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Oxaliplatin down-regulates survivin by p38 MAP kinase and proteasome in human colon cancer cells

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ABSTRACT

Oxaliplatin, a platinum derivative cancer drug, has been used for treating human colorectal cancers. Survivin has been proposed as a cancer target, which highly expressed in most cancer cells but not normal adult cells. In this study, we investigated the regulation of survivin expression by exposure to oxaliplatin in human colon cancer cells. Oxaliplatin (3–9 μM for 24 h) markedly induced cytotoxicity, proliferation inhibition and apoptosis in the human RKO colon cancer cells. The survivin protein expression of RKO cells is dramatically reduced by oxaliplatin; however, the survivin gene expression is slightly altered. The survivin blockage of oxaliplatin elevated caspase-3 activation and apoptosis in RKO cells. Over-expression of survivin proteins by transfection with a survivin-expressed vector resisted the oxaliplatin-induced cancer cell death. Meantime, oxaliplatin elicited the phosphorylation of p38 mitogen-activated protein (MAP) kinase. SB202190, a specific p38 MAP kinase inhibitor, restored the survivin protein level and attenuated oxaliplatin-induced cancer cell death. In addition, oxaliplatin increased the levels of phospho-p53 (Ser-15) and total p53 proteins. Inhibition of p53 expression by a specific p53 inhibitor pifithrin-α reduced the phosphorylated p38 MAP kinase and active caspase-3 proteins in the oxaliplatin-exposed RKO cells. In contrast, SB202190 did not alter the oxaliplatin-induced p53 protein level. Furthermore, treatment with a specific proteasome inhibitor MG132 restored survivin protein level in the oxaliplatin-treated colon cancer cells. Taken together, our results demonstrate for the first time that survivin is down-regulated by p38 MAP kinase and proteasome degradation pathway after treatment with oxaliplatin in the human colon cancer cells.

1. Introduction

Oxaliplatin is a third-generation of platinum derivative compound that has been used to treat human colorectal cancers [1–3]. Oxaliplatin displays a wide range of anticancer activities. It can induce DNA damage by the formation of cross-links between two bases of DNA leading to the blockade of replication and transcription [4,5]. Oxaliplatin also induces apoptosis in human cancer cells [6–10]. The underlying molecular responses of apoptosis to oxaliplatin remain largely unclear despite the fact that it can induce apoptosis. In addition, oxaliplatin is a good radiosensitizer by combination with radiotherapy for cancer therapy [11,12]. Furthermore, combined chemotherapeutic drugs such as paclitaxel and celecoxib with oxaliplatin can augment the anticancer abilities in cancer cells [13–15].

Survivin has been proposed as a cancer target for cancer therapy. It is highly expressed in a variety of human cancer cells [16–19]. Survivin belongs to the inhibitor of apoptosis (IAP) family, which exhibits anti-apoptotic activity by inhibiting the activity of caspases [16,20]. Anticancer agents have been shown to down-regulate survivin expression for inducing cancer cell death [21–23]. Blockage of survivin provides the important strategy of cancer therapy. Previously, oxaliplatin has been shown to reduce survivin protein expression [13,14]. However, the regulation and mechanism of survivin inhibition by oxaliplatin are valuable for further investigation. Understanding the blocking survivin pathways of oxaliplatin may contribute the novel strategies for cancer therapy.

Chemotherapeutic drugs can induce apoptotic pathways or block survival pathways to trigger cancer cell death. p38 mitogen-activated protein (MAP) kinase is an important signal molecule in

Abbreviations: MAP, mitogen-activated protein; MTT, 3-(4,5-dimetnyl-thiazol-2-yl)2,5-diphenyl tetrazolium bromide; FITC, fluorescein isothiocyanate; FBS, fetal bovine serum; PBS, phosphate-buffered saline; ERK, extracellular signal-regulated kinase; RT-PCR, reverse transcription-polymerase chain reaction; M-MLV, Moloney murine leukemia virus; GFP, green fluorescence protein; ANOVA, analysis of variance.

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the regulation of apoptosis [23–26]. The activation of p38 MAP kinase by anticancer agents leads to apoptosis in cancer cells [23,26]. The regulation of survivin by p38 MAP kinase is still unknown although oxaliplatin can activate the phosphorylation of p38 [9]. Currently, p53 is an important therapeutic target for cancer treatment [27,28]. p53 is a cellular gatekeeper for the purpose of regulating apoptosis, cell cycle arrest, and DNA repair [28–31]. The apoptotic induction of p53 is mediated by its downstream genes or proteins [31–34]. p38 MAP kinase-regulated apoptosis has been shown to correlate with the p53 accumulation [35].

In this study, we investigate the role of p38 on the regulation of survivin expression and apoptosis following treatment with oxaliplatin in human colon cancer cells. Oxaliplatin markedly reduces the survivin protein expression but has no affect on the survivin gene expression. The blockage of p38 MAP kinase and proteasome restores the protein level of survivin in the oxaliplatin-treated colon cancer cells. We provide that down-regulation of survivin protein expression by oxaliplatin is mediated by p38 MAP kinase and proteasomal degradation pathway.

2. Materials and methods

2.1. Reagents and antibodies

Oxaliplatin, pifithrin-α, Hoechst 33258, 3-(4,5-dimetnylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT), and cycloheximide were purchased from Sigma Chemical Co. (St. Louis, MO). SB202190 and MG132 (N-carbobenzoxyl-Leu-Leu-leucinal) were purchased from Calbiochem (San Diego, CA). Phospho-p53 (Ser-15), phospho-p38, and XIAP antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). p53 (DO-1), p38α (C-20), survivin (FL-142), GFP (FL), ERK-2 (C-14), and BCL-2 antibodies were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). The Cy5-labeled goat anti-rabbit IgG was purchased from Amersham Pharmacia Biotech (Little Chalfont Buckinghamshire, UK). BODIPY FL phallacidin (B-607) was purchased from Invitrogen (Carlsbad, CA). Caspase-3 antibody was purchased from BioVision Inc. (Mountain View, CA). Actin antibody was purchased from Millipore Corp. (Beverly, MA).

2.2. Cell culture

RKO was a colon carcinoma cell line. HEK293 was an embryonic kidney cell line. RKO and HEK293 cells were maintained in complete DMEM (Invitrogen) supplemented with 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). The cells were cultured at 37 °C and 5% CO_2 in a humidified incubator (310/Thermo, Forma Scientific Inc., Marietta, OH).

2.3. Cell viability assay

The cell viability was analyzed by MTT assay. RKO cells were plated in 96-well plates at a density of 1×10^4 cells/well for 16–20 h. Then the cells were treated with or without drugs. At the end of treatment, the cells were washed with phosphate-buffered saline (PBS), and then re-cultured in complete medium for 2 days. Thereafter, the cells were incubated in complete medium containing 0.5 mg/ml MTT at 37 °C for 4 h. Finally, the cells were dissolved by adding dimethyl sulfoxide. The viable cells converted MTT to formazan that presented blue-purple color when dissolved in dimethyl sulfoxide. The intensity of formazan was measured at 545 nm using a plate reader VERSAmax (Molecular Dynamics). The relative percentage of cell viability was calculated by dividing the absorbance of drug-treated samples (from the average of 6 wells) by that of the control.

2.4. Apoptosis analysis by Annexin V staining

The level of apoptosis induced by oxaliplatin was determined by Annexin V analysis. The Annexin V staining was used to examine the cells by incubating with fluorescein isothiocyanate (FITC)-conjugated-Annexin V (BioVision, Mountain View, CA). After treatment with oxaliplatin, the 1×10^6 cells were collected by centrifugation at $200 \times g$ for 5 min. Thereafter, the cells were incubated with Annexin V-FITC labeling solution at 25 °C for 5 min. Finally, the samples were immediately analyzed by flow cytometer (FACSCalibur, BD Biosciences, San Jose, CA). The Annexin V-FITC intensity of staining cells was quantified by CellQuest software (BD Biosciences).

2.5. Western blot

Western analyses of survivin, XIAP, BCL-2, ERK-2, GFP, phospho-p38, p38, caspase-3, phospho-p53 (Ser-15), and p53 were performed using specific antibodies. Briefly, proteins were separated on 10–12% sodium dodecyl sulfate-polyacrylamide gels, and transferred electrophoretically onto polyvinylidene difluoride membranes. The membranes were sequentially hybridized with primary antibody and followed with a horseradish peroxidase-conjugated secondary antibody. Thereafter, the protein bands were visualized on the X-ray film using the enhanced chemiluminescence detection system (PerkinElmer Life and Analytical Sciences, Boston, MA). The protein intensities of scanned images were semi-quantified by using Un-Scan-It gel software (ver. 5.1; Silk Scientific Inc., Orem, UT).

2.6. Immunofluorescence staining and confocal microscope

To analyze the protein expression and location of survivin and phospho-p53 (Ser-15) after treatment with oxaliplatin, the cells were subjected to immunofluorescence staining and confocal microscopy. The procedure was according to a previous study [19]. At the end of drug treatment, the cells were fixed with 4% paraformaldehyde solution. After fixation, the cells were washed three times with PBS, and non-specific binding sites were blocked in PBS containing 10% FBS and 0.25% Triton X-100 for 1 h. Thereafter, the samples were incubated with rabbit anti-survivin (1:50) or anti-phospho-p53 (Ser-15) (1:50) antibodies in PBS containing 10% FBS at 4 °C for 12–16 h. Thereafter, the cells were further incubated with goat anti-rabbit Cy5-labeled IgG (1:50) in PBS containing 10% FBS at 37 °C for 2.5 h. The actin filament (F-actin) and nuclei were stained with BODIPY FL phallacidin and Hoechst 33258, respectively. The samples were examined under a Leica confocal laser scanning microscope (Leica, Wetzlar, Germany).

2.7. Reverse transcription-polymerase chain reaction (RT-PCR)

RKO cells were plated at a density of 3×10^6 cells per a 100-mm Petri dish in culture medium for overnight. After treatment with or without oxaliplatin, the cells were washed twice with PBS. PCR assay cells were harvested and the total RNA was isolated using TRIzol reagent (Invitrogen Co., CA, USA) according to the manufacturer's recommendations. cDNA was synthesized from 1 μg/ml of total RNA using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen, CA, USA) and random Hexamer. First stranded cDNAs were synthesized by M-MLV reverse transcriptase with oligo $(dT)_{15}$ primer (Promega, Madison, WI). Each reverse transcript was amplified with glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as an internal control. The following primer pairs were used for amplification of survivin [36]: sense 5′-GGCATGGGTGCCCCGACGTTG-3′ and antisense 5′-CAGAGGCCTCAATCCATGGCA-3′, and for GAPDH:

sense 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and antisense 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′. RT-PCR was performed by a DNA thermal cycler, 5331/Mastercycler gradient (Eppendorf, Hamburg, Germany). The initial denaturation was performed at 95 °C for 5 min, followed by 35 cycles at 95 °C for 1 min, 55 °C for 1 min, and finally 72 °C for 1 min. The PCR products were visualized on 2% agarose gels with ethidium bromide staining under UV transillumination, and photograph was taken by a camera (Ezcatcher EZC-2002, Medclub Scientific Co., Taipei, Taiwan). The amplification products of survivin and GADPH were 439 and 306 bp, respectively.

2.8. Transfection

A survivin-green fluorescence protein (GFP) fusion vector (pCT-GFP-sur8) was constructed in a previous study [37]. The survivin cDNA fragment was cloned into a CT-GFP TOPO vector by using a CT-GFP fusion TOPO expression kit (K4820-101, Invitrogen) according to the manufacturer's recommendations. Control pCT-GFP2 vector and pCT-GFP-sur8 vector were employed for transfection using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. After transfection, the cells were subjected to MTT assay or Western blot analysis as described above.

2.9. Statistical analysis

Data were analyzed by one-way or two-way analysis of variance (ANOVA), and further post hoc tests using the statistic software of GraphPad Prism 4 (GraphPad software Inc., San Diego, CA). Differences between control and oxaliplatin were compared by one-way ANOVA with post hoc Tukey's tests. Differences between control and inhibitors in the oxaliplatin-treated samples were compared by two-way ANOVA with Bonferroni post-tests. A p value of <0.05 was considered statistically significant in the experiments.

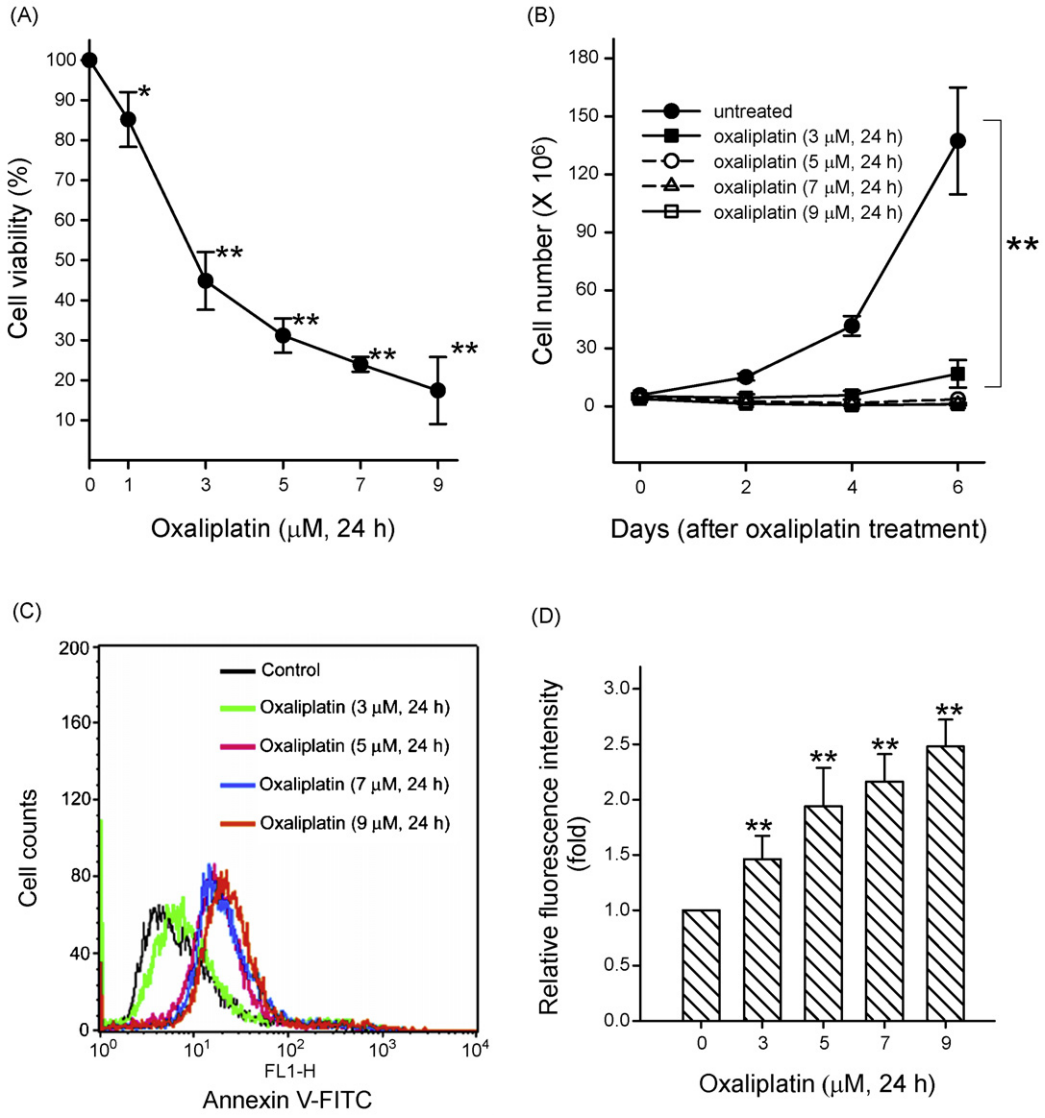

Fig. 1. Effect of oxaliplatin on the cell viability and apoptosis in the colon cancer cells. (A) RKO cells were treated with 1–9 μM oxaliplatin for 24 h. The cell viability was measured by MTT assay. Results were obtained from 4 experiments. (B) RKO cells were plated at a density of 1×10^6 cells/p100 Petri dish for 18 h. Then the cells were treated with 0–9 μM oxaliplatin for 24 h. After drug treatment, the cells were incubated for various times before they were counted by a hemocytometer. Results were obtained from 3 independent experiments. (C) Apoptosis was determined by Annexin V-FITC staining using flow cytometry analysis. The population of Annexin V⁺ cells represents cells undergoing apoptosis. (D) Populations of apoptotic cells were quantified using CellQuest software. Data were obtained from 4 independent experiments. The bar represents the mean ± S.D. $^*p \leq 0.05$ and $^{**}p \leq 0.01$ indicate significant difference between control and oxaliplatin.

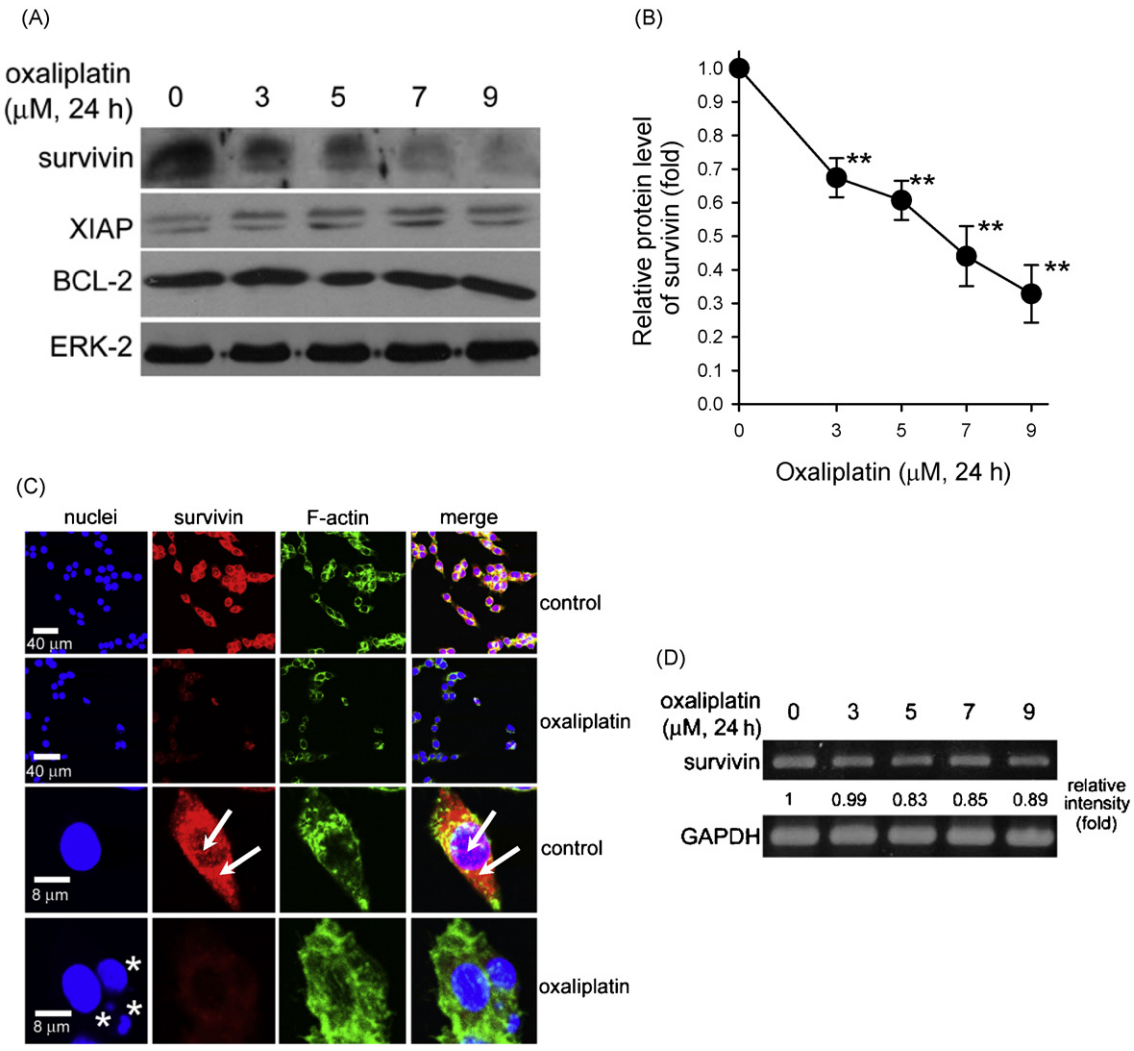

Fig. 2. Effect of oxaliplatin on the survivin expression in the colon cancer cells. (A) RKO cells were treated with 3–9 μM oxaliplatin for 24 h. The total protein extracts were subjected to Western analysis using specific antibodies for survivin, XIAP, BCL-2 and ERK-2. (B) The protein intensities of survivin were semi-quantified. Results were obtained from 4 independent experiments. $^{**}p \leq 0.01$ indicates significant difference between control and oxaliplatin. (C) RKO cells were treated with or without 9 μM oxaliplatin for 24 h. The red color indicated the protein location of survivin. The nuclei were stained with Hoechst 33258 that displayed the blue color. F-actin of cytoskeleton was stained with BODIPY FL phallacidin that displayed the green color. The stars show the apoptotic nuclei. (D) RKO cells were treated with 0–9 μM oxaliplatin for 24 h. Finally, the cells were harvested and analyzed by RT-PCR for examining the survivin mRNA levels. Results were obtained from 3 independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3. Results

3.1. Oxaliplatin induces cytotoxicity, proliferation inhibition, and apoptosis in the human colon carcinoma cells

Treatment with oxaliplatin (3–9 μM for 24 h) significantly reduced the cell viability via concentration-dependent manner in RKO cells (Fig. 1A). Moreover, the cell proliferation was inhibited by oxaliplatin when the treated cells were re-cultured for 2–6 days (Fig. 1B). The oxaliplatin-induced apoptosis was further analyzed by Annexin V staining assays. Fig. 1C shows that the fluorescence intensities of Annexin V-FITC (cells undergoing early apoptosis) were increased following oxaliplatin treatment. After quantification of Annexin V-FITC intensities, oxaliplatin significantly increased the level of apoptosis in RKO cells (Fig. 1D).

3.2. Oxaliplatin inhibits survivin protein expression but slightly reduces survivin mRNA level in the human colon carcinoma cells

To examine the effects of oxaliplatin on the protein expression of survivin following treatment with oxaliplatin in human colon cancer cells, the oxaliplatin-treated cells were subjected to Western blot analyses. Treatment with oxaliplatin (3–9 μM for 24 h) reduced survivin proteins via a concentration-dependent manner in RKO cells (Fig. 2A). Semi-quantification shows that oxaliplatin significantly decreased survivin protein levels (Fig. 2B). Nevertheless, the protein levels of XIAP and Bcl-2 were not decreased by oxaliplatin (Fig. 2A). ERK-2 protein has been used as an internal control protein in several studies [18,19]. The ERK-2 protein levels were not altered by oxaliplatin (Fig. 2A). Survivin proteins were presented in the nuclei and cytoplasm of RKO cells analyzed by immunofluorescence staining and confocal microscopy (Fig. 2C, the arrows). However, the fluorescence intensity of survivin was markedly inhibited following treatment with oxaliplatin (9 μM for 24 h) (Fig. 2C). Apoptosis was presented when the cells were declined survivin protein expression (Fig. 2C, the stars). The stars indicated the location of apoptotic nuclei formation. To examine the effects of oxaliplatin on the survivin gene expression, the oxaliplatin-treated colon cancer cells were subjected to RT-PCR analysis. GAPDH was used as internal control gene. Treatment with oxaliplatin (5–9 μM for 24 h) slightly reduced survivin gene expression in RKO cells (Fig. 2D).

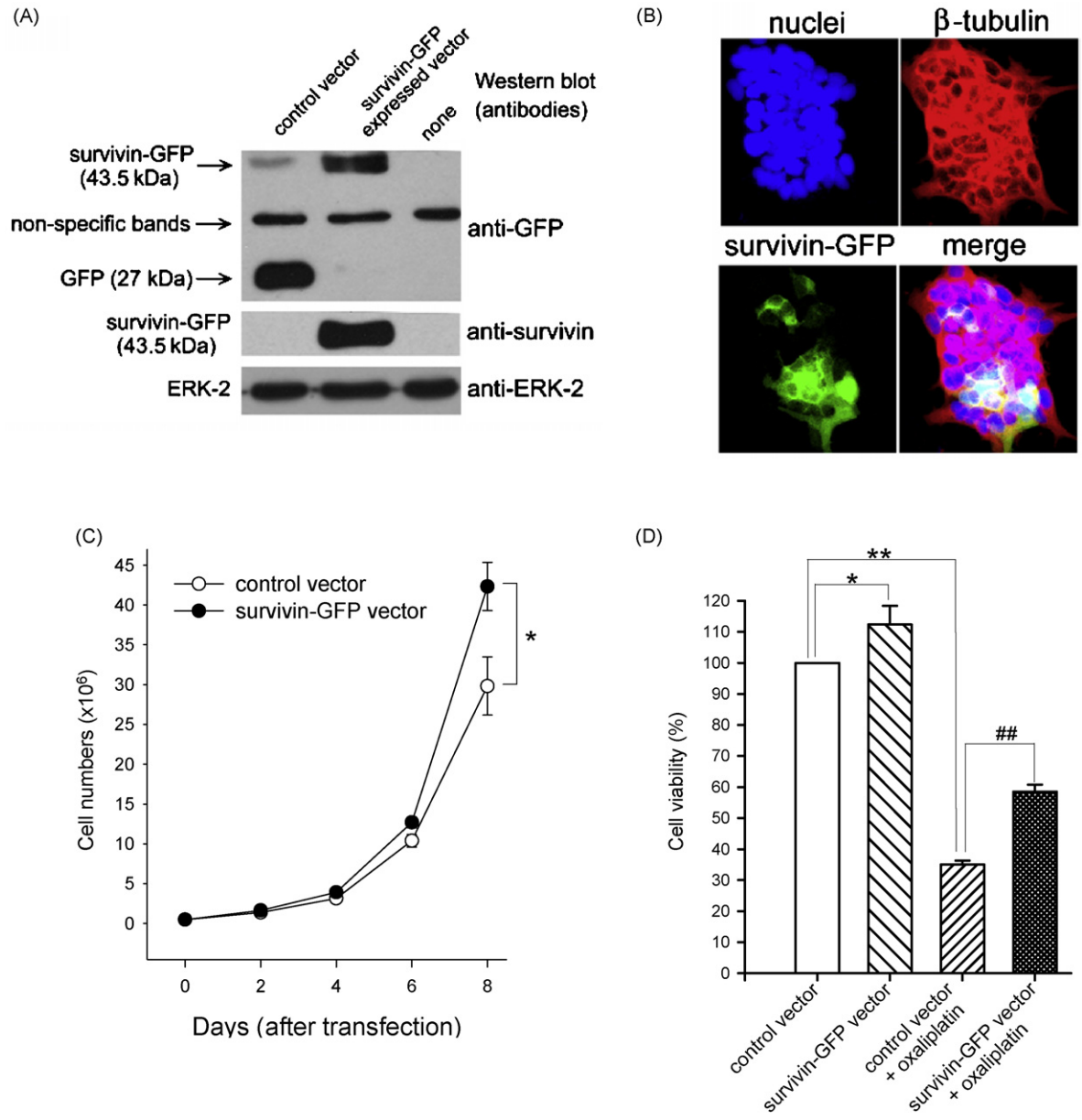

Fig. 3. Effect of over-expression of survivin by a survivin-expressed vector on cell survival and the oxaliplatin-induced cytotoxicity. (A) HEK293 cells were transfected with 10 μg control vector (pCT-GFP-2) or survivin-GFP expressed vector (pCT-GFP-sur8) for 24 h. Total protein extracts were subjected to Western blot analysis. (B) At the end of transfection, the β-tubulin of cells was stained with the Cy3-labeled anti-β-tubulin that displayed the red color. The nuclei were stained with Hoechst 33258 that displayed the blue color. The GFP-survivin proteins displayed the green color. (C) HEK293 cells were transfected with 10 μg pCT-GFP-2 or pCT-GFP-sur8 and then subjected to the cell growth assay. Results were obtained from 3 to 4 experiments. The bar represents the mean ± S.D. $^{*}p < 0.05$ indicates significant difference between control and survivin vector treated samples. (D) RKO cells were transfected with 30 μg pCT-GFP-2 or pCT-GFP-sur8 for 24 h and then treated with 3 μM oxaliplatin for 24 h. Cell viability was measured by MTT assay. Results were obtained from 3 separate experiments. The bar represents the mean ± S.D. $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate significant difference between control and survivin-GFP vector or oxaliplatin treated samples. $^{\#\#}p < 0.01$ indicates significant difference between control vector and survivin-GFP vector following treatment with oxaliplatin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

3.3. Transfection with a survivin-expressed vector increases the cell viability and reduces oxaliplatin-induced cytotoxicity in the human colon carcinoma cells

The role of survivin on the oxaliplatin-induced cytotoxicity was investigated by transfected with a survivin-expressed vector pCT-GFP-sur8. Transfection of pCT-GFP-sur8 vector expressed survivin-GFP-fusion protein (43.5 kD) in HEK293 cells (Fig. 3A). The control pCT-GFP2 vector expressed a 27 kD GFP protein (Fig. 3A). The green fluorescence from survivin-GFP proteins was observed by transfection with pCT-GFP-sur8 vector (Fig. 3B). Comparison between control and pCT-GFP-sur8, pCT-GFP-sur8 vector increased the total cell population of HEK293 cells (Fig. 3C). In addition, CT-GFP-sur8 vector significantly increased the cell viability of RKO cells (Fig. 3D). Moreover, the pCT-GFP-sur8 vector-transfected cells were more resistant to cytotoxicity than the control vector-transfected cells after treatment with oxaliplatin (Fig. 3D).

3.4. Oxaliplatin activates the p38 MAP kinase phosphorylation and caspase-3 in the human colon carcinoma cells

Fig. 4A shows the phosphorylated p38 MAP kinase and active caspase-3 proteins that were elevated by treated with oxaliplatin (3–9 μM for 24 h) in RKO cells (Fig. 4A). Semi-quantification shows that oxaliplatin significantly increased the protein levels of phosphorylated p38 MAP kinase and activated caspase-3 (Fig. 4B and C).

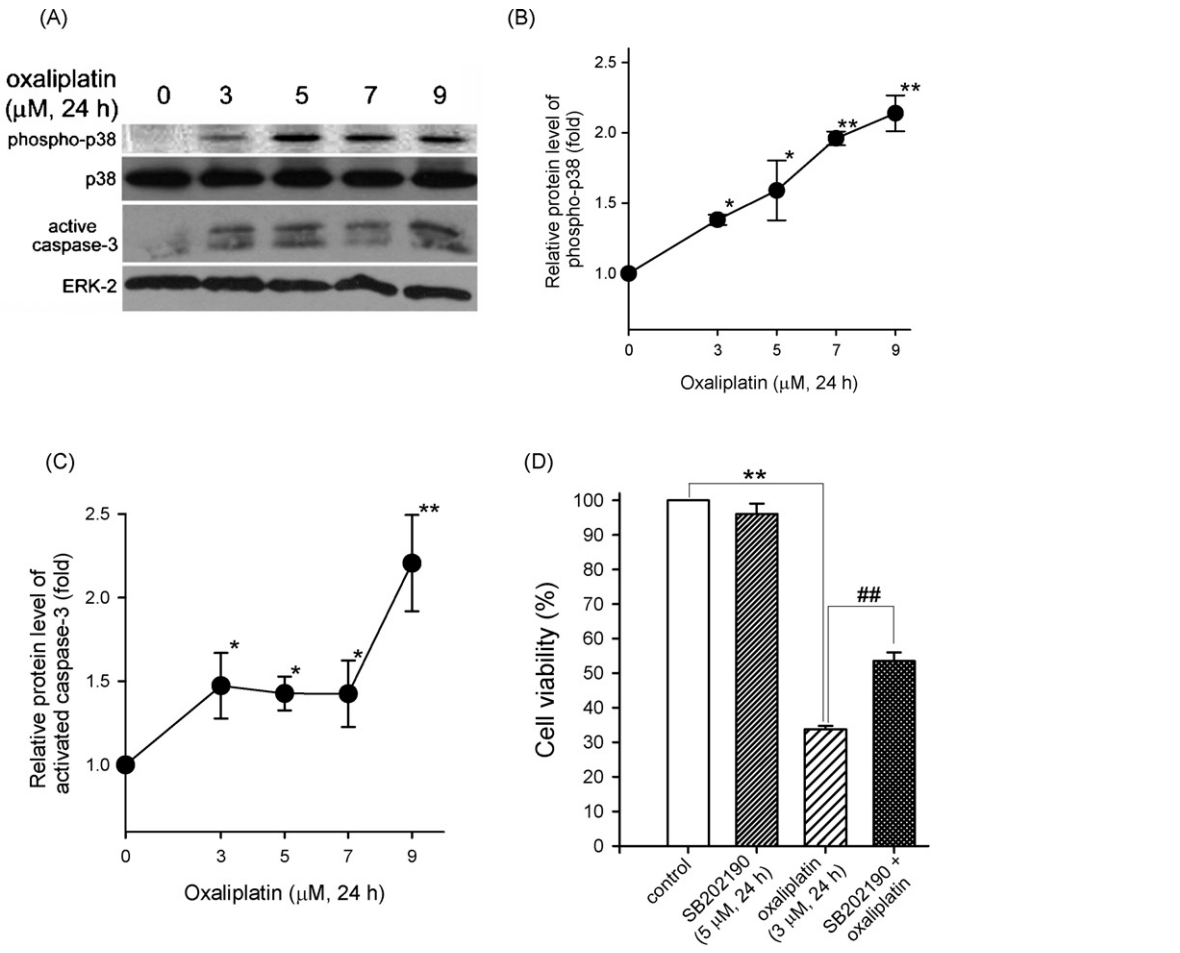

Fig. 4. Effect of oxaliplatin on the protein levels of phospho-p38 MAP kinase and active caspase-3 in the colon cancer cells. (A) RKO cells were treated with 3–9 μM oxaliplatin for 24 h. The total protein extracts were subjected to Western blot analysis. (B) and (C) The protein intensities of phospho-p38 and activated caspase-3 were semi-quantified. Results were obtained from 4 independent experiments. $^{**}p \leq 0.01$ indicates significant difference between control and oxaliplatin. (D) RKO cells were co-treated with or without 5 μM SB202190 and 3 μM oxaliplatin for 24 h. Cell viability was measured by MTT assay. Results were obtained from 3 to 4 experiments. The bar represents the mean ± S.D. $^{**}p < 0.01$ indicates significant difference between untreated and oxaliplatin-treated samples. $^{\#\#}p < 0.01$ indicates between oxaliplatin alone and co-treatment with SB202190.

However, the total p38 MAP kinase and ERK-2 proteins were not altered by oxaliplatin (Fig. 4A). Treatment with a specific p38 MAP kinase inhibitor SB202190 significantly reduced the oxaliplatin-induced cytotoxicity of RKO cells (Fig. 4D). Additionally, treatment with a general caspase inhibitor, Z-VAD-FMK, can attenuate the oxaliplatin-induced cancer cell death (data not shown).

3.5. A specific p38 MAP kinase inhibitor SB202190 restores the survivin protein expression and reduces caspase-3 activation in the oxaliplatin-treated colon carcinoma cells

To study the effect of p38 MAP kinase on the expression of survivin and active caspase-3 proteins, a p38 MAP kinase inhibitor, SB202190, was investigated in the oxaliplatin-treated cells. Treatment with SB202190 restored the survivin level and conversely reduced active caspase-3 proteins in the oxaliplatin-exposed RKO cells (Fig. 5A). After semi-quantification of Western blot, Fig. 5B and C shows the significant difference between oxaliplatin and oxaliplatin combined with SB202190 on the levels of survivin and active caspase-3, respectively. Nevertheless, the BCL-2 and XIAP proteins were not altered by oxaliplatin or SB202190 in RKO cells (Fig. 5A).

3.6. Activation of p53 mediates oxaliplatin-induced cell death in the human colon carcinoma cells

Treatment with 3–9 μM oxaliplatin for 24 h significantly increased the levels of phospho-p53 (Ser-15) and total p53 proteins in RKO cells (Fig. 6A). The phosphorylated p53 (Ser-15) proteins were located in the nucleus following oxaliplatin treatment (Fig. 6B, the arrows). Treatment with a specific p53 inhibitor, pifithrin-α, attenuated the levels of phosphorylated p53 (Ser-15), total p53, and active caspase-3 proteins in the oxaliplatin-treated RKO cells (Fig. 6C). After semi-quantification, the protein levels of phosphorylated p53 (Ser-15) and active caspase-3 were significantly reduced when combining oxaliplatin and pifithrin-α (Fig. 6D and E). Besides, pifithrin-α attenuated the oxaliplatin-induced cytotoxicity in RKO cells (Fig. 6F).

3.7. Inhibition of p53 reduces oxaliplatin-induced phosphorylated p38 MAP kinase proteins in the human colon carcinoma cells

Treatment with pifithrin-α significantly attenuated the protein level of oxaliplatin-induced phospho-p38 MAP kinase in RKO cells (Fig. 7A and B). In contrast, SB202190 did not alter the protein levels

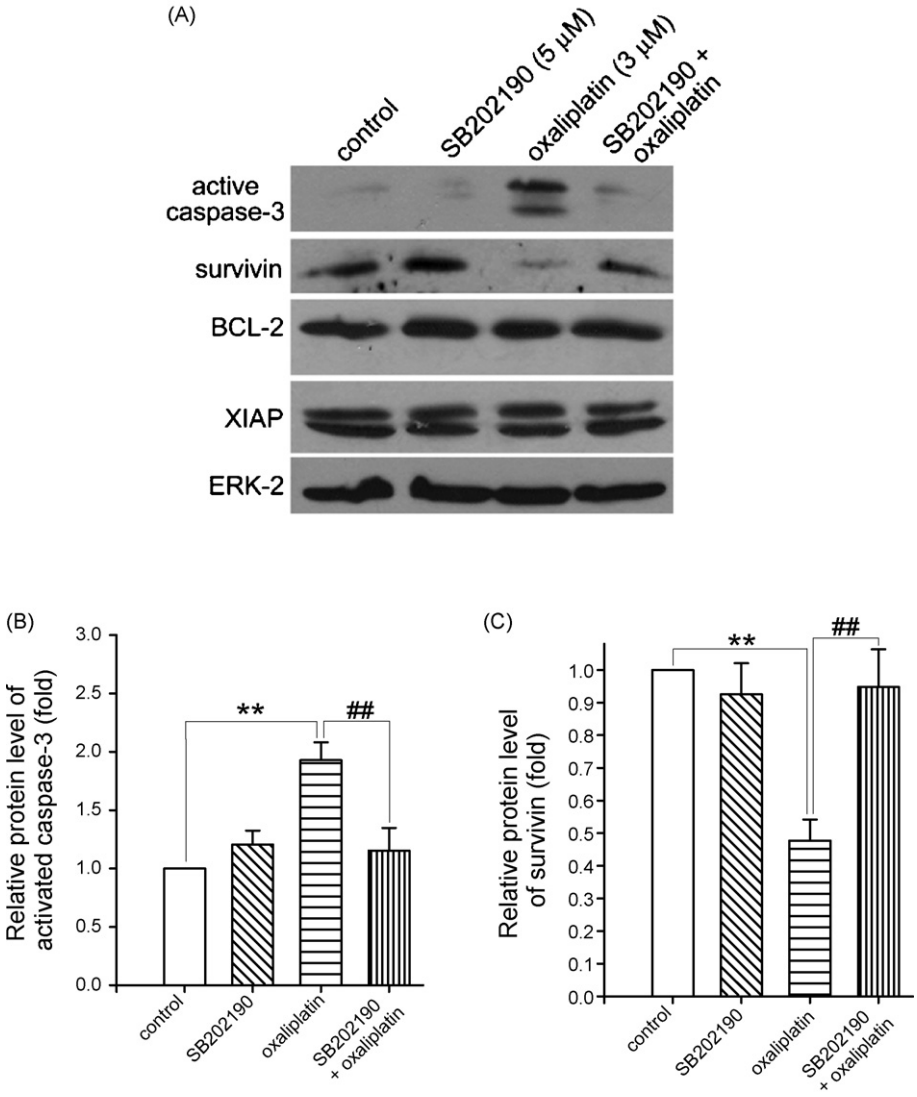

Fig. 5. Effect of a p38 inhibitor SB202190 on the protein expression of survivin and active caspase-3 in the oxaliplatin-exposed colon cancer cells. (A) RKO cells were co-treated with 5 μM SB202190 and 3 μM oxaliplatin for 24 h. At the end of treatment, the total protein extracts were subjected to Western analysis using specific antibodies for active caspase-3, survivin, BCL-2, XIAP, and ERK-2. (B) and (C) The protein intensities of activated caspase-3 and survivin were quantified from (A). $^{**}p \leq 0.01$ indicates significant difference between control and oxaliplatin. $^{\#\#}p < 0.01$ indicates significant difference between oxaliplatin alone and co-treatment with SB202190.

of phospo-p53 (Ser-15) and total p53 in the oxaliplatin-treated cells ($p > 0.05$) (Fig. 7C and D).

3.8. Blockage of proteasome pathway restores the oxaliplatin-inhibited survivin protein expression

MG132, a specific proteasome inhibitor, was combined with oxaliplatin in examining the effect of proteasome on survivin protein expression. Treatment with MG132 restored the survivin protein level in the oxaliplatin-treated cells (Fig. 8A). Actin is a loading control protein. After semi-quantification, MG132 significantly recovered the survivin protein expression in RKO cells (Fig. 8B). We have further examined the protein stability and half-life of survivin proteins in RKO cells by a protein synthesis inhibitor, cycloheximide. Fig. 8C shows that the survivin proteins were reduced approximately half of the amount following treatment with cycloheximide at 6–24 h period. After co-treatment with oxaliplatin and cycloheximide, the survivin protein degradation rate was more quick when comparing with cycloheximide alone in RKO cells (Fig. 8C). The protein level of survivin was completely inhibited when combining cycloheximide and oxaliplatin at 24 h (Fig. 8C).

4. Discussion

Survivin proteins are highly expressed in human cancer cells that prevent cancer cell death and apoptosis [16,17]. In the present study, oxaliplatin markedly decreased the survivin protein expression and induced the caspase-3 activation and apoptosis in the human colon cancer cells. Survivin belongs to IAP family, which exhibits anti-apoptotic activity by inhibiting the activity of caspases [16,20]. However, oxaliplatin did not affect the other IAP family proteins of XIAP and BCL-2. Accordingly, oxaliplatin displays unique ability on the survivin protein inhibition in colon cancer cells. Besides, we have demonstrated that over-expression of survivin by a survivin-expressed vector can increase the cell viability in colon cancer cells. Furthermore, transfection of survivin-expressed vector can partially resist the oxaliplatin-induced cancer death. It has been reported that oxaliplatin can reduce survivin protein expression [13,14]. Therefore, we suggest that down-regulation of survivin plays an important role in preventing cancer cell survival by oxaliplatin treatment during cancer therapy.

The p38 MAP kinase pathway has been associated with the induction of apoptosis by varying cellular stresses [25,37–39].

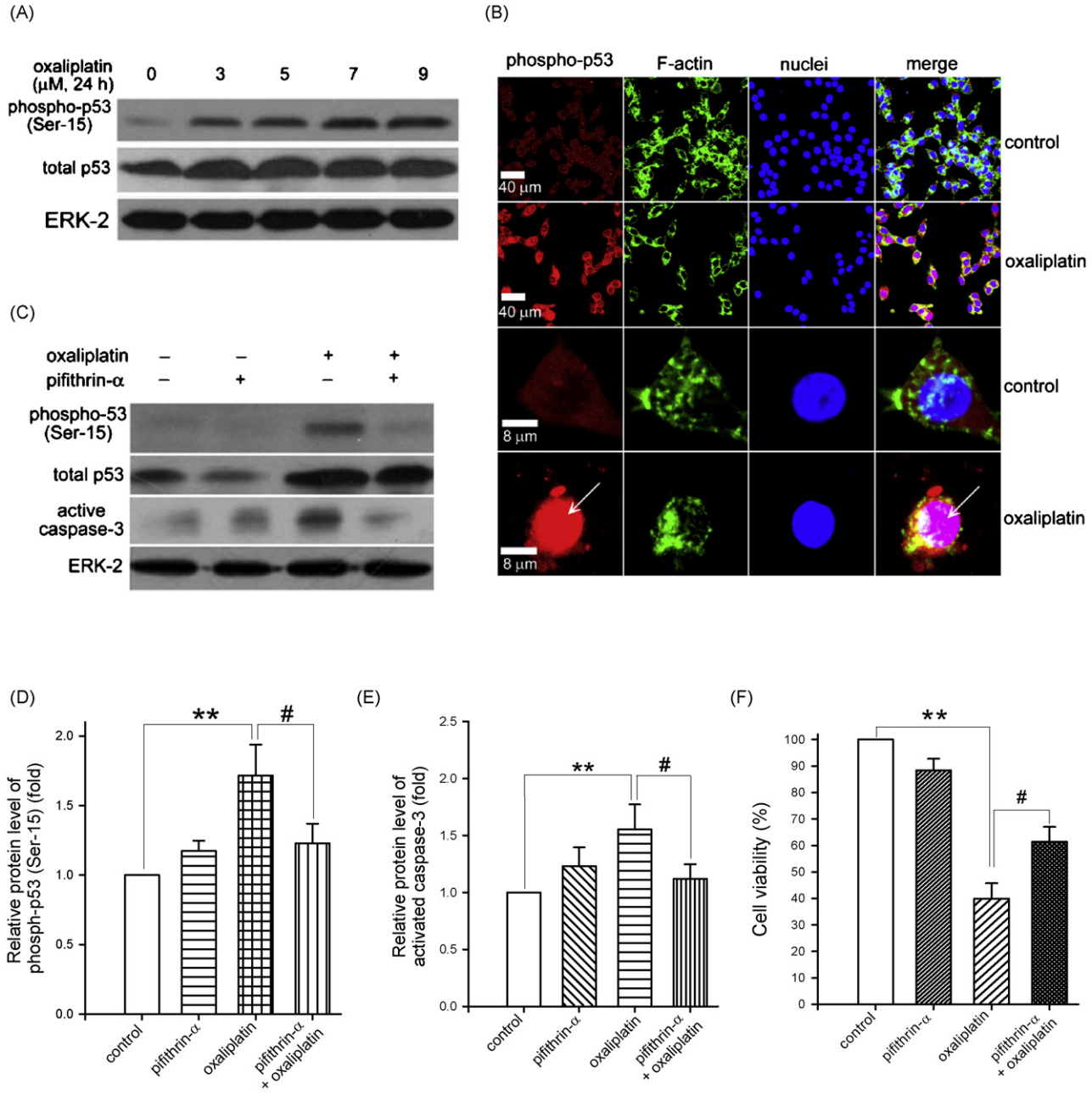

Fig. 6. Effect of oxaliplatin on the protein expression and location of phospho-p53 (Ser-15) in the colon cancer cells. (A) RKO cells were treated with 3–9 μM oxaliplatin for 24 h. Representative data were shown from 1 of 4 separate experiments with similar findings. (B) RKO cells were treated with or without 9 μM oxaliplatin for 24 h. After treatment, the cells were incubated with rabbit anti-phospho-p53 (Ser-15) and then incubated with goat anti-rabbit Cy5. F-actin and nuclei were stained with BODIPY FL phallacidin and Hoechst 33258, respectively. The arrow indicates that the phospho-p53 (Ser-15) proteins were located in the nucleus. (C) RKO cells were pre-treated with 20 μM pifithrin-α for 2 h prior to treatment with 3 μM oxaliplatin for 24 h. At the end of treatment, the total protein extracts were subjected to Western analysis. (D) and (E) the protein intensities of phospho-p53 (Ser-15) and activated caspase-3 were quantified from (C). (F) RKO cells were pre-treated with 20 μM pifithrin-α for 2 h prior to treatment with 3 μM oxaliplatin for 24 h. Cell viability was measured by MTT assay. Results were obtained from 5 to 6 experiments. The bar represents the mean ± S.D. $^{**}p < 0.01$ indicates significant difference between untreated and oxaliplatin-treated samples. $^{\#}p < 0.05$ indicates significant difference between oxaliplatin alone and pre-treatment with pifithrin-α.

It has been shown that p38 MAP kinase is involved in the down-regulation of survivin by baicalein treatment [23]. The phosphorylation of p38 MAP kinase mediates the activation of caspases [35,40,41]. Oxaliplatin can activate p38 MAP kinase phosphorylation [9]. It has been shown that oxaliplatin can induce the activation of casapse-3 and caspase-9 [42,43]. The phosphorylation of p38 MAP kinase was elicited by oxaliplatin in colon cancer cells. A specific p38 MAP kinase inhibitor, SB202190, attenuated active caspase-3 and cytotoxicity in the oxaliplatin-exposed cells; concomitantly, the survivin protein expression was restored. We found that oxaliplatin reduced the survivin protein expression, which was reversed by p38 MAP kinase inhibitor. Thus, the down-regulation of survivin by p38 MAP kinase can induce the activation of caspase-3 for apoptotic induction in human colon cancer cells.

p53 is a tumor suppressor, which regulates apoptosis in cancer cells and prevents tumor development [29–31]. The activation of p53 is mediated by phosphorylation at various sites [27,44–47]. The phosphorylated site of p53 at serine 15 plays an important role for the p53 activation [44] and stabilization [45,46]. Previously, we have found that oxaliplatin can activate p53 for apoptosis

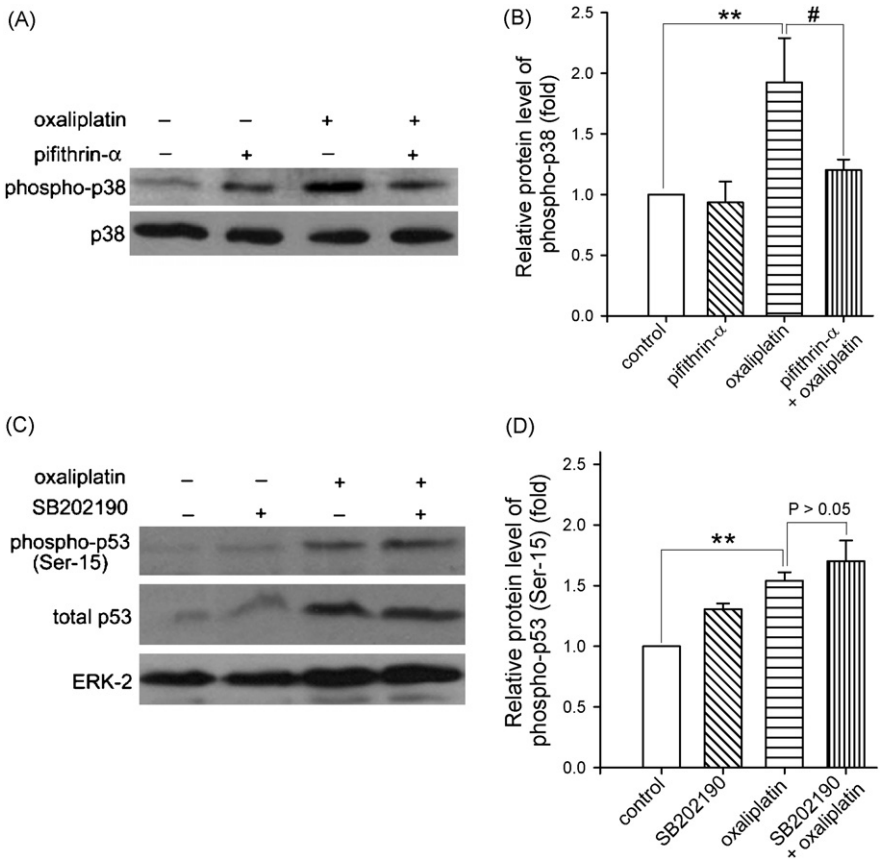

Fig. 7. Effect of pifithrin-α on the phosphorylation of p38 MAP kinase in the oxaliplatin-treated colon cancer cells. (A) RKO cells were pre-treated with 20 μM pifithrin-α for 2 h prior to treatment with 3 μM oxaliplatin for 24 h. (B) The protein intensity of phospho-p38 was quantified from (A). (C) RKO cells were co-treated with 5 μM SB202190 and 3 μM oxaliplatin for 24 h. (D) The protein intensity of phospho-53 (S-15) was quantified from (C). Results were obtained from 3 separate experiments. $^{**}p < 0.01$ indicates significant difference between untreated and oxaliplatin-treated samples. $^{\#}p < 0.05$ indicates significant difference between oxaliplatin alone and combined treatment with pifithrin-α.

and cell cycle arrest [8]. We observed that oxaliplatin increased phospho-p53 (Ser-15) proteins, which were translocated into the nuclei of cancer cells. Interestingly, treatment with p53 inhibitor pifithrin-α reduced the protein levels of p38 MAP kinase and active caspase-3 in the oxaliplatin-exposed colon cancer cells. It has been reported that p53 can down-regulate the survivin protein expression for inducing apoptosis [48]. We suggest that p53 mediates the oxaliplatin-inhibited survivin expression may be from the up-

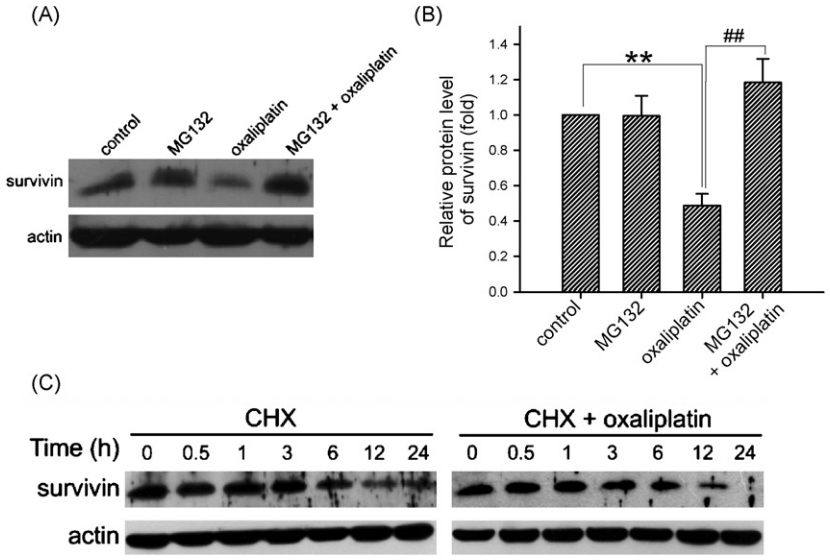

Fig. 8. Effect of MG132 and cycloheximide on the protein expression of survivin in the oxaliplatin-treated colon cancer cells. (A) RKO cells were pre-treated with 5 μM MG132 for 1 h prior to treatment with 3 μM oxaliplatin for 24 h. (B) The protein intensity of survivin was quantified from (A). Results were obtained from 4 separate experiments. $^{**}p < 0.01$ indicates significant difference between untreated and oxaliplatin-treated samples. $^{\#\#}p < 0.01$ indicates significant difference between oxaliplatin alone and combined treatment with MG132. (C) RKO cells were co-treated with or without 3 μM oxaliplatin and 20 μg/ml cycloheximide (CHX) for 0–24 h. The treated cells were harvested at the indicated times for Western blot assays. Representative data were shown from one of 3 separate experiments with similar findings.

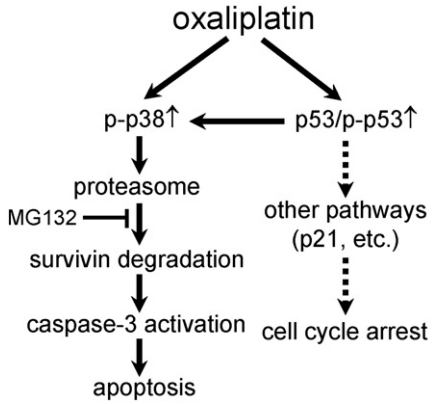

Fig. 9. Proposed model of the regulation of survivin protein expression by p38 MAP kinase and proteasome on the oxaliplatin-induced apoptosis.

regulation of phosphorylation of p38 MAP kinase in colon cancer cells.

Subsequently, we found that treatment with relative high concentration of oxaliplatin did not markedly reduce the level of survivin mRNA in colon cancer cells. However, we found that oxaliplatin induced survivin protein instability when blocked protein synthesis pathway by cycloheximide. Indeed, inhibition of survivin protein expression by oxaliplatin in esophageal cancer cells was partially due to the proteasome-mediated protein degradation pathway [10]. Thus, we can conclude that the oxaliplatin-decreased survivin protein level is not correlated with survivin gene expression in various cancer cells. The proteasomal degradation pathway has been shown to relate with kaempferol inhibited survivin protein expression in glial cancer cells [49]. Inhibition of proteasome by a specific proteasome inhibitor MG132 selectively increased phosphorylated p38 MAP kinase levels without affecting the mRNA or protein levels of p38 MAP kinase that is tightly coupled to protein degradation [50]. Moreover, p38 MAP kinase is involved in regulating MG132-induced apoptosis of myeloma cells [51]. We find that inhibition of p38 MAP kinase by SB202190 can restore the survivin protein level and attenuate the oxaliplatin-induced cancer cell death. Furthermore, treatment with MG132 can completely restore the survivin protein level by oxaliplatin treatment in colon cancer cells. Taken together, we suggest the protein down-expression of survivin that is regulated by p38 MAP kinase and proteasomal pathway.

In conclusion, we propose a model that oxaliplatin down-regulates survivin protein expression by p38 MAP kinase and proteasome pathway (Fig. 9). This study also provides that p53 can regulate the protein phosphorylation of p38 MAP kinase. In addition to apoptosis, oxaliplatin may mediate the p53–p21 pathway for cell cycle arrest [8]. Understanding the mechanisms of blocking survivin pathways and regulating other signal molecules following oxaliplatin treatment may contribute the novel strategies for cancer therapy.

Conflict of interest statement

None declared.

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