Biomedical Research 2019; 30 (1):

ISSN 0970-938X
www.biomedres.info

Paclitaxel induces apoptosis in osteosarcoma *via* promoting the production of reactive oxygen species and inhibiting the expression of cyclooxygenase-2.

Ming Li[#], Liming Yin[#], Lili Wu[#], Yunsen Zhu, Xi Wang[*]

The First People's Hospital of Wenling, Wenling, China

[#]These authors contributed equally to this work

Abstract

Objective: The present study was designed to investigate the possible molecular mechanisms of apoptosis in osteosarcoma induced by paclitaxel.

Study design: Cell viability was detected in human osteosarcoma cell line HOS-732 treated with paclitaxel at different concentrations. The apoptosis and reactive oxygen species (ROS) production rates were determined by flow cytometry. COX-2 expression at mRNA and protein levels in osteosarcoma tissues were measured by quantitative real time polymerase chain reaction (RT-PCR) and Western blotting.

Results: Paclitaxel can obviously inhibit the proliferation of HOS-732 cells in a dose-dependent manner. The production of ROS in HOS-732 cells was remarkably increased with the increasing concentration of paclitaxel. Moreover, paclitaxel can significantly inhibit the expression of COX-2 at mRNA and protein levels in rat osteosarcoma tissue. Furthermore, the inhibitory effects of paclitaxel on COX-2 expression at mRNA and protein levels were dose-related.

Conclusion: Paclitaxel treatment can induce the apoptosis and increase the production of ROS in HOS-732 cells. Moreover, paclitaxel can decrease the expression of COX-2 in a dose-dependent manner. These findings suggested that paclitaxel-induced apoptosis in osteosarcoma could be ascribed to the increased production of ROS and the reduction of COX-2 expression.

Keywords: Paclitaxel, Reactive oxygen species, COX-2, Osteosarcoma.

Accepted on February 21, 2019

Introduction

Osteosarcomais the common primary malignant bone tumor occurring frequently in adolescents. Early symptoms of osteosarcoma include pain, lumps, junction dysfunction, fractures and other irritative symptoms [1]. Currently, the main treatments for osteosarcoma are neoadjuvant chemotherapy, surgical resection and chemotherapy again following surgery. The 5-year survival rate in patients with osteosarcoma is approximately 60-70%, and there are about 40-50% of patients having pulmonary metastasis [2,3]. Hence, it is very urgent to develop new therapies improving the prognosis of osteosarcoma patients.

Generally, chronic inflammation is considered as an important pathological cause of cancer. Cyclooxygenase-2 (COX-2), one isoform of cyclooxygenase that catalyse the conversion of arachidonic acid to prostanoids, is normally detected in a few of tissues, including the brain, placenta and kidney. However, the expression of COX-2 is increased rapidly as an inflammatory reaction in response to extracellular stimuli [4]. Furthermore, COX-2 expression plays a critical role in the process of tumorigenesis [5,6]. Overexpression of COX-2 has been documented in many human cancers of various organs including lung, colon, breast, esophagus, stomach, pancreas, prostate, and is closely associated with tumor proliferation, migration, and invasion [4,7,8]. Reactive oxygen species (ROS) are chemically active molecules containing oxygen. Accumulating literatures demonstrated that ROS can cause the apoptosis of cells through a number of molecular mechanisms including the activation of apoptosis signal regulating kinase 1 (ASK1) and mitogen activated protein kinase (MAPK), the inhibition of the activity of topoisomerase II [9-12]. Moreover, it has been reported that overexpression of COX-2 is involved in the mechanisms of apoptotic cell death induced by ROS [13].

Paclitaxel is a chemotherapeutic drug for cancer, which is isolated from the Pacific yew tree. It is well-documented to have cytotoxic activity against several tumors including breast cancer, lung cancer, ovarian cancer etc. [14,15]. In the present study, human osteosarcoma cell line HOS-732 and rat osteosarcoma tissues were used to assess the effects of paclitaxel on the apoptotic cell death in osteosarcoma. Then, the production of ROS and the expression of COX-2 were investigated to determine the potential mechanism of the apoptosis induced by paclitaxel.

Materials and Methods

Animals

Adult male Sprague Dawley rats (200 ± 10 g) were obtained from Shanghai SLAC Laboratory Animal Company. The experimental protocols were approved by the Committee of Laboratory Animals of The first people's hospital of Wenling.

Cell viability test

Human osteosarcoma cell line HOS-732 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI-1640 medium containing 10% FBS (all purchased from Gibco). Cell viability test was carried out using CCK-8 assay. Briefly, cells seeded in 96-well plates at a density of 1×10^4 cells per well were treated with paclitaxel (SihuanPharm, Beijing) at different concentrations (0.5 μg/ml, 1 μg/ml and 2 μg/ml, dissolved in DMSO) and 0.1% DMSO (vehicle) for 48 h. After washed three times with fresh RPMI-1640 medium, the cells were incubated with 110 μl RPMI-1640 medium containing 10 μl CCK-8 (Beyotime Biotechnology) for 4 h. The absorbance at 450 nm was determined by a microplate reader (ELX800UV, Bio-Tek Instruments, Inc.). Three parallel replicates of each sample were prepared for statistical analysis.

Detection of apoptosisby flow cytometry

HOS-732 cells at a density of 1×10^5 cells per well were cultured in 12-well plates. Cells were treated with paclitaxel at different concentrations (0.5 μg/ml, 1 μg/ml and 2 μg/ml) and 0.1% DMSO for control for 48 h. Then, cells were collected and assessed the apoptosis induced by paclitaxel by using Annexin V-APC/7-AA Dapoptosis Kit (Biolegend). The apoptosis rates were detected by flow cytometry (Beckman Coulter, FC500).

Measurement of intracellular ROS by flow cytometry

Cells treated with paclitaxel at different concentrations were collected and washed twice in 0.01 M phosphate-buffered saline (PBS, pH=7.4). Then the cells were incubated with 10 μmol/LDCFH-DA (the probe of the reactive oxygen species) at 37°C for 20 minutes according to the instructions provided by the manufacturer. DCF fluorescence was detected by a Beckman-Coulter XL flow cytometer.

Preparation of rat osteosarcoma model

HOS-732 cells were collected and washed with 0.01 M PBS (pH=7.4) for three times. Cell at a density of 1×10^7 cells in 100 μl medium were injected subcutaneously into the right forelimb of SD rats.

Quantitative real-time PCR analysis of COX-2 expression

Total RNA of osteosarcoma tissues in rats were extracted by using a RNA extraction kit (RNAiso Kit, Takara). The concentrations and purity of total RNA were determined by UV absorbance at 260 nm using NanoDrop2000 (Thermo Fisher Scientific). The purified mRNA was used as a template to synthesize complementary DNA (cDNA) by a Takara RNA PCR kit (Takara, Japan) according to the manufacturer's protocol. The following primers were used: COX-2 (forward): 5-CCAGAGCAGGCAGATGAAATA-3; COX-2 (reverse): 5-CAGCATCGATGTCACCATAGAG-3; GAPDH (forward): 5'-CGCGAGAAGATGACCCAGAT-3'; GAPDH (reverse): 5'-GCACTGTGTTGGCGTACAGG-3'. The relative mRNA expression was quantified using the $2^{-\Delta\Delta Ct}$ method.

Western Blotting analysis of COX-2 expression

Total protein was extracted from rat tumor tissue by RIPA lysis and extraction buffer (Thermo). Protein content was determined by BCA assay (Beyotime, Shanghai, CHN). Equal amount of protein lysates were fractionated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, USA). The membranes were blocked with 5% fat-free milk in Tris-buffered saline (TBS) for 1 h, then incubated with a primary antibody anti-COX-2 (1:500) (Abcam) diluted in TBS at 4°C overnight. On the secondary day, the membranes were incubated with a secondary horse-radish peroxidase (HRP)-conjugated antibody (1:20000) for 1 h at room temperature. After washed by TBS, the blots were visualized using ECLPlus Kit reagents (GE Healthcare, USA).

Statistical analysis

All data were expressed as a mean ± SD. Two-tailed Student's t test and one way analysis of variance (ANOVA) were used to analyse the statistical significance. P-values of <0.05 were considered as statistical significance.

Results

Effects of paclitaxel on the cell proliferation of HOS-732 cells

As shown in Table 1, HOS-732 growth was significantly suppressed by paclitaxel at different concentrations compared to the control cells (P=0.0001 at 0.5 μg/ml, P=0.000009 at 1 μg/ml, P=0.0000007 at 2 μg/ml). Different concentrations of paclitaxel (0.5 μg/ml, 1 μg/ml and 2 μg/ml) had their corresponding growth suppression rates, which were 23.67 ± 2.52%, 33.33 ± 4.51% and 46.00 ± 2.65, respectively. Compared with cells treated with DMSO, P=0.0001 at 0.5 μg/ml, P=0.00001 at 1 μg/ml, P=0.0000008 at 2 μg/ml. Compared with cells treated with paclitaxel at concentration of 0.5 μg/ml, P=0.04 at 1 μg/ml, P=0.0002 at 2 μg/ml. Compared with cells treated with paclitaxel at concentration of 1 μg/ml, P=0.01 at 2 μg/ml. So, paclitaxel inhibited the HOS-732 cells proliferation in a dose-dependent manner.

Effects of paclitaxel on apoptosis in osteosarcoma cells

As shown in Figure 1, the apoptosis rates in HOS-732 cells were increased gradually, which were 18.53 ± 0.60% (P=0.01),

27.80 ± 2.03% (P=0.000017), and 45.37 ± 1.36% (P=0.00000004), respectively, with the increase of paclitaxel concentration (0.5 μg/ml, 1 μg/ml and 2 μg/ml). There was a significant difference of the apoptosis rates between the control cells and cells treated with paclitaxel at different concentrations (P<0.05).

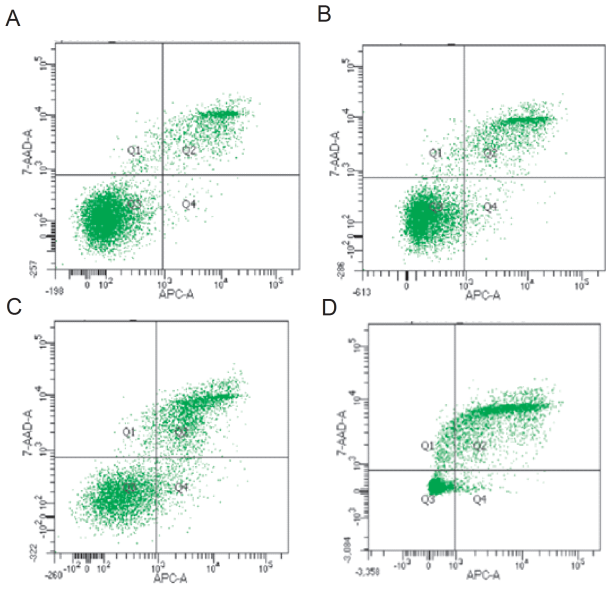

Figure 1. *The apoptosis in osteosarcoma cells treated by different concentrations of paclitaxel was analysed by Annexin V-APC/7-AAD method. (A) Cells treated with DMSO (B) Cells treated with paclitaxel at concentration of 0.5 μg/ml (C) Cells treated with paclitaxel at concentration of 1 μg/ml. D. Cells treated with paclitaxel at concentration of 2 μg/ml.*

Table 1. *Inhibitory effects of paclitaxel with different concentrations on HOS-732 cells growth.*

Group	Paclitaxel (μg/ml)	Growth-suppression rate (%)
Cells treated with DMSO	0	0
Cells treated with paclitaxel	0.5	23.67 ± 2.52$^{\#}$
	1	33.33 ± 4.51$^{\#\$}$
	2	46.00 ± 2.6$^{\#\$\&}$

Note: $^{\#}$P<0.05, compared with cells treated with DMSO, $^{\$}$P<0.05, compared with cells treated with paclitaxel at concentration of 0.5 μg/ml, $^{\&}$P<0.05, compared with cells treated with paclitaxel at concentration of1 μg/ml.

Effects of paclitaxel on ROS production in osteosarcoma cells

As shown in Figure 2, paclitaxel can significantly induce the production of ROS compared to the control group (P<0.05). Furthermore, paclitaxel at different concentrations (0.5 μg/ml, 1 μg/ml and 2 μg/ml) can induce different ROS-positive rate which is 12.10 ± 2.07% (P=0.0000005), 21.63 ± 0.75% (P=0.0000004) and 44.30 ± 1.61% (P=0.000000001), respectively. Thus, the effects of paclitaxel on the production of ROS exhibited a marked dose-dependent manner.

Paclitaxel decreased COX-2 expression in osteosarcoma tissues

Compared with the control group, paclitaxel significantly decreased the expression of COX-2 at mRNA levels in osteosarcoma tissues. Different concentrations of paclitaxel (5 mg/kg, 10 mg/kg and 20 mg/kg) had different inhibitory effects on the expression of COX-2 (3.57 ± 0.12 (P=0.0003), 2.53 ± 0.15 (P=0.0000006), 1.11 ± 0.14 (P=0.000000006), respectively) (Figure 3A). In accordance with RT-PCR results, the expression COX-2 at protein levels in osteosarcoma tissues was also reduced after paclitaxel treatment and negatively correlated to the concentration of paclitaxel (Figure 3B).

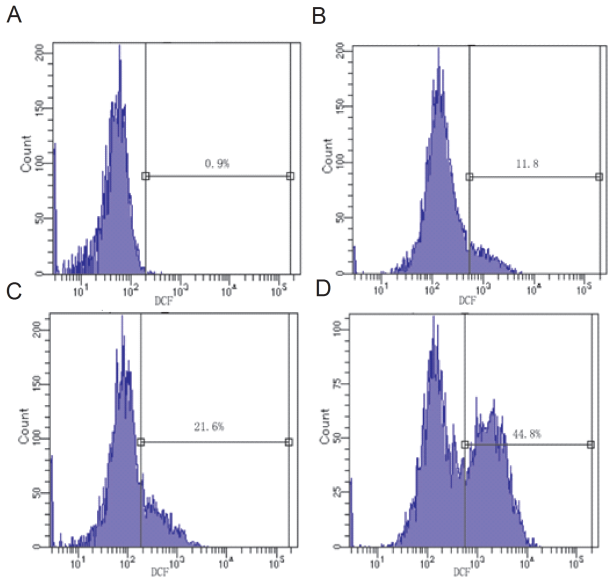

Figure 2. *The production of ROS in osteosarcoma cells treated by different concentrations of paclitaxel was analysed by flow cytometry (A) Cells treated with DMSO (B) Cells treated with paclitaxel at concentration of 0.5 μg/ml (C) Cells treated with paclitaxel at concentration of 1 μg/ml (D) Cells treated with paclitaxel at concentration of 2 μg/ml.*

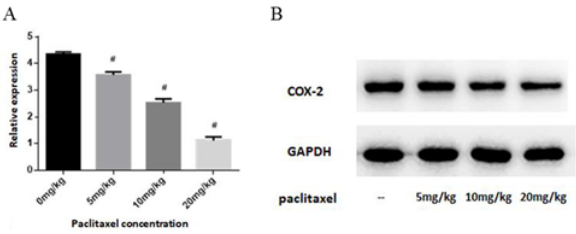

Figure 3. *Paclitaxel significantly reduce the expression of COX-2 at mRNA and protein levels in rat osteosarcoma tissues (A) RT-PCR showed the decreased expression of COX-2 at mRNA levels in rat osteosarcoma tissues treated with paclitaxel. $^{\#}$P<0.05, compared with cells treated with DMSO (B) Western blotting showed the expression of COX-2 at protein levels in rat osteosarcoma tissues treated with paclitaxel.*

Discussion and Conclusion

Our present results showed that paclitaxel can significantly inhibit the cell viability of human osteosarcoma cells HOS-732 and promote cell apoptosis. Moreover, paclitaxel can remarkably increase the production of ROS in human osteosarcoma cells HOS-732 and decrease the expression of COX-2 in rat osteosarcoma tissue.

Paclitaxel, a complex diterpenoid alkaloid compound derived from Pacific yew tree, is widely used for the treatment of many types of cancer [14,15]. Paclitaxel can disrupt microtubules dynamics, blocking mitosis, ultimately leading to the apoptotic cell death [16,17]. It has been shown that paclitaxel can induce apoptosis in several lines of cancer cells such as breast cancer cell, ovarian cancer cell and small cell and non-small cell lung cancer cell [18]. Similarly, our present results show that paclitaxel also significantly induce the apoptosis of osteosarcoma cells in a dose-dependent manner.

Multiple studies have shown that paclitaxel induce apoptosis by multiple mechanisms [16]. Here, we showed that paclitaxel can remarkably increase the production of ROS in human osteosarcoma cells HOS-732 with the increase of dosage. ROS is a byproduct of cell metabolism, mainly derived from the metabolism of molecular oxygen in the mitochondrial respiratory chain [11]. Excessive ROS can cause the occurrence of oxidative stress which plays a vital role in apoptosis [12]. Thus, the elevated production of ROS could be an important molecular mechanism of apoptosis induced by paclitaxel in human osteosarcoma cells HOS-73.

Moreover, COX-2 expression was determined in rat osteosarcoma tissues after paclitaxel treatment. Our results showed that paclitaxel can significantly decrease the expression of COX-2 at mRNA and protein levels in a dose-dependent manner in rat osteosarcoma tissues. COX is a key enzyme for the synthesis of prostaglandin. There are two predominant isoforms, known as COX-1 and COX-2. COX-2 is an inducible enzyme for the production of prostaglandin E2, and expressed in most normal tissues at low levels. However, increased COX-2 expression was found to be closely associated with the inflammatory disease and cancer. Several lines of evidence showed the overexpression of COX-2 in a variety of human malignancies. Paclitaxel has been shown to inhibit the expression of COX-2 in breast, ovarian and lung cancer cells, which is in accordance with our results [19-21]. Furthermore, it has been reported that tumor cells overexpressing COX-2 become resistant to apoptosis [22]. So, the down-regulation of COX-2 expression may be another molecular mechanism of apoptosis in rat osteosarcoma tissues induced by paclitaxel.

In conclusion, our present results demonstrated that paclitaxel can induce apoptosis and increase the ROS production inhuman osteosarcoma cells HOS-732. Moreover, paclitaxel can significantly reduce the expression of COX-2 in rat osteosarcoma tissues. Our data may offer a potential application paclitaxel in the treatment of human osteosarcoma patients that are resistant to other chemotherapeutic agents.

References

1. Gibbs CP Jr, Weber K, Scarborough MT. Malignant bone tumors. Instr Course Lect 2002; 51: 413-428.
2. Mcguire J, Utset-Ward TJ, Reed DR. Re-calculating! Navigating through the osteosarcoma treatment roadblock. Pharmacol Res 2017; 117: 54.
3. Vos HI, Coenen MJ, Guchelaar HJ, Te Loo DM. The role of pharmacogenetics in the treatment of osteosarcoma. Drug Discov Today 2016; 21: 1775-1786.
4. Williams CS, Mann M, DuBois RN. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 1999; 18: 7908-7916.
5. Eberhart CE, Coffey RJ, Radhika A, Giardiello FM, Ferrenbach S, DuBois RN. Up-regulation of cyclooxygenase 2 gene expression in human colorectal adenomas and adenocarcinomas. Gastroenterology 1994; 107: 1183-1188.
6. Gupta S, Srivastava M, Ahmad N, Bostwick DG, Mukhtar H. Over-expression of cyclooxygenase-2 in human prostate adenocarcinoma. Prostate 2000; 42: 73-78.
7. Su CW, Yuan Z, Zhu Y T. Stromal COX-2 signaling are correlated with colorectal cancer: A Review. Cri Rev Oncol Hematol 2016; 107: 33.
8. Markosyan N, Chen EP. Mammary carcinoma cell derived cyclooxygenase 2 suppresses tumor immune surveillance by enhancing intratumoral immune checkpoint activity. Breast Cancer Res 2013; 15: 75.
9. Imoto K, Kukidome D, Nishikawa T, Matsuhisa T, Sonoda K, Fujisawa K, Yano M, Motoshima H, Taguchi T, Tsuruzoe K, Matsumura T, Ichijo H, Araki E. Impact of mitochondrial reactive oxygen species and apoptosis signal-regulating kinase 1 on insulin signaling. Diabetes 2006; 55: 1197-1204.
10. Kim SJ, Hwang SG, Shin DY, Kang SS, Chun JS. p38 kinase regulates nitric oxide-induced apoptosis of articular chondrocytes by accumulating p53 via NFkappa B-dependent transcription and stabilization by serine 15 phosphorylation. J Biol Chem 2002; 277: 33501-33508.
11. Moloney JN, Cotter TG. ROS signalling in the biology of cancer. Sem Cell Develop Biol 2017.
12. Kovviok A, Piosik J, Gwizdek-Wisniewska A, Stosilojc G, Bigda J, Lojkowska E. Induction of apoptosis through reactive oxygen species mediated inhibition of topoisomeraseII by plumbagin from Drosera. Planta Medica 2007; 73: 806-807.
13. Bobbili VVP, Mubarak Ali A, Leela Kumari A, Pallu R, Ashok K. Phycocyanin-mediated apoptosis in AK-5 tumor cells involves down-regulation of Bcl-2 and generation of ROS. Mol Cancer Ther 2003; 2: 1165-1170.
14. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. Cell 2008; 132: 27-42.
15. Howat S, Park B, Oh IS. Paclitaxel: biosynthesis, production and future prospects. N Biotechnol 2014; 31: 242-245.

16. Javeed A, Ashraf M, Riaz A. Paclitaxel and immune system. Eur J Pharmac Sci Off J Eur Feder Pharm Sci 2009; 38: 283-290.
17. Wang H, Li D, Li X. Mammalian target of rapamycin inhibitor RAD001 sensitizes endometrial cancer cells to paclitaxel-induced apoptosis via the induction of autophagy. Oncol Lett 2016; 12: 5029-5035.
18. Chen C, Hu H. Anti-tumor activity of paclitaxel through dual-targeting lipoprotein-mimicking nanocarrier. J Drug Target 2015; 23: 311-322.
19. Sicking I, Rommens K. Prognostic influence of cyclooxygenase-2 protein and mRNA expression in node-negative breast cancer patients. BMC Cancer 2014; 14: 952.
20. Rizzo MT. Cyclooxygenase-2 in oncogenesis. Clin Chim Acta 2011; 412: 671-687.
21. Gregorio H, Raposo T, Queiroga FL. High COX-2 expression in canine mast cell tumours is associated with proliferation, angiogenesis and decreased overall survival. Veter Compar Oncol 2016.
22. Tsujii M, Dubois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995; 83: 493-501.

*Correspondence to

Xi Wang

The First People's Hospital of Wenling

Wenling

China