Resveratrol-Mediated Reduction of Collagen by Inhibiting Proliferation and Producing Apoptosis in Human Hypertrophic Scar Fibroblasts

Guofang ZENG,* Fang ZHONG,* Jin Li, Shaojun LUO,† and Peihua ZHANG†

Institute of Plastic Surgery, Affiliated Hospital of Guangdong Medical College, Zhanjiang 524001, China

Received June 19, 2013; Accepted September 11, 2013; Online Publication, December 7, 2013

Hypertrophic scar (HS) is a dermal fibroproliferative disorder characterized by excessive deposition of extracellular matrix. Here, to investigate the regulatory effects of resveratrol, a natural antioxidant compound, on fibroblasts from human skin HS tissue, a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was used to evaluate the inhibitory effect of resveratrol on cells. Cell cycle progression and apoptosis were measured by flow cytometry and Hoechst 33258 staining respectively. The hydroxyproline content and mRNA expression levels of type I and III procollagen were measured separately by ELISA and reverse transcription-polymerase chain reaction (RT-PCR). The results indicated that resveratrol significantly inhibited cell growth, arresting the cell cycle at the G1 phase and inducing apoptosis in the fibroblasts, decreasing hydroxyproline (or collagen) levels, and downregulating the expression levels of type I and III collagen mRNA. Taken together, these data indicate that resveratrol-mediated reduction of collagen in fibroblasts is at least partially effected by causing inhibitory cell growth, cell cycle arrest, and apoptosis, and they suggest that resveratrol is a potential agent for HS treatment.

Key words: resveratrol; apoptosis; collagen; fibroblasts; hyperplastic scar

The healing of skin wounds is a complicated process in inflammation, cell migration and proliferation, and the synthesis of extracellular matrix (ECM) components such as collagen, mechanical forces, and tissue reconstruction.1) Additionally, hypertrophic scarring is a common problem characterized by excessive deposition of ECM, including collagen, and an abnormal invasive growth of fibroblasts2,3) after deep dermal injuries such as abrasions, burns, and deep donor-site excisions. Previous studies suggest that a delicate balance between cell proliferation and apoptosis plays an important role in the normal wound healing process. Generally, hypertrophic scars present as erythematous, raised, pruritic lesions in the healing skin, which results in both cosmetic deformities and functional impairment that seriously impact the quality of life in those affected patients.4,5) Although there are many treatment choices, including corticosteroid injections, skin grafting, splinting, pressure therapy, and surgical excision, hypertrophic scars are still a major problem in plastic surgery. Currently, there is no definitive universal therapeutic mode fielding complete and permanent improvement of hypertrophic scars with no side effects.6,7) In view of an unbalance between cell proliferation and apoptosis when hypertrophic scars occur, induction of apoptosis in fibroblasts contributes to the alleviation hypertrophic scar formation.8) Accordingly, we search for generally recognized dietary agents to investigate their effects on the growth of fibroblast cells from hypertrophic scar tissue.

It is recognized that resveratrol (3,5,4-trihydroxy-trans-stilbene) is a natural plant polyphenol and phytoestrogen, widely present in grape skins, peanuts, and red wines.9,10) It has been identified as a key biologically active ingredient in red wine, and credited with mediating a number of beneficial effects in the cardiovascular system that accompany moderate red wine consumption.11) Its chemical structure is shown in Fig. 1. It has been reported that resveratrol shows many biological activities, including anti-inflammation,12) anti-oxidant,13) anti-cancer,14) anti-platelet, and vasorelaxant activities.15) A previous study indicated that it exerted anti-mouse skin tumors by induction of apoptosis, cell cycle arrest, activation of p53 activity, and alteration of apoptosis-related proteins.16) Additionally, it has been found to attenuate doxorubicin-induced cellular damage in kidney cells by regulation up of nitric oxide and the induction of apoptosis.17) More recently, there is increasing evidence to confirm the hypothesis that resveratrol has anti-proliferative effects in various cell types, including cancer cells, leiomyoma cells, malignant natural killer cells, normal rat fibroblast cells, and smooth muscle cells, by induction of cell cycle arrest and/or apoptosis7,18-21). However, to the best of our

1 To whom correspondence should be addressed. Peihua ZHANG, Tel: +86-759-238-7087; Fax: +86-759-238-7087; E-mail: zhangpeihua128@126.com; Shaojun LUO, Tel: +86-756-238-7087; Fax: +86-759-238-7087; E-mail: shaojunluo@yahoo.com
* Guofang ZENG and Fang ZHONG contributed equally to this work.
† Abbreviations: DMSO, dimethyl sulfoxide; ECM, extracellular matrix; RT-PCR, reverse transcription-polymerase chain reaction; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
knowledge, to date, no literature pertaining to the effects of resveratrol on hyperplastic scar fibroblasts is available.

Hence, in this study we investigated to determine whether resveratrol can affect cell growth, cell cycle progression, apoptosis, or type I or III pro-collagen mRNA expression in fibroblasts from hyperplastic scar tissue. Hydroxyproline (or collagen) levels were also determined by ELISA. Our data indicate that resveratrol can inhibit the proliferation of fibroblasts, causing cell cycle arrest and leading to apoptosis. In addition, mRNA expression of type I and III pro-collagen was regulated down, and hydroxyproline (or collagen) levels were also decreased in a concentration-dependent manner.

Materials and Methods

Reagents. Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO), dissolved in dimethyl sulfoxide (DMSO), and diluted with culture medium. The final content of DMSO was kept to less than 0.5% in all cell cultures, and this did not show any effect on cell proliferation. A hydroxyproline ELISA kit was purchased from the Nanjing Jianchen Bioengineering Institute (Nanjing, China), TRIZol reagent (Carlsbad, CA) and Hoechst 33258 solution (Haimen, China) respectively were purchased from Life Technologies and Beyotime Institute of Biotechnology.

Isolation fibroblasts and cell culture. Human skin hypertrophic scar tissues, normal skin samples, and informed consent were obtained from two 20–30 year old female donors. Specifically, hypertrophic scar tissues were obtained from one patient who was not treated with glucocorticosteroids or radiotherapy, and underwent plastic surgery for scar excision, confirmed by pathology, and defined as raised above skin level (>1 mm). There was no local infection or ulceration in the areas of hypertrophic scarring. This study was approved by the Human Research and Ethical Committee of the hospital. Fibroblasts from hypertrophic scar tissues and normal skin samples were isolated according to a previously described article,22) with some modifications. Briefly, about 10 g of hypertrophic scar tissue or 5 g of normal skin samples was cut into 0.5–1 mm2 pieces with scissors in 10-cm culture plates with 4 mL of PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KHPO4, pH 7.4). After isolation from the epidermis by digestion with 25 U/mL of dispase II (Invitrogen, Carlsbad, CA) overnight at 4°C, the minced dermal tissues were incubated in 400 U/mL of collagenase (Invitrogen, Carlsbad, CA) for 10 h at 37°C, and cells were then harvested by centrifugation. Then the cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA) at 37°C in a humidified incubator with 5% CO2. When the primary fibroblasts grew to 80–90% confluence, the cells were digested with 0.25% trypsin with 0.02% EDTA, and passed 4–7 times by serial cell expansion for subsequent experiments.

Cell viability assay. Effect of resveratrol on cell viability was evaluated by MTT assay. Briefly, fibroblasts (passage 4) were seeded into 96-well plates at a density of 1 × 104 cells/well and incubated for 12 h to allow cell attachment, followed by resveratrol treatment at various concentrations (0, 25, 75, 150, 300, and 400 μM) for the indicated hours. Then 20 μL of MTT solution (5 mg/mL) was added to each well for other 4 h of incubation. Then the culture medium was removed and 150 μL of DMSO was added. The absorbance for each well was measured at 570 nm with automated spectrophotometric plate reader (PerkinElmer, Waltham, MA). The experiments were performed in duplicate 3 times.

Cell cycle analysis. The effect of resveratrol treatment on cell cycle distribution was examined by a reported method,23) with some modifications. Briefly, cells (passage 5) grown in six-well plates were treated with resveratrol at increasing concentrations (0, 75, 150, and 300 μM) for the indicated h. The cells were harvested by trypsinization and centrifugation, and resuspended in PBS to a concentration of 2 × 105 cells/mL. They were fixed in cold 75% of the ethanol for 1 h, and then centrifuged to remove 75% ethanol. The fixed cells were incubated in cell cycle staining solution containing propidium iodide (50 μg/mL) for 30 min in the dark at room temperature according to the operation instructions (Beyotime, Haimen, China). The stained cells were measured for cell cycle distribution by flow cytometry (Becton Dickinson, NJ), and the experiments were carried out in duplicate 3 times.

Hoechst 33258 staining. Hoechst 33258 staining was used to evaluate the effect of resveratrol on apoptosis in fibroblasts by a published method,24) with some modifications. Briefly, about 106 fibroblasts (passage 5) on the cover slips in a six-well plate were treated with various concentrations of resveratrol for 24 h or 150 μM of resveratrol for 24, 48, and 72 h. The cells were then washed twice with PBS and fixed in 75% ethanol for 15 min. The fixed cells were incubated in 2.5 μg/mL Hoechst 33258 solution for 30 min at room temperature in the dark. They were observed and photographed were taken under an inverted fluorescence microscope (Olympus, Tokyo). The experiments were carried out 5 times.

RT-PCR analysis for type I and III procollagen mRNA expression. Fibroblasts (passage 7) were incubated with resveratrol at various concentrations for 24 h or with 150 μM resveratrol for 24, 48, and 72 h, and then harvested by centrifugation. Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) was used to analyze changes in gene expression at the mRNA level of types I and III procollagen. Total cellular RNA was extracted with TRIzol reagent (Life Technologies, Carlsbad, CA) following the instructions of the manufacturer. The concentration and the purity of isolated total RNA were measured with an ultraviolet spectrophotometer (Beckman Coulter, Lawrence, KS) at an absorption ratio of OD260/OD280. The total RNA (2 μg) of each sample was used to produce first-strand cDNA in a total volume of 25 μL with a TaqMan RT Reagents kit (Life Technologies, Carlsbad, CA) containing oligo (dT) 16 primer and M-MLV reverse transcriptase. The specific primers for the expression analysis of types I and III procollagen followed previously published articles.24,25) They were synthesized by Shanghai Sangon Biotech Company, and are listed in Table 1. Amplification was performed under specific reaction conditions: 3 min initial denaturation at 94°C, 35 cycles of 94°C for 1 min, 55°C for 2 min, and 72°C for 2 min, and a final extension of 5 min at 72°C. Finally, 10 or 15 μL of PCR products for type I and III procollagen were separated by electrophoresis on 1.5% agarose gels and stained with ethidium bromide, and images were captured under UV illumination. GAPDH served as loading control. The experiments were carried out 3 times.

CTCGTCTCCAAGGAATACAG-3' 5'-GCCAGGAGACCAGCTTC-3'
CAGCACCAGAAGCTTGAGG-3' 5'-CCCATCACCACCTTCCAG-3'
GAPDH (+) 5'-CCATCTCCAGAAGCTTGAGG-3' 577
GAPDH (--) 5'-CCTGCTTCCACCTTCT-3'

Table 1. Specific Primers Used in This Study

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primers</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I procollagen</td>
<td>5'-CTGGTCTCCAAGGAATACAG-3'</td>
<td>266</td>
</tr>
<tr>
<td>Type I procollagen</td>
<td>5'-GCCAGGAGACCAGCTTC-3'</td>
<td></td>
</tr>
<tr>
<td>Type II procollagen</td>
<td>5'-CAGCACCAGAAGCTTGAGG-3'</td>
<td>447</td>
</tr>
<tr>
<td>Type III procollagen</td>
<td>5'-CCATCTCCAGAAGCTTGAGG-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH (+)</td>
<td>5'-CCATCACCACCTTCCAG-3'</td>
<td>577</td>
</tr>
<tr>
<td>GAPDH (--)</td>
<td>5'-CCTGCTTCCACCTTCT-3'</td>
<td></td>
</tr>
</tbody>
</table>

(+) and (−), sense and antisense strand
Determination of hydroxyproline level and calculation of collagen level. Cells (passage 6) \((5 \times 10^5)\) were seeded in culture dishes with 5 mL of culture medium, and then treated with resveratrol at the indicated concentrations for 24, 48, or 72 h. Sample preparation was done according to a previously published report. The hydroxyproline level was determined with a human hydroxyproline ELISA kit (Nanjing Jianchen, Nanjing, China) according to the instructions of the manufacturer. Hydroxyproline concentrations from 0.25 to 6.50 mg/mL were used to construct a standard curve. The spectrophotometric absorbance at a wavelength of 450 nm was compared to known concentrations of the standard curve to measure the hydroxyproline levels in the samples. The collagen level was calculated from the hydroxyproline concentration assuming that hydroxyproline constitutes 12.5% of collagen. These experiments were carried out 3 times.

Statistical analyses. Data were presented as mean values ± standard deviation. Statistical analyses were performed using SPSS 17.0, and statistical significance was evaluated by two-tailed Student’s t test. The significance level was defined at values of \(p < 0.05\).

Results

**Resveratrol inhibited the proliferation of fibroblasts**

To evaluate the effect of resveratrol on cell viability, an MTT assay was used to analyze the effect of resveratrol on the proliferation of fibroblasts. The results indicated that cellular morphology was markedly changed after the fibroblasts were incubated with 150 \(\mu\)M resveratrol for 24, 48, and 72 h (Fig. 2A), and cell proliferation was apparently inhibited in a time-dependent manner (Fig. 2B). However, the control fibroblasts, which were treated with the same volume of culture medium instead of resveratrol, demonstrated typical characteristics of fibroblast cells, such as spindle-shaped morphology and an ability to adhere to the plastic walls of the culture plates, similarly to fibroblasts from normal skin tissues. In addition, cellular morphology hardly changed, and no cytotoxicity of resveratrol appeared when fibroblasts were treated with 75 \(\mu\)M resveratrol for up to 24 h (Fig. 2A), though resveratrol at this concentration significantly inhibited cell proliferation \((p < 0.01)\) (Fig. 2C). On the other hand, resveratrol also demonstrated significant inhibitory effects on fibroblast growth in a dose-dependent manner (from 25 to 400 \(\mu\)M) after resveratrol treatment for 24 h (Fig. 2C).

**Effect of resveratrol on cellcycle distribution**

To investigate further the mechanism by which treatment with resveratrol caused growth inhibition in fibroblasts, cellcycle progression was measured by flow cytometry. The results showed that resveratrol treatment of fibroblasts resulted in a cellcycle arrest at the G1/S phase. For 75 or 300 \(\mu\)M resveratrol treated cells for up to 24 h, the percentage of cells in the G1 phase increased from 59.20 ± 1.03% to 76.80 ± 2.00% or 94.73 ± 0.23% (Table 2). Furthermore, as shown in Table 3,
concomitant decrease in the percentage of cells in the resveratrol treatment from 24–72 h. Additionally, a incubation with 150 $\mu$M resveratrol (Fig. 3C).

Table 2. Resveratrol at Various Concentrations Affected Cell-Cycle Distribution in Fibroblasts

<table>
<thead>
<tr>
<th>Concentration (µM)</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>59.20 ± 1.03</td>
<td>18.93 ± 3.13</td>
<td>21.53 ± 2.32</td>
</tr>
<tr>
<td>75</td>
<td>76.80 ± 2.00**</td>
<td>8.43 ± 0.85**</td>
<td>14.53 ± 1.41**</td>
</tr>
<tr>
<td>300</td>
<td>94.73 ± 0.23**</td>
<td>2.46 ± 0.76**</td>
<td>2.83 ± 0.76**</td>
</tr>
</tbody>
</table>

Fibroblasts were treated for 24 h with resveratrol at the indicated concentrations, and then collected and stained with propidium iodide and analyzed by flow cytometry. Values are presented as means ± SD, and statistical analysis was analyzed by one-way ANOVA (n = three independent experiments). Data denoted *$p < 0.01$ is significant as compared to control, and $\Delta p < 0.05$ is significant as compared to the cells treated with 75 $\mu$M resveratrol.

Table 3. Cell-Cycle Distribution Changed in Fibroblasts after Incubation with 150 $\mu$M Resveratrol for 24, 48, and 72 h

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2/M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>87.63 ± 0.30</td>
<td>5.90 ± 1.47</td>
<td>6.70 ± 1.73</td>
</tr>
<tr>
<td>48</td>
<td>91.40 ± 0.43*</td>
<td>4.13 ± 0.40*</td>
<td>4.50 ± 0.26*</td>
</tr>
<tr>
<td>72</td>
<td>94.33 ± 0.40**</td>
<td>3.26 ± 1.28**</td>
<td>2.43 ± 0.94**</td>
</tr>
</tbody>
</table>

Fibroblasts were treated with 150 $\mu$M resveratrol for 24, 48, and 72 h, and the percentage of cell cycle was determined by flow cytometry. Values are presented as means ± SD, and statistical analysis was analyzed by one-way ANOVA (n = three independent experiments). Data denoted *$p < 0.05$ and **$p < 0.01$ are significant compared to the cells treated for 24 h, and $\Delta p < 0.05$ is significant compared to the cells treated for 48 h.

The G1 phase gradually increased with prolongation of resveratrol treatment from 24–72 h. Additionally, a concomitant decrease in the percentage of cells in the S and G2/M phases was observed after incubation with resveratrol (Tables 2 and 3).

**Resveratrol induced apoptosis in fibroblast cells**

Cell cycle checkpoints represent an intersection of cell survival and cell death where conditions for successful interphase and mitosis must be favorable for complete cell division or the cell commits to death. Therefore, according to the above results demonstrating resveratrol-mediated cell cycle arrest, analysis of apoptosis by Hoechst 33258 staining was used to measure the effects of resveratrol treatment on apoptotic induction in fibroblasts. The results indicated that the cells presented typical morphological changes following treatment with resveratrol, and that apoptotic cells showed chromatin condensation and nuclear fragmentation (Fig. 3A). We measured the rate of apoptosis in the fibroblasts, and found that cells treated with resveratrol showed higher apoptosis rates than the control ($p < 0.01$) (Fig. 3B). Furthermore, the percentage of apoptosis rose after resveratrol treatment at increasing concentrations. On the other hand, the apoptosis rate also gradually increased from 24 h after incubation with 150 $\mu$M resveratrol (Fig. 3C).

**Resveratrol decreased hydroxyproline and collagen levels**

Hydroxyproline is a unique imino acid to collagen constituent that is traditionally used to quantify collagen levels. Hence, to investigate whether resveratrol can affect collagen production, we decided to measure hydroxyproline levels using an ELISA kit, and calculated the collagen. As shown in Fig. 4A, the hydroxyproline was significantly decreased after 24 h of incubation with resveratrol at concentrations from 75–300 $\mu$M, as compared with control. Furthermore, it was obvious that the decrease in hydroxyproline was occurred in a time-dependent manner when fibroblasts were treated with 150 $\mu$M resveratrol for 24, 48, and 72 h (Fig. 4B). On the other hand, the calculated collagen level was consistent with the hydroxyproline level, indicating an obvious decreasing trend in a dose- and time-dependent manner (Fig. 4C and D).

To determine whether the decrease in collagen levels in culture supernatants as detected previously was associated with regulation down of mRNA expression of collagen, semi-quantitative RT-PCR analysis was done for the mRNA expression levels of type I and III procollagen. The results are shown in Fig. 5, showing representative images of PCR products for the mRNA expression profiles of type I (Fig. 5A and B) and III (Fig. 5E and F) procollagen in fibroblasts treated with resveratrol at specific concentrations for 24, 48, and 72 h. It is obvious that resveratrol treatment led to relatively lower expression levels of type I and III procollagen mRNA as compared to the control, as assessed by the weak intensity of the bands. The mRNA expression of a housekeeping gene of GAPDH was
Discussion

Hypertrophic scar formation often occurs after cutaneous injury, including traumas, burns, and surgical procedures. Although the exact etiology remains unclear, an imbalance of fibroblast proliferation and apoptosis is thought to be involved. In the process of hypertrophic scar formation and development, fibroblasts were found to exhibit excessive cell proliferation, abnormal secretion of cytokine, and deposition of redundant ECM. Furthermore, hypertrophic scar fibroblasts can change into myofibroblasts and facilitate collagen synthesis and wound contraction. Accordingly, it is crucial to inhibit fibroblast proliferation, decrease collagen synthesis, and induce apoptosis in the treatment of hypertrophic scars. In the present study, we isolated and cultured primary fibroblasts from hypertrophic scar tissues by surgical excision, and separately calculated as the ratio of type I and of type III procollagen to GAPDH, and the results showed that there was a marked decrease in the abundance of type I and of type III procollagen mRNA in the fibroblasts treated with resveratrol at the indicated concentrations in a dose-dependent manner (Fig. 5C and G). However, when the fibroblasts were treated with 150 μM resveratrol for 24, 48, and 72 h, the expression levels of type I and III procollagen mRNA slightly decreased (Fig. 5D and H), and no significant reduction was found until incubation with resveratrol for 72 h.

Fig. 4. Concentration of Hydroxyproline and Collagen Levels.

Fibroblasts were incubated in the absence and the presence of resveratrol at various concentrations for 24 h, or with 150 μM resveratrol for 24, 48, and 72 h. Then sample preparation was carried out following a previous report, and subjected to determination of hydroxyproline levels with an ELISA kit. A, Resveratrol at increasing concentrations was applied to treat cells for 24 h, and the hydroxyproline levels were decreased gradually in a time-dependent manner. B, Fibroblasts were treated with 150 μM resveratrol for 24, 48, and 72 h, and the hydroxyproline fell in a time-dependent manner. C and D, The collagen calculated from the corresponding hydroxyproline concentration as collagen was assumed to contain 12.5% hydroxyproline, and showed a consistent reduction. Values are expressed as means ± SD, n = 3. * p < 0.05, ** p < 0.01 compared to control.

Fig. 5. RT-PCR Analysis of Types I and III Procollagen mRNA Expression.

Total RNA was isolated from fibroblasts treated without (control) and with various concentrations of resveratrol for 24 h, or with 150 μM resveratrol for 24, 48 and 72 h. The expression levels of types I and III procollagen mRNA were analyzed by semi-quantitative RT-PCR. Housekeeping gene GAPDH was amplified and served as internal control. A and B, PCR products amplified for type I procollagen mRNA were separated on 1.5% agarose gel, and representative images were shown. C and D, Relative expression levels of type I procollagen mRNA for three independent experiments as quantified by densitometry (the ratio of the density of type I procollagen to that of GAPDH). E and F, Representative images of PCR products of type III procollagen mRNA separated on 1.5% agarose gel. G and H, Relative expression levels of type III procollagen mRNA. Values are expressed as means ± SD, n = 3. * p < 0.05 and ** p < 0.01 are significant compared to control.

employed the cells to evaluate the effects of resveratrol on cell proliferation and apoptosis. Additionally, we isolated primary fibroblasts from normal skin samples, and made a morphological comparison of fibroblasts isolated from normal skin samples and hypertrophic scar tissues. We found that they had very similar morphol-
of cell cycle progression, suppressing cell growth, triggering apoptosis in a dose- and time-dependent manner, and down-regulating the mRNA expression of type I and III procollagen in fibroblasts, resulting in significant decreases in hydroxyproline and collagen.

Previous studies indicate that resveratrol inhibits cell growth in diverse cell types, B16 melanoma cells,32) human hepatocellular carcinoma Huh-7 cells,33) breast cancer MCF-7 cells,30) human K562 chronic myeloid leukemia cells,35) and bladder carcinoma BTT739 and T24 cells.36) In the present study, we found that resveratrol significantly suppressed the proliferation of fibroblasts in a dose- and time-dependent manner (Fig. 2B and C). Furthermore, cellular morphological changes characterized by a swollen shape were observed by an inverted phase contrast microscope when fibroblasts were treated with 150 μM resveratrol for 24, 48, or 72 h (Fig. 2A), but cellular shape did not change after 24 h of incubation with 75 μM resveratrol, although cell proliferation was significantly inhibited. We speculate that the inhibition of cell growth caused by resveratrol at low concentrations does not result from a cytotoxic effect. On the other hand, our data indicate that the anti-proliferation effect of resveratrol in fibroblasts might be associated with cell cycle arrest and the induction of apoptosis. It has been reported that resveratrol and black tea polyphenol combination synergistically suppressed mouse skin tumors growth by inhibition of activated MAPKs and p53, and that increased phosphorylation of p53 (Ser15) might contribute to the stabilization and activation of p53.10)

We found that resveratrol treatment arrested the cell cycle at the G1 phase and concurrently decreased the S and G2/M phases in fibroblasts (Tables 2 and 3). These findings are consistent with previous studies,19,37) although the treated cells were not of the same cell lines. On the other hand, resveratrol induced human neuroblastoma SH-SY5Y cell cycle arrest at the S phase, concomitantly reducing the percentage of cells in the G1 and G2/M phases.38) We speculate that the reason for these discrepancies in different cell lines is the diverse mechanisms involved in the cell cycle modulated by resveratrol. But further investigation is needed to confirm this hypothesis.

A previous MTT assay showed that resveratrol can act as an anti-proliferation agent against fibroblasts. Hence we further investigated whether resveratrol-mediated cell growth inhibition is triggered via induction of apoptosis besides cell cycle arrest. Our data clearly showed by inverted fluorescent microscope that the apoptosis in fibroblasts following resveratrol treatment for 24, 48, and 72 h occurred (Fig. 3A). Furthermore, the percentage of apoptosis increased in a dose- and time-dependent manner (Fig. 3B and C). In situ apoptosis was assessed using an apoptosis detection kit, which allowed for visualization of apoptotic nuclei. The results showed that the percentage of apoptotic cells was significantly increased in a dose- and time-dependent manner (Fig. 3C). The induction of apoptosis was confirmed by determining the expression levels of cleaved caspase-3 and PARP in fibroblasts treated with resveratrol. The expression levels of these proteins were significantly increased in a dose- and time-dependent manner (Fig. 3D). These findings suggest that the resveratrol-mediated induction of apoptosis is via a caspase-dependent pathway.

Growing evidence indicates that resveratrol can mediate a wide range of biological activities with no obvious toxicity.12,14) A high dose of resveratrol, 700 or 600 mg/kg/d, was administrated to rats and dogs for 3 months, and no systemic toxicity and genotoxicity or other adverse effects was observed.40,41) Even at a dose of 750 mg/kg/d, resveratrol did not induce any adverse reproductive effects in an embryo-fetal toxicity study on rats.42) When beagle dogs received a daily oral dose of 1, 200 mg of resveratrol/kg for 3 months, only minimal toxicity, such as dose-related reductions in body weight gain, was evident, and no other biologically significant evidence of resveratrol toxicity was observed.43) These in vivo studies suggest the overall safety of resveratrol in rodents. In the present study, we found that resveratrol significantly suppressed the proliferation of fibroblasts, which is consistent with previous studies, indicating that resveratrol-mediated inhibition of cell proliferation is associated with effects on cell cycle progression.

In the present study, we also tested the effect of resveratrol on collagen synthesis. Downing regulation in type I and III procollagen mRNA expression was observed after 24 h of treatment in fibroblasts with resveratrol of various concentrations (Fig. 5), although the decrease did not show obvious time-dependence. The effect of resveratrol on collagen synthesis appeared to be associated with effects on cell cycle progression and apoptosis, although the relative expression level of type I procollagen mRNA was not significantly reduced by 75 μM resveratrol at 24 h of incubation.44) These findings, which are similar to previous reports,2) suggest that resveratrol-mediated inhibition of type I procollagen expression is more significant than inhibition of type III procollagen. However, the hydroxyproline level in fibroblasts treated with 150, 300, and 400 μM resveratrol for 24, 48, and 72 h demonstrated swollen shapes or apoptotic and necrotic characteristics (Fig. 2).

Previous study has found that increased deposition of collagen is a typical characteristic of hypertrophic scar. In the present study, we also tested the effect of resveratrol on collagen synthesis. Downing regulation in type I and III procollagen mRNA expression was observed after 24 h of treatment in fibroblasts with resveratrol of various concentrations (Fig. 5), although the decrease did not show obvious time-dependence. The effect of resveratrol on collagen synthesis appeared to be associated with effects on cell cycle progression and apoptosis, although the relative expression level of type I procollagen mRNA was not significantly reduced by 75 μM resveratrol at 24 h of incubation (Fig. 5C). These findings, which are similar to previous reports,2) suggest that resveratrol-mediated inhibition of type I procollagen expression is more significant than inhibition of type III procollagen. However, the hydroxyproline level in fibroblasts treated with 150, 300, and 400 μM resveratrol for 24, 48, and 72 h demonstrated swollen shapes or apoptotic and necrotic characteristics (Fig. 2).

Although further investigation of the mechanisms responsible for decreasing the mRNA expression levels of type I and III procollagen in fibroblasts are needed, previous studies indicate that resveratrol can inhibit healing. Therefore, the induction of apoptosis caused by resveratrol found in this study might contribute to inhibition of fibroblast in hypertrophic scars.

Growing evidence indicates that resveratrol can mediate a wide range of biological activities with no obvious toxicity.12,14) A high dose of resveratrol, 700 or 600 mg/kg/d, was administrated to rats and dogs for 3 months, and no systemic toxicity and genotoxicity or other adverse effects was observable.40,41) Even at a dose of 750 mg/kg/d, resveratrol did not induce any adverse reproductive effects in an embryo-fetal toxicity study on rats.42) When beagle dogs received a daily oral dose of 1, 200 mg of resveratrol/kg for 3 months, only minimal toxicity, such as dose-related reductions in body weight gain, was evident, and no other biologically significant evidence of resveratrol toxicity was observed.43) These in vivo studies suggest the overall safety of resveratrol in rodents. In the present study, we found that resveratrol significantly suppressed the proliferation of fibroblasts, which is consistent with previous studies, indicating that resveratrol-mediated inhibition of cell proliferation is associated with effects on cell cycle progression.
advanced glycation end-product (AGE)-induced proliferation and collagen synthesis in vascular smooth muscle cells by suppression of prollyl hydroxylase activity, a marker for collagen synthesis, and decreasing the mRNA expression of transforming growth factor beta 1 (TGF-β1). Some reports have proposed a role for TGF-β1 and insulin-like growth factor 1 (IGF-I) in mediating abnormal cell proliferation and increasing synthesis of ECM or collagen.\textsuperscript{35,46} and the estrogen receptor antagonist ICI 182780 can partly reverse resveratrol-induced inhibitory effects on AGES-mediated cell proliferation and prollyl hydroxylase activity. Another study found that resveratrol caused cellcycle arrest and decreased the expression of procollagen types I and III mRNA in rat smooth muscle cells, and that this was associated with attenuation of the stimulatory effects of TGF-β1 and IGF-I on cell proliferation and collagen production.\textsuperscript{18} In addition, resveratrol treatment was found to reduce acetylation of Smad3, an important mechanism by which TGF-β1 stimulates the fibrotic process.\textsuperscript{37,48} Proinflammatory cytokine interleukin (IL)-17 stimulated net collagen production in mouse cardiac fibroblasts, but pretreatment with resveratrol inhibited PI3K-, Akt-, and ERK-dependent IL-17 expression.\textsuperscript{49} We speculate that resveratrol-mediated downregulation of procollagens I and III is involved in the interplay of the various complex pathways.

In conclusion, our data indicate that resveratrol treatment of human hypertrophic scar fibroblasts can significantly decrease hydroxyproline levels in cell culture supernatants, downregulating the expression levels of types I and III procollagen mRNA. Additionally, we found that resveratrol-mediated collagen reduction is closely associated with strong inhibition of resveratrol in fibroblast proliferation, triggering cellcycle arrest and promoting apoptosis in fibroblasts. Thus, resveratrol has potential for the development of an inhibitory agent for hypertrophic scar treatment.

Acknowledgments

We thank MS, Marilyn Ann Foster for the critical reading and English proofreading of the manuscript. This work was supported by the Medical Scientific Research Foundation of Guangdong Province, China (grant no.: A20133434), and by the Open Project Program of the State Key Laboratory of Meat Products for Security and Production Technology in Xiamen city (grant no.: 2011XYGZ008).

References