Two-Dimensional (Polyacrylamide) Gel Electrophoresis Analysis of Apoptosis Induced by Harringtonine in K562 Cells

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OBJECTIVE The anti-tumor drug, harringtonine (HT), has been extensively used with satisfactory results in the treatment of acute or chronic myeloid leukemia. Previous studies have shown that the anti-tumor activity of the drug is related to induced apoptosis of tumor cells, but the molecular mechanism still remains unclear. The main purpose of this research was to analyze the protein profiles formed during HT-induced apoptosis in K562 cells and to screen the apoptotic-related proteins.

METHODS Annexin V and PI double staining was used in combination with flow cytometry to examine the early and the late stages of HT-induced apoptosis in K562 cells. In addition two-dimensional gel electrophoresis and computer-assisted image analysis were employed to separate and compare the HT-induced apoptotic proteins of the K562 cells and the controls.

RESULTS When a concentration of 10 μg/ml HT was used to treat K562 cells, the percentage of the early-apoptotic cells (Annexin V^+/PI^-) was found to be 28.3% and 18.1% at 5 and 24 h, respectively ($P<0.01$), while the rate of late-apoptotic cells (Annexin V^+/PI^+) was at a level of 9.1% and 20.2%, respectively ($P<0.01$). Matching analysis of the proteome among the control group and the early- and late-apoptotic groups showed 1,300 ± 50 protein spots which were identified in the control K562 cells with a matching rate of 88.3 ± 2.0 % for the protein spots in the two treated groups. Ten protein spots showed overt and steady changes in both quality and quantity in the cells of the late-apoptotic group ($P<0.01$), among which the level of expression for eight of the ten protein spots was up-regulated after apoptosis, one was down-regulated and one was merely expressed as in the control cells.

CONCLUSION The proteins with differential expression might be important proteins involved in the process of apoptosis in K562 cells induced by HT.

KEYWORDS: harringtonine, K562 cells, proteome, two-dimensional gel electrophoresis.

Chronic myeloid leukemia (CML) is a malignant clonal disease originating in hematopoietic stem cells. Numerous findings suggest that enhancement of an anti-apoptotic function of CML is the major reason why the CML cells display chemotherapy-resistant properties.[1] Harringtonnine (HT) is a non-specific anti-tumor drug with an alkaloid structure, which has been widely used with

satisfactory results in the treatment of acute and chronic myeloid leukemia. HT can rapidly decrease the peripheral white blood count of patients with CML and inhibit the effect of the Ph (+) chromosomal translocation, with a high rate of clinical remission. Some of the patients develop genetic changes that result in induction of apoptosis, but the detailed mechanism is unclear at present. [2] The technique of proteome analysis has developed in recent years. It can examine the nature of transient expression of all proteins under different cellular conditions, and it has become one of the most effective technologies for differential display and analysis of proteins. [3] Our research used this technology to determine the integral changes of the proteins during HT-induced apoptosis of K562 cells, and to look for proteins relating to HT-induction of apoptosis in these cells.

MATERIALS AND METHODS

Reagents and instruments

HT was supplied by the Pharmacy Institute of Peking Union Medical University. Annexin V/FITC was purchased from the Bender Medsystems Co and IPG (immobilized pH gradient) dry adhensive tape (pH 3~10L, L=18 cm) and low molecular number markers were bought from the Amersahm Pharmacia Co. Propidium iodide (PI), dithiothreitol (DTT), leupeptin, aprotinin, CHAPS, RNAase and DNAase were obtained from Sigma. The flow cytometer (FACS Calibur) was the product of the BD Co. The IPGphor IEF system, ImageScannner and ImageMaster 2D Elite v3.01 and two-dimensional electrophoresis image analysis software were all the products of Amersham Pharmacia Co. PROTEAN II xi Cell vertical electrophoresis units and its accessories were the products of the Bio-Rad Co. Trypsin (modified, sequencing grade) was purchased from the Roche Co. BIFLEX III matrix-assistant laser desorption/ ionization time-of-flight mass spectrometer, MALDI-TOF were the products of the Bruker Co.

Cell culture and grouping

Human chronic myeloid leukemia K562 cells were cultured in RPMI-1640 nutrient solution with 10% calf serum in various groups in an humidified 5% CO_2 atmosphere at 37℃ to a cell density of 1×10^6/ml. Ten μg/ml of HT was chosen as the test concentration based on our previous work. [4] Since we wished to study the effect of HT at various times, the cells were divided into normal control (A), 5-h HT treated (B), 12-h HT treated (C) and 24-h HT treated group (D). The cells in logarithmic growth were used for the experiments.

Annexin V/PI double staining analysis by flow cytometry

The cells were collected, washed twice in precooled PBS and was adjusted to $(2\sim5)\times 10^5$/ml with binding buffer. Five μl of Annexin V/FITC was added to 195 μl of the above cell suspension producing a uniform mixture which was incubated for 10 min in the dark followed by washing of the cells with binding buffer. After centrifugation 190 μl of RPMI was added to the cell suspension. After addition of 10 μl of PI staining solution (20 μg/ml), and incubation for 10 min in the dark, the cells were analyzed by flow cytometry.

Preparation of 2-DE protein samples of K562 cells

Normal K562 cells and K562 cells (1×10^6/ml) treated with HT for 5 and 24 h were collected by centrifugation, washed three times in precooled PBS and suspended in 60~100 μl of lysate (7 mol/L urea, 2 mol/L thiourea, 3% CHAPS, 65 mmol/L dithiothreitol, 2~8 mmol/L phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μl leupeptin, 10 U DNAase and 100 μg/ml RNAse). After 3~4 cycles of fast freezing and thawing the cell mixture was centrifuged at 12,000 rpm for 30 min and the supernatants stored at −70℃. Proteins were determined by the Bradford method. [5] Centrifugation of the lysed cells was conducted at 4℃ at 12,000 rmp for 15 min before electrophoresis.

First-dimension IPG electrophoresis

Samples containing 700 mg of protein were spotted in the gel with an appropriate amount of rehydratio buffers (8.0 mmol/L urea, 2% CHAPS, 65

mmol/dithiothreitol, 0.5% IPG buffer with a trace amount of bromphenol blue), with a total volume of 350 μl (18 cm IPG strips, pH3~10L). An IPG conversion kit was used and the IPG strips were carefully put in the apparatus to avoid production of air bubbles. Then the kit was sealed with IPG covering fluid to prevent evaporation of the samples and the electrophoresis conducted at 20℃ , with a total voltage-hour of 72,000 Vh. Upon completion of the electrophoresis, the IPG strips were put into 20 ml of balanced buffer solution (1.5 mol/L Tris, 6.0 mmol/L urea, 30% glycerol, 2% SDS and the trace bromphenol blue) and shook twice on a rocking bed for 15 min each time. The first balanced buffer solution contained 200 mg of DTT and in the second buffer solution, DTT was replaced by 0.148 g of iodoacetamide.

Second-dimension vertical flat plate SDS-PAGE

A 12% uniform gel was prepared, with a size of 200 mm × 200 mm × 1 mm. The balanced IPG strips were placed over the gel and sealed with 0.5% agarose. The electrophoretic buffer solution was tris-glycine-SDS. When the temperature was at 16 ℃, after 30 min of constant-current flowing electrophoresis with 20 mA, the constant current was changed into 30 mA, until the bromphenol blue reached the lower edge of the galss plate. The gel to be analyzed was put into a Coomassie brilliant blue staining solution (50% absolute alcohol, 10% glacial acetic acid, 0.1% Coomassie brilliant blue R-250) for 2~3 h, after electrophoresis. The staining solution was discarded and the destaininng solution was used (50% absolute alcohol, 10% glacial acetic acid) and shook for decolorization until the backgroud was clean.

Gel scanning and image analysis

ImageScanner photodensity scanning was performed after Coomassie brilliant blue staining. In the LabScan control, the resolution of 1,200 ppi was adopted to conduct the scanning. The electrophoretogram of the control K562 cells was used as a standard reference image. The other electrophoretograms were matched with it and the change in expression of proteinic spots judged by analyzing the changes in their photodensity with the ImageMaster 2D Elite v3.01 software. Fixed quantitation and statistical parameters were used to analyze the difference between the control and experimental groups.

Statistical analysis

The data were analyzed by SPSS 10.0 software and the chi square test was used to judge statitical significance.

RESULTS

HT-induced apoptosis of K562 cells

Upon treatment of the K562 cells with HT, the early-apoptotic cells (Annexin V^+/PI^-) were elevated at 5 h and then gradually decreased, while the late-apoptotic cells (Annexin V^+/PI^+) were significantly increased, with effect of HT becoming greater with time (Table 1). Statistical analysis showed that there was no significant difference between the 12-h and the 24-h group with regard to the effect on the early-apoptotic cells (P=0.23), but there was a significant difference between the late-apoptotic groups at those times (P<0.01). These results indicate that the apoptotic cells were changing from the early apoptosis to the late apoptosis. For the K562 cells treated with HT for 5 h, the Annexin V^+/PI^- cell fraction appeared in quadrant 4, which did not form visible cell groups and failed to be separated with the cell mass of quadrant 3, as seen on the scatterplot. When the continuous HT treatment reached 12 h, the cell mass of Annexin V^+/PI^- and of Annexin V^+/PI^+ were gradually formed into cell groups. When the HT treatment reached 24 h, cells in both the ealry- and the late-apoptotic stages were formed into groups, with a considerably high fluorescence intensity, thus explaining that the cell mass had been in a steady apoptotic state (Fig.1).

Two-dimensional protein profiles

Three samples of cell lysate were used and the tests repeated 2 to 3 times for each batch of samples, with a (88.3 ± 2.0) % matching rate of the protein spots. Based on good reproducibility and comparisons, the

protein profiles from cells treated with HT for 5 h and 24 h were contrasted, with the profiles from the normal control cells as the standard reference image. Gel imaging analysis showed, through ImageMaster 2D Elite v3.01 software analysis, that there were about 1,300 (± 50) distinguishable protein spots in the controls plus the early-apoptotic and late-apoptotic groups. In samples from the same batch, the intragroup coefficient of correlation in the controls was (92.1 ± 2.5) %, while that from the early- and late- apoptotic groups was (88.4 ± 5.4) %. In the samples from different batchs, the intragroup coefficient of correlation in the controls was (90.3 ± 4.1) %, but that in the early- and late- apoptotic groups was (82.2 ± 9.1) % (Figs.2,3).

Table 1. Percentage of apoptotic K562 cells treated with HT ($\%, \bar{x} \pm s$)

Items	A	B	C	D
Annexin V^+/PI^-	0.9 ± 0.2	28.3 ± 3.0	20.1 ± 2.5	18.1 ± 2.3
Annexin V^+/PI^+	3.6 ± 1.7	9.1 ± 1.6	15.3 ± 1.2	20.2 ± 1.3

Group A: control; Group B: HT treatment for 5 h; Group C: HT treatment for 12 h; Group D: HT treatment for 24 h.

Analysis of the differences between the expression of proteins of K562 cells in the treatment and the control groups

The results from the control, the early- and late-apoptotic groups were compared with each other using the software and statistical data. No definite protein disappeared in the early-apoptotic group but there was a significant decrease in two spots, namely apo-5 and apo-10, and a significant increase for another 5 spots, apo-3, apo-4, apo-7, apo-8 and apo-9 (the criterion for significance was based on increase or decrease of the relative amounts one time or more in the protein spots as compared to the controls). Apo-10 protein, a spot in the late-apoptotic group, disappeared, a significant decrease occurred for apo-5 and there were significant increases in eight spots, i.e., apo-1, 2, 3, 4, 5, 6, 7 and 8. Table 2 shows the isoelectric points, molecular weights and the relative amounts of the protein spots.

DISCUSSION

Annexin V/PI staining is a good method for differentiating stages of cellular apoptosis. In the early stage of the apoptosis when chromosomal DNA has not degraded, phosphatidyl serine (PS) in the inner side of the cellular membrane moves to the outer side, where it can bind with high affinity with Annexin V, a phosphatide binding protein. Therefore Annexin V can be taken as a probe for detection of the PS on the surface of cells, to detect early-apoptosis of cells. It can easily differentiate dead cells (Annexin V^-/PI^+) from early-apoptotic cells (Annexin V^+/PI^-), and from both late-apoptotic (Annexin V^+/PI^+) and normal cells (Annexin V^-/PI^-) when combined with PI, a membraneous non-permeable DNA stain.

The results of our study showed that K562 cells treated with HT for 5 h displayed early apoptotic features (Annexin V^+/PI^-) and their apoptotic rate was 28.3% . With time, late-apoptotic cells gradually increased. After 24 h of HT treatment, the K562 cells were in a steady apoptotic state and the apoptotic rate reached 48.3% , with the late-apoptotic cells accounting for 20.2%. Consideration the above results and that protein synthesis requires transcription and translation, a 24 h time period of HT treatment was used to study in order to provide enough time for protein synthesis and degradation and development of enough apoptotic cells. Treatment of HT for 5 h was considered as a time point for early apoptosis and a time to reflect changes in proteins during the course and stages of apoptosis.

Proteomics is a field that has become more important in recent years. This technique has the ability to investigate modifications and interactions and the level of expression of protein, providing a powerful tool for elucidating gene function and regulation and metabolic activity. As the main technique of proteomics, the two-dimensional gel electrophoresis technology can concurrently show hundreds of proteins on one gel, an isolation technique with the highest resolution. Especially, the application of the IPG strip, with solid phase pH gradients as its base, greatly simplified the process of gel formation

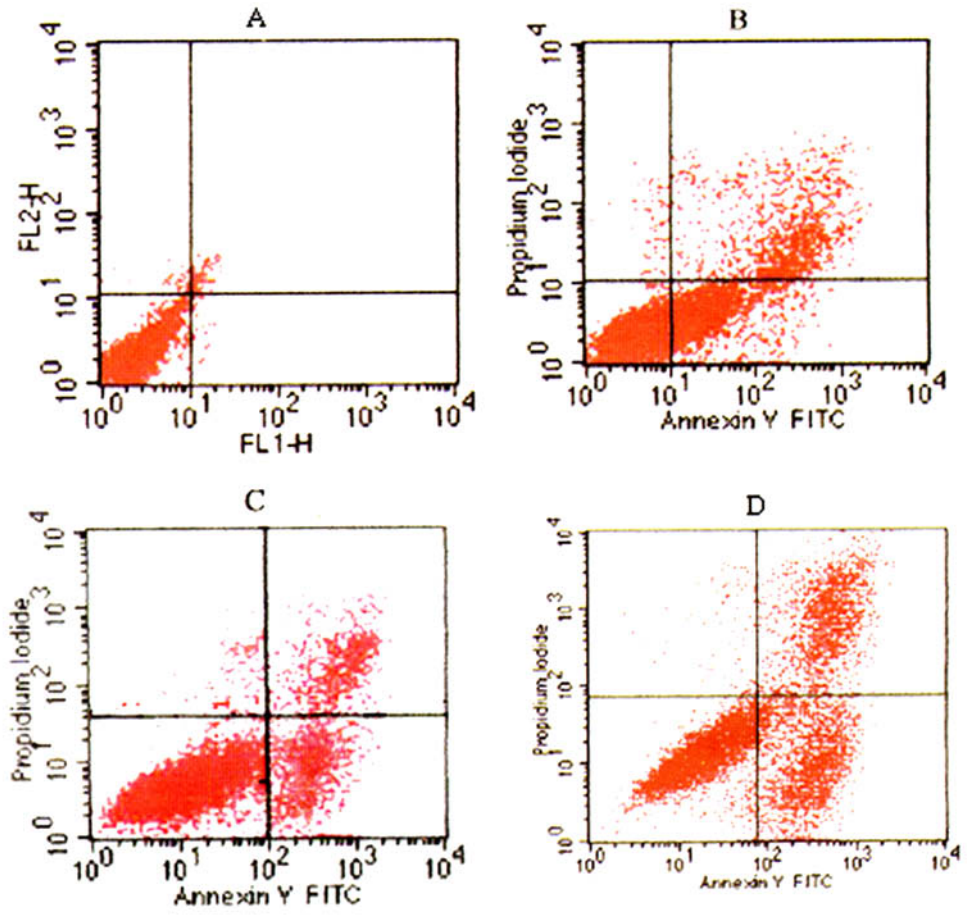

Fig.1. Flow cytometric analysis of Annexin V/PI staining. A: Control group; B, C, D: treatment with HT for 5, 12, 24 h, respectively.

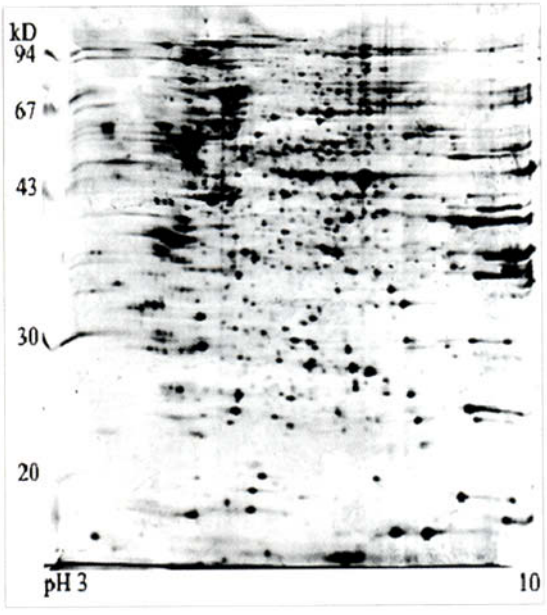

Fig.2. A two-dimensional electrophoretic proteinogram from control K562 cells. The gel was stained with Coomassie brilliant blue.

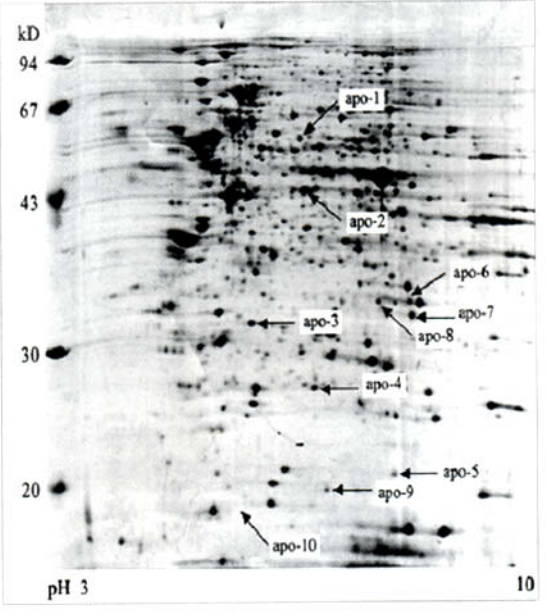

Fig. 3. A two-dimensional electrophoretic proteinogram from K562 cells treated with HT for 24 h. The gel was stained with Coomassie brilliant blue. The arrowheads show significant differences in the amount of protein in the spots.

Table 2. The isoelectric points, molecular weight and relative amount of differential protein spots

Spot	PI	Mr/kDa	Relative spot intensity (%)			*P* value
			A	B	D	
1	5.92	59.25	0.325 ± 0.021	0.452 ± 0.019	0.621 ± 0.036	0.002
2	6.14	59.12	0.515 ± 0.013	0.710 ± 0.019	0.914 ± 0.009	0.000
3	5.51	30.90	0.128 ± 0.008	0.442 ± 0.015	0.515 ± 0.022	0.000
4	6.37	22.48	0.276 ± 0.033	0.493 ± 0.017	0.526 ± 0.025	0.005
5	7.34	17.90	0.674 ± 0.011	0.315 ± 0.009	0.139 ± 0.006	0.000
6	7.69	35.32	0.317 ± 0.023	0.454 ± 0.014	0.612 ± 0.024	0.005
7	7.72	33.61	0.166 ± 0.015	0.472 ± 0.017	0.725 ± 0.013	0.000
8	6.93	34.92	0.089 ± 0.007	0.283 ± 0.011	0.328 ± 0.009	0.005
9	6.52	18.89	0.075 ± 0.012	0.454 ± 0.011	0.319 ± 0.015	0.001
10	5.29	18.70	0.688 ± 0.011	0.276 ± 0.009	0	0.000

A group: control; B group: HT treated for 5 h; D group: HT treated for 24 h; *P*: compared A with D.

and there has been considerable improvement in both resolution and reproducibility.

The IPG strip bought from Amersham Pharmacia Biotech was used in our work to avoid the cathodal drift caused by use of ampholytes. Isoelectric focusing and rehydration were conducted in ceramic trays of the IPGphor holder, which used little of the rehydration, enhanced the solubility of the samples, and prevented cross-contamination. The specimen cup was not utilized here, thus avoiding the emergence of protein sediments caused by spotting on the surface of the gel. Then the application of an 8,000 V power source and the setting of a constant temperature and enhancement of the maximum voltage 2 or 3 times, compared to the previous multi-use electrophoresis apparatus (Multiphor II), thus increased the efficiency to obtain an electrophoretogram with a high resolution. At the same time, the two-dimensional electrophoretogram matching analysis of the treatment group and the controls was performed, in combination with the ImageMaster 2D Elite v3.01 and the 2-DE analyzing software, which produced high reproducibility. Therefore it ensured an acquisition of a higher degree of resolution and reproducibility, as well as a larger sample size.[6]

Previously isoelectric focussing using Multiphor II has been conducted within pH 3 to 10L, then the second dimensional separation was carried out. Generally the number of spots obtained was only 800.[7] In our experiments the number of the protein spots reached approximately 1,300, through the second dimensional separation. Up to now, the problem of reproducibility in two-dimensional electrophoresis experiments has been a problem requiring special attention. For 2-DE electrophoresis with high resolution, which can separate hundreds of protein spots, it has been difficult to guarantee reproducibility. By developing optimized experimental conditions, we achieved reliable intra- and inter-batch reproducibility of analyses. The comparative analyses of the electrophoretograms for the three groups of cells were conducted, through ImageMaster 2D Elite v3.01, the imaging analysis software. The initial results showed that after removal of spurious, low-expression and uncertain protein spots, the mean number remaining in the three groups was 1,300 ± 50, with a small standard deviation. The intragroup accordance among the samples from same batch was high. It was noted that the accordance in the controls was higher than that in the apoptotic groups and reached 92.1% ; the intragroup accordance among the samples from batch to batch was somewhat less, especially, observed in the apoptotic groups. The reasons for these results were as follows: a) it was mainly due to different

degrees of extraction of the proteins from different batches of cells. b) mainly due to the effect of disruption in the apoptotic cells; c) during apoptosis, the changes of quality and quantity of proteins with a low-abudance may increase the heterogenicity of the cell proteins. Specific expression of some proteins were appraised by further photodensitometry of the specific spot. It was found that although there were statistically significant changes in some of the spots, the intragroup variation of different between batches was still big, which was probably caused by the differences in conditions during extraction of the samples. The drift itself was large, because the proteins were lying at the acidic or alkaline ends of the electrophoretogram. Therefore, although there was a quantitative change, temporarily it may be due to accidental error in the experimental conditions and/or the effect of some other factors.

Apoptosis is an extremely complicated biological process, in which a number of protein families and members participate. Through our observation of the early- and late-apoptotic cells, our study clearly revealed the dynamic changes of the proteins in the process of apoptosis. The spectrum of expression of the early- and late-apoptotic K562 cells induced by HT, and of the gross protein in normal control cells were compared, revealing that 10 changes occurred in the expression which may be proteins that participate in HT-induced apoptosis. The molecular weights and the isoelectric points were initially determined but further work is necessary to identify and determine whether they are known constitutive proteins or new unknown proteins. Information needed includes assessment of the mass spectra, analysis of the amino acid sequence and the retrieval of data on the internet for comparion. Investigations of the role proteins play during HT-induced apoptosis is still a research subject of importance in our laboratory, and studies of this phenomenon will continue in the future.

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