# Characterization of cell death induced by ethacrynic acid in a human colon cancer cell line DLD-1 and suppression by *N*-acetyl-L-cysteine

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**Since ethacrynic acid (EA), an SH modifier as well as glutathione S-transferase (GST) inhibitor, has been suggested to induce apoptosis in some cell lines, its effects on a human colon cancer cell line DLD-1 were examined. EA enhanced cell proliferation at 20–40 μ*M*, while it caused cell death at 60–100 μ*M*. Caspase inhibitors did not block cell death and DNA ladder formation was not detected. Poly(ADP-ribose) polymerase, however, was cleaved into an 82-kDa fragment, different from an 85-kDa fragment that is specific for apoptosisis. The 82-kDa fragment was not recognized by antibody against PARP fragment cleaved by caspase 3. *N*-Acetyl-L-cysteine (NAC) completely inhibited EA-induced cell death, but 3(2)-*t*-butyl-4-hydroxyanisole or pyrrolidinedithiocarbamate ammonium salt did not. Glutathione (GSH) levels were dose-dependently increased in cells treated with EA and this increase was hardly affected by NAC addition. Mitogen-activated protein kinase (MAPK) kinase (MEK) 1, extracellular signal-regulated kinase (ERK) 1 and GST P1-1 were increased in cells treated with 25–75 μ*M* EA, while c-Jun N-terminal kinase (JNK) 1 and p38 MAPK were markedly decreased by 100 μ*M* EA. NAC repressed EA-induced alterations in these MAPKs and GST P1-1. p38 MAPK inhibitors, SB203580 and FR167653, dose-dependently enhanced EA-induced cell death. An MEK inhibitor, U0126, did not affect EA-induced cell death. These studies revealed that EA induced cell death concomitantly with a novel PARP fragmentation, but without DNA fragmentation. p38 MAPK was suggested to play an inhibitory role in EA-induced cell death. (Cancer Sci 2003; 94: 886–893)**

Mammalian cytosolic glutathione *S*-transferases (GSTs, EC 2.5.1.18) are a family of dimeric enzymes which perform multiple functions, including conjugation of glutathione (GSH) with a number of electrophilic compounds.[1)] Among the seven classes of GSTs, Pi-class GSTs, such as rat GST-P (7-7) and human GST P1-1, are especially interesting because their expression appears to be associated with neoplastic development and anticancer drug resistance.[2, 3)] Transfection studies with cDNAs encoding GSTs[4–6)] and studies utilizing inhibitors for GSTs[7)] have supported an involvement of the enzyme in resistance to alkylating agents, doxorubicin and *cis*-diamminedichloroplatinum (II) (CDDP).[8)] GST inhibitors induce apoptosis in the Jurkat human T cell line[9)] and other cell lines,[10)] suggesting that GST plays a role in prevention of apoptosis. GST has been demonstrated to form complexes with c-Jun N-terminal kinase (JNK), a member of the mitogen-activated protein kinase (MAPK) family, and to inhibit the kinase activity.[11)] The MAPK family includes the extracellular signal-regulated kinase (ERK) and p38 MAPK, as well as JNK. The ERK pathway plays a major role in regulating cell proliferation and differentiation,[12, 13)] while the JNK and p38 MAPK pathways are associated with stress response or apoptosis induced by oxidative stress or anticancer drugs.[14–17)] Several GST inhibitors, such as ethacrynic acid (EA), have been shown to release JNK from the GST complexes, and then free JNK can activate the pathway for apoptosis.[11)] In our previous study we demonstrated that EA induced apoptosis in a mouse colon carcinoma cell line.[10)] Since EA, used as a diuretic drug in the past, is a modifier of SH residues of many proteins[18, 19)] as well as a GST inhibitor,[20, 21)] these actions of EA may be partly involved in apoptosis induction.

In the present study, we examined the effects of EA on the cell viability of a human colon cancer cell line, DLD-1. We found that EA induced cell death concomitantly with fragmentation of poly(ADP-ribose) polymerase (PARP), though the fragment was different from the cleavage product by caspase, but without DNA fragmentation. To characterize this cell death, the effects of antioxidants and alterations in components of the MAPK signaling pathways were also investigated.

## Materials and Methods

**Materials.** Ethacrynic acid (EA), 3(2)-*t*-butyl-4-hydroxyanisole (BHA) and *N*-acetyl-L-cysteine (NAC) were purchased from Wako Pure Chemical (Osaka). Pyrrolidinedithiocarbamate ammonium salt (PDTC) was from Sigma (St. Louis, MO). Alamar Blue was from Alamar Biosciences, Inc. (Sacramento, CA). U0126 (MAPK kinase (MEK) inhibitor) and SB203580 (p38 MAPK inhibitor) were from Promega (Madison, WI). SP600125 (JNK inhibitor), Ac-DEVD-CMK (caspase 3 inhibitor) and Z-VAD-FMK (inhibitor for caspases 1, 3, 4 and 7) were from Calbiochem (San Diego, CA). FR167653 {1-[7-(4-fluorophenyl)-1,2,3,4-tetrahydro-8-(4-pyridyl)pyrazolo[5,1-*c*][1,2,4]triazin-2-yl]-2-phenylethanedione sulfate monohydrate} (p38 MAPK inhibitor)[22, 23)] was a generous gift from Fujisawa Pharmaceutical Co. (Osaka). CDDP was kindly donated by Bristol-Myers Squibb Co. (New York, NY).

**Cell culture and measurement of cell number.** A human colon cancer cell line DLD-1 was supplied by the Japanese Cancer Research Resources Bank (JCRB, Tokyo). Cells were grown in Dulbecco's modified Eagle's medium (Nissui, Tokyo) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS) at 37°C under 5% $CO_2$ in air. Cell number was evaluated by Alamar Blue assay.[24)] Equal numbers of cells were seeded into 96-well plates at the density of $5\times10^2$ cells per well and incubated for 2 days. These cells were further exposed to various concentrations of EA, MAPK inhibitors (U0126, SP600125, SB203580 and FR167653) for 3 days. At the indicated time points, 10 μl Alamar Blue working solution was added to each well and the plates were further incubated for 3 h at 37°C. The fluorescence of each well was measured at ex 544 nm and em 590 nm using Fluoroskan Ascent CF (Thermo Labsystems, Vantaa, Finland). It was confirmed that the reaction proceeded linearly within the range of $1\times10^2$–$6\times10^3$ fluorescence intensity, corresponding to $3\times10^2$–$1.8\times10^4$ viable cells in a well.

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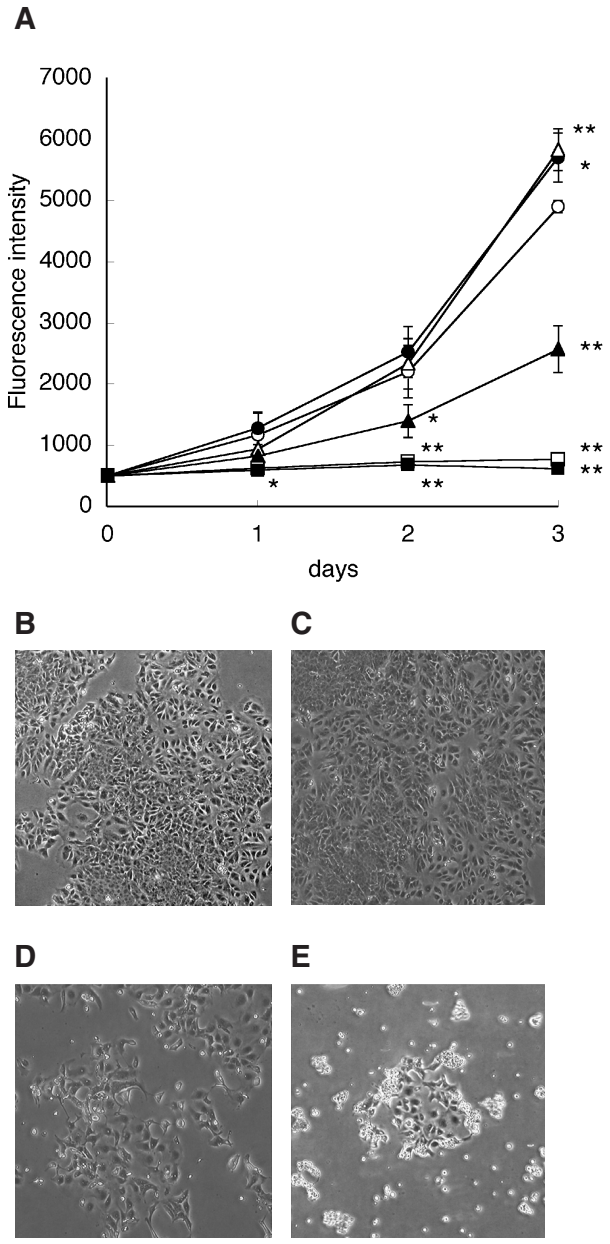


**Fig. 1.** Induction of cell death of DLD-1 cells by EA. (A) Fluorescence intensity of DLD-1 cells treated with EA. DLD-1 cells (500 cells/well) were cultured for 48 h and then further incubated for 1–3 days with (●, 20 μ*M*; △, 40 μ*M*; ▲, 60 μ*M*; □, 80 μ*M*; ■, 100 μ*M*) or without (○) EA. At the indicated time points, cell numbers were estimated by Alamar Blue assay with the data expressed as fluorescence intensity. Each point and bar represent the mean and SD from triplicate assays. * indicates $P<0.05$ versus control on the same day; **, $P<0.01$ versus control. Photographs of DLD-1 cells treated without (B) or with 40 μ*M* (C), 60 μ*M* (D) and 80 μ*M* EA (E) for 3 days. Original magnification, ×100.

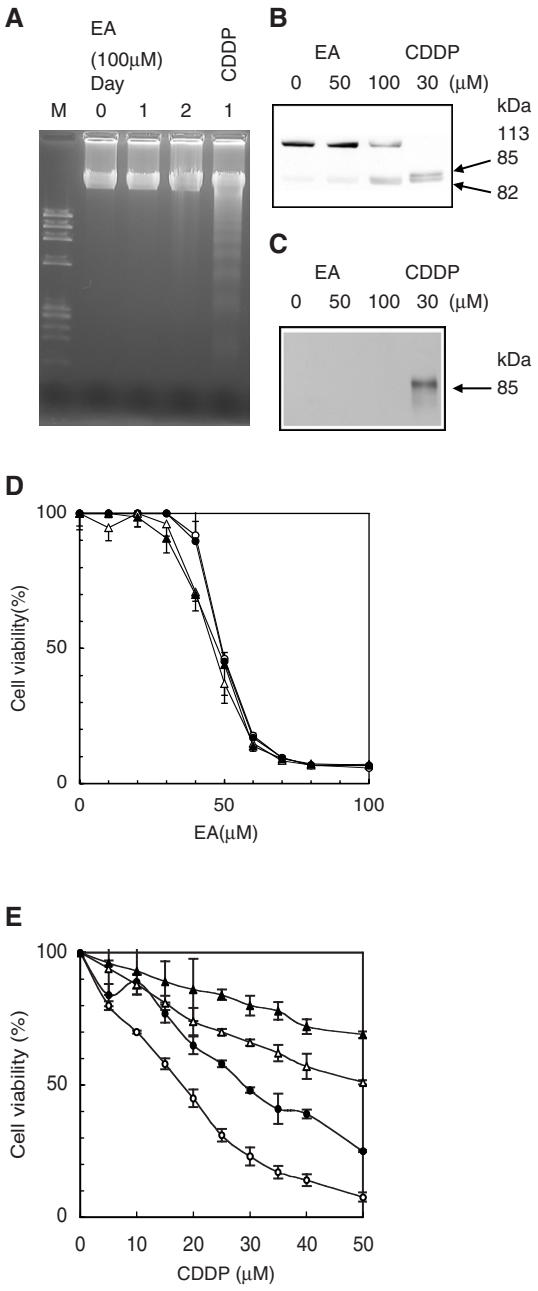


**Fig. 2.** Induction of cell death without DNA fragmentation by EA in DLD-1. (A) DNA was extracted from DLD-1 cells ($5\times10^5$ cells) incubated in the presence of 100 μ*M* EA for 0–2 days or 30 μ*M* CDDP for 1 day and then separated by 2.5% w/v agarose gel electrophoresis (5 μg/lane). After having been stained with ethidium bromide, DNA bands were visualized under UV light. M, DNA size markers, $\phi$x174-*Hae*III digest. (B and C) DLD-1 cells ($5\times10^5$ cells/dish) were cultured for 48 h and then further incubated for 3 days with 0–100 μ*M* EA or 30 μ*M* CDDP. Cell extracts were analyzed for PARP cleavage by western blotting with anti-PARP antibody (B) and anti-cleaved PARP antibody (C). Each lane contains 40 μg of protein. (D) DLD-1 cells (500 cells/well) were cultured for 48 h and then further incubated for 3 days with 0–100 μ*M* EA in the presence of 0–100 μ*M* Ac-DEVD-CMK (caspase 3 inhibitor) (○, 0 μ*M*; ●, 25 μ*M*; △, 50 μ*M*; ▲, 100 μ*M*). Viable cell number was estimated by Alamar Blue assay and percentage values of fluorescence intensity without EA at the respective caspase 3 inhibitor concentrations were expressed as cell viability. Each point and bar represent the mean and SD from triplicate assays, respectively. (E) Cultured DLD-1 cells were also incubated for 3 days with 0–50 μ*M* CDDP in the presence of 0–100 μ*M* Ac-DEVD-CMK (○, 0 μ*M*; ●, 25 μ*M*; △, 50 μ*M*; ▲, 100 μ*M*). Cell viability was assayed as described above. Each point and bar represent the mean and SD from triplicate assays, respectively.

**Analysis of DNA fragmentation.** Total DNAs were extracted from $5\times10^5$ cells following the method of Sambrook *et al.*[25] and 5 μg aliquots were separated by 2.5% w/v agarose gel electrophoresis in 40 m*M* Tris-5 m*M* sodium acetate-1 m*M* EDTA (pH 7.8) and stained with ethidium bromide. DNA bands were visualized under UV light.

**GSH measurement.** Cells were lysed with lysis buffer (255 μl of 0.1 *M* sodium phosphate-5 m*M* EDTA buffer, pH 8.0, and 60 μl of 25% $HPO_3$). The homogenates were centrifuged at 4°C at 100,000*g* for 30 min to obtain the supernatant for the assay of

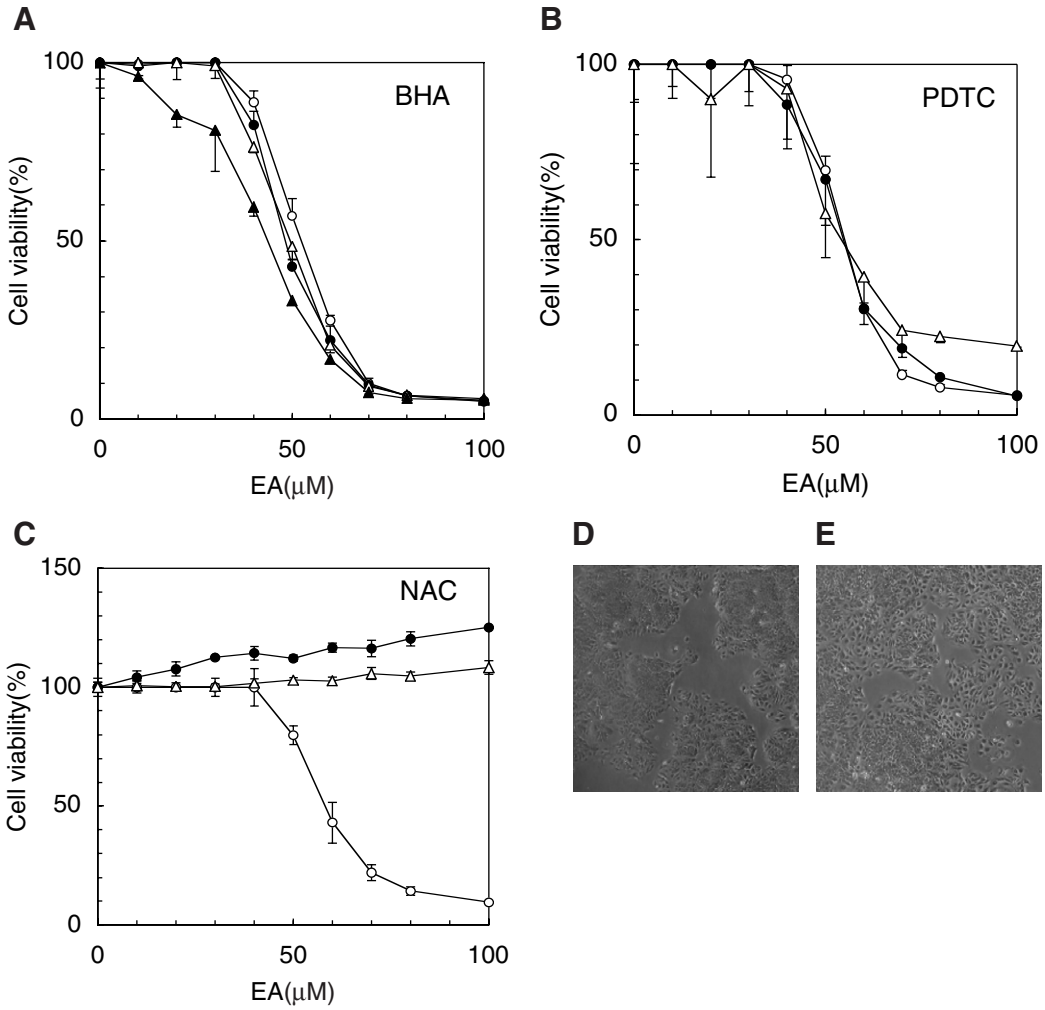


**Fig. 3.** Effects of antioxidants on EA-induced cell death of DLD-1 cells. DLD-1 cells (500 cells/well) were cultured for 48 h and then further incubated for 3 days with 0–100 μ*M* EA in the presence of BHA (○, 0 μ*M*; ●, 25 μ*M*; △, 50 μ*M*; ▲, 100 μ*M*, A), PDTC (○, 0 μ*M*; ●, 25 μ*M*; △, 50 μ*M*, B) or NAC (○, 0 m*M*; ●, 1 m*M*; △, 10 m*M*, C). Viable cell number was estimated as described for Fig. 2D and percentage values of fluorescence intensity without EA at the respective antioxidant concentrations were expressed as cell viability. Each point and bar represent the mean and SD from triplicate assays, respectively. Photographs of DLD-1 cells treated for 3 days with 1 m*M* NAC in the absence (D) or presence of 100 μ*M* EA (E). Original magnification, ×100.

GSH according to the method of Hissin and Hilf.[26] The final assay mixture contained 0.1 ml of the diluted cell supernatant, 1.8 ml of 0.1 *M* sodium phosphate-5 m*M* EDTA buffer, pH 8.0, and 0.1 ml of 0.1% *o*-phthalaldehyde solution. After thorough mixing and incubation at room temperature for 15 min, the fluorescence of the solution at 420 nm was determined with excitation at 350 nm.

**Western blot analysis.** Cells were lysed with lysis buffer (50 m*M* Hepes-NaOH, pH 7.0, containing 0.1% Nonidet P-40, 250 m*M* NaCl, 5 m*M* EDTA, 50 m*M* NaF, 1 m*M* dithiothreitol, 50 mg of aprotinin per ml, and 1 m*M* PMSF). Cellular protein was separated by SDS-PAGE using 8–12.5% acrylamide gels and electroblotted onto Hybond-ECL nitrocellulose membrane (Amersham, Piscataway, NJ).[27,28] Equal loading of protein was confirmed by staining the membrane after detection. After blocking with 5% non-fat dry milk in Tris-buffered saline, the membranes were incubated for 1 h with appropriate antibodies. Anti-PARP antibody (#9542) and anti-cleaved PARP (Asp214) antibody (#9541) were purchased from Cell Signaling Technology, Inc. (Beverly, MA). Anti-apoptosis signal-regulating kinase 1 (ASK1) antibody (H-300), anti-p38 MAPK antibody (N-20), anti-p38 MAPK β antibody (C-16), anti-c-Jun antibody (H-79) and anti-NF-E2-related factor 2 (Nrf2) antibody (C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-MEK1 antibody, anti-ERK1 antibody and anti-JNK1 antibody were from BD Biosciences (San Jose, CA). Anti-GST P1-1 antibody was prepared as reported previously.[29] The blots were then probed on an ECL western blotting detection system (Amersham).

**Statistical analysis.** Data were expressed as mean±SD. Differences between groups were examined for statistical significance using two-tailed Student's *t* test.

## Results

**Induction of cell death of DLD-1 cells by EA.** To study the effects of EA on the viability of DLD-1 cells, viable cell number was estimated by Alamar Blue assay and expressed as fluorescence intensity. When DLD-1 cells were treated with 20–40 μ*M* EA, the fluorescence intensity at day 3 was higher than that of cells without EA treatment (20 μ*M* EA, $P<0.05$; 40 μ*M* EA, $P<0.01$). When the cells were treated with 60 μ*M* EA, however, the fluorescence intensity was a half of the control at days 1–3, and at 80–100 μ*M* EA it did not increase at all (Fig. 1A).

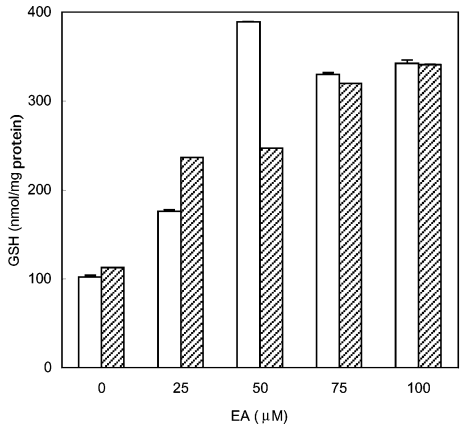


**Fig. 4.** Effects of EA and NAC on GSH levels in DLD-1 cells. DLD-1 cells ($5\times10^5$ cells/dish) were cultured for 48 h and then further incubated for 3 days with 0–100 μ*M* EA in the absence (open bars) or presence of 1 m*M* NAC (hatched bars). GSH level was measured by a fluorometric method, as described in "Materials and Methods." Each point and bar represent the mean and SD from triplicate assays, respectively.

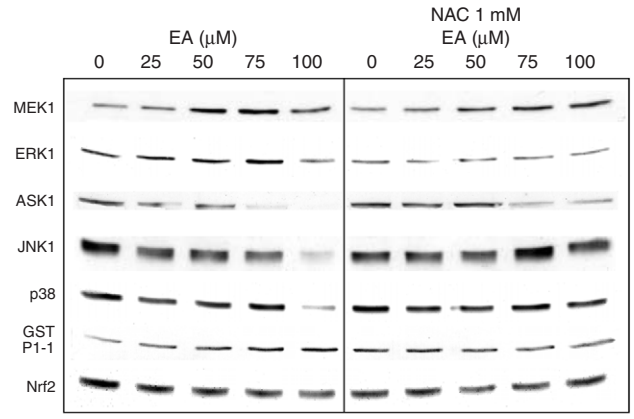


**Fig. 5.** Effects of EA on MAPK and GST P1-1 protein levels in DLD-1 cells. DLD-1 cells ($5\times10^5$ cells/dish) were cultured for 48 h and then further incubated for 3 days with 0–100 μ*M* EA in the absence or presence of 1 m*M* NAC. Cell extracts were analyzed for MEK1, ERK1, ASK1, JNK1, p38 MAPK, GST P1-1 and Nrf2 expression by western blotting, as described in "Materials and Methods." Each lane contains 40 μg of protein.

On microscopic examination, DLD-1 cells treated with up to 40 μ*M* EA for 3 days did not show any alterations in cell morphology (Fig. 1, B and C). When the concentration was 60 μ*M* or more, cells showed irregular shapes, suggesting the induction of cell death (Fig. 1, D and E). These results suggested that cell death was responsible for the decrease of fluorescence intensity by EA treatment.

To examine whether EA-induced cell death was due to apoptosis, DNA fragmentation was analyzed by electrophoresis. When treated with 100 μ*M* EA for 2 days, cells showed complete cell death, but DNA ladder formation was not observed (Fig. 2A). On the other hand, cells treated with 30 μ*M* CDDP exhibited ladder formation. Immunoblot analysis with anti-PARP antibody revealed that PARP with a $M_r$ 113-kDa subunit was cleaved into a dominant 85-kDa fragment and a minor 82-kDa fragment in cells treated with CDDP (Fig. 2B). Cells treated with 100 μ*M* EA exhibited no production of the 85-kDa band, but the 82-kDa band appeared, accompanied with a decrease in the intact PARP 113-kDa band. An antibody against a PARP fragment generated by cleavage with caspase (anti-cleaved PARP antibody) was used to examine whether the 82-kDa band is a such fragment. This antibody did not recognize the 82-kDa band, but recognized the 85-kDa band in CDDP-treated cells (Fig. 2C). Next we examined whether a caspase 3 inhibitor could block EA-induced cell death. Cells were treated with various concentrations of EA in the presence of 0–100 μ*M* Ac-DEVD-CMK for 3 days, then viable cell number was estimated by Alamar Blue assay, and fluorescence intensity at the individual caspase inhibitor concentrations was expressed as a percentage of the value without EA. The inhibitor did not block EA-induced cell death (Fig. 2D), but dose-dependently blocked CDDP-induced apoptosis (Fig. 2E). Another caspase inhibitor, Z-VAD-FMK, at 0–40 μ*M* concentration did not block EA-induced cell death (data not shown). These results indicated that EA-induced cell death occurred concomitantly with PARP cleavage but without DNA fragmentation, and caspases were unlikely to be involved in this cell death.

**Effects of antioxidants on death of DLD-1 cells induced by EA.** Since EA is an SH reagent,[18,19] this raised the possibility that its cytotoxic effects may be mediated by its modulation of SH residues of proteins or GSH, or by resultant oxidative stress. Therefore, we examined whether the addition of antioxidants could repress EA-induced cell death. The cells were treated with BHA, PDTC or NAC for 3 days in the presence of various concentrations of EA. The addition of 0–100 μ*M* BHA or 0–50 μ*M* PDTC did not exert significant effects on EA-induced cell death (Fig. 3, A and B), while 1 m*M* NAC completely inhibited EA-induced cell death (Fig. 3C). On microscopic examination, DLD-1 cells treated with 1 m*M* NAC and 100 μ*M* EA did not show any change in cell shape (Fig. 3E). Furthermore, alterations in intracellular GSH level were examined in cells treated with different concentrations of EA with and without NAC. GSH levels were dose-dependently increased in cells treated with EA in the range of 0–50 μ*M*, but were not further increased at higher EA concentrations (Fig. 4). Although addition of NAC completely repressed EA-induced cell death, it hardly affected the alteration in GSH level induced by EA. These results indicated that GSH level was not decreased in EA-treated cells and not responsible for cell death.

**Effects of EA on MAPK and GST P1-1 protein levels in DLD-1 cells.** To examine whether the MAPK signaling pathways or GST P1-1 are involved in EA-induced cell death, alterations in protein levels of these kinases and GST were studied in cells treated with EA in the absence or presence of 1 m*M* NAC. When cells were treated with 25–75 μ*M* EA, MEK1, ERK1 and GST P1-1 were increased, while JNK1 and p38 MAPK were not altered and ASK1 was decreased (Fig. 5). JNK1 and p38 MAPK were markedly decreased by treatment with 100 μ*M* EA, but GST P1-1 was further increased. It was unlikely that the decrease of these proteins reflected loss of proteins owing to cell death, because most protein bands were not altered on protein staining (data not shown). The addition of NAC repressed the increases of ERK1 and GST P1-1 induced by EA, but not the increase of MEK1. NAC also blocked the decreases of JNK1 and p38 MAPK induced by high concentrations of EA. The Nrf2 protein level was not changed by EA treatment. p38 MAPKβ and c-Jun were not detected by immunoblotting (data not shown).

**Inhibition of cell proliferation of DLD-1 cells by MAPK inhibitors.** Alterations in ERK1, JNK1, p38 MAPK and GST P1-1 levels induced by EA were blocked by NAC, raising the possibility that such proteins may mediate the signal for EA-induced cell death. To study this possibility, the effects of MAPK inhibitors on cell proliferation in the absence of EA were examined at first. After cells were treated with 0–20 μ*M* MAPK inhibitors for 3 days, viable cell number was estimated by Alamar Blue assay. Compared with the control, U0126 and SP600125 dose-dependently repressed the fluorescence intensity, to about a half at 10 μ*M* U0126 (Fig. 6A) and to 20% at 10 μ*M* SP600125

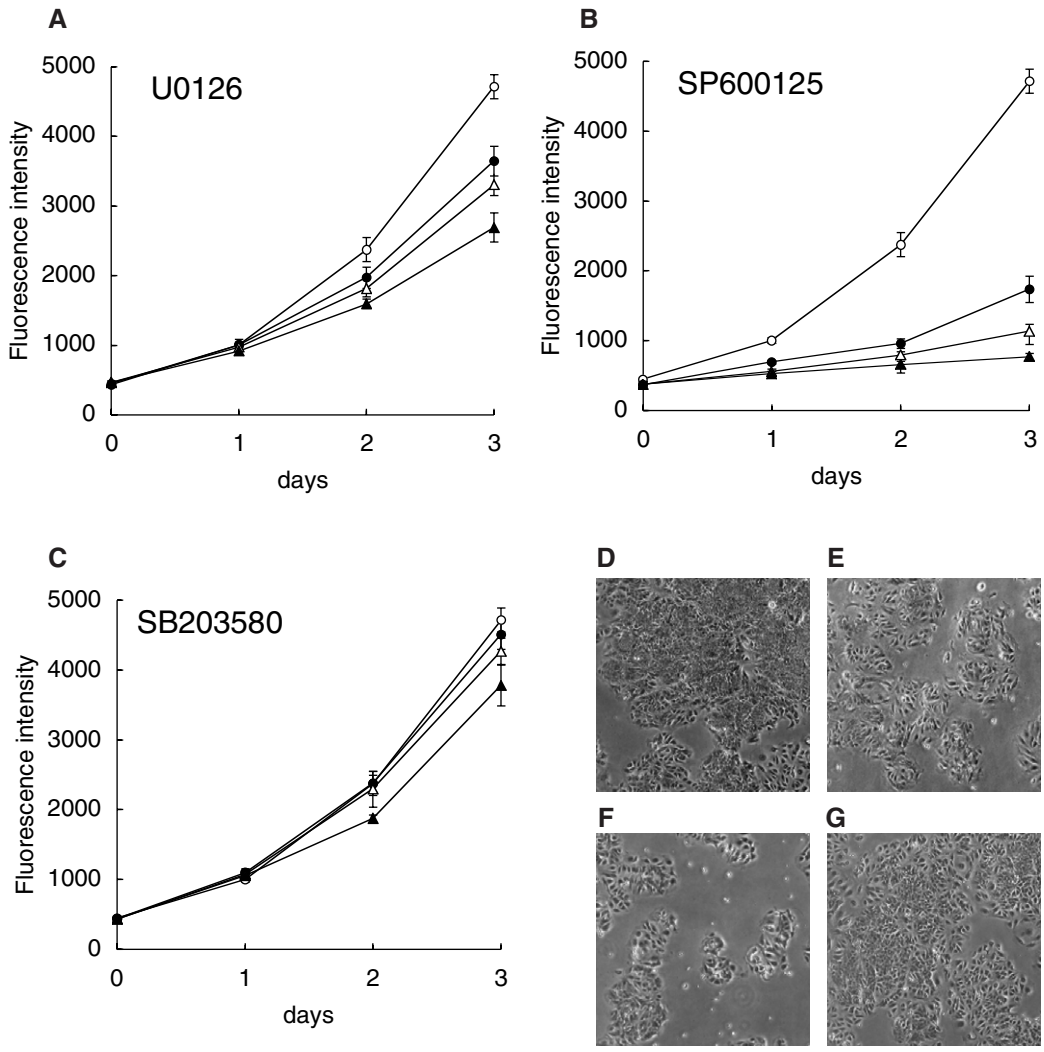


**Fig. 6.** Fluorescence intensity of DLD-1 cells treated with MAPK inhibitors. DLD-1 cells (500 cells/well) were cultured for 48 h and then further incubated for 3 days with (●, 5 μ*M*; △, 10 μ*M*; ▲, 20 μ*M*) or without (○) U0126 (MEK inhibitor, A), SP600125 (JNK inhibitor, B) or SB203580 (p38 MAPK inhibitor, C). Symbols in panels B and C represent the same concentrations of the respective inhibitors as in panel A. At the indicated time points, cell numbers were estimated by Alamar Blue assay with the data expressed as fluorescence intensity. Each point and bar represent the mean and SD from triplicate assays, respectively. Photographs of DLD-1 cells treated without (D) or with 10 μ*M* U0126 (E), 10 μ*M* SP600125 (F) or 10 μ*M* SB203580 (G). Original magnification, ×100.

(Fig. 6B). In contrast, SB203580 (Fig. 6C) or FR167653 (data not shown) did not affect the fluorescence intensity. On microscopic examination, cell shapes were not altered, but cell numbers were decreased by U0126 or SP600125 treatment (Fig. 6, E and F). SB203580 (Fig. 6G) or FR167653 did not result in alterations in cell shape or cell number. These results suggested that the decrease of fluorescence intensity by U0126 and SP600125 was due to inhibition of cell proliferation rather than induction of cell death, suggesting that MEK and JNK, but not p38 MAPK are responsible for proliferation of DLD-1 cells.

**Effects of MAPK inhibitors on EA-induced cell death of DLD-1 cells.** Next we examined whether MAPK inhibitors could block EA-induced cell death. Cell viability was evaluated in the same way as in the study on the effects of antioxidants. SB203580 (Fig. 7A) and FR167653 (Fig. 7B) dose-dependently enhanced EA-induced cell death. The EA concentration required to induce 50% cell death was 55 μ*M* in the absence of FR167653, and the value was decreased to 28 μ*M* at 20 μ*M* FR167653 (Fig. 7B). DNA fragmentation was not observed in cells treated with both EA and FR167653 (Fig. 7D). Although U0126 inhibited cell proliferation (Fig. 6A), it did not affect EA-induced cell death (Fig. 7C). These results suggested that p38 MAPK played an inhibitory role in EA-induced cell death, while MEK was not involved.

## Discussion

In the present study, EA enhanced cell proliferation of DLD-1 cells at low concentrations (20–40 μ*M*) while caused cell death at high concentrations (60–100 μ*M*) (Fig. 1). Low doses of reactive oxygen species (ROS) are known to promote cell proliferation, while severe oxidative stress causes cell death.[30] Thus, EA mimics ROS in eliciting multiple responses at different doses. Although EA usually decreases tissue GSH levels,[18] it increased GSH in DLD-1 cells and the addition of NAC hardly affected this increase (Fig. 4). EA induced cell death without DNA fragmentation and this cell death was not blocked by caspase inhibitors (Fig. 2). Furthermore, PARP was cleaved into an 82-kDa fragment, different from the 85-kDa fragment, which is a digestion product generated by caspase 3 and a marker for apoptosis.[31] The 82-kDa fragment was not recognized by anti-cleaved PARP antibody (Fig. 2). A significant

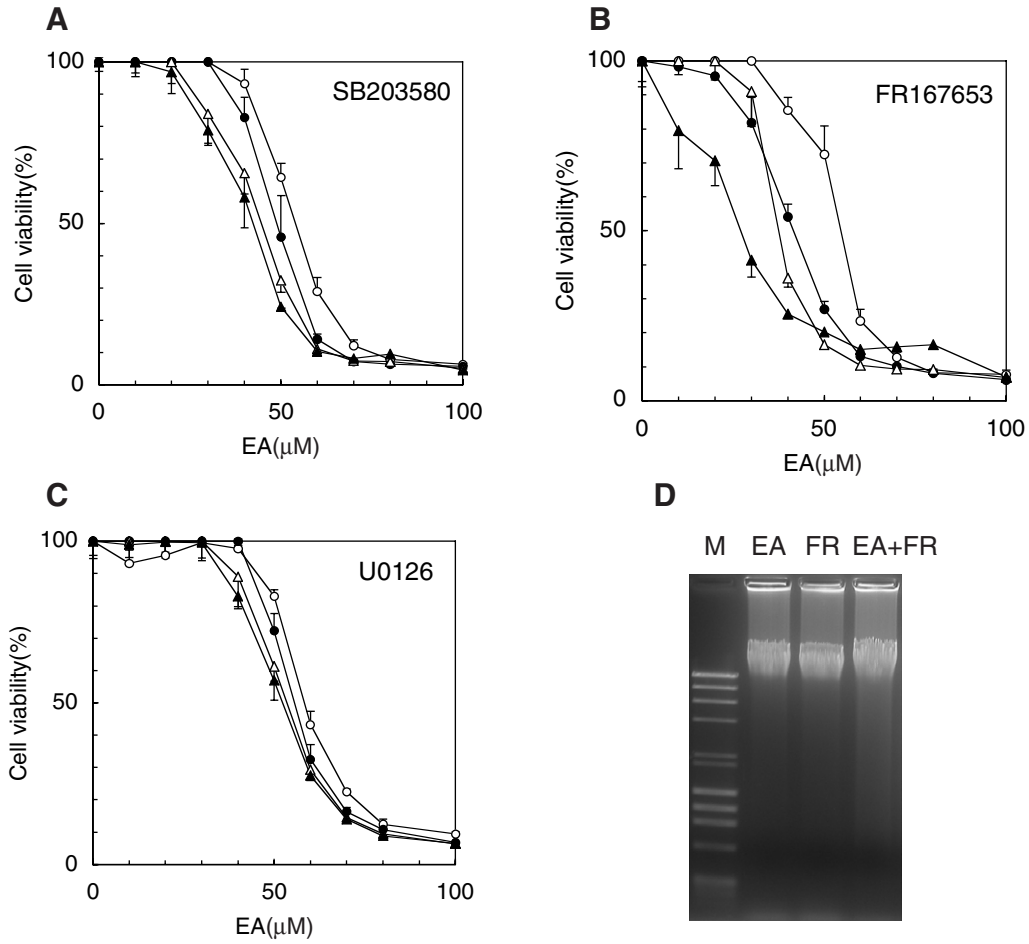


**Fig. 7.** Effects of MAPK inhibitors on EA-induced cell death of DLD-1 cells. DLD-1 cells (500 cells/well) were cultured for 48 h and then further incubated for 3 days with 0–100 μ*M* EA in the absence or presence of MAPK inhibitors (SB203580, A; FR167653, B; U0126, C) (○, 0 μ*M*; ●, 5 μ*M*; △, 10 μ*M*; ▲, 20 μ*M* respective MAPK inhibitor). Viable cell number was estimated by Alamar Blue assay and percentage values of fluorescence intensity without EA at the respective MAPK inhibitor concentrations were expressed as cell viability. Each point and bar represent the mean and SD from triplicate assays, respectively. (D) DNA was extracted from DLD-1 cells ($5\times10^5$ cells) incubated with EA or FR167653 for 3 days and then separated by 2.5% w/v agarose gel electrophoresis as described for Fig. 2. M, DNA size markers, $\phi$x174-*Hae*III digest; EA, DNA from cells treated with 100 μ*M* EA; FR, DNA from cells treated with 20 μ*M* FR167653; EA+FR, DNA from cells treated with 100 μ*M* EA and 20 μ*M* FR167653.

amount of intact PARP was retained in DLD-1 cells, raising the possibility that EA caused cell death only in a small proportion of DLD-1 cells. However, this is unlikely, because cell death was almost complete at 100 μ*M* EA. These findings suggested that EA-induced cell death is non-apoptotic rather than apoptotic, and caspases are unlikely to be involved in this effect. Although DNA fragmentation was not detected, fragmentation of the nuclear protein PARP suggested a possible alteration in the nucleus or DNA in EA-induced cell death. The result in DLD-1 cells is in clear contrast with apoptosis induction accompanied with DNA fragmentation in mouse colon 26 cells after EA treatment.[10] DNA fragmentation is considered as a late event in the processes of apoptosis, and the appearance of internucleosomal DNA cleavage is regulated by many factors.[32] The occurrence of cell deaths that do not fulfill the criteria for either apoptosis or necrosis has been documented.[33] Autophagic cell death and cytoplasmic cell death are known as types of non-apoptotic cell death.[34, 35] However, recent studies have revealed that apoptosis without DNA fragmentation occurs in certain cells.[36] PARP is reported to be cleaved by not only caspases, but also other cysteine proteases such as cathepsin B and calpain.[37] Cathepsin cleaves PARP into 50- to 62-kDa fragments in necrosis.[38, 39] To our knowledge, the 82-kDa fragment is a novel PARP cleavage product, although the protease(s) involved has not been identified yet. Further studies are clearly needed to characterize the nature of this fragment and to establish whether EA-induced cell death is apoptotic or non-apoptotic.

EA-induced cell death was completely inhibited by 1 m*M* NAC. This raises the possibility that NAC may chemically interact with EA in culture medium rather than inside cells and block the effects of EA. However, this seems unlikely, because intracellular GSH levels were increased in cells treated with both EA and NAC in the same way as in the case of EA alone (Fig. 4). Although NAC is reported to maintain intracellular GSH levels and to inhibit the accumulation of ROS,[40, 41] this is not the case in EA-treated DLD-1 cells. BHA inhibits apoptosis and non-apoptotic cell death by blocking the action of ROS.[42, 43] PDTC represses non-apoptotic cell death induced by Fas.[32, 44] In the present study, EA-induced cell death was not blocked by BHA or PDTC (Fig. 3). Thus, GSH or ROS are not responsible for this cell death, but SH modification of some protein(s), so far unidentified, is likely to be involved.

Many previous studies have supported the general view that the ERK pathway delivers a survival signal[45] and the JNK and p38 MAPK pathways are associated with apoptosis induction under stress conditions.[14–17] In most cases, these pathways are activated by cascades of phosphorylation reaction and their reg-

ulation by alteration of the protein amount has not been fully clarified. EA caused increases in MEK1, ERK1 and GST P1-1 at 25–75 μ*M*, while marked decreases in JNK1 and p38 MAPK occur at 100 μ*M* (Fig. 5). From the result of experiments using inhibitors, p38 MAPK seems to play an inhibitory role against EA-induced cell death (Fig. 7). In the absence of EA, MEK1, ERK and JNK are suggested to be involved in cell proliferation of DLD-1 cells (Fig. 6), while MEK or ERK were not involved in EA-induced cell death. Increase in MEK1 and ERK1 by low concentrations of EA may be responsible for enhanced cell proliferation. GST P1-1 is suggested to inhibit apoptosis in several cell lines,[46] but it may enhance cell death of leukocytes[47] and mouse liver cells.[48] At concentrations sufficient to induce cell death, EA completely inhibits GST P1-1 activity.[10] However, cell death of DLD-1 cells is not due to GST inhibition by EA, because NAC addition repressed cell death, but it did not block GST inhibition (data not shown). Although GST P1-1 is reported to bind JNK to inhibit kinase activity,[11] we could not evaluate the role of JNK in EA-induced cell death, since a JNK inhibitor alone almost completely repressed cell proliferation. In conclusion, the present study has revealed that EA causes cell proliferation in DLD-1 cells at low concentrations and cell death at high concentrations. This cell death occurs concomitantly with a novel PARP fragmentation, but without DNA fragmentation. EA also exerts different effects on multiple MAPK pathways, depending on its concentration. Further studies on EA-induced cell death may provide a new target for cancer treatment.

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