Intracellular Glutathione Regulates Sesquiterpene Lactone-induced Conversion of Autophagy to Apoptosis in Human Leukemia HL60 Cells

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Abstract. A sesquiterpene lactone, eupalinin A, found in Eupatorium chinense L., exhibited a marked inhibitory effect on cell growth in HL60 cells. In a previous study, it was indicated that the intracellular ROS generation accompanying mitochondrial dysfunction was closely related to the autophagic cell death induced by the treatment with eupalinin A. By glutathione (GSH) pre-treatment, eupalinin A-induced cell growth inhibition was markedly reduced in a time- and dose-dependent manner. Eupalinin A reduced the intracellular GSH level in the early phase, but the GSH pre-treatment reduced this depression. Interestingly, the supplementation of GSH changed the cell death type from autophagic cell death to apoptotic cell death. Pre-treatment with GSH plus p38 MAP kinase inhibitor (SB203580) strongly diminished the eupalinin A-induced autophagic cell death compared with GSH pre-treatment, suggesting a negative regulation of p38 MAP kinase in this cell death type conversion. Taken together, intracellular ROS levels, including GSH, are crucial for the susceptibility to cell death and the determination of type of eupalinin A-induced cell death.

Materials and Methods

Materials. Eupalinin A used in this study was separated by column chromatography and identified by $^1$H-, $^13$C-NMR and mass spectra. The 25 x Complete®, a mixture of protease inhibitors mixture was obtained from Roche (Penzberg, Germany). The antibodies to anti-human p44/42 MAP kinase (ERK), anti-human phospho-p44/42 MAPK (Thr202/Tyr204) (p-ERK), anti-human SAPK/JNK (JNK), anti-human phospho-SAPK/JNK (Thr183/Tyr185) (p-JNK), anti-human p38 MAP kinase (p38), anti-human phospho-p38 MAP kinase (Thr180/Tyr182) (p-p38), anti-human Bad, anti-human phospho-Bad (Ser112, Ser 136) (p-Bad) and anti-human Bid were from Cell Signaling Technology (MA, USA). The antibodies to anti-human Bcl-xl (H-5) were obtained from Santa Cruz Biotechnology (CA, USA). The antibodies to anti-human caspase-2, anti-human caspase-8 and anti-human caspase-9 were obtained from MBL (Nagoya, Japan). The antibodies to anti-human endonuclease G and anti-human...
β-actin were from Sigma (St. Louis, MO, USA). The antibody to anti-human AIF was from ProSci Inc (CA, USA). The antibody to anti-human cytochrome c was from Upstate Biotechnology (NY, USA). The antibody to LC-3 was from Nano Tools antikoerper technik (Teningen, Freiburg, Germany). The antibodies to anti-human β-actin were from Sigma. Anti-rabbit and anti-mouse antibodies conjugated with horseradish peroxidase and the chemiluminescence (ECL) kit were obtained from GE Healthcare Sci. (Buckinghamshire, England). All other reagents were of the highest quality available and obtained from WAKO Pure Chemical Industries, Ltd (Osaka, Japan).

**Cell culture and treatment.** Human M2-type leukemia cell line HL60 was provided by RIKEN Cell Bank (Tsukuba, Ibaraki, Japan). HL60 cells were cultured in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). Eupalinin A dissolved in DMSO was added to the cell culture at a final concentration of DMSO (<0.3%) that showed no significant effect on the growth and differentiation of HL60 cells (data not shown). HL60 cells were pre-incubated with GSH (0.5 or 1 mM) or buthionine sulfoximine (BSO) prior to eupalinin A exposure for 1 h. Viable cell number was measured by trypan-blue dye exclusion test using a Burker-Turk type cell count chamber.

**Measurement of intracellular GSH/GSSG levels in eupalinin A-treated HL60 cells.** Eupalinin A- or eupalinin A with GSH-treated cells were harvested and washed with PBS. The cell pellets were suspended in 500 μL of ice-cold 5% (w/v) metaphosphoric acid and then sonicated on ice. The suspensions were centrifuged at 12,000 × g for 5 min at 4°C and transferred the supernatants into a clean 1.5 mL tube. Measurements of intracellular GSH/GSSG (oxidized glutathione) levels were followed in a HT Glutathione assay Kit (TRAVIGEN, Inc., Gaithersburg, MD, USA).

**Morphological change in HL60 cells.** For the morphological examination of cell death, the cells were double-stained with Hoechst 33342 (Calbiochem, SanDiego, CA, USA) and PI (Invitrogen). Hoechst 33342 and PI were added to the cultured medium at a concentration of 5 μg/mL. After incubation for 30 min, the cells were collected and washed with phosphate-buffered saline (PBS) and then observed under a fluorescence microscope, Olympus BX-50 (Olympus, Tokyo, Japan).

**Measurement of intracellular ROS level by CM-H2DCF-DA fluorescent probe.** The amount of intracellular ROS was measured by using 5-(and-6-)carboxy-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H2DCF-DA; Invitrogen). CM-H2DCF-DA is a fluorogenic freely permeable tracer specific for ROS assessment. It is diacetylated by intracellular esterases to the non-fluorescent 2',7'-dichlorohydrofluorescein (DCFH), which is oxidized to the fluorescent compound 2',7'-dichlorofluorescein (DCF) by ROS. HL60 cells were incubated with 10 μM CM-H2DCF-DA for 30 min at 37°C after eupalinin A treatment. Cells were washed twice with PBS to remove the excess of CM-H2DCF-DA and were mounted onto glass slides. Photomicrographs of the mounted cells were taken with a fluorescent microscope equipped with UV supply system (Olympus BX-50). Cells stained with CM-H2DCF-DA were incubated with 100 μL of lysis buffer for 5 min on ice and then measured with excitation at 490 nm and emission at 530 nm with a fluorometer (MTP-600F, CORONA ELECTRIC Co. Ltd. Hitachinaka, Japan).

**Measurement of mitochondrial membrane potential by Mito-Tracker probe.** Mitochondrial membrane potential was measured by use of fluorescent dye, MitoTracker Red (Invitrogen), which accumulates selectively in active mitochondria and become fluorescent when oxidized. Mito-Tracker Red was added to the culture medium at a concentration of 10 nM. After the cells were treated with Mito-Tracker Red and washed twice with PBS, the cells were resuspended with PBS and observed under fluorescence microscopy.
DNA extraction and agarose gel electrophoresis. The cultured cells were treated with eupalinin A and the control cells were treated with DMSO alone. The cells were collected and washed with PBS. Lysis buffer (100 mM Tris-HCl (pH 7.4), 5 mM EDTA, 200 mM NaCl, 0.2% SDS and 200 μg/mL Proteinase K (Takara Bio Co., ltd, Ohtsu, Shiga, Japan)) was added to the cell pellet and incubated at 55˚C for 3 hours. After incubation, DNA was extracted with phenol/chloroform from the cell lysate. DNA was precipitated with ethanol and dissolved with Tris-EDTA buffer. RNase A (Sigma) was added to the DNA solution at a final concentration of 20 μg/mL. DNA (3 μg) was analyzed by electrophoresis on 2% agarose gel.

Measurement of autophagy. Klionsky et al. developed the method for the step of autophagy (6). The formation of autolysosomes and/or autophagosomes (AV) was measured by immunofluorescence staining of LC-3, monodancylcanaverin (MDC) staining and LC3 C-terminal processing (7, 8).

Immunofluorescence staining. Cells were washed twice with PBS and then fixed with 4 % paraformaldehyde for 10 min. Fixed cell were suspended with 3% BSA blocking solution for 10 min. Treated cells were incubated with the primary antibody which was diluted with PBS containing 0.1% Triton X-100 for 30 min at room temperature. After incubation for 30 min, the cells were washed twice with 0.1% BSA solution and then incubated with FITC-conjugated anti-mouse IgE for 30 min. The cells were collected and washed with PBS and then observed under a fluorescence microscope, Olympus BX-50.

LC3 C-terminal processing. The LC3 C-terminal processing was measured by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis.

MDC staining. Cells were incubated with 50 μM MDC (Sigma) for 60 min, 37˚C. After incubation cells were washed twice with PBS and immediately observed under fluorescence microscopy (356 nm excitation filter and 545 nm barrier filter). Moreover, MDC-treated cells were washed twice with PBS and lysed with RIPA buffer. Intracellular MDC was measured by fluorocount microplate reader (excitation wavelength 360 nm, emission wavelength 530 nm). To normalize the measurements to the number of cells present in each well, protein content of lysate was measured with a DC Protein assay kit.

Antibodies and Western blotting. For the preparation of cell lysate, HL60 cells were washed twice with PBS and harvested. The cell pellet was resuspended and washed twice with PBS, and lysed in RIPA buffer including protease inhibitor. Protein content was measured with a DC Protein assay kit (BIO RAD, Hercules, CA, USA). Five micrograms of protein of each cell lysate was separated by SDS-PAGE by using an adequate percent of polyacrylamide in the gel and electroblotted onto a PVDF membrane (Du Pont, 1451.
Figure 4. Eupalinin A-induced ACD accompanied by the depletion of intracellular GSH in the early phase. The cells were treated with vehicle control (●: GSH, ○: GSSG) or eupalinin A (4 μM) + GSH (1.0 mM) (▲: GSH, △: GSSG) or 4 μM of eupalinin A (■: GSH, □: GSSG) for the indicated times. Each point represents the mean of three independent experiments. Intracellular GSH/GSSG levels were measured by using a HT Glutathione Assay Kit.

Figure 5. Nucleosomal DNA fragmentation was enhanced by GSH pretreatment. DNA fragmentation in HL60 cells treated with 4 μM of eupalinin A or eupalinin A plus GSH; cells were cultured for 6 h in the absence of eupalinin A (lane 1) or in the presence of eupalinin A (4 μM) (lane 2), eupalinin A (4 μM) + GSH (1.0 mM) (lane 3). M indicates DNA size marker.

Figure 6. Eupalinin A-treated cells were mainly observed as swollen or fragmented swollen cells by Hoechst 33342-PI double staining. Cells were stained by Hoechst 33342 and PI (magnification ×400). White arrows indicate swollen and fragmented swollen cells. White arrow heads indicate fragmented and condensed cells.
Boston, MA, USA). After blockage of nonspecific binding sites for 1 h by 5% non-fat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated overnight at 4˚C with various antibodies. The membrane was then washed three times with TPBS, incubated further with alkaline phosphatase-conjugated goat anti-mouse antibody or anti-rabbit antibody at room temperature and then washed three times with TPBS. Proteins were detected with enhanced ECL kit and chemilluminescence detector (LAS-1000, Fuji, Japan).

**Statistical analysis.** All data were analyzed first by one-way ANOVA and subsequently by Fisher’s-multiple range test. The differences among the means were considered significant at p<0.01.

**Results**

Glutathione abrogated eupalalin A-induced cell growth inhibition in HL60 cells. It has been previously reported that eupalalin A-induced ACD was closely associated with ROS production accompanied by mitochondrial dysfunction (5). In order to clarify the association of intracellular ROS with cell death, the effect of GSH on eupalalin A-induced cell death was examined. GSH pre-treatment remarkably prevented eupalalin A-induced cell growth inhibition in a time- and dose-dependent manner (Figure 2). On the other hand, BSO, which is a specific inhibitor of GSH synthesis, and BSO plus eupalalin A treatments enhanced the cell growth inhibition in HL60 cells. Moreover, the number of abnormal karyotic cells, ROS production and attenuation of mitochondrial membrane potential induced by eupalalin A were significantly improved by GSH pre-treatment (Figure 3).

**Intracellular GSH level was decreased by eupalalin A treatment.** To further investigate the mechanism of eupalalin A-induced ACD, the intracellular GSH/GSSG levels were examined. Eupalalin A treatment decreased intracellular GSH as compared with the control cells. However, GSH pre-treatment moderated the reduction of intracellular GSH level by eupalalin A in the early phase but this effect was modest in the late phase (Figure 4). These results suggested that...
intracellular GSH level in the early phase of these pre-treatments plays the critical role for cell growth. GSH pre-treatment converted autophagy to apoptosis. Although the growth inhibition was attenuated by GSH pre-treatment, the nucleosomal DNA fragmentation was observed at 6 h after the treatments and the intensity of fragmented bands were significantly enhanced by GSH pre-treatment (Figure 5). Morphological changes of the cells by eupalinin A-treatment were mainly observed as a swollen or swollen fragmented cell by Hoechst 33342-PI double staining. When the swollen and fragmented swollen cells in GSH pre-treatment were measured, their population was significantly lower. On the other hand, the number of fragmented or condensed cells which were observed in apoptosis were unusually increased (Figure 6). Furthermore, lipidation of microtubule-associated protein light chain 3 (LC3-II), which is a potential marker of activation of the autophagic pathway, was decreased (Figures 7, 8). Additionally, fluorescence microscopic study of autophagosomes labeled with MDC indicated that the MDC levels decreased in GSH pre-treated HL60 cells, as compared with those in eupalinin A-treated cells (Figure 9 (i, ii)). Thus, these results suggested that eupalinin A-induced ACD was prevented and converted, in part, to apoptosis by GSH pre-treatment.

Apoptosis is well known to be executed by the cascade activation of initiator caspases such as caspase-8 and executioners such as caspase-3 (9). The relation of caspase activity to the conversion of the cell death type was also examined. The caspase-2, -3, -8 and -9 activities by Caspase Colorimetric Protease Assay indicated no activation by the GSH treatment (data not shown). Furthermore, no active

**Figure 9. GSH pre-treatment converted autophagic cell death to apoptosis in eupalinin A-induced cell death.** (i) Mature autophagic vacuoles by eupalinin A or eupalinin A + GSH were stained by MDC. (Magnification ×400). (ii) Cells were incubated with 50 μM monodansylcana verin (MDC) for 60 min at 37°C. Intracellular MDC was measured by fluorescence photometry as indicated under Materials and Methods. The data represent the mean ±SEM of three different experiments. Those not sharing a common superscript letter are significantly different at p<0.01 by Fisher’s multiple range test. Control cells were treated with DMSO alone.
forms of caspases-2, 3, 8, and 9 were detected by Western blot analysis (data not shown). These results suggested that the apoptotic cell death by GSH pre-treatment was induced via a caspase-independent pathway.

In order to elucidate the conversion mechanism of cell death, several apoptosis-related molecules were investigated. p38 MAP kinase was significantly activated by the GSH pre-treatment compared with that in eupalinin A treatment (Figure 10; the upper panel), but other molecules were not affected in the same way. Notably, the co-treatment of GSH with p38 MAP kinase inhibitor SB203580 further increased the number of viable cells (Figure 10; the lower panel). As to death factors, Western blot analysis showed that the amount of released EndoG from mitochondria increased in a time-dependent manner, but AIF, Smac/diablo and cytochrome c were not detected (Figure 12).

Figure 10. p38 MAP kinase was closely related to the cell growth by eupalinin A plus GSH pre-treatment. HL60 cells were pre-incubated with SB203580 (10 μM) prior to eupalinin A exposure for 1 h. Viable cell number was measured by trypan-blue dye exclusion test using a Burker-Turk type cell count chamber. The phosphorylation levels of p38 MAPK in eupalinin A or eupalinin A+ GSH-treated HL60 cells were measured by Western blot analysis. Data are expressed as means±SEM of three different experiments.

Figure 11. Detection of apoptogenic factors from mitochondria in GSH-treated HL60 cells. The death factors (such as EndoG, AIF, Smac/Diablo and cytochrome c) were released from mitochondria in PCD. EndoG was clearly detected by eupalinin A+ GSH-treatment.
Discussion

In a previous study, it was reported that eupalillin A-induced ACD has a close relation to the intracellular ROS production (5). It was assumed that intracellular increase in ROS level triggered eupalillin A-induced ACD. In the present study, the effect of the supplementation of GSH on eupalillin A-induced ACD was examined. The GSH pre-treatment significantly suppressed eupalillin A-induced ACD. Moreover, the elevation of intracellular ROS level and the decrease in mitochondria membrane potential by eupalillin A were clearly abrogated by GSH pre-treatment. Interestingly, the GSH pre-treatment has converted the type of cell death: i.e., from ACD to APO. From these data, it is concluded that eupalillin A-induced cell death was modulated by the intracellular redox state. Possibly, the intracellular GSH level is closely associated with the determination of the two types of programmed cell death.

Recently, Scherz-Shouval et al. reported that ROS are essential for starvation-induced autophagy (10-12). In autophagosome formation process, the phagophore (isolated membranes) is extended to surround the cytosome including the organelle. In this autophagosome/autolysosome formation stage, microtubule-associated-protein light chain 3 (LC3), the human homologue of \emph{S. cerevisiae} Atg8, are cleaved by the cysteine protease Atg4 to leave a conserved glysine residue. Cleaved LC3 is then transiently linked to the Atg7 protein, then to Atg3, and finally to phosphatidylethanolamine (PE) (13). Scherz-Shouval et al. reported that ROS, especially H$_2$O$_2$, inactives Atg 4 by oxidation of a critical cysteine residue and accompanied with accumulation of Atg8-PE on the phagophore membrane in autophagosome formation (10-12). It was considered that the ROS, which are generated by eupalillin A treatment, also play an important role in the eupalillin A-induced ACD. Additionally, eupalillin A-induced ROS generation co-localized with MitoTracker Red-stained mitochondria (Figure 12). DiMauro et al. reported that defective mitochondria and peroxisome produce more H$_2$O$_2$ than normal ones (14). Therefore, it was suggested that the mitochondrial dysfunction by eupalillin A treatment further accelerated ROS generation, which mainly led to the ACD.

GSH pre-treatment induced not only attenuation of cell growth inhibition but also conversion of cell death type (ACD to APO). Inbal et al. reported that DAPk and DRP-1 may act as regulators in the switching of apoptosis/autophagy signal (15). However, the mechanism of this switching between apoptosis and autophagy is currently obscure. When MAP kinase, PI3K/Akt and Bcl-2 family proteins were investigated in GSH pre-treated cells, p38 MAP kinase, which is well known as a stress response signal, was activated, supporting...
the notion that the cell growth and/or the conversion of the cell death type were modulated by intracellular redox state. Moreover, treatments of GSH plus SB203580 further canceled eupalalin A-induced cell growth inhibition compared with GSH pre-treatment. Thus, activation of p38 MAP kinase must be closely related to GSH treatment-induced conversion of autophagic cell death to apoptosis.

Schafer et al. reported that changes of the half-cell reduction potential (E(hc)) of the GSSG/2GSH couple appear to correlate with the biological status of the cell (proliferation E(hc) approximately –240 mV; differentiation E(hc) approximately –200 mV; or apoptosis E(hc) approximately –170 mV) (16). Previously, it has been shown that there is no contribution by caspases, Bcl-2 family proteins, MAP kinases (p44/42 MAP kinase, SAPK/JNK, p38 MAP kinase) or PI3K/Akt to eupalalin A-induced ACD. Therefore, the reduction of intracellular GSH could be a trigger of eupalalin A-induced ACD. As a cause of depletion of intracellular GSH, inhibition of γ-glutamylcysteine synthetase and glutathione synthetase and binding of exocyclic double bond of eupalalin A (Figure 1) to the thiol group in GSH were considered. Further study to clarify the depletion mechanism of intracellular GSH and the signaling pathways leading to the ACD to APO conversion will be needed.

References

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