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• *BASIC RESEARCH* •

Apoptosis of pancreatic cancer BXPC-3 cells induced by indole-3-acetic acid in combination with horseradish peroxidase

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Abstract

AIM: To explore the mechanisms underlying the apoptosis of human pancreatic cancer BXPC-3 cells induced by indole-3-acetic acid (IAA) in combination with horseradish peroxidase (HRP).

METHODS: BXPC-3 cells derived from human pancreatic cancer were exposed to 40 or 80 μmol/L IAA and 1.2 μg/mL HRP at different times. Then, MTT assay was used to detect the cell proliferation. Flow cytometry was performed to analyze cell cycle. Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay was used to detect apoptosis. 2,7-Dichlorofluorescin diacetate uptake was measured by confocal microscopy to determine free radicals. Level of malondialdehyde (MDA) and activity of superoxide dismutase (SOD) were measured by biochemical methods.

RESULTS: IAA/HRP initiated growth inhibition of BXPC-3 cells in a dose- and time-dependent manner. Flow cytometry revealed that the cells treated for 48 h were arrested at G_1/G_0. After exposure to 80 μmol/L IAA plus 1.2 μg/mL HRP for 72 h, the apoptosis rate increased to 72.5‰, which was nine times that of control. Content of MDA and activity of SOD increased respectively after treatment compared to control. Meanwhile, IAA/HRP stimulated the formation of free radicals.

CONCLUSION: The combination of IAA and HRP can inhibit the growth of human pancreatic cancer BXPC-3 cells *in vitro* by inducing apoptosis.

Key words: Indole-3-acetic acid; Horseradish peroxidase; BXPC-3 cells; Apoptosis; Free radical

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INTRODUCTION

Indole-3-acetic acid (IAA) is an important plant growth hormone found in higher plants, and plays a role in the regulation of plant cell division, elongation and differentiation[1]. It is present in human urine[2], blood plasma[3], and central nervous system[4]. IAA is well tolerated in humans[5] and not oxidized by mammalian peroxidases. Recent researches suggest that the combination of IAA and horseradish peroxidase (HRP) is cytotoxic to mammalian cells, and can be used as a novel anticancer drug[6-10], while neither IAA nor HRP alone shows any cytotoxic effect[6]. HRP is a heme-containing peroxidase and can oxidize a wide variety of substrates including IAA in the presence of hydrogen peroxide. It has been reported that IAA activated by HRP produces free radicals, such as indolyl, skatolyl, and peroxyl radicals, which can cause lipid peroxides[11-13]. There are differences in endurance to the combination of IAA and HRP among the different types of cells[14]. In the present study, we investigated the effects of IAA/HRP on BXPC-3 cells, a cell line derived from human pancreatic cancer, and found that IAA/HRP treatment could induce than increase of free radicals within the cells and cause apoptosis of BXPC-3 cells.

MATERIALS AND METHODS

Drug and reagents

IAA, HRP, and 3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Sigma Chemical Co. 2,7-Dichlorofluorescin diacetate (DCFH-DA) was from Molecular Probes Co., and *in situ* cell apoptosis detection kit was from Sino-American Biotechnic Co. Commercial kits used for determining lipid peroxide and superoxide dismutase (SOD) activity were obtained from the Jiancheng Institute of Biotechnology (Nanjing, China).

Cell culture

Cells (1.0×10^5 cells/mL) were cultured in RPMI supplemented with 100 mL/L fetal bovine serum containing 2.0 mmol/L glutamine and 20 μg penicillin-streptomycin/mL in 50 mL/L CO_2 at 37 ℃.

Experimental design

The experiments were divided into three groups: control group, 40 μmol/L IAA+1.2 μg/mL HRP (40 μmol/L IAA/HRP) and 80 μmol/L IAA+1.2 μg/mL HRP (80 μmol/L IAA/HRP) treatment groups. All tests were carried out in triplicate.

MTT assay for cell viability

Cells (2×10^4 cells/well) were seeded onto 96-well plates and incubated with test substances for an indicated time at 37 ℃ in 50 mL/L CO_2. Then, 20 μL/well of MTT solution (5 mg/mL) was added and incubated for another 4 h. Supernatants were removed and formazan crystals were dissolved in 200 μL of dimethylsulfoxide. Finally, optical density was determined at 540 nm by a POLARstar$^+$ OPTIMA (BMG Labtechnologies).

Detection of apoptosis

Apoptotic cells were identified using an *in situ* cell apoptosis detection kit. The cells were treated with IAA/HRP for a given time, and processed following the manufacturer's instruction. At least 1 000 cells were counted in each test, and the percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL)-positive cells was calculated.

Measurement of MDA content and SOD activity in cells

The number of cells was adjusted to 1×10^6 cells/mL, and broken by ultrasonic with PBS. Homogenates were centrifuged (1 000 r/min, 10 min, 4 ℃) and the supernatant was used immediately for the assays of malondialdehyde (MDA) and SOD according to the instructions of the kits. MDA content was determined by the thiobarbituric acid method[15] and expressed in nanomole per milligram protein. The assay for total SOD was based on its ability to inhibit the oxidation of oxyamine by the xanthine-xanthine oxidase system. The red product (nitrite) had an absorbance at 550 nm. One unit of SOD activity was defined as the quantity reducing the absorbance at 550 nm by 50%. The activity of SOD was expressed in nanounit per milligram protein.

Quantitative image analysis of intracellular ROS in live cells by confocal microscopy

Cells (2×10^4 cells/well) were seeded onto 24-well plates with cover slide and incubated with test substances for an indicated time at 37 ℃ in 50 mL/L CO_2. The cells adhered to the glass flake were incubated with 50 μmol/L of DCFH-DA for 5 min in dark in a CO_2 incubator. DCFH-DA is a nonpolar and nonfluorescent compound, which diffuses into the cells and is hydrolyzed into a polar 2',7'-dichlorofluorescin (DCFH)[16]. The intracellular DCFH was rapidly oxidized to produce highly fluorescent 2', 7'-dichlorofluorescein (DCF) at the presence of ROS or hydrogen peroxide within the cells. After incubation, the remaining dye was removed and the cells were washed twice with RPMI-HEPES before imaging collection. All confocal imaging analyses were performed under a Leica confocal laser scanning microscope using the 488-nm excitation laser line and simultaneous dual display mode (522 nm emission and phase-contrast) of the BioRad LaserSharp imaging program. Five random images were collected to determine the average fluorescence intensity.

Cell cycle analysis by flow cytometry

DNA content per duplicate was analyzed using a FACStar flow cytometer (Becton Dickinson, Mountainview, CA). The adherent cells were harvested by brief trypsinization, and washed with PBS, fixed in 70% ethanol, stained with 20 μg/mL propidium iodide containing 20 μg/mL RNase (DNase free) for 30 min, and analyzed by flow cytometry. The populations of G_0/G_1, S, and G_2/M cells were quantitated.

Statistical analysis

The data were expressed as mean±SD and analyzed by software of SPSS10.0. $P<0.05$ was considered statistically significant.

RESULTS

Inhibition of cell growth and arrest of cell cycle

The growth of human pancreatic cancer BXPC-3 cells was measured using MTT assay at varying time points after treatment. As shown in Figure 1, the combination of IAA and HRP could inhibit the growth of BXPC-3 cells. The inhibition of cell growth after treatment with 80 μmol/L IAA/HRP was higher than that after treatment with 40 μmol/L IAA/HRP, suggesting that the inhibition was in an IAA dose-dependent manner. Because the inhibition of cell growth was the highest 2 d after the treatment, we checked the cell cycle using a flow cytometer at the time of 2-d treatment. The cell cycle distribution of BXPC-3 cells in control group was 62.28% at G_1/G_0, 0.43% at G_2/M and 37.28% at S. In the IAA/HRP treatment group, the cell cycle distribution of BXPC-3 cells significantly increased at G_1/G_0 phase, and decreased greatly at S phase, implying that IAA/HRP could induce the arrest of cells at G_1/G_0 phase.

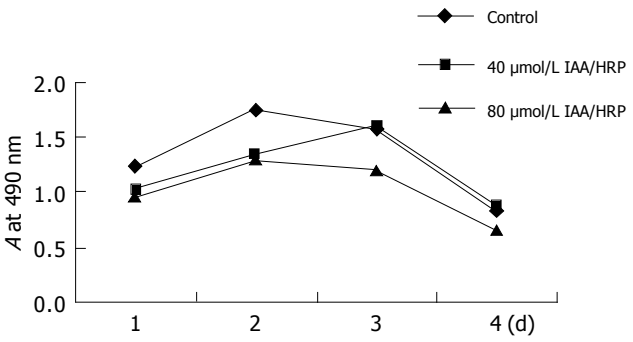

Figure 1 Effect of IAA and HRP treatment on growth of BXPC-3 cells.

Effect of IAA/HRP treatment on apoptosis of BXPC-3 cells

After TUNEL staining, the apoptotic cells markedly increased after IAA/HRP treatment. When the cells were treated with IAA/HRP for 72 h, the apoptosis rate increased to 72.5‰, which was nine times that of the control ($P<0.05$, Figure 2).

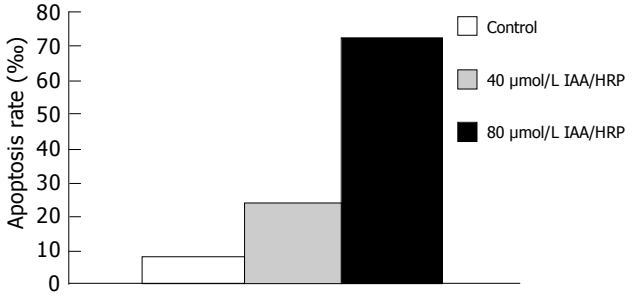

Figure 2 Apoptosis rate of BXPC-3 cells 3 d after IAA and HRP treatment.

Production of free radicals in BXPC-3 cells induced by IAA/HRP

The formation of ROS in BXPC-3 cells treated with IAA/HRP could be detected by confocal microscopy as the product was able to show fluorescence. As shown in Figure 3, DCF fluorescence in cells exposed to IAA/HRP for 3, 6, 24, and 48 h was much brighter compared to that in control group, suggesting that free radicals were formed in a IAA dose- and time-dependent manner (Table 1, Figure 4).

Level of MDA and activity of SOD in BXPC-3 cells treated with IAA/HRP

To investigate the damage of free radicals induced by IAA/HRP in BXPC-3 cells, MDA content and SOD activity were measured. The content of MDA and activity of SOD in BXPC-3 cells increased after IAA/HRP treatment (Table 2). There were significant differences between treatment group and control group ($P<0.01$).

Table 1 Cell cycle distribution of BXPC-3 cells 2 d after IAA plus HRP treatment

Treatment	G_1/G_0	G_2/M	S
Control	62.28±8.34	0.43±0.57	37.28±8.91
40 μmol/L IAA/HRP	76.68±5.06[a]	0.85±1.47	22.47±5.83
80 μmol/L IAA/HRP	76.91±5.78[a]	18.77±1.87[a]	4.32±6.66[a]

[a]$P<0.05$ between control *vs* treated group.

Table 2 SOD activity and MDA content in BXPC-3 cells 3 d after IAA and HRP treatment (mean±SD)

Treatment	Control	40 μmol/L IAA/HRP	80 μmol/L IAA/HRP
SOD (nU/mg protein)	105.60±41.04	135.98±1.46	193.34±37.89[b]
MDA (μmol/L/mg protein)	20.80±0.33	22.29±1.04	99.53±30.45[b]

[b]$P<0.01$ control group *vs* treatment group.

DISCUSSION

Although IAA/HRP-induced cell death and its potential application in cancer therapy has been recognized[8,17,18], the sensitivity of different cell types to IAA/HRP might not be the same[14]. We chose human pancreatic cancer BXPC-3 cells to evaluate IAA/HRP, which could be used in treatment of human pancreatic cancer. In this study, the MTT assay showed that the viability of BXPC-3 cells decreased with increase of IAA in the presence of HRP (Figure 1). In addition, TUNEL analysis showed that IAA/HRP induced apoptosis of BXPC-3 cells (Figure 2). In agreement with our results, it was also reported that the photoproducts of IAA induce apoptosis of human HL-60 and murine tumor cells[19].

There are two important arrest points in cell cycle at G_1/G_0 and G_2/M[20-23]. Flow cytometric analysis of propidium iodide-stained cells showed that cells accumulated at the G_1/G_0 phase compared to control (Table 1). These cell cycle changes suggest that pancreatic cancer cells have oxidative stress response to IAA/HRP treatment with DNA damage leading to apoptosis.

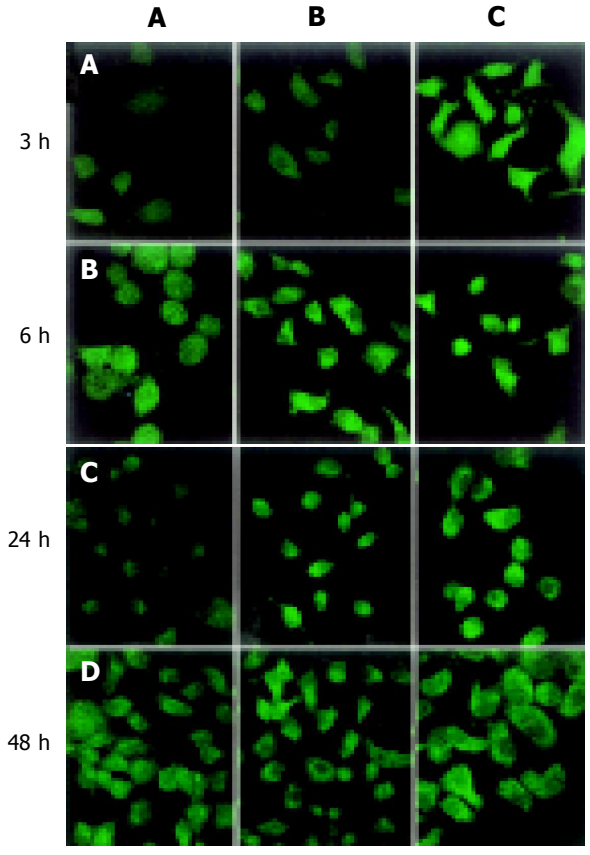

Figure 3 Confocal images of intracellular ROS generation after 3, 6, 24, and 48 h exposure of BXPC-3 cells to IAA/HRP. **A**: Control; **B**: 40 μmol/L IAA/HRP; **C**: 80 μmol/L IAA/HRP.

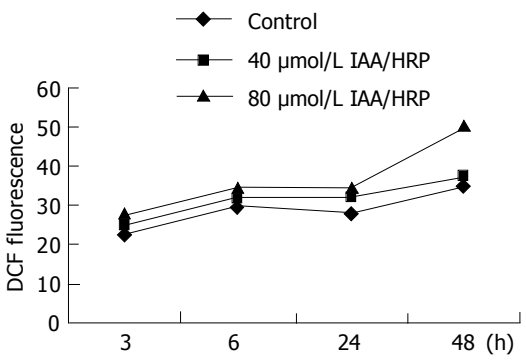

Figure 4 Production of free radicals in BXPC-3 cells exposed to IAA and HRP.

DCFH is widely used to measure the oxidative stress in cells[24-26]. When the diacetate form of DCFH is added to cells, it diffuses across the cell membrane and is hydrolyzed by intracellular esterases to liberate DCFH, which upon reaction with oxidizing species forms its two-electron oxidation product, the highly fluorescent compound DCF. The fluorescence intensity can be easily measured by confocal microscopy and is the basis of the popular cellular assay for oxidative stress. In this study, DCF was used to investigate the production of free radicals in BXPC-3 cells treated with IAA/HRP. The fluorescence intensity increased in the treatment group in comparison to control group (Figure 3), suggesting that the formation of free radicals increases in an IAA dose- and time-dependent manner (Figure 4).

It is known that tumor cells exhibit metabolic characteristics in terms of oxygen consumption and oxidative metabolism. The fact that tumor tissues are more susceptible to oxidative stress than the surrounding normal cells is supported by the increase of lipid peroxidation and DNA damage[27] and the decrease of antioxidant enzyme activities[28]. Folkes *et al*[6], found that lipid peroxidation in liposomes is stimulated by the IAA/HRP treatment, but it cannot be measured in mammalian cells. To investigate the damage of free radicals in cells, MDA content, and SOD activity were measured in this study. The results showed that the content of MDA and the activity of SOD in BXPC-3 cells increased when treated with IAA/HRP (Table 2). In agreement with our results, it was also reported that incubation of neutrophils for 24 h in the presence of IAA increases the activities of SOD, catalase (CAT) and glutathione peroxidase[29]. Recently, it was reported that ROS activates the ERK signaling cascade[30-32]. Kim *et al*[33], proved that IAA/HRP can activate p38 mitogen-activated protein (MAP) kinase and c-Jun N-terminal kinase (JNK) in G361 human melanoma cells, which are almost completely blocked by antioxidants.

In summary, the combination of IAA and HRP can inhibit the growth of human pancreatic cancer BXPC-3 cells *in vitro* by inducing apoptosis, which is associated with the increase of free radicals.

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