Amino Acids (2005) 28: 21–27
DOI 10.1007/s00726-004-0155-8

Amino Acids
Printed in Austria

Involvement of polyamines in evening primrose extract-induced apoptosis in Ehrlich ascites tumor cells

T. Arimura[1], **A. Kojima-Yuasa**[1], **Y. Tatsumi**[1], **D. O. Kennedy**[2], and **I. Matsui-Yuasa**[1]

[1] Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, Osaka, Japan
[2] Department of Environmental Health Sciences, Mailman School of Public Health, Columbia University, New York, U.S.A.

Received August 30, 2004
Accepted November 18, 2004
Published online February 10, 2005;

Summary. We previously demonstrated that evening primrose extract (EPE) induced apoptosis and inhibited the DNA synthesis in Ehrlich ascites tumor cells (EATC) and suggested that EPE-induced inhibition of the growth of EATC are via at least two pathway differentially modulated by reactive oxygen species, notably intracellular peroxides. These are (a) the EPE-induced apoptosis pathway which is dependent on increases in hydrogen peroxide and (b) the EPE-induced inhibition of cell proliferation which is hydrogen peroxide independent. In this study, EPE brought about a significant decrease in intracellular polyamine levels. Furthermore, the addition of polyamines reversed the EPE-induced decrease in cell viability and suppressed the EPE-induced increase in intracellular hydrogen peroxides. However, the addition of polyamines did not reverse EPE-induced decrease in DNA synthesis and phosphorylation of Rb protein, and EPE-induced translocation of AIF. These results suggest the involvement of polyamines in the EPE-induced apoptosis pathway which is dependent on increase in hydrogen peroxide.

Keywords: Evening primrose extract – Apoptosis – Polyamines – Hydrogen peroxide – DNA synthesis – Ehrlich ascites tumor cells

Introduction

Oenothera biennis L., one species of evening primroses, is a herbal plant. The edible oil from the seeds of evening primrose oil has been shown to have several pharmacological effects such as anti-diabetic (Takahashi et al., 1993; Jack et al., 2002), anti-inflammatory (Mera, 1994; Dirks et al., 1998), and also to be anti-tumoric (Yoshida et al., 1991; Muñoz et al., 1998, 1999). We previously demonstrated that an extract from evening primrose (EPE) induced apoptosis in Ehrlich ascites tumor cells (EATC), and this effect is specific on tumor cells. Furthermore, our results demonstrated that EPE exposure elicited the rapid increase in the activity of superoxide dismutase (SOD) and intracellular peroxides levels. These changes caused translocation of Bax to mitochondria, and then mitochondrial cytochrome *c* was released. However, in EPE-treated EATC no activation of caspase-3 was observed (Arimura et al., 2003a). On the other hand, apoptosis-inducing factor (AIF) was translocated from mitochondria to nuclei. The EPE-induced translocation of AIF was suppressed with the addition of catalase, suggesting that the rapid intracellular peroxide levels after addition of EPE triggers off induction of apoptosis, which is AIF-mediated and caspase-independent (Arimura et al., 2003b). Furthermore, we have shown that EPE elicited a dose-dependent accumulation of cells in the G1 phase and inhibited DNA synthesis. Our results demonstrate that cell cycle arrest and proliferation in EATC by EPE are associated with decreased Rb phosphorylation. Also, inhibitions of Rb phosphorylation and DNA synthesis by EPE were not suppressed with the addition of catalase. A previous study also suggested that intracellular peroxides, which triggers off induction of apoptosis, are not the trigger of EPE-induced G1 arrest in cell cycle (Arimura et al., 2004).

The polyamines (putrescine, spermidine and spermine) are indispensable for proliferation and differentiation of cells. Depletion of polyamines is associated with reduced cell proliferation, and the levels of polyamines rapidly increase during cell growth (Pegg and McCann, 1982; Tabor and Tabor, 1984; Casero and Pegg, 1993; Marton and Pegg, 1995; Pegg, 1998). The importance of polyamines in cell growth is further evidenced in the observations that dysregulation of ornithine decarboxylase

and spermidine/spermine N^1-acetyltransferase, the rate-limiting enzymes in the synthesis and biodegradation, respectively, of polyamines, affects the concentration of polyamines which modulates polyamines synthesis, degradation, uptake and excretion (Matsui et al., 1981; Poulin et al., 1995). These biochemical and physiological modifications can eventually control vital cell events such as apoptosis (Marton and Morris, 1987; Min et al., 1995; Grassilli et al., 1995; Dypbukt et al., 1994; Tobias and Kahana, 1995; Pacham and Cleveland, 1994). Polyamines may be involved in the onset of DNA degradation, as spermidine and spermine stabilize chromatine- and polyamine-depleted cells in which chromatine and DNA structural changes occur (Marton and Morris, 1987; Porter and Janne, 1987).

In this study, we investigated the involvement of polyamines in EPE-induced increase in apoptosis as well as in EPE-induced decrease in DNA synthesis.

Materials and methods

Materials

Evening primrose extract (EPE) was prepared by Oryza Oil & Fat Chemical Co. Ltd., Japan. The seeds of evening primrose, *Oenothera biennis* L. defatted with hexane were extracted with 70% ethanol and evaporated to dryness *in vacuo*. Fetal calf serum (FCS) was purchased from Thermo Trace Ltd. (Melbourne, Australia). Mouse anti-Rb antibody and mouse anti-AIF antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, USA). Biotinylated goat anti-mouse IgG and horseradish peroxidase-coupled streptavidin were obtained from DAKO (Kyoto, JAPAN). Other chemicals used in this study were special grade commercial products.

Methods

Cell culture

Ehrlich ascites tumor cells (EATC) were cultured in humidified atmosphere of 5% CO_2 in air at 37°C for 3–4 days in Eagle's minimum essential medium containing 10% FCS. Then the cells were washed and cultured again at a concentration of 1×10^6/ml in fresh medium. EPE was dissolved in dimethyl sulfoxide (DMSO) and diluted in cultured medium immediately before use (final DMSO concentration <0.5%). In all the experiments control cultures were made up of medium, DMSO and the cells. In experiments involving the addition of polyamines in cell culture, 1 mM aminoguanidine was added as an inhibitor of amine oxidase derived from FCS. Aminoguanidine had no effect on various parameters of the cell measured in this study.

Assay of cell viability

Cell viability of Ehrlich ascites tumor cells was determined by the Trypan blue exclusion analysis. The tumor cells (1×10^6/ml) treated with EPE were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 24 h in Eagle's minimum essential medium containing 10% FCS. To a cell suspension was added an equal volume of 0.4% Trypan blue reagent (Sigma) and percentages of viable cells were evaluated under the field microscope. Assays were performed in triplicate.

Measurement of DNA synthesis

Cells were labeled with [methyl-^{3}H]thymidine from 23 h to 24 h after EPE addition. The labeled cells were washed twice in phosphate buffered saline (PBS) and radioactivity of acid-insoluble fraction was measured as the amount of DNA synthesis (Matsui-Yuasa et al., 1987).

Preparation of protein for western blot analysis of Rb

EPE-treated cells were washed twice PBS and resuspended in 100 μl of lysis buffer (150 mM NaCl, 50 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% sodium deoxycholate, 1% Nonidet P-40, 10 μg/ml pepstatin, 1 mM sodium vanadate, 50 μg/ml leupeptin, 20 μg/ml aprotinin, 100 μg/ml phenylmethylsulfonate, pH 7.5) for 20 min on ice followed by freeze-thawing three times. Lysates were centrifuged at 17500 × g for 20 min at 4°C and supernatant was collected for western blot (Kennedy et al., 2002). Protein concentrations were determined by the Bradford method (Bradford, 1976).

Preparation of protein in nuclear fraction for western blot analysis of apoptosis-inducing factor (AIF)

EPE-treated cells were washed twice PBS and resuspended in 100 μl of lysis buffer (20 mM Hepes-KOH, 1 mM EGTA, 1 mM EDTA, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5% Nonidet-p 40, 250 mM sucrose, 0.5 mM PMSF and 1 mM DTT, pH 7.5). Lysates were centrifuged at 750 × g for 10 min at 4°C and pellet was resuspended in 400 μl of lysis buffer as nuclear fraction (Ghribi et al., 2002). Protein concentrations were determined by the Bradford method.

Western blotting analysis of Rb and AIF

Protein (20 μg) for Rb assay and protein (20 μg) from the nuclear fraction for AIF assay were loaded onto each lane of a 7.5% and 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and the separated proteins were blotted to 0.45 μm polyvidyline fluoride membranes (PVDF) membranes (Amersham Pharmacia Biotech). After an overnight blocking with 5% non-fat milk, 0.1% Tween-20, in PBS, each membrane was stained with anti-Rb antibody or anti-AIF antibody for 1 h at room temperature. After washing, the membrane was reincubated with 1:1500 diluted biotinylated mouse IgG and for 1 h at room temperature. The membrane were washed several times, and then incubated with 1:400 diluted horseradish peroxidase-coupled streptavidin for 1 h at room temperature. After several washing steps the color reaction was developed with 3-amino-9-ethylcalbazole (Kennedy et al., 2002; Tanaka et al., 1998).

Analysis of intracellular ROS formation

2′,7′-Dichlorodihydrofluorescein diacetate (DCFH-DA), a relative specific probe for the presence of hydrogen peroxide, was used as a probe for intracellular ROS formation (LeBel et al., 1990). Cells were incubated with DCFH-DA (8 μM) for the last 15 min of EPE treatment. Cells were washed with PBS twice and resuspended in Hanks solution. Fluorescence intensity was measured using a micro-plate reading fluoroscan plate-reader (Wallac 1420 ArVOsx, Amersham Pharmacia Biotech) with the excitation wavelength at 485 nm and the emission wavelength at 535 nm. The amount of intracellular ROS was calculated from a standard curve derived from 2′,7′-dichlorofluorescein (DCF) (Sigma). Protein concentration was measured by the Bradford method. For observation of fluorescence microscope, DCFH-DA (20 μM) and EPE were added simultaneously for 15 min and washed with PBS. After staining, cells were visualized immediately under a fluorescence microscope.

Determination of intracellular polyamines

Cells (2×10^6) collected by centrifugation were extracted with 0.3 ml of 0.4 N perchloric acid. The polyamines were separated on an ODS column (CrestPak C18S, 4.6 × 150 mm, particle size 5 μm, JASCO, Tokyo, Japan) using solvents A (10 mM 1-hexane sulfonic acid (sodium salt), 10 mM sodium acetate, pH 4.5) and B (methanol). The sample was eluted with 100% solvent A for 8 min, and then with a programmed solvent gradient using a gradient curve. The gradient changed from 0% to 20% solvent B from 8.1 min to 30 min and from 20% to 40% solvent B from 30.1 min to 40 min at a flow rate 1.0 ml/min. Eluted fractions were mixed with 6 mM *O*-phthalaldehyde (0.4 ml/min) and the fluorescence was measured at excitation and emission wavelength of 365 nm and 455 nm, respectively, for assay of polyamines with an FP1520 fluorescence detector (JASCO). The DNA content of the perchloric acid-precipitable materials was determined as previously described (Schneider et al., 1957), using calf thymus DNA as a standard.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity in the suspension buffer was measured as described previously (Bergmeyer et al., 1965) to monitor the degree of cell membrane injury. The assay solution contained 0.6 mM sodium pyruvate, 0.18 mM NADH and suitable volume of enzyme solution at 25°C in a total volume of 3.15 ml. The initial rate of NADH loss, measured as a reduction in absorbance at 340 nm, was used as an indication of lactate dehydrogenase activity. Under this assay condition, the loss of NADH was linear with respect to time and enzyme concentration over the range of enzyme activity monitored. The percentage of LDH leakage from cells was calculated according to the following formula: % LDH leakage = (LDH activity present in the medium after incubation/total LDH activity in cells) × 100.

Statistical analysis

Data are represented as mean ± SD (standard deviation from the mean) and statistical evaluations of cell viability, DNA synthesis and polyamines contents were made using analysis of variance with Fisher's *post hoc comparison* test and differences in all other parameters were tested using Student's *t*-test. $P < 0.05$ was used to indicate a statistically significant difference.

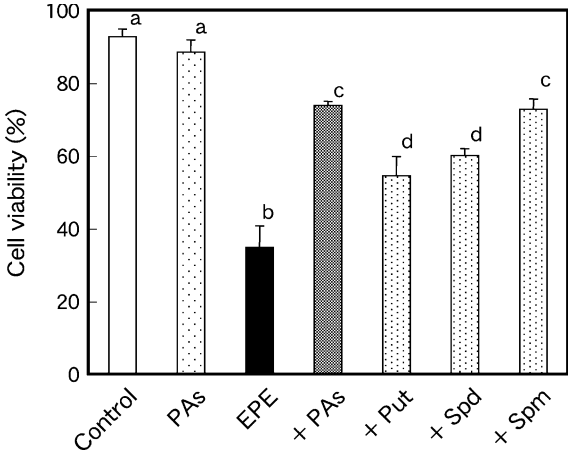

Fig. 1. Effect of exogenous polyamines on cell viability in EPE-treated Ehrlich ascites tumor cells. Cells were cultured in Eagle's minimum essential medium containing 10% FCS for 3–4 days, were diluted and incubated again in fresh medium with or without EPE. Putresine (*Put*), spermidine (*Spd*), spermine (*Spm*) or their polyamines (*PAs*) at the concentration of 0.1 mM each were added at the same time as EPE (200 μg/ml) addition and cell viability was measured 24 h later. Results show means ± SD of three experiments. Data not sharing common alphabet are significantly different ($p < 0.05$) using Fisher's test

Results

The effect of polyamines on cell viability in EPE-treated EATC was examined. Cells were incubated in fresh medium with or without EPE. Putresine (Put), spermidine (Spd), spermine (Spm) or their polyamines (PAs) at the concentration of 0.1 mM each were added at the same time as EPE (200 μg/ml) addition. This concentration of polyamines was determined from dose-dependent studies

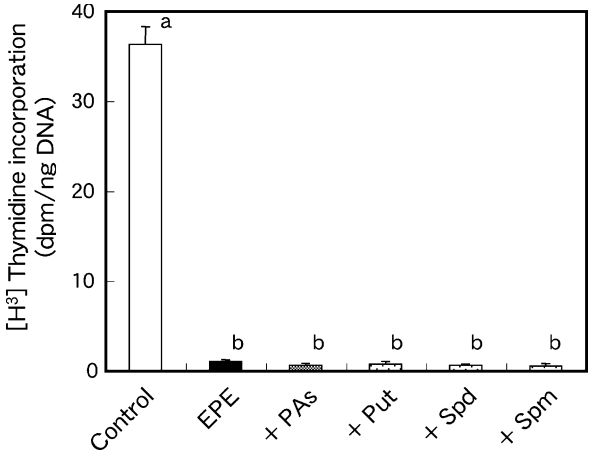

Fig. 2. Effect of exogenous polyamines on DNA synthesis in EPE-treated Ehrlich ascites tumor cells. Cells were cultured in Eagle's minimum essential medium containing 10% FCS for 3–4 days, were diluted and incubated again in fresh medium with or without EPE. Putresine (*Put*), spermidine (*Spd*), spermine (*Spm*) or their polyamines (*PAs*) at the concentration of 0.1 mM each were added at the same time as EPE (200 μg/ml) addition. Cells were labeled with [^{3}H]-thymidine from 23 h to 24 h after EPE addition. Radioactivity of acid-insoluble fraction was measured as the amount of DNA synthesis. EPE extract was dissolved in dimethyl sulfoxide (DMSO) (final concentration of DMSO was <0.5%). Results show means ± SD of three experiments. Data not sharing common alphabet are significantly different ($p < 0.05$) using Fisher's test

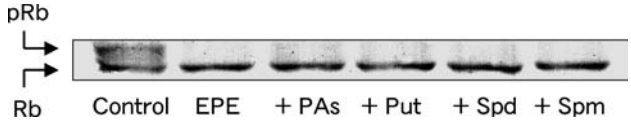

Fig. 3. Effect of exogenous polyamines on EPE-induced changes in Rb phosphorylation. Putrescine (*Put*), spermidine (*Spd*), spermine (*Spm*) or their polyamines (*PAs*) at the concentration of 0.1 mM each were added at the same time as EPE (200 μg/ml) addition, and cells were harvested at 8 h. Lysates were analysed by Western blot. Results are representative of three separate determinations. *pRb* hyperphosphorylated Rb; *Rb* hypophosphorylated Rb

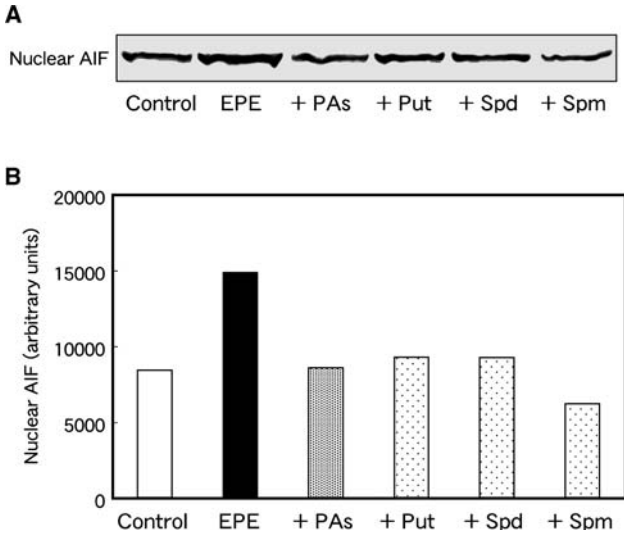

Fig. 4. Effect of exogenous polyamines on EPE-induced translocation of AIF in Ehrlich ascites tumor cells. Putresine (*Put*), spermidine (*Spd*), spermine (*Spm*) or their polyamines (*PAs*) at the concentration of 0.1 mM each were added at the same time as EPE (200 μg/ml) addition, and cells were harvested at 6 h. (**A**) Western blot, (**B**) quantification of nuclear AIF levels by densitometer. Results are representative of three separate determinations

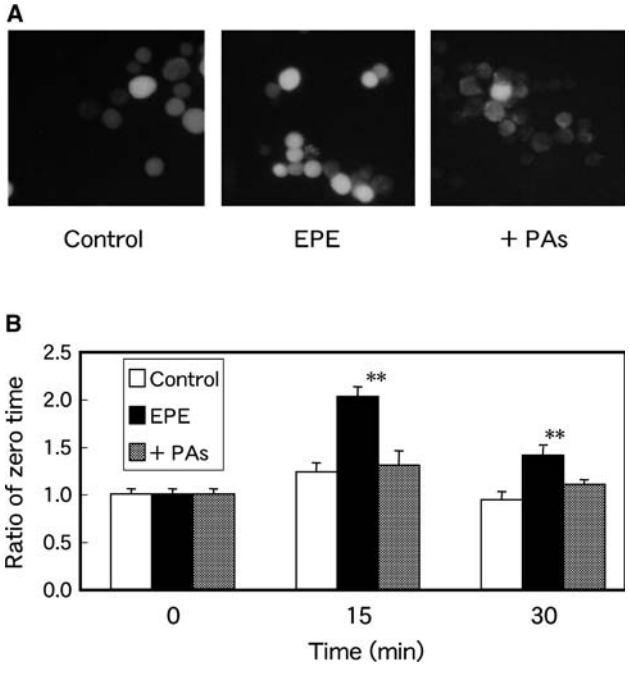

Fig. 5. Effect of exogenous polyamines on EPE-induced changes in intracellular peroxides levels in Ehrlich ascites tumor cells. Peroxides were measured by the DCFH-DA method. The fluorescence intensity of DCF was monitored at 535 nm with excitation wavelength set at 485 nm and used to indicate the level of intracellular peroxides formation. Polyamines (*PAs*) at the concentration of 0.1 mM each were added at the same time as EPE (200 μg/ml) addition. **A** Cells were treated with and without EPE and polyamines (PAs) for 15 min and undergone fluorography. **B** DCF fluorescence intensity of cells was measured at 0, 15 and 30 min after treatment with EPE and polyamines (*PAs*). Results show means ± SD of three experiments. $^{**}p < 0.01$, compared to the control group at the same time point using Student's *t*-test

in our previous studies involving polyamines in this particular cell line and thus the continuity of use as the effective dose. As presented in Fig. 1, EPE decreased cell viability and this effect was significantly improved with addition of polyamines.

In a previous study, we demonstrated that EPE caused a decrease in DNA synthesis, an accumulation of cells in the G1 phase, and a decrease in the phosphorylation of Rb protein, in its growth inhibitory effect in EATC. We examined the effect of polyamines on EPE-induced inhibition of cell proliferation. EPE (200 μg/ml) inhibited DNA synthesis (Fig. 2) and phosphorylation of Rb (Fig. 3), but the addition of polyamines (0.1 mM each) did not reverse these effects.

We previously reported that EPE-induced apoptosis in EATC is AIF-mediated and caspase-independent. To determine whether the effect of polyamines was related to EPE-induced translocation of AIF to nuclei, we carried out Western blot analysis of the nuclear extract. The

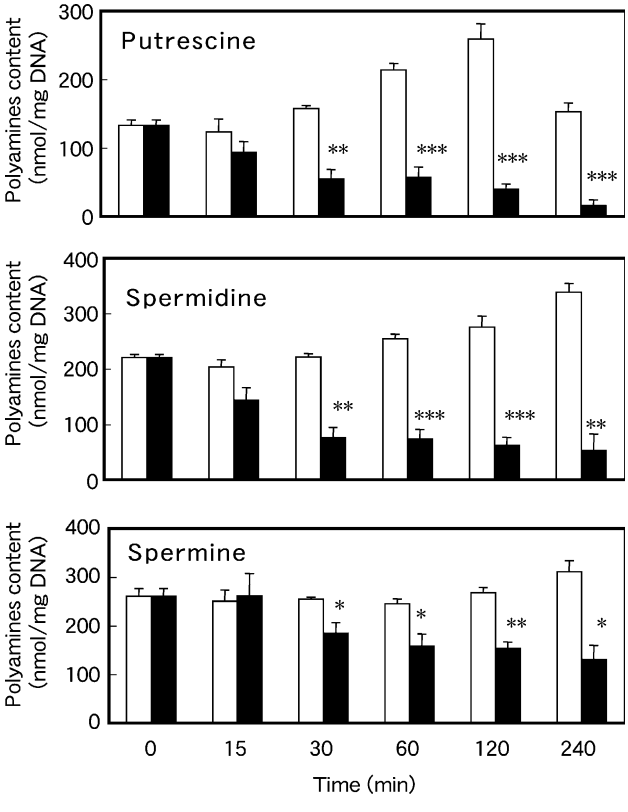

Fig. 6. Time-dependent effect of EPE on intracellular polyamines contents in Ehrlich ascites tumor cells. Cells were incubated with (black bars) or without (white bars) EPE (200 μg/ml) in Eagle's minimum essential medium containing 10% FCS. The cells were harvested and then intracellular polyamines contents were determined as described in Materials and methods. Results show means ± SD of three experiments. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.005$, compared to the control group at the same time point using Student's *t*-test

tumor cells exposed to EPE (200 μg/ml) showed a translocation of AIF to nuclei and the translocation was suppressed with the addition of polyamines (0.1 mM each) at the same time as EPE addition (Fig. 4).

We previously demonstrated that a rapid increase in intracellular peroxide levels after addition of EPE triggers off induction of apoptosis. The effect of polyamines on intracellular peroxides levels in EPE-treated EATC was examined. As shown in Fig. 5, the tumor cells exposed to EPE (200 μg/ml) showed significant increase in intracellular peroxides 15–30 min after incubation with EPE as compared with non-treated cells. The increase was suppressed with the addition of polyamines (0.1 mM each) at the same time as EPE addition.

To determine the effect of EPE on intracellular polyamines concentration, their levels were assayed by HPLC. EPE-treated cells showed significant decrease in putrescine, spermidine and spermine more than 30 min after incubation with EPE as compared with non-treated cells at the same time point (Fig. 6) and the decrease was also dose-dependent at 1 h (Fig. 7). To examine whether the decrease in polyamines was related to EPE-induced damage of cell membrane, LDH leakage was investigated. Table 1 showed that EPE did not induced a release of LDH, suggesting that EPE-induced decrease in intracellular polyamines was not caused by release from the cell membrane injured by EPE.

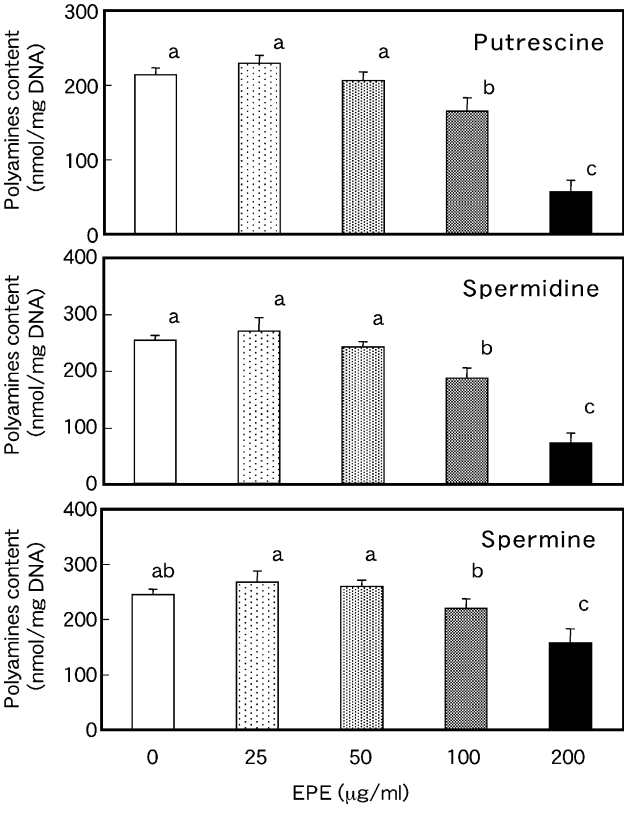

Fig. 7. Dose-dependent effect of EPE on intracellular polyamines contents in Ehrlich ascites tumor cells. Cells were cultured in Eagle's minimum essential medium containing 10% FCS with various concentrations of EPE (0–200 μg/ml) and harvested at 1 h. The cells were harvested and then intracellular polyamines contents were determined as described in Materials and methods. Results show means ± SD of three experiments. Data not sharing common alphabet are significantly different ($p < 0.05$) using Fisher's test

Table 1. Effect of EPE on leakage of LDH in Ehrlich ascites tumor cells

Time (min)	Leakage of LDH (% of total activity)	
	Control	EPE
0	15.49 ± 1.75	15.49 ± 1.76
15	14.98 ± 1.59	18.03 ± 1.16
30	15.49 ± 0.88	18.28 ± 2.64
60	18.28 ± 2.75	17.27 ± 3.17

Cells were incubated with or without EPE (200 μg/ml). Lactate dehydrogenase (LDH) leakage was determined at indicated times after EPE addition. Results show means ± S.D. of three experiments. Data not sharing common alphabet are significantly different ($p < 0.05$) using Fisher's test

Discussion

The significant finding in this study is that EPE, an extract from the Evening primroses used as a herbal plant, brought about a significant decrease in the polyamines, putrescine, spermidine and spermine, levels within 30 min after culture. Furthermore, the addition of these polyamines reversed the EPE-induced decrease in cell viability and suppressed the EPE-induced increase in intracellular hydrogen peroxides. On the other hand, EPE-induced decrease in DNA synthesis and phosphorylation of Rb protein and EPE-induced translocation of AIF were not reversed with the addition of the polyamines. These results suggest that polyamines are involved in the EPE-induced apoptosis pathway which is dependent on the increase in hydrogen peroxide.

We have demonstrated previously that EPE induced apoptosis in EATC, while mouse embryo fibroblast cells (NIH3T3), used as a normal cell model, were completely resistant to the cytotoxic activity of EPE. Furthermore, we demonstrated that EPE exposure elicited a rapid increase in the activity of hydrogen peroxide levels. These changes caused translocation of Bax to mitochondria, and then mitochondrial cytochrome c was released. One of the main consequences of mitochondrial cytochrome c release is the activation of caspase-3. However, no caspase activation was observed (Arimura et al., 2003a). On the other hand, apoptosis-inducing factor (AIF) was

translocated from mitochondria to nuclei. Hydrogen peroxide induces AIF translocation and apoptosis, which has been shown to be caspase-independent. EPE-induced translocation of AIF was suppressed with the addition of catalase, suggesting that the rapid increase in intracellular peroxide levels after addition of EPE triggers off induction of apoptosis (Arimura et al., 2003b). We also demonstrated that EPE caused a decrease in DNA synthesis, an accumulation of cells in the G1 phase, and a decrease in the phosphorylation of Rb protein, in its growth inhibitory effect of EATC. However, the rapid increase in intracellular peroxide levels did not trigger off inhibition of cell growth arrest, because inhibitions of Rb phosphorylation and DNA synthesis by EPE were not suppressed with the addition of catalase (Arimura et al., 2004). These results suggested that EPE-induced inhibitions of the growth of EATC are via at least two pathways differentially modulated by reactive oxygen species, notably intracellular peroxides. These are (a) the EPE-induced apoptosis pathway which is dependent on increases in hydrogen peroxide and (b) the EPE-induced inhibition of cell proliferation which is hydrogen peroxide independent.

ROS are usually generated in response to diverse external stimuli, such as growth factors or cytokines (Schreck and Baeuerle, 1991, Sundaresan et al., 1995). At low concentrations ROS may play the role of an intracellular messenger of various molecular events (Bhunia et al., 1997), including cell proliferation and apoptosis. However, the generation of large amounts of ROS represents multiple molecular species, including singlet oxygen, superoxide (O^{2-}), H_2O_2, NO, peroxynitrite ($ONOO^-$), the thioperoxyradical ($RSOO^-$), and the hydroxyl radical (HO^-). Superoxides are the early molecular species of ROS that are generated as a consequence of the interaction of cells with external stimuli that in turn generate H_2O_2, HO^-, etc. Recent studies indicate that ROS, such as H_2O_2, can be generated by anticancer drugs, can damage cells, and then induce apoptotic cell death (Ueta et al., 1999, Inoue et al., 2000). Although various sources for ROS have been described (Thannickal et al., 2000), plasma membrane-associated NADPH oxidases are considered as the main source of ROS acutely produced upon growth factor or cytokine stimulation (Rhee et al., 2000, Lambeth, 2002, Finkel, 2003). Whereas these enzymes have been originally discovered in phagocytes, homologues have found in non-phagocytic cells. On the other hand, Ogata et al. have demonstrated the inhibition by spermine of receptor-mediated activation of NADPH oxidase in neutrophils (Ogata et al., 1992, 1996). In our experiments, polyamines suppressed the EPE-induced increase in intracellular hydrogen peroxides and reversed the EPE-induced decrease in cell viability.

Polyamines play a critical role in cell growth, proliferation and differentiation. The importance of polyamines in cell growth is evidenced in the observation that dysregulation of ornithine decarboxylase and spermidine/spermine N1-acetyltransferase, the rate limiting enzymes in the synthesis and biodegradation, respectively, of polyamines, affects the concentration of polyamines. Furthermore, the intracellular polyamine pool is highly regulated, and influx and efflux of polyamines are thought to be controlled as part of cell growth, with increased influx and decreased efflux during periods of rapid cell growth (Wallace & Keir, 1981, 1986). In this study, we observed that EPE caused a significant decrease in putrescine, spermidine and spermine levels within 30 min after the addition. However, studies on the precise mechanism of the decrease in intracellular polyamines in EPE-treated cells is ongoing.

References

Arimura T, Kojima-Yuasa A, Watanabe S, Suzuki M, Kennedy DO, Matsui-Yuasa I (2003a) Role of intracellular reactive oxygen species and mitochondrial dysfunction in evening primrose extract-induced apoptosis in Ehrlich ascites tumor cells. Chem Biol Interact 145: 337–347

Arimura T, Kojima-Yuasa A, Suzuki M, Kennedy DO, Matsui-Yuasa I (2003b) Caspase-independent apoptosis induced by evening primrose extract in Ehrlich ascites tumor cells. Cancer Lett 201: 9–16

Arimura T, Kojima-Yuasa A, Kennedy DO, Matsui-Yuasa I (2004) Reactive oxygen species-independent G1 arrest induced by evening primrose extract in Ehrlich ascites tumor cells. Cancer Lett 207: 19–25

Bergmeyer HU, Bernt E, Hess B (1965) Lactate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis. Academic Press, New York, pp 736–743

Bhunia AK, Han H, Snowden A, Chatterjee S (1997) Redox-regulated signaling by lactosylceramide in the proliferation of human aortic smooth muscle cells. J Biol Chem 272: 15642–15649

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal Biochem 72: 248–254

Casero RJ, Pegg AE (1993) Spermidine/spermine N^1-acetyltransferase, the turning point in polyamine metabolism. FASEB J 7: 653–661

Dirks J, Van Aswegen CH, Du Plessis DJ, Snyman JR (1998) Effect of evening primrose oil on monocyte chemotactic protein-1 and tumour necrosis factor-alpha levels during delayed hypersensitivity immune responses. Med Sci Res 26: 567–569

Dypbukt JM, Ankarcrona M, Burkitt M, Sjoholm A, Strom K, Orrenius S, Nicotera P (1994) Different prooxidant levels stimulate growth, trigger apoptosis or produce necrosis of insulin secreting RINm5F cells. The role of intracellular polyamines. J Biol Chem 269: 30553–30556

Ghribi O, Herman MM, Savory J (2002) The endoplasmic reticulum is the main site for caspase-3 activation following aliminum-induced neurotoxicity in rabbit hippocampus. Neurosci Lett 324: 217–221

Finkel T (2003) Oxidant signals and oxidative stress. Curr Opin Hematol 15: 247–254

Grassilli E, Desiderio MA, Bellesia E, Salomoni P, Benetti F, Franceschi C (1995) Is polyamine decrease a common feature of apoptosis-evidence from gamma ray-induced and heat shock-induced cell death? Biochem Biophys Res Commun 216: 708–714

Inoue M, Sakaguchi N, Isuzugawa K, Tani H, Ogihara Y, (2000) Role of reactive oxygen species in gallic acid-induced apoptosis. Biol Pharmaceutical Bulletin 23: 1153–1157

Jack AM, Keegan A, Cotter MA, Cameron NE (2002) Effects of diabetes and evening primrose oil treatment on responses of aorta, corpus cavernosum and mesenteric vasculature in rats. Life Sci 71: 1863–1877

Kennedy DO, Kojima A, Moffatt J, Yamagiwa H, Yano Y, Hasuma T, Otani S, Matsui-Yuasa I (2002) Cellular thiol status-dependent inhibition of tumor cell growth via modulation of retinoblastoma protein phosphorylation by (-)-epigallocatechin. Cancer Lett 179: 25–32

Lambeth JD (2002) Nox/Duox family of nicontinamide adenine dinucleotide (phosphate) oxidases. Curr Opin Hematol 9: 11–17

LeBel CP, Bondy SC (1990) Sensitive and rapid quantitation of oxygen reactive species formation in rat synaptosomes. Neurochem Int 17: 436–441

Marton LJ, Morris DR (1987) Molecular and cellular functions of polyamines. In: McCann PP, Pegg AE, Sjoerdsma A (eds) Inhibition of polyamine metabolism: biological significance and basis for new therapies. Academic Press, New York, pp 79–105

Marton LJ, Pegg AE (1995) Polyamines as target for therapeutic invention. Annu Rev Pharmacol Toxicol 35: 55–91

Matsui I, Wiegand L, Pegg AE (1981) Properties of Spermine *N*-acetyltransferase from livers of rats treated with carbon tetrachloride and its role in the conversion of spermine into putrescine. J Biol Chem 256: 5454–5459

Matsui-Yuasa I, Otani S, Morisawa S, Kageyama K, Onoyama Y (1987) Effect of hyperthermia on spermidine/spermine N1-acetyltransferase activity in Ehrlich ascites cells. Eur J Cancer Clin oncol 23: 201–204

Mera SL (1994) Diet and disease. Br J Biomed Sci 51: 189–206

Min A, Hasuma T, Yano Y, Matsui-Yuasa I, Otani S (1995) Apoptosis of interleukin-2-dependent mouse T cell line by protein tyrosine phosphorylation and polyamines. J Cell Physiol 165: 615–623

Muñoz SE, Lopez CB, Valentich MA, Eynard AR (1998) Differential modulation by dietary *n*-6 or *n*-9 unsaturated fatty acids on the development of two murine mammary gland tumors having different metastatic capabilities. Cancer Lett 126: 149–155

Muñoz SE, Piegari M, Guzmán CA, Eynard AR (1999) Differential effects of dietary *Oenothera*, *Zizyphus mistol*, and corn oils, and essential fatty acid deficiency on the progression of a murine mammary gland adenocarcinoma. Nutrition 15: 208–212

Ogata K, Tamura M, Takeshita M (1992) Spermine down-regulates superoxide generation induced by fMet-Leu-Phe in electropermeabilized human neutrophils. Biochem Biophys Res Commun 182: 20–26

Ogata K, Nishimoto N, Uhlinger DJ, Igarashi K, Takeshita M (1996) Spermine suppresses the activation of human neutrophil NADPH oxidase in cell-free and semi-recombinant systems. Biochem J 313: 549–554

Pacham G, Cleaveland JL (1994) Ornithine decarboxylase is a mediator of c-Myc-induced apoptosis. Mol Cell Biol 14: 5741–5747

Pegg AE (1998) Polyamine metabolism and its importance in neoplastic growth and a target for chemotherapy. Cancer Res 48: 759–774

Pegg AE, McCann PP (1982) Polyamine metabolism and function. Am J Physiol 243: C212–C221

Porter CW, Janne J (1987) Modulation of antineoplastic drug action by inhibitors of polyamine synthesis. In: McCann PP, Pegg AE, Sjoerdsma A (eds) Inhibition of polyamine metabolism: biological significance and basis for new therapies. Academic Press, New York, pp 203–248

Poulin R, Pelletier G, Pegg AE (1995) Induction of apoptosis by excessive polyamine accumulation in ornithine decarboxylase-overproducing L1210 cells. Biochem J 311: 723–727

Rhee SG, Bae YS, Lee SR, Kwon J, Yang KS (2000) Hydrogen peroxide in peptide growth factor signaling. FASEB J 14: A1505–A1505

Schreck R, Baeuerle PA (1991) A role for oxygen radicals as second messengers. Trends Cell Biol 1: 39–42

Schneider WC, Colowick SA, Kaplan NO (1957) Determination of nucleic acids in tissues by pentose analysis. Methods Enzymol 3: 368–384

Sundaresan M, Yu ZX, Ferrans V, Irani K, Finkel T (1995) Requirement for generation of H_2O_2 for platelet-derived growth-factor signal-transduction. Science 270: 296–299

Tabor CW, Tabor H (1984) Polyamines. Annu Rev Biochem 53: 749–790

Takahashi R, Inoue J, Ito H, Hibino H (1993) Evening primrose oil and fish oil in non-insulin-dependent-diabetes. Prostag Leukotr Ess Fatty Acids 49: 569–571

Tanaka M, Sawada M, Miura M, Marunouchi T (1998) Insulin-like growth factor-I analogue prevents apoptosis mediated through an interleukin-I (converting enzyme (caspase-I)-like protease of cerebellar external granular layer neurons: developmental stage-specific mechanisms of neuronal cell death. Neuroscience 84: 89–100

Thannickal VJ, Fanburg BL (2000) Reactive oxygen species in cell signaling. Am J Physiol 279: L1005–L1028

Tobias KE, Kahana C (1995) Exposure to ornithine results in excessive accumulation of putrescine and apoptotic cell death in ornithine decarboxylase overproducing mouse myeloma cells. Cell Growth Differ 6: 1279–1285

Ueta E, Yoneda K, Yamamoto T, Osaki T (1999) Manganese superoxide dismutase negatively regulates the induction of apoptosis by 5-fluorouracil, peplomycin and gamma-ray in squamous cell carcinoma cells. Jpn J Cancer Res 90: 555–564

Wallace HM, Keir HM (1981) Uptake and excretion of polyamines from baby hamster kidney cells (BHK-21/C13). The effect of serum on confluent cell cultures. Biochim Biophys Acta 676: 25–30

Wallace HM, Keir HM (1986) Factors affecting polyamine excretion from mammalian cells in culture. Inhibition of polyamine biosynthesis. FEBS Lett 194: 60–63

Yoshida T, Chou T, Matsuda M, Yasuhara T, Yazaki K, Hatano T, Nitta A, Okuda T (1991) Woodfordin D and oenothein A, trimeric hydrolysable tannins of macro-ring structure with antitumor activity. Chem Pharm Bull 39: 1157–1162

Authors' address: Dr. Isao Matsui-Yuasa, Department of Food and Human Health Sciences, Graduate School of Human Life Science, Osaka City University, 3-3-138 Sugimoto, Sumiyoshi-ku, Osaka 5585-8585, Japan,

Fax: +81-6-6605-2810, E-mail: yuasa@life.osaka-cu.ac.jp