Combination of aloe-emodin with radiation enhances radiation effects and improves differentiation in human cervical cancer cells

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Abstract. The aim of the present study was to investigate the effects of aloe-emodin (AE) on the radiosensitivity and differentiation of HeLa human cervical cancer cells. Cell proliferation was assessed in the HeLa cervical cancer cell line by a methylthiazolyldiphenyl-tetrazolium bromide assay. Radiosensitivity was determined by a colony-forming assay. Flow cytometry was used for analysis of cell cycle distribution and apoptosis. The expression of γ-H2AX and cyclin B was assessed by western blotting. Alkaline phosphatase (ALP) activity was measured by an ALP activity kit. It was demonstrated that AE inhibited the proliferation of HeLa cells in a concentration- and time-dependent manner, induced G2/M and S phase cell cycle arrest and enhanced the radiosensitivity of HeLa cells. The combination of AE and radiation induced apoptosis, upregulated cyclin B and γ-H2AX expression and further improved ALP activity compared with treatment with AE or radiation alone. AE enhanced the radiosensitivity of HeLa human cervical cancer cells in vitro, inhibited the proliferation of HeLa cells, induced G2/M phase cell cycle arrest and, in combination with radiation, induced the apoptosis and improved the differentiation of HeLa cells.

Introduction

Cervical cancer is the second most common type of malignancy in females, with over half a million cases occurring annually worldwide (1). Early stage cervical cancer can be treated with surgery or radiation with equivalent results. For locally advanced disease; however, radiation offers the only initial option for curative therapy (2). The dose of radiation is increased to enhance the effects of radiotherapy, which inhibits cervical cancer growth and development; however, this also leads to adverse reactions. In order to improve the local control and diminish side effects, radiation is combined with a radiosensitizer.

Aloe-emodin (AE), an Aloe vera leaf exudate (3), is used in traditional Chinese medicine for its laxative, antiviral and hepatoprotective properties (4-6). Its ingredients are of great value for the food, modern pharmaceutical and cosmetic industries (7). Previous studies have demonstrated that AE exerts anti-proliferative effects by inhibiting cell cycle progression in certain types of cancer cells, including cervical cancer (8), KB oral cancer cells (9), bladder cancer (10), leukemia (11), HK-2 human kidney cells (12) and nasopharyngeal carcinoma cells (13). AE is hypothesized to inhibit progression at the G2/M phase of the cell cycle. In addition, AE has been reported to inhibit the proliferation of gastric cancer and tongue squamous cancer cells by inhibiting the cell cycle at S phase (14,15).

Radiation affects the cell cycle, and radiosensitivity depends on the phase of the cell cycle and its progression (16), however, the effect of AE as a radiosensitizer in HeLa cells has not been reported. The present study hypothesized that the combination treatment of AE and radiation may enhance the radiosensitivity and differentiation of cervical cancer cells. In order to assess this hypothesis, the present study analyzed the effects of combination treatment with AE and radiation in the HeLa human cervical cancer cell line and examined the underlying mechanisms of the inhibition of HeLa cells.

Materials and methods

Cells and cell culture. The HeLa cell line was purchased from Jilin Tumor Institute (Changchun, China). Cells were cultured under a humidified atmosphere containing 5% CO2 at 37°C in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were subcultured every 2-3 days. Exponentially growing cells were used for the experiments.

Reagents and equipment. AE was purchased from Aladdin Chemical Co., Ltd. (Shanghai, China). RPMI-1640 medium (Gibco-BRL, Carlsbad, CA, USA), fetal bovine serum
(FBS; Gibco-BRL), methylthiazolyldiphenyl-tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA), enhanced chemiluminescence (ECL) substrate (Pierce Biotechnology, Inc., Rockford, IL, USA), mouse anti-human β-actin monoclonal antibody (Sigma), anti-cyclin B and anti-γ-H2AX monoclonal antibody (Abnova Corporation, Taipei, Taiwan), goat anti-mouse IgG/horseradish peroxidase (HRP; Beijing Dingguo Changsheng Biotechnology Co. Ltd, Beijing, China), electrophoresis system (Bio-Rad, Hercules, CA, USA), an alkaline phosphatase (ALP) activity assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and an X-ray linear accelerator (Varian Medical Systems, Palo Alto, CA, USA) were used in the present study. The dose rate was 200 c Gy/min and the distance to the radiation source was 100 cm.

**MTT assay for cell proliferation.** HeLa cells were seeded in 96-well plates at a density of 4.0x10^3 cells/well for 24 h. The cells were treated with various concentrations of AE (0, 5, 10, 25, 50, 100, 200 and 500 µM) for 24, 48 and 72 h. Dimethylsulfoxide (DMSO; 0.5%) was used as the control. MTT (5 mg/ml) was added to each well and incubated for 4 h at 37°C, following which DMSO was added to each well to dissolve the dark blue crystal product. The absorbance was measured at a wavelength of 590 nm using a microplate reader (Bio-Rad, Shanghai, China).

**Colony-forming assays.** A single-cell suspension was plated onto 6-well culture plates at different densities (1x10^3, 2x10^3, 5x10^3 and 1x10^4 cells/ml). Following incubation for 24 h, the cells were treated with AE in medium at different concentrations (0, 50, 100 and 200 µM) and cells were then exposed to different irradiation doses (0, 2, 4, 6, 8 and 10 Gy). The cells were washed with normal medium and incubated in fresh, drug-free medium (10% FBS in RPMI-1640) for 14 days, 24 h after radiation treatment. The cells were then washed with phosphate-buffered saline (PBS), fixed with methanol and stained with Giemsa solution. The number of colonies containing at least 50 cells was counted manually under a phase-contrast microscope (Olympus, Tokyo, Japan) by two independent investigators. The plating efficiency (PE) and survival fraction (SF) were calculated as follows: PE = colony-forming number / inoculating number x 100. SF = PE (tested group) / PE (control group) x 100. GraphPad Prism software 5.01 and the multi-target single-hit model (using the equation SF = 1 - (1 - e^{-0.05D})^n) were applied to generate the dose-survival curve and calculate the radiobiological parameters, including the mean lethal dose (D0), quasi-threshold dose (D0.5), fraction dose of 2 Gy in clinical practice (SF2), extrapolation number (N), sensitizing enhancement ratio (SER) and SERQ. SER is one of the radiobiological parameters and AE increases the radiosensitivity in a concentration-dependent manner. Three replicates were set at each radiation dose.

**Cell cycle distribution and apoptosis analysis by flow cytometry.** Single-cell suspensions at a density of 1x10^6 cells/well were seeded in 6-well plates and incubated for 24 h. The cells were treated with AE (0 and 50 µM) for 30 min, irradiated with doses of 0 and 4 Gy and incubated for 12, 24, 48 and 72 h. The cells were harvested by trypsinization, washed twice with PBS and sedimented by centrifugation at 1,500 x g for 5 min. The supernatant was removed and cells were fixed in 70% ice-cold ethanol overnight. Cells were washed twice with cold PBS, incubated with 20 µg/ml RNase and stained with propidium iodide. Cell cycle distribution and apoptosis were determined by flow cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

**Western blot analysis of cyclin B and γ-H2AX expression.** A single-cell suspension was incubated for 24 h, following which the cells were treated with AE (0 and 50 µM) for 30 min followed by radiation treatment at doses of 0 and 4 Gy. Subsequently, the cells were washed, scraped with ice-cold PBS and centrifuged. The cell lysate was harvested and protein concentration was determined with a protein assay kit (Ye zhou, Shanghai, China). A total of 50 µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes (Mai bio, Shanghai, China) and incubated overnight with PBS containing 3% skimmed milk. The membranes were incubated with primary antibodies against β-actin, cyclin B and γ-H2AX for 2 h and washed with PBS. The membranes were then treated with HRP-conjugated rabbit anti-mouse IgG and detected with ECL substrate (Pierce Biotechnology, Inc., Rockford, IL, USA). Chemiluminescence was captured and analyzed using a Chemidoc system (Bio-Rad, Hercules, CA, USA). The blots were stripped and re-probed with antibodies against β-actin to ensure equal protein loading.

**Results.**

1. **Radiosensitivity of HeLa cells with AE.** HeLa cells were treated with different concentrations (0, 5, 10, 25, 50, 75, 100, 200 and 500 µM) of aloe-emodin for 24, 48 and 72 h. Dimethylsulfoxide solution served as a control. The mean ± standard deviation of three independent experiments are shown (P<0.05).

2. **Cell cycle distribution.** HeLa cells were treated with aloe-emodin (0, 50, 100 and 200 µM) and radiation (0, 2, 4, 6, 8 and 10 Gy) for 24 h. Experiments were repeated three times. The error bars represent standard error.

3. **Western blot analysis.** A total of 50 µg of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred onto nitrocellulose membranes, and subjected to chemiluminescence detection. The membranes were then treated with HRP-conjugated rabbit anti-mouse IgG and stained with alkaline phosphatase (ALP) activity assay kit (Jiancheng Bioengineering Institute, Nanjing, China). The blots were stripped and re-probed with antibodies against β-actin to ensure equal protein loading.
secondary antibody. Antibody-reactive bands were visualized by the ECL detection system (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Representative data were from an individual experiment repeated three times.

**Determination of relative ALP activity.** A single-cell suspension was incubated for 24 h, following which the cells were treated with AE (0 and 50 µM) for 30 min followed by radiation treatment at doses of 0 and 4 Gy. Subsequently, the cells were cultured for 24, 48 and 72 h. The cells were washed, lysed and centrifuged. The supernatant was used for the measurement of ALP activity with an ALP activity assay kit (Nanjing Jiancheng Bioengineering Institute). The absorbance at 520 nm was measured on a microplate reader. Three independent experiments were performed in this analysis.

**Statistical analysis.** Statistical analysis was performed using SPSS version 18.0 (SPSS, Inc., Chicago, IL, USA). Student’s t-test or one-way analysis of variance were used for all comparisons. Data are presented as the mean ± standard error of three independent experiments. P<0.05 was considered to indicate a statistically significant difference.

**Table I. Radiobiological parameters of the dose-survival curve.**

<table>
<thead>
<tr>
<th>Concentration</th>
<th>$D_0$</th>
<th>$D_q$</th>
<th>N</th>
<th>$SF_2$</th>
<th>SERD$_0$</th>
<th>SERD$_q$</th>
</tr>
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<tbody>
<tr>
<td>0 µM + RT</td>
<td>1.756</td>
<td>2.177</td>
<td>3.454</td>
<td>0.538</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 µM + RT</td>
<td>1.437</td>
<td>1.444</td>
<td>2.731</td>
<td>0.435</td>
<td>1.222</td>
<td>1.508</td>
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<tr>
<td>100 µM + RT</td>
<td>1.158</td>
<td>0.987</td>
<td>2.345</td>
<td>0.324</td>
<td>1.517</td>
<td>2.207</td>
</tr>
<tr>
<td>200 µM + RT</td>
<td>1.072</td>
<td>0.283</td>
<td>1.302</td>
<td>0.286</td>
<td>1.638</td>
<td>7.695</td>
</tr>
</tbody>
</table>

Radiobiological parameters of the dose-survival curve. RT, radiation dose 4 Gy; $D_0$, mean lethal dose; $D_q$, quasi-threshold dose; $SF_2$, daily fraction dose of 2 Gy in clinical practice; N, extrapolation number; SER, sensitizing enhancement ratios. SERD$_0$ = $D_0$ control group / $D_0$ AE group; SERD$_q$ = $D_q$ control group / $D_q$ AE group.

**Figure 3. Cell cycle distribution by flow cytometry.** (A) Control (dimethylsulfoxide) at 12 h; (B) AE (50 µM) treatment at 12 h; (C) irradiation (4 Gy) at 12 h; (D-G) combination treatment with AE (50 µM) and irradiation (4 Gy) at 12, 24, 48 and 72 h, respectively. AE, aloe-emodin.
Results

Growth inhibitory effects of AE on HeLa cells. The effect of AE on the proliferation of HeLa cells was evaluated by an MTT assay (Fig. 1). AE inhibited the proliferation of HeLa cells in a concentration- and time-dependent manner, particularly when the concentration of AE was >50 µM. Based on these results, 50 µM AE was selected as the dose for subsequent experiments.

Effect of AE on the radiosensitization of HeLa cells. As shown in Fig. 2 and Table I, the radiobiological parameters $D_0$, $D_{eq}$, $N$ and $S_{eq}$ decreased, and the SERD$_0$ and SERD$_{eq}$ increased in response to AE in a concentration-dependent manner in vitro. These effects were enhanced by combination treatment compared with the effect of radiation alone.

Effects of AE and radiation on HeLa cell cycle distribution and apoptosis assessed by flow cytometry. As shown in Fig. 3, HeLa cells treated with radiation or AE alone and the combination of radiation with AE demonstrated an increase in the number of cells in the G$_2$/M phase. Investigation demonstrated that the S phase was increased with AE alone at 48 and 72 h and a sub-G$_1$ peak clearly appeared (apoptosis) at 24, 48 and 72 h in cells treated with the combination of radiation and AE. Apoptotic peaks were significant with the extension of time.

Cyclin B and γ-H2AX expression by western blotting. Figs. 4 and 5 show the expression of cyclin B and γ-H2AX by western blotting. The cells treated with radiation (4 Gy) or AE (50 µM) alone and a combination of radiation and AE expressed cyclin B and γ-H2AX. The expression of cyclin B was highest at 12 h in cells treated with the combination and the expression of γ-H2AX in the combination group was higher than that following AE or radiation treatment alone.

Relative activity of ALP. As shown in Fig. 6, the ALP activity of HeLa cells was increased by the treatment with radiation or AE alone and the combination of radiation and AE in a time-dependent manner. This effect was enhanced by combination treatment compared with the effect of radiation or AE alone.

Discussion

Although the anticancer effect of AE has been reported in numerous studies, its effect on enhancing the radiosensitivity of cancer cells has not been investigated to date. The present study investigated the effect of AE on the radiosensitivity and differentiation of HeLa cells in vitro and examined its underlying mechanisms.
The results demonstrated that AE inhibited the proliferation of HeLa cells in vitro in a concentration- and time-dependent manner. Notably, AE concentrations ≥50 μM demonstrated significant cytotoxicity in the MTT assay. The radiosensitivity of cells treated with AE increased in a dose-dependent manner compared with that of cells treated without AE in colony-forming assays. These results demonstrated that AE enhanced the radiosensitivity of HeLa cells. It has been reported that the G2/M and M phases are the most sensitive to radiation in the cell cycle (17-19). Flow cytometry results demonstrated that treatment with AE or radiation alone and AE in combination with radiation inhibited cell cycle progression at the G2/M phase. Furthermore, the experiments also demonstrated that the cells arrested in the S phase with AE alone at 48 and 72 h and that there was a sub-G1 peak at 24, 48 and 72 h, which was particularly clear 72 h after combination treatment with AE and radiation in a concentration-dependent manner. The underlying mechanism of apoptosis (sub-G1 peak) in HeLa cells caused by S phase arrest was not further investigated in the present study. However, previous studies have demonstrated that AE is able to inhibit the growth of gastric cancer cells and induce apoptosis in squamous cell carcinoma of the tongue by inhibiting cell cycle progression at the S phase (14,15). The findings of the present study therefore, support the results of the contribution of S phase arrest to apoptosis. The present study hypothesized that S phase arrest may be one of the factors triggering apoptosis in HeLa cells, although it may not be the only one.

The cell cycle is a strictly ordered process that is controlled by multiple cell cycle regulatory proteins, including cyclin and cyclin dependent kinase (CDK) protein kinases. The main cell cycle regulatory proteins that control the G2/M phase are known as cyclin B and CDK1. The levels of CDK1 remain relatively constant during the cell cycle, while the levels of cyclin B undergo cyclical changes. Cyclin B is synthesized at late G1 phase and its expression levels increase as the cycle progresses, reaching a maximum value at the G2 phase. As cyclin B accumulates, the activity of CDK1 kinase is observed and reaches a maximum value at G1 phase, which is maintained throughout the M phase. Cyclin B is rapidly degraded at late M phase, concomitant with the inactivation of CDK1, and the cell enters the next cycle (20,21). CDK1 regulates the cell cycle by modulating the expression of cyclin B. Therefore, cyclin B was selected for analysis in the present study. Western blot analysis demonstrated that cyclin B expression was upregulated in cells treated with AE or radiation alone. Furthermore, combination treatment with AE and radiation further upregulated γ-H2AX expression at 1 and 6 h, indicating the further increase of DSBs. Histone H2AX phosphorylation is crucial in apoptosis and the appearance of strong H2AX phosphorylation is concurrent with the initiation of DNA fragmentation (30). Flow cytometry also demonstrated sub-G1 peaks (apoptosis) at 24, 48 or 72 h.

Cellular ALP is increasingly recognized as an important marker for monitoring tumor cell differentiation (31-33). AE inhibited the growth of cervical cancer cells, KB oral cancer cells and gastric cancer cells by inducing cancer cell differentiation (8,9,14). The present study demonstrated that the activity of ALP in HeLa cells is increased by treatment with radiation or AE alone and further increased by a combination of radiation and AE in a time-dependent manner. The differentiation of HeLa cells may also be improved by combination treatment with radiation and AE compared with the treatment with radiation or AE alone.

An ideal radiosensitizer enhances the radiosensitivity of tumor cells and is harmless to or protects normal tissue. Previous studies demonstrated that AE has no toxic effects on normal cells (34). However, whether or not it protects normal tissue remains to be determined.

The present findings, together with those of previous studies, suggest that AE has the following antitumor effects: i) AE inhibits the growth of HeLa cells; ii) AE arrests HeLa cells at the S and G2/M phases, inducing tumor cell apoptosis; iii) AE increases the radiation sensitivity of HeLa cells by inducing G2/M phase arrest; iv) AE increases radiation-induced DNA damage; and v) the combination of radiation and AE further improves the differentiation of HeLa cells. The present results are consistent with the hypothesis that combination treatment with AE enhances the effects of radiotherapy on HeLa cells in vitro. The exposure of cells to the combination of two cytotoxic modalities, AE and radiation, demonstrated an increase in the cell death of HeLa cells. This indicated that AE may be an effective radiosensitizer and a potential therapeutic agent for the induction of tumor cell proliferation. Further in vivo studies are required to clarify the anticancer effect of AE and its mechanisms with the intention of clinical application.

References