Alterations in Activating Protein 1 Composition Correlate with Phenotypic Differentiation Changes Induced by Resveratrol in Human Melanoma

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ABSTRACT

Resveratrol has demonstrated preventive and therapeutic activities in a variety of tumors. However, the mechanistic basis of its pharmacological effects on human melanoma has not been well defined. Our results demonstrated that resveratrol significantly inhibited melanoma anchorage-independent growth, and even at high doses no distinct apoptosis or cell cycle arrest was observed. It is noteworthy that c83-2c (metastatic) and wm3211 (radial growth phase) melanoma cells became more dendritic shaped with resveratrol treatment. Major histocompatibility complex (MHC) class I antigen and Fas/CD95 constitutive surface expression levels were, respectively, increased by 2.7- and 1.6-fold of control in c83-2c cells. Resveratrol reduced both activator protein-1 (AP-1) DNA binding and transcriptional activities, and supershift assay revealed that AP-1 composition was shifted from c-Jun/JunD/Fra-1 to JunD/Fra-1/Fra-2, with markedly increased JunD, Fra-1, and Fra-2 pro-
tein expression levels in the nucleus. Furthermore, we overexpressed Fra-2 in human melanoma cells by using a Fra-2 expression construct and both AP-1 transcriptional activity and 12-*O*-tetradecanoylphorbol-induced transcriptional transactivation were reduced significantly, whereas MHC class I antigen and Fas/CD95 levels were elevated to 2.0 and 1.8 times of control, respectively. Addition of H_2O_2 (10 μM) partially reversed the inhibition of colony proliferation; however, no effects on either MHC class I antigen or Fas expression was evident. Although H_2O_2 restored participation of c-Jun in AP-1 complexes, H_2O_2 addition did not affect the induction of Fra-1 and Fra-2 by resveratrol nor the morphological changes. We propose that alterations in AP-1 transcription signaling, mediated by changes in AP-1 dimeric composition and reduced intracellular reactive oxygen species levels, substantially contribute to the phenotypic changes induced by resveratrol.

Many epidemiological studies have demonstrated an inverse correlation between a modest intake of wine and death resulting from coronary heart disease. Resveratrol (*trans*-3,4′,5-trihydroxystilbene), a natural polyphenolic, was identified in wine by Siemann and Creasy (1992), and they suggested that resveratrol might be the biologically active ingredient of red wine. Resveratrol is also a potent tumor chemopreventive agent both in vitro and in animal models and is able to block the initiation, promotion, and progression phases (Aziz et al., 2003). In addition, resveratrol has been shown to inhibit the growth of a variety of tumor cells, including leukemia, prostate, breast, and endothelial cells (Bhat and Pezzuto, 2002). Many studies have demonstrated that resveratrol has significant antioxidant activities both in vitro and in vivo, acting as a potent ROS scavenger, which also might contribute to its coronary protective and chemopreventive effects (Bhat et al., 2001; Wu et al., 2001)

Melanoma is the most aggressive of all skin tumors and is associated with a high risk of mortality from metastatic disease. In view of the increasing incidence of melanoma and its resistance to therapy, new melanoma prevention and therapy strategies are greatly needed. Our previous studies have shown that the redox status in metastatic melanoma cells compared with normal melanocytes is aberrant, with significantly elevated intracellular ROS levels and abnormal activator protein-1 (AP-1) regulation (Meyskens et al.,

ABBREVIATIONS: ROS, reactive oxygen species; AP-1, activator protein-1; MnSOD, manganese-superoxide dismutase; MHC, major histocompatibility complex; TPA, 12-*O*-tetradecanoylphorbol; PBS, phosphate-buffered saline; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; SEAP, secreted alkaline phosphatase; DCF, 2′,7′-dichlorodihydrofluorescein; HE, dihydroethidium; PARP, poly(ADP-ribose) polymerase; MAPK, mitogen-activated protein kinase; MAP, mitogen-activated protein; SAPK, stress-activated protein kinase; JNK, c-Jun NH_2-terminal kinase; ERK, extracellular signal-regulated kinase.

2001b). ROS can perform essential metabolic functions in regulating signal transduction pathways and transcription factor functions (Sauer et al., 2001). Soluble antioxidants or antioxidant enzymes such as Cu-Zn-superoxide dismutase or oxidoreductase normally counterbalance intercellular oxidative stresses (Holmgren, 1985; Oberley et al., 1995). However, a known feature of human melanoma cells is that their antioxidant capacity is depressed with decreased catalase enzymatic activity and low levels of glutathione and α-tocopherol compared with normal melanocytes (Picardo et al., 1996). Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells (Church et al., 1993), as well as other cancer cells (Oberley, 2001). These observations raised the question of whether restoration of intracellular redox status by antioxidant agents such as resveratrol would facilitate the therapy or prevention of human melanoma. In this study, we examined the effects of resveratrol on human melanoma cells systematically and its mechanisms.

AP-1 is very sensitive to redox changes and controls transcription of a whole set of target genes (Sen and Packer, 1996). There has been reported an inverse correlation between MnSOD activity, which is one of the primary antioxidant enzymes, and AP-1 DNA binding activity. Both AP-1 DNA binding and transcription activity were reduced by as much as 20-fold in a MnSOD-overexpression clone (Li et al., 1998). Using a transgenic mice model, overexpression of MnSOD delayed AP-1 activation and inhibited tumor promotion, resulting in the induction of tumors (Zhao et al., 2001). These results suggested that aberrant redox status in metastatic melanomas might be associated with a quantitative or qualitative difference in AP-1 signaling. Our previous in vitro investigations showed that during human melanoma progression AP-1 DNA binding pairs were distinctly altered and were associated with elevated drug resistance and cell malignancy (Yang et al., 2004). To investigate the mechanisms of resveratrol action, we extended our studies to its effects on AP-1 signaling.

Herein, we report that with resveratrol treatment human metastatic melanoma cells undergo "differentiation" as assessed by morphological changes, reduced anchorage-independent growth, elevated MHC class I antigen and Fas expression levels, and decreased AP-1 binding and transcriptional activities. Furthermore, the results have shown that resveratrol induced AP-1 composition alterations with increased Fra-2 expression and undetectable c-Jun. We hypothesized that alterations in AP-1 composition and reduction of intracellular oxidative status by resveratrol might contribute to the recruitment of normal AP-1 transcription signaling in melanoma and subsequently induces cellular biological features consistent with a more normal phenotype.

Materials and Methods

Cells and Treatments. Human neonatal foreskins were placed in 0.25% trypsin at 4°C overnight. After this incubation, the tissue was scraped to recover the melanocytes and cultured in MCDB153 (Sigma-Aldrich, St. Louis, MO) medium containing 2% fetal calf serum, 0.3% bovine pituitary extract (Cambrex Bio Science Walkersville, Walkersville, MD), 10 ng/ml TPA, 2 mM CaCl₂, 5 μg/ml insulin, and 0.1 mM 3-isobutyl-1-methylxanthine (Sigma-Aldrich). Primary radial growth phase-like melanoma cells (wm3211) were cultured in RPMI 1640 medium with 5% fetal calf serum, 5% calf serum, peni-

cillin (100 units/ml), streptomycin (0.1 mg/ml), and insulin. These cells were a generous gift from Dr. M. Herlyn (Schaider et al., 2003). Metastatic melanoma cell strains (c81-46A and c83-2c) were cultured in F-10 with 5% fetal calf serum, 5% calf serum, 1% glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg /ml) and designated as melanoma complete medium. Cell strains c81-46A and c83-2c have been described previously and were initially cloned through soft agar and will form tumors in nude mice (Yohem et al., 1987). The passage number for the various cell strains used in these experiments was <8. A375 human melanoma cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium with 5% fetal calf serum, 5% calf serum, 1% glutamine, penicillin (100 units/ml), and streptomycin (0.1 mg /ml). In all experiments, cells were treated at 70% confluence, and fresh media and drug were added simultaneously.

Cloning Survival Formation Efficiency and Soft Agar Anchorage-Independent Growth Assay. To access the effect on cell survival and malignant potential, human melanoma cells were treated with resveratrol or dimethyl sulfoxide for 72 h. After treatment, cells were harvested and seeded into 100-cm² dishes (500 cells/ml) for 10 days, after which cell colonies were stained with methylene blue solution for counting. For soft agar assay, different melanoma cells were grown in the presence or absence of resveratrol, trypsinized, washed in PBS, and a single cell suspension at 1000 cells/ml was obtained. Cell suspension (9.4 ml) was added to 0.6 ml of 5% soft agar/medium, mixed well, and pipetted on the basal agar layer and incubated for about 10 to 14 days. Colonies in unstained cultures were counted with the aid of a low-power microscope. More than 30 aggregated cells were scored as a colony. 20 high-power fields were randomly chosen, and the average surviving colonies per high-power field were scored to assess cloning formation.

Cytofluorometric Analysis. Cells were grown in 100-cm² dishes to 70% confluence, washed with PBS, and collected with PBS/20 mM EDTA. Cells were washed 2 times with cold PBS and direct immunofluorescence analyses of surface antigen were performed as described previously. In brief, the cell pellets were suspended in 95 μl of PBS, specific monoclonal antibodies were added for surface staining, and pellets were incubated in the dark for at least 30 min. The cells were washed with cold PBS three times before assessment by flow cytometry. The following immunoreagents were used: phycoerythrin-labeled anti-human CD95/Fas and fluorescein isothiocyanate-labeled anti-human HLA-A, B, C/MHC class I antibodies (BD Biosciences PharMingen, San Diego, CA, and 1:20 dilution).

Apoptosis and Cell Cycle Assay. Human normal melanocytes or melanoma cells were incubated with resveratrol at different doses for 72 h and analyzed for apoptosis by Annexin V apoptosis detection kit, following the manufacturer's protocol (BD Biosciences PharMingen). Cell cycle parameters were detected as described previously (Tiemann et al., 2003).

Cell Protein Extraction. Nuclear extracts from cells were prepared as described previously with minor changes (Harant et al., 1996; Ausubel et al., 1999). In brief, cells were scraped and suspended in ice-cold PBS, centrifuged (1200 rpm × 5 min), and PBS was aspirated completely. Cells were resuspended in 400 μl of ice-cold buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.150 mM MgCl₂, 0.5 mM DTT, 0.2 mM PMSF) and allowed to swell on ice for 10 min. Then, 25 μl of a 10% Nonidet P-40 solution was added, and cells were lysed by vortexing for 10 s. After 30-s centrifugation, buffer was completely aspirated, and the pelleted nuclei were suspended in 25 μl of buffer C (20 mM HEPES, 20% glycerol, 0.42 M NaCl, 0.15 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and incubated on ice with shaking for >15 min. After centrifugation for 30 min at 14,000g, the extract was removed and diluted with 75 μl of buffer D (20 mM HEPES, 20% glycerol, 50 mM KCl, 0.5 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF). Using a detergent-compatible protein assay kit (Bio-Rad, Hercules, CA), the protein concentration of each nuclear extract sample was precisely measured three times, and the correlation coefficient of standard curve was at least 0.99 to

confirm equivalent protein loading in each lane. Nuclear extracts were stored at $-80°C$. Whole-cell lysates were prepared using cell lysis buffer (Cell Signaling Technology Inc., Beverly, MA) following the manufacturer's protocol. After protein concentration assay, all the samples were stored at $-80°C$.

Electrophoretic Mobility Shift Assay of AP-1 DNA Binding Activity. AP-1-specific binding activity was determined by Gel Shift Assay Systems (Promega, Madison, WI) with optimizations. In brief, nuclear extract was incubated in a final volume of 10 μl containing 2 μl of Gel Shift binding buffer (five times) and 1 μl of ^{32}P-labeled AP-1 consensus oligonucleotides ($\sim 6 \times 10^5$ cpm). Variable additions to the reaction mixture included 2 μl of antibody to individual AP-1 proteins for supershift assay. For oligonucleotide competition experiments, the reaction mixture was preincubated with a 50-fold excess of unlabeled oligonucleotide probes for 20 min before the addition of hot probes. The reaction mixture was separated on 4 to 5% nondenaturing polyacrylamide gels. By using autoradiography, the shift bands were qualified by densitometry. All the antibodies used in EMSA supershift assay were the products of Santa Cruz Biotechnology, Inc. (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1, and Fra-2).

Measuring the Binding of Activated AP-1 Transcription Factor. To study AP-1-dependent gene transcription, we used a reporter vector system containing the secreted alkaline phosphatase (SEAP) gene (BD Biosciences Clontech, Palo Alto, CA). The reporter construct pAP1-SEAP is designed to measure the binding of transcription factors to AP-1, providing a direct measurement of activation for this pathway. After transcription activators bind AP-1, transcription is induced and the reporter gene is activated. The secreted SEAP enzyme is assayed directly from the culture medium using Great EscAPe chemiluminescence detection kits (BD Biosciences Clontech). For the transient transfection, we plated different melanoma cells at a density of 1×10^5/well in a 24-well plate. After 24 h, the cells were transfected with 1 μg of the reporter constructs with the use of the LipofectAMINE Plus reagent kit (Invitrogen, Carlsbad, CA), according to the manufacturer's directions. Sixteen hours later, cells were treated with TPA (50 ng/ml) for 8 h and then changed to fresh media. Forty-eight hours later, the media were collected for SEAP activity assay, which was performed according to the manufacturer's instructions (BD Biosciences Clontech) using microplate illuminometer.

Immunoblot Analysis. Equal amounts of nuclear protein (25 μg) were loaded and electrophoresis was performed in a 10% polyacrylamide separating gel/5% stacking gel. Proteins were transferred to polyvinylidene difluoride membrane, and incubated in blocking solution for 2 h. The membrane was incubated for 1 h at room temperature with primary rabbit antibodies (anti-c-Jun, anti-JunD, anti-Fra-1, and anti-Fra-2 respectively, $\sim 1:2001:1000$). Blots were washed in 0.1% Tris-buffered saline/Tween 20 and incubated with second antibody (peroxidase-conjugated anti-rabbit IgG, $\sim 1:500-1:4000$) for 1 h at room temperature. After extensive washing, the second antibody was visualized by chemiluminescence reagents. Tubulin expression as an equal loading control is also performed with a mouse anti-human tubulin antibody (Sigma-Aldrich).

Reactive Oxygen Probes. Relative intracellular hydrogen peroxide and superoxide anion levels were determined based on methods adapted from those described previously (Meyskens et al., 2001b) using 6-carboxy-2'7'-dichlorodihydrofluorescein diacetate (5 μM) and dihydroethidium (10 μM), respectively (Molecular Probes, Eugene, OR). 6-Carboxy-2'7'-dichlorodihydrofluorescein diacetate diffuses through cell membranes and is hydrolyzed by intracellular esterase, which remains trapped within cells, reacts with hydrogen peroxide and generates the fluorescent 2'7'-dichlorodihydrofluorescein (DCF). The fluorescence intensity is proportional to the amount of peroxide produced by the cells. Dihydroethidium (HE) is the sodium borohydride-reduced form of ethidium bromide that is permeable to viable cells. HE is directly oxidized to ethidium bromide by superoxide anion, which then fluoresces. The red fluorescence is proportional to the intracellular superoxide anion levels. Cells were

incubated with the appropriate probe for 15 min, harvested, and analyzed by flow cytometry using a FACScan with Cell Quest software (BD Biosciences, San Jose, CA). DCF fluorescence was analyzed by using an excitation of 488 nm and filter of 530 nm, whereas HE fluorescence was analyzed using an excitation of 488 nm and filter of 585 nm.

Fra-2 Overexpression in Human Melanoma Cells. The Fra-2 expression vector was constructed by inserting the human Fra-2 cDNA (American Type Culture Collection) into the pcDNA3.1($-$) expression vector (Invitrogen). In brief, the recombinant Fra-2-pT7T3D-Pac plasmid was digested with EcoRI/HindIII, and the 1-kb base pair fragment was ligated to EcoRI/HindIII-digested pcDNA3.1($-$) plasmid and put into the sense direction. For transient transfection, human melanoma cells were plated in six-well plate and when cells were 90% confluent, they were transfected with 2 μg/well expression vector constructs with the use of LipofectAMINE 2000 reagent according to the manufacturer's directions. Forty-eight hours after transfection, cells were harvested, and MHC class I antigen expression levels were detected using flow cytometry as mentioned previously.

Statistical Analysis. All values are presented as the mean $\pm$ S.D. All experiments were repeated at least three times. Statistical significance was evaluated using the unpaired Student's t test for comparison between two means. Differences were considered to be statistically significant at $p \leq 0.05$.

Results

Resveratrol Decreases Intracellular ROS Levels of Human Melanoma Cells. Using the molecular probes DCF and HE we measured, respectively, the relative intracellular levels of hydrogen peroxide and superoxide anion in c83-2c melanoma cells after resveratrol treatment. The DCF fluorescence was decreased by resveratrol as much as 52% compared with control (Fig. 1), whereas HE fluorescence was unchanged after resveratrol treatment, even at high dose of 100 μM for 72 h.

Effects of Resveratrol on Malignant Phenotype of Human Melanoma Cells in Culture. The following properties were assessed: 1) morphological development of dendritic extensions, 2) intracellular melanin content, 3) anchorage-independent growth, and 4) cell surface expression of MHC I and Fas/CD95.

Normal melanocytes (Fig. 2A) have long dendrites that carry melanin to keratinocytes in the epidermis. Melanoma cells generally lack distinct dendrites. The c83-2c melanoma cell line is an extreme example of dendrite loss, and morphologically looks like rounded cells with the additional capacity of vertical growth in vitro and formation of three-dimensional colonies (Fig. 2B). After exposure to 75 μM resveratrol for 72 h, the c83-2c cells flattened, formed colonies two-dimensionally, and became dendritic (Fig. 2C). Similar morphological changes were also induced in primary radial growth phase-like wm3211 melanoma cells with resveratrol treatment (Fig. 2D, control; E, with resveratrol treatment). These alterations were associated with a more than 4-fold increase in total cellular melanin content, both in primary wm3211 and metastatic c83-2c melanoma cells (data not shown).

Reversal of malignant properties is usually accomplished by reduced anchorage-independent growth in soft agar. Resveratrol pretreatment of c83-2c cells for 3 days dramatically decreased cell soft agar colony formation (Fig. 3, A–C). Both the number and size of colonies were significantly reduced compared with control (15.5 $\pm$ 1.3 per high-power field, in

control; 4.9 $\pm$ 0.6, with resveratrol treatment) (Fig. 3C), which suggests that resveratrol pretreatment produced an irreversible phenotypic changes rather than a growth effect although more replating experiments would be required to prove this assertion.

The expression levels of MHC class I antigen and Fas were determined using intracellular cytofluorometric analysis after incubation of c83-2c and wm3211 melanoma cells with resveratrol. Constitutive surface levels of MHC class I were markedly enhanced after 72-h treatment with 75 μM resveratrol, both in wm3211 and c83-2c cells. The mean of the fluorescence density increased to 327.9 and 268.4%, respectively (control as 100%; Fig. 4, A and B, respectively). In wm3211 cells, a concentration of 10 μM or greater produced a significant induction of Fas expression compared with control, whereas in c83-2c cells such induction was only evident at a higher concentration (75 μM). At concentration of 75 μM, Fas/CD95 expression was elevated respectively to 198.8 and 162.4% of control in wm3211 and c83-2c cells (Fig. 4, C and D, respectively).

Antiproliferative Effect of Resveratrol on Human Melanoma Cells. c83-2c human melanoma cells were pretreated with resveratrol (75 μM) for 72 h, and followed by monolayer colony formation assay. After 8 days, the number of colonies was counted using a microscope. The capacity of colony formation was presented as mean of colonies per high-power field (Fig. 3D). Either with pretreatment or by coincubation with resveratrol, the cell colony formation was significantly reduced compared with control ($p < 0.05$).

Resveratrol Failed to Induce Apoptosis or Cell Cycle Arrest in Human Melanoma Cells. Flow cytometry and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium results revealed that at all the doses of resveratrol tested there was not a significant increase in apoptosis in different melanoma cells (c83-2c and c81-46A) compared with untreated control (data not shown). The addition of resveratrol (75 μM) to normal melanocytes for 3 days also had little effect on cell viability (data not shown). Cell cycle analysis also demonstrated that there is no distinct G1 or G2/M arrest with resveratrol (100 μM) treatment for 72 h (data not shown).

PARP can be cleaved by many ICE-like caspases in vitro and is one of the main cleavage targets of caspase-3. Cleavage of PARP facilitates cellular disassembly and serves as a marker of cells undergoing apoptosis. We found by Western

blot analysis that even after treated of c83-2c melanoma cells with a high dose of resveratrol (100 μM), no cleaved PARP bands were evident (Fig. 5A).

We also assessed the effect of resveratrol on the expression of apoptotic regulators such as Bcl-2 and Bax. In c83-2c cells treated with resveratrol, Bcl-2 was unaffected even at a dose of 100 μM. Proapoptotic Bax was unexpressed or in undetectable levels in c83-2c cells, which is consistent with reports that Bax expression was absent in most melanoma cell lines (Bowen et al., 2003). No induction of Bax was observed with resveratrol treatment (Fig. 5A).

AP-1 Activity Was Inhibited by Resveratrol Treatment. c83-2c human melanoma cells were treated with 75 μM resveratrol for 72 h, nuclear extracts were prepared, and EMSA assay was performed with a labeled oligonucleotide containing the TPA response element consensus sequence TGACTCA (Fig. 6, A and B). Two strong bands were observed and the upper one, altered with resveratrol treatment, was totally displaced by a 50-fold excess of unlabeled AP-1 consensus oligonucleotides, indicating that this band represented the specific binding of AP-1 binding protein to the AP-1 probe. Quantitated by densitometry, the result revealed that resveratrol reduced AP-1 DNA binding activity as much as 48% in c83-2c cells.

Because AP-1 DNA binding activity does not necessarily reflect AP-1 transcription activity (Karin and Hunter, 1995), we performed transfection with an AP-1-SEAP reporter vector system containing synthetic AP-1 element and a SEAP gene (Fig. 6C). Once AP-1 transcription is induced, SEAP gene is activated and detected. A pSEAP 2 vector was transfected as positive control. The results showed that both basal and TPA-induced binding of activated AP-1 transcription factor were significantly decreased by resveratrol (from 6.3- to 3.0-fold and from 7.0- to 1.7-fold of positive control, respectively)

Heterogeneous Composition Changes of AP-1 with Resveratrol Treatment. A major level of control of AP-1 activity is at the level of AP-1 composition and DNA binding activity. To investigate whether there are changes in the composition of AP-1 DNA binding complexes, we used the EMSA supershift assay using specific antibodies to c-Jun, JunB, JunD, c-Fos, Fos B, Fra-1, and Fra-2, respectively (Fig. 6B).

With nuclear extracts from untreated control c83-2c cells, AP-1 complexes were shifted by anti-c-Jun, anti-JunD, and
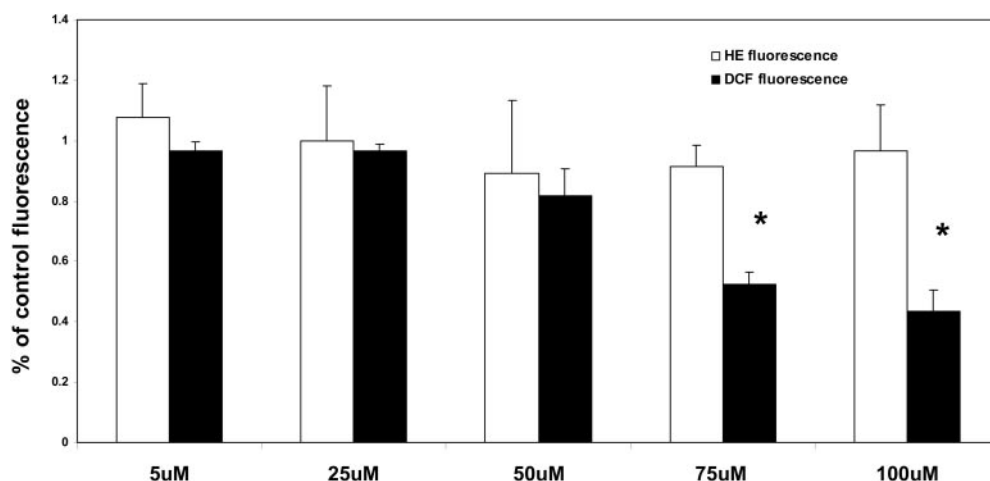
Fig. 1. Resveratrol reduced the intracellular ROS level in human melanoma c83-2c cells. Intracellular contents of peroxide and generation of superoxide were estimated as indicated under *Materials and Methods* and are expressed as fluorescence intensity as a percentage of control, with the control value set at 100%. Data presented as percentage of control intracellular DCF and HE fluorescence, respectively, means $\pm$ S.D. for three independent experiments. Significant difference from control is *, $p < 0.05$.

anti-Fra-1 antibodies, whereas in cells with resveratrol treatment, we observed that c-Jun antibody did not interact with these AP-1 complexes and anti-Fra-1 to a lesser extent. It is noteworthy that addition of anti-Fra-2 antibody unexpectedly shifted the AP-1 complex to a lower level compared with control.

Expression of AP-1 Family Members with Resveratrol Treatment. We further tested the protein expression levels of various AP-1 members in nuclear extracts from c83-2c cells with resveratrol treatment at different doses or for different times (Fig. 7). Western blot analysis showed that the nuclear expression of c-Jun was unchanged, even at high dose of resveratrol, whereas JunD levels were increased at doses of 75 and 100 μM (Fig. 7A). Fra-2 was expressed at a low level in control cells and greatly increased by 75 and 100 μM resveratrol. Fra-2 expression level was elevated by 12-h

treatment and reached a peak at 24 h, before any morphological changes occurred (Fig. 7B). Similar induction of Fra-2 was also found in primary wm3211 and metastatic c81-46A melanoma cells (Fig. 7C). JunB and FosB transcripts were undetectable, either in absence or presence of resveratrol, even after incubation at high concentration or prolonged duration (data not shown).

We also investigated the effect of resveratrol on MAPK, which has been reported as being depressed by resveratrol in many systems. With resveratrol treatment, phosphor-p54 SAPK/JNK level was decreased compared with control at the dose of 50 μM and greater (Fig. 5B). However, in c83-2c human melanoma cells, there are no significant changes of the p44/p42 MAP kinase, and only small decreases of phosphor-p44/42 MAP kinase expression occurred with resveratrol treatment compared with control (Fig. 5B).
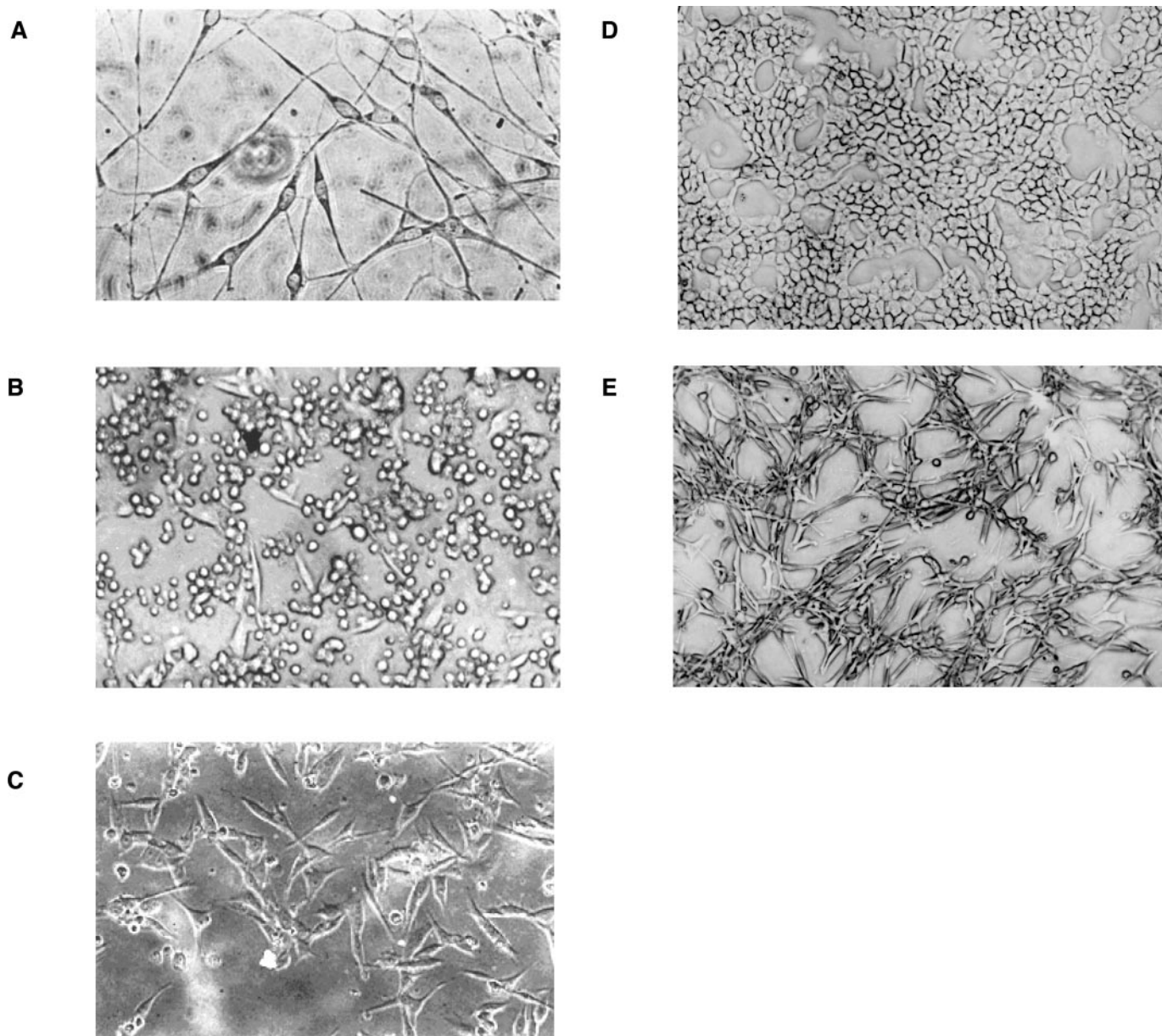

Fig. 2. Melanoma cells with resveratrol treatment undergo morphology changes. Photographs were taken after 3 days in culture. A, normal melanocytes (100×). B, control c83-2c melanoma cells (40×). C, c83-2c cells with 75 μM resveratrol treatment (40×). D, control w3211 primary melanoma cells (40×). E, wm3211 cells with 75 μM resveratrol treatment (40×).

Fra-2 Plays an Important Role in Resveratrol Treatment. Unlike c-Fos, Fra-2 proteins lack a transcriptional activation function. Therefore, the preferential induction of Fra-2-containing AP-1 complexes may explain the inhibitory effect of resveratrol. To examine this possibility, we transiently cotransfected Fra-2 expression construct and AP-1 reporter vector into c83-2c cells (Fig. 8A). Compared with control, this resulted in a significant reduction of AP-1 transcriptional activity and TPA-induced transcriptional transactivation activity ($p < 0.05$).

To investigate whether elevated Fra-2 could interfere with MHC class I and Fas/CD95 expression, we also transiently overexpressed Fra-2 in human melanoma A375 cells, which only has JunD detectable in the AP-1 complex (Yang et al., 2004). The expression level of MHC class I and Fas/CD95 were detected by cytofluorometric analysis. Results revealed that Fra-2 overexpression resulted in significant elevation of MHC class I and Fas expression (2.0- and 1.8-fold of control vector; Fig. 8, B and C, respectively), whereas no changes in cell viability or apoptosis were evident (data not shown). After a 48-h transfection, no significant morphologic changes were observed either.

Additional H_2O_2 (10 μM) Only Partially Reversed the Effects of Resveratrol in Human Melanoma Cells. To investigate the link between ROS reduction and cell proliferation inhibition, we incubated c83-2c cells in the presence of 75 μM resveratrol (which greatly inhibited melanoma cell proliferation) and/or 10 μM H_2O_2 (a low, nontoxic dose).

Under these conditions, controls formed 569 $\pm$ 21 colonies per dish and resveratrol reduced it to 121 $\pm$ 14; the colony number of c83-2c cells in presence of resveratrol and H_2O_2 was significantly elevated to 342 $\pm$ 25 compared with resveratrol alone (Fig. 9A). In contrast, no evident changes were observed in MHC class I and Fas expression when cells were coincubated with H_2O_2 and resveratrol in c83-2c (Fig. 9B) and wm3211 cells (data not shown) compared with resveratrol alone.

EMSA and supershift results revealed that neither AP-1 DNA binding activity nor composition was significantly changed with H_2O_2 (10 μM) treatment alone (Fig. 9C). However, compared with resveratrol treatment alone, H_2O_2 addition not only markedly increased its AP-1 DNA binding activity but also elevated c-Jun protein expression levels (Fig. 9D) and restored the participation of c-Jun in the AP-1 complex. Western blot also showed that the addition of H_2O_2 alone had no notable effects on Fra-1 and Fra-2 expression (Fig. 9D). When combined with resveratrol, H_2O_2 addition also did not affect the induced Fra-1 and Fra-2 levels. Compared with control, H_2O_2 alone did not inhibit JunD expression, whereas when combined with resveratrol, H_2O_2 significantly decreased the induced JunD. In toto, these results suggest that the reduction of intracellular ROS levels only partially contributed to the effects of resveratrol on AP-1 binding and its dimer composition.
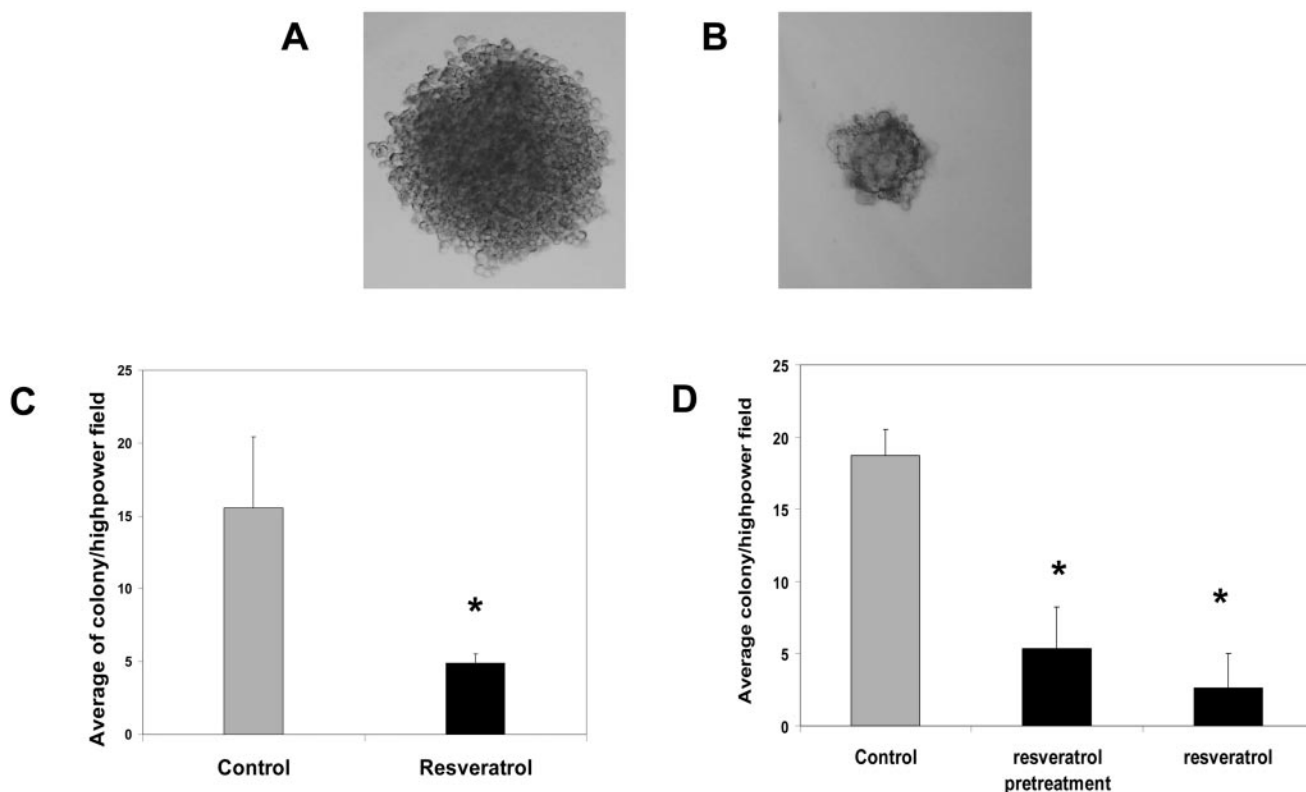

Fig. 3. A to C, inhibition of anchorage-independent growth of human melanoma cells with resveratrol pretreatment in soft agar. c83-2c cells were pretreated in presence of resveratrol (75 μM) or dimethyl sulfoxide for 72 h, and then seeded onto 60-mm dishes in medium containing 0.3% agar on top of a bed medium containing 0.5% agar. After 14 days, colony formation was suppressed by resveratrol both in size (B) and number (C) compared with control (A). The same original magnification (100$\times$) was used for taking both photos. D, resveratrol reduced colony proliferation of human melanoma cells in monolayer culture. Cells cultured in the presence or pretreated with resveratrol for 72 h were measured as described previously. The data in C and D are present as number of colonies per high-power field, means $\pm$ S.D. of 20 independent high-power fields. $*$, $p < 0.05$ compared with control.
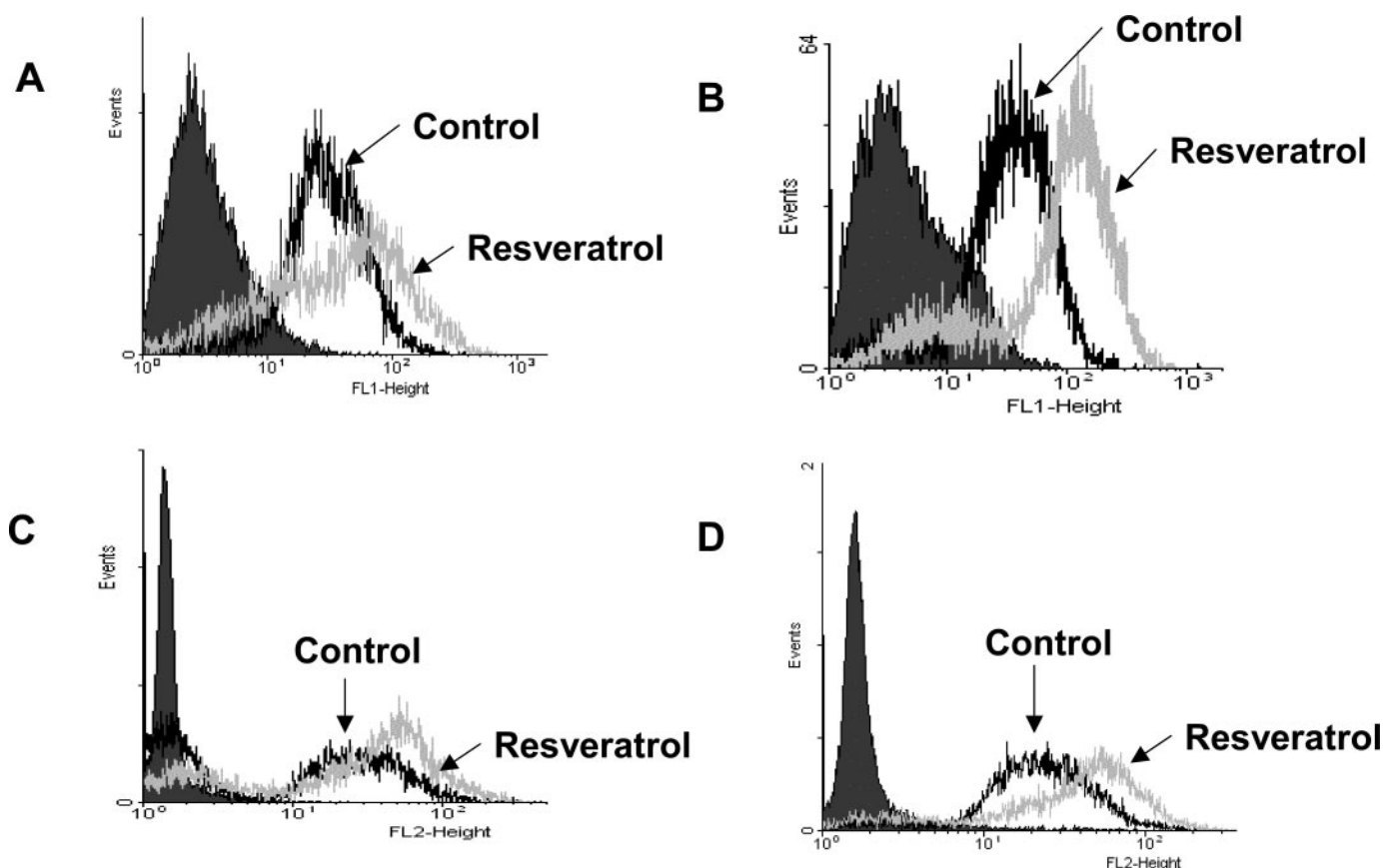
Fig. 4. Enhancement of MHC class I and Fas/CD95 expression by resveratrol in human melanoma cells. Melanoma cells were treated with resveratrol (75 μM) for 72 h, and the expression of MHC I and Fas was detected using monoclonal anti-human MHC I antibody and anti-human Fas/CD95 antibody, respectively, by cytofluorometric analysis. A and B, expression of MHC class I in wm3211 and c83-2c cells respectively. C and D, expression of Fas in wm3211 and c83-2c cells, respectively. Negative, black line with gray fill; control, black line without fill; resveratrol treatment, light line without fill.

Discussion

Resveratrol is one of the most interesting compounds being examined for its chemoprevention and possibly therapeutic capacities against human malignancy. Our investigations demonstrated that resveratrol significantly inhibited human melanoma cell proliferation in vitro. In this study, we have made two unique observations. First, resveratrol induced the immunological repertoire in melanoma to a more favorable phenotype, as measured by surface expression of MHC class I and Fas, an observation that lends itself to new immunological manipulations. Second, further mechanistic studies demonstrate that the shift of AP-1 composition to specific dimerization by resveratrol plays a critical role in the differentiation of human melanoma, thereby allowing future exploration of a unique molecular strategy involving manipulations of AP-1 dimers. We propose that restoration of AP-1 transcription signaling, mediated by changes in AP-1 dimeric composition and reduced intracellular ROS level, substantially contributes to the phenotypic changes induced by resveratrol.

It is well known that a reduction in tumor antigen presentation is one of several mechanisms by which tumors evade immune recognition. MHC class I expression is altered in 10 to 20% of primary melanoma and in ~60% of metastatic melanomas, which may therefore be an obstacle to cytotoxic T lymphocyte-based immunotherapy (Geertsen et al., 1998).
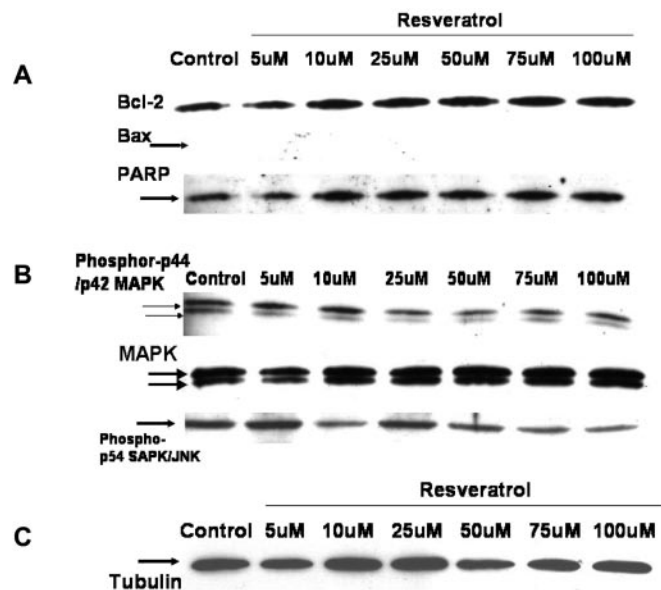

Fig. 5. No significant changes were evident with resveratrol treatment on the expression of apoptotic regulators (A) and MAPK (B). Whole cell extracts (50 μg) from c83-2c cells with resveratrol 72-h treatment at different doses were subjected to Western blotting using certain antibodies against Bcl-2, Bax, PARP, p44/42 MAP kinase (ERK1/2), phosphor-p44/42 MAP kinase, and phosphor-p54 SAPK/JNK. Representative blots were shown, respectively. Each blot was reblotted with antibody to tubulin to confirm equivalent protein loading in each lane (C).

During neoplastic progression, decreased MHC class I antigen expression may be selected to facilitate their escape from immune destruction by cytotoxic T lymphocytes, and associated with uncontrolled growth and metastatic progression in malignant cells. The restoration of MHC I expression seems to be correlated with restoration of growth control mechanisms in malignant cells. A functional Fas signal in tumor cells is therefore a prerequisite for effective Fas-mediated tumor cell destruction by immune system. Bullani et al. (2002) investigated Fas expression and function in 13 melanoma cell lines and in situ and found that the down-regulation of Fas expression and resistance to Fas-mediated apoptosis are frequent in human melanoma, which might contribute to immune evasion and tissue metastasis. The efficient induction of MHC class I and Fas expression by resveratrol may represent a very novel and useful therapeutic approach to the management of human malignant melanomas.

An important level of regulation of AP-1 occurs, though, controlling not only its activity but also the concentration of certain AP-1 member proteins in the nucleus. The specific subunit composition of AP-1 complex could reflect dynamic changes in AP-1 transcription activities and subsequently its target genes as well as the biological function that is dynamically altered (Eferl and Wagner, 2003). AP-1 DNA binding activity and transcriptional activity are markedly decreased by resveratrol with significant changes in its member proteins. Our data showed that Fra-1 and Fra-2 expression level is significantly elevated during the course of resveratrol treatment, and EMSA supershift assay also indicates that after treatment JunD dimerizing with Fra-1/Fra-2 becomes the major component of the AP-1 complexes instead of c-Jun and JunD with Fra-1. Although capable of heterodimerization with Jun proteins and binding to AP-1 sites, the Fra-2 protein is devoid of transcriptional activation function (Suzuki et al., 1991). Indeed, we found that transient transfection of Fra-2 significantly suppressed AP-1 transcription activity as well as TPA-induced transcriptional transactivation. These results suggest that inhibition of AP-1 by resveratrol might be secondary to the induction of Fra-2 expression. Furthermore, overexpression of Fra-2 in A375 cells, which only has JunD in the AP-1 complex, increased MHC class I antigen and Fas/CD95 expression significantly, whereas no induction of apoptosis was observed. Our results are consistent with the study of Andreucci et al. (2002). Their study confirms and extends our observation that during muscle differentiation, AP-1 function was dependent on the composition of the Fra-2 and c-Jun/JunD AP-1 dimers. In addition, the role of specific Fra-2 and JunD AP-1 complexes recently has been implicated in cellular differentiation in ovarian granulose (Sharma and Richards, 2000) and osteoblasts (McCabe et al., 1996). These studies demonstrated that JunD and Fra-2 were induced by luteinizing hormone and maintained as granulose cells terminally differentiated into luteal cells and that in fully differentiated osteoblasts Fra-2 and JunD are the
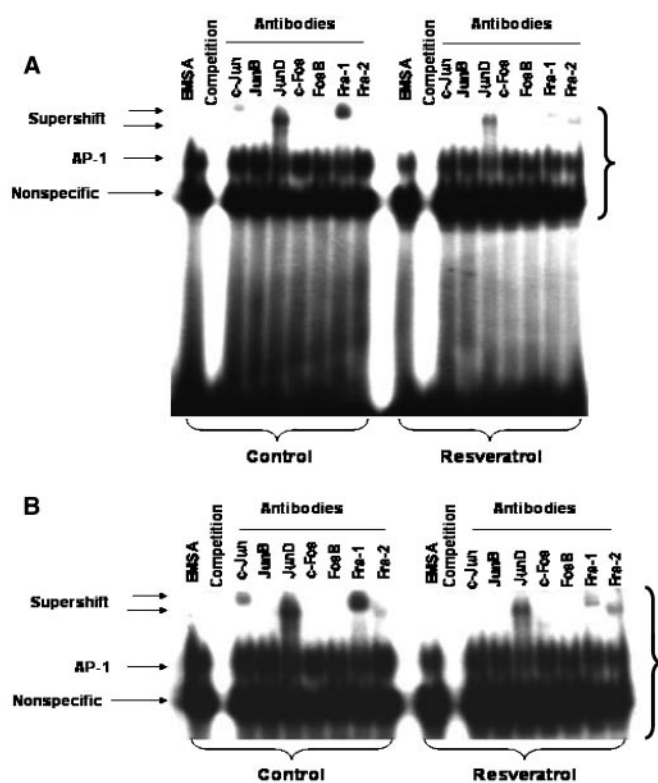

Fig. 6. Both AP-1 DNA binding and transcriptional activities were reduced by resveratrol in human melanoma c83-2c cells, associated with AP-1 composition changes. A, EMSA with ^{32}P-labeled AP-1 oligonucleotide probe was performed as described under *Materials and Methods*, and 50× excess unlabeled AP-1 oligonucleotides were added for the specific competition and 50× excess unlabeled nuclear factor-κB oligonucleotides were added for nonspecific competition. The nuclear extracts were subjected to EMSA in absence or presence of antibodies to the indicated AP-1 proteins, respectively. B, close-up view of the bracketed region of A with decreased intensity to improve visualization of the bands. C, inhibitory effect of resveratrol (75 μM) on AP-1 transcriptional activity and TPA-induced transcriptional transactivation activities in human melanoma cells using AP-1/SEAP reporter vector. Melanoma c83-2c cells were transiently transfected with AP-1/SEAP vectors containing the consensus AP-1 site. AP-1 binding or transcriptional transactivation by TPA result in activation of the SEAP gene, and protein activity was quantitated by luminometer. The relative SEAP activity was presented as folds of that of positive control vector pSEAP2, means ± S.D., of three independent experiments. Significant difference from AP-1 is ∗, $p < 0.05$ and from AP-1/TPA is #, $p < 0.05$.
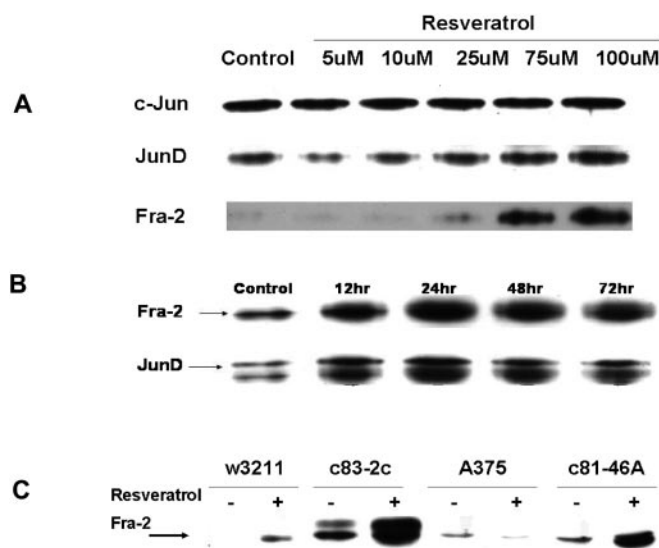

Fig. 7. Expression of AP-1 members in human melanoma cells with resveratrol treatment. Nuclear extracts (25 μg) prepared as described under *Materials and Methods* were subjected to Western blotting using certain antibodies against c-Jun, JunD, and Fra-2, respectively. A, c83-2c cells with resveratrol treatment at different doses for 72 h. B, c83-2c cells treated with 75 μM resveratrol for different time. C, different melanoma cell lines treated with 75 μM resveratrol for 72 h.

principal AP-1 members. We propose that with resveratrol treatment, a different set of AP-1 target genes might be activated subsequently by induction of Fra-containing AP-1 complexes forming new JunD/Fra-2 dimer, which is closely related to the cell differentiation process. It will thus be of considerable interest to determine how Fra-2/JunD affects downstream genes; future studies using stable transfected cell lines will specifically address the effects of modulating Fra-2 expression on differentiation in human melanoma.

It is noteworthy that Western blot showed that with resveratrol treatment no significant changes of c-Jun protein level was observed, whereas supershift results demonstrated that c-Jun was reduced to an undetectable level in the AP-1 complex. It has been well established that modification of c-Jun can be achieved by altering its phosphorylation status

by JNK and thereby enhanced Jun-dependent transcriptional activity (Vogt, 2002). Based on our observation, one possible explanation is that reduced phosphor-SAPK/JNK level by resveratrol leads to a reduction of c-Jun phosphorylation and inhibited its binding to nuclear TPA response element and transcription subsequently. It is well documented that JNK is activated by both exogenous and endogenously produced H_2O_2 (Iles and Forman, 2002). After coincubation with resveratrol, H_2O_2 effectively restored the involvement of c-Jun in AP-1 complexes by EMSA supershift assay, with elevated c-Jun protein expression level.

Our previous studies have revealed that melanoma cells produced large amounts of intracellular superoxide anion compared with normal melanocytes (Meyskens et al., 2001b). Recent studies have shown that trans-activation and the
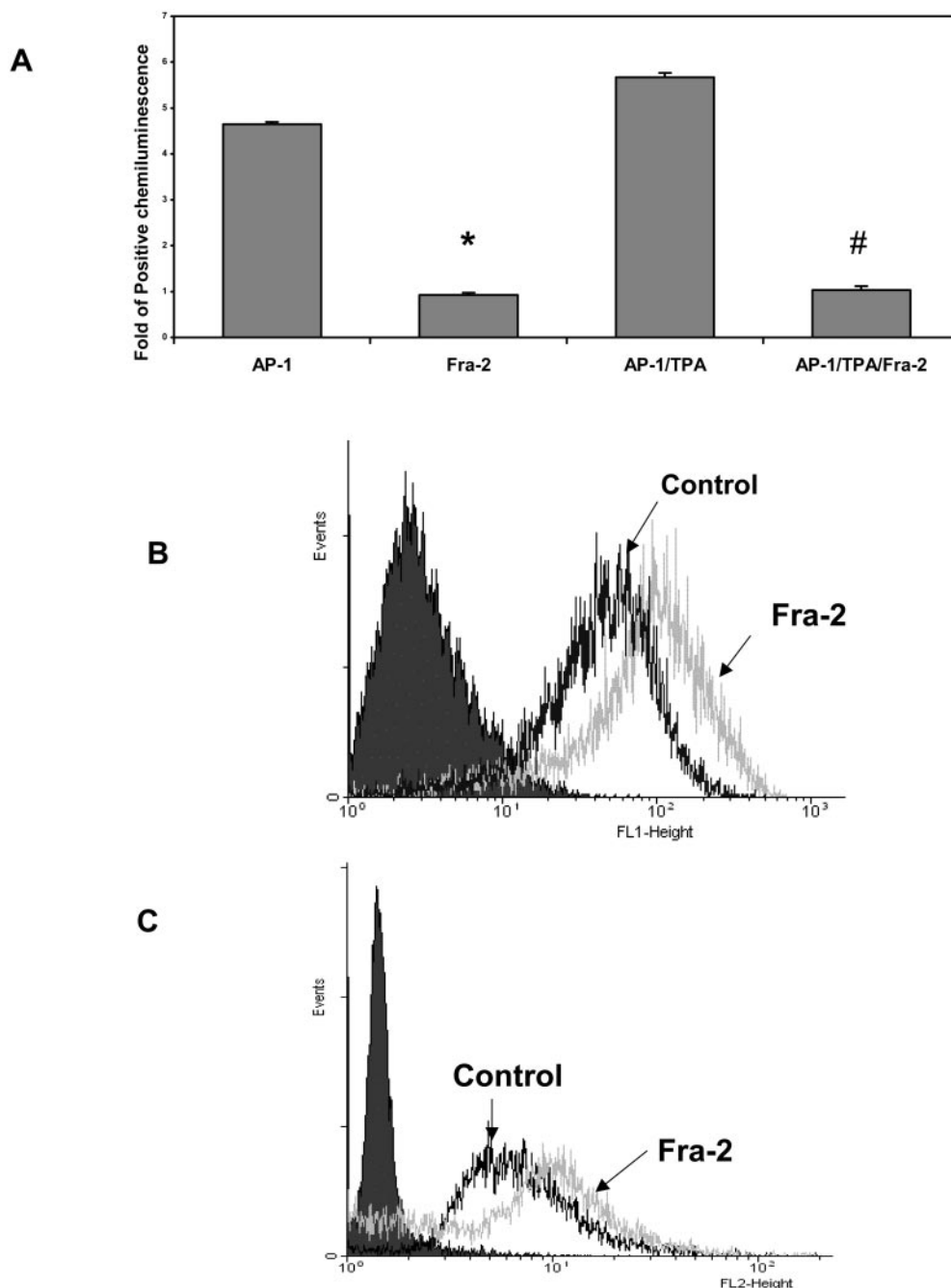

Fig. 8. A, Fra-2 overexpression inhibited AP-1 transcriptional activity and TPA-induced transcriptional transactivation in human melanoma c83-2c cells. Cells were cotransfected with AP-1/SEAP reporter vector and Fra-2 expression construct for 24 h, followed by changing fresh medium with or without TPA (50 ng/ml). Forty-eight hours later, collect culture supernatants for chemiluminescence assay as described under *Materials and Methods*. The relative SEAP activity was presented as folds of that of positive control vector pSEAP2, means ± S.D., of three independent experiments. Significant difference from AP-1 is *, $p < 0.05$ and from AP-1/TPA is #, $p < 0.05$. B and C, enhancement of MHC class I and Fas expression by Fra-2 overexpression in human melanoma A375 cells. Melanoma cells were transfected with 2 μg of Fra-2 expression or control vector for 48 h. The expression level of MHC class I (B) and Fas/CD95 (C) were detected using monoclonal anti-human MHC class I antibody and anti-human Fas/CD95 antibody, respectively, by cytofluorometric analysis. Black line with gray fill represents the negative control cells stained with an isotype-matched negative control antibody; black line without fill represents pcDNA3.1(−)-transfected cells; gray line without fill represents Fra-2-overexpressed cells.

DNA binding affinity of AP-1 can be modulated not only by posttranslational modifications such as phosphorylation or dephosphorylation but also by alterations of the intracellular redox status (Rosl et al., 1997). Our data showed that intracellular ROS levels in melanoma were significantly decreased by resveratrol treatment. In addition to it's being a potent ROS scavenger, another explanation for the antioxidant effect of this compound may be its induction of cellular melanin, because the ability of melanin to neutralize ROS has been well documented (Meyskens et al., 2001a); a superoxide dismutase-like activity has also been demonstrated for isolated melanin (Bustamante et al., 1993). AP-1 DNA binding activity and transcriptional activity were both reduced with resveratrol treatment, whereas addition of extra H_2O_2 in media significantly prevented the inhibitory effect of resveratrol on cell proliferation and increased AP-1 DNA binding activities. However, H_2O_2 had little effect on resveratrol-induced Fra-1 and Fra-2 protein levels, as well as MHC class-I and Fas expression or cell morphological changes. These results suggested that intracellular redox status only contributed partially to the effects of resveratrol on the various cellular properties.

Fra-1 and Fra-2 are substrates for phosphorylation by the ERK_S (Gruda et al., 1994). Our results also showed that there were no significant changes in ERK and only small decreases of phosphor-p44/p42 ERK expression levels with resveratrol treatment and indicated that the effects of resveratrol on Fra-1 and Fra-2 in human melanoma are not directly associated with ERK signaling, which is in contrast to some other studies in other cell types. Yu et al. (2001) demonstrated that resveratrol inhibited AP-1 activation by interfering with MAPK pathways in HeLa cells, and She et al. (2001) reported that in a mouse JB6 epidermal cell line MAP kinases and ERKs are involved in resveratrol-induced apoptosis and p53 activation. However, the regulation of AP-1 is clearly cell and tissue type specific (Eferl and Wagner, 2003).

Our data show that resveratrol exhibits a promising inhibitory effect on human melanoma cell growth and induction of biological features consistent with a more normal phenotype. However, the relationship between alterations in AP-1 composition and transcription of differentiation-related genes has been poorly explored. As reviewed by Kang et al. (1998), the potential role of AP-1 in human melanoma differentiation
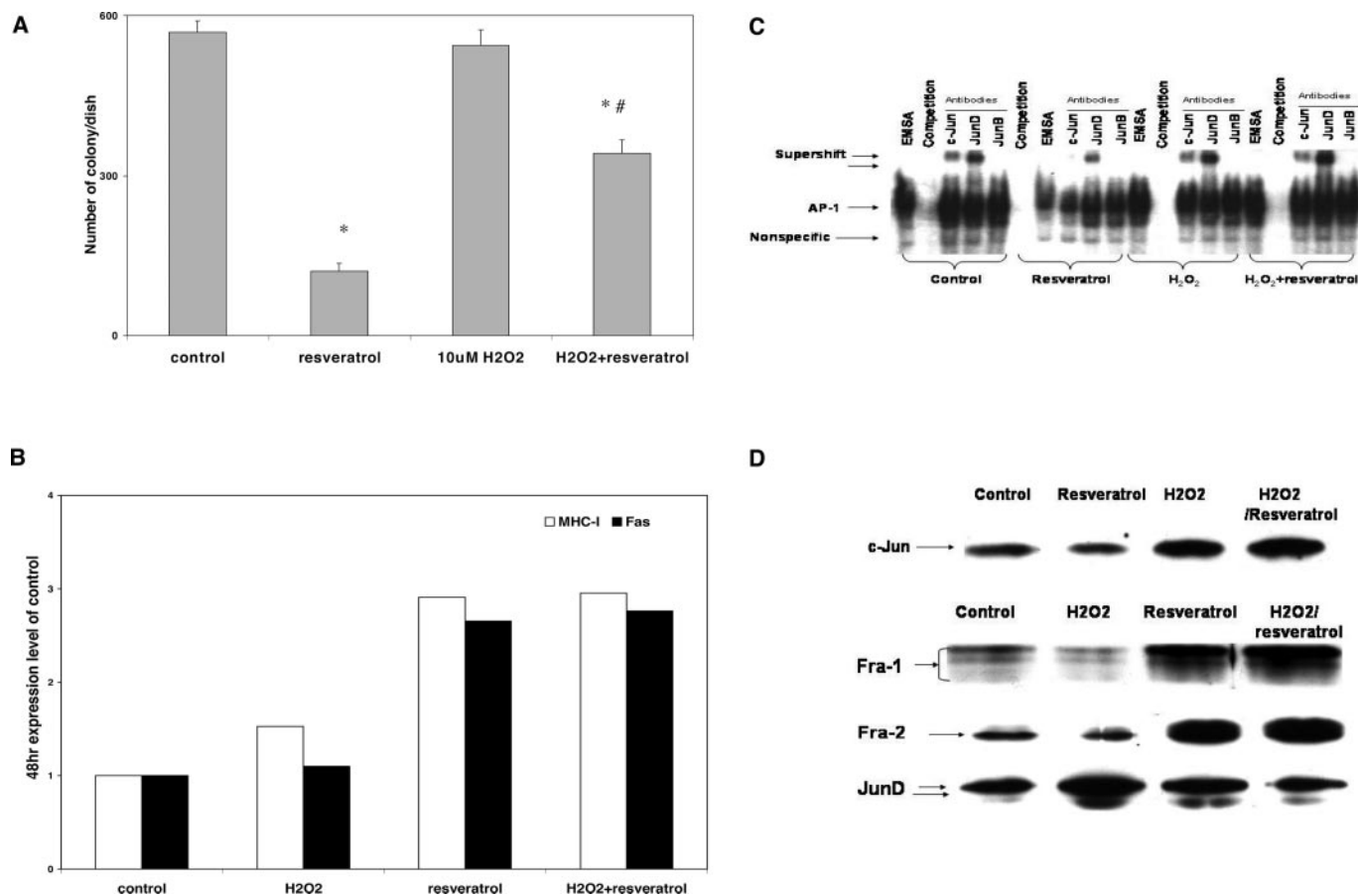

Fig. 9. Additional H_2O_2 (10 μM) only partially reversed the effects of resveratrol. A, c83-2c cells were incubated with 10 μM H_2O_2 and/or 75 μM resveratrol for 72 h before colony survival formation analysis. Significant difference from control is *, $p < 0.05$ and from resveratrol is #, $p < 0.05$. B, c83-2c cells were treated with 75 μM resveratrol in the presence or absence of H_2O_2 (10 μM) for 72 h, and then expression of MHC-I and Fas were detected using monoclonal anti-human MHC class I antibody and anti-human Fas/CD95 antibody, respectively, by cytofluorometric analysis. C, EMSA with ^{32}P-labeled AP-1 oligonucleotide probe was used as described under *Materials and Methods*, and 50× excess unlabeled AP-1 oligonucleotides were added for the specific competition and 50× excess unlabeled nuclear factor-κB oligonucleotides were added for nonspecific competition. The nuclear extracts were subjected to EMSA in absence or presence of antibodies to the indicated AP-1 proteins. Close-up view of the supershifted region with decreased intensity improves visualization of the bands. D, effect of H_2O_2 (10 μM) on the expression levels of AP-1 members alone or combined with resveratrol. Nuclear protein extracts (25 μg) prepared from c83-2c cells treated with resveratrol (75 μM) in the presence or absence of H_2O_2 for 72 h were subjected to Western blotting using specific antibodies against c-Jun, JunD, Fra-1, and Fra-2. Representative blots are shown.

remains to be explored in depth. Results from this current investigation, together with our previous observations that AP-1 composition has been altered during progression (Yamanishi et al., 1991; Yang et al., 2004), indicate that the abnormalities of AP-1 dimer composition in human melanoma cells may offer a unique preventive and therapeutic target for intervention of human melanoma.

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