Antitumor effect of violacein on colorectal cancer cells

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Abstract

Violacein is a blue-purple pigment extracted from soil bacteria and has been reported to have anticancer activity. In this study, we assessed the anticancer activity of violacein and confirmed the cytotoxicity and induction of apoptosis in colon cancer cell lines. It was confirmed that violacein-induced apoptosis follows two pathways. In the first pathway, activation of caspase-8 (an inducible caspase) activates caspase-3 (an executive caspase), which induces apoptosis in cancer cells. In the second pathway, cytochrome c is released from mitochondria and activates caspase-9 (an inducible caspase), which in turn activates caspase-3 (an executive caspase), which induces apoptosis in cancer cells. These findings suggest that violacein might be useful as a therapeutic agent for colorectal cancer in the future.

Keywords：violacein, colon cancer cell, apoptosis

Received December 12, 2022; accepted June 1, 2023

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1. Introduction

Cancer is the leading cause of death in Japan (27.6 %)[1]; in particular, colorectal cancer (CRC) has high morbidity and a high mortality rate[2-3]. Hence, there is an urgent need to identify novel therapeutic drug candidates for this disease. Therefore, we assessed the antitumor activity of violacein, which has various physiological activities such as antibacterial, antioxidant, and anticancer effects[4-7]. Violacein is a natural bluish-purple pigment compound (3*E*)-3-[5-(5-hydroxy-1*H*-indol-3-yl)-2-oxo-1,2-dihydro-3*H*-pyrrol-3-ylidene]-1,3-dihydro-2*H*-indol-2-one with a molecular weight of 343.34[8]. Natural pigments such as violacein lack vividness compared with synthetic pigments and have the drawback of low color stability (e.g., fading in sunlight), but they have physiological activities such as antibacterial and antioxidant properties and have attracted attention as functional pigments.

In this study, we assessed the antitumor effect of violacein on CRC cells and determined its mechanism of action.

2. Experimental

2.1 Cell culture. This study was performed using the COLO205 human CRC cell line[9]. COLO205 cells were cultured in RPMI 1640 Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10 % heat-inactivated fetal bovine serum (Cytiva, Marlborough, MA), 100 units/mL penicillin G, and 100 μg/mL streptomycin sulphate (Fujifilm Wako Pure Chemical Corporation, Osaka). The cells were cultured at 37°C in an incubator with 5 % CO_2. Violacein was dissolved in dimethyl sulfoxide (DMSO; Fujifilm Wako Pure Chemical Corporation) and the final concentration of DMSO in the controls was 1 %.

2.2 Cell proliferation assay. Cell proliferation was examined with Cell Counting Kit-8 (CCK-8) Kit (Dojindo Laboratories, Kumamoto). Briefly, cells were seeded in 96-well plates at a density of 5×10^4 cells/well. Subsequently, cells were treated with various doses of violacein (0, 5, 10, and 20 μmol/L). After 24 h of incubation, cells were incubated with 10 μL CCK-8 reagent at 37°C for 2 h. Absorbance was detected at 450 nm using the iMark™ Microplate Reader (Bio-Rad Laboratories, Hercules, CA).

2.3 Apoptotic analysis. COLO205 cells were seeded in 48-well plates at a density of 10^5 cells/well, followed by the addition of 1 % DMSO (control) or 10 μmol/L violacein to the cells. After 24 h, Annexin-V/PI Apoptosis Assay (Bio-Rad Laboratories, Hercules, CA) was conducted. Fluorescence intensity was measured using the BD FACSCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). For each measurement, at least 10,000 cells were counted.

2.4 Loss of MMP. COLO205 cells were seeded in 48-well plates at a density of 10^5 cells/well. Then, 1 % DMSO (control) or 10 μmol/L violacein was added to the cells. After 24 h, the mitochondrial membrane potential (MMP) was measured by adding JC-1 dye (Cell Technology, Davis, CA) to the cells and measuring fluorescence using the BD FACSCalibur Flow Cytometer.

2.5 Release of cytochrome c. COLO205 cells were seeded in 48-well plates at a density of 10^5 cells/well. Then, 1 % DMSO or 10 μmol/L violacein was added, and cytochrome c was measured after 24 h, using anti-cytochrome c antibody (Abcam, Cambridge, MA) according to the manufacturer's instructions. Fluorescence was measured using the BD FACSCalibur Flow Cytometer.

2.6 Detection of activated caspase. COLO205 cells were seeded in 48-well plates at a density of 10^5 cells/well. Then, 1 % DMSO or 10 μmol/L violacein was added to the cells. After 24 h, the activation of

caspase-3, caspase-8, and caspase-9 was measured with the Cleaved Caspase-3 FITC Staining Kit, Caspase-8 (active) FITC Staining Kit, and Caspase-9 (active) Red Staining Kit (all from Abcam), respectively, according to the manufacturer's instructions. Fluorescence was measured using the BD FACSCalibur Flow Cytometer.

2.7 Autophagy detection with DALGreen staining. Cells were plated in 24-well plates and cultured at 37°C with 1 mL of 0.1 μmol/L DALGreen (Dojindo Laboratories) for 30 min. After the cells were washed twice with culture medium, they were incubated with or without 10 μmol/L violacein for 48 h. Then, cells were washed twice with PBS(-), and fluorescence was measured using the BD FACSCalibur Flow Cytometer. For all samples, DALGreen fluorescence was collected through the FL1 channel and 10,000 events were acquired in at least three separate experiments.

3. Results and Discussion

3.1 Cytotoxicity test. The CCK-8 assay was performed to confirm that violacein is cytotoxic to COLO205 cells. As shown in Figure 1, violacein

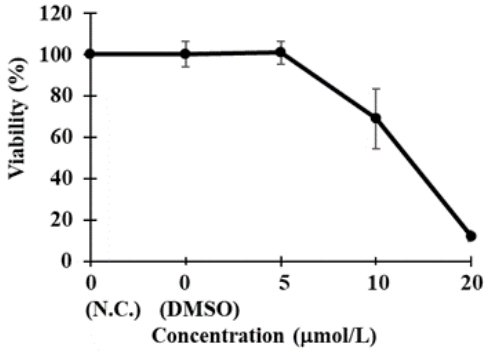

Fig. 1. The viability of COLO205 colon cancer cells was tested by the CCK-8 assay. COLO205 cells were seeded at a density of 5×10^4 cells/well and treated with 5 to 20 μmol/L violacein, and the absorbance was measured after 24 h. The experiments were performed three times each, and the data are presented as the mean ± standard deviation (SD).

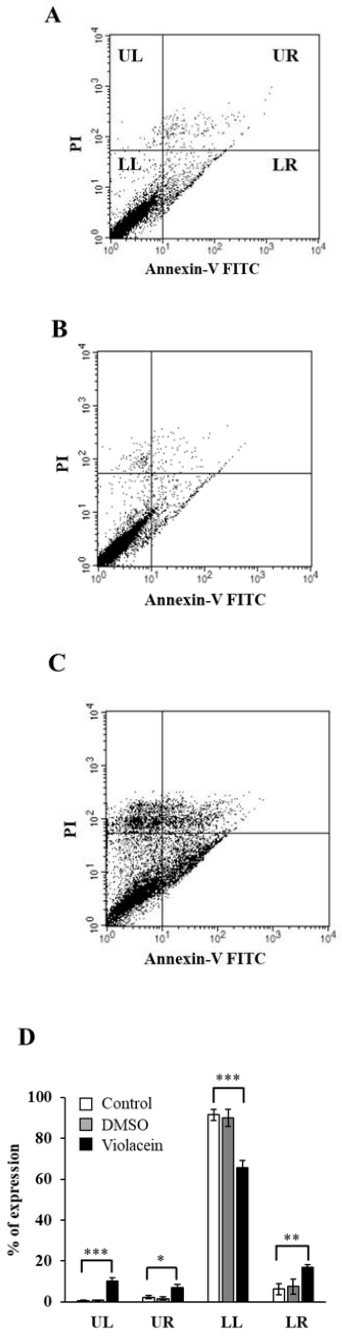

Fig. 2. Annexin V/PI assay of COLO205 cells at 24 h after the addition of violacein (10 μmol/L). Representative scatter plots of PI (y-axis) vs. Annexin V/FITC (x-axis). Dot plot of detected fluorescence control (A), DMSO (B), violacein (C). UL is cells stained only with PI, UR is necrotic cells or late apoptotic cells stained with both Annexin-V and PI, LL is living cells not stained with either Annexin-V and PI, and LR is early apoptotic cells stained only with Annexin V. Analysis of the detected fluorescence ratio and graphing (D). The experiments were performed three times each, and the data are presented as the mean ± standard deviation (SD). * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ versus control.

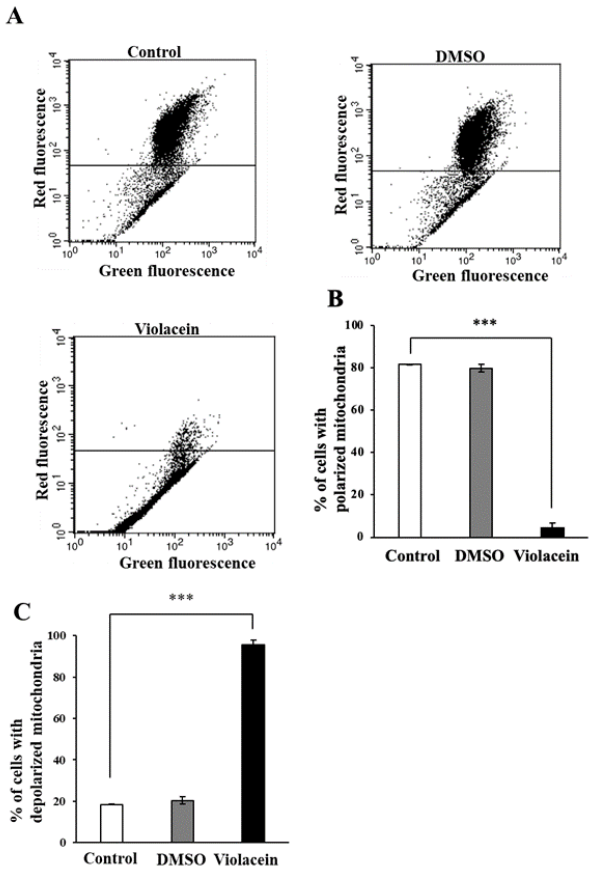

Fig. 3. Analysis of the mitochondrial membrane depolarization by JC-1 dye of COLO205 cells treated with and without 10 μmol/ L violacein for 24 h. Representative dot plots of JC-1 dye of COLO205 cells are shown. The lower panel shows COLO205 cells exhibiting green fluorescing monomers, representing polarized cells; and the upper panel shows cells exhibiting red fluorescence aggregates, representing depolarized cells (A). Percent decrease in normal cells and increase in apoptotic cells based on the dot plot of JC-1 dye (B and C). The experiments were performed three times each, and the data are presented as the mean ± standard deviation (SD). *** $P < 0.001$ versus control.

decreased the viability of cells in a concentration-dependent manner, with 50 % cell death occurring at 12.5 μmol/L. Therefore, subsequent experiments were performed at a violacein concentration of 10 μmol/L. Cytotoxicity did not change over time.

3.2 Analysis of apoptosis by fluorescence-activated cell sorting. In cells in which early apoptosis has been induced, the nuclear envelope remains normal, the asymmetry of the cell membrane is disrupted, and phosphatidylserine is exposed to the outside of the cell membrane. PI emits red fluorescence by binding to Annexin V and the double helix of DNA in the nucleus[10-16], and these two fluorescence signals were detected (Fig. 2A-D). The results showed that the population of dead cells increased about 3.7 fold in cells treated with violacein compared with the control, showing that violacein is cytotoxic. In addition, the proportion of early apoptotic cells increased about 2.7 fold, showing that violacein induces early apoptosis.

3.3 Loss of MMP and release of cytochrome c. In cells in which early apoptosis has been induced, the intracellular MMP disappears and cytochrome c, an apoptosis-inducing factor, is released into the cell[17] . Because the JC-1 dye has affinity for the MMP, it emits red fluorescence when the MMP is

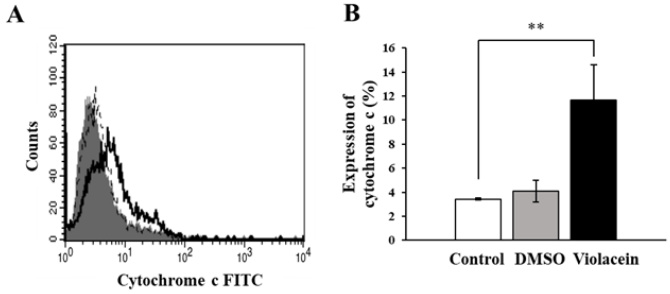

Fig. 4. Detection of cytochrome c in COLO205 cells by flow cytometry at 24 h after the addition of violacein (10 μmol/L). Graph showing the fluorescence intensity of cytochrome c FITC superimposed for the control (dotted line), DMSO (gray), violacein (solid line) (A). Graph showing the ratio of fluorescence intensity of cytochrome c FITC (B). The experiments were performed three times each, and the data are presented as the mean ± standard deviation (SD). ** $P < 0.01$ versus control.

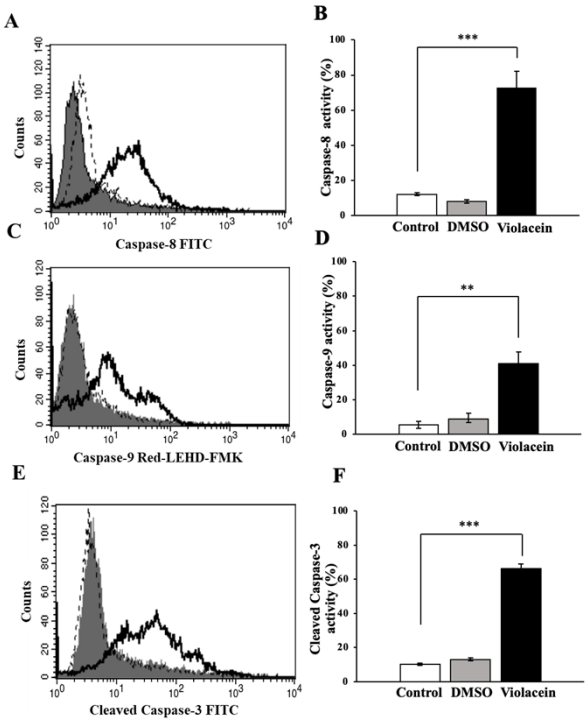

Fig. 5. Detection of caspase-8, caspase-9, and caspase-3 in COLO205 cells by flow cytometry at 24 h after the addition of violacein (10 μmol/L). Graphs showing the fluorescence intensity of caspase-8 FITC for the control (dotted line), DMSO (gray), and violacein (solid line) (A) and the ratio of the fluorescence intensity of caspase-8 FITC (B). Graphs showing the fluorescence intensity of caspase-9 Red-LEHD-FMK for the control (dotted line), DMSO (gray) and violacein (solid line) (C) and the ratio of fluorescence intensity of caspase-9 Red-LEHD-FMK (D). Graphs showing the detected fluorescence intensity of cleaved caspase-3 FITC for the control (dotted line), DMSO (gray), and violacein (solid line) (E), and the ratio of fluorescence intensity of cleaved caspase-3 FITC (F). The experiments were performed three times each, and the data are presented as the mean ± standard deviation (SD). ** $P < 0.01$ and *** $P < 0.001$ versus control.

normal and emits green fluorescence when the MMP decreases[17-21]. The results showed that the MMP was reduced to about 1/18 that of the control in cells treated with violacein (Fig. 3A-C).

Next, using the BD FACSCalibur Flow Cytometer, an antibody that specifically binds to cytochrome c and emits green fluorescence was used to detect the fluorescence[22-27]. The results showed that cells supplemented with violacein released about 3.4 times more cytochrome c compared with control

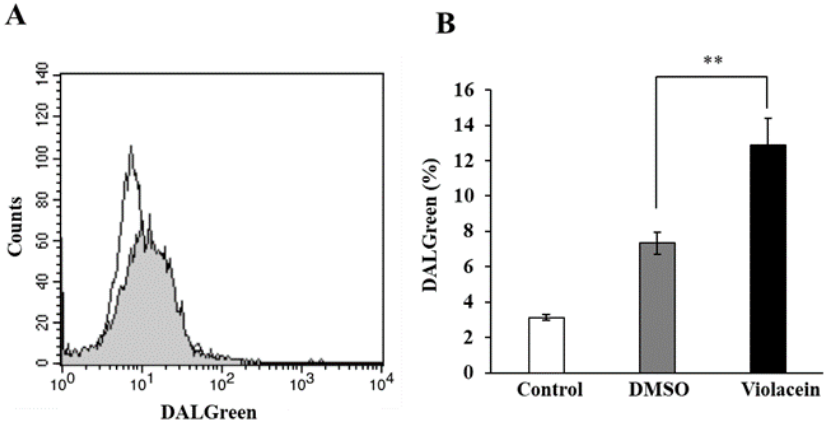

Fig. 6. Effects of violacein on autophagy. Flow cytometry analysis of autophagosome formation (autolysosome) in COLO205 cells treated with violacein for 48 h. Graph showing the fluorescence intensity of DALGreen DMSO (solid line) and violacein (gray) (A). Graph shows the ratio of the fluorescence intensity of DALGreen (B). The experiments were performed three times each, and the data are presented as the mean ± standard deviation (SD). ** $P < 0.01$ versus DMSO.

cells (Fig. 4A, B). These data suggest that the MMP disappeared due to the destruction of the intracellular mitochondrial membrane by violacein, and cytochrome c was released from the mitochondria into the cytoplasm.

3.5 Detection of activated caspase. To confirm the route by which violacein induces apoptosis, the activity of caspase was evaluated using an antibody that specifically binds to cleaved caspase-3, caspase-8, and caspase-9[28-34]. The results showed that cells supplemented with violacein had about a 6-fold higher caspase-8 activity compared with control cells (Fig. 5A, B). Cells supplemented with violacein had about an 8-fold higher caspase-9 activity compared with control cells (Fig. 5C, D). Cells supplemented with violacein had about a 6-fold higher caspase-3 activity compared with control cells (Fig. 5E, F). Together, these results showed that violacein activates inducible caspase-8, which stimulates caspase-3 activity, thus inducing apoptosis and the release of cytochrome c from the mitochondria. Thus, inducible caspase-9 and executive caspase-3 are activated to induce apoptosis by two routes that cause cancer cells to undergo apoptosis.

Kim *et al.*[7] reported that violacein induces nuclear condensation in Huh7 and Hep3B hepatocellular carcinoma cells, alters MMP, and increases reactive oxygen species (ROS), leading to activation of the caspase cascade and induction of apoptosis. In addition, Queiroz *et al.* [35] reported that violacein induces cell death in TF1 leukemia progenitor cells by inhibiting calpain and DAPK1 and activating PKA, AKT, and PDK through endoplasmic reticulum stress and Golgi apparatus collapse. These findings suggest that violacein induces apoptotic cell death via endoplasmic reticulum stress and Golgi apparatus collapse due to increased nuclear aggregation and ROS.

3.6 Effects of violacein on autophagy. Our previous study found that violacein induced cell death due to necrosis as well as apoptosis[5]. In addition, it is known that autophagy is a mechanism that can regulate apoptosis positively or negatively[36-39].
Therefore, we studied the effects of violacein on autophagy. Cells were treated with 10 μmol/L violacein for 48 h to label autophagosomes. Fluorescence-activated cell FACS analysis demonstrated that treatment with violacein significantly increased autophagy in COLO205 cells (Fig. 6A, B).

4. Conclusion

In this study, we investigated whether violacein extracted from soil bacteria induces the apoptosis in CRC cells. The CCK-8 assay showed that violacein exhibited cytotoxicity against COLO205 CRC cells in a concentration-dependent manner. In addition, it was confirmed that violacein induced apoptosis via caspase-3, which was activated by caspase-8 or caspase-9. This study elucidated the mechanism of action of cytotoxicity and apoptosis induction of violacein in COLO205 cells, showing that violacein induced a significant increase in apoptosis and autophagy associated with disruption of the MMP in COLO205 cells. Thus, violacein might be useful for the treatment of CRC in the future.

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