Caspase-8 has an essential role in resveratrol-induced apoptosis of rheumatoid fibroblast-like synoviocytes

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Objective. Resveratrol is a naturally occurring polyphenol, which possesses chemotherapeutic potential through its ability to trigger apoptosis. The objective of this study was to investigate the major determinant for the apoptotic cell death induction by resveratrol in fibroblast-like synoviocytes (FLS) derived from patients with RA.

Methods. The effect of resveratrol on apoptotic cell death was quantified in a population of subG1 in RA FLS by flow cytometry. The underlying signalling mechanism for apoptotic death was examined by analysing mitochondrial membrane potential, activation of the caspase cascade and translocation of Bid.

Results. We show that activation of caspase-8 is essential for triggering resveratrol-induced apoptotic signalling via the involvement of the mitochondrial pathway in RA FLS. Our findings also suggest that this enhanced apoptosis caused by resveratrol occurred in RA FLS irrespective of p53 status. Exposure to resveratrol caused extensive apoptotic cell death, along with a caspase-dependent (activation of caspase-9 and -3, poly ADPribose polymerase (PARP) cleavage and mitochondrial cytochrome c release) or caspase-independent [translocation of apoptosis-inducing factor (AIF) to the nucleus] signalling pathway. Analysis of upstream signalling events affected by resveratrol revealed that the activated caspase-8 triggered mitochondrial apoptotic events by inducing Bid cleavage without any alteration in the levels of Bax, Bcl-xL or Bcl2. The caspase-8 inhibitor or over-expression of crmA abrogated cell death induced by resveratrol and prevented processing of the downstream cascade.

Conclusion. The results suggest that resveratrol causes activation of caspase-8, which in turn results in modulation of mitochondrial apoptotic machinery to promote apoptosis of RA FLS.

KEY WORDS: Resveratrol, Fibroblast-like synoviocytes, Apoptosis, Caspase-8, Mitochondria membrane potential.

Introduction

RA is a chronic inflammatory disease characterized by inflammation of the synovial lining and destruction of the adjacent bone and cartilage [1]. The inflamed synovium consists of diverse cell populations, including B cells, T cells, macrophages and synovial fibroblasts, leading to abnormal immune phenomena in the joint, chronic inflammation and synovial hyperplasia [2, 3]. Although all populations of cells contribute in its own way to progression and perturbation of the disease, hyperplastic growth of fibroblast-like synoviocytes (FLS) is considered as a pathological hallmark of RA [4, 5]. However, the exact mechanism for the hyperplastic growth of RA FLS is yet to be determined. In fact, alterations in apoptosis in RA FLS have been suggested as a potential cause for the hyperplastic growth of RA FLS. While initial studies indicated increased levels of apoptosis in the rheumatoid synovium, recent investigations have demonstrated *in vivo* evidence of the resistance of RA FLS to apoptosis [6]. Indeed, the cells including macrophages and RA FLS, as found in the rheumatoid joint appear resistant to apoptosis [7–10], even though Fas and Fas ligand are strongly expressed [11–13]. Such resistance to apoptosis could be related to the expression of anti-apoptotic molecules including Fas-associated death domain-like interleukin-1β-converting enzyme-inhibitory protein (FLIP) and sentrin-1/

small ubiquitin-like modifier (SUMO-1) in RA FLS [10, 14] that can prevent apoptosis *in vivo*. Therefore, better understanding of the apoptotic signalling mechanisms in RA FLS will provide insights in developing more effective therapeutic approaches for inhibiting the destruction of cartilage and bone in RA.

Resveratrol (*trans*-3,4′,5-trihydroxystilbene) is a polyphenol found in various fruits and vegetables and is abundant in grape skins and red wines [15]. Resveratrol has a cardioprotective effect as an antioxidant, modulator of lipid metabolism and in platelet aggregation [15–19]. Moreover, it has been reported to elicit cellular responses, including cell cycle arrest and differentiation, and its anti-cancer properties are well documented. The anti-carcinogenic properties of resveratrol are closely associated with its antioxidant activity and ability to inhibit cyclo-oxygenase, hydroperoxidase, protein kinase C, Bcl-2 phosphorylation, Akt, NF-κB and matrix metalloprotease-9, and programmed cell death [20–23]. According to several studies, resveratrol may activate apoptotic signalling cascades through several mechanisms. It triggers apoptotic signalling via the involvement of mitochondria that mediate the essential caspase processing by the release of cytochrome c and other inter-membrane proteins into the cytosol, p53-dependent or p53-independent apoptosis, depending on the specific cell type and cellular environment [24–26].

In addition to these chemopreventive effects, although resveratrol possesses specific cytotoxic effects towards tumour cells with relative non-toxicity to normal cells [20, 27], little is known about its cytotoxic efficacy or underlying mechanisms against FLS derived from patients with RA. Here, we report that resveratrol can induce extensive apoptotic death in RA FLS by converging on the mitochondrial signalling pathway. Moreover, our results indicate that caspase-8 has an essential function upstream of mitochondria to trigger caspase-dependent (activation of caspase-9 and -3, poly ADPribose polymerase (PARP) cleavage and mitochondrial cytochrome c release) or caspase-independent [translocation of apoptosis-inducing factor (AIF) to the nucleus] apoptosis by inducing Bid cleavage in RA FLS. Thus, our findings

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reveal a possible intracellular signalling mechanism that causes apoptotic cell death in response to resveratrol.

Materials and methods

Reagents

Resveratrol, methotrexate (MTX), propidium iodide (PI), pifithrin-α (PFT-α), carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (Fccp) and rhodamine123 (Rho 123) were purchased from Sigma-Aldrich (St Louis, MO, USA). The active metabolite of leflunomide (A77 1726), the caspase-8 inhibitor z-IETD-FMK, the caspase-3 inhibitor z-DEVD-FMK and peroxidase-conjugated secondary antibodies were purchased from Calbiochem (San Diego, CA, USA). Anti-AIF, anti-Bcl2, anti-GFP, anti-COX4 and anti-tubulin antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-β-actin antibody was purchased from Sigma-Aldrich. Anti-caspase-8, anti-p21, anti-p53, anti- poly (ADP-ribose) polymerase (PARP) and anti-cytochrome-c antibodies were purchased from BD Biosciences PharMingen (San Diego, CA, USA). Anti-caspase-3, anti-caspase-9, anti-Bid, anti-Bax and anti-Bcl-xL antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA, USA). Anti-p84N5 antibody (clone 5E10) was purchased from GeneTex (San Antonio, TX, USA). MitoTracker Red was purchased from Molecular Probes (Eugene, OR, USA). Institutional review board approval of Chungnam National University Hospital and informed patient consent were obtained for this study before the study was commenced.

Isolation of primary human RA FLS and cell culture

RA FLS were isolated by the enzymatic digestion of synovial tissues obtained from RA patients undergoing total joint replacement surgery or knee synovectomy. After discarding fat and fibrous tissue, the synovium was minced into small pieces and treated for 2 h with 2 mg/ml of type II collagenase in Hank's balanced salt solution (HBSS) at 37°C in 5% CO_2. The tissue was then filtered using fine sterile gauze, washed and resuspended in DMEM supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Dissociated cells were then centrifuged at 800g, following 1000g and then plated in 10 cm dishes. After overnight culture, the non-adherent cells were removed and the adherent cells were cultivated in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were kept at 37°C in 5% CO_2 and the medium was replaced every day. When the cells approached confluence, they were passaged into fresh culture dishes after trypsin/EDTA treatment. RA FLS from passages four to nine were used in each experiment. The cells were morphologically homogeneous and exhibited the appearance of FLS, with typical bipolar configuration under inverse microscopy. Wild-type (wt) and p53 null mouse embryonic fibroblast (MEF) cells were cultured in DMEM supplemented with 10% (v/v) FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Determination of cell death

RA FLS and MEF cells were plated in six-well culture dishes at concentrations determined to yield 60–70% confluence within 24 h. Cells were then treated with resveratrol (50–200 μM) each time. And then cells were harvested by trypsinizing, washed with phosphate buffered saline (PBS), and fixed in 70% ethanol for 15 min on ice. Fixed cells were washed again with PBS and incubated in 50 μg/ml PI, 0.1 mg/ml RNaseA and 0.05% Triton X-100 for 40 min at 37°C. Cells were resuspended with PBS and then DNA content was then analysed using a flow cytometer (FACStation; Becton Dickinson, San Jose, CA, USA) equipped with computer software (CellQuestPro; Becton Dickinson).

Western blot analysis

After treatment with reagent as described in the figure legends, cells were collected and lysed in M2 buffer [20 mM Tris at pH 7.6, 0.5% NP-40, 250 mM NaCl, 3 mM EDTA (ethylenediaminetetraacetic acid), 3 mM EGTA (ethylene glycol tetraacetic acid), 2 mM DTT (dithiothreitol), 0.5 mM PMSF (phenylmethanesulphonylfluoride), 20 mM β-glycerol phosphate, 1 mM sodium vanadate, 1 μg/ml leupeptin]. Fifty micrograms of the cell lysates were subjected to SDS polyacrylamide gel and blotted onto polyvinylidene difluoride (PVDF) membrane. After blocking with 5% skim milk in PBS Tween-20, the membrane was probed with the relevant antibody and visualized by enhanced chemiluminescence, according to the manufacturer's instruction (Amersham; Piscataway, NJ, USA).

Cellular fractionation

RA FLS were seeded in a 10 cm dish at a density of 2×10^5 cells/ml and allowed to proliferate for 12 h before treatment with reagent as described. The cells were harvested using trypsin–EDTA, washed twice with PBS and then were suspended in 1 ml of isotonic buffer containing 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfuric acid (HEPES)–KOH, pH 7.4, 250 mM sucrose, 1 mM EDTA, 1 mM EGTA, 1 mM DTT and 1 mM PMSF on ice for 30 min. The cells were homogenized on ice using a Dounce homogenizer with a tight pestle, and then subjected to centrifugation at 3500g for 5 min to obtain post-nuclear supernatant. The cytoplasmic fraction was obtained by centrifugation of the post-mitochondrial supernatant at 19 500g for 60 min. About 20 μg for cytosol proteins were subjected to SDS–PAGE and western blotting as described above.

Evaluation of mitochondrial transmembrane potential

RA FLS were seeded in six-well plates and treated with the indicated concentrations of reagents as described in the figure legends. The cells were trypsinized and washed in PBS and then incubated with 10 μM Rho 123 in PBS at 37°C for 15 min. The incubated cells were washed twice with PBS, and suspended with 0.1% BSA. The mitochondrial transmembrane potential ($\Delta\psi$m) was estimated by counting the Rho 123 stained-cells with a FACScan flow cytometer (Becton Dickinson & Co., NJ, USA).

Caspase activity assay

The caspase colorimetric assay kits specific for caspase-3 or caspase-8 (Biovision, CA, USA) were used to detect caspase activation by measuring the cleavage of a synthetic colorimetric substrate. In brief, RA FLS were cultured in 10 cm dishes and treated with 100 μM resveratrol for 36 h or pre-treated caspase inhibitors for 30 min. Cell lysates were prepared in the lysis buffer from the assay kits, the lysates were centrifuged at 10 000g for 1 min and the supernatants were collected. With BSA as the standard, equal amounts of proteins from each sample were reacted with the synthetic colorimetric substrates at 37°C for 2 h and read at 405 nm on a microplate reader. Fold-increase in caspase-3 or -8 activities was determined by comparing the data of treatment samples with those of control samples.

Immunofluorescence analysis of AIF

For immunofluorescence analysis, RA FLS were grown on coverslips and treated with 100 μM resveratrol or pre-treated with caspase inhibitors for the indicated periods. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100 in PBS. Cells were then blocked with 5% goat serum for 1 h and washed in PBS. And then cells were incubated with rabbit anti-AIF, diluted 1:500 in 2% BSA/PBS for overnight at 4°C chamber. Excess antibody was removed by washing the coverslips three times with PBS. Cells, which were protected from light, were

then incubated with a goat anti-mouse IgG conjugated to phycoerythrin (PE) (BD PharMingen), diluted 1:100 in 2% BSA/PBS for 1 h at room temperature. After washing three times with PBS, cells were measured by a Fluoview500 confocal laser scanning microscope (CLSM) (Olympus, Japan).

Construction of recombinant plasmids and transfection

The cDNA encoding human amino acids of the Bid was obtained from cDNA of RA FLS. The following primer sets were designed for the amplification of the human Bid (Gene ID: NM_001196), sense: CGA ATT CTG ATG GAC TGT GAG GTC, antisense: CGG TGG ATC CCG GTC CAT CCC ATT TCT. The PCR product of human Bid was cloned to pEGFP-N1 vector using *Bam*H1 and *Eco*R1 to tag green fluorescence protein (GFP) at C-terminal and confirmed by sequence analysis. The Bid-GFP gene was nucleofected using pre-grogrammed settings on the AMAXA nucleofector® into the RA FLS. Each condition was tested at least twice. The expression of target gene was observed under fluorescence microscope and was measured by a Fluoview500 CLSM (Olympus).

Statistical analysis

Data are expressed as the mean ± S.E. from at least three separate experiments performed in triplicate. The differences between groups were analysed using a Student's *t*-test, and $P < 0.05$ is considered statistically significant. Statistical analyses were carried out using SPSS software (ver. 11.0; SPSS Inc., Chicago, IL, USA).

Results

Resveratrol-induced apoptotic cell death in RA FLS involves processing of caspase-9 and -3, cleavage of PARP and release of cytochrome c and AIF by disrupting the mitochondrial membrane potential

To investigate the cytotoxicity and mode of cell death of RA FLS following treatment with resveratrol, cell death was assessed by DNA fragmentation at the apoptotic sub-G1 peak, as indicated by PI staining, and then analysed by flow cytometry. As shown in Fig. 1A, resveratrol did not induce significant changes in apoptotic DNA fragmentation at 50 μM, but at 100 and 200 μM, it induced massive apoptotic DNA fragmentation in a time-dependent manner. Notably, under these conditions, methotrexate (a folate analogue) or A77 1726 (an active metabolite of leflunomide), standard treatments for patients suffering from active RA, did not enhance cell death in RA FLS. To clarify the death signalling pathway underlying the resveratrol-induced apoptosis in RA FLS, we investigated the activation of the caspase cascade, including caspase-9 and -3, and resultant cleavage of PARP. Caspase-9 and -3 were processed after resveratrol treatment, as shown by the degradation of inactive pro-enzymes in a dose- and time-dependent manner in western blot experiments (Fig. 1B and C).

Based on the ability of resveratrol to induce apoptotic cell death through caspase-3 activation, we evaluated the possibility that resveratrol would induce the loss of $\Delta\psi$m. Following cellular staining with Rho 123, we used flow cytometry to analyse the effects of resveratrol on cell membrane integrity (as indicated by $\Delta\psi$m). Apoptosis induced by treatment with resveratrol caused a rapid decline in $\Delta\psi$m to a similar extent as found in cells treated with a mitochondrial uncoupler, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (Fccp) (Fig. 2A). Next, we examined the release of apoptogenic factors, including cytochrome c and AIF from the mitochondria upon resveratrol treatment by using cell fractionation experiments. As expected, the release of cytochrome c and AIF from mitochondria was observed after treatment with resveratrol in a time-dependent manner
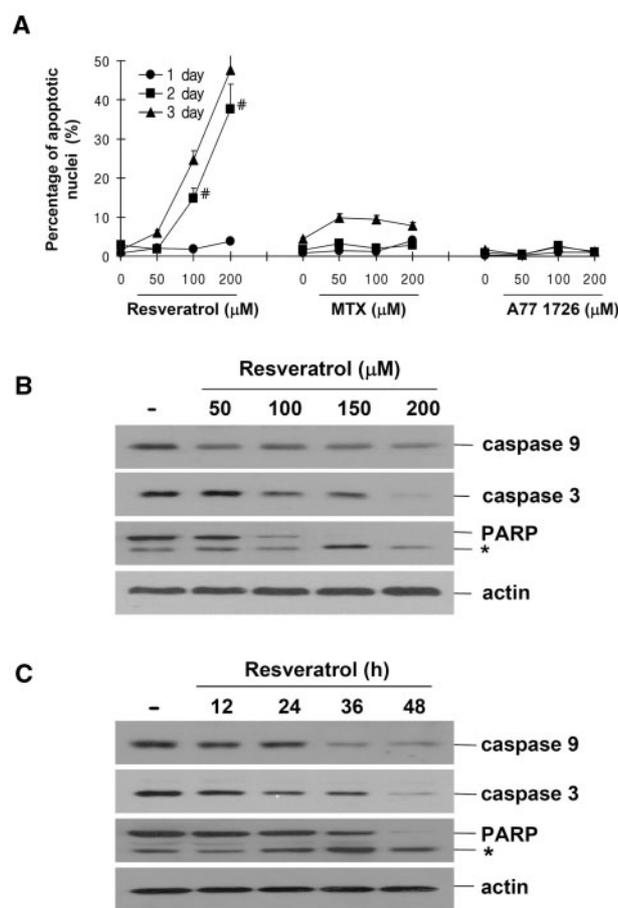

FIG. 1. Resveratrol induces apoptotic cell death involving processing of caspase-9, -3 and cleavage of PARP in RA FLS. (**A**) FLS from RA patients were treated with indicated concentrations of resveratrol, methotrexate and A77 1726 for various times as indicated. The cells were then stained with PI and analysed by flow cytometry as described in the Materials and methods section. The percentage of the sub-G1 population for each sample was plotted as a graph. Each data shows mean ± S.E. of at least three independent experiments. #P < 0.05, compared with non-treated group. RA FLS were treated with various concentrations of resveratrol for 36 h (**B**) or 100 μM of resveratrol for various times (**C**) as indicated. The whole cell extracts were analysed by SDS–PAGE and western blotting with antibodies against caspase-9, -3 and PARP. As a protein loading control, the same amounts of extracts were applied to SDS–PAGE for western blotting with anti-actin antibody. *Cleaved product of PARP upon resveratrol treatment.

(Fig. 2B), indicating that the resveratrol-induced apoptosis of RA FLS is associated with disruption of the mitochondrial membrane, and subsequent cytochrome c-dependent and -independent activation of signalling cascade.

Resveratrol-induced apoptosis is not linked to p53 signalling pathway

Because the activated p53 transactivates genes, many products of which are involved in the induction of apoptosis, we next examined the possibility that resveratrol might be acting by activating this signalling pathway in mitochondria-mediated apoptosis in RA FLS. To determine whether resveratrol affects the transcriptional activity of p53, we first analysed the expression of p53 target gene p21 in RA FLS exposed to 100 μM resveratrol. Immunoblotting of total cell extracts revealed that resveratrol induced p21 expression. However, unexpectedly, pre-treatment with PFT-α, an inhibitor of p53 transcriptional activity, had no effect on apoptotic cell death induced by resveratrol (Fig. 3A), even though PFT-α efficiently blocked p21 expression (Fig. 3B), indicating that p53 activity may not contribute in resveratrol-induced apoptosis.
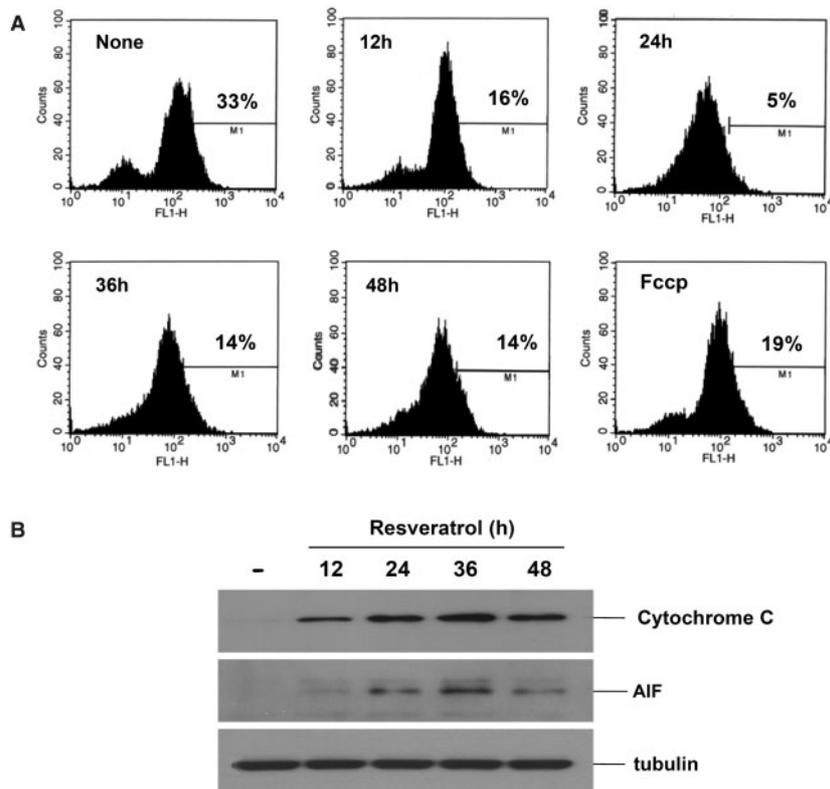
FIG. 2. Resveratrol enhances loss in $\Delta\psi$m and subsequent release of cytochrome c and AIF from mitochondria. (**A**) RA FLS were treated with resveratrol (100 μM) for the indicated times or Fccp (10 μM) for 36 h. After treatment, cells were stained with 10 μM Rho 123 and subjected to the flow cytometric analysis for $\Delta\psi$m. Each data point represent relative $\Delta\psi$m compared with the non-treated control level from three similar experiments. (**B**) RA FLS were treated as described in (A) and cells were disrupted, and separated cytosolic fractions excluded from mitochondrial fractions were obtained as described in the Materials and methods section. Lysates of cytosolic fractions were analysed by SDS–PAGE followed by western blotting with antibodies against cytochrome c, AIF and tubulin.
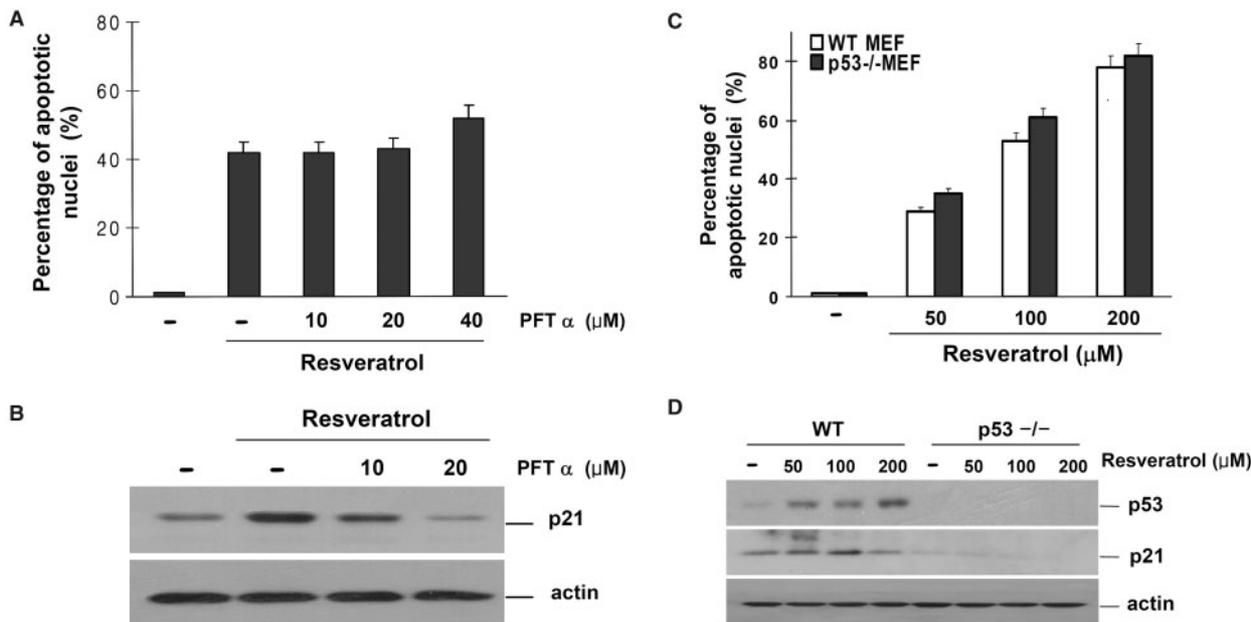

FIG. 3. Resveratrol-induced apoptotic death is not linked to p53 signalling pathway. (**A**) After 30 min pre-treatment with an inhibitor of p53 transcriptional activity PFt-α for various concentrations as indicated, RA FLS were treated with resveratrol (100 μM) for 36 h, and then apoptotic cell death was quantified by flow cytometry as described in Fig. 1A. Each data point shows mean $\pm$ s.e. of at least three independent experiments. (**B**) RA FLS were treated with resveratrol (100 μM) for 36 h in the absence or presence of PFT-α. Cell extracts were analysed by SDS–PAGE followed by western blotting with antibodies against p21 and actin. (**C**) Wild-type and p53−/− MEF cells were treated with resveratrol (100 μM) for 36 h, and then apoptotic cell death was quantified by flow cytometry as described in Fig. 1. Each data point shows mean $\pm$ s.e. of at least three independent experiments. (**D**) Wild-type and p53−/− MEF cells were treated with resveratrol for various concentrations as indicated, and cell extracts were analysed by SDS–PAGE and western blotting with antibodies against p53, p21 and actin.

To strengthen our findings, we further examined whether the activated p53 plays a role in resveratrol-induced apoptosis by comparing the responses of wt and p53 null MEF cells. As shown in Fig. 3C, resveratrol efficiently induced apoptosis in wt and p53 null MEFs in a dose-dependent manner, even though the extent of apoptosis in p53 null MEFs was slightly higher than in wt MEFs. To ensure that p53 signalling is indeed defective in p53 null MEFs, we confirmed that resveratrol-induced expression of p53 and its target p21 increased in wt MEFs but not in p53 null MEFs (Fig. 3D).

Caspase-8 has an essential role in resveratrol-induced apoptosis, as an upstream of mitochondria in RA FLS

To determine the upstream events of the pathway that lead to mitochondria-mediated apoptotic cell death induced by resveratrol, we investigated caspse-8 activation and Bid cleavage after treatment with resveratrol at different time points. As shown in the top and second panel of Fig. 4A, when RA FLS were treated with resveratrol, the degradation of the inactive form of caspase-8 started to be detected about 24 h after treatment, whereas the level of Bid appeared to be down-regulated, in accordance with resveratrol-induced activation of caspase-8. No change, however, occurred in the levels of Bax, Bcl-xL and Bcl2 after treatment with resveratrol. To investigate the mechanisms that regulate initial caspase activation during apoptosis induced by resveratrol, we sequentially analysed the activity of caspases in RA FLS, using fluorescent substrates after resveratrol treatment with or without the irreversible caspase-3 and -8 inhibitors z-DEVD-fmk and z-IETD-fmk, respectively. Resveratrol treatment resulted in a significant increase in enzymatic activity of caspase-8 and -3. Although the caspase-3 inhibitor failed to reduce caspase-8 activity, pre-treatment with the caspase-8 inhibitor significantly reduced caspase-3 activity (Fig. 4B), indicating that resveratrol-induced activation of caspase-3 is downstream of caspase-8 activity. Under these conditions, however, the caspase-3 inhibitor marginally inhibited resveratrol-induced apoptosis by up to 30%, whereas inhibition of caspase-8 dramatically reduced cell death (Fig. 4C). Further results showed that transient transfection of RA FLS with a vector encoding CrmA (which can prevent processing of caspase-8) was found to be markedly resistant to resveratrol-induced apoptosis as compared with mock vector-transfected cells (Fig. 4D).

To better understand the precise role of casapse-8 in mediating mitochondrial damage through Bid, we transiently transfected RA FLS with CrmA and exposed them to resveratrol for 36 h. Compared with vector-transfected cells, CrmA transfection completely prevented the resveratrol-induced degradation of Bid, although cleavage of Bid into truncated Bid (tBid) was not detected under our experimental conditions (Fig. 4E). To further support these results, we introduced Bid-GFP in RA FLS and examined resveratrol-induced tBid expression by western blot analysis using a GFP antibody. Consistent with the above results, Bid cleavage after resveratrol treatment was blocked by z-IETD-fmk but not by z-DEVD-fmk (Fig. 4F). Further approaches using Bid-GFP in combination with Mito Tracker dye showed that the translocation of Bid to mitochondria in resveratrol-treated apoptotic cells, and the translocation of Bid-GFP to mitochondria were also blocked by z-IETD-fmk but not by z-DEVD-fmk (Fig. 4G).

Caspase-8 is required for resveratrol-mediated AIF translocation to the nucleus in RA FLS

Since the above results show that the caspase-3 inhibitor suppressed resveratrol-induced apoptosis to a lesser extent as compared with the caspase-8 inhibitor (Fig. 4C), other factors in addition to caspase-dependent processing may participate in the resveratrol-induced apoptotic pathway. To examine whether certain caspase-independent death effectors, including AIF, contribute to resveratrol-induced apoptosis, translocation of AIF into the nucleus was analysed by immunofluorescent staining in RA FLS treated with resveratrol. Whereas quiescent cells demonstrated a lack of nuclear expression of AIF, resveratrol treatment induced demonstrable translocation of AIF to the nucleus at 24 h, which gradually increased up to 48 h (Fig. 5A). Consistent with Bid cleavage, pre-treatment of RA FLS with z-IETD-fmk, but not z-DEVD-fmk, prevented the translocation of AIF into the nucleus, as detected by fluorescent staining (Fig. 5B) and western blot analysis in cell fractionation experiments (Fig. 5C), indicating that caspase-8 is required for resveratrol-mediated translocation of AIF to the nucleus in RA FLS.

Discussion

RA is a chronic inflammatory disease of unknown origin that primarily affects the synovial joints, ultimately leading to their destruction [1]. Since RA FLS has the critical role for destructing the joints and are expanded possibly through the increased proliferation and/or insufficient apoptosis, studying the regulation of the cell death pathways in this cell type provides an attractive approach for treating RA patients, especially for achieving the long-term remissions [6, 28]. In this study, we demonstrated for the first time that resveratrol efficiently caused cell death via a mitochondria-mediated apoptotic signalling pathway in RA FLS. In addition, although resveratrol induces p53 and its transcriptional activation, these signalling processes appear to be dispensable for resveratrol-induced apoptosis. Moreover, caspase-8 may act upstream of mitochondrial processing that can be triggered by the cleavage and translocation of Bid; this subsequently results in the induction of a caspase-dependent or -independent pathway of apoptosis in RA FLS following resveratrol exposure. These results constitute direct evidence that resveratrol might have an essential role in targeting casapse-8-dependent cell death effectors in the FLS of patients with RA, producing a resultant decrease in synovial hyperplasia.

Resveratrol, a polyphenol found in red grape skins as well as other food products, possesses chemotherapeutic potential through its ability to trigger apoptosis in several cancer types, both *in vitro* and *in vivo*. Current studies have proposed that the anti-carcinogenic property of resveratrol is closely associated with its antioxidant activity and its ability to inhibit cyclo-oxygenase, cytochrome P450 isoenzymes, protein kinase C and matrix metalloproteinase [21, 22, 29–31]. In accordance with previous observations in a variety of tumour cell lines, we found that resveratrol induced cell death by apoptosis in RA FLS. The resveratrol-induced apoptosis in RA FLS appeared to accompany the release of cytochrome c and AIF together with activation of caspase-9 and -3, and subsequent PARP cleavage (Figs 1B and 2B), which indicate a prominent role for mitochondria in resveratrol-induced cell death. In the intrinsic mitochondria-initiated apoptotic pathway, p53-target gene products are involved in the release of cytochrome c, resulting in activation of caspase-9, which in turn activates execution-type casapase-3 [32, 33]. Furthermore, the results of recent studies suggest that resveratrol can engage selective apoptotic signals through activation of p53 in several cancer cell lines [25, 34–36]. However, we found that PFT-α, an inhibitor of p53 transcriptional activity, did not block the apoptotic cell death upon resveratrol treatment in RA FLS (Fig. 3A). These results were further confirmed by comparing the apoptotic responses of resveratrol in p53-null and wt MEF cells (Fig. 3B), indicating that, resveratrol induces apoptosis via p53-independent pathway in both RA FLS and MEFs.

Previously, it was reported that truncated Bid, tBid, generated from Bid protein by the active caspase-8, was rapidly translocated to mitochondria to allow mitochondrial cytochrome c release into the cytosol [37–39]. In RA FLS, following exposure to resveratrol,
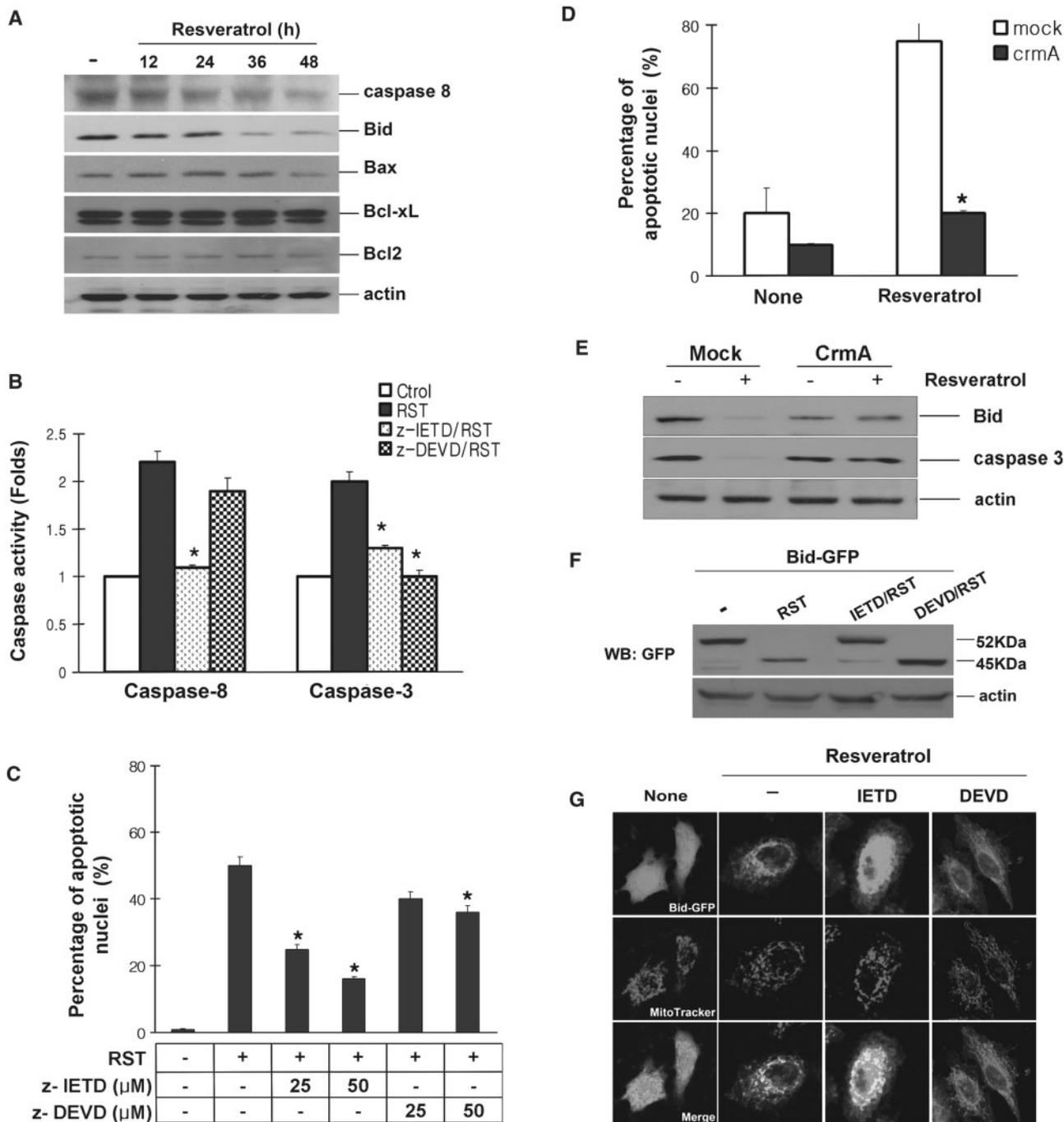
FIG. 4. Caspase-8 has an essential role in resveratrol-induced apoptosis, as an upstream of mitochondria in RA FLS. (**A**) RA FLS were treated with resveratrol (100 μM) for the indicated times, and cell extracts were analysed by SDS–PAGE and western blotting with antibodies against caspase-8, Bid, Bax, Bcl-xL, Bcl2 and actin. (**B**) RA FLS were treated with resveratrol (100 μM) for 36 h in the absence or presence of caspase inhibitors z-IETD-fmk (50 μM) or z-DEVD-fmk (50 μM). The enzymatic activity of respective caspases were analysed by using caspase activity assay kit as described in the Materials and methods section. The relative activity per milligram of protein was determined by calculating the fold increase of caspase-8 and -3 activities in untreated cells. Each bar shows mean ± S.E. of at least of three independent experiments. *P < 0.05, when compared with resveratrol-treated group. (**C**) After pre-treatment with caspase inhibitors, z-IETD-fmk or z-DEVD-fmk for various concentrations are indicated, and then apoptotic cell death was quantified by flow cytometry as described in Fig. 1A. RA FLS were transfected with pcDNA3 (mock vector) or an expression vector for crmA by using Amaxa nucleofector. After the cells were treated with 100 μM resveratrol for 36 h and apoptotic cell death (**D**) was quantified by flow cytometry as described in Fig. 1A, cell extracts were analysed by SDS–PAGE and western blotting (**E**) with antibodies against Bid, caspase-3 and actin. RA FLS were transiently transfected with GFP-tagged Bid plasmid. After 24 h of transfection, cells were treated with resveratrol (100 μM) for 36 h in the absence or presence of caspase inhibitors z-IETD-fmk or z-DEVD-fmk and cell extracts were analysed by western blotting (**F**) with anti-GFP and anti-actin antibodies. (**G**) For confocal microscopy analysis, cells were first stained with Mito Tracker dye as mitochondrial staining. The Bid (green) that translocated to mitochondria (red) is shown in yellow (Merge). Data were representative of three similar experiments.

we were unable to observe the endogenous generation of tBid, under our experimental conditions, presumably due to the short half-life or low level of tBid. However, we clearly detected a significant reduction in the level of Bid, along with

resveratrol-induced caspase-8 activation (Fig. 4A) and mitochondrial cytochrome c release (Fig. 2B), indicating that resveratrol-induced mitochondrial cytochrome c release was mediated through Bid cleavage by the active caspase-8. In agreement with
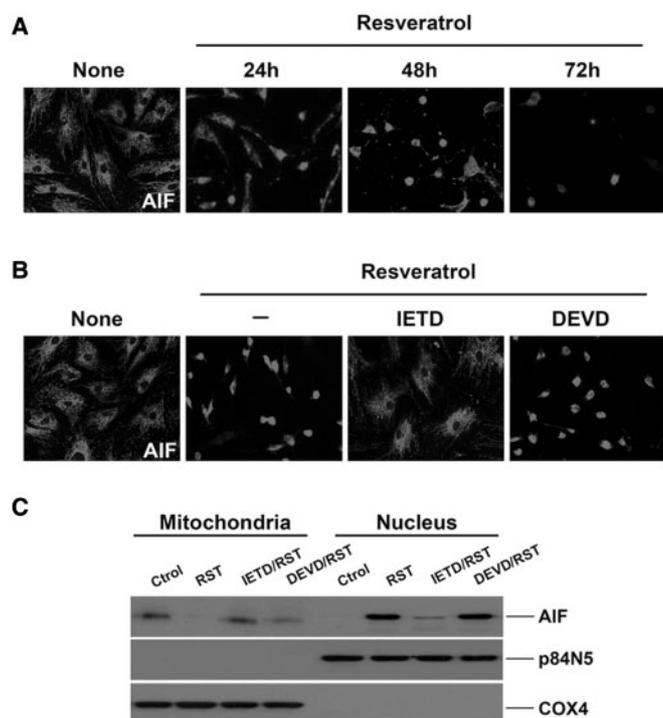
Fig. 5. Caspase-8 is required for resveratrol-induced AIF translocation into the nucleus in RA FLS. (A) RA FLS were treated with resveratrol (100 μM) for various times as indicated, and immunostained using antibody that recognized AIF following PE-conjugated secondary antibody. The cells were viewed using a Fluoview500 confocal scanning microscope (CLSM, Olympus). (B) RA FLS were treated with resveratrol (100 μM) for 36 h in the absence or presence of caspase inhibitors z-IETD-fmk or z-DEVD-fmk, and translocation of AIF was analysed as described in (A). Data were representative of three similar experiments. (C) RA FLS were treated with resveratrol (100 μM) for 36 h in the absence or presence of caspase inhibitors, z-IETD-fmk or z-DEVD-fmk, and then lysed. Mitochondria and nuclear fractions were obtained as described in the Materials and methods section, and each lysates were analysed by SDS–PAGE followed by western blotting with antibodies against AIF, p84N5 and COX-4. Ctrl, control; RST, resveratrol.

these data, our studies also revealed that GFP-Bid transfection in RA FLS induced the cleavage form of Bid (tBid) upon resveratrol treatment by western blot analysis (Fig. 4F). Furthermore, it appears that caspase-8 contributes to the cleavage and translocation of Bid at a point upstream of mitochondria in resveratrol-treated cells, since tBid expression and translocation were completely abrogated by pre-treatment with the caspase-8 inhibitor z-IETD-fmk (Fig. 4G). In addition, pre-treatment with the caspase-3 inhibitor z-DEVD-fmk, partially, but not completely, suppressed resveratrol-induced apoptosis while the activation of caspase-8 was not affected (Fig. 4B and C). These findings suggest that caspase activation via processing the apoptosome complex is not essential for resveratrol-induced apoptosis, although it may contribute to this process. This interpretation is supported by our finding that the z-IETD-fmk, but not z-DEVD-fmk, inhibits the translocation of AIF (Fig. 5B and C). Therefore, the apoptotic effect of resveratrol in RA FLS is likely mediated by the release of a caspase-independent death effector, including AIF from mitochondria.

In mechanistic analysis of resveratrol in RA synovial fibroblasts, we observed that activation of caspase-8 is the major determinant for apoptotic cell death induction by resveratrol upstream of tBid-mediated mitochondrial damage. Consistent with this conclusion, it was recently reported that resveratrol induced the mitochondrial death pathway through activation of casapse-8 in colon cancer cells [40]. Although it was proposed that caspase-8 activation has an upstream role as an apoptosis-initiating event in this study, the mechanism that accounts for resveratrol-induced activation of caspase-8 is still unclear. It has

been reported that, in human leucaemic and colon carcinoma cells, activation of caspase-8 induced by resveratrol could have occurred at the level of a death receptor through the up-regulation of FasL expression and a FasL/Fas interaction [27]. In our study, however, the mRNA transcriptional level and expression of FasL on the cell surface was unaffected following resveratrol treatment (data not shown). Therefore, it is unlikely that the Fas pathway plays a major role in resveratrol-induced caspase-8 activation in RA FLS. The reason for this discrepancy is currently unclear, although it is possible that the requirement for the Fas pathway is not only disease-specific but also cell type-specific. Recent observation indicates that the clustering of the Fas receptor and its redistribution into the membrane rafts, which may induce the formation of a death-inducing signalling complex with Fas-associated death domain (FADD) and procaspase to trigger caspase-8 processing by resveratrol [40], and thus whether apoptotic signal in response to resveratrol is initiated in the cell membrane, most likely, through a death receptor remains for future study.

Taken together, our results reveal that resveratrol can induce extensive apoptotic effects by converging on the mitochondrial signalling pathway in FLS derived from patients with RA. In addition, we suggest that caspase-8 could represent a potential molecular target for the cytotoxic activity of resveratrol in RA FLS. More importantly, very recent studies *in vivo* have demonstrated that intra-articular injection of resveratrol reduces the severity of cartilage lesions and synovial inflammation in an experimental inflammatory arthritis model [41]. Although further studies are warranted for evaluating any evidence of toxicity of resveratrol in clinical trials, our studies provide a novel idea of using resveratrol for long-lasting therapy in patients with RA via targeting the mitochondrial caspase pathway.

Rheumatology key messages

- Resveratrol induces extensive apoptotic cell death, along with a caspase-dependent or -independent pathway, by converging on mitochondrial signalling in RA FLS.
- Caspase-8 is the major determinant for apoptotic death induction by resveratrol upstream of mitochondrial damage.

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