RESEARCH ARTICLE

Effects of Aloe-emodin and Emodin on Proliferation of the MKN45 Human Gastric Cancer Cell Line

Takeshi Chihara[1]*, Kan Shimpo[1], Hidehiko Beppu[1], Naoki Yamamoto[2], Takaaki Kaneko[1], Kazumasa Wakamatsu[3], Shigeru Sonoda[1]

Abstract

Aloe-emodin (1, 8-dihydroxy-3-hydroxyl-methylanthraquinone; AE) and emodin (1,3,8-trihydroxy-6-methylanthraquinone; EM) are anthraquinone derivatives that have been detected in some medical plants and share similar anthraquinone structures. AE and EM have been shown to exhibit anticancer activities in various cancer cell lines; however, the inhibitory effects of these derivatives on the growth of cancer cells were previously reported to be different. Gastric cancer is the second most common cause of cancer cell death worldwide. In the present study, we examined the inhibitory effects of 0.05 mM AE and 0.05 mM EM on the proliferation of the MKN45 human gastric cancer cell line. The proliferation of MKN45 cells was significantly inhibited in AE- and EM-treated groups 24 h and 48 h after treatment. Furthermore, the inhibitory effects of EM were stronger than those of AE. The cell cycle of MKN45 cells were arrested in G0/G1 phase or G0/G1 and G2/M phases by AE and EM, respectively. However, an analysis of intracellular polyamine levels and DNA fragmentation revealed that the mechanisms underlying cell death following cell arrest induced by AE and EM differed.

Keywords: Aloe-emodin - emodin - MKN45 cells - cell cycle arrest - intracellular polyamines

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Introduction

The anthraquinone derivatives aloe-emodin (1,8-dihydroxy-3-hydroxyl-methylanthraquinone; AE) and emodin (1,3,8-trihydroxy-6-methylanthraquinone; EM) have been detected in some medicinal plants such as rhubarb (*Rheum palmatum*) (Huang et al., 2013) and Semen Cassiae (Xu et al., 2012), and share very similar anthraquinone structures (Figure 1).

AE has been shown to exhibit anticancer activity in various cancer cell lines (Harlev et al., 2012; Suboj et al., 2012a, 2012b; Chen et al., 2014), while EM has also been reported to have anticancer effects on several types of cancer cell lines (Lai et al., 2009; Chun-Guang et al., 2010; Hsu et al., 2010; Wang et al., 2010). Chen et al. (2010) previously demonstrated that the strength of the inhibitory effects of AE and EM on the growth of cancer cells differed.

Gastric cancer is the second most common cause of cancer death worldwide (Yasui et al., 2011). Furthermore, recurrence rates remain high at approximately 70% following successful surgery (Brenner et al., 2011). Peritoneal dissemination is the most frequent form of recurrent gastric cancer (Shiozaki et al., 2014), and has a poor prognosis. Therefore, the chemoprevention of gastric cancer is required.

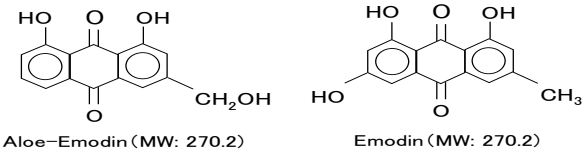

Figure 1. Chemical Structures of Aloe-emodin (AE) and Emodin (EM)

Polyamines are low molecular weight biogenic polycationic amines that exist in all living cells. The polyamines putrescine, spermidine, and spermine have been detected in mammalian cells and play important roles in cell growth and differentiation. Polyamine levels were previously shown to increase during cell cycle progression (Seidenfeld et al., 1981). The cell cycle is traditionally divided into four sequential phases (G1, G2, S, and M) and G0: G1, the first gap or growth phase; S, the DNA synthetic phase; G2, the second gap or growth phase; M, the mitotic phase; and G0, the quiescent state. The accumulation and depletion of polyamine levels during cell-cycle progression has been reported (Thomas and Thomas, 2001). Polyamines have been shown to increase in proliferating cells, including tumor cells, but also induce cell death in cancer cells (Thomas and Thomas, 2001).

In the present study, we examined the inhibitory effects of AE and EM on the proliferation of the human gastric cancer cell line MKN45 with cell cycle arrest and changes in intracellular polyamine levels.

*[1]Division of Biochemistry, Fujita Memorial Nanakuri Institute, Fujita Health University; 423, Oodori-cho, Tsu, Mie, [2]Laboratory of Molecular Biology & Histochemistry, Fujita Health University Joint Research Laboratory, [3]Department of Chemistry, Fujita Health University School of Health Sciences, Toyoake, Aichi, Japan *For correspondence: tchihara@fujita-hu.ac.jp*

Materials and Methods

Chemicals

AE was purchased from Sigma-Aldrich Co. (Saint Louis, MO, USA). It was dissolved in dimethyl sulfoxide (DMSO) in a stock solution at a concentration of 40 mM, stored at -20°C, and protected from light. DMSO never exceeded 0.13% and this concentration did not interfere with growth control cultures. EM was purchased from Fluka Chemie AG (Buchs, Switzerland). It was dissolved in a similar manner to that described for AE.

Cell line and cell culture

The human gastric cancer cell line MKN45 was kindly provided by Dr. Tadashi Watanabe. Exponentially growing MKN45 cells (2×10^6) were cultured in 25 cm^2 plastic flasks containing 4 ml RPMI 1640 (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with 10% heat-inactivated fetal bovine serum (Nichirei Biosciences Inc., Tokyo, Japan), 100 units/ml penicillin, and 100 μg/ml streptomycin (Sigma-Aldrich Co.). Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. After being incubated for 24 h, cells were then incubated in a final concentration of 0.05 mM AE or 0.05 mM EM. Control cells cultured in medium containing 0.13% DMSO. After 24 h or 48 h of the AE or EM treatment, the medium was removed and the cell surface was washed once with 4 ml of PBS. Cells were harvested with 0.25% trypsin in phosphate-buffered saline (Ca^{2+} and Mg^{2+} free; PBS) with 0.02% EDTA -4Na. The cells were pelleted by centrifuging at 1000 × g for 5 min, and the isolated cells were used in subsequent experiments.

Cell viability assay

The total number of viable cells was determined at each time point by the trypan blue exclusion test.

Cell cycle analysis

In the flow cytometry analysis, cells were fixed with 70% ethanol and then stained with propidium iodide (PI) using a CycleTest Plus DNA Reagent Kit (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA), according to the instructions of the manufacturer, and samples were analyzed by flow cytometry (FACSVantage SE, Becton Dickinson Immunocytometry Systems). The population of cells in each cell cycle phase was determined with CellQuest™ software.

Measurement of intracellular polyamine levels

To analyze intracellular polyamine levels, isolated cells were washed with PBS. Cell pellets were then homogenized in 0.2 M ice-cold perchloric acid solution (PCA), incubated at 4°C for 16h, and the acid-soluble polyamines extracted by centrifugation at 17400 × g for 10 min. Benzoyl derivatization, which was performed according to the method of Verkoelen et al. (1988), was used to analyze polyamine levels. In this experiment, the benzoyl polyamines, spermidine and spermine were measured by high-performance liquid chromatography with UV detection as described by Verkoelen et al. (1988) with some modifications. Separation was carried out on an Inertsil ODS-2 column (4.6 mm i.d.×150 mm, 5 μm, GL Sciences, Tokyo, Japan) by isocratic elution with 55% methanol and a flow rate of 1.2 ml/min. Acid-insoluble pellets were dissolved in 0.1 M NaOH and their protein contents were assayed using the BCA protein assay kit (Pierce Biotechnology Inc., Rockford, IL, USA). Values for spermidine and spermine were reported as nmol/mg protein.

DNA fragmentation analysis

After 48h of the AE or EM treatment, cells were harvested and pelleted according to a previously described method. A DNA fragmentation analysis was performed according to the procedure described by Yanagihara et al. (2005) with minor modifications. Briefly, cell pellets were incubated in lysis solution [10 mM Tris-HCl (pH 7.4), 10 mM EDTA (pH 8.0), and 0.5% (V/V) Triton X-100] at 4°C for 15 min. Ribonuclease A (0.2 mg/ml) was added to the lysates, which were incubated at 37°C for 1h, followed by proteinase K (0.4 mg/ml) at 50°C for 1h. The lysate was added to 5 M NaCl and isopropanol and left to stand overnight at -20°C for DNA precipitation. After centrifugation at 17400 × g for 10 min at 4°C, precipitated DNA was dissolved in TE buffer (10 mM Tris-HCl, pH7.4, 1 mM EDTA) and applied to 2% agarose gel electrophoresis. The gels were stained with ethidium bromide, and the DNA bands were visualized under ultraviolet light and photographed.

Statistical analysis

All data were expressed as the mean±S.E.M. Statistical analyses were performed using a one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test with InStat version 3.0 for Windows (GraphPad Software, Inc., San Diego, CA, USA).

Results

Growth inhibitory effects of AE and EM

The results in Table 1 show that the number of viable cells was significantly lower in the AE- and EM-treated groups than in the control group 24 h and 48 h after the treatment. Furthermore, the inhibitory effects of EM were stronger than those of AE 24 h and 48 h after treatment.

Changes in cell-cycle distribution of MKN45 cells 24 h after the AE or EM treatment

As shown in Table 2, relative to the control, 0.05 mM AE significantly increased the population of cells in the G0/G1 phase from 53.2 to 63.5%, which was accompanied by a decrease in the proportion of cells in the S phase from 37.1 to 26.7%. This effect was enhanced when MKN45 cells were treated with 0.05 mM EM (75.2% of the cell population in the G0/G1 phase and 13.8% of the cell population in the S phase). Furthermore, the population of cells in the G2/M phase was significantly increased from 9.8 to 11.1% by the 0.05 mM EM treatment. These results indicated that the cell cycle of MKN45 cells treated with 0.05 mM AE or 0.05 mM EM was arrested at the G0/G1 phase or G0/G1 and G2/M phases, respectively.

Table 1. Variable Number of MKN45 Cells 24 h and 48 h after the AE or EM Treatment

Groups	Cell number (1×10^6)	
	24 h	48 h
Control	4.20±0.05	4.86±0.15
0.05 mM AE	2.93±0.08[a] (30.2)[b]	1.93±0.09[a] (60.5)
0.05 mM EM	1.61±0.04[a] (61.7)	0.32±0.06[a] (93.4)

[a]Significantly different from the cntrol group ($p<0.01$; Dunnett's multiple comparisons test). [b]Numbers in parentheses are the inhibition rates

Table 2. Cell cycle distribution of MKN45 cells 24 h after the AE or EM treatment

Groups	Cell cycle (%) 24 h		
	G0/G1	S	G2M
Control	53.2±0.5	37.1±0.4	9.8±0.2
0.05 mM AE	63.5±1.8[a]	26.7±1.8[a]	9.8±0.3
0.05 mM EM	75.2±1.0[a]	13.8±0.7[a]	11.0±0.5

[a]Significantly different from the cntrol group ($p<0.01$; Dunnett's multiple comparisons test)

Table 3. Intracellular Polyamine Levels in MKN45 Cells 24 h and 48 h after the AE or EM Treatment

Groups	24 h		48 h	
	Spd	Spm	Spd	Spm
Control	9.15±0.69	7.01±0.33	12.55±0.34	7.94±0.32
0.05 mM AE	9.92±0.55	6.61±0.26	9.36±0.79[a]	7.45±0.49
0.05 mM EM	6.55±0.34[a]	6.48±0.22	7.90±0.58[a]	13.25±0.48[a]

Abbreviations: Spd, spermidine; Spm, spermine; [a]Significantly different from the cntrol group ($p<0.01$; Dunnett's multiple comparisons test)

Intracellular polyamine levels of MKN45 cells 24 h and 48 h after the AE or EM treatment

The results are shown in Table 3. Spermidine levels 24 h after the treatment were significantly lower in EM-treated cells than in control cells. Spermidine levels 48 h after the treatment were significantly lower in AE- and EM-treated cells than in control cells. On the other hand, spermine levels were significantly higher in EM-treated cells than in control cells, whereas no significant difference was observed between AE-treated and control cells.

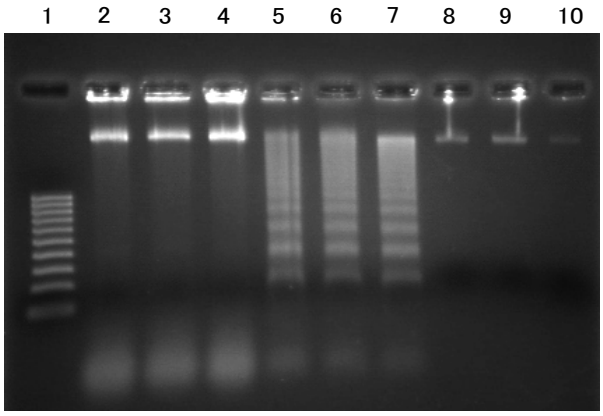

Figure 2. DNA Fragmentation Analysis of MKN45 Cells 48h after the AE and EM Treatments. Lane 1, 100bp DNA marker; Lanes 2-4, untreated cells; Lanes 5-7, AE treated cells; Lanes 8-10, EM treated cells

Analysis of DNA fragmentation by agarose gel electrophoresis

Figure 2 shows a DNA fragmentation analysis was used to determine the type of cell death. The control cells showed an intact genome (lanes 2, 3, and 4). However, DNA fragmentation was detected in AE-treated cells (lanes 5, 6, and 7), but was absent in EM-treated cells (lanes 8, 9, and 10).

Discussion

Chen et al. (2007) previously reported using the gastric cancer cell line AGS that the IC_{50} value of AE 72h after the treatment was below 0.07 mM. Based on these findings, we examined the inhibitory effects of 0.05 mM AE and 0.05 mM EM on the proliferation of the human gastric cancer cell line MKN45. The results obtained clearly demonstrated that AE and EM both significantly inhibited the proliferation of MKN45 cells. Additionally, the inhibitory effects of EM were approximately 2 or 1.5-fold stronger than those of AE 24 h or 48 h after the treatment, respectively. Chen et al. (2010) also previously showed that the proliferation of human tongue cancer cell line SCC-4 was more strongly inhibited by EM than by AE due to the induction of DNA damage and inhibition of DNA repair gene expression. One of the reasons for this difference may be the anthraquinone structure. Badria and Ibrahim (2013) reported that bioactivities were influenced by the position of the hydroxyl group on the anthraquinone structure.

The cell cycle has been divided into four sequential phases (G1, G2, S, and M) and G0. All cells undergo this cell cycle. Cancer has primarily been attributed to the loss of cell cycle regulation (Evan and Vousden, 2001). Therefore, a cell cycle analysis is considered important in the screening of anticancer agents. In the present study, the cell cycle of MKN45 cells treated with 0.05 mM AE or 0.05 mM EM was arrested at the G0/G1 phase or G0/G1 and G2/M phases, respectively, 24 h after treatment. Takahashi et al. (2014) showed that metformin, which is a biguanide drug, induced G0/G1 phase arrest after a 24 h incubation and G0/G1 and G2/M phase arrests after a 48 h incubation in an endometrial cancer cell line (Ishikawa cell). Based on these findings, they speculated that a low concentration of metformin caused G0/G1 phase arrest, whereas a higher concentration caused G2/M phase arrest. Lam and Ng (2010) also found that G0/G1 phase and G2/M phase arrests occured in a dose-dependent manner in the breast cancer cell line MCF-7 in response to a treatment with haemagglutinin, a carbohydrate-binding protein, 24h after the treatment. In the present study, MKN45 cells treated with 0.05 mM EM showed G0/G1 and G2/M phase arrests. This result was attributed to the stronger inhibitory effects of the 0.05 mM EM treatment than the 0.05 mM AE treatment. A cell cycle analysis may be useful for investigating the model of cell death. Hsiao et al. (2014) showed that pterostilbene, a natural dimethylated analog of resveratrol, simultaneously induced G0/G1 phase arrest and apoptosis in human acute myeloid leukemia cell lines 24 h after the treatment. Lam

and Ng (2010) also reported that a low concentration of haemagglutinin induced G0/G1 phase arrest and the early phase of apoptosis, while a high concentration induced G2/M phase arrest and a late apoptotic/necrotic stage. In the present study, G0/G1 phase arrest and DNA ladder formation was only observed in the 0.05 mM AE-treated group. AE at a concentration of 0.05 mM may impair the growth of MKN45 cells via G0/G1 phase arrest and concomitant apoptosis.

Regarding intracellular polyamine levels in MKN45 cells 24h after the treatment, no significant differences were observed in spermidine levels between the AE-treated group and control group. However, spermidine levels were significantly lower in the EM-treated group than in the control group. Spermine levels were also lower in the AE- and EM-treated group than in the control group. These results are may have been due to cell cycle arrest by the treatment with AE and EM. Ray et al. (1999) showed that the depletion of polyamines lead to cell cycle arrest in normal rat intestinal epithelial cells. On the other hand, regarding intracellular polyamine levels in MKN45 cells 48h after the treatment, spermidine levels were significantly lower in both treated groups than in the control group. No significant differences were observed in spermine levels between the AE-treated group and control group, but were significantly higher in the EM-treated group than in the control group. Nemoto et al. (2001) reported that spermine levels were elevated in MKN45 cells treated with DL-α-difluoromethylornithine, an inhibitor of polyamine biosynthesis, but did not induce apoptosis. In the present study, DNA ladder formation was not detected in the 0.05 mM EM-treated group. We also analyzed lactate dehydrogenase (LDH) activity in the culture medium 48 h after the treatment in order to confirm the mechanism responsible for cell death. The results obtained revealed that the 0.05 mM EM treatment induced the release of LDH (data not shown). Necrotic cell death is defined by the breakdown of the plasma membrane, resulting in the leakage of LDH, a cytoplasmic enzyme, from damaged cells (Chan et al., 2013). EM at a concentration of 0.05 mM may impair the growth of MKN45 cells via G0/G1 and G2/M phase arrests and concomitant necrosis.

In conclusion, the 0.05 mM AE and 0.05 mM EM treatments significantly inhibited the proliferation of the human gastric cancer cell line MKN45 after 24 h and 48 h, respectively. Although AE and EM share very similar anthraquinone structures, the inhibitory effects of EM were stronger than those of AE. Furthermore, our results suggested that the mechanisms underlying cell death by AE and EM differed. We intend to elucidate the molecular mechanisms underlying cell death in more detail in future.

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