DIFFERENTIAL EXPRESSIONS OF MICRO TUBULE-ASSOCIATED PROTEINS IN MULTIDRUG RESISTANT LEUKEMIA CELLS

BY

JIE HUANG

A Thesis Presented to
The Hong Kong University of Science and Technology
in Partial Fulfilment of the Requirements for
the Degree of Master of Philosophy
in Biology

Hong Kong, February 1995

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ACKNOWLEDGMENT

I would like to thank Dr. Mun Fai Leung, my supervisor, for his patient guidance.

I am grateful to Ms. Frances Chan for her helping me to conduct the Cell Cycle Analysis and P-glycoprotein Expression experiments using the flow cytometer.

I am indebted to Dr. Lewis. M. Slater for his supplying me with the L₀, L₁₀₀ and L₁₀₀₀ cell lines and the MDR reversal drug, cyclosporin A.

Also I want to thank Dr. Karl Tsim and Dr. Maria Lung, members of my advisory committee, for giving me helpful suggestions on this project.
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ABSTRACT

Two multidrug resistant human acute lymphocytic leukemia cell lines (L_{100} and L_{1000}) were developed by exposing parental cells (L_{0}) to progressively increased concentrations of vincristine sulfate (VCR). These cells have a novel multilobulated nucleus and a distinct microtubule (MT) organization that may be due to the perturbation of the cytoskeleton induced by VCR. The expression of MT and microtubule-associated proteins (MAPs) of the L_{100} and the L_{1000} cells were compared to that of the L_{0} cells. A 28 KD protein of pI 6.9 and a 31 KD protein of pI 4.4 were found to be overexpressed in L_{100} and L_{1000} cells. Moreover, a 38 KD protein was found to be expressed in L_{0} cells but not can be detected in L_{100} and L_{1000} cells. The distinct MT organization and nuclear morphology of these cells may be due to the differential expression of the MAPs. The differential expression of these MAPs is correlated to the multidrug resistance (MDR) phenotype of these cells. These data suggest that these MAPs may be associated with the mechanism of MDR.
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LISTS OF ABBREVIATIONS

at-MDR: Atypical multidrug resistance
BSA: Bovine serum albumin
CHO: Chinese hamster ovary
COL: Colchicine
DMSO: Dimethylsulfoxide
DTE: Dithioerytheritol
EGTA: Ethylene glycol-bis(b-aminoethyl ether) N,N,N',N'-tetra acetic acid
FBS: Fetal bovine serum
FITC: Fluorescein-5-isothiocyanate
GST: Glutathione-S-transferase
GTP: Guanosine triphosphate
HCHO: Formaldehyde
Hsp: Heat shock protein
MAPs: Microtubule-associated proteins
MDR: Multidrug resistance
MRP: Multidrug resistance-associated protein
MTOC: Microtubule organization center
MTs: Microtubules
PBS: Phosphate buffered saline
Pgp: P-glycoprotein
PI: Propidium iodide
pI: Isoelectric focusing point
PIPES: Piperazine-N,N'-bis-(2-ethanesulfonic acid)
PMSF: Phenylmethysulfonyl fluoride
SDS-PAGE: Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis
TEMED: Tetramethylethylenediamine
Topo II: Topoisomerase II
VCR: Vincristine Sulfate
1. Introduction

1.1 The structure and polymerization dynamics of microtubules

Microtubules, intermediate filaments and microfilaments are the three major components of cytoskeleton in the cell (Bernhardsky and Vasiliev, 1988). The core of microtubules is a hollow cylinder 25 nm in diameter and 5 nm in thickness consisting of heterodimers of α- and β-tubulin subunits (Mandelkow and Mandelkow, 1989). The nonidentical polypeptide chains α- and β-tubulin subunits have about 40% amino acid sequence homology (Luduena et al., 1992). The α-tubulin is slightly more basic (pI 5.3) and heavier (about 50 kDa) than β-tubulin (pI 5.1 and about 50 kDa) (Field et al., 1984). Tubulin is highly conserved during evolution. Considerable homologies are found between the nucleotide sequences of tubulins from very distant organisms (Burns and Surridge, 1990). Due to the conservation of tubulin structures, antibodies against microtubules of different organisms cross react. The α- and β-tubulins have different isoforms which are encoded by the tubulin multi-gene family (Sanchez and Natzle, 1980). Extensive post-translational modification like acetylation (L'Hernault and Rosenbaum, 1985; Le Diczedt and Piperno, 1987), tyrosination/detyrosination (Schulze et al., 1987; Wehland and Weber, 1987; Kumar and Flavin, 1982), phosphorylation/dephosphorylation (Gard and Dirschner, 1985; Serrano et al., 1987; Hargreaves et al., 1986; Edde and Gros, 1981; Luduena et al., 1988; Diaz-Nido et al., 1990) and glutamylation (Edde et al., 1990) are found in these isoforms. Microtubules have a plus (+) end and a minus (-) end. The minus end is usually inserted into a specific organelle called the microtubule organizing center (MTOC or centrosomes). The formation and breakdown of microtubules are mostly dictated by the kinetics of assembly and disassembly at the plus end through a process called dynamic instability (Gelfand and Bershadsky, 1991).
The assembly and disassembly of microtubules, with exchange of tubulin subunits between polymers and soluble tubulin, is one of the most prominent features of microtubules. Conversion from one polymerization state to another is regulated by the amount of tubulin-GTP at the plus end of the microtubules. Tubulin assembly is very sensitive to many environmental factors (Vallee, 1986). It is inhibited by low temperature and the presence of calcium ions. In contrast, its assembly requires the presence of GTP and magnesium ions.

1.2 Microtubule-associated proteins

The potential diversification of microtubule functions is achieved through the interaction between the microtubule-associated proteins and microtubules. Microtubule-associated proteins (MAPs) are defined as proteins associated with microtubules in vivo; therefore, an operational definition includes proteins that copurify with microtubules or tubulins. Microtubule-associated proteins can be divided into two groups: structural microtubule-associated proteins and the dynamic microtubule-associated proteins. The structural microtubule-associated proteins have an architectural role, modulating the spatial organization of microtubules and thereby influencing their function. Early work discovered the neural microtubule-associated proteins which consist of high molecular weight (>200 kDa) MAP1 and MAP2 families as well as the tau proteins with a molecular weight of about 60 kDa (Vallee, 1990). The MAP1, MAP2 and tau proteins are abundant in neuronal cells (Wiche, 1991) and all have the ability to stimulate tubulin assembly in vivo and in vitro. The MAP2 and tau promote the assembly of the tubulin by conferring an 18 amino acids repeats at the carboxy terminus to bind to tubulin (Goedert et al., 1991; Chapin and Bulinski, 1992). They stabilize the microtubule by forming cross-bridges between microtubules and serve as spacers between microtubules (MacRae,
1992) and other cellular constituents. This spacer function may be necessary to allow unimpeded transport of vesicles or organelles by certain vehicles using them as tracks (Campbell et al., 1989). Apart from being spacers, they play a role in morphogenesis by specifying the interaction partners of microtubules in different types and compartments of cells (Wiche, 1989). MAP2 and tau also contain intermediate filament binding sites regulating the interaction of microtubules and intermediate filaments (Leterrier et al., 1982). In the neuron, they play a role in the growth, differentiation, and plasticity of neurons. They are also involved in the neuronal responses to growth factors, neurotransmitters, synaptic activity, and neurotoxins (Johnson and Jope, 1992). Besides the neural microtubule-associated proteins, many microtubule-associated proteins were discovered in other cell types. MAP4, containing large family members, are found in various organisms. They are proposed to be involved in the destruction of cytoplasmic microtubules at the end of G2 phase by phosphorylation at the proline-rich region (Mori et al., 1991). Like MAP2 and tau, MAP4 also contains the repeated carboxy-terminal assembly-promoting and microtubule binding regions to promote the nucleation and elongation of tubulin polymerization. Adligin, a 32-kDa protein from Caenorhabditis elegans, cross-links microtubules in vitro (Aamodt et al., 1989). A microtubules cross-linking protein of 49 kDa was discovered in brine shrimp Artemia (Campbell et al., 1989). Hidrokawa et al discovered the 75 kDa microtubule-associated protein, buttonin, in sea urchin eggs (Hirokawa and Hisanaga, 1987). Syncolin is a microtubule-associated protein discovered in chicken erythrocytes by Feick et al. (Feick et al., 1991). Recently some heat shock proteins (Hsp) were suggested to be microtubule-associated proteins. The heat shock proteins are a group of proteins that enable the cell to survive and recover from stressful conditions such as heat shock and pathophysiologic stresses (Ciocca et al., 1993). The Hsp 70, which is responsible for the transport among different cellular membrane
compartments and the stabilization of the unfold protein (Tsang, 1993) has a microtubule binding region which is related to MAP1B (Sanchez et al., 1994). Another heat shock protein, Hsp 27 was also demonstrated to be related to the microtubule (Ciocca et al., 1993). Newly finding suggested that these heat shock proteins were involved in the development of drug resistance (Ahmad et al., 1990; Ciocca et al., 1993; Huot et al., 1991; Oesterreich et al., 1993; Lee et al., 1992).

The dynamic microtubule-associated proteins are proposed to play a role in translocation of membrane bound vesicles, elements of the endoplasmic reticulum, mitochondria, and chromosomes (Gelfand and Bershadsky, 1991). They modulate the cytoplasmic organization and take advantage of microtubule organization imposed by the structural microtubule-associated proteins, or chemical signals in the tubulin, to carry out vectorial movement. Dynamic microtubule-associated proteins include kinesin, dynein and dynamin. Kinesin and dynein are abundant in nerve axons. They also contribute to some forms of movement of mitosis (Cande, 1982) and they have a role in the transport of membranous vesicles along the microtubules (MacRae, 1992). Its association with the endoplasmic reticulum implicates kinesin in the movement and positioning of cytoplasmic organelles. The discovery of kinesin superfamilies expands kinesin functions to include segregation of meiotic chromosomes, fusion of nuclei during karyogamy, and separation of spindle pole bodies. Dynamin, a 100 kDa protein, is involved in the protein sorting during endocytosis (Gelfand and Bershadsky, 1991).

Apart from being a part of the cytoskeleton to maintain cell shape, the microtubules also play an important role in vital cell functions: cell division, vesicle and organelle transport (Gelfand and Bershadsky, 1991; Burgess et al., 1991) organization of cellular components during morphogenetic events (e.g., polarization, directed migration, and process outgrowth), programmed
modifications of cell shape during both normal morphogenesis and neoplastic transformation (Anand and Chou, 1993) and distribution of antigen and receptors on cell surface and regulation of the transmission of information from membrane to the nucleus (Edelman et al., 1973). It is not surprising that the microtubule apparatus serves as a target for the antineoplastic drugs and it was suggested that the alterations in microtubule-associated proteins were involved in the formation of malignant cells and sensitization of malignant cells to anticancer agents (Anand and Chou, 1993).

1.3 Effects of drugs on polymerization of tubulin

Several groups of drugs can bind to tubulin and affect its polymerization in vitro and in vivo. They are widely used in the study of function and assembly of microtubules. These drugs include colcemid, colchicine, nocodazole, vinblastine, vincristine, podophyllotoxin, griseofulvin, maytansine, and taxol. Most of these cause the depolymerization of the microtubules, by various mechanisms. Colchicine, isolated from the plants Colchicum autumnale or Colchicum speciosum, inhibits the polymerization of tubulin in vitro and causes rapid disassembly of most types of cellular microtubules in vivo by binding to the tubulin dimer (John et al., 1985). It is believed that the colchicine- or colcemid-bound tubulin dimer binding to the assembly end of the microtubule, can cause the inhibition of further elongation. Vinca alkaloids such as vincristine and vinblastine can depolymerize the microtubule by binding to polymerized tubulins (Bryan, 1972). Griseofulvin was suggested to react with the microtubule-associated protein to inhibit the polymerization of microtubules (Grisham et al., 1973; Roobol et al., 1977). Taxol is derived from the bark of the pacific yew, Taxus brevifolia. Different from a number of other microtubule-disrupting or dispolymerizing drugs, taxol enhances both the yield and rate of microtubules assembly. It binds to the microtubule polymer
in a 1:1 stoichiometry with tubulin and the binding is very strong (Parness and Horwitz, 1981). The polymerization of microtubules promoted by taxol does not need the presence of microtubule-associated proteins and also be resistant to low temperatures and the presence of calcium (Vallee, 1986).

1.4 Mechanism of multidrug resistance

Multidrug resistance (MDR) is a generic term for the variety of strategies tumor cells use to evade the cytotoxic effects of anticancer drugs (Sanford and Melvin, 1994). It is characterized by a decreased sensitivity of tumor cells not only to the drug employed for chemotherapy but also to a broad spectrum of drugs with neither obvious structural homology nor common targets. This pleiotropic resistance is one of the major obstacles to successful treatment of tumors. To date, many mechanisms are proposed to elucidate the MDR. They are involved in the modifications in accelerated removal or secretion of drug detoxification, DNA repair pathways, changes in cellular sites of drug sequestration, decreases in drug-target affinity, synthesis of specific drug inhibitors within cells, altered or inappropriate targeting of proteins and alterations in cellular processes that compensate for the action of the drug. The best studied mechanism involved in the increasing efflux of the drug is the overexpression of a 180 kDa protein called P-glycoprotein (Pgp). The Pgp is said to be a cytoplasmic membrane transporter which acts as an ATP-dependent efflux pump to pump out the drugs which are different in structure and function (Kartner and Ling, 1989). The Pgp-mediated MDR cells can be reversed by agents such as verapamil (calcium channel blocker) (Slater et al., 1986b) and cyclosporin A (calmodulin inhibitor) (Slater et al., 1986a). Schilber proposed that verapamil affects the membrane permeability to or transport of a wide variety of hydrophobic drugs (Schilber et al., 1988). However, the mechanism for this MDR reversal action is still unknown. Overexpression of the Pgp can not explain all MDR phenomenon because
some MDR cells do not express Pgp. Other MDR-associated phenotypes include the overexpression of MDR-associated protein (MRP), increased glutathione S-transferase (GST), and decrease expression of topoisomerase II (Topo II). The overexpression of a 190 kDa intracellular membrane protein, MDR-associated protein (MRP) (Grant et al., 1994; Barrand et al., 1994), has been found to be associated with the acquirement of MDR in many cells. The transfection of an MRP expression vector conferred to the transfectant an MDR phenotype similar to that mediated by Pgp. The experiment of reduced nuclear drug content in MRP-overexpressing MDR cells when compared to that in the drug sensitive parental cells support the postulation that the MRP induce the MDR phenotype by shifting of drug subcellular localization (Sanford and Melvin, 1994). Increased efficiency in detoxification of the drug inside the cell is another mechanism proposed to elucidate the MDR. Glutathione S transferase (Kano et al., 1987; Moscow et al., 1989) are a family of enzymes that play an important role in detoxication by catalyzing the conjugation of many hydrophobic and electrophilic compounds with reduced glutathione. Overexpression of GST modifies the glutathione metabolism to induce the drug resistance. Topoisomerase II is a nuclear enzyme having a role in DNA replication and RNA transcriptions (DiNardo et al., 1984; Albers et al., 1989). Anti-tumor drugs which act on Topo II to increase the half life of the Topo II-DNA covalent intermediate, called the cleavable complex, interfere with DNA replication. The down expression or alterations in structure of Topo II may lead to the decrease in drug-affinity resulting in the MDR activity (Andoh et al., 1993; Jong et al., 1990). Besides these changes, there are many other protein changes associated with MDR which may be involved in the mechanisms of the MDR. These mechanisms may separately or synergistically contribute to the MDR phenotype (McCLean and Hill, 1992). However, the mechanisms regulating most of the changes
associated with MDR phenomenon are still unknown. Whether the associated changes and MDR phenotype are related is still to be investigated.

1.5 Involvement of microtubules and microtubule-associated proteins in multidrug resistance

The alterations in microtubules and microtubule-associated proteins are usually found to be associated with MDR activity suggesting the involvement of microtubules and microtubule-associated proteins in the MDR mechanism. The involvement of microtubules and microtubule-associated proteins in cells exhibit a limited pattern of drug resistance and is well studied. For example, cells selected for resistance to colcemid which inhibits microtubule assembly, are cross-resistant to other microtubule destabilizing drugs such as vinblastine, but are hypersensitive to taxol which stabilizes microtubules and promotes tubulins assembly (John et al., 1985). These cells have no alteration in drug uptake, drug accumulation, drug retention or expression of Pgp. For example, the Col-2 cell is a mutant selected by and resistant to colchicine. It is also resistant to other microtubule depolymerizing drugs without classic MDR phenotype. Alterations in the β-tubulin subcomponent (Schibler and Huang, 1991; Cabral et al., 1980; Pain et al., 1988), α-tubulin subcomponent (Silflow et al., 1993; Schibler and Cabral, 1986) and microtubule-associated proteins were reported in these mutants (Keates et al., 1981). The alterations in tubulin subunit include the phosphorylation site, acetylation site, and electrophoretic pattern. Altered microtubule polymerization dynamics was demonstrated in CHO cell mutants suggesting that tubulin assembly properties are affected (Minotti, 1991). Mechanisms were proposed that alteration of tubulin assembly may lead to resistance to microtubule drugs; hyperstabilization or hyperdestabilization of polymerization of tubulin could counteract the result from the depolymerizing drugs like cochicine or microtubule polymerizing drugs like taxol (Schibler
and Cabral, 1986; Cabral and Barlow, 1989). The mechanism involved in the alteration in drug binding site of tubulin was demonstrated by Ling (Ling et al., 1979) in a CHO cell mutant, suggesting that decrease in intracellular binding for drugs accounts for the drug resistance in this mutant.

Microtubules were also demonstrated to be related to some types of classic MDR. The development of MDR is found to be accompanied by significant alterations in cellular morphology and physiology, e.g. changes in cell spreading, growth rate, membrane traffic, decrease of tumorigenicity and metastasis capability, etc (Bielder et al., 1975; Slater et al., 1992; Bradley et al., 1988; Ganapathi et al., 1987). These changes are mediated, at least in part, by the cytoskeleton (Erokhina et al., 1994). For example, in the MCF-7 breast MDR cancer cells, the MTOC disappeared and there were no paracortical bundles of microtubules when compared to the sensitive cancer cells (Mujagic H and Mujagic Z, 1991). Two independent colchicine-resistant sublines of Rous sarcoma virus-transformed Syrian hamster fibroblasts were found with restored stress fibers when compared to the dispersed form in the drug sensitive parental cell lines (Erokhina, 1994).

Recently, the heat shock proteins have been found to be associated with the development of multidrug resistant. The overexpression of heat shock protein Hsp 70 and Hsp 27, which are suggested to be microtubule-associated proteins, have been demonstrated to be correlated to the development of multidrug resistance. Although the mechanism is still unknown.

1.6 Multidrug resistant acute lymphocytic leukemia

The multidrug resistance phenotype has been found in many acute lymphocytic leukemia cells. The mechanisms involved also include the P-glycoprotein, multidrug resistance-associated protein (MRP), glutathione-S-transferase (GST) and topoisomerase (Topo II) which have been mentioned

Slater developed multidrug resistant cell lines, L\textsubscript{100} and L\textsubscript{1000} cells, from a human acute lymphocytic leukemia cell line L\textsubscript{0} cell by exposure to increasing concentrations of vincristine (Slater et al., 1992). L\textsubscript{100} and L\textsubscript{1000} are resistant to 100 nM vincristine and 1000 nM vincristine, respectively. The continued growth of L\textsubscript{100} cells requires vincristine. Apart from resistance to vincristine, L\textsubscript{100} cells also showed the ability to be cross resistant to VP-16 and daunorubicine (Slater et al., 1992). Thus, L\textsubscript{100} cells display classical multidrug resistance activity. Like many classical multidrug resistant cells, L\textsubscript{100} cells overexpress Pgp when compared to L\textsubscript{0} cells. According to the studies by Slater, the multidrug resistant activity of L\textsubscript{100} cells can be reversed by the multidrug resistant reversal drugs, cyclosporin A and verapemil (Slater et al., 1986a; Slater et al., 1986b).

In addition to over-expression of Pgp, L\textsubscript{100} cells exhibit a different nuclear morphology and microtubular distribution (Slater et al., 1992; Leung et al., 1992). It was found that the nucleus of L\textsubscript{100} cells was multilobulated while that of L\textsubscript{0} cells was round. Studies also revealed that while the microtubules were localized between the lobes of the nucleus in L\textsubscript{100}, in L\textsubscript{0} cells the microtubules were distributed evenly around the nucleus (Leung et al., 1992). The multilobulated nuclear morphology of L\textsubscript{100} cells may be regulated by the microtubules. When the L\textsubscript{100} cells were treated with colchicine to depolymerize the microtubules, the multilobulated nucleus assumed a rounded morphology. With the removal of the colchicine through washing, the round nucleus resumed a multilobulated shape with the repolymerization of microtubules.
1.7 Significance of the study

The development of multidrug resistance is the major reason for the failure of chemotherapy for cancer remission. Many cytosolic proteins including tubulins and microtubule-associated proteins have been found to be associated with the development of multidrug resistance (McCLean and Hill, 1992). These protein changes may be involved in the multidrug resistance mechanisms.

It has been shown that the nuclear and microtubular morphology between the drug sensitive parental L₀ cells and the drug resistant L₁₀₀ cells are different. And the novel nuclear morphology in L₁₀₀ cells is regulated by the microtubule. The expression of microtubules and microtubule-associated proteins of these multidrug resistant cells is studied in this project to understand their roles in regulation of nuclear morphology and multidrug resistance activity. This study may contribute to the understanding of the role of cytoskeleton in multidrug resistance.
2. Materials and methods

2.1 Cell cultures

L₀, an acute lymphocytic leukemia cell line, was maintained in RPMI 1640 medium with 15% fetal bovine serum (FBS, GIBCO labs., Grand Island, NY). L₁₀₀₀ and L₁₀₀₀₀ cells are vincristine-induced multidrug resistant cell lines. These cells were developed by Slater (Slater et al., 1992) from L₀ cells and were routinely maintained in growth medium containing 100 nM and 1000 nM vincristine sulfate (Sigma Chemical Co., St. Louis, MO), respectively. Cells were incubated in a humidified 37°C atmosphere containing 5% CO₂. Stock cells were kept in liquid nitrogen at 2 x 10⁶ cells/ml freezing medium consisting of RPMI 1640 supplemented with 20% FBS and 8% dimethylsulfoxide (DMSO). For the experiments studying cell growth, cells were seeded in the fresh medium at the density of 0.2 x 10⁶/ml. Every 24 hours, the number of cells in 200 μl of cell suspension was counted using a Coulter Counter (ZM) equipped with a channel analyzer (Model 256) (Coulter Electronics Limited, England). Cells in the log phase of growth were used in the experiments and had not passaged more than 30.

2.2 Cell cycle analysis

2 x 10⁶ cells were washed 3 times with cold phosphate buffered saline (PBS) at 4°C for 5 minutes and were then fixed in 2 ml of 95% ethanol/PBS. The fixed cells were treated with 100 μg/ml of RNase I (Sigma) at 37°C for 20 minutes and were stained with 50 μg/ml of propidium iodide (Sigma) at 4°C for 30 minutes. The stained cells were analyzed using a flow cytometer (Vantage, Becton Dickinson, San Jose, CA).
2.3 Determination of P-glycoprotein expression

2 x 10^6 cells were washed with cold PBS followed by fixation with 3.7% formaldehyde (HCHO) in PBS for 15 minutes. After being washed with PBS once, the cells were incubated with 10 μl of mouse anti-human P-glycoprotein (Kamlyla Biomedical Company, Thousand Oaks, CA, USA) in 1 ml PBS for 30 minutes at 4°C. Then the cells were washed with PBS 3 times followed by incubation with 1:1000 fluorescein-5-isothiocyanate (FITC) conjugated goat anti-mouse antibody (Cappel, Durham, NC, U.S.A.) for 30 minutes at 4°C. The cells were washed with PBS for 3 times and analyzed using a flow cytometer.

2.4 Light microscopy of nuclear morphology

Cells were washed with PBS once and adjusted to a cell density of 0.2 x 10^6 cells/ml. 200 μl of the cell suspension were collected onto a glass slide using a Cytospin II (Shandon Scientific Limited, England) centrifuge and the nuclear morphology of cells is visualized using a Wright-Giemsa Staining kit (Baxter Healthcare Corporation, McGaw Park, IL) followed by washing with deionized water. The slides were air-dried and mounted using DPX Mountant (Fluka, Switzerland). The stained cells were examined using a Zeiss Axiophot inverted microscope (Zeiss, West Germany) equipped with a 100 x objective. Photographs were taken using a UFX exposure system (Zeiss) with Fuji RD 135 Chrome (Japan).

2.5 Fluorescent microscopy of microtubules

The MTs were visualized by indirect fluorescent staining (Leung, 1992). Cells were washed with phosphate buffered saline (PBS) and then were collected through a Cytospin II centrifuge onto a glass coverslip. The cells were incubated in PM2G buffer (100 mM PIPES, 2 mM EGTA, 1 mM MgSO₄, 2 M glycerol, pH 6.9) for 1 minute followed by fixation with 3.7%
HCHO in PM2G buffer for 30 minutes at room temperature. The cells were washed once with PBS for 5 minutes followed by washing with 0.1 M glycine in PBS, pH 7.4 for 5 minutes. Cells were then permeabilized with 0.3% Nonidet P-40 in PBS and incubated for 10 minutes, followed by washing with PBS for 3 times. The extracted cells were then incubated with mouse anti-β-tubulin antibody (Sigma) for 30 minutes in a moistened dish. After the cells were washed with PBS 4 times, the cells were then stained with rhodamine-conjugated goat anti-mouse IgG antibody Cappel (Organon Teknika-Cappel, West Chester, PA). Finally the cells were washed with PBS 4 times, once with water then mounted with Airvol (Air Products and Chemicals, Inc., Allentown, PA) on a glass slide. All steps were conducted at room temperature and all reagents were warmed to room temperature before use. A confocal microscope (Bio-Rad MRC-600, Bio-Rad, Richmond, C.A.) was used to scan different sections of the cells and the pictures were taken directly from the screen of the confocal microscope using Fuji RD 135 Chrome and a Nikon camera (F 601 M, Japan).

2.6 Extraction of microtubules and microtubule-associated proteins (MAPs)

Microtubules and MAPs were purified using a method that induce the microtubules to assemble with the aid of taxol (Vallee, 1986). 500 x 10⁶ of cells were harvested and were washed with cold PBS followed by homogenization in 1 ml cold PMED buffer (100 mM PIPES, 1 mM MgSO₄, 1 mM EGTA, 2 mM dithioerytheritol, pH 6.4) at 4°C using a ultrasonicator (B. Braun Labsonic U., B.Braun Diessel Biotech GmbH, Germany) with a needle probe (40T) for 1 minute. The lysate was transferred to a TLS-55 centrifuge tube (Beckman Instrs., Inc., Fullerton, CA) and spun at 100,000 g for 1 hour using a tabletop ultracentrifuge (Beckman, TLS-55 rotor) at 4°C. The supernatant was then transferred to a 5 ml glass test tube and incubated in the
presence of 20 nM taxol (Sigma) and 200 µM GTP (Sigma) at 37°C for 30 minutes with occasional shaking. The samples were then aliquoted to 200 µl centrifuge tubes (Beckman, TLA-100 rotor). The samples were spun at 100,000 g for 30 minutes at 37°C and the pellets were washed once with PMED buffer. The pellet containing microtubules and MAPs was then stored at -80°C and later used for determination of the protein content. For SDS-PAGE analysis, sample buffer (0.0625 M Tris, 1% SDS and 1% 2-mercaptoethanol) was used to dissolve the pellet by boiling for 5 minutes. The protein content was determined by Bradford micro-assay using a Beckman, DU 600 spectrophotometer. Samples for two-dimensional gel electrophoresis were dissociated in Triton X-100 dissociation buffer (9 M Urea, 2% 2-2-Mercaptoethanol, 2% Pharmalyte 3-10, 2% Triton X-100 and 8 µM PMSF). The protein content was determined by Lowry assay (Lowry et al., 1951). The BSA standard for both Bradford and Lowry assays was obtained from Bio-Rad. A control sample was prepared in the absence of taxol, GTP and Mg²⁺. Under this condition, microtubule self-assembly does not occur (Vallee, 1986)

2.7 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

Proteins were separated by SDS-PAGE gel electrophoresis as described by Laemmli (Laemmli, 1970) using a slab gel electrophoresis system (SE 600, Hoefer Scientific Instruments, San Francisco, CA). The sample to be analyzed by SDS-PAGE was dissolved or diluted in sample buffer as described above, but with addition of bromophenol blue and the mixture was centrifuged at 14,000g for 1 minute to spin down the undissovled particles. The proteins were resolved on a 10% SDS-PAGE slab gel (10% polyacrylamide, 0.3% N,N'-methylene-bis-acrylamide, 0.1% SDS, 0.0625 M Tris-HCl, 0.5% ammonium persulfate, 0.02% TEMED, pH 8.8) with 4%
acrylamide stacking gel (4% polyacrylamide, 0.12% N', N'-methylene-bis-acrylamide, 0.1% SDS, 0.0625 M Tris-HCl, 1% ammonium persulfate, 0.04% TEMED, pH 6.8). The resolving gel was left to polymerize overnight and the stacking gel was set 30 minutes before electrophoresis. The gel was electrophoresed at 80 V for the stacking gel and x120 V for the resolving gel. After electrophoresis, the gel was fixed with 25% ethanol and 10% acetic acid for 30 minutes followed by incubation with the incubation solution (6.8% sodium acetate, 0.13% glutardialdehyde and 0.2% sodiumthiosulfate) for 20 minutes. After overnight rinsing, the gel was stained with 0.1% silver nitrate for 40 minutes and developed using 25% Na₂CO₃ and 0.01% formaldehyde. Protein molecular weight standards were coomassie blue prestained standards from Bio-Rad. The gel was preserved in 2% glycerol overnight followed by heat drying at 80°C using a gel dryer (Bio-Rad, Model 543).

2.8 Western blot analysis

Tubulins were visualized by the chemi-luminescent technique using indirect labelling with horseradish peroxidase (HRP) conjugated antibody after the proteins were separated by SDS-PAGE and transferred to nitrocellulose paper. 10 μl of 1 mg/ml proteins were separated by SDS-PAGE on a 10% mini gel using a slab gel system (Bio-Rad, Model 422) and then were electrophoretically transferred (80 mA/cm² for 1 hour) from the acrylamide gel to a nitrocellulose membrane (Pharmacia LKB Biotechnology AB, Sweden) using NovaBlot Electrophoretic Transfer System (Pharmacia). After electrophoretic transfer, the membrane was blocked in TBS-T (20 mM Tris, 137 mM NaCl and 0.1% Tween-20, pH 7.6) with 5% dry milk for 2 hours followed by incubation with 0.03% mouse anti-α and anti-β tubulin antibodies (Sigma) in TBS (20 mM Tris, 137 mM NaCl and pH 7.6) for 1 hour. The membrane was then washed with TBS-T buffer 3 times with 15
minutes each followed by incubation with 0.1% HRP labelled goat anti-mouse IgG in TBS for 30 minutes. The HRP-indirectly bound proteins were visualized using chemi-luminescent technique (ECL Kit, Amersham, UK) and followed by autoradiography using Kodak X-OMAT AR film. The film was developed by an X-ray film processor (Fuji RG II, Japan).

2.9 Immunoprecipitation of microtubules and microtubule-associated proteins

$2 \times 10^6$ cells were metabolically labelled for 4 hours with 100 $\mu$Ci/ml of $[^{35}S]$methionine (>1000 Ci/mmol, Amersham) in a 1 ml centrifuge tube with rocking every half hour. The cells were then collected, washed with cold PBS 3 times, and lysed on ice by single detergent lysis buffer (50 mM Tris, 150 mM NaCl, 0.02% sodium azide, 100 $\mu$g/ml PMSF, 1 $\mu$g/ml aprotinin and 1% Nonidet p-40, pH 7.5) for 1 hour. The lysed cells were centrifuged at 4°C at 16,000 g for 10 minutes. The lysate was incubated with 20 $\mu$l of rabbit anti-tubulin antibody (Sigma) with slowly rocking for 12 hours at 4°C, followed by incubation with 200 ul of 12.5% (W/V) Protein A Sepharose CL-4B (Sigma) for 1 hour at 4°C. The sample was then washed with wash buffer (50 mM Tris and 150 mM NaCl) three times and the protein A was collected by centrifugation at 16,000 g for 20 seconds. Then sample buffer was added to the pellet which was boiled for 10 minutes followed by transfer of the sample buffer-protein A mixture to a 0.45 $\mu$M microfuge (Micron Separations Inc., MA, Westborough) and centrifuged at 16,000 g for 20 seconds. After filtration, 5 $\mu$l of protein sample was transferred to a scintillation vial containing 5 ml of scintillation cocktail (Wallace Oy., Tarku, Finland) and the radioactivity was quantitated using a LS 6500 scintillation counter (Beckman). Proteins containing 50,000 cpm were used analysis on 12% SDS-PAGE. After electrophoresis, the gel was fixed as described above followed by incubation in Amplify (Amersham) for 30 minutes. After drying
as above, the gel was exposed to an X-ray film (Kodak, X-OMAT AR) in the presence of an intensifying screen (OKAMOTO MFG. Co. LTD, Japan) at -80°C for 48 hours.

2.10 Two-dimensional gel electrophoresis

The sample to be analyzed by 2-D electrophoresis was first diluted to 1 mg/ml using sample solution as previously described with the addition of bromophenol blue. The sample solution was centrifuged at 14,000 g for 1 minute. 10 μg of proteins were used in the 2-D gel electrophoresis. The 2-D gel electrophoresis was performed using the modified method of O'Farrell (O'Farrell, 1975). Briefly, first dimension pH 3-10 isoelectric focusing was carried out at 300 V for 8 hours in the first phase and 2,000 V for 8 hours in the second phase with a total of 22,650 V-h at 15°C using a precast gel strip (Pharmacia) which had been prehydrated with prehydration buffer (8 M urea, 0.5% Triton X-100, 0.15% DTT and 0.01% acetic acid). The isoelectric focusing gel strip was then incubated in equilibration solution (0.05M Tris, 6 M urea, 30% glycerol and 1% SDS) for 10 minutes twice and was placed on top of a 0.5 mm 8-18% gradient precast gel (Pharmacia). The gel strip was removed after the gel had been electrophoresed at 20 V for about 30 minutes followed by switching to 50 V for about 45 minutes. The SDS-PAGE running buffer is precast buffer strips from Pharmacia. The whole process of 2-D gel electrophoresis was run on the Multiphor II Electrophoresis System (Pharmacia) with a 15°C constant temperature regulator. The gel was then fixed and stained as described and dried at 60°C. The isoelectric focusing standards were purchased from Pharmacia. The pI standard proteins include amyloglucosidase, methyl red, soybean trypsin inhibitor, b-lactoglobulin A, bovine carbonic anhydrase B, human carbonic anhydrase B, horse myoglobin-acidic band, horse myoglobin-basic band, lentil lectin-acidic band, lentil lectin-middle band, lentil-basic band and trypsinogen.
2.11 Reversal of multidrug resistance activity

The VCR resistance of L100 and L1000 cells was reversed by cyclosporin A and verapermil (Slater, 1986a and Slater, 1986b). Cells were resuspended in duplicate at 0.2 x 10^6/ml. L0 cells were cultured in the presence of 100 nM VCR. L100 and L1000 cells were cultured in the presence of 100 nM VCR and serial concentrations of cyclosporin A (kindly supplied by Dr. Lewis M. Slater, University of California, Irvine) and verapermil (Sigma) in the culture condition as described earlier. The concentrations of cyclosporin A were 5, 10 and 15 μg/ml and the concentrations of verapermil were 10, 20 and 30 μM. After 4 days incubation, 10 μl of aliquots were taken from each duplicate culture and counted with a hemocytometer, and the cell viability was determined by trypan blue dye exclusion assay (Trypan Blue, GIBCOL). For the morphology study of MDR reversed cells, cells were cultured in the presence of 10 μg/ml cyclosporin A and 10 μM verapermil in the absence of VCR. After a 4-day culture, the cells were collected. The nucleus and MTs of the cells were visualized using Wright Giesma stain and fluorescence staining, respectively, as described previously.
3. Results

3.1 Growth and cell cycle analysis of L₀, L₁₀₀ and L₁₀₀₀ cells

The characteristics of the growth of L₀, L₁₀₀ and L₁₀₀₀ cells were determined (Figure 1). The doubling times of L₀, L₁₀₀ and L₁₀₀₀ cells during log phase are about 24, 37 and 60 hours, respectively. The log phase of L₀, L₁₀₀ and L₁₀₀₀ cells are 2-5 days, 3-7 days and 4-9 days after beginning of culture, respectively. Log phase cells were used throughout the study.

Cell cycle analysis of L₀, L₁₀₀ and L₁₀₀₀ cells was conducted (Figure 2 and figure 3). Cells were stained with propidium iodide after RNase digestion and analysed by flow cytometry. The growth phase distribution among the L₀, L₁₀₀ and L₁₀₀₀ cells is 46%, 47% and 47% for G₀+G₁, 45%, 41% and 43% for S phase and 7%, 9% and 9% for G₂+M, respectively. These results indicated that although the three cell lines have very different growth rates as shown in figure 1, these cells have similar cell cycle distribution.

3.2 P-glycoprotein expression of L₀, L₁₀₀ and L₁₀₀₀ cells

The expression of the P-glycoprotein in L₀, L₁₀₀ and L₁₀₀₀ leukemia cells was determined by using anti-P-glycoprotein antibody and FITC-conjugated secondary antibody staining followed by analysis using a flow cytometer (Figure 4). Both L₁₀₀₀ and L₁₀₀ cells were shown to overexpress P-glycoprotein compared to L₀ cells. The relative mean fluorescence intensity of L₀ cells is 6. However, the relative mean fluorescence intensity of L₁₀₀ and L₁₀₀₀ cells is 32 and 48, respectively.
Figure 1. Growth curves of L₀, L₁₀₀ and L₁₀₀₀ leukemia cells. L₀ cells (solid circle), L₁₀₀ cells (open circle) and L₁₀₀₀ cells (open triangle) were grown in fresh medium in the presence of 0 nM, 100 nM and 1000 nM VCR, respectively. Data represent three experiments ± S.D.
% G0/G1 : 46
% S : 45
% G2 + M : 7

% G0/G1 : 47
% S : 41
% G2 + M : 9
Figure 2. DNA histogram of L₀, L₁00 and L₁000 cells. The DNA content of the ethanol fixed L₀ (a), L₁00 (b) and L₁000 (c) leukemia cells was determined by flow cytometry after staining with propidium iodide and RNase digestion.
Figure 3. Cell cycle analysis of L0, L100 and L1000 cells. The DNA content of the ethanol fixed cells stained with propidium iodide was analyzed by a flow cytometry. The data represent three separate experiments ± S.D.
Figure 4. P-glycoprotein expression of L₀, L₁₀₀ and L₁₀₀₀ cells. The P-glycoprotein was stained by anti-Pgp antibody and FITC-conjugated secondary antibody. The fluorescence intensity of FITC-conjugated secondary antibody control (red), L₀ cells (brown), L₁₀₀ cells (green) and L₁₀₀₀ cells (grey) was analyzed by flow cytometry.
3.3 Nuclear and microtubular morphology of L₀, L₁₀₀ and L₁₀₀₀ cells

The nuclear and microtubular morphology of L₀ and L₁₀₀ cells has been shown by Slater (Slater, 1992; Leung, 1992). However, once the L₁₀₀₀ cells were developed only recently, the nuclear and microtubular morphology of L₁₀₀₀ cells were determined and compared to that of L₀ and L₁₀₀ cells. The nucleus of the cells were visualized by Wright Giemsa staining. The nucleus of L₀ is rounded and the nuclei of L₁₀₀ and L₁₀₀₀ cells are multilobulated (Figure 5). The novel nuclear morphology of L₁₀₀ and L₁₀₀₀ cells is maintained up to 50 passage levels.

The microtubules of L₀, L₁₀₀ and L₁₀₀₀ cells were stained with mouse anti-tubulin antibody and rhodamine-conjugated secondary antibody and viewed by scanning confocal microscopy (Figure 6). The microtubular distribution in L₁₀₀₀ is similar to that of L₁₀₀ in which the microtubules are localized between the lobes of the nucleus. However, in L₀ cells, the microtubules distribute evenly around the nucleus.

3.4 Microtubules and microtubule-associated protein expression of L₀, L₁₀₀ and L₁₀₀₀ cells

The microtubule (MT) and microtubule-associated proteins (MAPs) of these three cell lines were extracted in the presence of taxol and GTP. It should be noted that the extracted MAPs are associated with the polymerized tubulin. A western blot analysis (Figure 7) was conducted to ensure this method did effectively extract cytoplasmic tubulins. The taxol/GTP extracted proteins contained high contents of tubulins (Figure 7, a-c). No tubulin was detected in the remaining fractions (Figure 7, d-e). The results showed that taxol extraction almost completely removed tubulin from the total cellular extraction.
Figure 5. Nuclear morphology of L₀, L₁₀₀ and L₁₀₀₀ cells. Cells were collected onto slides using a cytopsin. The nuclear morphology was visualized by Wright Giemsa Stain. (a) L₀, (b) L₁₀₀ and (c) L₁₀₀₀ cells Bar = 5 μm.
Figure 6. Microtubular distribution of L₀, L₁₀₀ and L₁₀₀₀ cells. The MT was visualized by immunofluorescent staining using an anti-β-tubulin antibody and examined by a confocal microscope. (a) L₀, (b) L₁₀₀ and (c) L₁₀₀₀ Cells. Bar = 5 μm.
Figure 7. Western blot analysis of the tubulins extracted by taxol and the GTP-dependent method. The extracted proteins were separated by SDS-PAGE followed by Western blotting using anti-α tubulin and anti-β tubulin antibodies. The tubulins were visualized by the chemi-luminescent technique. 10 μg of taxol extracted L₀, L₁₀₀ and L₁₀₀₀ lysates were loaded in lanes a, b and c, respectively. 10 μg of tubulin depleted extracts of L₀, L₁₀₀ and L₁₀₀₀ cells were loaded in lanes d, e and f, respectively. In lane g, 10 μg of total cellular proteins of L₁₀₀₀ cell was loaded. The tubulin bands were pointed out by the arrow.
The tubulin and MAPs were resolved in 12% SDS-PAGE (Figure 8). Two differential expressions of MAPs were obtained among these cells. A 28 kDa and a 31 kDa protein were both overexpressed in L_{100} and L_{1000} cells as compared to L_0 cells.

These proteins were then resolved by 2-D gel electrophoresis (Figure 9). The pI of the 28 kDa protein is 6.9 and the pI of the 31 kDa protein is 4.4. To demonstrate the 28 kDa and 31 kDa proteins are MAPs, the taxol method without addition of Mg^{2+}, GTP and taxol was used to extract proteins were analysed by 2-D gel electrophoresis (Figure 10). The 28 kDa and 31 kDa proteins were not present in this extract suggesting that these differentially expressed proteins are not specific sediment (contaminant) with MTs and MAPs.

To further study the microtubules and MAPs expression of these cell lines, immunoprecipitation using anti-tubulin antibody was employed. It should be noted that the MAPs purified using this method are attached to the unpolymerized tubulin subunit which is different from the MAPs purified using the taxol method. To verify the precipitation of tubulin, the extraction was examined using Western blotting (Figure 11). Figure 12 indicated that the tubulin was precipitated by this method. Proteins precipitated by anti-tubulin antibodies were then resolved by 12% SDS-PAGE. A 38 kDa protein which is undetected in L_{100} and L_{1000} cells, was found to be expressed in L_0 cells.

3.5 Reversal of multidrug resistance of L_{100} and L_{1000} cells

The MDR activity of L_{100} cells and L_{1000} cells was reversed by cyclosporin A and verapamil. The effects of reversing VCR sensitivity with increasing concentrations of cyclosporin A and verapamil on L_{100} cells and L_{1000} cells are shown in Figure 13. After a 4-day incubation with 10, 20 and 30 μM of verapamil, the L_{100} and L_{1000} cells display decreasing cell
Figure 8. Analysis of microtubules and microtubule-associated protein expression of L₀, L₁₀₀ and L₁₀₀₀ cells using SDS-PAGE. The taxol and GTP-dependent extracted proteins of L₀, L₁₀₀ and L₁₀₀₀ cells were separated by 12% SDS-PAGE and visualized by silver staining. The two arrows point to the 28 kDa and 31 kDa protein bands, respectively.
Figure 9. Two dimensional gel electrophoresis analysis of the microtubules and microtubule associated proteins of L₀, L₁₀₀ and L₁₀₀₀ cells. The taxol and GTP-dependent extracted proteins were separated by two-dimensional gel electrophoresis. Protein samples were separated by isoelectric focusing at pH 3-10 followed by separation by 8-18% SDS-PAGE. The proteins were visualized by silver staining. L₀ cells (a), L₁₀₀ cells (b) and L₁₀₀₀ cells (c). The proteins pointed out by arrows are the 28 kDa and 31 kDa proteins as described above.
Figure 10. Two-dimensional gel electrophoresis analysis of L1000 cells extracted in the absence of Mg$^{2+}$, GTP and taxol. The arrows point out the localizations of the 28 kDa and 31 kDa as described.
Figure 11. Western blot analysis of the tubulins extracted by immunoprecipitation using rabbit anti-α and anti-β tubulin antibodies. The extracted proteins were separated by SDS-PAGE followed by Western blotting using mouse anti-α and anti-β tubulin antibodies. The tubulins were visualized by chemi-luminescence techniques. The arrow points to the tubulin bands.
Figure 12. Analysis of immunoprecipitated microtubules and microtubule-associated proteins of L₀, L₁₀₀ and L₁₀₀₀ cells. The cells were metabolically labeled with ³⁵S-methionine. The extracted proteins were immunoprecipitated by using anti-tubulin antibodies. The microtubules and microtubule-associated proteins were separated by 12% SDS-PAGE and visualized by autoradiography. The arrow points to the 38 kDa protein band.
Figure 13. Reversal of VCR resistance of L_{100} and L_{1000} cells by cyclosporin A and verapamil. The cells were cultured with 2.5, 5 and 10 ug/ml of cyclosporin A (a) and 10, 20 and 30 uM of verapamil (b) in the presence of 100 nM of VCR for 4 days.
viability. The relative viability reached 21% and 25% for L₁₀₀₀₀ and L₁₀₀₀₀₀ cells, respectively after exposure to 30 μM verapamil. In the 4-day cyclosporin A treatment, L₁₀₀₀₀ and L₁₀₀₀₀₀ cells also display decreasing cell viability in the presence of increasing concentrations of cyclosporin A. The relative cell viability of L₁₀₀₀₀ and L₁₀₀₀₀₀ cells are 18% and 25% after incubation with 10 μg/ml of cyclosporin A.

The nuclear morphology of L₁₀₀₀₀ and L₁₀₀₀₀₀ cells (Figure 14) and the microtubular morphology of L₁₀₀₀₀₀ cells (Figure 15) after reversal of MDR by cyclosporin A and verapamil are shown. There were no changes in nuclear morphology and microtubular distribution in the VCR resistance of L₁₀₀₀₀ and L₁₀₀₀₀₀ cells after reversal by 10 μg/ml of cyclosporin A and 10 μM of verapamil. The nuclei are still multilobulated and the MT localized between the lobes of the nucleus.
Figure 14. Nuclear morphology of L100 and L1000 cells after reversal of VCR resistance by cyclosporin A and verapermil. The cells were cultured with 10 μg/ml of cyclosporin A (a) and 10 μM verapermil (b) without VCR for 4 days followed by visualization of nuclei using the Wright-Giesma Stain. Bar = 5 μm.
Figure 15. Microtubular distribution of L_{100} and L_{1000} cells after reversal of MDR by cyclosporin A and verapemil. The cells were cultured with 10 \mu g/ml cyclosporin A (a) and 10 \mu M verapemil (b) without VCR for 4 days followed by visualization of microtubules by immunofluorescent staining using an anti-\beta-tubulin antibody and examined by a confocal microscope. Bar = 5 \mu m.
4. Discussion

4.1 The novel nuclear morphology and microtubule distribution of $L_{100}$ and $L_{1000}$ cells and the differential expression of microtubule-associated proteins.

The alterations in microtubule organization have been reported in other MDR cell lines (Mujagic H. and Mujagic Z., 1991). Slater discovered that the multidrug resistant $L_{100}$ subline showed a novel nuclear morphology and microtubule distribution changes compared to the parental cell $L_0$ (Slater et al., 1992). Through morphological studies, the $L_{1000}$ cells display a $L_{100}$-like novel microtubule (MT) distribution (Figure 6) and multilobulated nuclear morphology (Figure 5). The multilobulated nucleus has been demonstrated to be regulated by microtubules (Leung et al., 1992) Since the microtubule assembly is regulated by microtubule-associated proteins (MAPs). This suggests that the novel microtubule distribution in $L_{100}$ and $L_{1000}$ cells may be due to the differential expression of tubulins and MAPs.

The tubulin content of $L_0$ and $L_{100}$ cells is quite similar by Leung's study (Leung, 1992). In our study, the Western blot analysis indicates that the tubulin expression, in term of the cellular content and molecular weight size, are quite similar among these three cell lines, $L_0$, $L_{100}$ and $L_{1000}$. The tubulin seems have no electrophoretic alteration through the two dimensional electrophoresis analysis. These studies suggested that the novel microtubule distribution in $L_{100}$ and $L_{1000}$ cells are not due to the changes in tubulins themself.

The identification of MAPs by the selective extraction base on the criteria that the co-polymerization of MAPs with microtubules or tubulins. The MAPs extracted by taxol method is compared to that of immunoprecipitation can be different. The taxol extraction method takes
advantage of that the microtubules polymerized with taxol are very stable. Hence, taxol will increase the microtubules and MAPs extraction yield. The MAPs extracted by taxol method are attached to the polymerized tubulin. However, the MAPs extracted using immunoprecipitation of the tubulin base on the principle that MAPs bind to tubulin subunit are pulled down by the anti-tubulin antibody. Therefore, the MAPs extracted by immunoprecipitation are associated with unpolymerized tubulin subunit. The SDS-PAGE and 2-D gel electrophoresis analysis of the taxol-extracted microtubules and microtubule-associated proteins revealed a 31 kDa protein with pI 4.4 and a 28 kDa protein with pI 6.9 were overexpressed in L100 and L1000 cells (Figures 8, 9). The SDS-PAGE analysis of the immunoprecipitated tubulins and MAPs indicated that there is a missing 38 kDa MAP in both L100 and L1000 cells (Figure 12). The presence of Mg$^{2+}$, GTP and taxol is very important for microtubule assembly, or in the other word, the absence of Mg$^{2+}$, taxol and GTP would fail to extract the tubulin and MAPs. The absence of the 31 kDa protein with pI 4.4 and 28 kDa protein with pI 6.9 proteins in the control extraction (without Mg$^{2+}$, GTP and taxol) further revealed that these two proteins are MAPs, and not the contaminant proteins cosediment with microtubules and microtubule-associated proteins (Figure 10). The association of the novel microtubule organization and the alterations in the microtubule-associated protein expression in L100 and L1000 cells suggested that these MAPs may be responsible for the novel microtubule distribution and the nuclear morphology in L100 and L1000 cells.

Since L0 cells grows faster than L100 and L1000 cells, the differential expression of microtubule-associated proteins may be due to the differential growth of cells. Cell cycle analysis of the cells show that they have similar cell cycle distribution suggesting that the differential expression of these microtubule-associated proteins are not the cell cycle related.
Since the long-term removal of VCR from the L\textsubscript{100} cells would lead to the death of the cells which indicate that the L\textsubscript{100} cells are VCR dependent (Slater \textit{et al.}, 1992). It is possible that the binding of VCR to microtubule confer the novel microtubule organization in L\textsubscript{100} and L\textsubscript{1000} cells. However, we shortly withdraw the VCR from the L\textsubscript{100} and L\textsubscript{1000} cells which does not offer the differential expression of these MAPs and the novel morphology still persist (data not shown), indicating that the novel morphology and the differential expression of these MAPs is not VCR-induced.

Although changes of other proteins can be seen in figures as shown, the changes of the 28, 31 and 38 kDa proteins as described are consistently found in the repeat experiments.

4.2 Differential expressions of microtubule-associated proteins and its relationship with multidrug resistance

The microtubules and microtubule-associated proteins are important in the transmission of information from the membrane to cytoplasm. It also has been demonstrated that microtubule morphology and microtubule-associated protein changes were associated with the MDR development in other cell lines (Mujagic H. and Mujagic Z., 1991; Lee \textit{et al.}, 1992). This suggests that the microtubules and microtubule-associated proteins may be involved in the MDR mechanism (Song \textit{et al.}, 1993; Ciocca \textit{et al.}, 1992; Lee \textit{et al.}, 1992). In L\textsubscript{100} and L\textsubscript{1000} MDR cells, the microtubule morphological changes (Figure 6) and the altered microtubule-associated proteins expressions (Figures 8, 9, 12) were also found to be correlated to the VCR resistant activity which also suggested that the cytoskeletal changes in these cells may play a role in the drug resistance mechanism.

Many MDR cells overexpressed Pgp which is said to be an efflux membrane pump for drugs (Kartner and Ling, 1989). The Pgp is also found to
be overexpressed in L100 and L1000 cells when compared to that of L0 cells as determined by the Pgp expression analysis (Figure 4). This data suggests that the overexpression of Pgp is responsible for, at least in part, the MDR mechanism in L100 and L1000 cells. However, the ratio of the relative fluorescence intensity of Pgp expression of the L1000 and L100 cells is not more than two-fold (32/24). This is quite different from the ratio of their ability to be resistant to the VCR which is 10-folds. This significant difference in the non-corresponding relationship between the Pgp expression and the drug resistance suggested that the Pgp overexpression only accounts for part of the drug resistance mechanism in L100 and L1000 cells. There may be other pathways employed by L100 and L1000 cells to exhibit MDR. The altered expression of microtubule-associated proteins as shown in this study may be related to the drug resistance in L100 and L1000 cells.

The alterations in microtubules and microtubule-associated proteins have been suggested to play a role not only in their lack of regulation of controlled proliferation but also their sensitivity to microtubule disrupting chemotherapeutic agents in other acute leukemia cell lines (Anand and Chou, 1993). In Anand and Chou's study, decreased expression of a MAP of 30 kDa, pI 4.4 is involved in the activation of normal human peripheral blood T cells (Anand and Chou, 1992). An overexpressed 31 kDa, pI 4.6 MAP (Figure 9) found in L100 and L1000 cells has similar pI and molecular weight to the MAP detected in the Anand and Chou study. The overexpression of this MAP may play a role in the desensitization of L100 and L1000 cells to the anticancer drug which in turn are responsible for the MDR activity.

Another altered MAP detected in the taxol method is the protein of 28 kDa and pI 6.9. According to its molecular weight and isoelectric focusing point, it is quite similar to that of the heat shock protein, Hsp 27 which is about 27 kDa and pI 6.5. The overexpression of Hsp 27 have been shown to
down regulate the cell proliferation (Pechan, 1991; Spector et al., 1994) and be involved in the development of drug resistance. The Hsp 27 is an actin-associated protein and the localization of Hsp 27 is microtubule dependent (Ciocca et al., 1993). The heat shocked cells, in which the Hsp 27 and other heat shock proteins were induced to overexpressed, had altered microtubule organization [ ]. In the MDR sublines L_{1000} cells and L_{100} cells, the altered microtubule organization (Figure 6) and altered expression of MAPs (Figure 9, 12) are demonstrated when compared to the drug sensitive parental cells L_{0}. The increasing expression of the MAP (28 kDa, pI 6.9) are correlated to increasing drug resistant ability among these cells. It is speculated that this MAP, which has a similar molecular weight and pI to Hsp 27, is actually the Hsp 27. Based on the previous study, if this MAP is the Hsp 27, the altered expression of this MAP may contribute to the decreased growth rate, altered microtubular organization and the development of drug resistance additional to Pgp mediated MDR in L_{100} and L_{1000} cells. The mechanism of conferring the drug resistance by Hsp 27 is still unclear. However, it is apparent that proteins other than the Pgp, can mediated MDR (Huot et al., 1991).

To examine whether the novel microtubule distribution is related to the multidrug resistance (MDR) phenomenon directly, a set of reversal experiments was conducted. The MDR of L_{100} and L_{1000} cells can be reversed by verapermil and cyclosporin A. However, the nuclear morphology (Figure 14), microtubule morphology (Figure 15) and the microtubule and microtubule-associated protein expression (data not shown) of L_{100} and L_{1000} cells do not change after reversal of drug resistance. Since the mechanism of MDR reversal by cyclosporin A and verapermil is unknown, these data suggested that the action of cyclosporin A and verapermil is unrelated to the microtubule and microtubule-associated protein expression and distribution.
The VCR is an antimitotic agent which binds to tubulin specifically with subsequent depolymerizing of microtubule. One possible mechanism by the altered MAPs confer the VCR resistance in L100 and L1000 cells is to stabilize the polymerized microtubule which counteract the VCR depolymerizing effect on microtubule.

The possibility that the alterations in MAPs detected (Figure 9) are the changes resulted from other fundamental physiological changes can not be ruled out. The Hsp 27 is suggested to be the downstream target in signaling pathway (Ciocca et al., 1993). If the MAP of 28 kDa and pl 6.9 is the Hsp 27, it is not surprising that the alterations in the upstream pathway contribute to the alterations in downstream target.
5. Conclusion

5.1 The morphological studies indicated that L\textsubscript{1000} cells have a L\textsubscript{100} like nucleus and microtubule distribution. The nucleus of these two cell lines are both multilobulated and the microtubules localize between the lobes of the nucleus.

5.2 The sodium dodecyl sulfate polyacrylamide gel electrophoresis and two-dimensional gel electrophoresis analysis of the taxol extracted microtubules and microtubule-associated proteins revealed that there are two proteins, 31 kDa with pI 4.4 and 28 kDa with pI 6.9, while are overexpressed in both L\textsubscript{100} and L\textsubscript{1000} cells. A 38 kDa protein is expressed in L\textsubscript{0} cells but can not be detected in L\textsubscript{100} and L\textsubscript{1000} cells.

5.3 The association of the novel microtubule distribution, which regulated the unique nuclear morphology in L\textsubscript{100} and L\textsubscript{1000} cells, and the altered expressions of the microtubule-associated proteins suggest that these altered proteins may regulate the unique nuclear morphology and the microtubule distribution.

5.4 The correlation of these altered proteins and the multidrug resistant activity of L\textsubscript{100} and L\textsubscript{1000} cells suggested that these altered proteins may be involved in the mechanism of multidrug resistance.
6. Reference


Krishnamachary, N. and Center, M.S. The MRP Gene Associated with a Non-P-glycoprotein Multidrug Resistance Encodes a 190-kDa Membrane Bound Glycoprotein. *Cancer Res.* 53: 3658-3661, 1993


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