Resveratrol Enhances Chemosensitivity in Mouse Melanoma Model Through Connexin 43 Upregulation

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ABSTRACT: Although current studies indicate that resveratrol exhibits potential antitumor activities, the precise mechanisms of its beneficial effects combined with chemotherapy are not fully understood. This work is warranted to elucidate the underlying mechanism of antitumor effects by the combination therapy of resveratrol and cisplatin. The presence of functional gap junctions is highly relevant for the success of chemotherapy. Gap junctions mediate cell communication by allowing the passage of molecules from one cell to another. Connexin (Cx) 43 is ubiquitous and reduced in a variety of tumor cells. Cx43 may influence the response of tumor cells to treatments by facilitating the passage of antitumor drugs or death signals between neighboring tumor cells. Following resveratrol treatment, dose-dependent upregulation of Cx43 expressions was observed. In addition, gap junction intercellular communication was increased. To study the mechanism underlying these resveratrol-induced Cx43 expressions, we found that resveratrol induced a significant increase in mitogen-activated protein kinases (MAPK) signaling pathways. The MAPK inhibitors significantly reduced the expression of Cx43 protein after resveratrol treatment. Specific knockdown of Cx43 resulted in a reduction of cell death after resveratrol and cisplatin treatment. Our results suggest that treatment of resveratrol in tumor leads to increase Cx43 gap junction communication and enhances the combination of resveratrol and cisplatin therapeutic effects. Environ Toxicol 30: 877–886, 2015.

Keywords: gap junctions; connexin 43; resveratrol; cisplatin

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INTRODUCTION

Gap junctions mediate cell communication by allowing the passage of molecules from one cell to another. The major role of gap junction intercellular communication (GJIC) is considered to be the maintenance of homeostasis in organisms (Sharrow et al., 2008). Gap junctions are formed by two hemichannels, called connexons, each made of six connexin (Cx) proteins. Cx43 is ubiquitous and reduced in a variety of tumor cells (Wang et al., 2007). Cx43 may influence the response of tumor cells to treatments by facilitating the passage of antitumor drugs or death signals between neighboring tumor cells (Mancuso et al., 2011). Many tumor cells are characterized by dysfunction of Cx43 (Chang et al., 2013a).

Resveratrol is found in grapes and red wine that exerts several biological activities. Resveratrol may play important role in anti-inflammation and targets for the NF-κB signaling pathway could be used as effective therapies for inflammatory-mediated disease. Previously, we found that the resveratrol exerts therapeutic effects on reducing tumor metastasis and prolonging survival of the tumor-bearing mice (Chen et al., 2012). However, little is known about the relationship between resveratrol and Cx43 expression in molecular mechanisms. In this study, we want to elucidate the underlying mechanism of antitumor effects by the combination therapy of resveratrol and cisplatin.

MATERIAL AND METHODS

Cell Lines, Reagents, and Mice

Murine K1735 melanoma (Lee et al., 2010), murine B16F10 melanoma (Lee et al., 2004), were cultured in Dulbecco's modified Eagle's medium supplemented with 50 μg/mL gentamicin, 2 mM L-glutamine, and 10% heat-inactivated fetal bovine serum at 37°C in 5% CO_2. Murine K1735 cells were kindly provided by Dr. MC Hung (The University of Texas M. D. Anderson Cancer Center). Resveratrol, and DMSO were purchased from Sigma Aldrich (Sigma Aldrich, St. Louis, MO).The working concentrations of the various inhibitors were as follows: 25 μM SB203580 (Sigma-Aldrich), 20 μM PD98059 (Sigma), or 10 μM SP600125 (Sigma). Cells were pretreated various inhibitors for 1 h, then resveratrol (4μg/mL) was added to cells for 24 h. Male C57BL/6 mice at the age of 6 to 8 weeks were purchased National Laboratory Animal Center of Taiwan. The animals were maintained in specific pathogen-free animal care facility under isothermal conditions with regular photoperiods. The experimental protocol adhered to the rules of the Animal Protection Act of Taiwan, and was approved by the Laboratory Animal Care and Use Committee of the China Medical University (the permit number: 99-20-N).

Immunoblot Analysis

The protein content in each sample was determined by bicinchoninic acid (BCA) protein assay (Pierce Biotechnology, Rockford, IL). Proteins were fractionated on SDS-PAGE, transferred onto Hybond enhanced chemiluminescence nitrocellulose membranes (Amersham, Little Chalfont, UK), and probed with antibodies against Cx43 (Sigma-Aldrich), extracellular signal-regulated kinase (ERK; Abcam, Cambridge, UK), phosphor-ERK(Abcam), p38 (Abcam), phosphor-p38 (Abcam), c-jun N terminal kinase (JNK; Abcam), phosphor-JNK (Abcam) or monoclonal antibodies against β-actin (AC-15, Sigma Aldrich). Horseradish peroxidase-conjugated goat anti-mouse IgG or anti-rabbit IgG (Jackson, West Grove, PA) was used as the secondary antibody and protein-antibody complexes were visualized by enhanced chemiluminescence system (Amersham). The signals were quantified with ImageJ software (rsbweb.nih.gov/ij/; Liu et al., 2013).

Analysis of Cx43 Transcriptional Activity

Cells grown in 24-well plates were cotransfected with luciferase reporter plasmids driven by Cx43 promoters (0.66 μg) and pTCYLacZ (0.34 μg), a β-galactosidase (β-gal) expression plasmid driven by the β-actin promoter, by lipofectamine 2000 (Invitrogen, Carlsbad, CA). At 6 h post-transfection, cells were treated with resveratrol and cell lysates were harvested at different concentrations. The cell lysates were assessed for their luciferase activities determined by a dual-light luciferase and β-gal reporter gene assay system (Promega, Madison, WI) using a luminometer (Minilumate LB9506, Bad Wildbad). Relative luciferase activity was measured as luciferase activity divided by β-gal activity to normalize transfection efficiency per microgram protein. The protein content in each sample was determined by the BCA protein assay (Pierce).

Knockdown of Cx43

The specific shRNA of Cx 43 plasmids were purchased from Santa Cruz Biotechnology (sc-35091-SH, Santa Cruz Biotechnology, Santa Cruz, CA). K1735 or B16F10 cells were transfected with Cx43 shRNA plasmids by lipofectamine 2000 (Invitrogene).

Scrape Loading and Dye Transfer Analysis

Levels of gap junctional intercellular communication in control and treated cell in culture were determined scrape loading and dye transfer technique using a fluorescent dye, lucifer yellow (Sigma-Aldrich). Scrape loading was performed applying cuts on cell monolayer with a razor blade, and then 0.5% lucifer yellow was added to the cells. The dye was rinsed away after 5 min. Cells were washed PBS, fixed with 4% paraformaldehyde, and cells stained with lucifer yellow were detected by fluorescence microscope at magnification

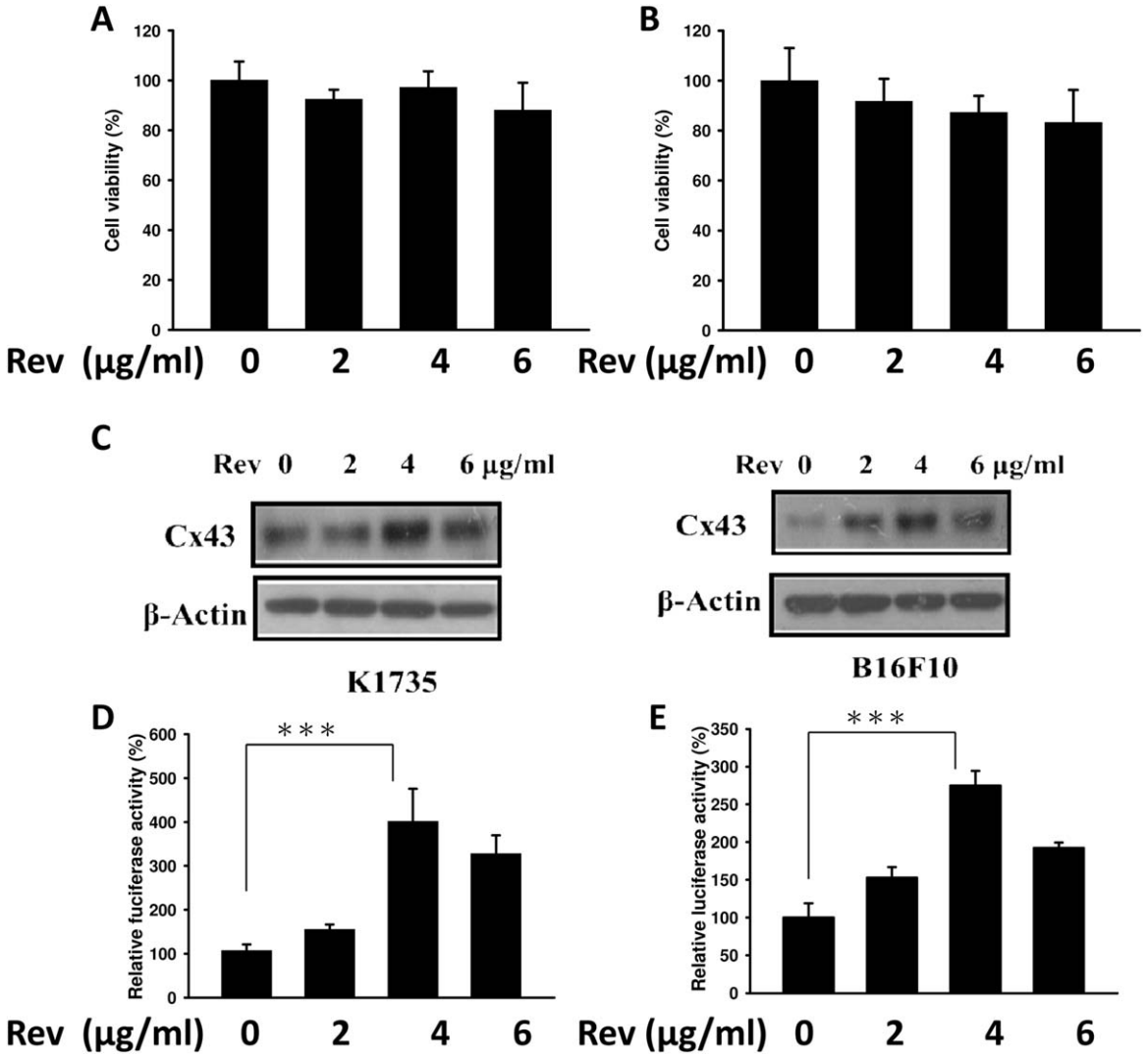

Fig. 1. Resveratrol (Rev) induced Cx43 expression in melanoma cells. The effects of resveratrol on the cell growth. The (A) K1735 or (B) B16F10 (10^3) cells were treated with resveratrol (0–6 μg/mL). The cell viability was then assessed by the WST-8 assay. (means ± SD, $n = 6$). C: Resveratrol induced Cx43 protein expression in K1735 or B16F10 cells. After exposure to resveratrol (0–6 μg/mL) for 24 h, the expression of Cx43 levels in K1735 or B16F10 cells were determined by immunoblot analysis. Resveratrol induced Cx43 transcriptional activity in melanoma cells. The (D) K1735 or (E) B16F10 cells transfected with luciferase gene under the control of Cx43 promoter were treated with resveratrol (0–6 μg/mL) for 24 h. The transcriptional activity of Cx43 was determined by the luciferase reporter assay and is expressed as the fold of the relative luciferase activity relative to that in the control tumor cells. ***, $P <$ 0.001. Data are expressed as mean ± SD of hexaplicate determinations. Each experiment was repeated three times with similar results.

of ×200. The dye-spreading area was quantified by measuring the fluorescent area in three flied at the center of the scrape line using QCapture Pro 6.0 (QImaging, Surrey, BC, Canada).

Cell Viability Assay

Resveratrol-treated or non-treated cells were exposed to 0–20 μg/mL of cisplatin under for 48 h. Cell viability was determined with a colorimetric WST-8 (Dojindo Labs, Tokyo, Japan) assay and expressed as the mean of the percentage of surviving cells relative to that of the cells in the absence of cisplatin.

Animal Studies

C57BL/6 a mice were inoculated subcutaneously (s.c.) with 10^6 B16F10 cells on day 0, and on day 7 nodules developed at all injection sites with approximately tumor volume of 100

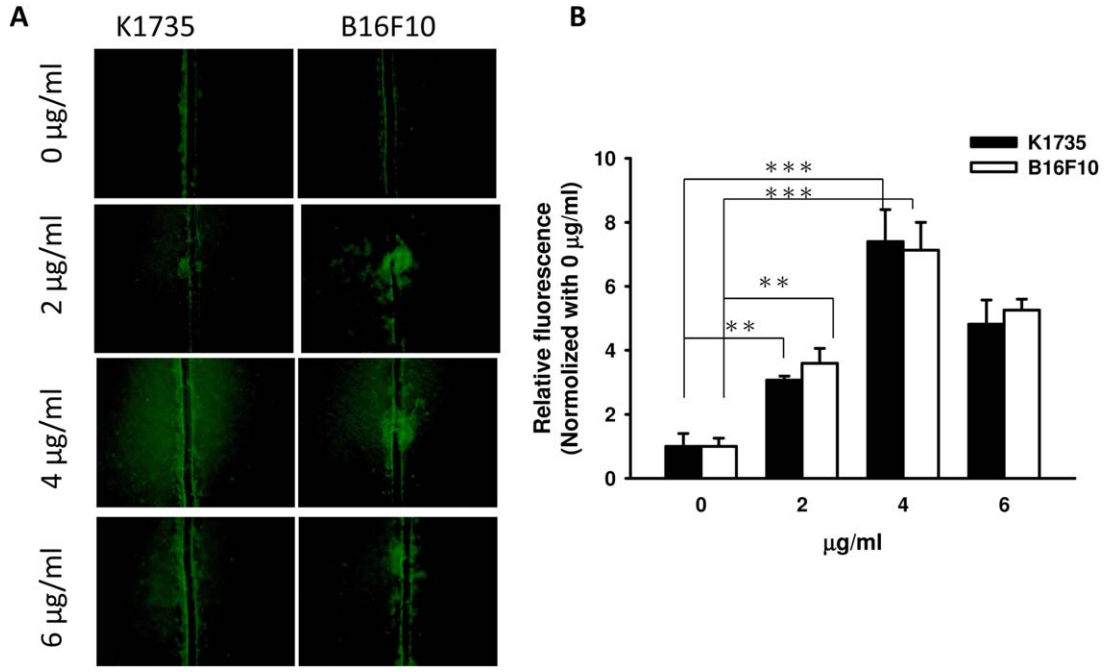

Fig. 2. Resveratrol induce GJIC in tumor cells. A: K1735 or B16F10 cells treated for 21 h with different concentrations of resveratrol were determined by scrape loading and dye transfer analysis. B: GJIC was expressed as fold of the control. Data are expressed as mean ± SD of hexaplicate determinations. **, $P < 0.01$; ***, $P < 0.001$. Each experiment was repeated three times with similar results. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

and 450 mm^3, respectively. Groups of tumor-bearing mice were oral administrated with resveratrol (10 mg/kg), with cisplatin (4 mg/kg) intraperitoneally (i.p.) three times a week or both. All of the mice were monitored for tumor growth and survival as previously described (Lee et al., 2013).

Terminal Deoxynucleotidyltransferase-Mediated Deoxyuridine Triphosphate Nick End-Labeling (TUNEL) Assay

Mice were inoculated with 10^6 B16F10 cells at day 0. Groups of tumor-bearing mice were orally administrated with resveratrol (10 mg/kg) at day 7 for a week followed by cisplatin (4 mg/kg) treatment at days 10, 13, and 15, or with either treatment alone. Then, tumors were excised and snap frozen at day 16. TUNEL assay was used to detect cell apoptosis within tumors and was performed according to the manufacturer's instructions (Promega, Madison, WI). Nuclei were stained with 50 μg/mL of DAPI. TUNEL-positive cells were counted under the microscope. We counted three high-power (×200) fields that showed highest density of positive-stained cells per field to determine the average percentage of apoptotic (TUNEL positive) cells in each section (Lee et al., 2008).

Statistical Analysis

The unpaired, two-tailed Student's t test was used to determine differences between groups. The survival analysis was performed using the Kaplan-Meier survival curve and log-rank test. Any P value <0.05 is regarded statistically significant.

RESULTS

Resveratrol Increased Cx43 Expression in Melanoma Cells

First, we evaluated the potential cytotoxic effects of resveratrol in the range of 0–6 μg/mL by using proliferation assay. At concentration up to 6 μg/mL, resveratrol showed no cytotoxic effects on K1735 [Fig. 1(A)] or B16F10 [Fig. 1(B)] cells treated for 24 h. Therefore, we performed the following experiments using concentration less than or equal to 6 μg/mL. To examine the effect of resveratrol on Cx43 levels in melanoma cells (K1735 and B16F10), cells were incubated with different concentrations of resveratrol, and then analyzed by immunoblot analyses. Treatment of melanoma cells with 0, 2, or 4 μg/mL of resveratrol caused a dose-dependent increase in Cx43 levels compared with controls [Fig. 1(C)]. The expression of Cx43 protein was reduced after resveratrol (6 μg/mL) treatment. Meanwhile, we examined the effect of resveratrol on the Cx43 transcriptional activity by the luciferase reporter assay [Fig. 1(D,E)]. The extent of Cx3 transcriptional activity of resveratrol treatment in melanoma cells varied, ranging from 1.2 to 4 folds. The ratio of luciferase activity in cells was higher in 4 μg/mL resveratrol treatment than that in 6 μg/mL resveratrol treatment (Fig. 1). The

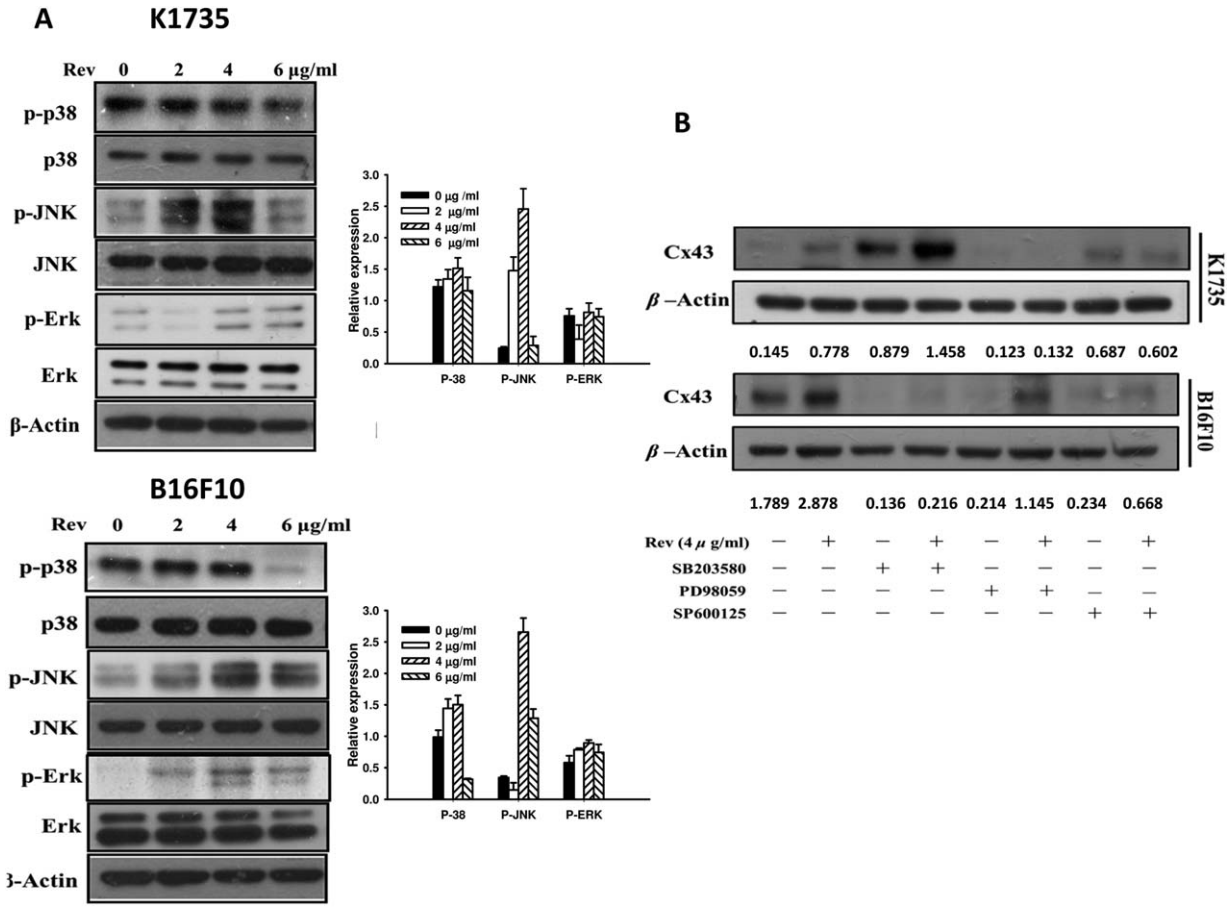

Fig. 3. MAPK inhibitors prevented resveratrol (Rev)-induced Cx43 expression. A: K1735 or B16F10 cells were treated with resveratrol (0–6 μg/mL) for 24 h. The cells were lysed and protein expression of p-ERK, p-P38, and p-JNK was examined. B: After treatment of cells with inhibitor for ERK (PD98059), p38 (SB203580), and JNK (SP600125) for 1 h, K1735 or B16F10 cells were treated with resveratrol (4 μg/mL) for 24 h. The cells were lysed and protein expression of Cx43 was examined. The expression of β-actin served as the quantitative control. Inserted values indicated relative proteins expression in comparison with β-actin. Each experiment was repeated three times with similar results.

transcriptional activity is consist with protein levels. This result points out that resveratrol did not influence melanoma growth and enhanced Cx43 expression under 4 μg/mL treatment.

Resveratrol Increased GJIC

To determine the extent to which Cx43 expression was related to GJIC in melanoma cells, we performed scrape loading dye transfer assay using the gap junction permeable fluorescent dye lucifer yellow. The scrape loading/dye transfer assay for gap junction function showed an increased level of dye transport in melanoma cells, consistent with the presence of Cx43 in these cells. In resveratrol treated cells, considerable dye transfer was observed [Fig. 2(A)]. Furthermore, our results show that degrees of GJIC in tumor cells were correlated with the expression of Cx43 induced by resveratrol [Fig. 2(B)]. After treatment of resveratrol, increasing effect of resveratrol on GJIC was observed.

Resveratrol Activated the Expression of Cx43 Through Mitogen-Activated Protein Kinases (MAPK) Signaling Pathways

We further investigated the potential signaling pathways in resveratrol-induced Cx43 expression in melanoma cells. Resveratrol significantly induced the phosphorlyation of ERK, JNK, and p38 [Fig. 3(A)]. However, resveratrol-induced Cx43 protein expression was blocked by inhibitor of ERK (PD98059) and JNK (SP600125) in K1735 cells. There was no significant effect on Cx43 expression after the inhibitors of p38 [Fig. 3(B)] in K1735 cells. Furthermore, we found that the inhibitor of p38 (SB203580) and inhibitor of JNK (SP600125) influenced resveratrol-induced Cx43 expression in B16F10 cells [Fig. 3(B)]. The JNK play an impartment role in resveratrol induced Cx43 expression. These results suggest that MAPK signaling pathway might play a role in the resveratrol-induced expression of Cx43.

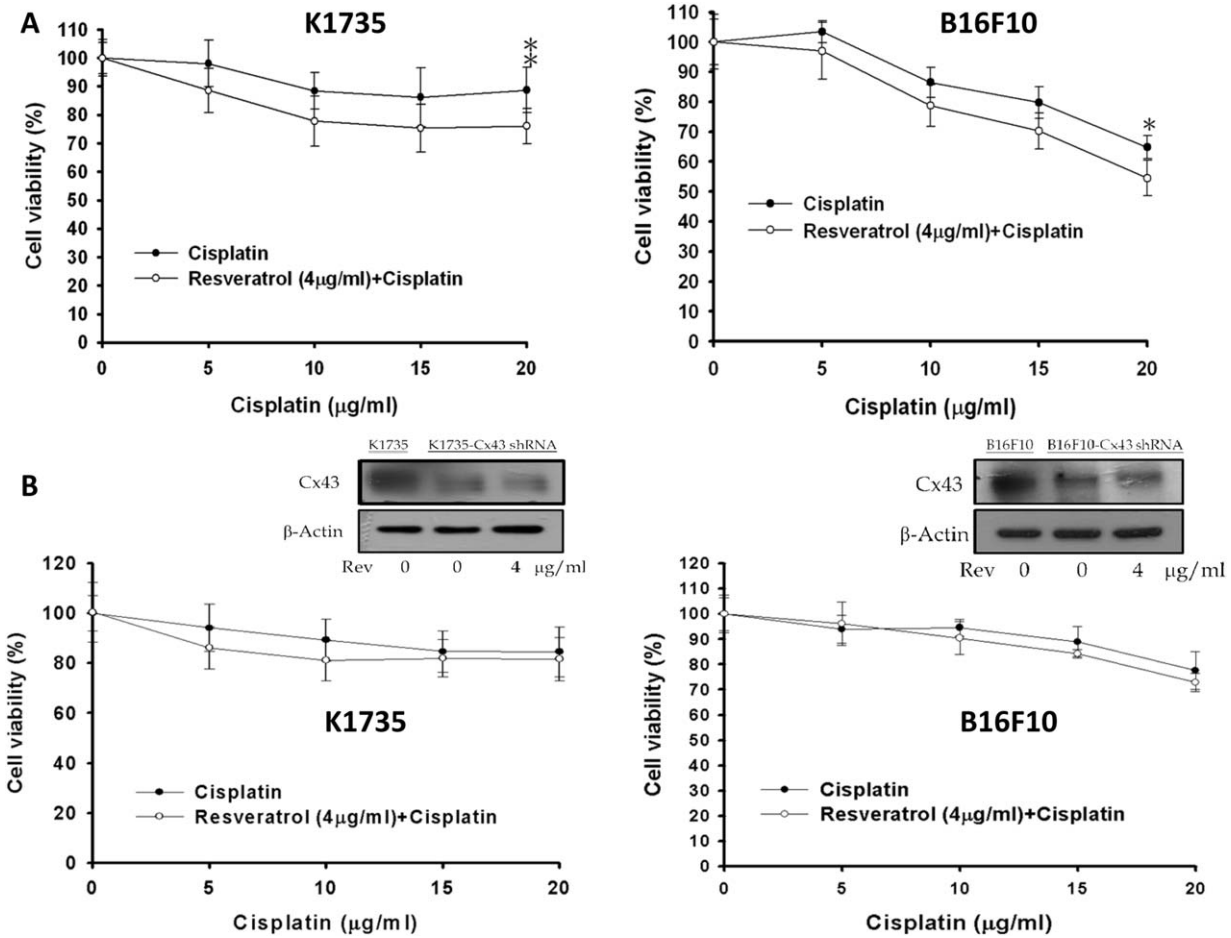

Fig. 4. Resveratrol (Rev)-induced Cx43 expression in conjunction with cisplatin exerted cytotoxic effects on melanoma cells. A: Resveratrol-treated or control cells were exposed to cisplatin (0–20 μg/mL) for 24 h followed by determination of their viability by the WST-8 assay. Data are expressed as mean ± SD of hexaplicate determinations. B: Cx43 shRNA plasmid downregulates the cytotoxic effect of cisplatin. Resveratrol-treated or control cells were transfected with Cx43 shRNA plasmid. The expression of Cx43 was determined by immunoblot analysis. The expression of β-actin served as the quantitative control. Cells were treated with cisplatin (0–20 μg/mL) for 24 h followed by determination of their viability by the WST-8 assay. **, $P < 0.05$; **, $P < 0.01$. Each experiment was repeated three times with similar results.

Resveratrol Increased Susceptibility of Tumor Cells to Cisplatin Induced Cell Death

We examined the effects of Cx43 expression upon the sensitivity of the tumor cells to killing by cisplatin that induce apoptosis. Previously, to show whether cisplatin can upregulate Cx43, the protein expression was determined by Western blotting. Relative to controls, expression of Cx43 declines with treatment of K1735 cells with cisplatin at 2, 4, and 6 μg/mL for 48 h (Chang et al., 2013a). Cytotoxicity was quantified using a WST-8 assay. K1735 cells were highly resistant to killing by cisplatin [Fig. 4(A)]. Treatment with cisplatin, significantly reduced cell viability in resveratrol treated cells as compared with untreated cells. To obtain more insight into the role of Cx43 in resveratrol induced additive cytotoxic effect, we knocked down Cx43 using shRNA plasmid. Initially, K1735 cells were transfected with shRNA plasmids for Cx43. The protein expression of Cx43 was determined by immunoblot analysis after resveratrol treatment [Fig. 4(B)]. Cx43-shRNA decreased the expression of Cx43 protein induced by resveratrol [Fig. 4(B)]. The viability of cells treated with resveratrol significantly reduced than that of control cells after cisplatin treatment [Fig. 4(A)]. On the contrary, Cx43 shRNA antagonized the effects of resveratrol treatment (Fig. 4). The same phenomenon was observed in B16F10 cells. These results suggest that resveratrol-induced Cx43 expression exerted additive cytotoxic effects following cisplatin treatment.

Additive Antitumor Effects by the Combination Therapy of Resveratrol and Cisplatin

Groups of mice that had been inoculated s.c. with B16F10 cells at day 0 were treated orally with resveratrol (10 mg/kg) at day 7 for a week followed by cisplatin (4 mg/kg)

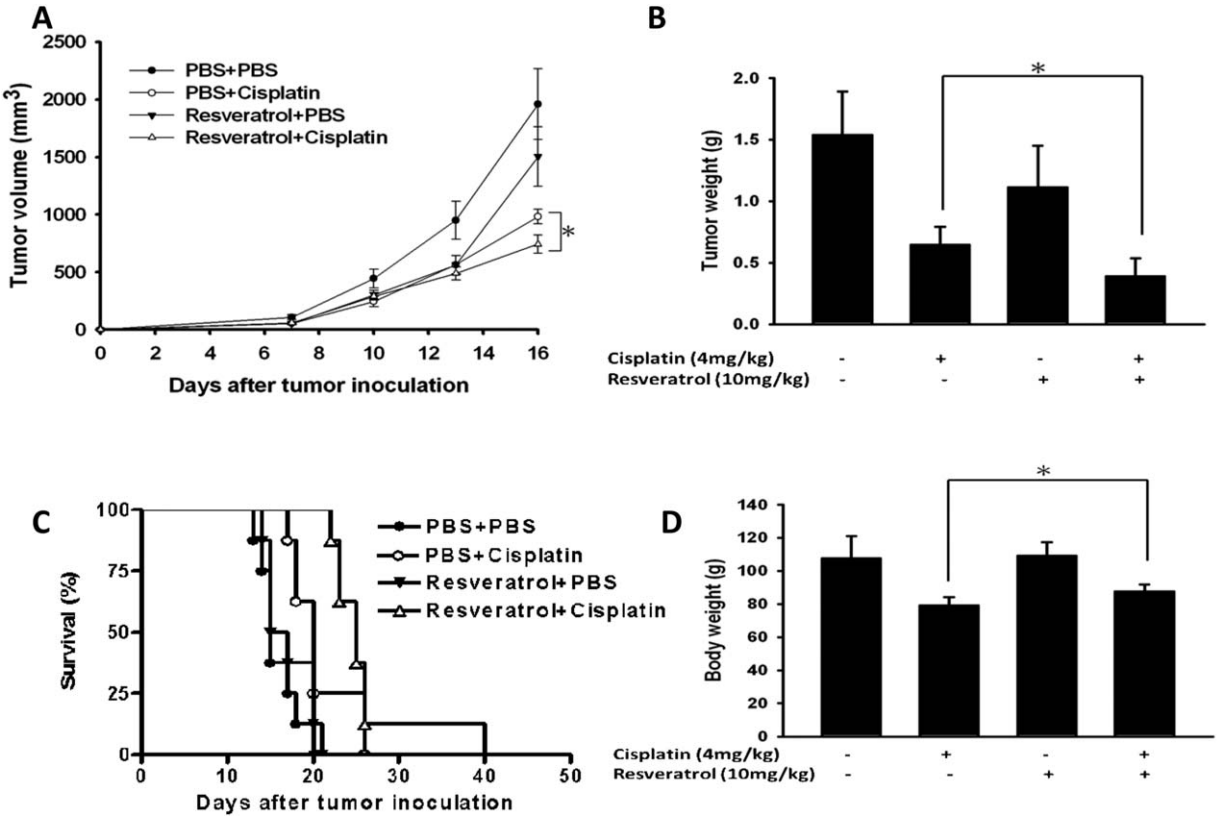

Fig. 5. Additive antitumor effects of resveratrol in combination with cisplatin on subcutaneous B16F10 tumors. A: Groups of 8 mice that had been inoculated s.c. with B16F10 cells (10^6) at day 0 were treated orally with resveratrol (10 mg/kg) at day 7 for a week followed by cisplatin (4 mg/kg) at days 10, 13, and 15, or with either treatment alone. Vehicle control mice received PBS. Tumor volumes (mean ± SEM, $n = 8$) among different treatment groups were compared in mice bearing B16F10 tumors ($P < 0.05$ for Resveratrol + Cisplatin versus Cisplatin; $P < 0.01$ for Resveratrol + Cisplatin versus Resveratrol or PBS; $P < 0.01$ for Cisplatin versus PBS). B: The tumor weight from these mice were measured on day 16 (mean ± SD; $n = 4$). *, $P < 0.05$. C: Kaplan-Meier survival curves of the mice bearing B16F10 tumors ($P < 0.05$ for Resveratrol + Cisplatin versus Cisplatin, Resveratrol or PBS; $P < 0.05$ for Cisplatin versus PBS) with different treatments are shown. D: The body weight from these mice were measured on day 16 (mean ± SD; $n = 4$). *, $P < 0.05$. Each experiment was repeated two times with similar results.

treatment at days 10, 13, and 15, or with either treatment alone. Vehicle control mice received PBS. Antitumor effects of resveratrol alone or combined with cisplatin were evaluated in terms of tumor growth and survival of the mice bearing B16F10 tumors. In mice bearing B16F10 tumors, treatment of resveratrol alone did not significantly retard tumor growth [Fig. 5(A,B)] and prolong the survival time [Fig. 5(C)] compared with PBS treatment. Tumor-bearing mice treated with cisplatin exerted significant antitumor effect [Fig. 5(A,B)] and prolonged the survival time [Fig. 5(C)] compared with PBS treatment. The antitumor effects of resveratrol combined with cisplatin were evaluated in terms of tumor growth and survival in mice bearing B16F10 tumors [Fig. 5(A,B)]. Interestingly, the combination therapy significantly reduced tumor size and tumor weight compared with cisplatin in tumor-bearing mice [Fig. 5(A,B)]. The mean tumor volume for mice in the combination therapy group decreased by 32% compared with those in the cisplatin-treated group. The survival of the mice treated with resveratrol plus cisplatin was significantly prolonged compared with that of the mice treated with cisplatin [Fig. 5(C)]. We further monitored symptomatic health parameters including body weight caused by cisplatin after starting cisplatin administration. Treatment of mice with resveratrol significantly attenuated the weight loss induced by cisplatin [Fig. 5(D)]. Oral resveratrol did not influence the body weight and survival of mice. The results suggested that resveratrol was safety for mice. Furthermore, the tumors from B16F10-bearing mice treated with resveratrol or cisplatin alone, or in combination were analyzed for apoptotic

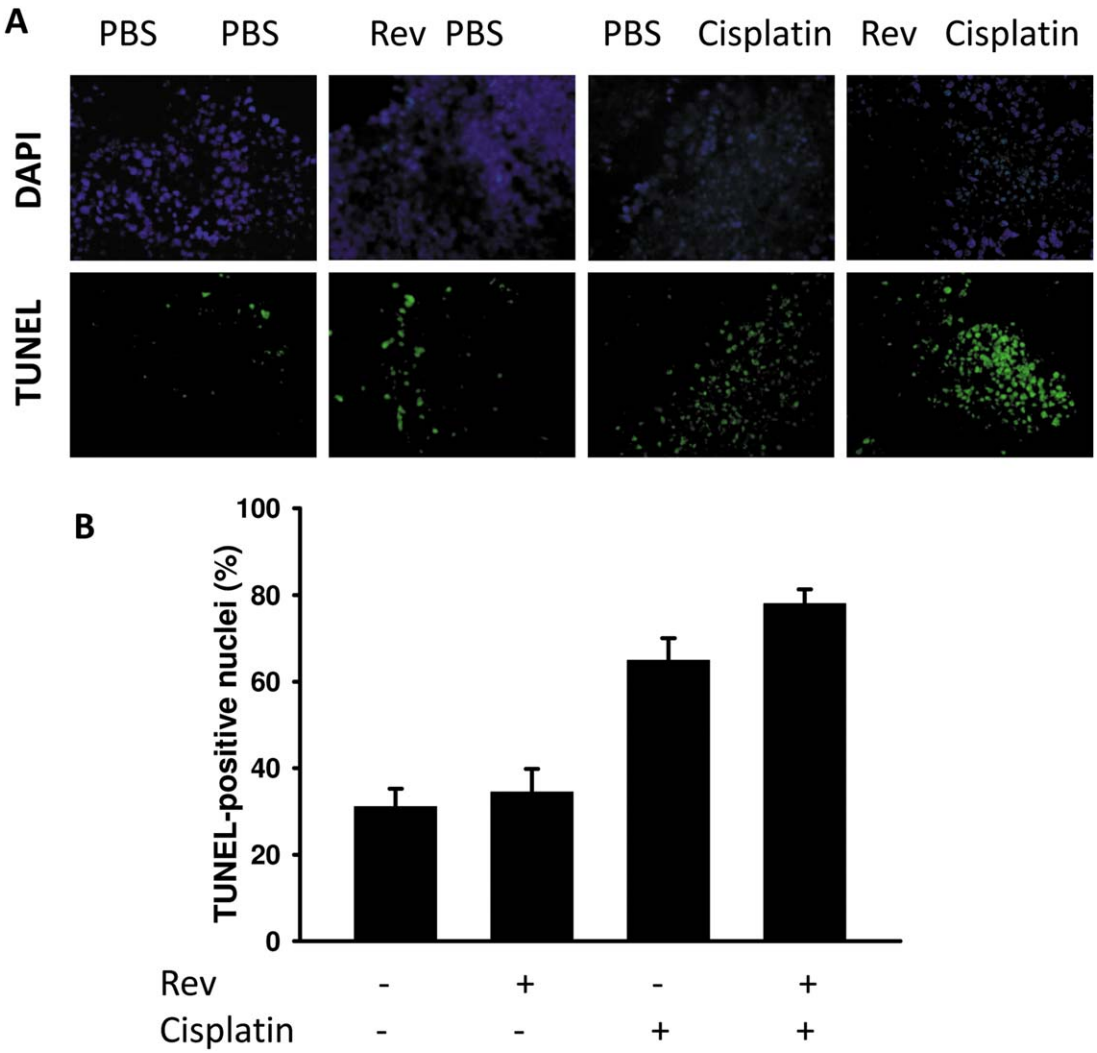

Fig. 6. Increase in tumor cells undergoing apoptosis in B16F10 tumor-bearing mice treated with resveratrol in combination with cisplatin. A: Groups of 4 mice that had been inoculated s.c. with B16F10 cells (10^6) at day 0 were treated orally with resveratrol (10 mg/kg) at day 7 for a week followed by cisplatin (4 mg/kg) at days 10, 13, and 15, or with either treatment alone. Vehicle control mice received PBS. Tumors were excised at day 16, and TUNEL assay was used to detect apoptotic cells (×400). B: TUNEL-positive cells were counted from three fields of highest density of positive-stained cells in each section to determine the percentage of apoptotic cells (mean ± SEM, $n = 4$). ($P < 0.05$ for Resveratrol + Cisplatin versus Cisplatin, Resveratrol or PBS; $P < 0.05$ for Cisplatin versus PBS). Each experiment was repeated two times with similar results. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

cells by the TUNEL assay. Representative results for immunohistochemistry are shown in Figure 6(A). There was a 1.2-fold in the number of apoptotic cells induced by resveratrol plus cisplatin compared with that induced by cisplatin alone [Fig. 6(B)]. Taken together, these results indicate that the combination therapy with resveratrol and cisplatin resulted in retarding tumor growth, increasing apoptosis in the tumors. Taken together, Cisplatin as a single-agent therapy could retard tumor growth and enhance survival in murine melanoma tumor models. More strikingly, additive antitumor effects could be achieved with the combination therapy.

DISCUSSION

This study used melanoma models to determine the effects of the combinational treatment of cisplatin and resveratrol *in vitro* and *in vivo*. The results showed a decrease in tumor growth with resveratrol in combination with cisplatin compared to control groups. The combination of resveratrol and cisplatin reduced tumor cell growth and tumor size compared to cisplatin alone, pointing out that resveratrol can increase the efficacy of cisplatin and reduce the side effects induced by cisplatin treatment. Cisplatin cytoxicity is

dependent on GJIC. Some studies point out that resveratrol induced Cx43 expression: Resveratrol induces a delay in cell cycle progression and both alone and in combination with X rays is able to enhance GJICs (Leone et al., 2008). In lung injury model, resveratrol enhanced the gap junction communication in lung tumor and endothelial cells (Ma et al., 2013). Resveratrol can protect the retinal pigment epithelial cells against hyperglycemia-induced low-grade inflammation and GJIC degradation (Losso et al., 2010). However, the detailed mechanism of resveratrol-induced Cx43 expression remained uncertain. We investigated mechanisms of resveratrol-induced signaling and the consequences of therapeutic in mouse tumor models. Herein, resveratrol was demonstrated the ability to enhance GJIC activity through an increase in Cx43 expression via MAPK pathway. Some studies also demonstrated upregulation of Cx43 expression and GJIC in cells via activation of MAPK pathway (Ho et al., 2013). There is a growing evidence that an increase in cisplatin-mediated response with GJIC enhancement (Shishido and Nguyen, 2012).

There is a significant increase of apoptosis in cisplatin-treated cell. Proliferation analysis and TUNEL assay showed a significant increase in cell death of resveratrol combined with cisplatin treated tumors compared to control. The cell death signal induced by cisplatin is transmitted to neighboring cell via GJIC (Jensen and Glazer, 2004). Enhancement of GJIC may allow transmission of the death signal to neighboring cell. Gap junction expression in cancers may influence the clinical response to cisplatin. In addition, resveratrol manipulate gap junction in conjunction with cisplatin treatment may provide new approaches to cancer therapy.

Previous studies demonstrated that Cx43 enhanced the cisplatin-induced cytotoxicity in mesothelioma (Sato et al., 2009) and in breast cancer (Shishido and Nguyen, 2012). Then considering the involvement of Cx43, molecular mechanism of Cx43, which contributes to cisplatin cytotoxicity was investigated via gap junction dependent manner or gap junction independent manner. Herein, we found that resveratrol induced Cx43 expressions and enhanced GJIC, as revealed by the fluorescent dye scrape loading assay (Fig. 2). Meanwhile, Cx43 regulates Src kinase through interaction of the Cx43 C-terminal region, irrespective of GJIC function (Giepmans et al., 2001). Inhibition of Src by Cx43 and induction of apoptosis pathway by cisplatin could induce additive antitumor effects.

Cisplatin is one of the most used chemotherapeutic drugs. Meanwhile, the major limiting factor in the use of cisplatin is the side effects in normal tissue including kidney. Renoprotective approaches are being discovered, but the effects are partial. This study for a new combination treatment for melanoma using cisplatin is to reduce dose to prevent renal toxicity (Chang et al., 2013b). This would allow the use of lower cisplatin concentration, thus decreasing the side effects. Resveratrol provides to accelerate cell death in melanoma cells while increasing Cx43 expression.

By taking advantages of the tumoricidal effect of cisplatin and the pleiotropic activities of resveratrol, we can come to a conclusion that resveratrol combining with cisplatin appears to hold promise for the treatment of solid tumors.

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