ETOPOSIDE ACTIVATES CASPASE–3 VIA A CASPASE–1 INDEPENDENT MECHANISM IN CANCER CELLS

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ЭТОПОЗИД–ЗАВИСИМАЯ АКТИВАЦИЯ КАСПАЗЫ–3 В ОПУХОЛЕВЫХ КЛЕТКАХ НЕ СВЯЗАНА С АКТИВАЦИЕЙ КАСПАЗЫ–1

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It is known that caspase-1 is activated before caspase-3 activation when the cells were treated with anti-Fas-antibody. In present study the activities of these two proteases in etoposide-treated cells were examined. It has been shown that whilst treatment of U937 and Jurkat cells with etoposide results in caspase-3 activation it did not influence caspase-1 activity. At the same time treatment of U937 cells with anti-Fas antibody induces caspase-1 activity. These results demonstrate that etoposide transmits the death signal to caspase-3 directly, suggesting that caspase-3 was activated by a caspase-1 independent mechanism during etoposide–induced apoptosis.
***Key Words*: caspase-1, caspase-3, apoptosis, etoposide.**

Известно, что при обработке клеток анти-Fas-антителами активация каспазы-1 предшествует активации каспазы-3. Мы определяли активность указанных протеаз в клетках, в которых с помощью обработки этопозидом был индуцирован апоптоз. Было установлено, что инкубация клеток линий U937 и Jurkat с этопозидом приводила к активации каспазы-3, но не оказывала влияния на индукцию активности каспазы-1. В то же время анти- Fas-антитела индуцировали активность каспазы-1 в клетках линии U937. Полученные данные продемонстрировали, что при этопозид-индуцированном апоптозе происходит прямая активация каспазы-3, не требующая предварительной активации каспазы-1.
***Ключевые слова*: каспаза-1, каспаза-3, апоптоз, этопозид.**

Apoptosis, a morphologically distinguished form of programmed cell death, is critical not only during development and tissue homeostasis, but also in the pathogenesis of a variety of diseases, including cancer, autoimmune disease, viral infection, and neurodegenerative disorders [1]. Whereas numerous genes that control apoptosis have been identified and partially characterized, the precise mechanisms by which these genes interact to execute the apoptotic program remains poorly understood [2]. In mammals, a family of cysteine proteases that is designated as caspases appears to represent the effector arm of the apoptotic program. Up to date, more than 14 caspases have been identified and partially characterized [3, 4]. Each caspase contains characteristic conserved sequences important for proteolytic activity and induction of apoptosis. During apoptosis, many cellular proteins undergo caspase–dependent degradation [5, 6]. It is well known that caspase–3 is responsible for the cleavage of their substrates at the final onset of apoptosis. Caspase activation is induced by a wide array of death signals and leads to precipitous cleavage of protein substrates and execution of the apoptotic program. Some substrates of these caspases including polyadenosyl ribose polymerase (PARP), inhibitor of caspase–activated deoxyribonuclease (ICAD), DNA fragmentation factor (DFF), actin, gelsolin, fodrin, and lamin were cleaved by caspases during apoptosis [7], and the cleaved products can accelerate further activation of apoptotic transducers, although the activation mechanism remains unclear.

In this study, we examined the peculiarities of etoposide–induced apoptotic signaling in cancer cells. The major finding is that etoposide activates caspase–3 via a caspase1–independent mechanism in U937 cells, whereas anti–Fas antibody triggers caspase–1 activation prior to caspase–3 activation.

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Abbreviations used: YVAD–AMC — acetyl–L–tyrosyl–L–valyl–L–alanyl–L–aspart–7–amino–4–methylcoumarin; DEVD–AMC — acetyl–L–aspartyl–L–glutamyl–L–valyl–L–aspart–7–amino–4–methyl–coumarin.

MATERIALS AND METHODS

Cell culture. Human hematopoietic Jurkat cells and human histiocytic lymphoma U937 cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air in RPMI 1640 medium supplemented with 10% heat–inactivated fetal bovine serum, 100 μg/ml of penicillin and streptomycin.

Chemicals. The fluorogenic substrates of proteases (acetyl–L–tyrosyl–L–valyl–L–alanyl–L–aspart–7–ami–

no–4–methylcoumarin (YVAD–AMC) and acetyl–L–aspartyl–L–glutamyl–L–valyl–L–aspart–7–amino–4–methylcoumarin (DEVD–AMC) as well as Z–VAD, a general caspase inhibitor, were obtained from Bachem AG (Bubendorf, Switzerland). Etoposide (VP–16) was commercially available.

MTT assay. MTT assay was carried out as follows: cells ($5 \cdot 10^5$ cells/ml) were plated in 96–well plates and incubated for 24 h in 100 μl of RPMI medium. Various concentrations of drugs were added, and the cells were incubated for additional 48 h. Then, 10 μl of MTT solution (5 mg/ml MTT in PBS) were added to each well and incubated for 4 h at 37 °C. In order to stop the reaction, 100 μl of 0.04 M HCl in isopropanol were then added with vigorous mixing. The absorbance was determined in a microtiter plate reader at wave length 564 nm.

Protease activity. The death protease activities were measured by modified method [8, 9]. Briefly, the cells were incubated with medium alone or medium containing etoposide (Sigma Chemical Co., St. Louis, USA) or anti–Fas monoclonal antibody (Clone CH–11, MBL Co, Nagoya, Japan) for 0–6 h. Then the cells were harvested and lysed with the lysis buffer (10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiothreitol). The cell lysate was then incubated with 20 μM of the fluorogenic substrate of protease (YVAD–AMC or DEVD–AMC) in ICE buffer (20 mM HEPES, pH 7.5, 10% glycerol, and 2 mM dithiothreitol) for 15 min at 37 °C. The AMC released from the fluorogenic substrates was measured with the excitation at 380 nm and the emission at 460 nm using a spectrophotometer [8, 9].

DNA fragmentation. The DNA fragmentation assay was carried out as previously described [8–11]. In brief, cells ($1 \cdot 10^5$ cells/ml) were seeded in 24 wells, and cultured with or without etoposide (2, or 20 μg/ml). The cells were suspended in 20 μl of 50 mM Tris–HCl, pH 8.0, 10 mM EDTA, and 0.5 μg/ml proteinase K (Sigma, USA). After incubation at 50 °C for 1 h, 10 μl aliquot of 0.5 mg/ml RNase A solution was added to a mixture of 10 μl of the 70 °C–preheated solution containing 10 mM EDTA, pH 8.0, 1% (w/v) low–melting point agarose (Sigma, USA), 0.25% bromophenol blue, and 40% sucrose. The DNA was analyzed by electrophoresis in 2% agarose gels followed by ethidium bromide staining and photographing on an UV illuminator.

RESULTS AND DISCUSSION

In this study we examined whether or not etoposide activates caspase–1. It is known that anti–Fas antibody triggers the induction of caspase–1 activation, and sequentially activates other caspases [12–14] and that caspase–3 is the universal death executioner regulated by various stimuli. Because some anticancer drugs act through activation of the death proteases in cancer cells, we assume that, by activating other protease that is upstream of caspase–3, we can get the important data concerning mechanisms of apoptosis control. By MTT assay different anticancer drugs (etoposide, UCN–01, and staurosporine (protein kinase C inhibitors), camptothecin and cisplatin) were screened for ability to induce apoptosis in U937 and Jurkat cells. Finally, etoposide was selected and optimal concentration of that drug and time course of apoptosis induction was determined. When Jurkat and U937 cells were treated with etoposide at a concentration of 1 μg/ml the cell viability was about 14.5% and 20.0%, respectively (Fig. 1, *a*). The research of DNA fragmentation in the cells treated with 2 and 20 μg/ml of etoposide has shown (Fig. 1, *b*, lanes 8–13) that 0.5–6 h treatment with 2 μg/ml of etoposide did not induce apoptosis, but 4–6 h –long treatment with 20 μg/ml of etoposide induced apoptosis in the U937 cells (Fig. 1, *b*, lanes 2–7) and in Jurkat cells (Fig. 1, *c*). It is well known that caspases are playing a pivotal role in stimuli– or drug–induced apoptosis. Caspase activation is a universal event in apoptosis and consists in cleavage of cellular substrates, for example, PARP, actin, lamin, and fodrin [10, 11]. Therefore, we have examined the caspase–3 activity by a substrate cleavage assay. It is known that caspase–3 specifically cleaves aspartic residue of DEVD–AMC, whereas caspase–1 cleaves YVAD–AMC. Our data have shown that in etoposide–treated cell lysates DEVD–AMC cleavage activity is increased (closed triangles) whereas in

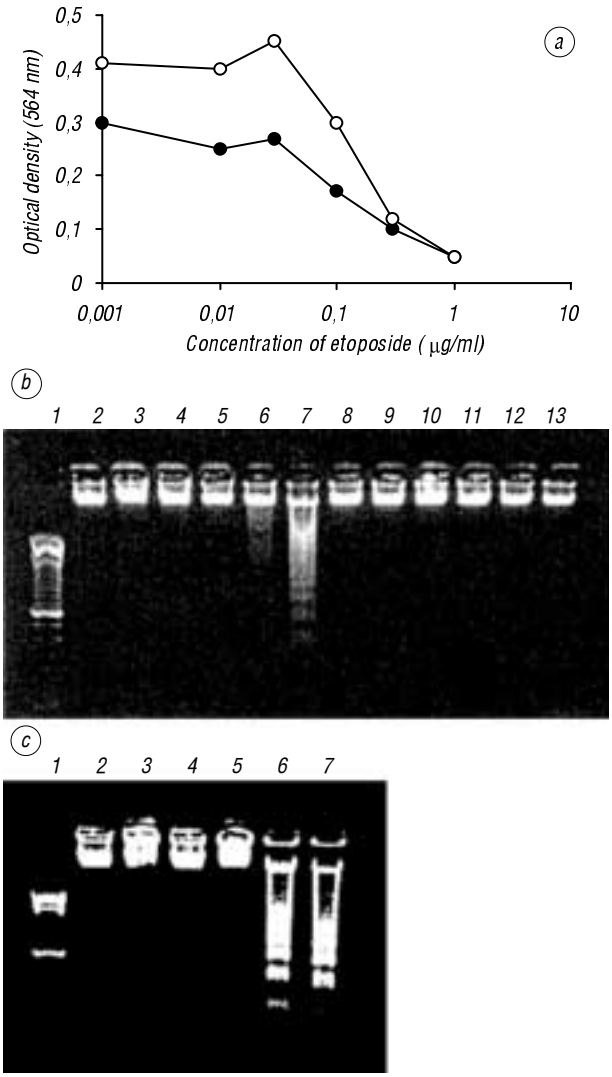

Fig. 1. VP–16 induces apoptosis in cancer cells. MTT–assay of U937 (open circles) or Jurkat cells (closed circles) treated with various concentrations (0.001–10 μg/ml) of etoposide (*a*). Time course of DNA fragmentation in Jurkat (*b*) or U937 (*c*) cells, cultured with etoposide (20 μg/ml) for 0 (lane 2), 0.5 (lane 3), 1 (lane 4), 2 (lane 5), 4 (lane 6), or 6 (lane 7) h

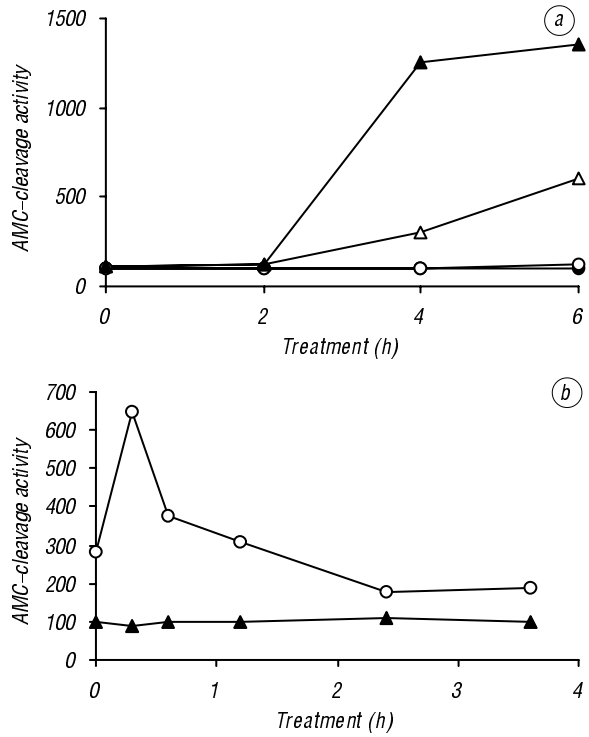

Fig. 2. Caspase–3 and caspase–1 substrate cleavage assay. AMC cleavage activity of caspase–3 in the lysates of U937 cells treated with etoposide (closed triangles) or Z–VAD (open triangles). Etoposide alone (without cell lyzates, open circles) and Z–VAD alone (closed circles) did not show any activities (*a*). AMC–cleavage activity of caspase–1 in the lysates of U937 cells treated with etoposide (closed triangles) or anti–Fas antibody (open circles) (*b*)

Z–VAD treated cell lysates its activity is decreased (open triangles). These results suggested that etoposide induces apoptosis via a caspase–3–dependent mechanism. Next, specific caspase–1 activity in etoposide–treated U937 cells has been studied. We have shown (Fig. 2) that treatment with anti–Fas antibody (open circles) resulted in quick induction of caspase–1 activity, but etoposide did not induce caspase–1 activation in U937 cells (Fig. 2, *b*, closed triangles). These results suggest that the signal pathway of etoposide is different from that of anti–Fas antibody [12].

In conclusion, we have demonstrated that etoposide activates caspase–3 death protease via a caspase–1–independent mechanism in U937 cells, while anti–Fas antibody activates caspase–1.

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REFERENCES

1. **Thompson CB**. Apoptosis in the pathogenesis and treatment of disease. Science 1995; **267**: 1456–62.

2. **Vaux DL, Strasser A.** The molecular biology of apoptosis. Proc Natl Acad Sci USA 1996; **93**: 2239–44.

3. **Yuan J**. Transducing signals of life and death. Curr Opin Cell Biol 1997; **9**: 247–51.

4. **von Mering M, Wellmer A, Michel U, Bunkowski S, Tlustochowska A, Bruck W, Kuhnt U, Nau R.** Transcriptional regulation of caspases in experimental pneumococcal meningitis. Brain Pathol 2001; **11**: 282–95.

5. **Cohen GM**. Caspases: the executioners of apoptosis. Biochem J 1997; **326**: 1–16.

6. **Meier P, Evan G.** Dying like flies. Cell 1998; **95**: 295–8.

7. **Nunez G, Benedict MA, Hu Y, Inohara N.** Caspases: the proteases of the apoptotic pathway. Oncogene 1998; **17**: 3237–45.

8. **Lee S-H, Fujita N, Mashima T, Tsuruo T.** Interleukin-7 inhibits apoptosis of mouse malignant T-lymphoma cells by both suppressing the CPP32-like protease activation and inducing the Bcl-2 expression. Oncogene 1996; **13**: 2131–9.

9. **Mashima T, Naito M, Fujita N, Noguchi K, Tsuruo T**. Identification of actin as a substrate of ICE and an ICE-like protease and involvement of an ICE-like protease but not ICE in VP-16-induced U937 apoptosis. Biochem Biophys Res Commun 1995; **217**: 1185–92.

10. **Mashima T, Naito M, Tsuruo T.** Caspase-mediated cleavage of cytoskeletal actin plays a positive role in the process of morphological apoptosis. Oncogene 1999; **18**: 2423–30.

11. **Gunji H, Kharbanda S, Kufe D.** Induction of internucleosomal DNA fragmentation in human myeloid leukemia cells by 1-beta-D-arabinofuranosylcytosine. Cancer Res 1991; **51**: 741–3.

12. **Enari M, Talanian RV, Wong WW, Nagata S.** Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 1996; **380**: 723–6.