Effects of paclitaxel on proliferation and apoptosis in human acute myeloid leukemia HL-60 cells

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ABSTRACT

AIM: To investigate the regulatory effect of paclitaxel on proliferation and apoptosis in human acute leukemia HL-60 cells. **METHODS:** HL-60 cell growth was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tertrazolium bromide (MTT) colorimetric assay. Cell cycle kinetics and apoptosis were analyzed by flow cytometry and microscopic examination. In addition, DNA microarrays containing 14400 EST elements were used to investigate the gene expression pattern of HL-60 cells exposed to paclitaxel 1 μmol/L. **RESULTS:** Paclitaxel inhibited HL-60 cell growth significantly in a dose-dependent and time-dependent manner ($P<0.01$). Marked cell accumulation in G_2/M phase and multinucleated cells were also observed after treatment with paclitaxel 0.1 and 1 μmol/L. Among 14400 EST elements, 277 genes were found to be markedly up- or down-expressed in the HL-60 cells treated with paclitaxel 1 μmol/L for 0.5 h, comprising 210 known genes and 67 unknown genes. **CONCLUSION**: Paclitaxel suppresses the growth of HL-60 cells *in vitro* by causing cell-cycle arrest and apoptosis. The results of microarray suggest that paclitaxel initiates apoptosis through multiple mechanisms.

INTRODUCTION

Most chemotherapeutic drugs exert their anti-tumor effects by inducing apoptosis. A powerful new approach to facilitate the discovery of unique genes is the recently developed microarray technology[1]. This process allows the expressions of thousands of genes to be compared and profiled simultaneously. The development of microrobotics and a computer-enhanced detection system allow up to 1×10^4 separate gene elements to be placed on a glass slide. This slide can be hybridized to two different probes, each labeled with a distinct fluorescent dye. Each probe consists of any single-stranded genetic element capable of hybridizing to the cDNA placed on the glass slide.

Paclitaxel exerts its cytotoxic effect by binding and stabilizing microtubules[2] and induces apoptosis in a variety of cell types[3,4]. It was demonstrated that paclitaxel released cytochrome c by direct action on the mitochondria[5], activated small GTP-binding proteins[6], and activated the NF-κB/IκB-α signaling pathway[7]. Akt (protein kinase B, PKB), a serine/threonine protein kinase, has a pivotal role in exerting an antiapoptotic effect against various stimuli. Overexpression of Akt was reported to confer resistance to paclitaxel[8].

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However, it was reported that paclitaxel independently induced apoptosis in human ovarian carcinoma cells of Akt[9]. Thus, it is unclear whether paclitaxel causes apoptosis through modulation of the Akt survival pathway. The JNK/SAPK (c-Jun N-terminal kinases/ stress-activated protein kinases) is frequently associated with apoptosis. It was demonstrated that activated JNK/SAPK was required for the early phase of paclitaxel-induced apoptosis[10]. However, it was reported that paclitaxel induced prolonged activation of the JNK/SAPK signaling pathway independently of activating the programmed cell death machinery[11]. The precise roles of these activated kinases in this context remain obscure. Although these actions were implicated as the mechanisms through which paclitaxel induced apoptosis, these studies were impaired by the treatment of high concentrations of paclitaxel and by the long exposed time (over 16 h). And the upstream expressing molecules of the apoptotic-signaling pathway activated by paclitaxel are unclear.

In an attempt to identify the genes responsive to or responsible for paclitaxel-induced apoptosis and to reveal the apoptosis signaling pathways triggered by paclitaxel, we have utilized the microarray technology to simultaneously display changes of gene expression. Characterization of these paclitaxel-responsive genes may lead to the identification of novel target (s) for the discovery of anticancer drugs.

METERIALS AND METHODS

Agents Paclitaxel, MTT, propidium iodide (PI) and RNase were purchased from Sigma Chemical Co; TRIzol and RPMI-1640 medium were the products of Gibco. Human cDNA expression arrays, representing 14 400 human genes, were obtained from United Gene Scientific Technology Company, Ltd (Shanghai, China). All other reagents were analytical reagents.

Cell culture and cytotoxicity assay *in vitro* Human leukemia HL-60 cell line was from Shanghai Institute of Cell Biology, Chinese Academy of Sciences. Cells were cultured in RPMI-1640 medium, supplemented with 10 % heat-inactivated calf serum, streptomycin 100 mg/L, benzylpenicillin 100 kU/L, in a 5 % CO_2 humidified incubator at 37 ºC.

Apoptosis and cell cycle assay HL-60 cells were planted in T-75 cm^2 flasks and treated for 24 h with either Me_2SO (dimethyl sulfoxide) or paclitaxel. Single-cell suspensions were collected, and pellets were fixed in ice-cold ethanol (70 %) for 30 min. After centrifugation of the samples, propidium iodide (50 mg/L) and RNase (30 kU/L) were added to the pellets at 37 ºC for 20 min. After filtration, samples were analyzed by flow cytometry (FACsalibur, Becton Dickinson, USA). For each sample, at least 1×10^4 cells were analyzed by flow cytometry. Results were analyzed by software of CELLQUEST and ModFIT for macV1.01 (Becton Dickinson, USA).

Morphology of apoptotic cells After treatment with or without paclitaxel, 5×10^4 cells were washed with PBS, pH 7.3, and resuspended in the same buffer. Cytospin preparations of the cell suspensions were fixed and then stained with Wright's stain. Cell morphology was determined by light microscopy. In all, five different fields were randomly selected for counting at least 500 cells. The percentage of apoptotic cells was calculated for each experiment. Cells designated as apoptosis were those that displayed the characteristic morphological features of apoptosis, including cell volume shrinkage, chromatin condensation, and the presence of membrane-bound apoptotic bodies.

Chip preparation The 14 400 EST elements were used in the cDNA chips. These genes were amplified through PCR using universal primers and purified by the standard method. The quality of PCR was monitored by agarose electrophoresis. Target genes were dissolved in 3×SSC spotting solution, and spotted on silylated slides (Telechem, Inc, USA) by Cartesian 7500 spotting robotics (Cartesian, Inc, USA). Each target gene was dotted twice. After spotting, the slides were hydrated (2 h), dried (0.5 h, room temperature), cross-linked under UV light and then treated with 0.2 % SDS, H_2O and 0.2 % $NaNBH_4$ for 10 min, respectively. The slides were then dried in the cold and ready for the probe preparation.

Total RNA in the treated samples with or without drug were extracted by a single step method[12]. The suspensions of HL-60 cells were centrifugated, then washed with PBS, pH 7.3, and homogenized in D solution plus 1 % mercaptoethanol. After centrifugation, the supernatant was extracted twice with phenol:chloroform (1:1), then once with NaAc and acidic phenol: chloroform (5:1), the aqueous phase was precipitated by equal volumes of isopropanol.

The precipitate was centrifuged and dissolved with milli-Q H_2O. After purification by a LiCl precipitating method, UV analysis and electrophoresis detection showed the good quality of purified RNA. mRNA were

isolated and purified using an Oligotex mRNA MidiKit (QIAGEN, Inc, USA). The fluorescein-labeled cDNA probe was prepared through reverse transcription and purified by the method of Schena[13]. Probes from the control samples were labeled with Cy3-dUTP; those from tested samples were labeled with Cy5-dUTP. The probes were mixed, then precipitated by ethanol and resolved in 20 μL hybridization solution (5×SSC+2 % SDS).

Hybridization and washing Hybridizing probe and chip were denatured in a 95 ºC bath for 5 min. The probe was added on the chip and covered with glass. The chip was hybridized in a sealed chamber at 60 ºC for 15-17 h. After removing the covered glass, the slide was washed in a solution of 2×SSC+0.2 % SDS, 0.1×SSC+0.2 % SDS, and 0.1 % SSC for 10 min each, respectively, then dried at room temperature.

Fluorescence scanning and analysis The chip was scanned with a Scan Array 3000 Scanner (General Scanning Inc, USA). Cy3 and Cy5 overall intensity were normalized and corrected by a coefficient according to the location ratios of the 40 housekeeping genes. The acquired image was analyzed by ImeGene 3.0 Software using a digital computer. Intensity of the fluorescent signal and each ratio of Cy3 to Cy5 were compared. The data were obtained on an average of two repeated spots. The standards to screen out each differentially expressed gene were defined: (1) the absolute value of the natural logarithm of the signal ratio of Cy5/Cy3 was greater than 0.69 (gene expression change >2-fold). (2) One of the signal values of Cy3 and Cy5 was greater than 600. (3) The results of PCR were good.

RESULTS

Cytotoxic effect of paclitaxel on HL-60 cells HL-60 cells at 4×10^8 cells/L were incubated with different concentrations of paclitaxel for different time (24 h, 48 h, and 72 h), and the effect of paclitaxel on the cell growth was examined by MTT assay. The growth of HL-60 cells was markedly inhibited by paclitaxel 1 nmol/L-0.1 mmol/L (Fig 1). Moreover, the cytotoxicity of paclitaxel was concentration-dependent. The time-effect analysis of paclitaxel showed that its cytotoxicity against the human leukemia cells was in a time-dependent manner (Fig 1).

Effect on cell-cycle kinetics of HL-60 cells Cell-cycle kinetics of HL-60 cells treated with paclitaxel was analyzed by flow cytometry. Paclitaxel 0.1 μmol/L and 1 μmol/L for 24 h caused an accumulation of the cells in G_2/M phase about 70.5 % and 78.5 %, respectively, and that of the control about 10.27 %. Moreover, the G_2/M cell cycle arrest of paclitaxel in HL-60 cells was concentration-dependent (Tab 1).

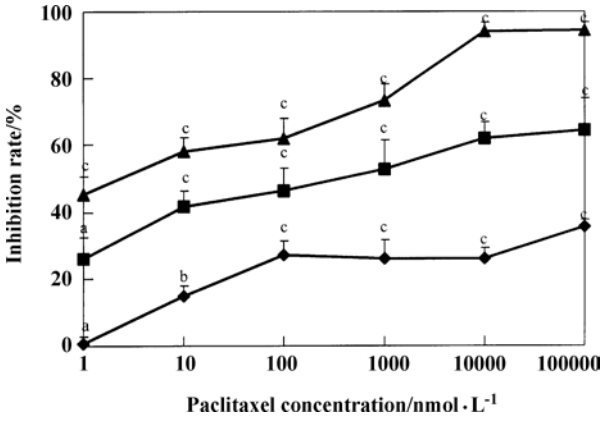

Fig 1. Effect of paclitaxel treatment on the cytotoxicity of HL-60 cells. *n*=3. Mean±SD. [a]*P*>0.05, [b]*P*<0.05, [c]*P*<0.01 *vs* control. (◆) 24 h; (■) 48 h; (▲) 72 h.

Tab 1. Effect of 24 h-paclitaxel treatment on the cell cycle of HL-60 cells. *n*=3. Mean±SD. [c]*P*<0.01 *vs* control.

Groups/μmol·L^{-1}	Cell cycle/%		
	G_0/G_1	S	G_2/M
Control	41.2±2.4	48.5±1.6	10.3±1.5
Paclitaxel 0.1	0.20±0.8[c]	29.3±2.0[c]	70.5±2.9[c]
1	0.7±0.8[c]	20.8±2.4[c]	78.5±3.0[c]

Induction of HL-60 cells apoptosis As analyzed by flow cytometry, paclitaxel induced the apoptosis of HL-60 cells. Apoptosis was induced with the cells exposed to paclitaxel 0.1 μmol/L and 1 μmol/L for 24 h, the apoptotic percentages were 40.5 % and 37.5 % respectively, compared with 2.27 % of the control (Tab 2). The results from light microscope were similar to

Tab 2. Effect of 24 h-paclitaxel treatment on inducing apoptosis of HL-60 cells. *n*=3. Mean±SD. [c]*P*<0.01 *vs* control.

Groups/μmol·L^{-1}	Apoptosis/%
Control	2.3±0.5
paclitaxel 0.1	40.5±1.4[c]
1	37.5±1.3[c]

those mentioned above (data not shown). Morphologic observation further confirmed paclitaxel-induced apoptosis in HL-60 cells. The results from light microscope displayed shrinkage and morphologic abnormality of the cells after treatment with paclitaxel 0.1 μmol/L for 48 h. On the contrary, the untreated cells did not show these apoptotic characteristics (photo not shown).

Identification of the genes responsive to apoptosis induced by paclitaxel: the use of microarray technology In an attempt to identify genes whose expressions changed during paclitaxel-induced apoptosis, we utilized microarray technology to obtain an overall profile of gene expression. In order to identify early apoptosis-responsive genes, poly (A+) mRNA was isolated from cells treated with paxlitaxel (1 μmol/L) for 0.5 h and subjected to cDNA chip hybridization. Cells treated with Me_2SO for 0.5 h were used as the control. The hybridization results from all chips were compiled and sorted on the basis of fold change compared to the control cells. Genes that displayed approximately two-fold or greater changes were scored as having significant changes[14]. Hybridization results obtained by parallel comparison of gene expression of the two samples demonstrated: 277 genes were differentially expressed between the tested samples and the control. Of these 277 mRNA species, comprising of 210 known genes and 67 unknown genes, 121 were significantly induced and 156 were significantly repressed. According to their functions, these genes could be divided into 13 groups (Tab 3). The biochemical function of the genes in this expression profile are diverse, including: transcription factors, protein kinases and phosphatases, cell cycle regulators, proteases, apoptotic and antiapoptotic factors, protein synthesis-related genes, proliferation-related genes, as well as a large number of metabolic genes. Altered expressions of the transcription factors included down-regulation of the general transcription factor II-I (GTF2I), the activating transcription factor 5 (ATF5), the transcription elongation factor B (TCEB), and the cyclin D binding myb-like transcription factor 1 (DMTF1). These changes seemed to be consistent with a general shutdown of transcription response to paclitaxel treatment. Changes in the expressions of a group of zinc finger transcription factors were also noted. Altered expressions of the protein kinases and phosphatases included down-regulation of the protein kinase B (PKB), the protein kinase C (PKC), the protein phophatase 1 regulatory subunit 9A (PPP1R9A), the serine/threonine protein kinase 18 (STK18), the dual specificity phosphatase 3, the adenylate cyclase 6 and the AMP-activated protein kinase 2 (PP2A), up-regulation of the protein tyrosine kinase 6 (PTK6), the Rho-associated coiled-coil containing protein kinase 1 (ROCK1) and the protein phosphatase 2. Different expressions of the cell cycle regulators included down-regulation of the cyclin E2, the exportin 1 (XPO1/CRM1), the quiescin Q6, the retinoblastoma-binding protein 9 (RBBP9) and up-regulation of the E2F transcription factor 5 (E2F5). Altered expressions of the apoptotic and antiapoptotic factors included down-regulation of gelsolin, the death effector domain-containing protein (DEDD), the MAP kinase-activating death domain (MEDD), the TNF receptor-associated factor 5 (TRAF5) and the partner of RAC1. Altered expressions of the protein synthesis-related genes and metabolic genes included down-regulation of the eukaryotic translation elongation factor 2 (EEF2), the eukaryotic translation initiation factor 4A (EIF4A), the adenine nucleotide translocation 1, the isicitrate dehydrogenase 2, COX15 and the NADH-ubiquinone oxidoreductase flavoprotein 1 (NDUFV1). We also found that the DNA ligase I, the DNA polymerase Kappa (POLK), the ubiquitin-specific protease 5 (USP5) and the prosaposin (PSAP) were decreased. In addition, the heterogeneous nuclear ribonucleoprotein H1 (HNRPH1), the heterogeneous nuclear ribonucleoprotein B1 (HNRPB1), the het-

Tab 3. Classification of gene function. Gene expression difference between the tested samples and the control.

Gene function classification	Number of different gene expression	
	Induced genes	Decreased genes
Cell cycle proteins	6	7
Metabolism-related proteins	8	16
Protein synthesis-related genes	0	13
DNA binding, transcription and its factor	6	14
DNA synthesis, repair and recombinant proteins	0	6
Ion-channel and transporters	4	7
Proliferation-related genes	0	7
Cell receptors	4	1
Cell surface antigen and adhesion proteins	4	5
Cell antiapoptosis-related proteins	6	14
Cell signs and transducing proteins	6	9
Extra-pressure reaction proteins	3	0
Other genes	27	37

erogeneous nuclear ribonucleoprotein A/B (HNRPAB), the oxysterol-binding protein and the valosin-containing protein (p97) were depressed. These changes were consistent with the cytotoxic effects of paclitaxel.

DISCUSSION

Recent accumulating reports have demonstrated that paclitaxel-induced microtubular bundling and mitotic arrest of human leukemia cells are followed by DNA fragmentation and morphological features of apoptosis[2,3,15]. We found that an increased apoptotic population in HL-60 cells caused by paclitaxel treatment was accompanied by a pronounced mitotic arrest. We also found that paclitaxel inhibited the cell cycle G_2/M of HL-60 cells in a dose-dependent manner (Tab 1), but the apoptosis was not increased after paclitaxel treatment (Tab 2), according to the previous report[16]. We speculate that the mitotic arrest induced by paclitaxel was not due to paclitaxel-induced apoptosis.

In regard to the biochemical events downstream of paclitaxel binding to microtubules, which lead to apoptosis, our results provide a genome-wide analysis of the cellular responses to paclitaxel. The cytotoxic response to paclitaxel in HL-60 cells involves the induction, as well as the repression, of genes of various biochemical and regulatory pathways (Tab 3). Paclitaxel exposure leads to the down-regulation of GTF2I, ATF5, TCEB, DMTF1 and HDAC1. Decreased expression of these genes may trigger a general repression of transcription in cells in response to the deadly insult of a cellular poison. Moreover, it is also intriguing that the expressions of a group of previously unknown putative zinc finger transcription factors, were prominently targeted for alterations in response to paclitaxel. Although the functional consequences of these alterations are unclear, we speculate that the downstream target genes of these zinc finger transcription factors may be a response to the cytotoxic insult.

Another cluster of genes that showed changes after paclitaxel treatment were the protein kinases and phosphatases, including PKB, PKCm, STK18, ROCK1 and PP2A, which were down or up-regulated after paclitaxel treatment. A role of PKCm in signal transduction pathways was related to growth control[17]. STK18 may be involved in the promotion or progression of cancers[18]. ROCK1 may have a role in the progression of the cell cycle[19]. PP2A modulated cell cycle progression[20] and apoptosis[21]. Whether these protein kinases and phosphatases have some influences on cell-cycle and apoptotic regulation in HL-60 cells treated by paclitaxel remains to be further explored.

DNA microarray also showed an interesting pattern of expression of cell cycle genes following exposure to paclitaxel. Cyclin E2, CRM1, and RBBP9 were depressed, and E2F5 was induced by paclitaxel. We suggested that these genes may determine the terminal fate of HL-60 cells in G_2/M after exposure to paclitaxel.

Cell death induces the activation of proapoptotic factors and the inactivation of antiapoptotic factors. One of the mechanisms that triggers cell death involves the release of cytochrome c from the mitochondria, which subsequently causes apoptosis by activation of caspases. Kamada *et al* ascribed the antiapoptotic activity of gelsolin to prevention of a step leading to cytochrome c release from the mitochondria into the cytosol[22]. The N terminus of DEDD induces apoptosis and the C terminus has antiapoptotic activity[23]. Overexpression of MADD activated the mitogen-activated protein (MAP) kinase ERK2 and JNK1[24]. TRAF5 specifically interacts with the lymphotoxin-beta receptor and activates the transcription factor NF-kappa-B[25]. Partner of RAC1 mediates synergy between the ARF and RHO family signaling pathways[26]. Our results showed repression of gelsolin, DEDD, MADD, TRAF5 and partner of RAC1 after paclitaxel treatment, which presumably lead to the eventual death of the cells treated with paclitaxel.

A subset of genes that showed striking changes after paclitaxel treatment were the genes involved in protein synthesis, DNA biosynthesis, repair and metabolism, including EEF2, EIF4A, adenine nucleotide translocation 1, isicitrate dehydrogenase 2, COX15, DNA ligase I, POLK, and hnRNPs, which were depressed after paclitaxel treatment. These results reveal that the drug exerts an inhibiting action on protein synthesis, DNA biosynthesis, repair and metabolism in tumor cell.

We also found that two interesting genes of USP5 and PSAP were depressed after paclitaxel treatment. USP5 is a member of the ubiquitin specific protease family (UBP). It may exhibit a role in the regulated catabolism of the polymeric ubiquitin, including the polyubiquitin protein degradation signal[27]. The precise mechanisms by which protein degradation may affect the drug effect remain to be determined. The sphingolipid ceramide is an important second signal molecule that regulates diverse signaling pathways involving apoptosis, the cell cycle, and differentiation. It has been

shown that PSAP is the ceramidase activator, and the protein-deficiency caused the ceramide accumulation[28]. Although the functional consequences of PSAP are unclear, we speculate that inhibition of PSAP and ceramide accumulation may be another important factors on the apoptosis of HL-60 after paclitaxel treatment.

The use of DNA microarrys containing 14 400 EST elements in our analysis has provided a global view of the response of HL-60 cells to paclitaxel at the genomic levels. The ultimate identification of patterns of responsive genes for any given treatment of agents will have a profound influence in the target identification and validation in future drug discovery.

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