent manner (Tab 1).

A 3 h-incubation in SM 100 μmol·L^{-1} and in drug-free DMEM for 6 h resulted in 6.3 % of apoptosis of Hela cells. The percentages of apoptotic cells were

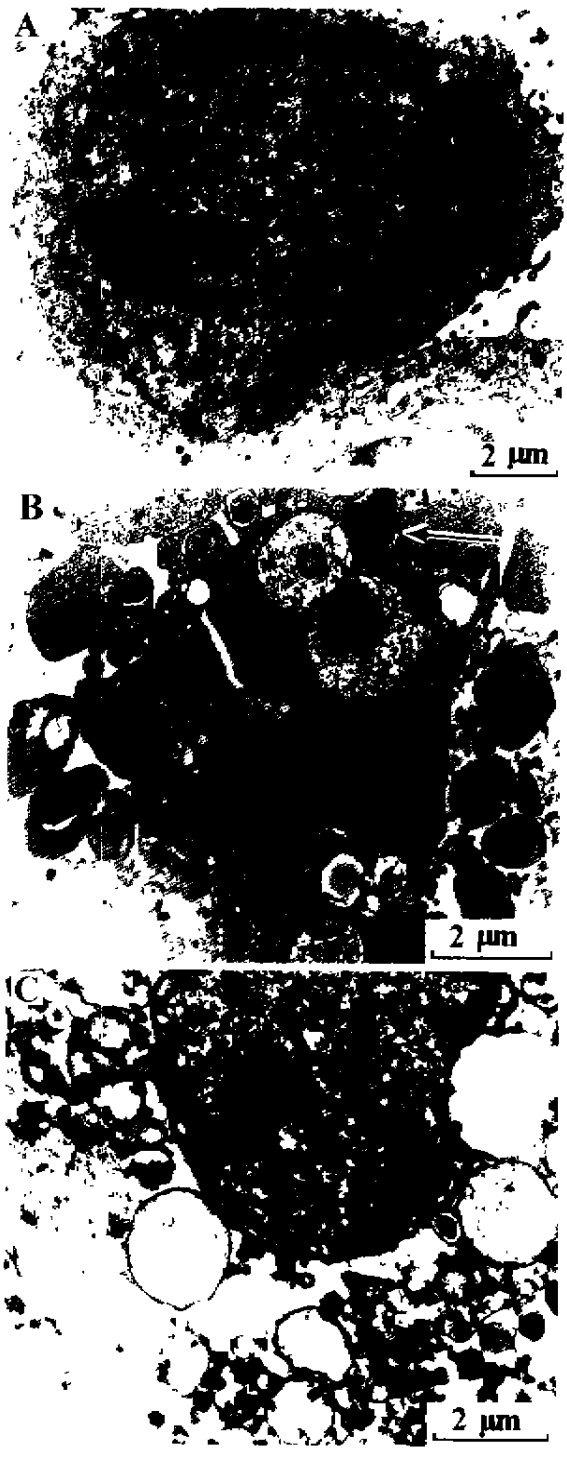

Fig 2. Electron microscopy of Hela cells, PI stain. A) Control, × 6000; B) treated with sulfur mustard 100 μmol·L^{-1} for 3 h and in fresh medium for 24 h, × 8000; C) 1000 μmol·L^{-1} for 3 h and in fresh medium for 24 h, × 8000.

increased within 24 h (33 % at 12 h and 49 % at 24 h).

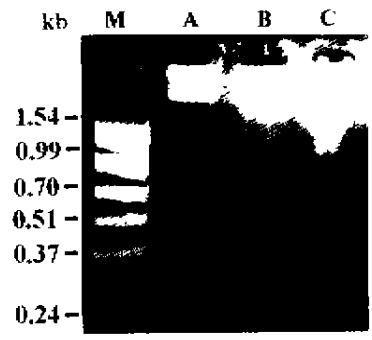

Fig 3. Agarose gel electrophoresis of DNA extracted from Hela cells treated with SM for 12 h. M: molecular weight markers. A) Control; B) sulfur mustard 10 μmol·L^{-1}; (C) 100 μmol·L^{-1}.

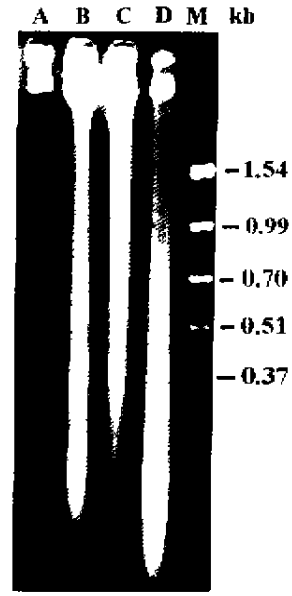

Fig 4. DNA smear induced by SM. M: PCR molecular weight markers. A) Control; B) SM 10 mmol·L^{-1} for 12 h; C) 1 mmol·L^{-1} for 12 h; D) 10 mmol·L^{-1} for 24 h.

Studies in this paper on the alkylation-induced cell death in the human cell line (Hela) showed that SM elicited either apoptosis or necrosis of cells in a concentration-dependent manner.

Besides SM, nitrogen mustard[6], ethylmethanesulfonate[7], and ethyl nitrosourea[7] are all able to induce apoptosis in human cells, suggesting that the pathological and biochemical apoptotic changes are a general characteristic of alkylating agents. The cell

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Tab 1. Effect of SM on the cell cycle of Hela cells. Exponentially growing cells were treated with SM 1 – 100 μmol·L^{-1} for 3 h and then incubated in drug-free medium for 12 h. $n = 3$ samples (10 000 cells in each sample). $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* control.

Sulfur mustard/ μmol·L^{-1}	Cells cycle distribution/% G_1	S	G_2/M	Apoptosis/ %
0	41 ± 6	51 ± 6	8 ± 1	1.9 ± 2
1	43 ± 6^a	49 ± 6^a	8 ± 4^a	7.4 ± 1^b
10	51 ± 6^a	38 ± 2^b	11 ± 2^a	15 ± 3^c
100	50 ± 4^a	35 ± 7^b	15 ± 5^a	33 ± 4^c

cycle and its biochemical events were well documented in the recent years, lots of factors accelerating or retarding the process of cell cycle have been discovered. It is tenable in prospect of potential use of some of the factors in prophylaxis of SM injury.

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硫芥诱导 Hela 细胞发生凋亡及坏死

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关键词 细胞凋亡; 细胞周期; 坏死; 芥子气; Hela 细胞; 培养的肿瘤细胞

目的: 研究硫芥诱导 Hela 细胞凋亡的作用. **方法**: 生长在 DMEM 培养基中的 Hela 细胞与不同浓度的硫芥作用 3 小时, 凋亡用电镜, 电泳及流式术检测. **结果**: 低浓度硫芥(1 μmol·L^{-1})抑制细胞生长; 较高浓度(1 – 100 μmol·L^{-1})使细胞主要在 G_1 期阻滞, 发生典型的凋亡形态改变, 提取细胞 DNA 进行琼脂糖凝胶电泳, 出现"DNA Ladder". 流式术观察表明硫芥处理 3 小时后, 细胞撤药培养 12 小时, 凋亡率达 33 %. S 期的细胞最敏感. 硫芥 1000 μmol·L^{-1}使 Hela 细胞发生明显的坏死. **结论**: 硫芥诱导 Hela 细胞发生两种不同的损伤: 坏死及凋亡. 硫芥的细胞毒有浓度依赖性.

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