Anticancer effect of thalidomide *in vitro* on human osteosarcoma cells

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Received May 6, 2016; Accepted September 5, 2016

DOI: 10.3892/or.2016.5158

Abstract. Osteosarcoma is a high-grade malignant tumor frequently found in children and adolescents. Thalidomide has been reported for treatment of various malignancies. Thalidomide was added to osteosarcoma cells and studied by cytotoxicity assay, evaluating apoptosis, cell cycle arrest, mitochondrial membrane potential (ΔΨm), and reactive oxygen species (ROS) levels and the expression of Bcl-2, Bax, caspase-3 and NF-κB. The results showed that thalidomide could inhibit the proliferation of MG-63 and U2OS cells in a concentration- and time-dependent manner. Morphological changes of apoptosis were also observed. Thalidomide increased the apoptosis rate of MG-63 cells and induced cell cycle arrest by increasing the number of cells in the G0/G1 phase and decreasing the percentage of S phase in MG-63 cells. Further investigation showed that a disruption of ΔΨm and upregulation of ROS were induced by thalidomide in high concentration. By western blot analysis, thalidomide resulted in the decreasing expression of Bcl-2 and NF-κB, and the increasing expression of Bcl-2/Bax and caspase-3. Here, we provide evidence that thalidomide could cause apoptosis in osteosarcoma cells. Taken together, these results indicate that thalidomide could be an antitumor drug in the therapy of osteosarcoma.

Introduction

Osteosarcoma is a high-grade malignant tumor frequently found in children and adolescents. The therapeutic method has evolved obviously in recent years, from surgery only to combined therapy of surgery, chemotherapy and radiation, and it also improved the long-term survival rate from 20 to 70% (1,2). However, most patients were first diagnosed as advanced and metastatic osteosarcoma, and their 5-year survival rate was <20%. New drugs to improve the survival rate are required.

Thalidomide has been reported to possess different cytotoxic activity towards different tumor cell lines, such as prostate, colorectal, non-small cell lung cancer, breast cancer, and renal cell carcinoma (3-9). Tsai *et al* (10) reported a clinical case that a relapse osteosarcoma patient was treated with a combination of thalidomide and celecoxib, then the tumors in the lung became smaller 1 month later. Apoptosis plays an important role in controlling tumorigenesis in many anticancer drugs (11-13). Unfortunately, very few studies have been carried on the inhibitory effect of thalidomide on osteosarcoma and its mechanisms. Therefore, the aim of the present study was to thoroughly investigate thalidomide-induced apoptosis, and to explore its potential mechanisms.

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Key words: thalidomine, cytotoxicity *in vitro*, apoptosis, cell cycle arrest, reactive oxygen species, mitochondrial membrane potential, western blot analysis

Materials and methods

Reagents. Thalidomide was purchased from Sigma. Annexin V-FITC/PI apoptosis detection kit, DNA content quantitation assay (cell cycle), reactive oxygen species (ROS) detection kit, apoptotic cell Hoechst 33258 detection kit were purchased from KeyGEN (China). Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Laboratories in Japan. Mitochondrial membrane potential assay kit with JC-1 was purchased from Beyotime Biotech (China). DMEM, McCoy's 5A medium, trypsin and fetal bovine serum were purchased from Gibco (USA). Rabbit anti-caspase-3, anti-Bcl-2, anti-Bax, anti-NF-κB and anti-GAPDH antibodies were purchased from Abcam. The secondary antibodies were purchased from Bioworld Technology, Inc.

Cell culture and treatments. MG-63 and U2OS (osteosarcoma cells) were purchased from the American Type Culture Collection. The cells were cultured in DMEM or McCoy's 5A medium supplemented with 10% FBS at 37˚C in 5% CO_2 and

95% air, respectively. Thalidomide was dissolved in DMSO, at concentration <0.1%.

Cytotoxicity assay in vitro. CCK-8 assay procedures were used to measure the cell viability. Cells were seeded in 96-well plates overnight before drug treatment. Thalidomide was added to the cells at various concentrations (0, 12.5, 25, 50, 100, 200 and 400 μg/ml), with 5 wells used for each concentration. The plates were incubated at 37˚C in a 5% CO_2 incubator. After 24, 48 and 72 h, 10 μl of CCK-8 solution were added to each well at 37˚C for 3 h and followed by a measurement of absorbance at 450 nm using a microplate reader. The IC_{50} values were calculated. Each experiment was repeated at least three times to obtain the mean values. The two tumor cell lines used in the this study were MG-63 and U2OS.

Cell apoptosis assay. The morphological changes of apoptosis were measured by Hoechst 33258 (Beyotime Biotech) after the cells were treated with thalidomide. After 48 h, cells were washed with 1X PBS three times and stained with 1 μg/ml of Hoechst 33258 nuclear dye for 10 min. Then the cells were observed and imaged by a fluorescence microscope.

Flow cytometry detecting FITC-Annexin V-positive apoptotic cells. Flow cytometry analysis was used to detect cell apoptosis. After drug treatment, cells were collected by trypsinization, and stained with FITC-Annexin V and propidium iodide (PI). Both early (Annexin V^+/PI^-) and late (Annexin V/PI^+) apoptotic cells were sorted by FCM (FACSCalibur; BD Biosciences).

Flow cytometry analysis on cell cycle arrest studies. Flow cytometry analysis was used to detect the distribution of cell cycle. After drug treatment, cells were collected by trypsinization, and washed twice with ice-cold PBS, suspended in 70% alcohol, and kept at 4˚C overnight. Then cells were stained with the Cycletest Plus. The cell cycle distribution was detected with FCM (BD FACSCalibur; BD Biosciences).

Mitochondrial membrane potential (ΔΨm) assay. The fluorescent dye JC-1 (Beyotime Biotech) was used to assess ΔΨm. After drug treatment, MG-63 cells in 6-well plates were collected and loaded with 1 μg/ml JC-1 at 37˚C for 20 min in the dark, and then rinsed twice with PBS. Then cell pellets were suspended in PBS and ΔΨm was monitored by flow cytometry.

Assay of intracellular ROS. The non-fluorescent probe 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to measure ROS. The probe diffused into cells and reacted with ROS to form the trapped fluorescent product DCF. After the treatment of thalidomide for 24 h, MG-63 cells in 96-plate were washed three times with PBS. DCFH-DA, diluted to a final concentration of 10 μM with RPMI-1640 medium, was added to cover the cells and incubated for 20 min at 37˚C. The treated cells were then washed with cold PBS twice, and involved in PBS. The fluorescence intensity was measured at an excitation wavelength of 488 nm and emission at 525 nm with Thermo Scientific Varioskan Flash. The increase in value compared to control was viewed as the increase of endocellular ROS.

Table I. The IC_{50} (μM) values of thalidomide on osteosarcoma cells.

Cells	24 h (μg/ml)	48 h (μg/ml)	72 h (μg/ml)
MG-63	>500	151.05±8.09	94.76±10.52
U2OS	>500	476.13±93.31	156.61±40.65

Western blot analysis. MG-63 cells were incubated with different concentrations of thalidomide in the presence of 10% FBS for 48 h. Total protein was extracted with RIPA and PMSF buffer and quantified using a bicinchoninic acid (BCA) Protein Assay kit (Beyotime Biotech). A total of 40 μg of protein was subjected to 10% SDS-PAGE electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane later. The membrane was incubated with Bax (1:1,000 dilution), Bcl-2 (1:250 dilution), caspase-3 (1:500 dilution), NF-κB (1:1,000 dilution), GAPDH (1:1,000 dilution) (all from Abcam) at 4˚C overnight and incubated with goat anti-rabbit second antibody (1:5,000 dilution; Bioworld Technology, Inc.) at room temperature for 1 h. The intensity of the specific immunoreactive bands was detected by enhanced chemiluminescence (ECL).

Statistical analysis. All data were analyzed with the statistical software GraphPad Prism 5.0, and all values are expressed as means ± SD. The differences between two groups were analyzed using Student's unpaired t-test, and differences between three or more groups were evaluated via one-way ANOVA with Bonferroni correction. A probability value of <0.05 was considered significant.

Results

Cytotoxicity assay in vitro. After exposure to the desired concentration ranged from 12.5 to 400 μg/ml for 24, 48 and 72 h, the cytotoxicity of thalidomide against MG-63 and U2OS cells was evaluated by cell viability using the CCK-8 assay. From the results (Fig. 1A and B), the inhibition of thalidomide to MG-63 and U2OS cells was observed to be time- and concentration-dependent, which indicates that thalidomide could effectively inhibit the cell proliferation. The IC_{50} values are also shown in Table I, which indicate 151.05±8.09 and 94.76±10.52 μg/ml for 48 and 72 h in MG-63 cells.

Apoptosis studies by Hoechst 33258 staining and by flow cytometry. After the treatment of thalidomide of different concentration (50-200 μg/ml) for 48 h, MG-63 cells were stained with Hoechst 33258 and imaged under a fluorescent microscope. As shown in Fig. 2, significant nuclear condensation and morphological changes, such as nuclear shrinkage and chromatin condensation, were observed in MG-63 cells.

In Annexin V-FITC/PI double staining by FACS analysis, Annexin V-FITC/PI double-positive cells significantly increased after treatment with thalidomide for 48 h in a

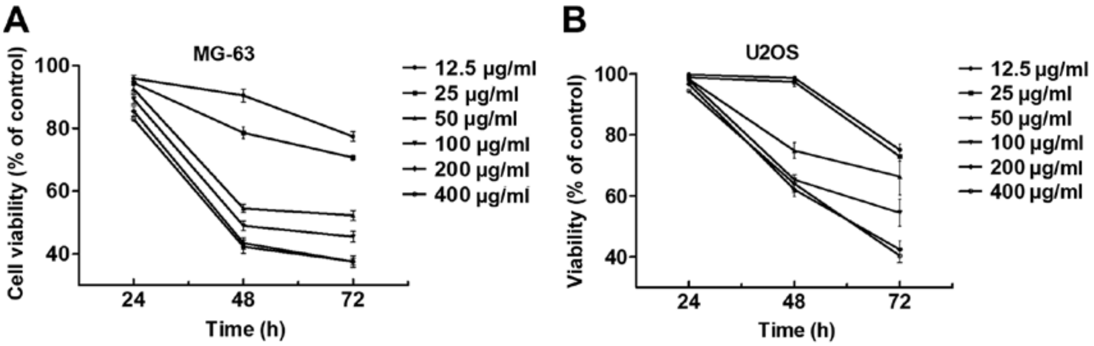

Figure 1. The cell viability induced by thalidomide in (A) MG-63 and (B) U2OS cells. Cell activities were detected by CCK-8 in various concentrations (12.5, 25, 50, 100,s 200 and 400 μg/ml) for 24, 48, 72 h; n=3; $^{*}p<0.05$. CCK-8, Cell Counting Kit-8.

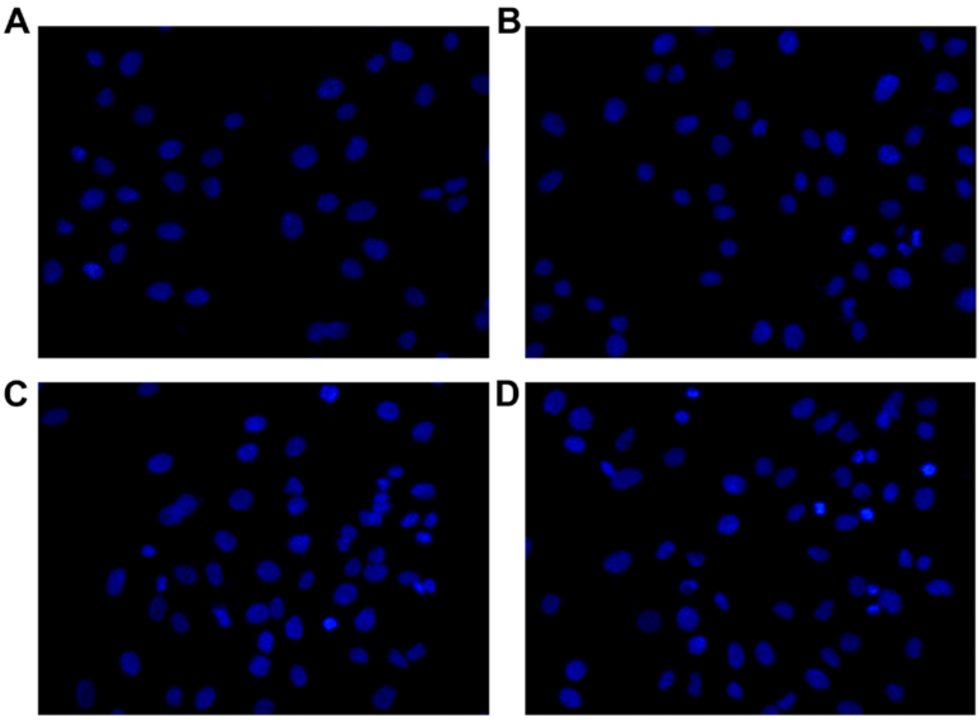

Figure 2. The morphological changes of MG-63 cells were recorded by Hoechst 33258. Cells were incubated with thalidomide of (A) 0 μg/ml, (B) 50 μg/ml, (C) 100 μg/ml, and (D) 200 μg/ml for 24 h.

concentration-dependent manner. As shown in Fig. 3, the percentage of apoptotic cells was 7.98±1.26% in negative control. Exposure to 50, 100 and 200 μg/ml of thalidomide, the percentage of apoptotic cells was 10.58±1.18, 28.74±6.08 and 38.00±6.40%, respectively. The data appeared to suggest that the apoptotic effect of thalidomide to MG-63 cells was concentration-dependent.

Cell cycle arrest. Flow cytometry was used to investigate the effect of thalidomide on the cell cycle arrest. After the treatment with different concentration (50-200 μg/ml) of thalidomide for 48 h, the DNA distribution histogram is shown in Fig. 4. The differences between thalidomide-treated culture and negative controls were significant. In the control, the percentage in G0/G1 phase was 63.68±1.76%. And the percentage was 71.9±0.83, 73.87±1.72 and 76.37±1.12%, respectively, after MG-63 cells were treated with 50, 100 and 200 μg/ml of thalidomide. The percentage in S phase was 15.08±3.35, 13.53±2.96 and 12.38±2%, compared with the control 19.95±3.11%, respectively. These data showed that thalidomide induced cell cycle arrest by increasing the number of cells in the G0/G1 phase and decreasing the percentage of S phase in MG-63 cells. The effect of thalidomide on the cell cycle arrest was also concentration-dependent.

$\Delta\Psi m$ assay. To study the initiation of apoptosis, JC-1 was used as a fluorescence probe in detecting the change of $\Delta\Psi$m induced by thalidomide. MG-63 cells were cultured with increasing concentration (50-200 μg/ml) of thalidomide for 24 h, and then analyzed by flow cytometry. As shown in Fig. 5, the percentage of cells showing an intact mitochondrial membrane decreased 92.37±0.97, 90.4±2.62, 85.53±4.70% by concentration (50, 100 and 200 μg/ml), compared with the negative control 95.17±0.31%, respec-

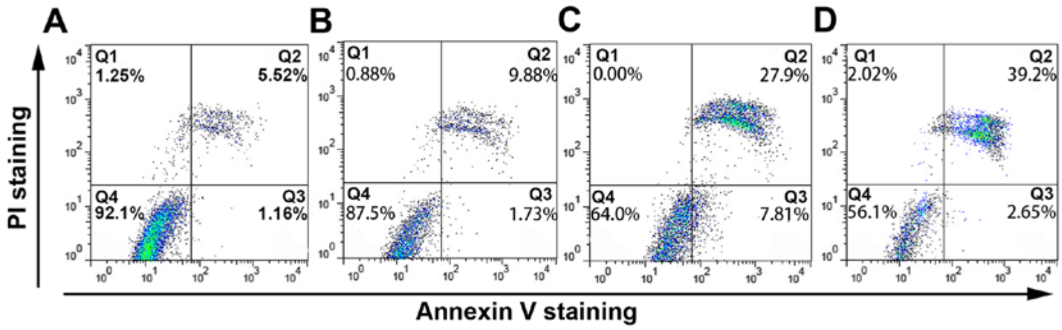

Figure 3. Thalidomide resulted in the change of apoptosis in MG-63 cells. Flow cytometry was used. Cells were incubated with thalidomide of (A) 0 μg/ml, (B) 50 μg/ml (C) 100 μg/ml and (D) 200 μg/ml for 48 h; n=3.

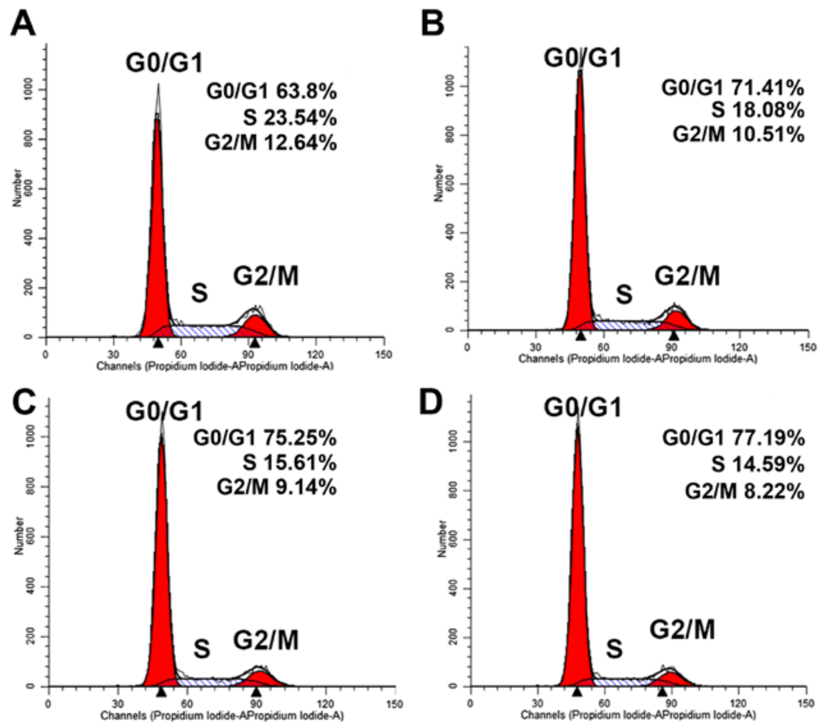

Figure 4. Thalidomide resulted in the change of cell cycle in MG-63 cells. Flow cytometry was used. Cells were incubated with thalidomide of (A) 0 μg/ml, (B) 50 μg/ml, (C) 100 μg/ml and (D) 200 μg/ml for 48 h; n=3.

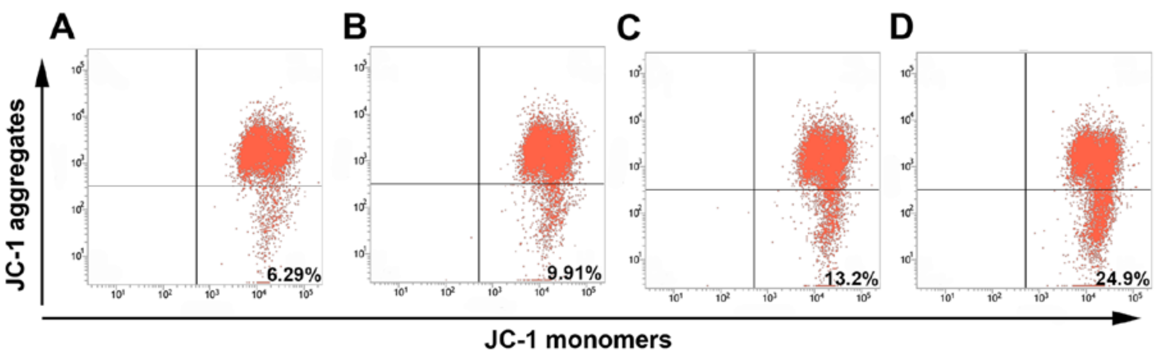

Figure 5. Thalidomide resulted in the change of ΔΨm in MG-63 cells. Flow cytometry was used. Cells were incubated with thalidomide of (A) 0 μg/ml, (B) 50 μg/ml, (C) 100 μg/ml and (D) 200 μg/ml for 24 h; n=3. ΔΨm, mitochondrial membrane potential.

tively. The number of cells showing loss of mitochondrial membrane increased 7.63±0.94, 9.57±2.64, 13.62±5.92%, compared with negative control 4.84±0.31%, respectively. This result suggested that thalidomide disrupted the ΔΨm in a

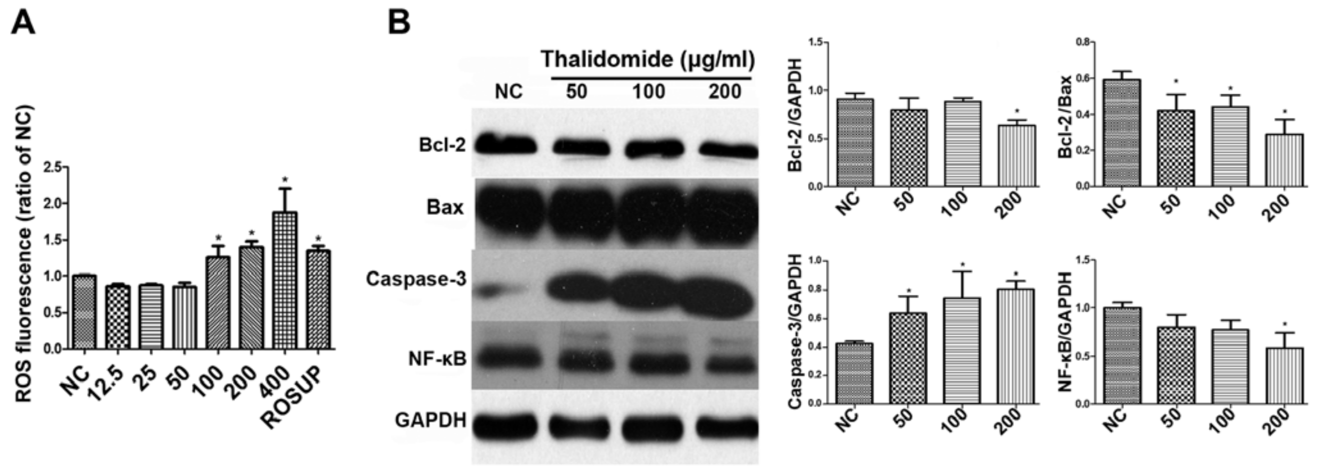

Figure 6. Thalidomide resulted in the change of ROS and apoptosis-related protein in MG-63 cells. (A) The level of ROS was detected by DCFH-DA after treatment with thalidomide (12.5, 25, 50, 100, 200 and 400 μg/ml) for 24 h; n=3. (B) Bcl-2, Bax, caspase-3 and NF-κB were detected by western blot analysis as described; n=3. ROS, reactive oxygen species. *P<0.05.

concentration-dependent manner. Taken together, these results indicated that thalidomide induced apoptosis in MG-63 cells through the mitochondrial pathway.

ROS level determination. Many potential anticancer agents induce apoptosis through ROS generation. DCFH-DA was used as a fluorescent probe to detect intracellular ROS production change. As shown in Fig. 6A, the result indicated that thalidomide could increase the levels of ROS in MG-63 cells at concentrations of 100-400 μg/ml. The fluorescent intensities of DCF increased 1.26±0.16, 1.40±0.08 and 1.88±0.32 times of the negative control in 100, 200 and 400 μg/ml of thalidomide group, respectively, while the positive control Rosup was 1.34±0.07 times. The differences of ROS between high concentration (100-400 μg/ml) and low concentration (12.5-50 μg/ml) were also statistically significant.

The expression of Bcl-2, Bax, caspase-3 and NF-κB assay. Apoptosis was the major reason of cell death produced by antitumor drugs. To clarify the underlying mechanism of apoptosis, the effects of thalidomide to the expression of Bcl-2, Bax, caspase-3 and NF-κB in MG-63 cells are shown in Fig. 6B. Bcl-2 family proteins play important roles in the regulation of apoptosis via the control of mitochondrial membrane permeability and the release of cytochrome *c* and/or Smac/DIABLO (14). The Bcl-2 is an oncogene and Bax is a cancer suppressor gene. An imbalanced Bcl-2/Bax ratio has been recognized as a signature of apoptosis acquisition in cancer cells (15,16).

Thalidomide treatment in MG-63 cells for 48 h resulted in a decreasing expression of Bcl-2 (0.91±0.07, 0.79±0.13, 0.89±0.04 and 0.63±0.05 of GAPDH for 0, 50, 100 and 200 μg/ml of thalidomide, respectively) and Bcl-2/Bax ratio (0.60±0.05, 0.42±0.09, 0.44±0.07 and 0.29±0.08 for 0, 50, 100 and 200 μg/ml of thalidomide, respectively). Caspases are known to mediate the apoptotic pathway (17,18), and processed effector caspase-3 can create damage to the organelles. In this study, caspase-3 was highly increased after the administration of thalidomide compared with negative control (0.42±0.02, 0.64±0.12, 0.74±0.19 and 0.80±0.06 of GAPDH for 0, 50, 100 and 200 μg/ml, respectively).

Constitutive NF-κB activation has been noted in 95% of all cancers (19-21). It plays an oncogenic role of in the promotion of cell proliferation, control of apoptosis, promotion of cell proliferation, control of apoptosis, stimulation of angiogenesis and invasion/metastasis in cancer cells (22-26). Significantly decreasing level of NF-κB is seen in Fig. 2C (1.00±0.05, 0.80±0.13, 0.77±0.11 and 0.59±0.16 of GAPDH for 0, 50, 100 and 200 μg/ml of thalidomide, respectively).

Discussion

Osteosarcoma, occur predominantly in adolescents and young adults, and is the most common malignant disease of primary bone. The curative rate is low, due to terminal prognosis at the first diagnosis and declining effects of cytotoxic drugs (27-29). Finding new therapeutic agents to osteosarcoma is important. Thalidomide, together with its anti-angiogenic, antiproliferative, and pro-apoptotic activities, is thought to regulate antitumor responses (30,31). Here we observed that thalidomide induced apoptosis in cultured osteosarcoma cells. Treatment of MG-63 cells with thalidomide, the cell viability decreased in time- and concentration-dependent manner. Morphological changes of apoptosis were observed as well. Thalidomide could effectively induce apoptosis of MG-63 cells and inhibit the cell growth at the G0/G1 phase. The high concentration of thalidomide could increase the levels of ROS. Thalidomide could also induce the decrease of ΔΨm, and thalidomide could downregulate the expression of Bcl-2, Bcl-2/Bax ratio and NF-κB, and simultaneously increase the level of caspase-3.

Apoptosis plays an important role in controlling tumorigenesis in many anticancer drugs (18). It is well known that two major pathways are involved in mammalian cells: the extrinsic and intrinsic pathway. The latter leads to ΔΨm disruption, the early event in mitochondrial-mediated apoptosis, and results in the release of cytochrome *c* and the activation of caspase-9 (31). Then the apoptosomes cleave pro-caspase-3 formed caspase-3, which plays a critical role in implementing apoptosis (32). It was also clear that Bcl-2 and Bax could regulate the release of apoptogenic factors and the opening of the mitochondrial permeability transition pore (33-35). In the present study, early and late apoptotic cells quantitated by Annexin V-FITC/PI double staining showed concentration-dependent apoptosis. The sub-G1 population during cell cycle analysis prompted the presence of apoptotic cells. The result of mechanistic studies showed that thalidomide-induced apoptosis in MG-63 cells was mediated by mitochondrial-mediated intrinsic pathway, followed by the increase of caspase-3 and decrease of Bcl-2 protein and the ratio of Bcl-2/Bax.

ROS at moderate levels represent significant signaling molecules, which are widely involved in physiological processes through oxidizing proteins, lipids and polynucleotides (36). Oxidative stress is one of the major causes for cell death and damage for oxidative damage to DNA and biomolecules. Overexpression made internal defense mechanism fail the fight against it. In the present research, ROS was observed to be increasing in high concentration of thalidomide in MG-63 cells. In addiction, ROS could reduce the level of Bcl-2 (37). ROS production might also increase independently the metabolic state of mitochondria.

In many cancer cells NF-κB was persistently active and located in the nucleus. The continuously expressing nuclear Rel/NF-κB activity could protect cancer cells from apoptosis and stimulate their growth. In this study, activation of NF-κB receded in a concentration-dependent manner with treatment of thalidomide. ROS stimulated the expression of NF-κB to activate MnSOD, which could clear free radicals and reduced the activation of NF-κB in return. In the present study, it was assumed that NF-κB pathway work to decrease the level of ROS in low concentration of thalidomide.

In conclusion, we found that thalidomide induced apoptosis in osteosarcoma cells, which was accompanied by ROS, disruption of $\Delta\Psi$m and regulating the expression of Bcl-2, Bax, caspases-3 and NF-κB. Therefore, thalidomide might play a role in the therapy of osteosarcoma disease.

Acknowledgements

This study was supported by the National Natural Science Foundation of China (no. 81272941) and the Guangdong Provincial Department of Science and Technology (2014A020212009). This study was also supported by the Guangdong Provincial Key Laboratory of Malignant Tumor Epigenetics and Gene Regulation, Sun Yat-Sen Memorial Hospital, Sun Yat-Sen University.

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