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Gene expressions in Jurkat cells poisoned by a sulphur mustard vesicant and the induction of apoptosis

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1 The sulphur mustard vesicant 2-chloroethylethyl sulphide (CEES) induced apoptosis in Jurkat cells.

2 Akt (PKB), a pivotal protein kinase which can block apoptosis and promotes cell survival, was identified to be chiefly down-regulated in a dose-dependent manner following CEES treatment. Functional analysis showed that the attendant Akt activity was simultaneously reduced.

3 PDK1, an upstream effector of Akt, was also down-regulated following CEES exposure, but two other upstream effectors of Akt, PI3-K and PDK2, remained unchanged.

4 The phosphorylation of Akt at Ser^{473} and Thr^{308} was significantly decreased following CEES treatment, reflecting the suppressed kinase activity of both PDK1 and PDK2.

5 Concurrently, the anti-apoptotic genes, Bcl family, were down-regulated, in sharp contrast to the striking up-regulation of some death executioner genes, caspase 3, 6, and 8.

6 Based on these findings, a model of CEES-induced apoptosis was established. These results suggest that CEES attacked the Akt pathway, directly or indirectly, by inhibiting Akt transcription, translation, and post-translation modification.

7 Taken together, upon exposure to CEES, apoptosis was induced in Jurkat cells *via* the down-regulation of the survival factors that normally prevent the activation of the death executioner genes, the caspases.

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Abbreviations: Akt, protein kinase B (PKB); Bad, Bcl-2 associated death agonist; CEES, 2-chloroethylethyl sulphide; Fak, focal adhesion kinase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; L32, ribosomal protein L32 (rpl32) genes; PDK, 3-phosphoinositide-dependent protein kinase; PH, pleckstrin homology; PI3-K, phosphatidylinositol 3 kinase; PTEN, phosphatase and tensin homologue; PIP3, phosphatidylinositol-3,4,5-triphosphate; RPA, RNase protection assay

Introduction

Apoptosis or programmed cell death is a self-destruction process characterized by stereotypical ultrastructural changes including condensation of the nucleus and cytoplasm, membrane blebbing, and external display of phosphatidylserine, a signal for recognition and engulfment of apoptotic cells by adjacent cells (Wyllie *et al*., 1980). Furthermore, fragmentation of the nuclear chromatin is a signature apoptotic event (Takahashi & Earnshaw, 1996; Sharma *et al*., 2000). The caspases, a family of cysteine proteases, play a critical role in apoptosis and are responsible for many of the biochemical and morphological changes associated with the phenomenon of cell death (Los *et al*., 2001). Caspases cleave crucial proteins of the nucleus and the cytoskeleton at the aspartate residues, resulting in phenotypic changes during apoptosis, including advanced chromatin condensation and internucleosomal DNA fragmentation (Cryns & Yuan, 1998).

The sulphur mustard 2-chloroethylethyl sulphide (CEES) is a sulphur mustard vesicant that alkylates a wide range of biological molecules (Ludlum *et al*., 1986). Because sulphur mustards can be used as weapons of mass destruction, there is a heightened interest in the development of an effective means of therapeutic treatment. In addition to mutagenic, tumorigenic, cytotoxic, and vesicating effects (Papirmeister & Davison, 1965; Somani & Babu, 1989; Wormser, 1991), sulphur mustards also constitute prototypes of cancer chemotherapeutic agents.

Associated with CEES-caused cell death are the enhancement of a DNA repair process that activates poly(ADP-ribose) polymerase (PARP), depletion of intracellular energy (Papirmeister *et al*., 1991), and vesication (Papirmeister *et al*., 1985). Among the more prominent effects of sulphur mustard is the alkylation of DNA (Fox & Scott, 1980) in the 7-position of guanine, the 3-position of adenine, and the *O*6 position of guanine (Ludlum *et al*., 1986). Mustard–DNA

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adducts induce the inhibition of DNA binding by preventing the formation of the ternary TFIIB–TBP–TATA element, hence disrupting the TBP : TFIIB complex (Gray *et al*., 1991; Pieper *et al*., 1989; Pieper & Erickson, 1990). Sulphur mustards can result in lung injury due to free radical-mediated reactions and oxidative stress (Elsayed *et al*., 1992). Additionally, sulphur mustards can react readily with cellular nucleophiles such as glutathione (GSH) (Ray *et al*., 1992). Specifically, CEES has been shown to inactivate Ca^{2+}-ATPase by alkylating the enzyme at a region other than the active site (Kim *et al*., 1995). It also decreases the stability of lysosomal membranes (Shin *et al*., 1995). Recently sulphur mustard has been shown to induce apoptosis *via* a Ca2+-calmodulin and caspase-dependent pathway (Rosenthal *et al*., 1998; Hur *et al*., 1998). However, there is a paucity of data regarding the precise mechanism of the molecular pathology induced by CEES. To elaborate the toxic mechanism of CEES, we examined the specific changes in gene expressions involved in the survival and death pathways of Jurkat cells treated with CEES.

Methods

Reagents

2-Chloroethylethyl sulphide (CEES) was purchased from Fisher and redistilled in-house. Foetal calf serum, RPMI 1640, and Trizol reagent were purchased from Invitrogen.

Cell culture

Jurkat cells were maintained in RPMI 1640 media with 10% foetal calf serum and incubated at 37°C in a humidified incubator under an atmosphere of 5% CO_2 : 95% air.

Treatment of cell with CEES

Jurkat cells were incubated with CEES dissolved in DMSO immediately before use. The final concentration of DMSO in cell suspensions was 0.1%. Unless indicated otherwise, the cells were treated with CEES for 14 h with various amounts of CEES. After incubation and washing with PBS, aliquots of cells were used for DNA fragmentation, Western blots, or RNA extraction.

Total RNA isolation

Total RNA from control and treated cells was isolated by using Trizol according to the manufacturer's instructions, and was followed by two rounds of ethanol precipitation.

DNA fragmentation assay

The procedure was slightly modified after a prior method (Kim *et al*., 1997). Jurkat cells (7.5×10^6) in 15 ml media were treated with CEES, and were centrifuged at $1000 \times g$ for 10 min and washed once with 1X Tris-buffered saline (TBS). Cells were re-suspended in 1 ml of an extraction buffer composed of (mM) Tris 1 (pH 8.0), EDTA 0.1 (pH 8.0), 20 μg ml^{-1} DNase-free RNase and 0.5% SDS and incubated at 37°C for 1 h. Next 8 μl proteinase K (25 mg ml^{-1}) was added to each lysate, which was then incubated for 3 h at 55°C. DNA was extracted three times with equal volumes of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), and once with an equal volume of chloroform. Two volumes of 100% ethanol were added to the supernatant and incubated for 15 min on ice. The DNA precipitates were collected by centrifugation ($12{,}000 \times g$ for 15 min) and washed with 1 ml of 70% ethanol. DNA was air dried and re-suspended in sterile water to a final concentration of 0.5 μg ml^{-1}. The DNA was electrophoresed in a 1.8% TBE agarose gel, stained with ethidium bromide and visualized by UV.

Electron microscopy

Jurkat cells treated with 200 μM CEES for 24 and 48 h were centrifuged at 2000 r.p.m. for 10 min. Cell pellets were re-suspended in 1X PBS and centrifuged again. The PBS was removed and the cells were fixed in 4F1G (4% formaldehyde, 1% flutaraldehyde) overnight at 4°C and processed for electron microscopy. Thin sections were stained with lead citrate and uranyl acetate and examined with a LEO 912 electron microscope.

Akt assay

Akt kinase activity was assayed using a peptide derived from glycogen synthase kinase-3 (GSK-3) as a substrate (after treatment and lysing of the cells as described above) using the Akt kinase assay kit from Upstate Biotechnology (Lake Placid, NY, U.S.A.) following the manufacturer's protocol.

Caspase-3 assay

The DEVD-cleaving activity of active caspase-3 was measured by using Caspase-3 Assay Kit from PharMingen (San Diego, CA, U.S.A.) following the manufacturer's protocol. Ac-DEVD-AMC was used as the fluorogenic substrate of assay. AMC fluorescence is quantified by UV spectrofluorometer using an excitation wavelength of 380 nm.

Microarray analysis

Single microarray experiments were performed for each time point and concentration from a pool of cultured cells, in which 50 μg of total RNA from CEES-treated and vehicle control cells were labelled by reverse transcription using SuperScript II (GibcoBRL) with either Cy3- or Cy5-dUTP (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, U.S.A.). The reverse transcription reaction was initiated with anchored oligo dT primer. Labelled DNA was hybridized overnight at 65°C to glass microarrays containing 2304 cDNA spots per slide (Advanced Technology Center, NCI, NIH, U.S.A.). After hybridization, the slides were washed for 2 min in 0.1% SDS/0.5X SSC, 2 min in 0.01% SDS/0.5X SSC, and 2 min in 0.06X SSC. Slide surfaces were dried by centrifugation at $1000 \times g$ for 10 min. Completed slides were scanned with the Axon GenePix 4000B Array Scanner. The images were acquired and the fluorescence intensities were analysed with the Axon GenePix Pro 3.0 software Suite. Resultant data was normalized by the ratio of medians and log2 transformed for vectorial analysis by cluster.

RNase protection assay (RPA)

Each RPA was performed with 10 μg of total RNA using the RiboQuant RPA kit (PharMingen, CA, U.S.A.). The following multiprobe template sets specific for human apoptosis were used: hAPO-1b, which detects transcripts for caspase 8, 3, 6, 5, 2, 7, 1 and 9; and hAPO-2, which detects bcl xL/S, bfl1, bik, bak, bax, bcl2 and mc11. Both template sets contained probes for the housekeeping genes L32 and GAPDH to serve as internal controls for the assays. Probes were labelled with [^{32}P]UTP. The RPA procedure was performed according to the manufacturer's instructions. Each assay included normal Jurkat cell RNA and yeast tRNA as controls. The protected fragments were precipitated, analysed with 5% polyacrylamide–urea gel electrophoresis and quantitated using Bio-Rad phosphorimager. Quantitation of the protected RNA samples was normalized against those for the L32 and GAPDH housekeeping genes in the corresponding lanes.

RT–PCR

RNA samples were treated with DNase I before RT–PCR, which was performed using ready-to-go RT–PCR beads (Amersham Pharmacia Biotech, NJ, U.S.A.). After reverse transcription, cDNA templates were denatured for 120 s at 95°C, then amplified 35 cycles of: annealing (55°C, 30 s), extension (72°C, 40 s), and denaturing (95°C, 30 s). The final step was incubation for 7 min at 72°C. Each PCR product was amplified in parallel using primers for β-actin as the RT–PCR internal control. The RT–PCR products were normalized against β-actin. Primers used for PCR amplification are listed in Table 1.

Western immunoblots

Cells were homogenized in a lysis buffer ((mM) Tris·HCl 25 (pH 7.4), EDTA 0.5, EGTA 0.5, PMSF 1, leupeptin 25 μg ml^{-1}, DTT 1, NaF 25, 0.5% Triton X-100) and centrifuged. The supernatant, each containing 10 ng of total protein, was separated on a sodium dodecyl sulphate (SDS) – 10% polyacrylamide gel coupled to a 4% polyacrylamide stacking gel. After transferring the separated proteins to polyvinylidene difluoride (PVDF) membranes, the membranes were soaked in 20 ml of 4% BSA in Tris-buffered saline–Tween 20 (TBST) buffer for 1 h at room temperature, and next probed overnight with 2 μl of first antibodies (0.15 μg ml^{-1}; Santa Cruz, CA, U.S.A.) in the same buffer. After three 20-min washes with TBST, the membranes were exposed for 1 h to secondary antibodies conjugated with horseradish peroxidase in 10 ml of blocking buffer. After washing three times with TBST, the membranes were exposed to a chemiluminescent reagent (ECL Plus; Amersham, Arlington Heights, IL, U.S.A.) for 1 min in the dark and were then exposed to Kodak X-ray film. Same membrane was used with phosphorylation analysis, i.e. after blotting with anti-Thr^{308}, the membrane was stripped, and re-exposed to X-ray film for background check. This membrane was next used for anti-Ser^{473} analysis.

Statistical analysis

All experiments were repeated at least three times, except the microarray analysis which was performed twice. The data in Figures 2D, 5A, and 6B were analysed with a one-way analysis of variance randomized block design, and the Dunnett's procedure was used to compare data from different concentrations to the control. Data were log10 transformed to stabilize the variance when needed. All other data were analysed with the Student's *t*-test and the significance was set at $P<0.05$. All controls were DMSO vehicle controls (final concentration in culture was 0.1% DMSO). This concentration of DMSO did not affect any results in our investigation.

Results

Induction of apoptosis by CEES

Initially, to investigate the molecular mechanism of CEES as an inducer of apoptosis, Jurkat cells were treated with different concentrations of CEES for various lengths of time. As shown in Figure 1A, in comparison to the untreated cells (lane 2), DNA fragmentation in typical ladder form was evident in CEES-treated cells for 72 h (lane 3). When examined by electron microscopy, perinuclear margination of chromatin was observed in the treated cells (Figure 1B). Since DNA fragmentation and chromatin condensation are two hallmarks of apoptosis, these observations were indicative that apoptosis was part of cell death caused by CEES.

Up-regulation of caspases by CEES

DNA fragmentation during apoptosis is due to the activation of caspases. To ascertain the importance of caspases following vesicant poisoning, microarray experiments (2.5 K gene per DNA chip) were performed using Jurkat cell RNA samples from six different CEES treatments. The data from these gene arrays were clustered using an agglomerative hierarchical method (clustering data not shown), which initially showed the up-regulation of caspases 3, 4, 6, 8 and

Table 1 Primers used for RT–PCR

Gene	*Sense Primer*	*Anti-sense Primer*	*Location in cDNA (residues)*
β-actin	5′-AGCAAGAGAGGCATCCTCACCC	5′-TTCTCCTTAATGTCACGCACG	178-644
Akt1	5′-AGCGACGTGGCTATTGTGAAG	5′-GCCGTCAGCCACAGTCTGGATG	4-327
PI3-K85α	5′-GGAATGAACGACAGCCTGCAC	5′-GAAGCCRTATTTCCCATCTCGRTG	892-1176
PI3-K110	5′GCATGCCAATTGGTCTGTATCC	5′TAGGATCTGGGTAATTACAGTC	1482-1828
PDK1	5′-TCATGTCGCGCCTGGATCACC	5′-GAGTTGGCCCTGGCTTGTTTGC	398-722
PDK2	5′-TGAGAGAGCTTCTCCGAGAG	5′-CACTCTTCATTGAAGTCCCTGC	431-639
Caspase4	5′-GCTGTTTACAAGACCCACGTG	5′-GTGGCTTCCATTTTCAATTGC	868-1147

9. These results were confirmed by RPA (Figure 2A) and RT–PCR (Figure 2B). After CEES treatment, the mRNA expression increased as follows: caspase 3 by 2.9 fold; caspase 4 by 2.4 fold; caspase 6 by 2.3 fold; caspase 8 by 3.4 fold, and caspase 9 by 1.5 fold. In contrast, caspase 1, 2 and 7 remained unaltered in the expression levels (Figure 2A). Western blots of caspase 3 and 4 showed that the protein level was also increased 2.3 and 2.6 fold, respectively, following CEES treatment (Figure 2C). The cleaving activity of caspase 3 was increased more than 10 fold after 24 h with 50 μM CEES (Figure 2D). Since the changes in the expression were statistically significant ($F(3,9)=1150$, $P<0.001$), these results emphatically point to the induced expression of caspases as a likely trigger for apoptosis in the cells poisoned by CEES.

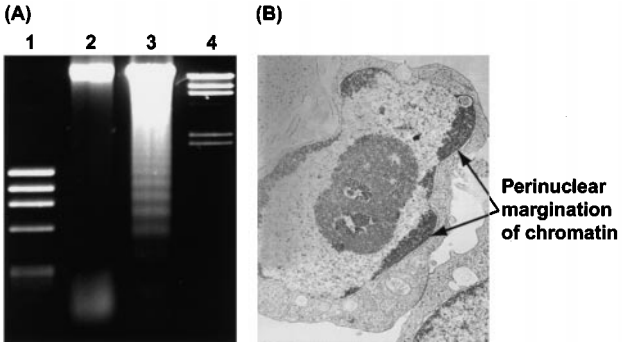

Figure 1 Apoptosis in Jurkat cells. (A) DNA fragmentation in cells treated with 200 μM CEES for 72 h (lane 1) *Hae*III digested ϕ-X174 DNA; (lane 2) Control cells genomic DNA; (lane 3) Genomic DNA from treated cells; (lane 4) *Hind*III digested λDNA. (B) Electron microscopy of Jurkat cells treated with CEES; arrows indicate the highly condensed chromatin bodies.

Down-regulation of Bcl family genes by CEES

To investigate the mechanism of caspase gene up-regulation, we examined the expression of the genes of the Bcl family, which consists of both antagonist and agonist genes that regulate apoptosis through dimerization. After CEES treatment the expression of some Bcl genes was drastically decreased (Figure 3): Bcl-2 by 90%, Bax by 80%, Bcl-X_L by 67%, Bak and Mcl-1 by 70%, and Bik by 57%. All the changes were significantly different from control ($P<0.01$). Our findings agreed with the observation that sulphur mustards could reduce the expression of Bcl-2 (Hur *et al*., 1998).

Inhibition of Akt expression by CEES

Since CEES induced the expression and activity of caspases and suppressed the expression of the Bcl family genes, we reasoned that a common factor that controls the expression of both might be involved. The protooncogene Akt, also known as protein kinase B (PKB), is a serine-threonine

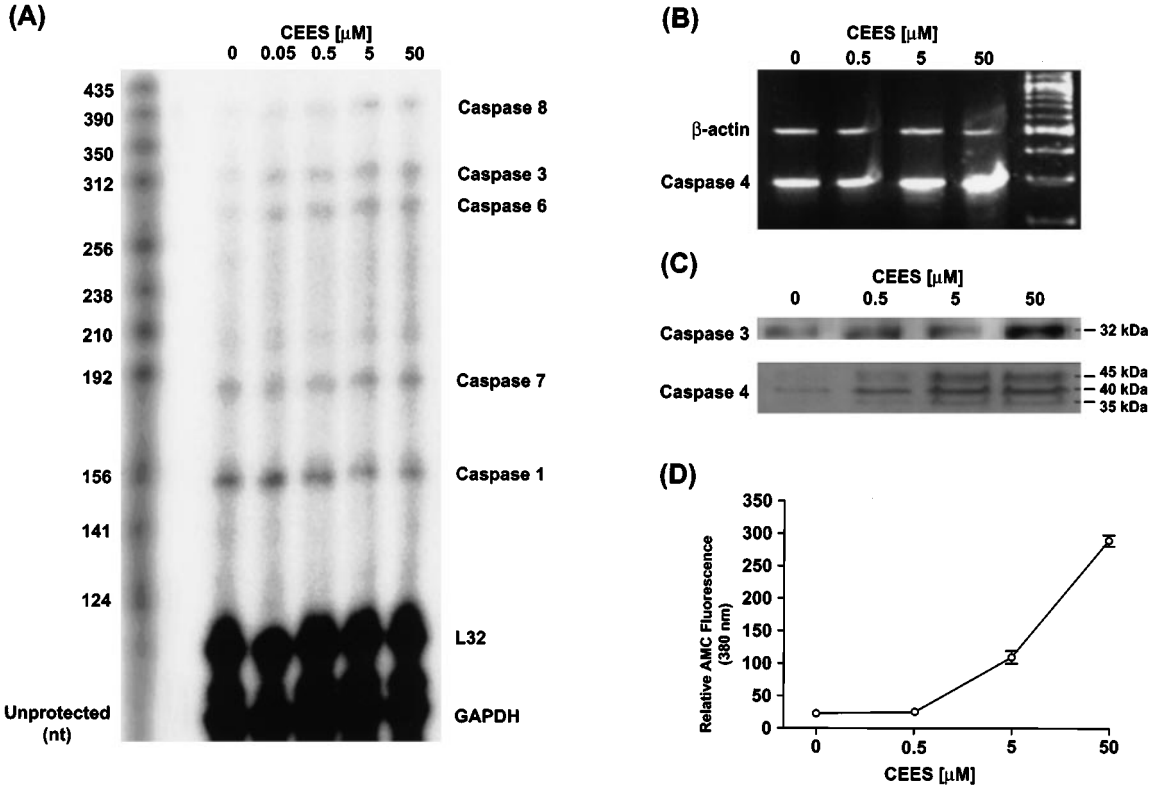

Figure 2 Induction of caspases by CEES. Jurkat cells were exposed to increasing concentrations of CEES at 37°C for 24 h. (A) RPA products separated by 5% polyacrylamide gel and analysed by autoradiogram. (B) RT–PCR of caspase 4 was performed as described under Materials and methods. (C) Western blot analysis of caspase 3 and 4. Total cellular protein was immunoblotted with anti-caspase 3 antibody (upper panel), and anti caspase 4 antibody (lower panel). Samples were run on a 10% polyacrylamide gel. (D) Caspase-3 activities.

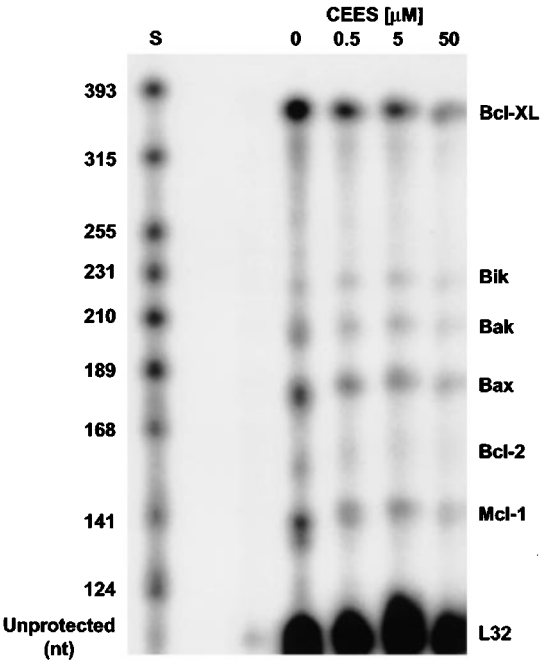

Figure 3 Down-regulation of Bcl family genes in Jurkat cells treated with CEES. RPA products were separated by 5% polyacrylamide gel. S refers to unprotected multiple probe set of hAPO-2.

kinase involved in the regulation of cell survival and apoptosis. Akt is activated by a variety of extracellular signals. Our initial microarray data revealed that Jurkat cells treated with 50 μM CEES reduced the expression of Akt by 50% (not shown). This important observation was further supported by RT–PCR (Figure 4A), and analysis by Western blot which demonstrated that Akt protein level decreased 80% in CEES-treated cells ($P<0.01$) (Figure 4B).

Down-regulation of PDK1

Since activation of Akt requires the phosphorylation of Thr^{308} by PDK1 and Ser^{473} by PDK2, we therefore examined the expression of RNA and protein level of PDK1 and PDK2. Figure 5 shows that high concentration of CEES led to reductions of PDK1 in both its gene expression and protein synthesis ($F(3,9)=692$, $P<0.001$). In contrast, the synthesis of PDK2 was less affected by CEES ($F(3,9)=6.99$, $P=0.01$).

Inhibition of Akt phosphorylation by CEES

Western blot analysis showed that both Thr^{308} and Ser^{473} phosphorylation was also inhibited by CEES treatment. (Figure 6). The phosphorylation of Thr^{308} was suppressed by more than 90%, and that of Ser^{473} by 50%, and was accompanied by over 90% drop ($F(3,9)=2009$, $P<0.001$) in the Akt kinase activity (Figure 6), indicating that changes in the Akt kinase activity were directly correlated with the Akt phosphorylation status in the cells.

Upstream regulators of Akt not affected by CEES

The major upstream regulator of Akt is PI3-K, and its expression at the RNA (Figure 7A) or protein level (Figure 7B) was untouched by CEES. Consistent with these results, the expression of PI3-K upstream factors, Fak and PTEN, remained unaltered ($P>0.05$) (data not shown).

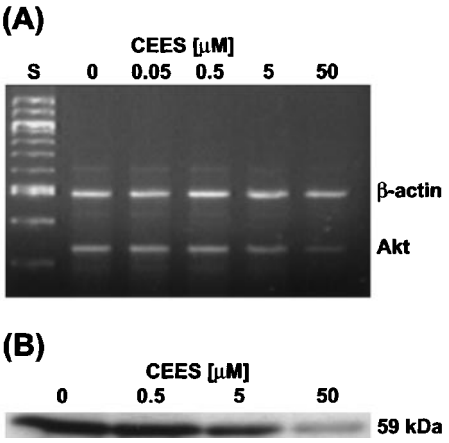

Figure 4 Akt expression in Jurkat cells treated with CEES. (A) RT–PCR of Akt-treated cells. *S* refers to the 100 bp ladder DNA standards. (B) Western blot analysis.

Discussion

Haematological complications such as leucopoenia and bone marrow depletion are observed in humans or rats after being poisoned by sulphur mustards (Fox & Scott, 1980; Pauser *et al*., 1984; Dacre & Goldman, 1996), indicating that leukocytes are part of the targets of sulphur mustards. To elucidate the toxic mechanism of sulphur mustards, we examined the expression of genes that are intimately involved in the regulation of survival and death pathway in Jurkat cells treated with CEES. One of the central regulators of eukaryotic cellular survival is the protooncogene Akt or protein kinase B (PKB), which is a serine/threonine kinase (Jones *et al*., 1991). The physiological significance of Akt is its ability to protect a variety of cell types from apoptosis (Ahmed *et al*., 1997; Dudek *et al*., 1997; Kauffmann-Zeh *et al*., 1997; Kulik *et al*., 1997). Akt can promote cell survival by inhibiting proteins that mediate apoptosis (Downward, 1999), and it could directly phosphorylate and inhibit the caspases, the key executioners of apoptosis (Cardone *et al*., 1998). The kinase activity of Akt is dependent upon the phosphorylation of Akt at Ser^{473} and Thr^{308} (Alessi *et al*., 1996; Andjelkovic *et al*., 1997). Akt is activated when phosphorylated at residues Thr^{308} and Ser^{473}, and its phosphorylation is controlled by at least two key factors: receptor-proximal lipid kinase phosphoinositide 3-kinase (PI3-K), and the lipid phosphatases PTEN. As a target of the PI3-K, Akt is required for the transduction of a variety of PI3-K generated signals including those that inhibit apoptosis and promote oncogenesis.

Phosphatidylinositol-3 kinase (PI3-K), which phosphorylates the 39-position of the inositol ring in phosphatidylinositol 4,5-bisphosphate to generate PIP3, functions upstream of Akt. Binding of the PH domain of Akt to membrane PIP3 causes the translocation of Akt to the plasma membrane, subsequently bringing it into contact with membrane-bound Akt kinase (PDK1 and 2), which phosphorylates and

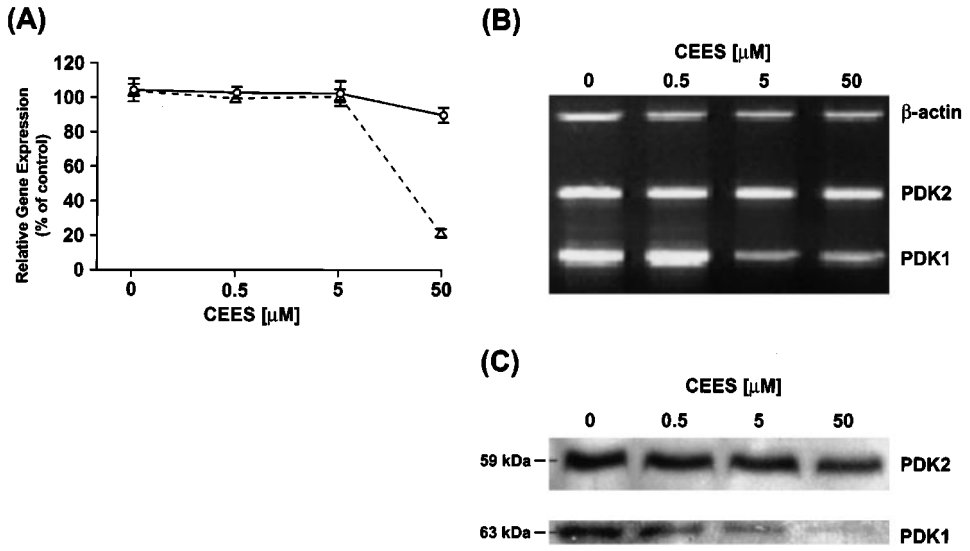

Figure 5 Induction of PDK genes by CEES. (A) PDK1 (dotted line) and PDK2 (solid line) expression determined from microarray. (B) RT–PCR of PDK2 (upper panel) and PDK1 (lower panel). (C) Western blot analysis of PDK2 (upper panel) and PDK1 (lower panel).

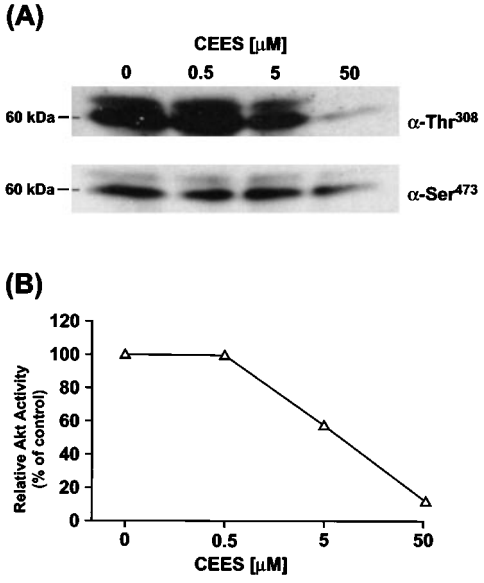

Figure 6 Inhibition of phosphorylation and kinase activity of Akt in Jurkat cells by CEES. (A) Western blot analysis of Akt phosphorylation. Total cellular protein was immunoblotted with anti-Thr^{308} antibody (upper panel), and anti-Ser^{473} antibody (lower panel). Lower panel was a re-blotting in the same membrane in the upper panel after stripping. (B) Relative kinase activities of Akt in CEES-treated Jurkat cells.

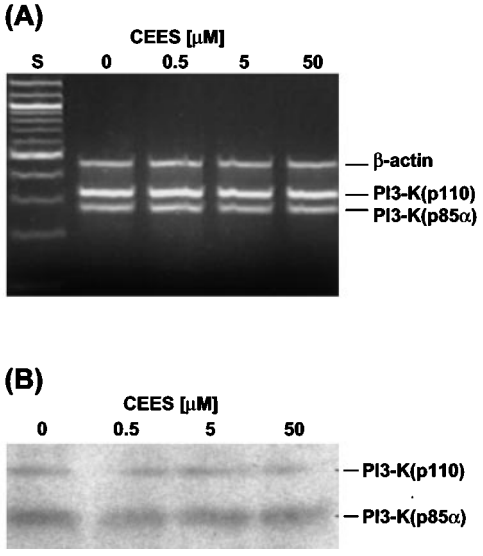

Figure 7 Upstream regulator expression in cells treated with CEES. (A) RT–PCR of PI3-K; *S* refers to the 100 bp ladder DNA standards. (B) Western blot analysis of PI3-K against anti-human PI3-K antibodies.

activates Akt. PTEN acts to limit Akt activation by dephosphorylating PIP3.

PDK1 (protein kinase, phosphatidylinositol 3-phosphate-dependent kinase-I) phosphorylates Akt selectively at Thr^{308} in a reaction completely dependent on added PIP3. The hypothetical kinase responsible for Ser^{473} phosphorylation is usually called PDK2. PDK1 is at the hub of many signalling pathways, activating Akt and protein kinase C isoenzymes, as well as p70 S6 kinase. PDK1 is a 63-kDa Ser/Thr kinase ubiquitously expressed in human tissues. It consists of an N-terminal kinase domain and a C-terminal PH domain. It was first identified by its ability to phosphorylate Thr^{308} of Akt (Stephens *et al*., 1998; Stokoe *et al*., 1997; Alessi *et al*., 1997). PDK1 regulates a number of pathways involved in responses to stress and in growth factor signalling. Our data suggest a novel mechanism of vesicant causing cell death, through which PDK1 and Akt activities are down-regulated.

Akt inhibits apoptosis by phosphorylating bad, thus promoting the binding of the latter to the cell survival factor Bcl-x_L, resulting in the blocking of its activity (Toker & Cantley, 1997). It is also intriguing that inducible inhibition of PI3-K (leading to a reduction in endogenous Akt activity) blocks cellular proliferation but does not induce apoptosis

(Craddock *et al*., 1999), suggesting that Akt might play a role in cell-cycle regulation and/or surveillance mechanisms. Two major upstream factors regulate PI3-K function; focal adhesion kinase (FAK) enhances it and phosphatase and tensin homologue (PTEN) inhibits it.

Bcl family genes regulate a distal step of cell death in a conserved evolutionary pathway, with some members functioning as suppressors and others as promoters of apoptosis. Although the exact mechanisms that confer these properties on the Bcl-2 family of proteins remain unknown, several of them are capable of physically interacting with each other through a complex network of homo- and heterodimers. The agonists, e.g. Bax, Bak, Bid and BAD, are distant members of the Bcl-2 family of proteins that are Bcl-2-interacting proteins. BAD bears the Bcl-2 homology domains 1, 2 and 3 (known as BH1, BH2 and BH3) and dimerizes with Bcl-2 and Bcl-x_L, hence neutralizing their protective effects and promoting cell death (Ottilie *et al*., 1997). The exposure of cell lines to hormones induces the phosphorylation of Bad at two sites, Ser-112 and Ser-136. Phosphorylation of either of these sites causes Bad to dissociate from Bcl-x_L and to associate instead with cytoplasmic 14-3-3 protein. Association of Bad with 14-3-3 protein protects Bad itself from dephosphorylation or sequestration away from its targets. The phosphorylation of Bad disrupts its ability to bind to and inactivate Bcl-x_L. Thus, phosphorylation of Bad inactivates its ability to cause cell death and promotes cell survival. The inhibition of Bcl-x_L expression sensitizes normal human keratinocytes and epithelial cells to apoptotic stimuli (Taylor *et al*., 1999). Our results showed that, interestingly, all of Bcl family proteins, antagonists or agonists, were down-regulated by CEES.

Cells have different responses to toxic damage in different stages. In our preliminary experiments, 24 h after CEES treatment is the best time point to study apoptosis process in the Jurkat cells. To investigate the possible mechanism of the CEES-induced apoptosis, we chose sub-lethal doses under which cells started to exhibit molecular and function changes with marginal structural breakage. After the treatment of Jurkat cells with different concentration of CEES, we screened initially important genes that are involved in the molecular pathology that leads to vesication and cell death by the use of microarray technology in conjunction with RPA. Our results identified that Akt, a pivotal protein kinase that can block apoptosis and promote survival, was specifically down-regulated. After 50 μM of CEES for 24 h, Akt gene expression in the Jurkat cells was reduced by 60% along with a parallel decrease in its protein level by 80%. These results indicated that CEES affected Akt's transcription as well as translation. Whereas the upstream effector of Akt, PDK1, was down-regulated, the expression of the major upstream effectors of Akt, PI3-K, was unchanged in both its catalytic subunit (p110) and regulatory subunit (p85α). Phosphorylation analysis showed that function-related phosphorylation in both Thr^{308} and Ser^{473} of Akt was suppressed by CEES, regardless of the expression of its upstream effectors. The change of phosphorylation in Akt is dependent on at least two factors: the quantity and function of PDK1 and PDK2. Whereas PDK1 expression was down-regulated by CEES as well as its kinase activity, CEES only inhibited PDK2's function but not its gene expression and protein level in cells. It is known that the function of Akt is correlated with its phosphorylation level (Alessi *et al*., 1996; Andjelkovic *et al*., 1997). Our results demonstrated that the decrease of Akt function was correlated with the reduction in its phosphorylation, and this might be a key factor for the CEES-induced apoptosis. Concomitantly, the genes of the Bcl family, which include Bcl-x_L, Bcl-2, Bfl 1, Blk, Bax and Mc11, were also down-regulated. Some canonical death executioner genes, which include caspases 3, 6 and 8, were strikingly up-regulated. Meanwhile the caspase 4 and 5, which belong to the ICE subfamily involved in inflammation, were also up-regulated. These data point out that because of the CEES insult, cells went through the apoptosis cascade. All of the genes that support cell survival were suppressed while some death genes were induced. Thus, a possible mechanism of apoptosis induced by CEES in Jurkat cells was by triggering the inhibition of the Akt pathway. However, what primary cellular targets CEES alkylates remain to be defined, and we intend to pursue further this goal.

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