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2-Chloroethylethyl Sulfide Induces Apoptosis and Necrosis in Thymocytes

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2-chloroethylethyl sulfide (CEES) is an alkylating agent that readily reacts with a wide variety of biological molecules causing metabolic abnormality. The mechanism of cell death during CEES injury is poorly understood. We have examined the effect of exposure of thymocytes with various concentrations of CEES to determine the pattern of cell death in thymocytes injury induced by CEES. In the present study, we show that two patterns of cell death occurred by either one of two mechanisms: apoptosis and necrosis. Exposure to low levels of CEES (100 μM) for 5 h caused an induction of apoptosis on thymocytes, as identified by the following criteria: DNA fragmentation visualized by the characteristic "ladder" pattern was observed upon agarose gel electrophoresis and morphological features were revealed by microscopical observations. In contrast, exposure to high levels of CEES (500 μM) induce necrotic features such as cell lysis. Thus, depending on the concentrations, CEES can result in either apoptotic or necrotic cell damage. Our findings suggest that thymocytes which are not killed directly, but merely injured by low levels of CEES, are able to activate an internally-programmed cell death mechanism, whereas thymocytes receiving severe damages apparently can not.

Keywords: Apoptosis, Cell death pattern, 2-Chloroethylethyl sulfide, Necrosis.

Introduction

2-Chloroethylethyl sulfide (CEES) or 2,2′-dichlorodiethyl sulfide, two sulfur alkylating agents, are vesicant or blistering chemical warfare agents of which no effective treatment are available to prevent or minimize injury induced by exposure to them. In addition to skin damage, these agents exert other toxic effects including eye and respiratory track damage, systemic injuries, nervous system effects, and reproductive system damage (Papirmeister *et al.*, 1991).

It has been proposed that alkylating agent-induced DNA damage causes cell death according to the following biochemical cascade of events (Fax *et al.*, 1980; Gross *et al.*, 1985; Martens, 1991; Papirmeister *et al.*, 1991): alkylation of DNA → activation of poly (ADP-ribose) polymerase (PADPRP) to repair DNA → overuse of nicotinamide adenine dinucleotide (NAD^+) by PADPRP → depletion of NAD^+ → change in intracellular energy metabolism → cell death. Another mechanism for the cytotoxicity of these compounds has been proposed to involve the release of lysosomal hydrolytic enzymes, which may destroy the cytoskeletal component in cells (Choi *et al.*, 1995; Shin *et al.*, 1995). An additional mechanism of Ca^{2+}-mediated cytotoxicity has been recently proposed in which sulfur alkylating agents induce the increase of intracellular free calcium and arachidonic acid release from cell membranes (Ray *et al.*, 1995). However, these proposed pathway inhibitors could not properly protect the cells from the 2-chloroethylethyl sulfide or 2,2′-dichlorodiethyl sulfide toxicity (Mol *et al.*, 1989; Martens *et al.*, 1993), indicating that the mechanism of toxicity of these compounds may involve events other than hypotheses already proposed.

Up to the present, most studies have been focused on the effects of sulfur alkylating agent on skin tissue cells (Willems 1989; Momeni *et al.*, 1992; Dabrowska *et al.*, 1996). Recently, it was observed that 2,2′-dichlorodiethyl sulfide affected the derangement of the immune system (Meier 1996; Gross *et al.*, 1997). For example, significant decrease of cellularity of the spleen and thymus was found in 2,2′-dichlorodiethyl sulfide-treated lymphocytes (Coutelier *et al.*, 1991). In addition, dramatic decrease in the number of B- and T-helper lymphocytes was observed in sulfur alkylating agent-exposed patients (Venkateswaran

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et al., 1994). These observations suggest the hypothesis that sulfur alkylating agents also attack the immune cells to alter some immune mechanisms. To better understand the effect of sulfur alkylating agents on immune cells, in the present study, we have investigated the mode of cell death thymocytes after damage initiated by CEES.

Necrosis and apoptosis are distinct mechanisms of cell death with very different characteristics. Necrosis is characterized by cell swelling, injury to cytoplasmic organelles including mitochondria and endoplasmic reticulum, chromatin flocculation, and disruption of membrane integrity, followed by cell lysis (Swartz *et al.*, 1993). In contrast, apoptosis is an active process of cell death with specific morphological and molecular features. Apoptotic cell death is characterized by cell shrinkage, membranes blebbing, and DNA fragmentation into integer multiples of nucleosome-sized units (Bursch *et al.*, 1992). Membrane integrity is not lost in the initial stage of apoptosis, which is a marked difference from what is seen in necrosis (Ueda and Shah, 1994).

In this study, we observed two patterns of cell death mode in thymocytes that was dependent on the concentrations of CEES.

Materials and Methods

Materials 2-Chloroethylethyl sulfide (CEES) was purchased from Aldrich Chemical Co. (Milwaukee, USA). Fetal calf serum was from Difco Lab. (Detroit, USA). RPMI 1640 medium, agarose, RNase A, ethidium bromide and all other reagents were obtained from Sigma Chemical Co. (St. Louis, USA). All reagents were of analytical grade.

Preparation of the cell suspensions ICR mice between the weights of 27–30 g were used as thymocyte donors. Thymus glands were removed aseptically and placed in cold RPMI 1640 medium. Single-cell suspensions were prepared by gently teasing the thymus in RPMI 1640 medium followed by filtration through 4 layers of gauze. After two washing steps, the cells were resuspended in the culture medium.

Cell culture Thymocytes were diluted to a final concentration of 10×10^6 cells in 1ml of culture medium containing RPMI 1640 medium supplemented with 10 U/ml of penicillin, 10 μg/ml streptomycin, and 10% fetal calf serum. The cells were incubated at 37°C in a humidified incubator under an atmosphere of 5% CO_2 : 95% air in the absence or presence of CEES.

Exposure of thymocytes to CEES 2-Chloroethylethyl sulfide (1 mM final concentration) was dissolved in ethanol just before being added to the cell culture medium at room temperature under a hood. The final concentration of ethanol in the culture medium was 0.25%. Control cultures were exposed to the same amount of ethanol. The samples were incubated in an environmentally-controlled incubator (37°C and 5% CO_2). After incubation, aliquots of cells were used for DNA fragment assay or terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay.

DNA fragmentation quantitation The assay method for quantitation of fragmented DNA was based on the sandwich-enzyme-immunoassay measuring principles using mouse monoclonal antibodies directed against DNA and histones (Bonfoco *et al.*, 1995). This ELISA (Boehringer Mannheim kit, Cat. No. 1544 675) provided quantitative determination of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasmic fraction of cell lysates. After incubation with CEES, cells were collected by centrifugation at $200 \times g$ for 5 min. The cell pellet was mixed with lysis buffer for 30 min at 4°C. The lysate was centrifuged at $20{,}000 \times g$ for 10 min and the supernatant (cytoplasmic fraction) carefully removed. Cytoplasmic fractions were incubated in anti-histone antibody-fixed microtiter plates. In this incubation step, the nucleosomes contained in the sample bind via their histone components to the immobilized anti-histone antibody. In the second incubation step, anti-DNA-peroxidase (POD) reacts with the DNA-part of the nucleosome. After removal of unbound peroxidase conjugate by washing, the amount of peroxidase retained in the immunocomplex was determined by a spectrophotometer at 405 nm with ABTS (2,2-azino-di-[3-ethylbenzthiazoline sulfonate]) as a substrate.

Agarose gel electrophoresis Cells were incubated as described above and harvested by centrifugation at $200 \times g$ for 10 min. The pellet was lyzed with ice-cold hypotonic lysis buffer (5 mM Tris-HCl, 20 mM EDTA, 0.5% Triton X-100, pH 8.0) and incubated at 4°C for 20 min. The lysate was centrifuged for 20 min at $27{,}000 \times g$. After centrifugation, DNA from the supernatant fraction (containing fragmented DNA) was extracted with Tris pH 8.0-buffered phenol. The DNA was then serially extracted with equal volumes of phenol:chloroform (1 : 1) and chloroform. The aqueous phase of chloroform was then precipitated by 0.5 M NaCl and ethanol. The samples were kept overnight at −20°C. They were then centrifuged at $13{,}000 \times g$ for 20 min. The pellets were resuspended in TE buffer (10 mM Tris-HCl, 5 mM EDTA, pH 8.0) containing RNAse A (type I-A; Sigma), incubated for 10 min at 65°C, followed by 15 min at 37°C. Loading buffer containing 50% glycerol, $5 \times$ TAE buffer ($1\times = 40$ mM Tris-acetate, pH 8.5, 2 mM EDTA) and 0.1% bromophenol blue was added to the samples in a ratio of 1:4 (v/v). Electrophoresis was carried out in 1.8% agarose gel at 10 V/cm in TAE buffer. DNA was visualized on an ultraviolet (UV) transilluminator after ethidium bromide staining.

TUNEL assay Apoptotic cells were visualized by the method of a commercial apoptosis detection kit (*In Situ* Apoptosis Detection Kit: Peroxidase, Oncor, Gaithersburg, USA). Isolated thymocytes were fixed in microscope slides with 4% neutral buffered formalin for 10 min in room temperature. The microscope slides were dried and washed twice in pH 7.4 phosphate-buffered saline (PBS). After washing, the samples were processed for immunocytochemical detection of incorporated digoxigenin-dUTP by anti-digoxigenin-peroxidase, followed by an enzyme reaction using diaminobenzidine (DAB) as a substrate. Counterstaining was made with methylgreen for histological evaluation (Constan *et al.*, 1966).

Morphological evaluations Apoptotic cells were distinguished morphologically from other cells by the presence of condensed brown nuclei under the light microscope (×200 magnification).

Quantification of apoptotic cells (apoptotic index) was performed on minimum 1000 consecutive cells from five standard regions of each smear. The proportion of cells with condensed brown nuclei was expressed as a percentage of total nuclei.

Results and Discussion

The determination of whether a thymocyte dies by apoptosis as opposed to necrosis is most easily made on the basis of distinct structural changes in nuclear chromatin that occurs prior to the lysis of cell membranes. These changes include fragmentation of DNA and condensation of chromatin (Ueda and Shah, 1994).

To detect the DNA fragmentation, the ELISA method described in Materials and Methods was used to evaluate the histone-associated DNA fragment. The ELISA uses monoclonal antibodies to detect an increase in histone proteins that are associated with the fragmented DNA. Thymocytes were exposed to various concentrations (0–500 μM) of CEES for 5 h. At 5 h after exposure to these concentrations of CEES, an increase of DNA fragmentation was observed. As shown in Fig. 1, this increase was observed even with 5 μM CEES and became even more prominent with 100 μM CEES (a 40 % increase compared to untreated control). Meanwhile, at higher concentrations (>100 μM) of CEES, the amount of fragmented DNA was markedly decreased. For example, 500 μM CEES produced the same amount of histone-associated DNA fragment as in control.

Therefore, two different patterns of DNA fragmentation dependence on CEES concentrations were observed. At lower concentrations of CEES (10–100 μM), DNA fragmentation increased in a dose-dependent manner. However, higher concentrations of CEES (>100 μM) did not further enhance the DNA fragmentation. Cleavage of host chromatin into oligonucleosome-length DNA fragments (200 bp) is considered as characteristic biochemical markers for apoptosis (Bortner *et al.*, 1995). Our results clearly showed the CEES-induced DNA fragmentation to be a characteristic of apoptosis. It also suggested that the concentration of CEES was important to induce DNA fragmentation.

To confirm the morphologic evidence of nuclear fragmentation, DNA was extracted and analyzed by agarose gel electrophoresis. The "DNA ladder" of multiples of the 200 bp nucleosomes, characteristic for endonucleolytic cleavage of DNA, was clearly observed in CEES-treated cells. As in Fig. 2, at lower concentration (100 μM) of CEES, cells displayed a sharp increase in the number of small DNA fragments, whereas in the presence of high concentration (500 μM) of CEES, the degree of DNA fragmentation was not significantly different from that in control cells.

Therefore, the results of our agarose gel analysis of DNA fragmentation are well consistent to the ELISA results. Both methods clearly revealed that low concentrations of CEES-exposed cells induced the internucleosomal cleavage typical of apoptosis. Our results further suggested that in higher concentrations of CEES, thymocytes did not show increase of DNA fragmentation as an apoptotic characteristic, indicating that in this level of CEES, cell death was not via apoptosis.

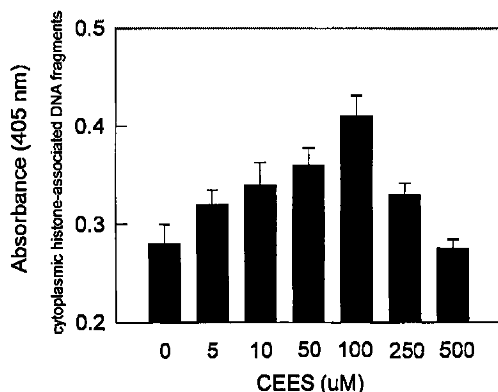

Fig. 1. ELISA quantification of histone-associated DNA fragments in various concentrations of CEES-treated thymocytes. Thymocytes were incubated in the presence of various concentrations of CEES at 37°C for 5 h as described in Materials and Methods. The data represent the mean ±SE.

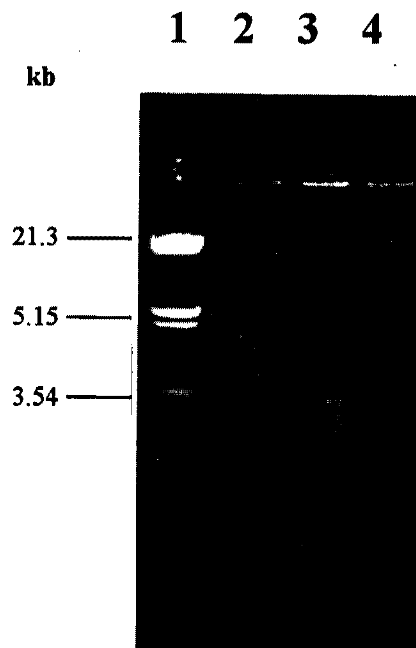

Fig. 2. Induction of internucleosomal DNA cleavage by CEES. Thymocytes were incubated for 5 h either alone (lane 2, control) or in the presence of CEES (100 and 500 μM, lanes 3 and 4, respectively). The cells were then examined by agarose gel electrophoresis for evidence of DNA laddering. Lane 1 contains molecular weight markers.

As reported earlier (Ueda and Shah, 1994), there are many distinct features rather than a single characteristic that should be present to suggest the occurrence of apoptosis in thymocytes. For this reason, we performed the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end labeling (TUNEL) assay as an immunochemical method. The TUNEL assay was able to discriminate morphologically the apoptotic cells from other cells. TUNEL methods take advantage of fragmented nuclear DNA in apoptotic cells by assembling groups of tagged nucleotides at strand breaks, allowing for the discrimination of apoptotic cells from normal cells using immunochemical staining methods (Gavrieli *et al.*, 1992).

With this technique, the apoptotic cells were easily identified and quantified. Microscopic examination confirmed the increase in number of apoptotic nuclei in 100 μM CEES-treated cells compared to untreated control cells (Figs. 3A and 3B). Table 1 shows quantification of the number of nuclei observed by the TUNEL assay. At low levels of CEES, the percentage of apoptotic cells increased with increasing CEES concentrations. After 5 h of incubation with 100 μM CEES, approximately 30% of cells were positively stained, whereas only 13% of untreated cells were stained. As shown in the DNA fragmentation assay and agarose gel electrophoresis, the percentage of apoptotic nuclei sharply decreased in high concentrations of CEES-treated thymocytes. By 5 h after the exposure to 500 μM CEES, the percentage of apoptotoc nuclei was decreased to 14%.

Further evidences that CEES induced a biphasic pattern of cell death in thymocytes came from the microscopic study of the morphological change of chromatin. Previous work as suggested that necrotic cells showed typical features of the chromatin such as chromatin flocculation and chromatin margination as small aggregates, eventually giving rise to karyolysis (Searle *et al.*, 1982; Duvall and Wyllie, 1986). Cells in the final stages of necrosis undergo cell lysis. By a direct contrast, chromatin of apoptotic cells rapidly forms dense crescent-shaped aggregates lining the nuclear membranes, and nuclear pores are absent adjacent to the nuclear membranes (Wyllie *et al.*, 1984; Oberhammer *et al.*, 1993). Complex invaginations develop in the nuclear membrane, leading to a segmented nucleus (Sun *et al.*, 1994; Nakamura *et al.*, 1995). As shown in Fig. 4, two typical types of apoptotic features were observed at low concentrations of CEES-treated cells. Normal thymocytes have nuclei with highly condensed heterochromatin that is typically distributed around the membrane of the nucleus with the euchromatin condensed into the nuclear interior (Bortner *et al.*, 1995). In contrast, 100 μM CEES-exposed cells showed that chromatin was condensed and coalesced against one pole of the nuclear membrane (Fig. 4A). In addition, another typical morphological feature of apoptosis, a nuclear breakage into several fragments, was also observed (Fig. 4B). However, the higher concentration (500 μM) of CEES-exposed cells showed typical characteristics of necrosis such as loss of

Table 1. Effect of CEES on apoptosis. Thymocytes were exposed to various concentrations of CEES for 5 h. Apoptotic cells were identified by nuclei staining positive in the TUNEL assay.

CEES Concentrations (μM)	Apoptotic Cells[a] (%)
0.00	13.4 ± 1.8
0.01	19.6 ± 2.8
0.10	29.7 ± 3.0
0.50	14.3 ± 1.4

[a]Values are expressed in percentage of total cells as described in Materials and Methods. Data represent the mean ±SE.

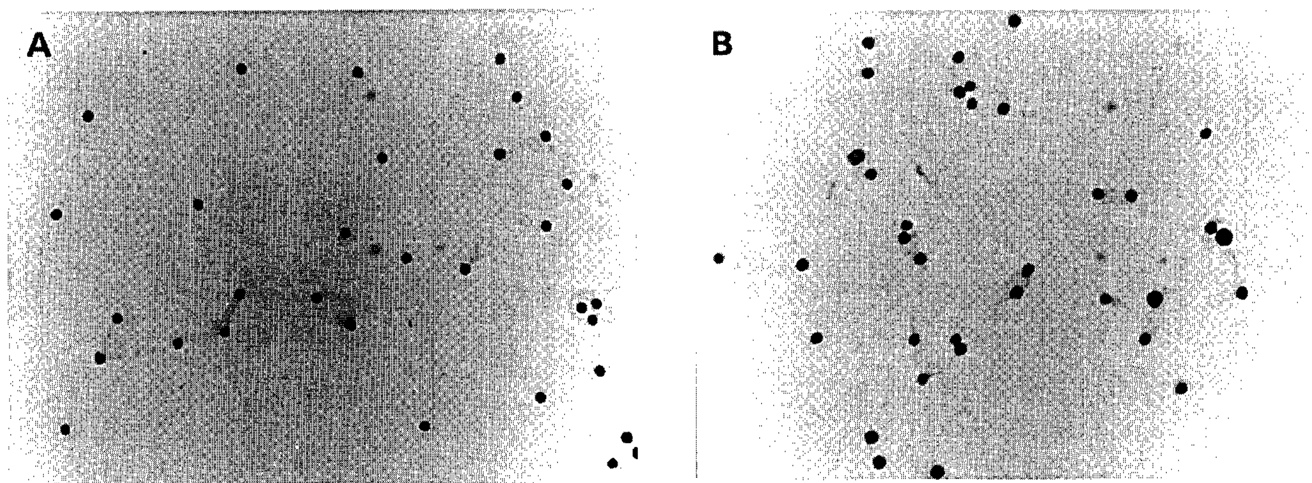

Fig. 3. Labeling DNA strand breaks by the TUNEL assay in thymocyte cultures exposed to CEES. (A) Control culture. (B) Culture after 5 h exposure to 100 μM CEES. Apoptotic cells were stained with dark brown color. Magnifications ×200.

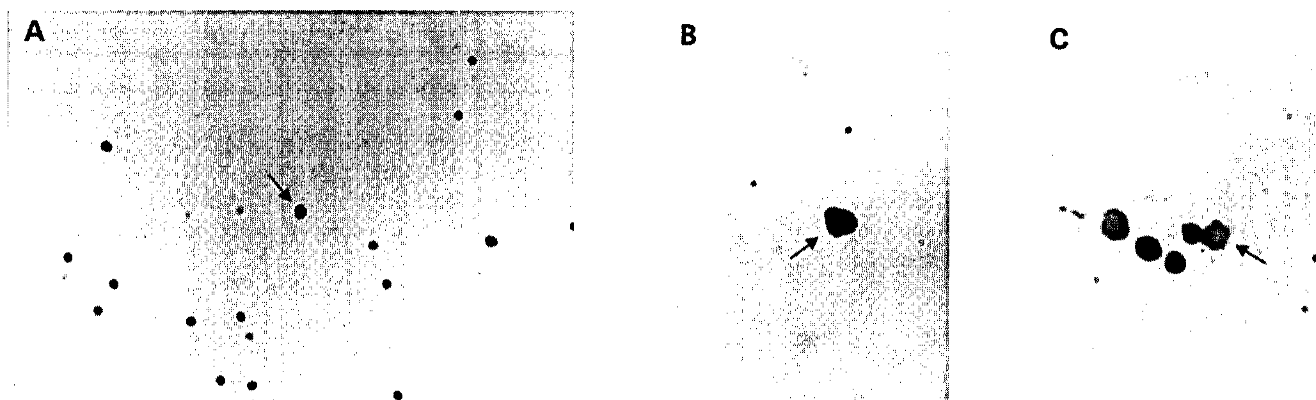

Fig. 4. Demonstration of the morphological changes of cells in various concentrations of CEES-treated thymocytes. Apoptotic cells display typical morphological characteristics of nuclei. The arrows indicate condensation of the chromatin at the nuclear membrane (A) and segmentation of nucleus into small pieces (B). Cells show features of necrosis, including cell swelling and plasma membrane disruption (C). Magnifications ×800.

plasma membrane integrity (Fig. 4C). This observation supports the results of the DNA fragment assay and TUNEL assay showing inhibition of apoptotic cell death under higher concentrations of CEES.

In the present study, the hypothesis, that sulfur alkylating agents also attack the immune cells, was tested by exposing thymocytes to various concentrations of CEES. We have investigated the mode of cell death by CEES in thymocytes, using biochemical and morphological methods. At low concentrations of CEES, cells underwent biochemical change typical of apoptosis: namely, DNA fragmentation. In addition, examination of the fragmented DNA of these cells by agarose gel electrophoresis revealed internucleosomal cleavage, which is typical of apoptosis. Moreover, these cells had additional typical morphological features of apoptosis such as condensation of chromatin and segmentation of nuclei that were confirmed by microscopy. In direct contrast, cells exposed to higher concentrations of CEES underwent morphological changes typical of necrosis. These changes included cell swelling and plasma membrane disruption. Hence, the induction of cell death mode in the thymocytes by CEES was found to be dependent on the dose of this agent. Although the findings of this study were observed in immune cells, both apoptotic and necrotic cell death by alkylating agents have also been reported in other cell types such as endothelial cells (Dabrowska *et. al*, 1996).

Therefore, all of these results suggest that the dose of sulfur alkylating agents may determine the ensuing pathway to either apoptotic or necrotic cell death. Our results would be helpful in developing therapeutic methods for reducing the toxicity of sulfur alkylating agents.

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