Molecular mechanism of apoptosis induced by ricin in HeLa cells

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KEY WORDS ricin；HeLa cells；cysteine proteinases；scanning electron microscopy；Western blotting；electron microscopy；apoptosis；flow cytometry；cell cycle

ABSTRACT

AIM：To study the morphological changes and molecular mechanism of HeLa cell apoptosis induced by ricin. METHODS：HeLa cells were coincubated with ricin 0.05 $\mu mol \cdot L^{-1}$ for 1，2，3，6，12，18，and 24 h，then scanning electron microscopy (SEM)，transmission electron microscopy (TEM)，Western blot，cell cycle，cell cytotoxicity，and cell viability were assayed. RESULTS：The typical apoptosis was induced by ricin 0.05 $\mu mol \cdot L^{-1}$ and necrotic cells increased after being cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ for more than 12 h. The apoptotic cells mainly showed cytoplasmic membrane blebbing，chromatin condensation and fragmentation，and crescentic nuclear and membrane bound apoptotic bodies formation. No detectable levels of p53，Bax，Bcl-2 and the subunit p20 of interleukin-1 beta-converting enzyme (ICE) were found by Western blot，but the active subunit p17 of 32-kDa putative cysteine protease (CPP32) was detected at 3，6，and 9 h after ricin treatment. The activity of CPP32 in HeLa cells increased 4 to 5 folds after being treated with ricin 0.05 $\mu mol \cdot L^{-1}$ and reached the peak at 6 h of treatment. There was no significant difference of ICE activity between the ricin treated cells and control cells. The percentage of G_2/M cells increased from 13.9 % ± 0.5 % to 33.2 % ± 0.5 % after 24 h of ricin 0.05 $\mu mol \cdot L^{-1}$ treatment. CONCLUSION：CPP32 but not ICE was involved in the ricin-induced apoptosis in HeLa cells. Ricin 0.05 $\mu mol \cdot L^{-1}$ had no effect on the G_0/G_1 phase of cell cycle，but induced G_2/M arrest.

INTRODUCTION

Ricin is a cytotoxic protein which can be easily isolated from castor seeds (*Ricinus communis*) by standard biochemical methods. It is one of the most toxic plant toxin. Ricin is a heterodimetric protein which consists of a 34-kDa A chain (RTA) linked by a disulfide band to a 32-kDa B chain (RTB). The B chain binds to beta-galactoside-terminated oligosaccharides on the surface of mammalian cells. After binding，the toxin is internalized to cells，and RTA enters the cytosol. RTA is a *N*-glycosidase. It attacks ribosomes，removing a specific adenine base from the 28s rRNA (A4324 in rat) and thereby inhibits protein synthesis[1]. It induced cell death and apoptosis at some instances[2].

A new class of protease has been implicated in apoptotic cell death belonging to caspase family of cysteine protease. These consist of at least 10 different homologues，including CPP32/YAMA/apopain[3]，Nedd2 (mammalian Ich-1)/Ich-1 [a gene related to the C. elegans cell death gene (Ced-3) and the mammalian homolog of Ced-3][4]，TX/Ich-2/ICE_{rel}II[5]，ICE_{rel}III[6]，Mch2 (mammalian Ced-3 homologue gene)[7]，Mch3/ICE-LAP3/CMH-1 (CPP32/Mch2 homolog 1)[8]，Mch-4，and Mch-5[9]. Common features of certain members of this family include a conserved active site QACRG pentapeptide and require proteolytic cleavage of the precursor at aspartic acid residues for activation.

Among the caspase homologues，32-kDa putative cysteine protease (CPP32) has been deemed an attractive candidate as a putative mediator of the mammalian apoptosis. Following the induction of apoptosis CPP32 can be maximally activated when the proenzyme is cleaved from a 32-kDa isoform into active 17- and 13-kDa fragments[10]. Activated CPP32 can proteolytically cleave several important intracellular，membrane，and nuclear proteins that can facilitate cell death during apoptosis[11] when they degraded.

In this study，we tried to study the morphological changes，mechanism of apoptosis induced by ricin and

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explore the role of two caspase proteases, interleukin-1 beta-converting enzyme (ICE) and CPP32 following ricin induced cell apoptosis in HeLa cells.

MATERIALS AND METHODS

Reagents The fluorogenic substrate *N*-acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) and *N*-acetyl-Tyr-Val-Ala-Asp-7-amino-4-methylcoumarin (Ac-YVAD-AMC) were purchased from PharMingen (a Becton Dickinson Co, USA). SDS, acrylamide, and 3, 3′-diaminobenzidine (DAB) were purchased from Sigma Chemical Co. (St Louis, MO, USA). The Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco, BRL (Grand Island, NY, USA). Ricin was isolated by our laboratory and the purity was identified by SDS-PAGE electrophoresis. The polyclonal goat anti-ICE p20, ICH-1_L, c-Fos, Bcl-2, Bax, CPP32, and mouse IgG2α monoclonal anti-p53 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture The human epithelial HeLa cell line was preserved by our laboratory and cultured in DMEM containing 10 % fetal bovine serum. Hanks' solution containing 0.02 % edetic acid was used for digesting cells in cell passage.

Scanning electron microscope and transmission electron microscope observation The HeLa cells cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ were collected and fixed in 2.5 % glutaraldehyde at 0 ℃ for 4 h. Following fixation cells were washed twice with PBS, fixed in osmic acid, and samples were prepared. SEM and TEM observations were performed.

Agarose gel electrophoresis DNA was extracted from cells incubated with ricin 0.05 $\mu mol \cdot L^{-1}$ for 9 and 12 h. The DNA was electrophoresed using a 0.8 % agarose gel with ethidium bromide staining.

Cytotoxicity and cell viability assays HeLa cells were seeded at 1×10^4 cells/well in 200 μL medium into 96-well plates and allowed to grow for 24 h before addition of the different substances. Then the HeLa cells were cultured with ricin 0.001, 0.1, and 10 $\mu mol \cdot L^{-1}$ for 0, 3, 6, 9, 12, 18, and 24 h. Tetrazolium salt (MTT) was added 20 μL/well and incubated for 4 h. A 10 % SDS 100 μL was added and incubated at 37 ℃ for 16 h. The optical density was read at 570 nm wavelength. All of the cytotoxicity and cell viability assays were performed in triplicate.

Cell cycle analysis The HeLa cells were collected after being cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ for 0, 6, 12, 18, and 24 h. Cell cycle analysis was performed by fixing cell with 75 % alcohol in PBS. The cells were collected by centrifugation and resuspended in propidium iodide (PI 50 $mg \cdot L^{-1}$, 0.05 % Triton-X 100, edetic acid 18 $mg \cdot L^{-1}$, RNase A 1×10^5 $IU \cdot L^{-1}$) in PBS. After a 30-min incubation at 4 ℃ the cells were centrifuged and the pellet was resuspended in PBS. DNA content was determined by quantitative flow cytometry[12].

Preparation of whole cell lysates for Western blot HeLa cells 2×10^7 were lysed in 500 μL boiling 2 × SDS-PAGE loading buffer. The lysates were sheared by passing them 5 times through a 4-gauge needle[13]. Lysates 50 μL were separated via SDS-10% PAGE, and transferred onto nitrocellulose membrane. Blots were incubated overnight at 4 ℃ with primary antibody. Membranes were washed 3 times in PBS and incubated with horseradish peroxidase-conjugated second antibody (dilution 1∶1000) at 37 ℃ for 1 h. DAB was used as a detecting reagent. The primary antibodies were used at the following dilutions: anti-p53 (1∶1000), anti-CPP32 (1∶100), anti-ICE p20 (1∶100), anti-Ich-1L (1∶500), anti-Bcl-2 (1∶1000), anti-Bax (1∶1000), anti-c-Fos (1∶1000).

The activity of CPP32 and ICE assay The HeLa cells were collected after being cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ for 0, 3, 6, 9, and 12 h and washed with PBS. Cells 2×10^6 were lysed in cell lysis buffer (Tris 10 $mmol \cdot L^{-1}$ pH 7.5, NaCl 130 $mmol \cdot L^{-1}$, 1 % Triton-X 100, Na_3PO_4 10 $mmol \cdot L^{-1}$, $Na_4P_2O_7$ 10 $mmol \cdot L^{-1}$). For each reaction, Ac-DEVD-AMC (or Ac-YVAD-AMC) 20 $\mu mol \cdot L^{-1}$ and cell lysate 50 μL were added to 1 mL protease assay buffer (HEPES 20 $mmol \cdot L^{-1}$, pH 7.5, glycerol 10 %, dithiothreitol 2 $mmol \cdot L^{-1}$). The reaction mixtures were incubated at 37 ℃ water for 2 h. The fluorescence was measured at an exitation wavelength 380 nm, emission wavelength 439 nm (Ac-DEVD-AMC) or 441 nm (Ac-YVAD-AMC)[14].

Data analysis Data were expressed as $\bar{x} \pm s$ obtained from at least 3 independent experiments. The paired *t*-test was used.

RESULTS

Cell death and morphological changes of apoptotic cells induced by ricin The cytotoxicity and

cell viability assay showed that ricin 0.001 $\mu mol \cdot L^{-1}$ was enough to cause cell death. The cell viability was 61.0 % ± 1.6 % after 24 h of ricin 0.001 $\mu mol \cdot L^{-1}$ treatment. The toxicity increased when the ricin concentration was increased. The cell viability was 39.6 % ± 2.7 % after 24 h of ricin 10 $\mu mol \cdot L^{-1}$ treatment (Fig 1).

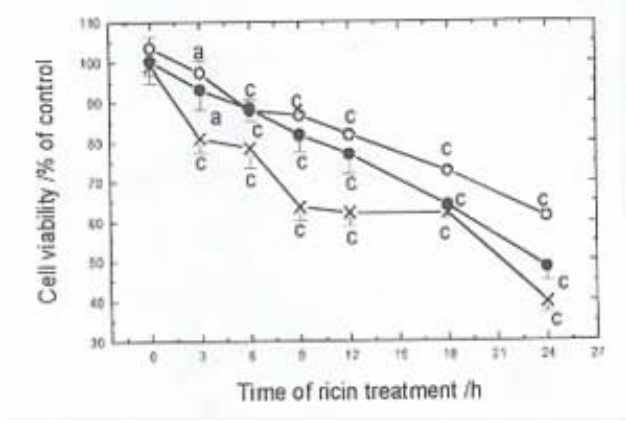

Fig 1. Effect of ricin on cytotoxicity and cell viability. Ricin 0.001 $\mu mol \cdot L^{-1}$(○), 0.1 $\mu mol \cdot L^{-1}$(●), 10 $\mu mol \cdot L^{-1}$ (×). $n = 3$. $\bar{x} \pm s$. $^{a}P > 0.05$, $^{c}P < 0.01$ *vs* 0 h.

The typical apoptosis was induced by ricin 0.05 $\mu mol \cdot L^{-1}$. There were a lot of blebs and spikes (Fig 2) observed under phase-contrast microscope on the surface of apoptotic cells when being cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ as early as 3 to 4 h. SEM observation showed shrinkage of apoptotic cells, blebs, and spikes on their surface after ricin 0.05 $\mu mol \cdot L^{-1}$ treatment for 6 h (Fig 3A). The cells were disintegrated by losing the blebs and spikes when the incubation time was prolonged. TEM observation showed cytoplasmic membrane blebbing, chromatin condensation and fragmentation, crescentic nuclear (Fig 3B) and membrane bound apoptotic bodies formation (Fig 3C) of apoptotic cells. Agarose gel eletrophoresis showed that DNA was cleaved in apoptotic cells, but no typical DNA ladder was found (Data not shown). The necrotic cells increased when being cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ for more than 12 h. The HeLa cells mainly showed necrosis when the ricin concentration was higher than 1 $\mu mol \cdot L^{-1}$. The necrotic cells showed no specific structure and there were many vacuoles in the nucleus (Fig 3D).

Cell cycle G_2/M arrest induced by ricin The flow cytometric analysis showed that there was no effect on G_0/G_1 phase of cell cycle when cultured with ricin 0.05 $\mu mol \cdot L^{-1}$ for 0, 6, 12, 18, and 24 h. The percentage of S phase cells increased and G_2/M phase cells

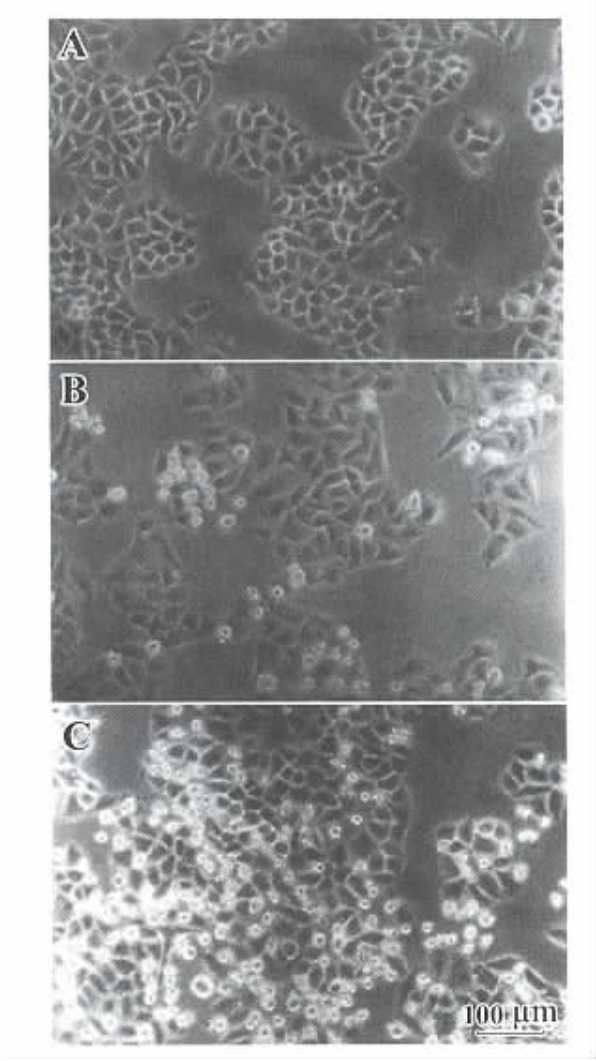

Fig 2. The phase-contrast micrograph of HeLa cells. A, normal HeLa cells; B, the HeLa cells treated with ricin 0.05 $\mu mol \cdot L^{-1}$ for 6 h; C, for 18 h. ×100.

dropped a little at 6 and 12 h of incubation, but there was no significant difference compared with 0 h group ($P > 0.05$). While the percentage of S phase cells decreased andG_2/M phase cells increased at 18 and 24 h of incubation with ricin 0.05 $\mu mol \cdot L^{-1}$ and there were significant differences ($P < 0.01$) compared with 0 h group. The % of S phase cells was 5.2 % ± 4.8 % and G_2/M phase cells 33.2 % ± 0.4 % at 24 h of incubation (Fig 4, 5).

Activation of CPP32 c-Fos was expressed at early as after 30 min of incubation. Strong expression was seen at 1, 2, 3, 6, and 9 h of ricin 0.05 $\mu mol \cdot L^{-1}$ treatment and decreased at 12 h. The CPP32 was activated and the 32-kDa proenzyme was cleaved. The active subunit p17 was detected at 3, 6, and 9 h of incubation and reached the peak at 6 h. The amount of p17 subunit decreased at

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9 h. The 45 kDa ICE was detected

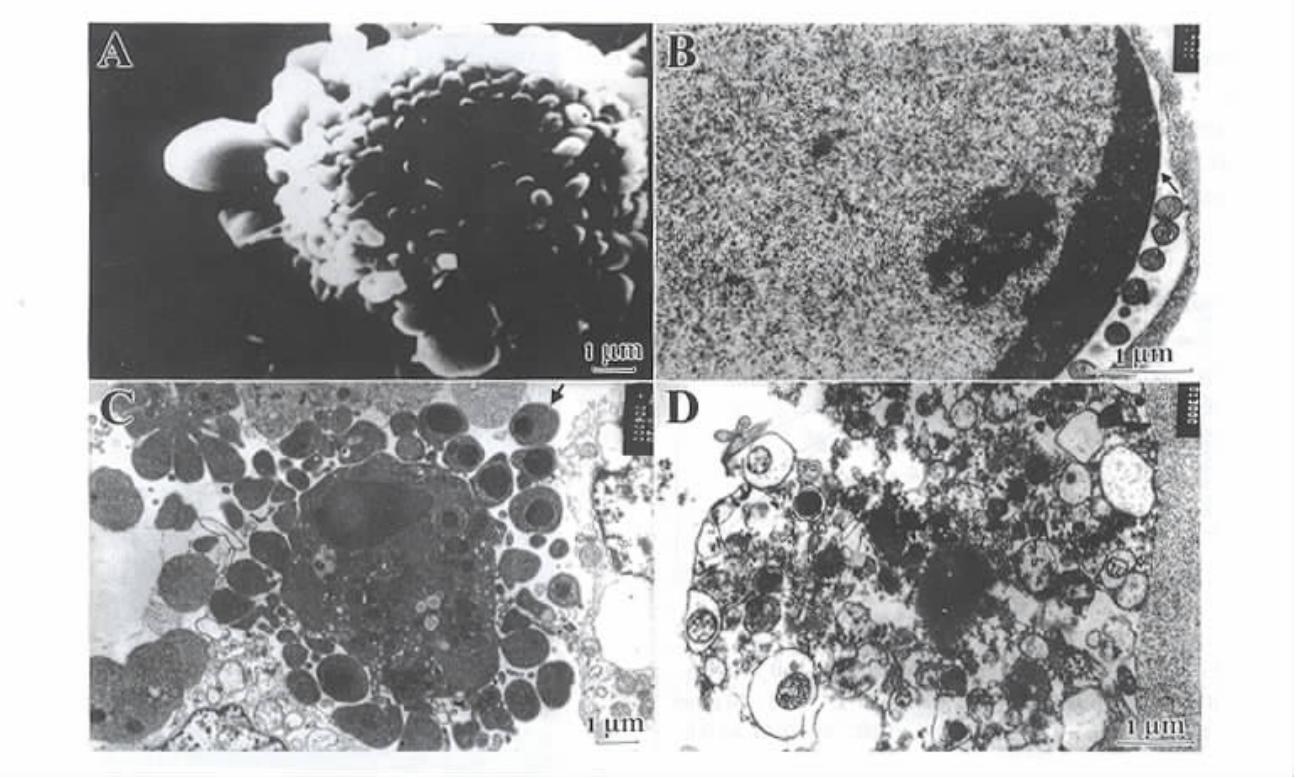

Fig 3. SEM (A) and TEM (B, C, D) electron micrograph of HeLa cells treated with ricin 0.05 μmol·L^{-1}. **A, HeLa cells treated with ricin 0.05 μmol·L^{-1} for 6 h and the blebs on the surface of the apoptotic cells.** × 5700; **B, The chromatin of apoptotic cells condensed and formed crescentic nucleus (↑, × 17 000) and C, membrane bound apoptotic bodies (⇑, ×6000) after ricin 0.05 μmol·L^{-1} treatment for 9 h; D, the HeLa cell undergoing necrosis when treated with ricin 1 μmol·L^{-1} for 9 h.** × 13 000.

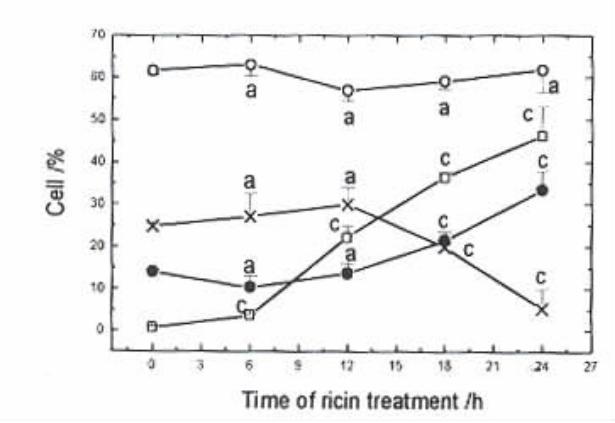

Fig 4. Effects of ricin 0.05 μmol·L^{-1} on G_0/G_1 (○), G_2/M (●), S (×) phase of HeLa cells. (□) showed the apoptotic cells induced by ricin 0.05 μmol·L^{-1}. $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^cP < 0.01$, *vs* 0 h.

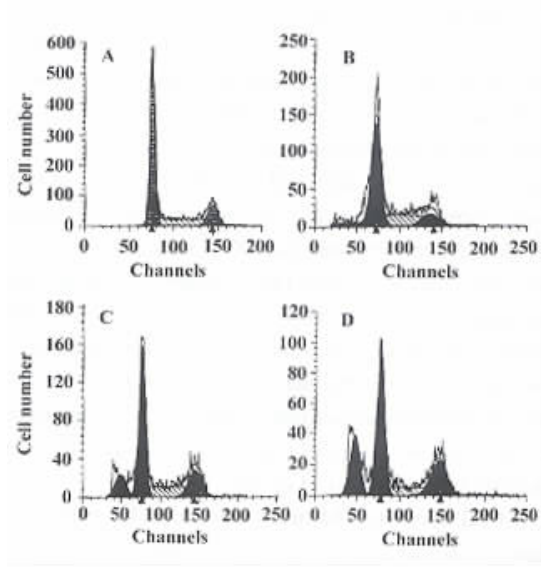

Fig 5. The flow cytometric analysis of cell cycle. A, normal HeLa cells. B, C, D showed 6, 12, and 24 h of ricin 0.05 μmol·L^{-1} treatment, respectively.

at 0 and 1 h of incubation. The active subunit p20 was only detected at 0 h. No 45 kDa ICE or p20 subunit

were found after 1 h of incubation. The caspase-2 Ich-1_L (mRNA encodes a protein product of 435 amino acids that is homologous to both the p20 and p10 subunits of ICE and the entire CED-3 protein) was detected at 3, 6, and 9 h of incubation. The peak was at 3 h and decreased at 9 h. No detectable p53, Bax, and Bcl-2 were found in this study (Data not shown).

CPP32 activity induced by ricin The activity of CPP32 began to increase at 3 h of incubation with ricin 0.05 μmol·L^{-1}. The fluorescence densities were 4.4 ± 0.5 at 3 h, 9.1 ± 0.5 at 6 h, 3.8 ± 0.3 at 9 h, 2.6 ± 0.2 at 12 h compared with 0 h of incubation (2.1 ± 0.2), $P < 0.01$. The ICE activity did not increase throughout the process of observation (Fig 6).

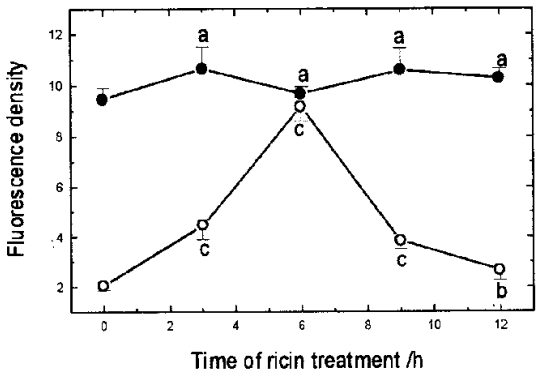

Fig 6. CPP32 and ICE activity assay. The CPP32 substrate Ac-DEVD-AMC was cleaved and the fluorescence increased (○); the ICE substrate Ac-YVAD-AMC was not cleaved and the fluorescence had no change (●). $n = 3$. $\bar{x} \pm s$. $^aP > 0.05$, $^bP < 0.05$, $^cP < 0.01$ *vs* 0 h.

DISCUSSION

The cell death induced by ricin showed two forms, apoptosis at low concentration of ricin and necrosis at high concentration. In this study, we observed that the typical apoptosis was induced by ricin 0.05 μmol·L^{-1}. We knew that many of the changes in apoptosis were highly dynamic and potentially asynchronous. We may well explain why ricin-induced apoptosis showed no typical DNA ladder. The presence of laddering in DNA gels implies an apoptosis process, but its absence does not rule it out.

Damage to the rRNA by ricin leads to the activation and transcription of the immediate-early gene c-Fos. In this study, two bands of c-Fos were observed. The band with greater molecule may be c-Fos combined with DNA[14,15]. No detectable level of p53 was found throughout the course of experiment. This may agree with the G_2/M arrest of cell cycle induced by ricin, since the p53 induces G_0/G_1 arrest.

The results from Western blot experiments suggested that Bax, Bcl-2, ICE and its active subunit p20 were not involved in the ricin-induced apoptosis. The presence of the active subunit p17 of CPP32 in cells undergoing apoptosis induced by ricin suggested an active role for CPP32 protease. Cleavage of Ac-DEVD-AMC offered another proof that the CPP32 was involved in the apoptosis process[10]. The fluorogenic substrate cleaved by cell lysates from ricin-treated HeLa cells for 6 h was 4 to 5-fold higher than that of the untreated control. However, The 32 kDa and 17 kDa subunits were not detected after 9 h of incubation. It may be that the inhibition of protein synthesis induced by ricin led to their disappearance or the apoptotic cells reached necrotic stage and the protease released digested them.

Interestingly, Ich-1_L was expressed at 3, 6, 9 h of treatment, and decreased after 12 h of incubation. This may suggest that the Ich-1_L was also involved in the apoptosis process induced by ricin. We know that the caspase family proteases may trigger the activation of each other as well as of themselves, and the current notion is that the proteases are activated sequentially, generating an amplified signal[16]. For example, in Fas-induced apoptosis, recent studies have shown that the protease FLICE/MACH (caspase-8) is activated first, by interaction through its death domain with the Fas-associated death domain-containing protein (FADD), leading in turn to the activation of proteases CPP32 and Mch3 (caspase-7)[9,17]. We think that the Ich-1_L may be a factor in the pathway of CPP32 activation. We donot know whether the Ich-1_L is only a caspase and what function it exerts in the CPP32 activating pathway. In all, our experiments suggest that CPP32 is predominantly involved in ricin-induced apoptosis. The exact nature of this caspase remains to be determined.

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蓖麻蛋白诱导 HeLa 细胞凋亡的分子机制

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关键词 蓖麻蛋白；HeLa 细胞；半胱氨酸蛋白酶类；扫描电子显微镜检查；蛋白质印迹；电子显微镜检查；细胞凋亡；流式细胞术；细胞周期

目的：研究蓖麻蛋白引起的 Hela 细胞凋亡的形态变化及机制. **方法**：扫描电镜，透射电镜，Western blot，细胞周期分析、细胞毒性和细胞相对存活率测定. **结果**：蓖麻蛋白 0.05 μmol·L^{-1}引起 HeLa 细胞发生典型的凋亡. 凋亡细胞主要表现为胞浆膜起泡，核染色质浓缩，形成新月状核或膜包裹核染色质的凋亡小体；Western blot 未检测到 p53、Bax 和 ICE 的 p20 活性亚基，而检测到 CPP32 的 p17 活性亚基，CPP32 活性升高，而 ICE 活性无显著改变. **结论**：CPP32 参与了蓖麻蛋白诱导的 HeLa 细胞凋亡过程.

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