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Cytotoxic and Apoptotic Effects of Echinomycin on Murine Leukemia Cells

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Abstract: A number of anticancer-chemotherapeutic agents induce cell death through the process of apoptosis. Effects of echinomycin, an anticancer agent on cancer progression, were investigated in P388 murine leukemia cells. First, according to the results of cytotoxicity measurement, IC_{50} of echinomycin was 1.12 nM, a relatively lower value than the other examined anticancer agents, mitomycin-C and etoposide. Second, the DNA fragmentation assay for echinomycin-treated cells exhibited that echinomycin was able to induce apoptosis in a shorter period of time and with a lower dose than mitomycin-C or etoposide. The data of DNA fragmentation were quite comparable to those of cytotoxicity measurement. Finally we showed that mitogen-activated protein (MAP) kinase, a key protein in cell mitosis, was translocated into the nucleus from the cytosol after treatment with echinomycin. These findings suggest that a MAP kinase-related process may be involved in apoptosis induced by echinomycin.
Key words: apoptosis, cytotoxicity, echinomycin, mitogen-activated protein (MAP) kinase.

Echinomycin is an antitumor agent which acts by binding to DNA via the mechanism of bifuctional intercalation (Waring *et al.*, 1974; Keith *et al.*, 1990). Echinomycin consists of two planar quinoxaline moieties connected by an octapeptide bridge (Martin *et al.*, 1975). This anticancer agent is derived from *Streptomyces echinatus*, and is in phase II clinical trials (Scott *et al.*, 1994). Up to date, it is reported that apoptosis is induced by anticancer agents including etoposide and mitomycin-C (Scott, 1989). Also the apoptosis occurs as deletion of autoreactive T cell clones during thymic maturation (Smith *et al.*, 1989), and the addition of physiologic regulatory hormones, glucocorticoids (Wyllie *et al.*, 1982). The apoptotic process is found in cells attacked by CTL and NK cells (Russel, 1981), and recently in cells infected by HIV-1 (Corbeil *et al.*, 1996). In fact, the molecular mechanism of apoptosis is not completely defined. However, until now it is widely assumed that DNA cleavage is the result of endogenous endonuclease activity (Duke *et al.*, 1986), and apoptosis is induced by PKA activated by cAMP and by influx of calcium from outside the cell (McConkey *et al.*, 1990). Also it was reported that PKC is a negative regulator of PKA and Ca^{2+} and therefore it prevents apoptosis (McConkey *et al.*, 1989). But it is unknown which enzymes are associated with the endonuclease directly in nucleus. From this it can be seen that apoptosis is controlled by many different, distinct signals, but recently it appears that different sinalling pathways converge to activate a common apoptotic program (Raff, 1992; Raff *et al.*, 1993). Anti-apoptotic proteins, such as Bcl-2 and baculovirus p35, can inhibit apoptosis in response to many different death-inducing signals (Sugimoto *et al.*, 1994; Thompson, 1995). Also, the *Drosophila reaper* gene may serve as a universal activator of apoptosis, a signal is transmitted to death-promoting proteins, ICE-like proteases including ICE (Cerretti *et al.*, 1992), nedd-2/Ich-1 (Kumar *et al.*, 1992), CPP32 (Fernandez-Alnemri *et al.*, 1993), granzyme B (Shi *et al.*, 1992) and so on. Recently, two nuclear proteins, poly (ADP-ribose) polymerase and lamins have been identified as substrates for proteolytic cleavage (Kaufmann *et al.*, 1993), but more substrate candidates remain to be discovered. At any rate, events downstream of ICE are unclear and it is unknown whether ICE or CPP32 enter the nucleus or if the actual substrate for ICE or CPP32 goes into the nucleus. In this point, we focused on MAP kinase, which activates enzymes in the cytosol and transcription regulators in the nucleus, shuttling between nucleus and cytoplasm (Mansour *et al.*, 1994). Also recent date suggests that MAP kinase family members, including ERK (extracellular signal-regulated kinase), JNK (c-Jun NH_2-ter-

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minal protein kinase) and p38 contributed to the regulation of neuronal apoptosis (Xia *et al.*, 1995).

In this study, we report the cytotoxic capacity of echinomycin in P388 cells by MTT assay and DNA fragmentation patterns, and show that echinomycin was able to induce apoptosis in a shorter period of time and with a lower dose than mitomycin-C or etoposide. And, to elucidate the involvement of MAP kinase during apoptosis induced by anticancer agents, we examined the translocation of MAP kinase into the nucleus after treatment with echinomycin. On the basis of these results, we suggest that MAP kinase, which delivers signals shuttling between nucleus and cytosol, moved to the nucleus after treating P388 cells with echinomycin.

Materials and Methods

Chemicals and cells

Echinomycin, mitomycin-C and etoposide were purchaed from Sigma Chemical Co. (St. Louis, USA). Chemiluminescencent immunoblotting chemicals (Western-Light Plus protein detection system) were from Tropix (Bedford, USA) and all other chemicals were of analytical grade. P388 murine leukemia cells from ATCC (Rockville, USA) were grown in DMEM supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 U/ml) and streptomycin sulfate (100 μg/ml). The cells were maintained in a humidified atmosphere of 5% CO_2 at 37℃.

Cytotoxicity assay

This assay is dependent on the cellular reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium-bromide (MTT) by the mitochondrial dehydrogenase of various cells to a blue formazan product, which can be measured spectrophotometrically. P388 cells growing exponentially were inoculated to 1×10^4 cells/well using 96 well plate and supplemented with 100 μl DMEM. Cells were treated with echinomycin, mitomycin-C or etoposide in various concentrations and incubated for 3 days. 50 μl MTT (1 mg/ml) was added to each well and incubated at 37℃ for 4 h, MTT solutions were separately removed from each well and then 150 μl DMSO was added to dissolve formazan produced from MTT. OD values of the solutions were measured at 540 nm by spectrophotometer.

DNA fragmentation

Several concentrations of echinomycin, mitomycin-C and etoposide were added to 5×10^5 P388 cells at the various time points (3~48 h). The cells were harvested by centrifugation at 350×*g* for 10 min. The pellets were lysed with 0.4 ml lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 100 mM NaCl) containing 0.1% SDS, 10 μl proteinase K (1 mg/ml), and incubated at 37℃ for 12 h. This solution was then dissolved in 10 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) containing 0.1 mg/ml RNase (DNase free) for 2 h at 37℃. Then electrophoresis was carried out in 1.0% agarose gels containing 50 μg/ml ethidium bromide and using TBE buffer for 3 h at 40 V. DNA was visualized by UV lamp and photographed.

Nucleus separation

Confluent P388 murine leukemia cells were maintained in serum-free DMEM media for 24 h. After the P388 cells (5×10^6) were treated with 1 μg echinomycin for 5, 10, 30, 60 and 240 min, cells were harvested at 350×*g* for 10 min at 4℃. The cell pellets were washed with STE solution (150 mM NaCl, 50 mM Tris-Cl, 1 mM EDTA, pH 7.2) and scraped into 500 μl hypotonic lysis solution I (10 mM β-glycerophosphate, 1 mM sodium orthovanadate, 2 mM $MgCl_2$, 10 mM KCl, 1 mM dithiothreitol, 40 μg of PMSF per ml, and 10 μg of both pepstatin and leupeptin per ml, pH 7.2) per 5×10^6 cells on ice for 30 min, and then dounce-homogenized (20 to 30 strokes) with a tight-fitting pestle. The homogenate was loaded onto 1 ml of 1 M sucrose in lysis solution I and centrifuged at 1,600×*g* for 10 min to pellet nuclei. To prepare the cytosol fraction, 500 μl of the supernatant was taken above the sucrose cushion. The nuclear pellet was solubilized in 300 μl of hypotonic lysis solution II (hypotonic lysis solution I+0.5% Igepal CA-630, 0.1% deoxycholate, 0.1% Brij-35) on ice for 30 min. The lysates were centrifuged at 15,000×*g*, 4℃ for 30 min, and then supernatant was used as nucleus solution. The purity of each fraction was monitored by measuring the activity of lactate dehydrogenase, a cytosol marker.

Immunoblot analysis

For a MAP kinase immunoblot assay, prepared nuclear extracts from echinomycin treated P388 cells were electrophoresed on 10% polyacrylamide gel in the presence of SDS. After electrophoresis, proteins were transferred to nitrocellulose membranes, blocked in TBST (10 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.15% Tween-20) containing 2% bovine serum albumin and the protein transferred membrane was washed 3 times in TBST. The membrane was incubated with anti-ERK1 antibodies at a 1:1,000 dilution in TBST containing 2% bovine serum albumin for 1 h and then washed 3 times in TBST. The blots were incubated with biotinylated anti-rabbit Ig antibodies at a 1:10,000 dilution in TBST contatining 2% bovine serum albumin for 1 h and then washed 3 times in TBST. The membrane

Table 1. Cytotoxic effect measurements of anticancer agents on P388 murine leukemia cells by MTT assay. P388 cells (1×10^4 cells/well) were plated and anticancer agents were diluted in different concentrations

Anticancer agents	IC_{50}[a]
Echinomycin	0.122 nM[b]
Mitomycin-C	0.124 μM
Etoposide	0.102 μM

[a] IC_{50} (50% inhibitory concentration) is equal to anticancer agents' concentration when sample absorbance-blank absorbance/control absorbance-blank absorbace$\times$100 is 50.
[b] This numerical value was acquired by simple linear regression.

was incubated with alkaline phosphatase conjugated streptoavidin at a 1:10,000 dilution in TBST containing 2% bovine serum albumin for 1 h, washed 3 times in TBST and then treated with Western-Light Plus protein detection system.

Results and Discussion

Cytotoxic effect of echinomycin

Prior to measurement of cytotoxic effects of different anticancer agents to P388 cells, we analysed the pattern of P388 cell growth. Since the cells were exponentialy growing during 3 days, P388 cells were incubated for 72 h after treatment with echinomycin, mitomycin-C or etoposide. Following appropriate incubation, live cells were analysed by MTT assay. According to the results of cytotoxicity measurements, IC_{50} of echinomycin was 0.122 nM, about a thousand times lower value than those of other examined anticancer agents, mitomycin-C and etoposide (Table 1).

DNA fragmentation induced by echinomycin

In order to determine the apoptotic effect of echinomycin, DNA fragmentation was induced for various incubation times and concentrations of anticancer agents. Mitomycin-C and etoposide (Scott, 1989), which have been reported to induce DNA fragmentation, were also tested to compare the apoptotic effects of the agents. Mitomycin-C was able to induce DNA fragmentation after 48 h treatment as reported previously (Rey *et al.*, 1994), and at higher concentration (over 100 μg/5 $\times10^5$ cells) (Fig. 1-A). Etoposide is known to be needed at 40 μg for DNA fragmentation and incubation times were variable for each cell type (Scott, 1989). Therefore in the case of etoposide, we performed the DNA fragmentation assay for various incubation times. The obtained result (Fig. 1-B) showed that induction of DNA fragmentation by etoposide occured after at least 24 h incubation, like mitomycin-C. We also examined DNA fragmentation patterns of echinomycin for

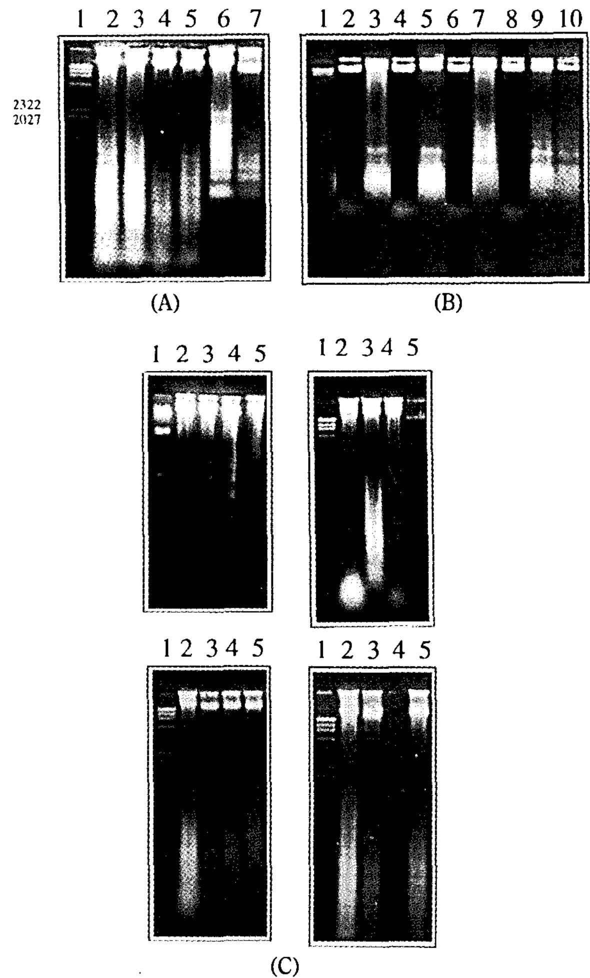

Fig. 1. DNA fragmentation induced by anticancer agents. (A) Mitomycin-C induced DNA fragmentation in P388 cells. P388 cells (5×10^5) were treated with 1, 5, 10, 100, 200 μg for 48 h (lane 3-7 respectively). The migrations of λ/*Hin*dIII size marker (lane 1) and control (lane 2) were shown for comparison. (B) Etoposide-induced DNA fragmentation in P388 cells. Cells (5×10^5) were treated with 40 μg etoposide for 3, 6, 12, 24, 48 h (lane 3, 5, 7, 9, 10, respectively). lane 2, 4, 6, 8 were controls for 3, 6, 12, 24/48 h and lane 1 is λ/*Hin*dIII. (C) Echinomycin-induced DNA fragmentation in P388 cells. P388 cells (5×10^5) were treated with 0, 0.1, 1, 5 μg echinomycin (lane 2~5, respectively) for 3 h (upper left), 6 h (upper right), 12 h (lower left), 24 h (lower right) and lane 1 is λ/*Hin*dIII.

longer incubation times (72 h, 96 h), but we could not detect any DNA fragmentation under those conditions. In contrast to the data for mitomycin-C and etoposide, echinomycin was able to induce apoptosis in a shorter period of time (within 3 h) and with a lower dose (0.1~5 μg) than mitomycin-C and etoposide (Fig. 1-C). The data for DNA fragmentation were quite comparable to those of cytotoxicity measurement.

MAP kinase translocation into Nucleus

MAP kinase, a key protein in cell mitosis, largely phosphorylates serine/threonine residues of target pro-

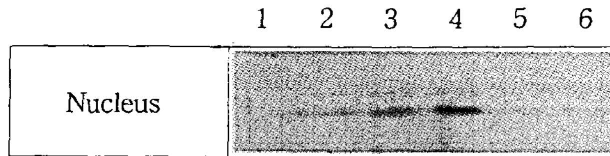

Fig. 2. Immunoblotting assay for MAP kinase translocation. After echinomycin treated to P388 cells, nucleus fractions were separated. P388 cells (5×10^6) were treated with 1 μg echinomycin for 5, 10, 30, 60, 240 min (lane 2, 3, 4, 5, 6, respectively) or without echinomycin (lane 1).

teins. Shuttling between the nucleus and cytoplasm, it activates enzymes in the cytosol and transcription regulators in the nucleus (Mansour *et al.*, 1994; Corbeil *et al.*, 1996). We examined translocation of MAP kinase in the nucleus by immunoblot analysis. P388 murine leukemia cell homogenates treated with echinomycin were fractionated into cytosolic and nuclear fractions, and localization of the MAP kinase was examined. As shown in Fig. 2, MAP kinase in P388 cells translocated from cytosol into nucleus about 5 min after treatment with echinomycin and showed maximal nuclear translocation after 30 min. The nuclear localization of MAP kinase is not due to contamination with cytosolic enzymes since those fractions were tested by the cytosol marker enzyme lactate dehydrogenase. Actually, it is well known that the cytosolic MAP kinase is activated within 2~5 min when treated with mitogen, such as PMA or serum (Chen *et al.*, 1992). These findings suggest that a MAP kinase-related process may be involved in apoptosis induced by echinomycin. More comprehensive studies are planned to verify this hypothesis.

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