The Mechanism of Cisplatin-induced Apoptosis in HeLa Cells

Youqing Liu
Hui Xing
Xiaobing Han
Xiaoyan Shi
Fengqi Liang
Gang Chen
Ding Ma

Department of Obstetrics and Gynecology, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, China.

Correspondence to: Ding Ma
Tel: 86-27-8366-2661
Fax: 86-27-8366-2475
E-mail: dma@tjh.tjmu.edu.cn

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OBJECTIVE To study the mechanism of apoptosis induced by cisplatin in vitro in HeLa cells cervical cancer cell line.

METHODS The inhibitory effect of cisplatin on HeLa cell growth was analyzed by the MTT assay. Cell apoptosis was measured with flow cytometry and Hoechst 33258 staining following cisplatin treatment. The effect of cisplatin on transcription of HPV E6 was analyzed by RT-PCR and protein expression of E6, P53, p21, Bax and Bcl-2 was studied by Western blots.

RESULTS Cisplatin inhibited cellular proliferation in a time and dose-dependant manner. The sub-G1 peak by flow cytometry showed a higher apoptotic rate in the experimental group compared to the controls and Hoechst 33258 staining indicated that apoptosis was induced by cisplatin. Results of RT-PCR demonstrated that cisplatin decreased transcription of E6. Western Blots showed that cisplatin decreased protein expression of E6 and increased protein expression of P53, p21 and Bax but had no effect on protein expression of Bcl-2.

CONCLUSION Cisplatin induces apoptosis and death of HeLa cells through the suppression of HPV E6 and restoration of p53 function.

KEYWORDS: cell apoptosis, cisplatin, flow cytometry, HPV E6, Bax, Bcl-2, cervix cancer.

High-risk human papillomavirus (HPV) is associated with the development of cancers, of which the most serious is cervical cancer. This malignancy is the second leading cause of cancer deaths in women worldwide.[1] The high-risk HPV E6 protein binds to the normal P53 protein, promotes its degradation through the ubiquitin pathway preventing P53 function. Cisplatin is a DNA damaging agent that is widely used for the treatment of a variety of tumors.[2] Cisplatin is also known to act synergistically when administered in combination with various chemotherapeutic drugs, and it has become a standard part of cancer therapy. In contrast to many other human tumors, most forms of HPV-positive cervical cancers possess a wild p53 gene. But its normal function is disrupted by expression of HPV E6. Therefore, restoration of P53 function by blocking the E6/P53 pathway might be a potential therapeutic target for these cancers.

MATERIALS AND METHODS

Materials

The HeLa cell line was obtained from the CCTCC (China Center for Type Culture Collection). Cisplatin was purchased from the Dezhou Pharmaceutical Co. in Shandong Province. Cisplatin was freshly prepared in distilled water and diluted in DMEM. Propidium iodide (PI)

was obtained from the Pharmingin Co. MMLV, oligo-dT and dNTP were purchased from Promega Co. 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) was obtained from the Sino-American Co. Fetal bovine serum (FBS), Dulbecco's Modified Eagle's medium (DMEM), trypsin and DNA markers were products of Gibco.

Methods

Cell culture

HeLa cells were cultured in DMEM containing 10% heat-inactivated (56℃, 30 min) fetal bovine serum. The cells were grown at 37℃, in 5% CO_2 and under saturation humidity.

MTT assay

Suspensions of 6×10^3 HeLa cells/100 μl of medium were added to each well of 96-well plates (5 duplicates) and incubated for 24 h after which the drugs were added at concentrations of 2.5, 5.0, 10 and 15 μg/ml) in a volume of 100 μl per well. The plates were then incubated for 1, 2 and 3 days after which 10 μl of a solution of 5 g/L MTT was added to each well followed by incubation for another 4 h at 37℃. The medium was carefully discarded and the formazan crystals dissolved in 200 μl of DMSO. Absorbance was read at 570 nm using μQuant (Bio-Rad Co.). The inhibition rate of cell growth =1-(absorbance in experimental groups/absorbance in control group)×100%.

Apoptotic cells staining

HeLa cells were seeded onto glass slides. After treatment, the cells were washed with PBS and then fixed with methanol:acetic acid (3:1) for 15 min, stained with Hoechst 33258 for 5 min and then examined by fluorescence microscopy.

Flow cytometric analysis

The cells were collected and washed twice with cold PBS and fixed with 80% ethanol at -20℃ overnight. After removing ethanol, the cells were stained with a mixture containing 50 μg/ml PI and 50 μg/ml RNase at room temperature in the dark for 30 min. The cells were then analyzed in triplicate by FACScan flow cytometry (Becton Dickinson). The percentage of cells in each phase of the cell cycle was determined by the ModFit and CellQuest programs.

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA at different times was extracted using a TRIZOL solution according to the manufacture's instructions. Total RNA (3 μg) was used to synthesize cDNA which then was used for 35 cycles of PCR amplification. Each cycle of PCR was run as follows: 1 min at 95℃, 30 s at 94℃, 45 s at 50℃, 1 min at 72℃. The primer sequences and sizes of amplified products are as follows: HPV18 E6 sense 5'-CAA CAC GGC GCC CTA-3', and antisense 5'-TCG CGT CGT TGG AGT-3', amplified PCR fragment, 314 bp; human GAPDH sense 5'-AGC CAT GTA CGT GCT ATC C-3' and antisense 5'-TTG GCG TAC AGG TCT TTG C-3' 498 bp. The PCR products were electrophoresed in a 2% agarose gel, and then examined under UV light.

Western blotting

The cells were washed twice with ice-cold PBS and treated for 20 min at 0℃ with lysing buffer containing 100 μg/ml PMSF, 50 mM Tris-base at pH 8.0, 150 mM NaCl, 1% NP-40, and 1 μg/ml aprotinine. After centrifugation at 12,000 rpm for 20 min, the supernatant containing the proteins was collected and the protein concentration determined by a Bio-Rad DC Protein Assay kit. Equal amounts of protein concentration were electrophoresed on 10% SDS-polyacrylamide gel and electro-transferred onto PVDF membranes. The membranes were probed with the appropriate antibody and the signals detected by an enhanced chemiluminescence (ECL) system.

Statistical analysis

All statistical data were processed by using a *t*-test for the mean difference between 2 samples with SPSS 11.0.

RESULTS

Effect of cisplatin on the growth of HeLa cells

Various concentration of cisplatin were incubated with the HeLa cells for different times. Fig.1 shows the inhibitory effect of increasing concentrations of cisplatin on HeLa cell growth. Cisplatin inhibited HeLa cell growth in a dose- and time-dependent manner.

Apoptosis induced by cisplatin

Hoechst 33258 staining was used to evaluate apoptosis which was characterized by cytoplasmic and nuclear shrinkage, chromatin condensation and apoptotic bodies (Fig.2).

Flow cytometric analysis of apoptosis

Cells treated with 10 μg/ml of cisplatin at various time points were analyzed for DNA content by means of FACS. With time the HeLa cell DNA histogram, sub-G1 peak was gradually raised (Fig.3).

The apoptotic rates were (0.82±0.27)%, (10.85±1.30)%, (26.68±1.92)%, (47.84±2.16)% and (67.16±3.75)% at 0, 12, 24, 36 and 48h, respectively. There were significant differences between treated groups and untreated group (t=12.76, 27.07, 43.16, 28.61, P< 0.01).

Effect of cisplatin on expression of HPV E6 mRNA

As shown in Fig.4, HPV E6 mRNA expression was down-regulated after treatment with 10 μg/ml cisplatin.

Effect of cisplatin on expression of HPV E6, P53, p21, Bax and Bcl-2 protein

Fig.5 shows that HPV E6 protein expression was down-regulated in a time-dependent manner after treatment with cisplatin. On the other hand, P53, p21 and bax proteins were up-regulated by cisplatin but Bcl-2 protein expression was not affected.

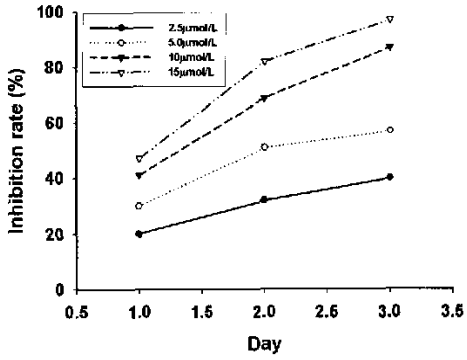

Fig.1. Effects of cisplatin on HeLa cell growth (in a dose- and time-dependant manner).

DISCUSSION

Apoptosis is an active complex pathophysiological process that requires metabolic activity by dying cells. It is generally considered that the mechanism by which chemicals kill cancer cells is via gene damage resulting in inhibition of DNA replication. In spite of a drug's effect, various chemotherapeutic agents kill tu-

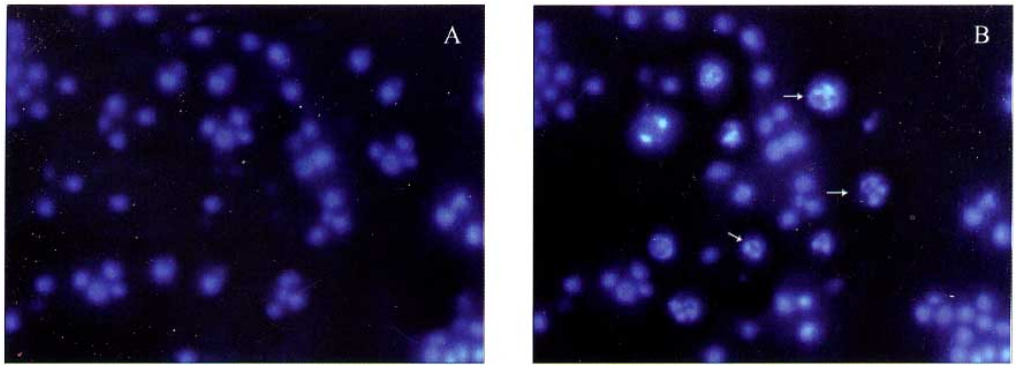

Fig.2. Microscopic appearance of Hoechst-stained nuclei of untreated HeLa cells (A) and those treated with cisplatin (B)(apoptotic cells are indicated with arrows.)

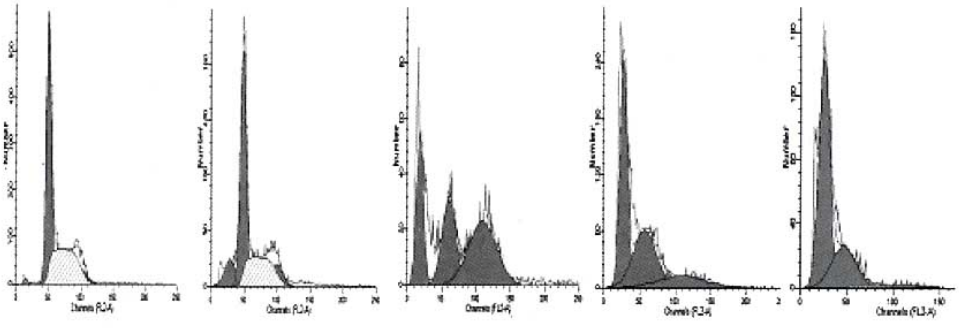

Fig.3. Apoptotic rates of HeLa cells after treatment with cisplatin for different times were evaluated by PI staining.

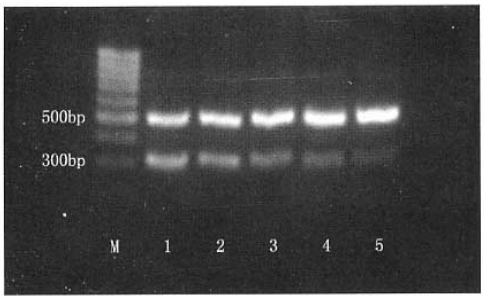

Fig.4. mRNA expression status of HPV E6 at different times after treatment with cisplatin. M: marker (100 bp); 1~5: HeLa cells after treatment of cisplatin at 0, 12, 24, 36, 48 h.

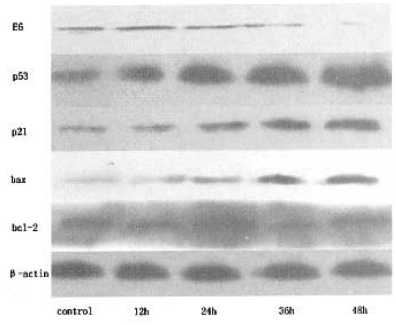

Fig.5. Protein expression status of HPV E6, P53, p21, Bax and Bcl-2 at different times after treatment with cisplatin.

mor cells through apoptosis and necrosis.[3] Cisplatin is primarily considered to be a DNA-damaging anticancer drug, mainly forming different types of bifunctional adducts in its reaction with DNA resulting in the induction of apoptosis.[4]

Our study showed that cisplatin significantly inhibited HeLa cell growth in a dose- and time-dependent manner. Flow cytometry and Hoechst 33258 staining determined that cisplatin inhibited HeLa cell growth through apoptosis. The mitochondrial pathway is mainly regulated by the Bcl-2 protein family which has more than 15 members. Based upon differences in pathways of regulation of apoptosis, members of this family can be divided into 2 subgroups. Each of them contains five or more function- and structure-related proteins. The first group is composed of the anti-apoptotic proteins such as Bcl-2, Bid, Bcl-X, Mcl-1, Bcl-w and A1. Members of the second group are pro-apoptotic including Bax, Bak, Bad, Bik, Hrk, Bid, Bcl-xs.[5] In our study, assessment of the Bax and Bcl-2 proteins showed that Bax protein expression was up-regulated, thus confirming that apoptosis occurred through Bax up-regulation.

Under normal growth conditions, P53 is turned over by the ubiquitin-proteasome system. But in cervical cancer cells infected with HPV, the degradation of P53 is completely switched to HPV E6-mediated ubiquitination. The p21 gene is located downstream from the p53 gene. Recently, several approaches have been proposed to study the control of growth of E6-expressing cancer cells. Hietanen et al.[6] used leptomycin B and actinomycin D to restore P53 activity in HPV-positive cervical carcinoma cells. Chou et al.[7] used sodium arsenite to suppress the expression of HPV E6 and restore p53 tumor suppressor pathway in human cervical carcinoma cells. In the present study, cisplatin significantly suppressed the expression of both HPV E6 mRNA and protein. Meanwhile, Western blots indicated that the expression of P53 and p21 was up-regulated confirming that cisplatin suppressed the expression of HPV E6 protein thereby restoring the function of P53. But the interaction between HPV E6 and cisplatin needs to be studied further.

In conclusion, cisplatin inhibits the expression of HPV E6 resulting in the restoration of the normal p53 tumor suppressor pathway and induction of apoptosis in HeLa cells. Therefore, cisplatin might be viewed as another potential therapeutic candidate to restore normal p53 functon and induce apoptosis in HPV-positive cervical cells. As an important anticancer drug, cisplatin has a vital role in treating cervical cancer.

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