

Resveratrol Induces Luminal Apoptosis of Human Colorectal Cancer HCT116 Cells in Three-dimensional Culture

TOSHIYUKI TSUNODA^{1,2}, SHUHEI ISHIKURA^{1,2}, KEIKO DOI^{1,2}, HIROSHI MATSUZAKI¹,
YURI IWAIHARA¹ and SENJI SHIRASAWA^{1,2}

¹Department of Cell Biology, Faculty of Medicine, Fukuoka University, Fukuoka, Japan;

²Central Research Institute for Advanced Molecular Medicine, Fukuoka University, Fukuoka, Japan

Abstract. *Background:* We have previously reported the crucial roles of oncogenic Kirsten rat sarcoma viral oncogene homolog (KRAS) in inhibiting apoptosis and disrupting cell polarity via the regulation of phosphodiesterase 4 (PDE4) expression in human colorectal cancer HCT116 cells in three-dimensional cultures (3DC). *Herein we evaluated the effects of resveratrol, a PDE4 inhibitor, on the luminal cavity formation and the induction of apoptosis in HCT116 cells. Materials and Methods:* Apoptosis was detected by immunofluorescence using confocal laser scanning microscopy with an antibody against cleaved caspase-3 in HCT116 cells treated with or without resveratrol in a two-dimensional culture (2DC) or 3DC. *Results:* Resveratrol did not induce apoptosis of HCT116 cells in 2DC, whereas the number of apoptotic HCT116 cells increased after resveratrol treatment in 3DC, leading to formation of a luminal cavity. *Conclusion:* Resveratrol induced apoptosis of HCT116 cells in 3DC, resulting in the formation of a luminal cavity, probably by inhibiting PDE4 activity.

Three-dimensional culture (3DC) of cells closely resembles the *in vivo* microenvironment of tissues. Cells grown in 3DC assemble into spherical aggregates called spheroids (1). A 3DC system provides the ability to directly investigate the importance of cell–cell and cell–extracellular matrix interactions (2), which are frequently de-regulated in tumorigenesis (3). We previously established HKe3 cells, which are derived from human colorectal cancer HCT116 cells and are disrupted at the oncogenic Kirsten rat sarcoma viral oncogene homolog (KRAS) gene by homologous recombination (4). HKe3 cells form polarised structures

resembling a colonic crypt in 3DC, whereas HCT116 cells grown in 3DC form structures without apical–basal cellular polarity (5). In this model, the oncogenic KRAS is involved in inhibiting luminal apoptosis and disrupting cellular polarity by regulating phosphodiesterase 4B (PDE4B) expression in 3DC, suggesting that PDE4B plays a critical role in maintaining cellular polarity in normal epithelial tissues (4–6).

PDE4 family members are hydrolytic enzymes responsible for degrading the second messenger cyclic AMP (cAMP), and the family comprises of four isoforms, PDE4A, PDE4B, PDE4C and PDE4D (7, 8). Disrupted cAMP metabolism caused by altered PDE4 expression and activity is involved in tumorigenesis (9). Indeed, many recent studies have indicated that rolipram, a PDE4 inhibitor, is effective in different types of cancer cells (9–14), suggesting a novel therapeutic strategy for targeting PDE4 isoforms for cancer. However, the clinical use of rolipram is limited because of its side-effects (15), hence, a PDE4 inhibitor with less toxicity is needed for cancer treatment and chemoprevention.

Resveratrol, which is contained in various natural products, has similar PDE4-inhibitory activity to that of rolipram (16). In the present study, a multicellular spheroid model comprising HCT116 cells was used to evaluate the effects of resveratrol on the formation of a luminal cavity and the induction of apoptosis under conditions closely mimicking the *in vivo* microenvironment.

Materials and Methods

Antibodies and reagents. Antibody against cleaved caspase-3 (5A1) was obtained from Cell Signaling Technology (Beverly, MA, USA). Alexa Fluor® 555 phalloidin was purchased from Life Technologies (Carlsbad, CA, USA). 4',6-Diamidino-2-phenylindole (DAPI) and resveratrol (*trans*-3,4',5-trihydroxysilbene) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. HCT116 cells were cultured in the presence or absence of resveratrol for six days in two-dimensional culture (2DC) or 3DC as previously described (4, 5, 17–22).

Correspondence to: Senji Shirasawa, MD, Ph.D., Department of Cell Biology, Faculty of Medicine Fukuoka University, 7-45-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan. Tel: +81 928011011, Fax: +81 928643865, e-mail: sshirasa@fukuoka-u.ac.jp

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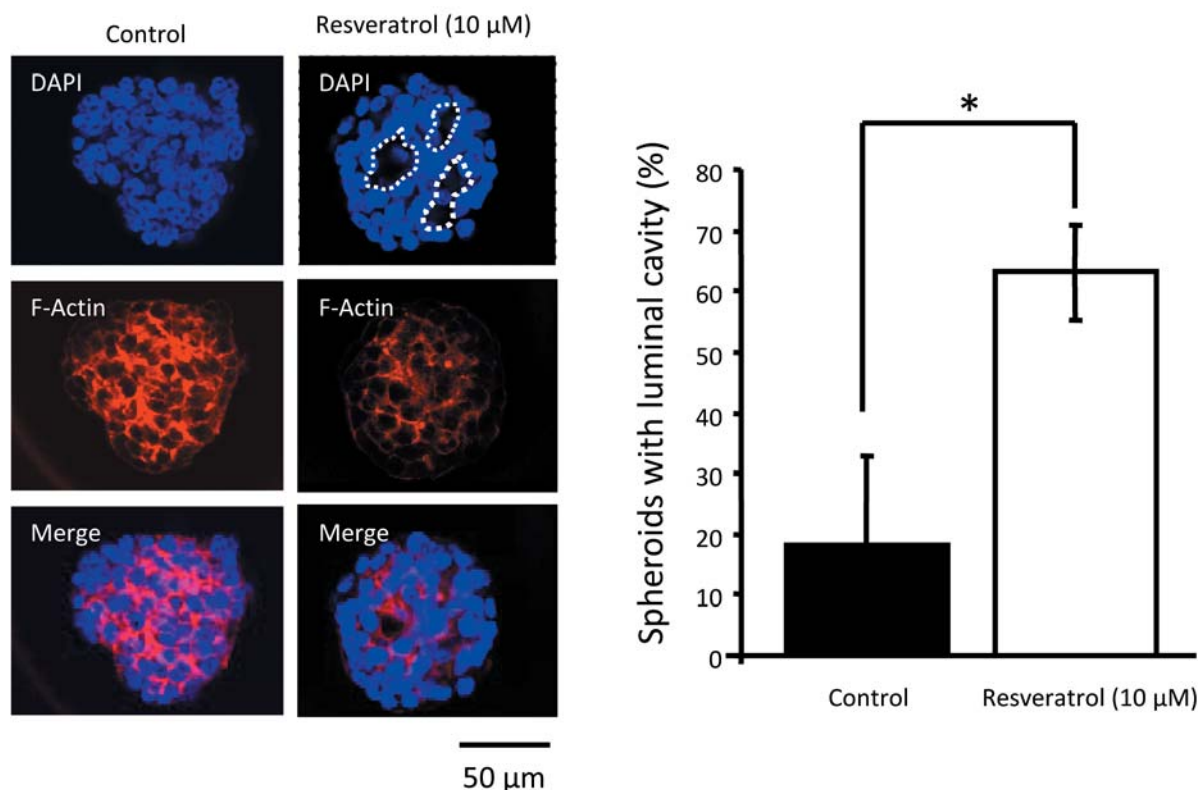


Figure 1. Resveratrol induces the formation of luminal cavities in HCT116 cells grown in three-dimensional culture. The 4',6-Diamidino-2-phenylindole (DAPI) and F-actin signals in HCT116 spheroids treated with ethanol-alone or resveratrol on day 6 are shown in the right panel. Nuclear DNA, blue; F-actin, red. Dots represent luminal cavities. Scale bar=50 μ m. The ratios of spheroids containing a luminal cavity in the left panel. Black bar, HCT116 spheroids treated with ethanol alone; White bar, HCT116 spheroids treated with resveratrol. * p <0.001.

Quantification of luminal cavities in spheroids. Cells were stained with phalloidin and DAPI on day 6, and imaged using CS-SP5 laser scanning confocal microscopy (Leica, Wetzlar, Germany). The ratio of spheroids containing a luminal cavity to the total number of spheroids was calculated as previously described (5, 6).

Quantification of apoptosis in spheroids. Apoptotic cells were detected by immunofluorescence using laser scanning confocal microscopy with antibody to cleaved caspase-3. The ratio of spheroids containing apoptotic cells to the total number of spheroids was calculated as previously described (5, 6).

Statistical analyses. Data are presented as means \pm standard deviations. Statistical analyses were performed using the unpaired two-tailed Student's *t*-test. *p*-Values of <0.05 were considered statistically significant.

Results

Resveratrol induces the formation of luminal cavities in HCT116 cells grown in 3DC. To address the effects of resveratrol on the formation of luminal cavity, HCT116 spheroids were treated with resveratrol or ethanol-alone, and immunostained with phalloidin to detect F-actin. The F-actin

assembly at the apical surface of acini was more clearly observed in spheroids treated with resveratrol compared to those treated with ethanol-alone (Figure 1, right panel). Next, quantitative assays were performed to confirm these results. The ratio of spheroids with luminal cavities increased 3.49-fold after resveratrol treatment (Figure 1, left panel; * p =0.001). These results indicate that resveratrol induced the formation of luminal cavities in HCT116 spheroids probably by inhibiting PDE4 activity, suggesting that PDE4 plays a critical role in cellular polarity.

Resveratrol induces luminal apoptosis of HCT116 cells grown in 3DC. To address the effects of resveratrol on induction of apoptosis, we examined whether resveratrol affects the number of apoptotic HCT116 cells when grown in 2DC or 3DC. The number of apoptotic cells in 2DC was not significantly different between HCT116 cells treated with resveratrol and those treated with ethanol-alone (Figure 2A), indicating that resveratrol did not affect apoptosis of HCT116 cells grown in 2DC. In contrast, apoptotic cells in 3DC were rarely observed in HCT116 spheroids treated without resveratrol, whereas resveratrol treatment increased

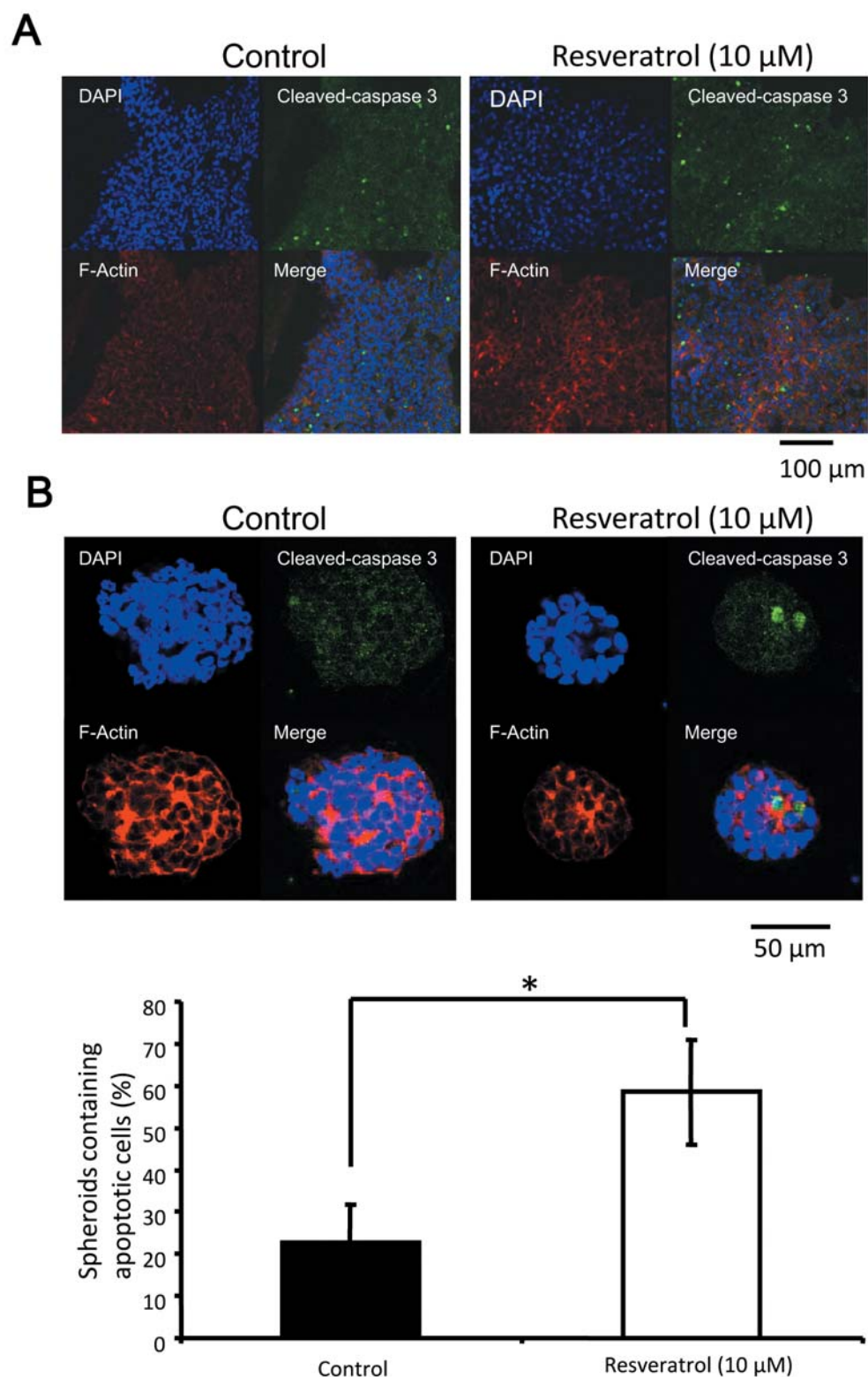


Figure 2. Resveratrol induces luminal apoptosis in HCT116 cells grown in three-dimensional culture (3DC). Cleaved caspase-3 signals in HCT116 cells treated with ethanol alone or resveratrol on day 6 in two-dimensional culture (A) and 3DC (B, upper panel). F-Actin, red; DAPI, blue; cleaved caspase-3, green. Scale bar=100 μ m. The ratio of spheroids containing apoptotic cells (B, lower panel). Black bar, HCT116 spheroids treated with ethanol alone; White bar, HCT116 spheroids treated with resveratrol. * p <0.05.

apoptotic cells specifically within the luminal cavity of spheroids. Next, quantitative assays were performed to confirm these results. The ratio of spheroids containing apoptotic cells increased 2.57-fold after resveratrol treatment (Figure 2B, bottom panel; $*p=0.015$), indicating that resveratrol induced apoptosis in the luminal cavity in HCT116 spheroids probably by inhibiting PDE4 activity.

Discussion

We found that resveratrol induced the formation of cavities and apoptosis in the lumen of HCT116 spheroids. Resveratrol is a phytoalexin produced by plants in response to stress, injury and fungal attack (23). Previous studies have shown that resveratrol has potential as a therapeutic drug for various diseases, including diabetes, obesity, Alzheimer's disease, Parkinson's disease and cancer (24). Indeed, resveratrol can suppress cell proliferation and induce apoptosis in various cancer cell lines (25, 26). Although many intracellular targets of resveratrol that affect cell proliferation and apoptosis have been reported (23), a recent study showed that resveratrol directly inhibits activity of PDE1, PDE3 and PDE4, leading to reduction of cAMP levels (16). Here, the effects of resveratrol on apoptosis and the formation of luminal cavity in 3DC HCT116 cells were highly similar to those of rolipram, which is a selective PDE4 inhibitor, suggesting that the inhibition of PDE4 activity by resveratrol resulted in the formation of cavities and the induction of apoptosis in the lumen of HCT116 spheroids. Interestingly, resveratrol was able to induce apoptosis of HCT116 cells only in 3DC but not in 2DC. Our observations are consistent with those of Fouad *et al.* who reported that 10 μ M resveratrol, the concentration used in our study, was insufficient for inducing apoptosis of HCT116 cells in 2DC (27). The effect of resveratrol on apoptosis was enhanced by the 3D microenvironment, indicating the usability of 3DC to validate the effects of drugs. Because of its low toxicity and its efficacy, resveratrol is a promising agent for chemoprevention and chemotherapy (28, 29). Further elucidation of the precise molecular mechanisms of the effects of resveratrol on apoptosis is required to establish a novel therapeutic strategy targeting PDE4 for cancer.

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