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ANALYSIS OF APOPTOSIS BY DNA END LABELING METHOD (TDT) IN LEUKEMIA CELL LINES HL-60 AND U937*

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Antitumor-drug-induced apoptosis in leukemia cell lines HL-60 and U937 was quantitatively analyzed with TDT (terminal deoxynuleotidyl transferase-mediated nick-end labeling) approach, which allows to label the ends of DNA broken strands in apoptotic cells by biotinlated dUTP which to avidin-FITC. In this way the apoptotic cells show fluorescent when the labeling cells were emitted by UV light microscope or laser-activated flow cell sorting at r=480. In our study, HL-60 and U937 cell lines were cocultured respectively with cis-diaminodichloroplatinum (CDDP), hydroxycamptothecinum (HCT) and vindesini sulfa (VCR) for 18 hours. By calculating percentages of apoptotic cells with TDT method, we were able to show that the two cell lines gave different sensitivity to the drugs. HL-60 showed high sensitivity to CDDP but U937 cells were more sensitive to other two drugs, HCT and VCR. Meanwhile we compared the results of obtained by DNA gel electrophoresis with that by TDT. We found that gel electrophoresis is not sensitive enough to reveal apoptosis since there was no ladder structure, a typical electrophoresis pattern for apoptosis, appeared until the apoptotic cells reached or over 13%. And we report in this paper as first time that three forms of apoptotic cells could be detected under fluorescent microscope, which we called as spot form and crescent form and assembling form in terms of distribution of light spots within cell nuclei. It seemed that the spot form was at an early stage of apoptosis and the crescent form represented a later stage of apoptosis.

Key words: Apoptosis, Leukemia cell line, Antitumor drug, TDT.

Apoptosis is a kind of cell death recognizable by a series of specialized changes in morphological, biochemical and molecular biological forms. Apoptosis is an active process of cell destruction, characterized by cell shrinkage, chromatin aggregation with extensive genomic fragmentation, crescent body forming by chromosome pyknosis along the nuclear margin. In the apoptosis, endouncleasease dependent Ca2+ and Mg2+ is activated, this enzyme make chromosome degraded at the linker sections to fragments equivalent to single and multiple nucleosomes. The latter are revealed as the typical "ladder" on agarose gel during electrophoresis which is know as one of the biochemical features. Apoptosis were identified according to their morphological features and the ladder on gel electrophoresis for decades.[1,2] But it was believed that there were some shortages in apoptosis studies using morphological characteristics and DNA gel electrophoresis as parameters as apoptosis should be studied meticulously and the methods described above could not analyze apoptosis quantitatively. In this paper, we analyzed quantitatively the chemosensitivity of two leukemia cell lines,

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HL-60 and U937, to three antitumor drugs using a method called as TDT (terminal deoxynuleotidyl transferase-mediated nick-end labeling) and indicated as first time that there were three forms in the apoptotic cells under fluorescent microscope.

MATERIALS AND METHODS

Cells

All experiments were performed on cell line HL-60 (early granular leukemia) and U937 (monocyte leukemia) during their exponential phase of growth. The cells were maintained in RPMI (Gibco) supplemented with 10% calf bovine serum, 100 unit/ml penicillin, 100 μg/ml, streptomycin, and 2 mM L-glutamine as described proviously.[3] The cells were split every second day and were kept Ig phase of growth when three antitumor drugs were added.

Drugs

Cis-diaminodichloroplatinum (CDDP, Qilu Medicine Co., Shandong Province) hydroxycampto-thecinum (HCT, Huangshi Feiyun Ltd. Co., Zhejiang Province), and vindesini sulfa (VCR, Guangzhou Mingxing Medicine Co., Guangdong Province) were adopted to treat HL-60 and U937 cells at a concentration of 20 μg/ml. The cells were suspended as 10^6/ml and co-cultured with the drugs for up to 18 h at 37 ℃, and then labeled with TDT.

TDT Labeling

The treated cells were labeled as described by Gorczyca[3] with our modification: After incubated with the drugs, the cells were fixed in 1% formaldehyde in 0.2 M PBS (pH 7.4) for 30 min in 4 ℃. Washing in PBS for 2 times, cells were resuspended in 50 μl of a solution containing 0.5 μg terminal transferease (SIGMA), 0.5 nmoles biotin-16-dUTP (SIGMA) and 15 μl TDT buffer. Then cells were incubated in this solution at 37 ℃ for 3 h rinsed in PBS and resuspended in 100 μl of the staining buffer containing fluoresceinated avidin which included 2.5 μg/ml fluoresceinated avidin (SIGMA), 4×SSC, 0.1% triton X-100 (Fluka) and 5% (w/v) bovine serum albumin (SIGMA) for 30 min at room temperature in the dark. Then the cells were washed using PBS for 2 times. In this experiment, the broken strands of DNA in apoptotic cells were labeled by b-16-dUTP-FITC, so these incorporated cells showed FITC positive and could be observed using flow cytometry or UV microscope easily.

Flow Cytometry and Photography under UV Light Microscope

Following incubation in staining buffer the cells were rinsed in 80% ethnol-PBS at 4 ℃ foe 30 min and washed in PBS. The pellet of cells were incubated with 200 μl PBS containing 5 μg/ml PI and 1% RNase at 40 ℃ for 15 min. Flow cytometry was performed on a FACScan flow cytometer (Becton Dicknson). The red (PI) and green (FITC) fluorescence emission from each cell were separated and measured using the standard optics of the FACScan. The data from 2×10^5 cells were collected at 800/sec speed, stored and analyzed using LYSYS 11 software. Fluorescence microscope (OLYMPUS): UV were emitted by — and filtered to form r=340 nm. The UV activated FITC labeled in apoptotic cells to form green fluorescence. The green cells were observed and photoed using auto-photo instrument.

DNA Gel Electrophoresis

Treated and non-treated cells were collected by centrifugation and assayed as described by Wesselborg et al.[4] Briefly, the collected cells were resined in 20 μl lysis solution containing 10 mmol/L EDTA, 50 nmol/L Tris-HCL, 0.5% SDS, 0.5% protein K (Boehringer Mannheim) and incubated at 50 ℃ for 1 h. Then the samples were added 10 μl RNase (10 mg/ml) and incubated at same temperature for another 1 h. The samples were added 8 μl loading buffer and 5 μl 1% low melt agarose and incubated at 65 ℃. After 5 min, 30 μl of each sample was loaded on 2% agarose. Horizontal 2% agarose gel electrophoresis was performed at 6 V/cm for 1 h and DNA in gels was visualized under UV light staining with 5 μg/ml of ethidium bromide (SIGMA).

RESULTS

Quantitative Analysis of Apoptosis Induced by Three Antitumor Drugs in HL-60 and U937 Cell Lines by Using FACScan

Being FITC-labeled by TDT method as described above, DNA strands breaks in apoptotic cells could be recognized by FACScan to indicate percentages of apoptotic cells induced by antitumor drugs. HL-60 and U937 gave different patterns of chemosensitivity to drugs as described in Table 1. HL-60 showed high response to CDDP and more than 90% cells became apoptotic with intensive labeling after incubated with the drug, while U937 cell line showed lower sensitivity and only 21.64% cells were of apoptosis by same treatment. Moreover, being treated with VCR and HCT, lower sensitivity for HL-60 and higher sensitivity for U937 could be observed. The apoptotic U937 cells were 41.06% and 53.08% respectively.

Table 1. Analysis of apoptotic HL-60 and U937 cell line induced by three antitumor drugs

Drugs (20 μg/ml)	Control (%)	CDDP (%)	HCT (%)	VCR (%)
HL-60	2.01	95.39*	18.08	13.24
U937	2.88	21.64	53.80*	41.06*

* $P<0.01$

Assay Apoptotic HL-60 and U937 Cell Lines on DNA Gel Electrophoresis

Apoptosis inducing in HL-60 and U937 cell lines was further confirmed by ladder structures in gel electrophoresis (Figure 1), which was considered traditionally as a typical feature of apoptosis. The ladder structures were observed in the DNA samples from HL-60 treated by CDDP (lane 2) and HCT (lane 3), as well as in those from U937 treated with CDDP (lane 6), HCT (lane 7) and VCR (lane 8). In control without treatment, there were no ladder appeared. Interestingly, however, the lane 4 in Figure 1 does not show the ladder structure even though the cells, from which DNA was extracted, have been treated with VCR at the dosage inducing typical changes of apoptosis assayed by TDT method. This suggests that for VCR-induced apoptosis in HL-60, the ladder pattern is not a sensitive criterion as that assayed by TDT for apoptosis detecting. It is likely that the percentage of apoptotic cells (13.24%) in this case was below the threshold, only above which the ladder structure could be revealed.

Morphology of Apoptotic Cells Labeled by TDT Method under Fluorescent Microscope

Using fluorescent microscope, the normal cells appeared to be negative even though they were labeled by b-dUTP-FITC, so that they were barely visible under UV light microscope. As to apoptotic cells, however, strong green fluorescence could be observed easily when emitted by UV light. In our experiment, the labeling apoptotic cells revealed three appearances which we called as sport form and crescent form and assembling form respectively.

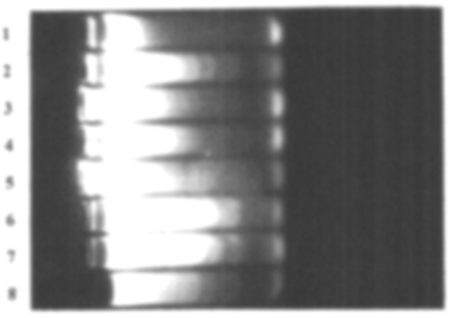

Fig. 1. Agarose gel electrophoresis of DNA from HL-60 and U937 treated with 20 μg/ml CDDP, HCP and VCR. Lane 1–4: group of HL-60 cell line incubated with no drug (lane 1), with CDDP (lane 2), HCT (lane 3) and VCR (lane 4). Lane 5–6: group of U937 cell line treated by CDDP (lane 6), HCT (lane 7) and VCR (lane 8).

1. Spot Form: In the early stage of apoptosis before cell condesensing, the broken DNA strands were labeled and showed spots scattering in the nuclear of apoptotic cells (that showed with arrow in Figure 2). We have found that the spot form was a most common visible one using TDT method in HL-60 and U937 cell lines treated by anti-tumor drugs.

2. Crescent form: The form appeared in the middle or late stage of apoptosis, labeling DNA of apoptotic cells condensed and created one to three crescent bodies (those showed by traingle in Figure 2) along the margin of nuclear membrane. These crescent forms are one of typical characters of apoptosis.

3. Assembling form: In our examination of labeling apoptotic HL-60 and U937 cells treated by three drugs, we also observed that there were aggregating crescents just like a bound of grapes, which suggested the crescent was consist of several

apoptotic bodies at late stage of apoptosis (Figure 3). Some times three forms could be found in same field under microscope.

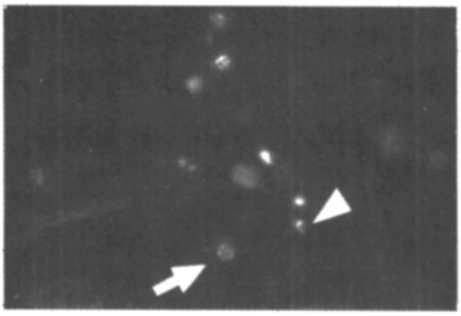

Fig. 2. Apoptotic cell characterized by DNA broken strands which show spot form (arrow showing), crescent form (traingle showing) after labeled with TDT method ×50, under fluorescent microscope).

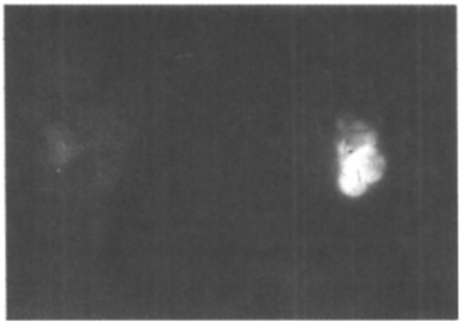

Fig. 3. Assembling form by gathering of several apoptotic bodies (× 200).

DISCUSSION

There are several methods of detection of apoptosis. Morphological changes involve a characteristic pattern of condensation of chromatin and cytoplasm, which makes it possible to identify these cells by microscopy and electron microscope.[5] DNA from apoptotic cells can create a typical ladder structure for bands on gel electrophoresis, which are equivalent to DNA fragments of approximately 200 bp and their multiples. The ladder structure has been as a token of apoptosis for a long time. In 1980, Langlosis analyzed chromosome of apoptotic cells by flow cytometry[6] and since then, more and more researchers investigated apoptosis using cytometry according to Langlosis describing. Now flow cytometry has been used to analyze apoptosis quantitatively.[7] This is commonly used method to quantitatively assay apoptosis called dubble lasers analysis.[7,8] Here alive cells including normal and apoptotic cells are stained by DNA dyes such as Hoechst 33342 firstly, then apoptotic cells are detected according to their forward light scatter verses side scatter, while dead cells are gated by PI stained.[7,8] But it is very difficulty to identify early apoptotic cells in which DNA has degraded but no condensation of chromatin and cytoplasm. Meanwhile a flow cytometry is too expensive to be used wildly. Although DNA gel electrophoresis has been used commonly in most laboratory, but it is hardly to analyze apoptosis quantitatively because the ladder structure is barely visible if apoptotic cells are less so that this method shows its shortage in studying apoptosis meticulously. Fehsel et al. reported firstly that DNA strand breaks that occurred in cells undergoing apoptosis could be labeled *in situ* with a biotinylated nucleotide in the reaction using exogenous terminal transferease. The incorporated precursor was detected by fluoresceinated avidin. The labeled cells were then identified directly by flow cytometry or UV light microscope.[9] Now this method has been used more and more wildly. HL-60 and U937 cells were treated and incubated with three antitumor drugs respectively for 18 h and labeled by biotinlated dUTP and fluoresceinated avidin by FACS and UV microscope. The percent of apoptotic cells were different in HL-60 from that in U937 although they were leukemia cell lines. This demonstrated that two cell lines gave different sensitivity to the drugs especially to CDDP, which may result from the difference nature of cell lines because the HL-60 comes from early granular leukemia while U937 is from monocyte leukemia. For example, in the CDDP treatment more than 90% HL-60 cells were going to apoptosis but only 21% of U937 cells were apoptosis. The different sensitivity of these two cells to CDDP was significant ($P<0.01$) in terms of percent of apoptotic cells. The different also existed in the sensitivity to other two drugs, HCT and VCR for example, HL-60 gave a lower sensitivity to VCR and HCP but U937 showed higher response to both of them in which apoptotic cells reached 41% and 53% respectively. Agarose gel electrophoresis was also

used to detect the apoptotic cells in this reporter. There were ladder appearance on agarose gel when DNA extracted from HL-60 and U937 cells except for HL-60 incubated with VCR. This indicated that gel electrophoresis was not sensitive enough to identify apoptosis as there was no ladder unless apoptotic cells had reach or over 13%. The TDT approach, however, can be used to obtain good results not only for its specific but also for its quantitative ability as we reported here. Besides, we found that there were three forms of apoptotic cells: spot form which might represent a morphology of early stage of non condenseing apoptotic cells, crescent form and assembling form which obviously emerged in middle and later stage of apoptosis. The apoptotic cells with spot form was not visible under non-UV microscope and was difficult to distinguish from mormal cells. In later stage of apoptosis, nuclear and cytoplasm were condensed and form crescent and apoptotic bodies that were easy to observe only under microscope. In the most situation, there were three forms observed at same time, a phenomenon so far no reports deal with. What we present here might be significant in studying on sensitivity of tumor cells to drugs and on cell pathology and physical metabolism.

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