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Resveratrol enhances the expression of death receptor Fas/CD95 and induces differentiation and apoptosis in anaplastic large-cell lymphoma cells

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ABSTRACT

Resveratrol (3,5,4′-trihydroxy-trans-stilbene), a phytoalexin found in grapes and other plants, plays a protective role in human atherosclerosis and carcinogenesis. We examined the effects of resveratrol on the anaplastic large-cell lymphoma (ALCL) cell line SR-786. Resveratrol inhibited growth and induced cellular differentiation, as demonstrated by morphological changes and elevated expression of T cell differentiation markers CD2, CD3, and CD8. Resveratrol also triggered cellular apoptosis, as demonstrated by morphological observations, DNA fragmentation, and cell cycle analyses. Further, the surface expression of the death receptor Fas/CD95 was increased by resveratrol treatment. Our data suggest that resveratrol may have potential therapeutic value for ALCL.

1. Introduction

Resveratrol, a phytoalexin found in grapes, fruits, and root extracts of the weed *Polygonum cuspidatum*, has long been an important constituent of Japanese and Chinese folk medicine [1]. In 1985, resveratrol was described to prevent coronary heart disease by inhibiting eicosanoid synthesis [2]. Further biological activities of resveratrol were uncovered subsequently. Resveratrol was found to block human platelet aggregation, act as an oestrogen receptor agonist, and exert direct and indirect vasodilatory effects, implicating a protective role in human atherosclerosis and coronary heart disease [1,3]. Furthermore, resveratrol has exhibited anti-inflammatory, anti-proliferative, anti-oxidative, anti-aging, and differentiation-promoting activities on various types of cells [1]. Therefore, resveratrol may be potentially useful in cancer chemoprevention and chemotherapy [4–6].

Anaplastic large-cell lymphoma (ALCL; also known as Ki-1 lymphoma) accounts for 2% of lymphomas in adults and 25% in children. Durable tumour remission has previously been achieved in nearly 50% of patients with refractory ALCL by treatment with 13-cis retinoic acid [7,8]. SR-786 – an ALCL cell line – is particularly sensitive to differentiation agents such as retinoids which lead to cellular apoptosis [9]. However, the chronic use of retinoids is associated with significant toxicity [10]. We thus attempted to identify alternative treatments for ALCL using naturally derived compounds with lower toxicity for humans. Since resveratrol presents lower toxicity than do retinoids for humans, the possibility of treating ALCL by

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resveratrol deserves to be seriously evaluated. In this study, therefore, we examined the biologic effects of resveratrol on SR-786 cells, focusing on its effects on the pro-apoptotic death receptor Fas/CD95.

2. Materials and methods

2.1. Cell culture and chemicals

The Ki-1 (+) ALCL cell line SR-786 [11], kindly given by professor Ann-Lii Cheng (National Taiwan University Hospital, Taipei, Taiwan), was cultured in RPMI 1640 medium containing 10% foetal calf serum in a humidified environment supplied with 5% CO_2. In culture, SR-786 cells appear as large-sized cells including multinucleated neoplastic cells resembling Reed-Sternberg giant cells which account for approximately 5% of all cells. Previous immunophenotypic studies of SR-786 have demonstrated the absence of CD2, CD3, and TCR-β expression on the cell surface or in the cytoplasm with concurrent expression of the activation markers CD30, CD25, CD71, HLA-DR, the T-cell marker CD4, and the B-cell markers CD19 and CD20 [11,12]. Resveratrol (Sigma–Aldrich, St. Louis, Missouri, USA) was added to the growth medium at the indicated concentrations.

2.2. Cytotoxicity assay

The viable SR-786 cells were evaluated by a tetrazolium-based colorimetric assay (MTS assay) (Promega, Madison, Wisconsin, USA). Briefly, SR-786 cells were seeded in 96-well plates at 2×10^4 cells/well and exposed to various concentrations of resveratrol for the indicated time. At the end of each set of experiment, 40 μl of the 20:1 (v/v) mixture of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) and phenazine methosulfate solutions was added to each well and incubated at 37 °C for 1.5 h. The absorbance at 492 nm for each well was determined, and the data were presented as mean ± standard deviation (bar).

2.3. Electron microscopy

SR-786 cells treated with resveratrol or 0.1% dimethyl sulfoxide (DMSO) as control were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for 15 min. Fixed cells were precipitated, mounted in 2% agarose gel, and then post-fixed in 1% osmium tetroxide for 30 min. After being washed with 0.1 M cacodylate buffer, samples were dehydrated in alcohol, stained with 0.015% lead citrate, and observed under an electron microscope (Hitachi H-600, Japan).

2.4. DNA fragmentation assay

To observe the DNA cleavage pattern, DNA samples extracted from cells were subjected to RNase A treatment prior to electrophoresis in a 1.8% agarose slab gel pre-impregnated with ethidium bromide. After electrophoresis at 100 V for 5 h, the fluorescence of the DNA was visualized under ultraviolet light.

2.5. Monoclonal antibodies and immunofluorescent flow cytometry

Mouse anti-human CD2-RPE (IgG2b, MoAb), mouse anti-human CD3-RPE (IgG1, MoAb), isotypic mouse IgG2b-RPE, and isotypic mouse IgG1-RPE were purchased from Serotec (Oxford, England). Mouse anti-human CD4-FITC (IgG1, MoAb), mouse anti-human CD8-FITC (IgG2a, MoAb), mouse anti-human CD95-FITC (IgG, MoAb), isotypic mouse IgG1-FITC, and isotypic mouse IgG2a-FITC were purchased from BD (New Jersey, USA).

SR-786 cells treated with resveratrol at different concentrations (1×10^6 cells) were harvested at the indicated time. After washing with phosphate buffered saline (PBS), cells were incubated with fluorochrome-conjugated MoAb at 4 °C for 1 h in the dark. After incubation, cells were washed, re-suspended in 1 ml Fix-PBS (Coulter, Hialeah, USA), and analyzed on a FACScan flow cytometer (BD).

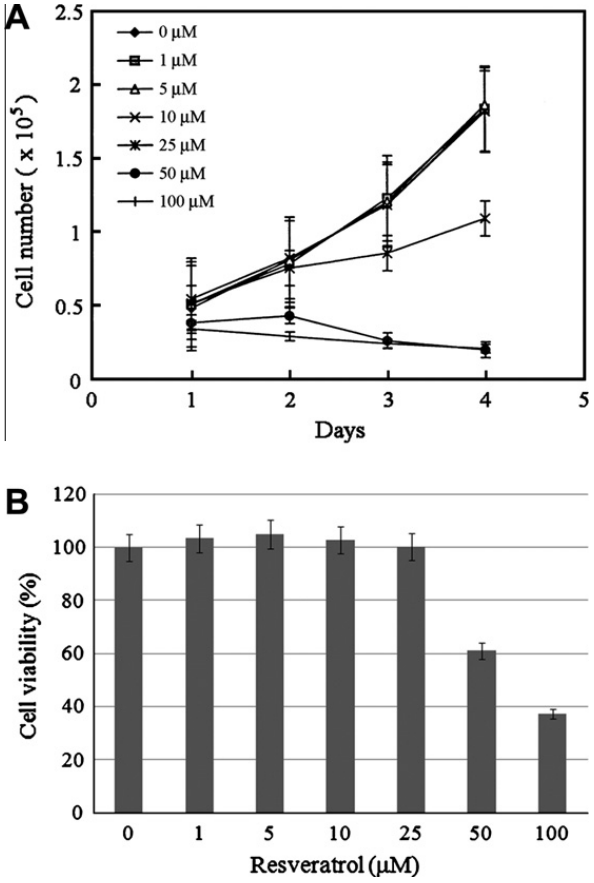

Fig. 1. Effect of resveratrol on the viability of SR-786 cells. (A) SR-786 cells were treated with different concentrations of resveratrol for various time points. Cell viability was evaluated by MTS assays in 96-well plates and represented by the cell number of each well. (B) Dose effect on the viability of SR-786 cells treated with resveratrol for 48 h. The viable cell numbers were estimated by MTS assays and presented as a percentage relative to untreated cells.

2.6. Cell cycle (sub-G1) analysis

For the sub-G1 analysis, control and resveratrol-treated cells were harvested, washed with PBS, and collected by centrifugation. After re-suspension in 0.5 ml of PBS containing 1% Triton X-100 and 50 μg RNase A, cells (1×10^6) were incubated at 37 °C for 30 min. For DNA staining, cells were incubated with 0.5 ml of propidium iodide solution (100 mg/ml) on ice for 15 min in the dark. The DNA content of the cells was assessed by measuring and analyzing the fluorescence intensity with a FACScan flow cytometer (BD) and the CellQuest software (BD).

3. Results

3.1. Resveratrol induced differentiation and death in SR-786 cells

First, we evaluated the biological effects of resveratrol on SR-786 cells. We observed significant growth inhibition in a dose-dependent manner when cells were treated with resveratrol for 48 h (Fig. 1). Cell viability was significantly decreased in response to resveratrol treatment over 50 μM with an IC_{50} of 66 μM.

To further examine the cell morphology before and after resveratrol treatment, SR-786 cells were observed with electron microscopy (Fig. 2). Cells treated with 25-μM resveratrol presented morphological features of differentiating T cells, including condensation of heterochromatin, disappearance of pseudopodia, and reduction of Golgi apparatus and mitochondria (Fig. 2A and B). Cells treated with a higher dose (50 μM) of resveratrol exhibited features of activation, characterized by prevalent occupancy of euchromatin in the nucleus with prominent nucleoli and vacuolization of the Golgi complex (Fig. 2C), followed by typical characteristics of apoptosis with the presence of condensed chromatin and fractured nucleus (Fig. 2D).

3.2. Resveratrol induced apoptosis in SR-786 cells

Agarose gel electrophoresis of genomic DNA from resveratrol-treated SR-786 cells showed a ladder pattern of DNA fragments consisting of multiple bands of approximately 180–200 base pairs demonstrating cellular apoptosis (data not shown). The extent of apoptosis was then evaluated by measuring the sub-G1 regions obtained from flow cytometric analysis (Fig. 3). The results showed that cellular apoptosis was induced dose-dependently by resveratrol treatment with an abrupt increase in the apoptotic cell number at 25 μM. These data, together with the microscopic observations (Fig. 2D), clearly demonstrated that resveratrol induced apoptosis in SR-786 cells.

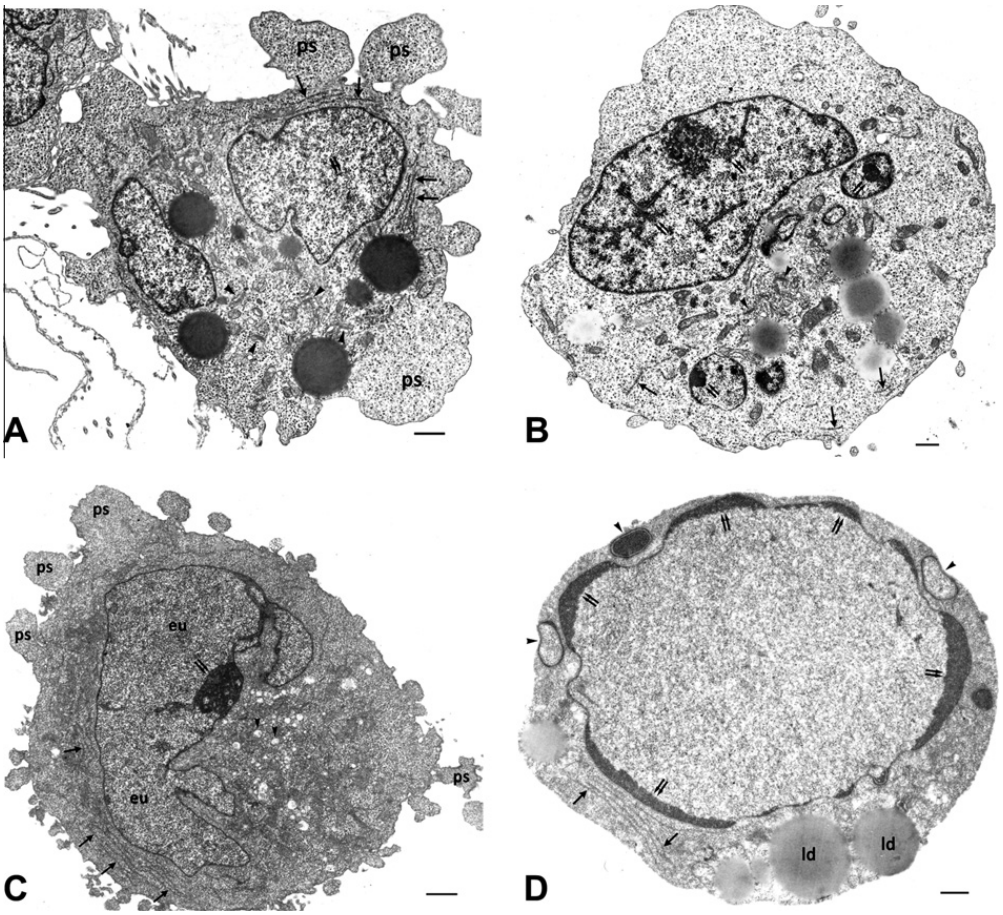

Fig. 2. Morphologic features of resveratrol-treated SR-786 cells observed by electron microscopy. (A) Untreated SR-786 cells were large lymphoid cells showing abundant pseudopodia (ps), rough endoplasmic reticulum (arrows), and prominent nuclei with presence of heterochromatin (double arrows). (B) SR-786 cells treated with 25 μM of resveratrol for 48 h showed morphologic features of cell differentiation revealed by increased heterochromatin condensation (double arrows), disappearance of pseudopodia, and reduced amounts of Golgi apparatus (arrowheads) and rough endoplasmic reticulum (arrows). (C) SR-786 cells treated with 50 μM of resveratrol for 48 h showed features of cellular activation with the presence of small pseudopodia (ps), vacuolization of the Golgi complex (arrowheads), prominent rough endoplasmic reticulum (arrows) and nucleolus (double arrows), and extensive euchromatin (eu) in the nucleus. (D) SR-786 cells treated with 50 μM of resveratrol for 48 h showed apoptotic features illustrated by the presence of compact heterochromatin at the periphery of the nucleus (double arrows), nuclear fragments (arrowheads), and prominent rough endoplasmic reticulum (arrows) and lipid droplets (ld). Scale bars = 1 μM.

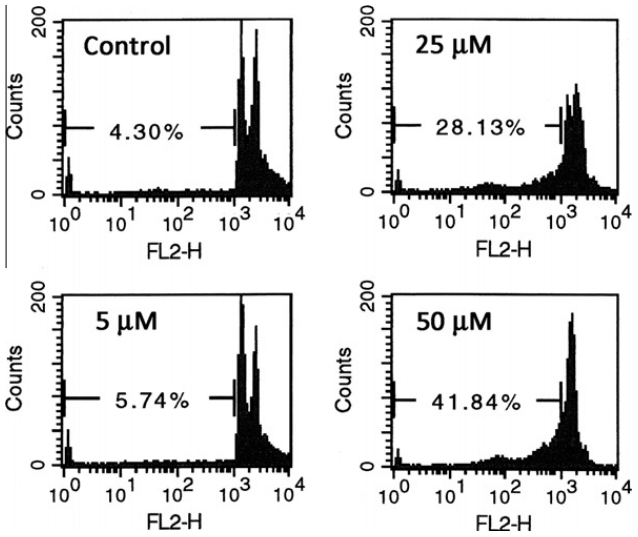

Fig. 3. Cell cycle analysis of SR-786 cells by flow cytometry. SR-786 cells were untreated or treated with 5, 25, and 50 μM of resveratrol as indicated for 48 h and then subjected to sub-G1 analysis. The sub-G1 population indicates the cells with sub-diploid DNA content representing fragmented apoptotic DNA and is indicated by a percentage.

3.3. Resveratrol up-regulated the expression of T cell differentiation markers in SR-786 cells

To further examine the cellular differentiation triggered by resveratrol, the expression levels of the T cell differentiation markers CD2, CD3, CD4, and CD8 were analyzed by immunofluorescent flow cytometry. In SR-786 cells, the expression levels of CD2, CD3, and CD8 were very low, whereas CD4 expression was substantial. The expression of CD2 and CD3 was significantly induced, but CD8 was induced to a lesser extent after 48 h resveratrol treatment at concentrations higher than 25 μM (Fig. 4). Resveratrol was also shown to effectively increase CD4 expression in SR-786 cells, while the effect was most pronounced with 25 μM of resveratrol treatment (Fig. 4). These results clearly revealed that resveratrol is capable of enhancing T cell differentiation.

3.4. Resveratrol enhanced the death receptor Fas/CD95 expression in SR-786 cells

To explore the possible mechanisms of resveratrol-induced apoptosis, the expression of the death receptor Fas/CD95 in SR-786 cells was examined. Our results demonstrated that Fas/CD95 expression was enhanced about twice folds when cells were treated with 25 μM resveratrol for 48 h (Fig. 5). These results suggested that the cellular apoptosis is likely triggered by resveratrol through a Fas/CD95-dependent mechanism.

4. Discussion

In this study, we demonstrated that resveratrol induced differentiation and apoptosis of SR-786 cells. The IC_{50} of 48-h resveratrol treatment was 66 μM, similar to reports from other groups using different cells [4,13,14]. The growth inhibition activity of resveratrol appears to be cancer cell-specific since it has been demonstrated that resveratrol, up to concentrations of 32 μM, did not significantly affect the survival of normal human peripheral blood lymphocytes [5]. Resveratrol has been shown to inhibit cancer cell growth by enhancing S and G1 phase arrest, down-regulating cyclin D1/D2 expression as well as cyclin-Cdk complexes formation, decreasing ERK and Rb phosphorylation, and reducing telomerase activity [15–18]. From our data, we showed that resveratrol inhibited SR-786 cell growth by inducing apoptosis, and verified this by DNA fragmentation, electron microscopy, and sub-G1 analyses.

Although our data provided evidences on one ALCL cell line SR-786, we showed not only cell marker expression studies but also detailed electron microscopic observations on this cell line. It is also documented that apoptosis can be induced by resveratrol in a number of other cell lines from different haematologic malignancies of both B-cell and T-cell origins, including Burkitt's lymphoma, mantle cell lymphoma, follicular lymphoma, diffuse large B-cell lymphoma, and T-cell acute lymphoblastic leukaemia [19–24]; therefore, it is expected and reliable that ALCL cells also respond to resveratrol in our study. Furthermore, ALCL is phenotypically heterogeneous, so are the cell lines. The SR-786 cell line which we used in our study represents specifically for the T-cell type, CD4 and CD30-positive, and ALK-positive large cell lymphoma, which also express some B-cell markers such as CD19 and CD20 [11,12]. Our data provide useful information for therapeutic strategy of ALCL diagnosed as this specific phenotype.

Studies in rats have revealed that resveratrol was absorbed effectively after oral administration, moved quickly into the bloodstream, and presented in substantial concentrations in several important organs, especially in the liver and kidney [25–27]. Bioavailability studies in humans have shown that, after oral administration, resveratrol was rapidly absorbed to reach peak plasma concentration within 30 min and was then quickly metabolized [28]. Toxicological studies in rats have reported that high dose of resveratrol (3 g/kg/day for 4 weeks) caused adverse events—mainly concerning nephrotoxicity [29]. Data from human studies have shown that resveratrol applications of up to a single dose of 5 g per person and followed by 13 doses of 150 mg per person every 4 h do not cause serious adverse events, although some mild short-term adverse effects including frontal headache and a small increase of blood bilirubin or alanine aminotransferase level have been described [30–32]. It was reported that approximately 3.3 μM of resveratrol in the plasma (2.7 mg in 3.6 l of total body plasma) can be detected in 1.5 h after oral ingestion of pure resveratrol (dissolved in 5 ml of whisky and mixed with 50 ml of water) in humans [26]. To reach the effective concentration of 50 μM as reported in our experiments, the amount of 15.2 mg/kg of resveratrol, which is far below the condition to cause the adverse events described above, should be taken. Therefore, resveratrol is an ideal candidate for chemoprevention in humans with minimal harmful effects.

Normal human thymocytes undergo intrathymic T cell differentiation during which they express CD2 and CD3 at the earliest stage, TCR-β at the intermediate stage, and TCR-α at the last stage [33,34]. In our study, resveratrol was noted to promote both cell differentiation and apoptosis on SR-786 cells. Our results showed that in the groups treated with vehicle alone, 5 μM, 25 μM, and 50 μM of resveratrol, apoptotic cells numbered 4.3%, 5.74%, 28.13%, and 41.84%, respectively. However, $CD2^+$ cells were 0.46%, 0.52%, 13.12%, and 42.64%, while $CD3^+$ cells numbered 0.66%, 1.71%, 12.84%, and 36.15% in the respective groups. From these data, we cannot conclude whether these cells

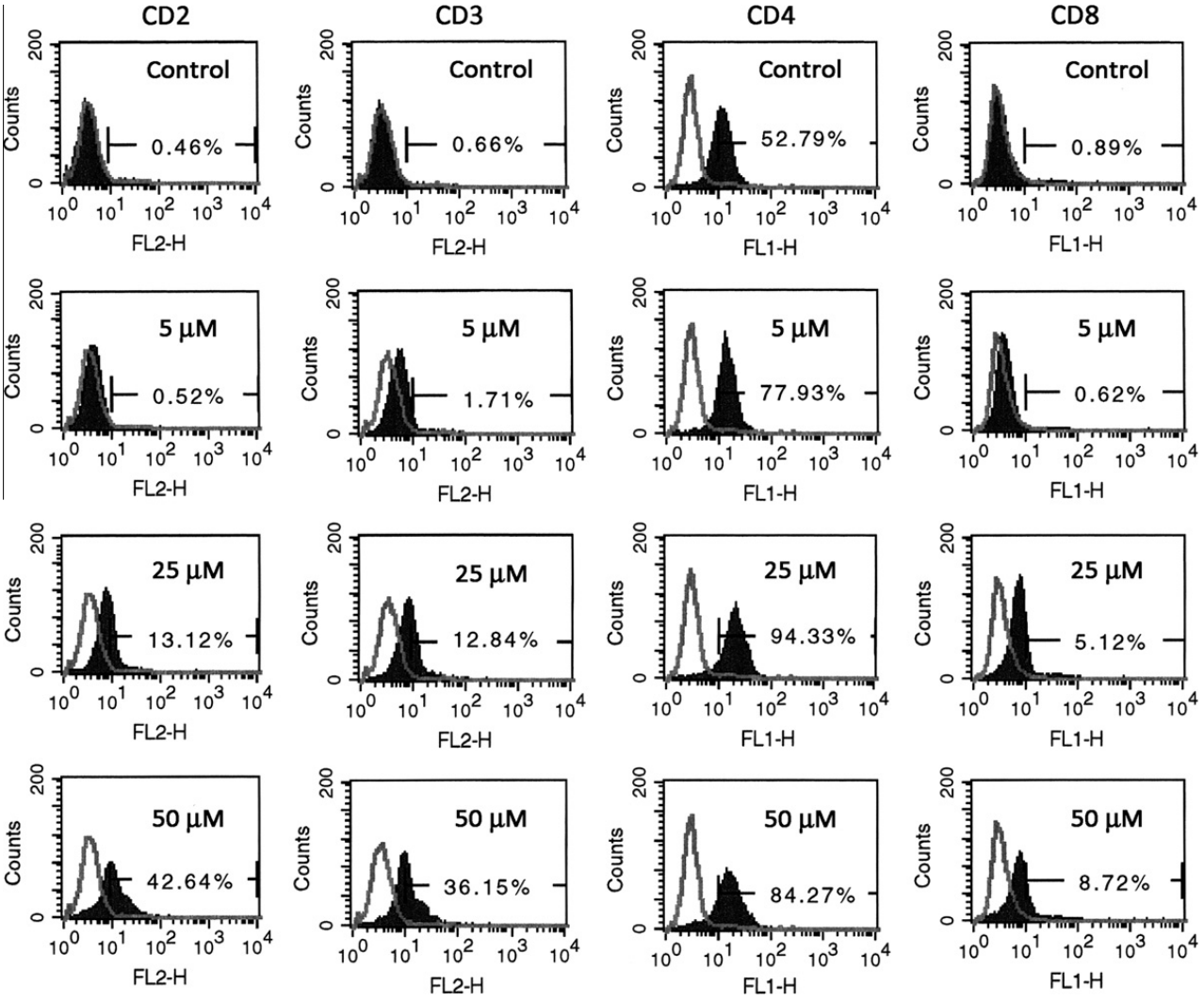

Fig. 4. Immunofluorescent flow cytometric analysis of the expression of T cell differentiation markers CD2, CD3, CD4, and CD8 in SR-786 cells. Cells were treated with different concentrations of resveratrol for 48 h and then analyzed by immunofluorescent flow cytometry using specific monoclonal antibodies. The levels of cell surface expression of the indicated markers were determined and are presented as filled histograms. Open histograms represent the binding of the isotypic control IgG. Similar results were obtained in two additional independent experiments.

underwent differentiation and apoptosis in sequential events. However, we hypothesize that some SR-786 cells were highly sensitive to resveratrol and subjected to apoptosis, while others differentiated and presented more mature phenotypes first and turned apoptotic later in the process.

Our flow cytometric study revealed that resveratrol induced the expression of CD2, CD3, and CD8 in the $CD4^+$ SR-786 cells. The substantial induction of CD2 and CD3, 42.64% and 36.15%, respectively, may represent the differentiation of T cells. The relatively lesser increase of $CD8^+$ cells (8.72%) suggested that, with resveratrol treatment, a subset of cells with CD4/CD8 double positive phenotype (high CD4 and low CD8) may emerge from the original $CD4^+CD8^-$ T cells. The double positive cells were described in Hodgkin lymphoma and a number of neoplastic and non-neoplastic conditions including normal peripheral blood lymphocytes [35–38]. They were suggested to perform as activated T cells linked with immunoregulatory activity, enhanced cytotoxicity, and repressed proliferative ability [36]. The potential of resveratrol to induce the formation of CD4/CD8 double positive cells is possibly beneficial for the disease progression of ALCL and needs to be further evaluated.

Resveratrol has been reported to induce apoptosis in various human cancer cell lines [39], chemically induced tumours on mouse skin [40], and tumour cell xenografts in nude mice [41–43] through the activation of intrinsic and/or extrinsic pathways [39]. It has been demonstrated that resveratrol triggered an extrinsic pathway via Fas/CD95-dependent [5,44,45] or Fas/CD95-independent [14,46–49] or both [50] mechanisms depending on the cancer cell types. Our results indicated that resveratrol enhanced Fas/CD95 expression in a dose-dependent manner, suggesting that the CD95-CD95L signalling pathway may contribute to resveratrol-induced apoptosis of SR-786 cells. In the reports which demonstrated that resveratrol induced a Fas/CD95-dependent apoptotic mechanism [5,44], it was noted that the expression of CD95L, but not of CD95, was enhanced by resveratrol in HL-60 leukaemia cells and

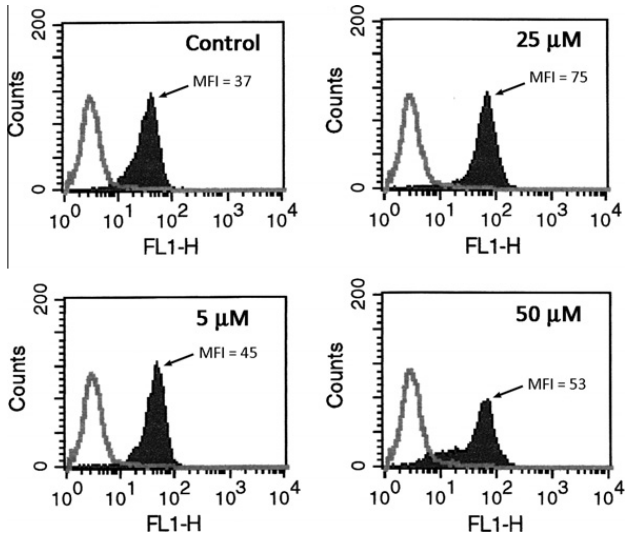

Fig. 5. Effect of resveratrol on the surface expression of Fas/CD95 in SR-786 cells as analysed by fluorescent flow cytometry. Cells were treated with DMSO only or with 5, 25, and 50 μM of resveratrol for 48 h and then subjected to fluorescent flow cytometry using monoclonal antibodies against human Fas/CD95. The levels of Fas/CD95 expression in cells treated with resveratrol of the indicated concentrations were shown as filled histograms and the mean fluorescence intensities (MFI) were determined. Open histograms represent the isotypic control IgG. Similar results were obtained in two other independent experiments.

T47D breast carcinoma cells. However, resveratrol-induced apoptosis attributed to Fas/CD95 up-regulation has been reported for SNU-1 human gastric adenocarcinoma and HCT116 human colon carcinoma cell lines [45,50]. Therefore, it is considered that the CD95/CD95L-dependent apoptosis pathway is differentially regulated by resveratrol depending on the cell types used. It has also been described that the clustering of the cell surface Fas/CD95 to membrane rafts is responsible for resveratrol-induced apoptosis of colon cancer cells, while the expression of CD95 and CD95L is not affected [51]. To clarify whether this mechanism occurs in ALCL cells, further investigations are needed.

Differential responses can be triggered by the activation of Fas/CD95 receptor [51,52]. Upon activation, Fas/CD95 aggregates with its adaptor FADD in the membrane rafts, leading to the recruitment of procaspase-8. The receptor complexes are then internalized to the endosomal compartment followed by the formation of the death-inducing signalling complex (DISC). In the so-called type I cells, abundant DISC formation results in large amount of activated caspase-8 to subsequently activate caspase-3 and directly initiate apoptosis. In the so-called type II cells, however, the membrane rafts clustering and internalization of Fas/CD95, the formation of DISC, and the activation of caspase-8 are very limited. Type II cells depend on the release of mitochondrial signals to activate and amplify the apoptotic activity. One previous study has demonstrated that the clustering of Fas/CD95 to the membrane rafts is responsible for resveratrol-induced apoptosis in colon cancer cells, while the expression of CD95 and CD95L is not affected [53]. Therefore, it is necessary to further identify whether the type I or the type II response is triggered by resveratrol in ALCL cells. Elucidation of the underlying molecular mechanism would be very helpful for the clinical application of resveratrol in ALCL.

SR-786 cells harbour t(2;5)(p23;q35) translocation to express a fusion protein of the nucleolar protein nucleophosmin (NPM) and the anaplastic lymphoma kinase (ALK), resulting in the constitutive expression and activation of ALK kinase activity [54]. Recent therapeutic strategies for this type of ALCL (the ALK(+) ALCL) include the application of ALK small-molecule inhibitors and inhibitory antibodies, ALK-mRNA silencing by ribozyme, inhibition of ALK-interacting proteins, as well as tumour vaccination [55,56]. Our study is the first to demonstrate that ALCL cells respond to resveratrol and thereby presents the potential of resveratrol in inducing cellular differentiation and apoptosis. Further in-depth studies are required to verify whether ALCL represents a tumour type specifically sensitive to resveratrol-induced Fas/CD95-mediated apoptosis and whether resveratrol can act synergistically with therapeutic applications targeting ALK. Therefore, the next step is to examine the effects of resveratrol combined with conventional chemotherapy or new target therapy agents not only on the cell lines but also on the NPM-ALK(+) transgenic mice and the ALCL patients. Recent studies have revealed that ALK inhibitor TAE684 and PF-02341066 are effective in inhibiting growth of ALK(+) ALCL cells by inducing cell cycle arrest and apoptosis [57–59]. Promising responses are obtained in the clinical trial of PF-02341066 on treating non-small-cell lung cancer patients with EML4-ALK rearrangement, which also results in ALK autophosphorylation [60]. The clinical trial of PF-02341066 on ALCL treatment is now underway. Since our study raises the possibility for resveratrol as a promising chemopreventive and chemotherapeutic agent for ALK(+) ALCL, it is of interested to examine how resveratrol treatment would benefit the target therapy for ALK(+) ALCL patients by playing a pro-apoptotic or an antioxidative role.

Conflict of interest

The authors indicate no potential conflict of interests.

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