

Original Research Article

Indole-3-acetate induces apoptosis and stimulates phosphorylation of p65NF- κ B in 143B and HOS osteosarcoma cells

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Abstract

Purpose: To investigate the effect of indole-3-acetate (IAA) on the proliferation of 143B and HOS osteosarcoma cells, and its mechanism of action.

Methods: Indole-3-acetate (IAA)-induced changes in cell proliferation and apoptosis were investigated using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay and flow cytometry, respectively. The effects of IAA on expressions of mRNAs for phosphatase and tensin homolog (PTEN), fas ligand (FasL), and fas receptor (FasR) were evaluated using western blot assay.

Results: Early apoptosis in 143B cell cultures due to addition of IAA (5 μ M) was 34.67 %, relative to 2.82 % in untreated cultures. In HOS cells, IAA caused 39.21 % apoptosis, relative to 3.53 % apoptosis in control. The addition of IAA to the cell cultures significantly enhanced the expressions of mRNAs for PTEN, FasL and FasR, compared to untreated cells ($p < 0.05$). Western blot analysis showed that IAA caused a significant decrease in the level of I κ B α expression in both cell lines ($p < 0.05$). In 143B and HOS cells, treatment with IAA led to accumulation of higher levels of NF- κ B in the nucleus than in the cytosol. The levels of cytosolic NF- κ B, and nuclear lamin B1 in IAA-treated cells were lower than the corresponding levels in untreated cells.

Conclusion: These results indicate that IAA inhibits proliferation, and induces apoptosis in 143B and HOS cells via activation of NF- κ B, and its translocation to the nucleus. Therefore, IAA may be a useful drug target in the treatment of osteosarcoma.

Keywords: Indole-3-acetate, Phosphatase, Fas receptor, Translocation, Proliferation, Tumoricidal activity

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INTRODUCTION

Osteosarcoma (OS) is a frequently-occurring malignant tumour of the bone in people of various age groups such as children,

adolescents and young adults. Osteosarcoma constitutes the primary bone tumours in more than 50 % of these patients. In Japan alone, the number of osteosarcoma patients diagnosed annually is about 100 [1,2]. Various studies have

been carried out in order to explain the mechanism underlying the pathogenesis of osteosarcoma so as to develop strategies for its treatment [1-6]. There are reports that osteosarcoma is caused by gene mutations which give rise to tumours of complicated nature [1,2].

The treatment for osteosarcoma involves multidisciplinary approach comprising surgical intervention followed by chemotherapy and radiotherapy [7]. Screening and investigation of chemotherapeutic agents for osteosarcoma has led to promising results, with 5-year survival rate of localized tumour patients being higher than 70 % [8]. The combined chemotherapeutic agents found to be effective against osteosarcoma include methotrexate, doxorubicin, cisplatin and ifosfamide [9–13]. These agents have been used for osteosarcoma treatment for a long time. Thus, there is need for the development of a more effective therapeutic strategy for the disease. Currently, a lot of interest is focused on the development of molecular-targeted strategies for treatment of various cancers [14].

The induction of apoptosis in carcinoma cells is considered to be of vital importance for the treatment of cancer [15,16]. Cellular apoptosis is regulated through the phosphorylation of various factors [17]. An important factor which has been found to play a role in the induction of apoptosis is nuclear factor-kappa B (NF- κ B) [18-22]. Translocation of NF- κ B from cytoplasm to the nucleus leads to activation of genes responsible for regulation of apoptosis. The translocation of NF- κ B is initiated by the activation and degradation of I κ B α [18-22].

Studies have shown that indole compounds present in cruciferous vegetables exhibit promising tumoricidal activities [23,24]. Indeed, it has been demonstrated that indole compounds induce apoptosis, arrest cell cycle in G1 phase in cancer cells, and inhibit the invasive and metastasis potential of tumour cells [25-27]. These effects are exerted through suppression of cell cycle protein expression (cyclin D1, E); inhibition of anti-apoptotic genes, and activation of caspases 3 and 9 [26,27]. The present study was designed to investigate the role of IAA in the inhibition of viability of osteosarcoma cells, and to unravel its mechanism of action.

EXPERIMENTAL

Cell culture

Osteosarcoma cell lines (143B and HOS) were supplied by Science Cell Research Laboratories

(Carlsbad, CA, USA). The cell lines were cultured at 37 °C in Dulbecco's Modified Eagle's Medium (DMEM) containing 10 % fetal bovine serum, 100 U/mL penicillin and 100 mg/mL streptomycin in a humidified atmosphere of 5 % CO₂.

Determination of proliferation of 143B and HOS cell lines

Changes in the proliferation of 143B and HOS cells due to IAA treatment were determined using MTT (Sigma-Aldrich) assay. Into 2 mL of D-MEM medium containing FBS (10 %) 143B and HOS cells were separately placed (2 x 10⁶ cells per well) in 96-well culture plates (Nunc A/S Plastfabrikation, Roskilde, Denmark). The cells were incubated for 12 h after which the medium was changed and replaced with a new medium containing IAA. The plates were then incubated for 48 h, after which 50 μ L MTT (5 μ g/mL) was added. Incubation was then continued for 2 h, following which the medium was removed, and dimethyl sulfoxide (DMSO, 150 μ L) was added to the wells. The absorbance of each well was read at 570 nm in EL800 Universal Microplate Reader (BioTek Instruments, Inc, Winooski, VT, USA).

Analysis of cell apoptosis

Apoptosis in 143B and HOS cells was analysed using Annexin V/PI apoptosis assay kit in accordance with the instructions of the manufacturer (Multi Sciences Biotech Co., Ltd., Hangzhou, China). Cell pellets of 143B and HOS were treated with IAA for 48 h and then placed in 1x binding buffer. The cells were incubated for 10 min with Annexin V (conjugated with FITC; 5 mL) and PI (10 mL) in the dark. Cell fluorescence was analysed using a Flow cytometer (Epics-XLII, Becton Coulter, Inc., Brea, CA, USA). The proportions of intact cells (Annexin V-/PI-), cells that showed early apoptosis (Annexin V+/PI-), and cells with evidence of late apoptosis (Annexin V+/PI+) were then determined.

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

Real-time RT-PCR was performed to study the effect of IAA on levels of expression mRNA in 143B and HOS cells. Each cell line was incubated at a density of 2 x 10⁷ cells in 100-mm dishes for 48 h in DMEM containing IAA. This was followed by RNA extraction using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Then, cDNA was synthesized from 2 mg of RNA using SuperScript reverse transcriptase (Bioneer, Daejeon, Korea) with oligo dT primers. The real-time PCR was

carried out with LightCycler 480 using SYBR green master mix (Roche). The primers used were PTEN: forward 5'-ACCGCCAAATTTAATTGCAG-3', backward 5'-GGTCCTGAATTGGAF FAAT-3'; FasL: forward 5'-TCTCAGACGTTTTTC GGCTT-3', backward 5'-AAGACAGTCCCC TTGAGGT-3'; FasR: forward 5'-CAAGGGATTG GAATTGAGGA-3', backward 5'-GACAAAGCCA CCCCAAGTTA-3'; GAPDH: forward 5'-ACCAC AGTCCATGCCATCAC-3' backward 5'-TCCACC ACCCTGTTGCTGTA-3'. The sequence of events consisted of incubation for 2 min at 50 °C, denaturation for 10 min at 50 °C with subsequent 50 cycles of 15 sec at 95 °C, followed by 2 min at 60 °C. The non-regulated housekeeping gene GAPDH was employed as the internal loading control. The products of PCR were analysed using agarose gel (2 %), ethidium bromide and a UV illuminator.

Western blot analysis

The 143B and HOS cells, with or without IAA treatment, were placed in DMEM. After washing in PBS, the cells were treated for half an hour with RIPA (Roche, Shanghai, China) under ice-cold condition. The lysates were centrifuged at 4 °C for 15 min at 12,000 x g, and the supernatants were collected. The concentrations of proteins in the supernatants were determined using Bradford method [12]. Samples of the protein were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories, Hercules, CA, USA) after separation using SDS-polyacrylamide gel (8 %) electrophoresis.

The blockage of non-specific sites in the membranes was achieved by incubation for 1 h with dry milk (10 %) in TBST at room temperature. The membranes were incubated overnight at 4 °C with primary antibodies corresponding to anti-IkB α , anti-phospho-Ser536 p65NF- κ B, anti-phospho-Ser529 p65NF- κ B, anti-lamin B1 and β -actin (1:1000; Cell Signalling, Shanghai, China). The membranes were then washed twice with PBS plus Tween-20 (0.05 %) and subsequently incubated for 1 h with horseradish peroxidase-conjugated polyclonal anti-rabbit secondary antibody (1:1,500; Cell Signalling Technology, Inc., Danvers, MA, USA). The blots were developed using enhanced chemiluminescence kit (Intron Biotechnology, Inc., Seongnam, Korea).

Construction and transfection of DNA

The 143B and HOS cells were cultured in DMEM containing FBS in plastic dishes. After achieving 80 % confluence, the cells were washed two times with PBS prior to treatment with DNA and

Opti-MEM mixture. The cells were then transfected with p65NF- κ B S536A in which alanine was incorporated at position 536 in place of serine. The transfection was achieved using Quick Change II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). The cell lines were then incubated for 48 h with IAA in DMEM supplemented with 10 % FBS.

Statistical analysis

All data are presented as mean \pm standard deviation (SD). Statistical analysis of the data was carried out using SPSS 13.0 statistical software (SPSS, Inc. Chicago, IL, USA). Comparison among multiple groups was performed using one-way analysis of variance (ANOVA), followed by Dunnett's test. $P < 0.05$ was taken as statistically significant.

RESULTS

Effect of IAA on viability of 143B and HOS cells

Treatment with IAA reduced the viability of 143B and HOS cells in a significant and concentration-dependent manner ($p < 0.05$). The concentration of IAA in 143B and HOS cell cultures were 1, 2, 3, 4, 5 and 6 μ M, and the plates were incubated for 48 h. The viabilities of 143B cells treated with 1, 2, 3, 4, 5 and 6 μ M IAA were 98, 81, 73, 49, 26 and 25 %, respectively (Figure 1). At IAA doses of 1, 2, 3, 4, 5 and 6 μ M, HOS cell cultures showed cell viabilities of 99, 79, 68, 43, 21 and 20 %, respectively (Figure 1). However, there was 100 % viability in 143B and HOS cells in the cultures without IAA. These results demonstrate that IAA exhibits inhibitory effect on the viability of osteosarcoma cells.

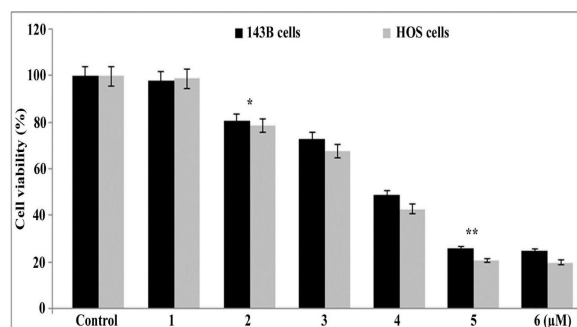


Figure 1: Effect of IAA on the viabilities of 143B and HOS cells. The cell lines were incubated for 48 h with 1, 2, 3, 4, 5 and 6 μ M of indole-3-acetate. Values are mean \pm SD (n = 3). Significant differences in comparison to the control cell cultures are marked as * $p < 0.05$ and ** $p < 0.02$

Effect of IAA on early apoptotic changes in 143B and HOS cells

The treatment 143B and HOS cells with IAA led to significant increases in the population of cells in early apoptosis in both cells ($p < 0.05$). There was 34.67 % apoptosis in 143B cell cultures, while 2.82 % apoptosis was seen in untreated cultures. In HOS cells, apoptosis in IAA-treated cells and control were 39.21 and 3.53 %, respectively. These results are shown in Figure 2.

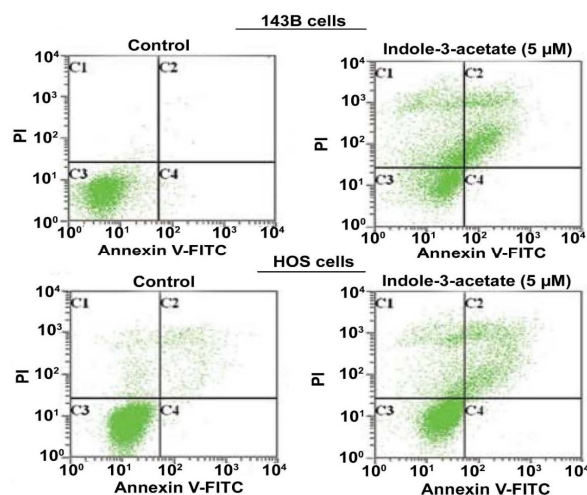


Figure 2: Quantification of effect of IAA on apoptosis in 143B and HOS cells using fluorescence microscopy. 143B and HOS cells were incubated for 48 h, with or without IAA (5 μ M). Fluorescence microscopy using Annexin V and propidium iodide staining was employed for determination of apoptotic cell population

Effect of IAA on the expressions of mRNAs of apoptosis-related proteins in 143B and HOS cells

Treatment of 143B and HOS cell cultures with IAA increased the expressions of mRNAs for phosphatase and tensin homolog (PTEN), fas ligand (FasL) and fas receptor (FasR), when compared to untreated cells (Figure 3).

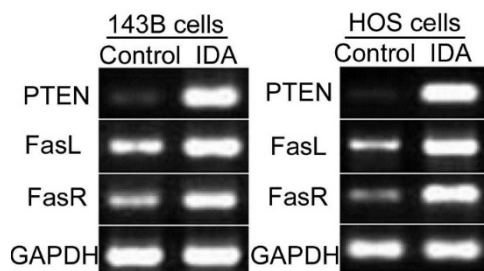


Figure 3: Effect of IAA on the expressions of mRNAs of apoptosis-related proteins in 143B and HOS cells. The 143B and HOS cells were incubated with or without 5 μ M IAA for 48 h. Thereafter, RT-PCR

analysis was used for the determination of changes in the levels of mRNAs for PTEN, FasL and FasR, with GAPDH mRNA expression as internal control

IAA induces decrease in the levels of I κ B α in 143B and HOS cells

Results from Western blot analysis (Figure 4) revealed that IAA caused significant decreases in the expressions of I κ B α in 143B and HOS cell lines ($p < 0.05$).

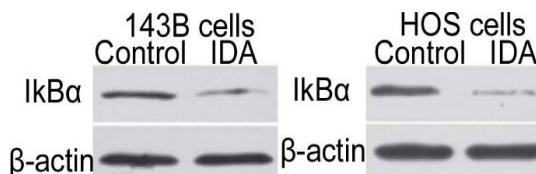


Figure 4: Effect of IAA on the levels of I κ B α in 143B and HOS cells. The cells after incubation for 48 h with 5 μ M IAA were analysed for I κ B α level by western blot assay

IAA increases NF- κ B phosphorylation in 143B and HOS cells

In 143B and HOS cells treated with IAA, a strong interaction was found between anti-phospho-Ser536 p65NF- κ B antibody and the protein, relative to the association in untreated cells (Figure 5). However, IAA exhibited no effect on the interactions of antiphospho-Ser529 p65NF- κ B and anti-p65NF- κ B with the protein.

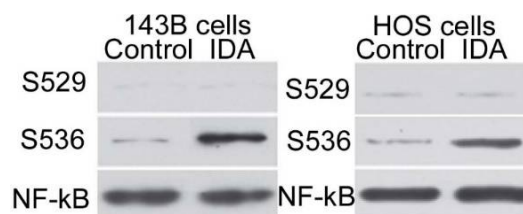


Figure 5: Effect of IAA on NF- κ B phosphorylation in 143B and HOS cells, as determined by western blot assay after treatment with IAA for 48 h

IAA enhances accumulation of NF- κ B in the nuclei of 143B and HOS cells

Treatment of 143B and HOS cells with IAA (5 μ M) led to accumulation of higher levels of NF- κ B in the nucleus, when compared to untreated cells. The level of NF- κ B in the cytosol of IAA-treated cells was also lower than corresponding levels in untreated cells. In addition, lamin B1 levels in 143B and HOS treated with IAA were higher in the nucleus than in the cytosol. These result are shown in Figure 6.

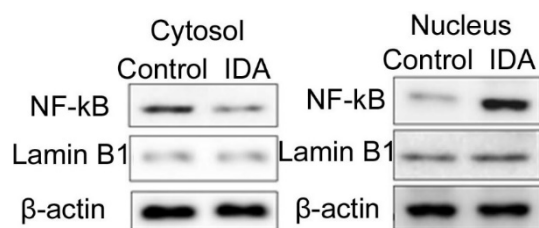


Figure 6: Effect of IAA on expression levels of NF- κ B in the nucleus of 143B and HOS cells, and its effect on nuclear and cytosolic levels of lamin B1

DISCUSSION

Osteosarcoma is an aggressive and frequently diagnosed bone tumour which affects the musculoskeletal system in children and adults [28,29]. The treatment strategy for osteosarcoma entails radiotherapy, chemotherapy and surgery, but the results obtained are often not satisfactory [30]. In the present study, the inhibitory effect of IAA on the viability of osteosarcoma cells, and the associated mechanism were investigated. The results reveal that indole-3-acetate decreased the viabilities of 143B and HOS osteosarcoma cells through induction of apoptosis. In order to understand the mechanism associated with the IAA-induced apoptosis in osteosarcoma cells, the expression of PTEN, FasL, and FasR were investigated. It is known that apoptosis induction via NF- κ B pathway is regulated by the expression of PTEN, FasR and FasL [31]. Up-regulation of expressions of PTEN, FasR and FasL has been shown to cause cell apoptosis through NF- κ B pathway [32,33]. The results of the present study revealed that addition of IAA to cell cultures of 43B and HOS significantly increased the expressions of PTEN, FasL and FasR. These results clearly demonstrate that IAA induces apoptosis in 143B and HOS cells through up-regulation of the expressions of PTEN, FasL and FasR mRNA. Western blot analysis showed that IAA caused a significant reduction in the level of I κ B α expression in the two cell lines. It has been observed that during apoptosis, p65NF- κ B is activated through phosphorylation of its serine 536 residue [34-36].

The results of the study also revealed that treatment of 143B and HOS cells with IAA led to strong interactions between anti-phospho-Ser536 p65NF- κ B antibody and the protein. Thus IAA treatment resulted in phosphorylation of p65NF- κ B in 143B and HOS cells. The activation of p65NF- κ B facilitated its migration from the cytoplasm into the nucleus. It has been reported that activation of NF- κ B induces the expression of I κ B α , which in turn down-regulates NF- κ B activation [37]. However, in the present study

exposure of 143B and HOS cells to IAA brought about inhibition of the expression of I κ B α .

CONCLUSION

The results obtained in this study strongly suggest that indole-3-acetate inhibits the viability of osteosarcoma cells via induction of apoptosis, and by increasing the expressions of PTEN, fas ligand and fas receptor. In addition, it inhibits I κ B α expression and up-regulates NF- κ B pathway. Thus, indole-3-acetate may be of vital importance in the treatment of osteosarcoma.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

The authors declare that this work was done by the named authors, and all liabilities pertaining to claims relating to the content of this article will be borne by them. Yanhui Zhang, Yang Li and Chao Wang performed the experimental work. Kewen Zheng and Chen Feng carried out the literature survey and compiled the data. Wenbo Wang designed the study and wrote the manuscript, which was thoroughly read by all the authors, and approved for publication.

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