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Basic Investigations

STUDY ON ANTITUMOR DRUG-INDUCED APOPTOSIS IN HUMAN CANCER CELLS BY TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE ASSAY

Tong Tong* 童彤 Sun Hanxiao 孙含笑 Liu Liying 刘丽影 Liang Jinfeng 梁金凤
Guo Suping 郭素萍 Han Naijun 韩乃君 Cheng Shujun 程书钧

Cancer Institute (Hospital), Chinese Academy of Medical Sciences, Beijing Union Medical College, Beijing 100021

Objective: Considerable evidence has showed that apoptosis is involved in both cancer development and inhibition. A new assay (terminal deoxynucleotidyl tansferase, TdT) was recently reported to have advantages in the detection of apoptosis. In this study, this assay was used to investigate antitumor drug-induced apoptosis in human cancer cells. Methods: TdT assay, DNA gel electrophoresis, electron and light microscopy were used to observe apoptosis. Results: Our results showed that cisplatin-induced apoptosis in both HL-60 and SV40T-transformed human bronchial epithelial cells was detected with a good dosage and time response. The occurrence of the apoptosis was preceded by the decrease of bcl-2 mRNA expression. With the TdT assay, apoptotic cells were observed in ovarian tumor of patients treated with carboplatin. Conclusion: TdT assay may be applicable to monitor apoptosis in human cancers induced by chemotherapy, and to evaluate tumor cell response during treatment.

Key words: Apoptosis, Terminal deoxynucleotidyl transferase, Ovarian neoplasms, Cisplatin, Oncogenes.

Apoptosis, or programmed cell death, is a cell death pathway which is characterized by a process of specialized morphological and biochemical changes. A series of apoptosis-associated genes (bcl-2, p53, c-myc, etc.) play an important role in the process of cell apoptosis regulation. We currently consider that there is a strong correlation between cell apoptosis and the occurrence of cancer. In recent years, some results suggest that cell apotosis induced by chemo-therapy is a possible mechanism for fighting cancer. These data are mainly derived from the haemopoietic system. Evidence derived from solid tumors in which chemical drugs induced cell death, *in vivo*, is limited. It is possible that the reason for the difficulties encountered in the detection of apoptotic cells is due to their rapid destruction by phagocytic cells and neighboring cells.[1,2] For this reason, a more sensitive method must be established in order to detect these apoptotic cells in earlier stages prior to their destruction. Gorczyca[3] established the TdT method, in which biotin-dUTP molecules are added to the 3'-OH end of a DNA strand breakage. The reaction is catalyzed by terminal deoxynucleotidyl transferase (TdT). This method, which is considered a sensitive enough means for detecting apoptotic cells in the early stages, can be applied to assess the apoptotic HL-60 cells. We compared the drug-induced apoptosis of HL-60 with that of immortal human bronchial epithelial cells (SV40T). It confirms the TdT method's sensitivity by

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*Present address: Institute of Medicinal Biotechnology, Chinese Academy of Medical Sciences, Beijing 100050

its identification of cellular apoptosis of human bronchial epithelial cells. We also used the TdT method to observe apoptotic cancer cells in ovarian tumor tissues obtained from patients treated with chemotherapy.

MATERIALS AND METHODS

Materials

Human promyelocyte HL-60 was maintained in an RPMI-1640 medium supplemented with a 10% fetal calf serum at 37 ℃ in a 5% Carbon Dioxide incubator. The cells were split every three days and cells densities in cultures did not exceed 5×10^5 cells/ml.

SV40T transformed human bronchial epithelial cells (HBEM) were constructed by the Department of Etiology at the Cancer Institute (CAMS) in Beijing, China.

The fresh tissues of ovarian cancer, treated or otherwise by chemotherapy, were offered by patients at the department of Gynecological Oncology at the Cancer Hospital (CAMS).

The plasmid probe for bcl-2 (4.3 kb) was supplied by Dr. Seeta R. Chaganti.

Methods

DNA Gel Electrophoresis[3]

Control & cisplatin-treated HL-60 cells were collected by centrifugation, washed in PBS, resuspended in 0.5 ml TBE (45 mM Tris-borate buffer, 1 mmol/L EDTA, pH 8.0) containing 0.25% Nonidet NP-40 and 1 mg/ml RNase A, incubated at 37 ℃ for 30 min, then treated with 1 mg/ml proteinase K and incubated for an additional 30 min at 37 ℃. After incubation, a horizontal 1.5% agarose gel electrophoresis was performed for 4 h. The DNA present in the gels were visualized under a UV light (254 nm) after being stained with 5 μg/ml ethidium bromide. The gel was then photographed.

The in Situ Terminal Deoxynuycleotidyl Transferase (TdT) Assay[3]

Cell smears were fixed in 4% buffered formaldehyde for 15 min. Fresh tissues were fixed in 4% buffered formaldehyde and left overnight. Paraffin-embedded tissue sections were then prepared. After treating the sections with 2% H_2O_2 for 5 min and washing them in water, the sections were then incubated with terminal deoxynucleotidyl transferase and biotin-16-dUTP at 37 ℃ for 1 h, then with 2% BSA for 10 min, and then again with biotin-avidin-peroxidase complex (dilution of 1:100) for 30 min. After immersing the sections in PBS, they were stained with 0.01% H_2O_2 -DAB for 5 min.

In Situ mRNA Hybridization[4]

Cell smears were fixed in 4% paraformaldehyde and then with Levamisole, at which point they were incubated with proteinase K and dehydrated. The air-dried smears were pre-hybridized with a solution containing 50% formamide for 3 h at 42 ℃ and were then hybridized with biotinylated bcl-2 probe (200 ng/ml) for 18 h at 42 ℃. After rinsing in SSC, containing 50% formamide, and incubating with 3% acetylated bovine serum album for 1 h and 1:100 streptavidin-alkaline phosphatase for 1 h, the smears were developed in an alkaline phosphatase substrate for 10 h. The cases where there were blue-purple granules in the cytoplasm were defined as the positive response for bcl-2 mRNA. Cases in which the cytoplasm was full of the blue-purple granules were labeled as +++. Cases where the cytoplasm was 1/3–1/2 full of the granules were labeled as ++ and in cases less than 1/3, were labeled as +. In cases where there were no blue-purple granules, were considered negative responses.

Detection of Apoptosis with Light Microscope and Electron Microscope

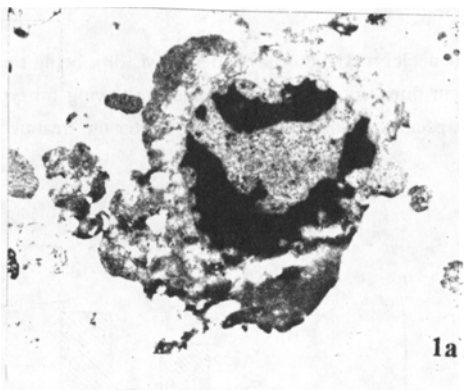
Tissue sections that were observed with the light microscope were stained with haemotoxylin and eosin. The sections observed with the transmission electron microscope were prepared according to standard methods.

RESULTS

Results from the light microscope and electron microscope showed that HL-60 cells and HBEM cells, incubated with cisplatin for different duration of time, appear to indicate typical apoptotic morphology. The

recognized morphological changes are compaction of nuclear chromatin, with the formation of sharply delineated, uniformly granular masses that become marginated against the nuclear envelope, with condensation of the cytoplasm and formation of apoptotic bodies (Figures 1a, 1b). This manifests that apoptosis can be induced by cisplatin in the two cell lines.

Gel electrophoresis of DNA from HL-60 cells, treated with cisplatin for 24–48 h, revealed a clear ladder pattern (Figure 2). In the same conditions, DNA from HBEM cells did not reveal the DNA ladder pattern.



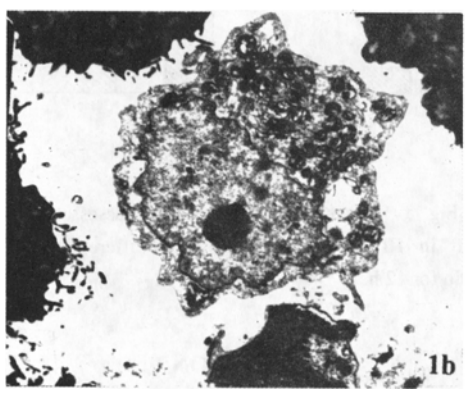


Fig. 1 Electron micrograph showing an apoptotic morphology of HBEM cell after being treated with 50 μmol/L cisplatin for 48 h (1a), normal HBEM cell (1b). x 7100

Brown precipitants in the cellular nuclei of HL-60 and HBEM, detected by the TdT method, were considered positive responses for cell apoptosis (Figure 3a, 3b). Figure 4 shows the incidence of apoptotic cells in the two cell lines, determined by either TdT or the morphological method. The rate of incidence greatly increased after being treated with 50 μmol/L cisplatin. The extent of the apoptosis revealed by the TdT method was considerably greater than that determined using nuclear morphology. The figure also shows the occurrence of apoptotic cells, assessed by the TdT method, having a good time response.

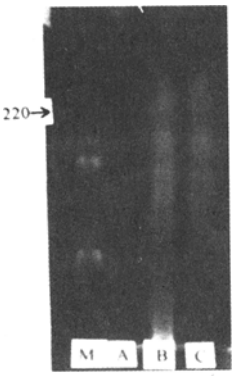


Fig. 2. Gel electrophoretic analysis of HL-60 cells with 50 μmol/L cisplatin for 24 h (B) or 48 h (C), untreated control (A), molecular size markers (M).

The apoptotic HL-60 cells, incubated with different doses of cisplatin and monitored by the TdT method, show a good dose response (Figure 5).

Results from the *in situ* mRNA hybridization assay show that the expression of bcl-2 mRNA in both HL-60 and HBEM cells decreases after treatment with cisplatin for 1 or 6 h, but the expression cannot be detected on the 12 h as shown on the Table 1. This indicates that the occurrence of apoptosis has an inverse relationship with the expression of bcl-2 mRNA.

*Table 1. Number of cell (%) with bcl-2 mRNA expression in HL-60 and HBEM cells treated with 50 μmol/L cisplatin for different duration**

Cell lines	Duration treated with cisplatin (h)			
	0	1	6	12
HL-60	30.8	21	15	0
HBEM	66	64.5	17	0

* Value of cells labeled only as +++ and ++ (See method) was included in this Table.

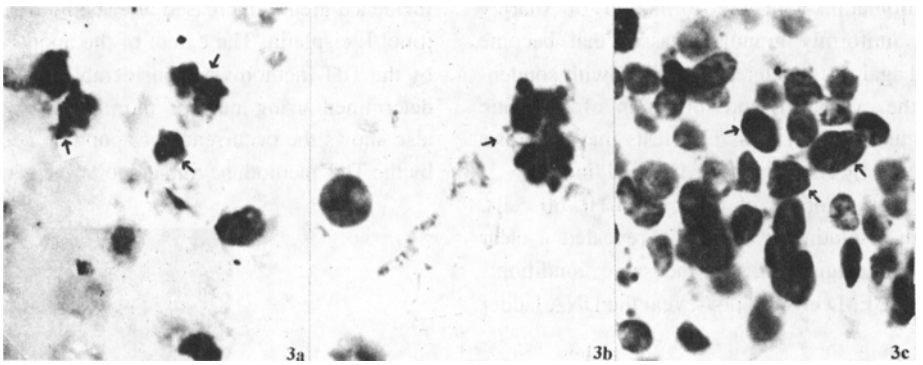
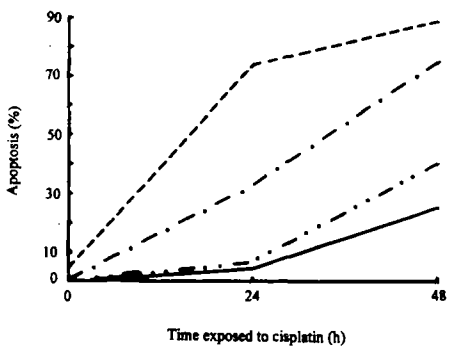


Fig. 3. TdT positive brown precipitants in the apoptotic cellular nuclei of HL-60 (3a) and HBEM (3b), being treated with 50 μmol/L cisplatin for 24 h. TdT positive brown precipitants in three apoptotic cellular nuclei showing no typical apoptotic morphology (3c, Arrows) from an ovarin papillary serous cystadenocarcinoma patient, 9 days after the treatment of 300 mg carboplotin i.p. x1000



--- Apoptosis in HL-60 cells by TdT method
—· Apoptosis in HL-60 cells by morphologic method
—·· Apoptosis in HBEM cells by TdT method
— Apoptosis in HBEM cells by morphologic method

Fig. 4. Quantification of apoptosis induced by the treatment of 50 μ mol/L cisplatin at different times.

Scattered early-apoptotic cancer cells, monitored by the TdT method, were found in tumor tissue sections obtained from ovarian papillary serous cystadenocarcinoma patients treated with 300 mg carboplatin i.p. (Figure 3c). Several cancer cells with typical apoptotic morphology were distinctly stained positive by the TdT method.

Fig. 5. Quantification of apoptosis assessed by TdT method in HL-60 cells treated with different doses of cisplatin for 12 h.

DISCUSSION

The question concerning whether or not chemotherapy can induce apoptosis *in vivo* in human solid-tumors has gained much attention in the cancer therapy community. This study clearly points out that chemotherapeutic drugs not only induce apoptosis in the HL-60 and HBEM cell line *in vitro*, but can also induce cancer cell apoptosis in cases of ovarian cancer.

Through much research, DNA strand breakage is one of the characteristic biochemical changes to occur in cell apoptosis.[5] Some reports indicate DNA ladders (Shown by gel electrophoresis of extracted apoptotic cell DNAs) to be the product of double-

strand cleavage at the linker regions between nucleosomes; leading to the formation of fragments that are multiples of units composed of 180–200 base pairs. Recent results indicate the occurrence of DNA internucleosomal cleavage, suggesting the presence of larger fragments.[6] This study showed that DNA extracted from HL-60 cells, treated with cisplatin for 48 h, exhibited characteristic apoptotic ladders. DNA ladders were not available for the HBEM cell line. Although the electron microscope revealed that both cell lines can undergo apoptosis in the same conditions mentioned above, a positive stain for both was achieved using the TdT method. Figure 4 indicated that cell apoptosis detected by the morphological method is preceded by that of the TdT method. Although the incidence of apoptotic cells revealed by the two increased synchronously, they both had a good dose and time response. This study also verified the sensitivity of the TdT method for the assessment of cell apoptosis, not only derived from that of the haemopoeitic system, but also from that of solid tumors. Variable number of apoptotic cancer cells were found in the cancer tissues obtained from ovarian cancer patients after being treated with the chemotherapeutic drug (Figure 3c). If the ovarian cancer tissues were cultured *in vitro* with the organic culture system and were then introduced to cisplatin, more apoptotic cells would have been available (data not shown).

Cell death is regulated by a series of genes. A recent study claims that the expression of the bcl-2 gene may block the occurrence of apoptosis and extend the cellular life span.[7] Our results demonstrated that the expression of bcl-2 mRNA in HL-60 and HBEM cell lines decreased rapidly after being treated with cisplatin, later becoming undetectable at 12 h, followed by a gradual increase of apoptotic cells. This manifested that the decrease of bcl-2 gene expression is relevant to the onset of apoptotic cells. This study notes that the TdT method may be applied to identify apoptotic human tumor cells treated *in vivo* with chemotherapy, therefore, proving its practical value in the evaluation of therapeutic effects.

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