ROLE OF PROTEIN TYROSINE PHOSPHORYLATION IN CHRONIC LYMPHOCYTIC LEUKEMIA CELL APOPTOSIS

By

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A Thesis
Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree of

MASTER OF SCIENCE

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Winnipeg, Manitoba

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Role of Protein Tyrosine Phosphorylation in Chronic Lymphocytic Leukemia Cell Apoptosis

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Keding Cheng

A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

of

Master of Science

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ABSTRACT

Chronic lymphocytic leukemia (CLL) is a malignant disorder characterized by the proliferation and accumulation of mature appearing, though dysfunctional lymphocytes. The malignant lymphocytes infiltrate the bone marrow, lymph nodes, liver and spleen, resulting in enlargement of these organs and in bone marrow failure. Better understanding of specific defects that contribute to the clonal expansion of this neoplasm may lead to improved treatment of this disease. Growing evidence suggests that programmed cell-death (apoptosis) plays a key role in the selection and differentiation of lymphoid cells. Since CLL lymphocytes accumulate rather than proliferate, this disease may involve defects in apoptosis. The molecular and biochemical basis for decreased susceptibility of CLL cells to drug-induced apoptosis remains largely unknown. Recently, protein tyrosine kinase activation has been shown to provide an early and requisite signal for Fas-induced apoptosis in Jurkat (human T cell leukemia), U937 (human histiocystic lymphoma), and K562 (human myelogenous leukemia) cell lines. In the present study, we investigated the role of tyrosine phosphorylation in CLL cell apoptosis. A cell line (WSU-CLL) derived from a CLL patient and the lymphocytes from several CLL patients and healthy volunteers were used to explore the role of protein tyrosine phosphorylation and its related signaling. Since tyrosine phosphorylation is important in lymphocyte signaling, including cell proliferation, differentiation and apoptosis, defects in tyrosine phosphorylation may contribute to the clonal expansion of this neoplasm. Tyrosine phosphorylation is maintained by a balance between the activities of the protein tyrosine

kinases and phosphatases. We hypothesized that the inhibition of protein tyrosine phosphatase by vanadate would increase protein tyrosine phosphorylation and apoptosis in CLL cells. Indeed, vanadate treatment of WSU-CLL cells increased the tyrosine phosphorylation in conjunction with enhanced cytotoxicity and cell death, as measured by trypan blue dye exclusion, soft agar clonogenic assay, and apoptosis assay. Vanadate-induced apoptosis in WSU-CLL cells was associated with the activation of c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK), release of cytochrome c from mitochondria, and cleavage/activation of effector caspases. In WSU-CLL cells, vanadate also attenuated the activities of Akt and PI 3-kinase responsible for providing survival signals. Thus, vanadate-induced protein tyrosine phosphorylation triggers apoptosis in WSU-CLL cells at several levels involving cytochrome c release from mitochondria, activation of caspases, and removal of cell survival signals. Preliminary studies with a limited number of patient samples showed that WSU-CLL cells and the cells from CLL patients exhibited lower levels of protein tyrosine phosphorylation as compared to normal lymphocytes and the cells less sensitive to a commonly used drug chlorambucil (CLB) had lower levels of tyrosine phosphorylation. These studies suggested that protein tyrosine phosphorylation plays an important role in determining the sensitivity of CLL cells to drug-induced apoptosis. Taken together, protein tyrosine phosphorylation appears to be an intrinsic part of the vanadate-induced apoptotic process in CLL cells.

ACKNOWLEDGMENTS

I would like to thank my supervisor Dr. Satya P. Saxena first for his supervision and knowledge. His great knowledge in signal transduction and sharp viewpoint in scientific research has left a deep impression on me. I have learned much from his constant supervision. This thesis would not be possible without his guidance and grant support.

Special thanks are due to my advisory committee members. Dr. Sabine Mai. and Dr. Lyonel G. Israels. for their constructive suggestions and help throughout my thesis. Dr. Mai's valuable help in immunofluorescence studies and Dr. Israels's efforts in providing clinical samples is highly appreciated.

Many thanks to my colleagues in the Manitoba Institute of Cell Biology and the Department of Physiology for their valuable help in a variety of laboratory techniques and course preparation.

Finally. I would like to thank my wife. Xiangguo Qiu for her unlimited support and help. both in the laboratory and at home. to complete this project successfully.

LIST OF ABBREVIATIONS

AIF	apoptosis inducing factor
Apaf-1	apoptosis activating factor
Ara-c	1-β-D-arabinofurano-sylcytosine
γ-ATP	phosphorus-^{32}-ATP
BCA	Bicinchoninic acid
BCR	B cell receptor
BH	Bcl-2 homology
BSA	bovine serum albumin
CARD	caspase recruitment domain
CDA	chorodeoxyadenosine
CDK	cyclin-dependent kinase
C. elegans	caenorhabditis elegans
CLB	chlorambucil
CLL	chronic lymphocytic leukemia
Dabco	1.4 diazabicyclo[2.2.2]octane
DAPI	4.6-diamidino-2-phenylindole
DBM	deleted in B cell malignancy
EBV	Epstein-Barr virus
ECL	enhanced chemiluminescence
FAK	focal adhesion kinase

F-ara-ATP	F-ara-adenine triphosphate
FCS	fetal calf serum
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
GST	glutathione S-transferase
HRP	horseradish proxidase
IC_{50}	drug concentration (μM for CLB in this paper) required for 50% inhibition of cell viability
IgH	immunoglobulin heavy chain
IL-1	interleukin 1
IR	ionizing radiation
JNK	c-jun N-terminal kinase
MAPK	mitogen-activated protein kinase
MCL	mantle cell lymphoma
MDR	multiple drug resistance
MKK	MAP kinase kinase
MKP	mitogen-activated protein kinase phosphatase
MMS	methylmethanesulphonate
MPT	mitochondria permeability transition
PAK	p21-activated kinase
PARP	poly(ADP-ribose) polymerase
PBS	phosphate buffer saline
PCR	polymerase chain reaction

PDK	phosphoinositide-dependent protein kinase
PH	pleckstrin homologue
PI	phosphatidylinositol
PI 3-kinase	phosphatidylinositol 3-kinase
PI 4-P	PI 4-phosphate
PI 3,4-P_2	PI 3,4-biphosphate
PI 3,4,5-P_3	PI 3,4,5-triphosphate
PKA	protein kinase A
PKCδ	protein kinase C-delta
PLC	phospholipase C
p-NPP	p-nitrophenyl phosphate
3-PPI	3-phosphorylated phosphoinositide
PTK	protein tyrosine kinase
PTP	protein tyrosine phosphatase
RFLP	restriction fragment length polymorphism
SAPK	stress-activated protein kinase
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SEK	SAPK/ERK kinase
SIg	surface immunoglobulin
TBS	Tris buffer saline
TBST	Tris buffer saline-tween
Tris	tris (hydroxymetyl) aminomethane

SSCP	single strand conformation polymorphism
STAT	signal transducer and activator of transcription
TE	Tris-EDTA
TGF	transforming growth factor
TM	transmembrane
TNF	tumor necrosis factor
Van	Sodium orthovanadate

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INTRODUCTION

V.1 ***Chronic Lymphocytic Leukemia (CLL)***: CLL is the most common type of leukemia in western countries (O'Brien et al., 1995; Rozman & Montserrat, 1995; Zwiebel & Cheson, 1998), accounting for 25-30% of the total leukemias. This disease is rare in Asian countries (Hoffman et al., 1991). The cause of CLL is not known (Rozman & Montserrat, 1995). In general, the average survival time of patients with CLL is about nine years (Rozman & Montserrat, 1995). Most of the CLL patients are over 55 years, and there are more male than female patients (Hoffman et al., 1991). A recent increase in CLL in younger patients has also been reported (Hoffman et al., 1991). The clinical characteristics of CLL vary from patient to patient (Zwiebel & Cheson, 1998). While some patients can have the disease for many years without apparent clinical symptoms, others die within a few years of diagnosis (Zwiebel & Cheson, 1998).

The various symptoms of CLL include tiredness, weight loss and infections (Hoffman et al., 1991; O'Brien et al., 1995; Zwiebel & Cheson, 1998). Since the disease is characterized by gradual accumulation of mature-looking but "immunologically incompetent" lymphocytes (Rozman & Montserrat, 1995; Zwiebel & Cheson, 1998), CLL can be suspected if the peripheral lymphocytes are over 5 x 10^9/L. About 95% of CLL are B-lymphocytic (Hoffman et al., 1991). Clinical diagnosis of CLL depends on the morphology and surface characteristics of the lymphocytes of CLL cells (Hoffman et al., 1991; Zwiebel & Cheson, 1998). Morphologically, the B-lymphocytes in CLL are mature-looking, small lymphocytes with strong staining of basophilic chromatin of the

nucleus (Hamblin & Oscier, 1997). The cell is fragile so "smudge" cells can be seen on the slides (Hamblin & Oscier, 1997). The cells resemble fetal B cells since they have very low surface immunoglobulin M (SIgM) and respond poorly to *in vitro* stimulation such as cross-linking or lectin treatment (Reed, 1998; Schroeder & Dighiero, 1994; Hamblin & Oscier, 1997). They express some B cell markers such as CD19, CD20, CD23 and CD40, but lack germinal center B cell marker CD10 (Reed, 1998). The most striking feature is the co-expression of the T cell marker CD5 (O'Brien et al., 1995; Hamblin & Oscier, 1997; Reed, 1998) and Fcε receptor CD23 (Hamblin & Oscier, 1997; DiGiuseppe & Borowitz, 1998; Reed, 1998). The commonly accepted clinical staging of CLL (Zwiebel & Cheson, 1998) is mainly dependent on the extent of cell growth and infiltration of the abnormal lymphocytes (lymphocytosis) in the body and the final consequence of complications caused by lymphocyte infiltration, such as malfunction of the bone marrow (anemia, low platelet count), and enlargement of lymph nodes, spleen and liver (Hoffman et al., 1991; O'Brien et al., 1995; Zwiebel & Cheson, 1998). From low risk to high risk, there are 5 Rai stages, stage 0 to stage IV, or 3 Binet stages, stage A to C (Zwiebel & Cheson, 1998). The staging does not reflect the complete pattern of the disease because of the heterogeneity of the disease but does have a significant bearing on prognosis.

V.2 ***<u>Genetic Abnormalities in CLL:</u>*** CLL is a complex disease involving many cytogenetic abnormalities of the lymphocytes, and the reason for most of the genetic changes and the effects related to these changes are largely unknown (O'Brien et al., 1995; Crossen, 1997; Hamblin & Oscier, 1997; Juliusson & Merup, 1998; Reed, 1998). Studies in this area are hampered by the difficulty in getting metaphase cells because of

the poor response of CLL cells to mitogens (Crossen, 1997). Chromosomal abnormalities can be found in half of the patient samples (Juliusson & Merup, 1998). The most common change is the deletion and/or translocation of chromosome 13q14 region, accounting for about 15-25% of patients and 65-75% of the patients with genetic abnormalities (O'Brien et al., 1995; Crossen, 1997; Hamblin & Oscier, 1997; Juliusson & Merup, 1998; Reed, 1998). There is about a 280 kb nucleotide loss (Crossen, 1997) or a 550 kb nucleotide loss (Zwiebel & Cheson, 1998) after chromosome 13q14 deletion and/or translocation, and this area is confined between genetic marker D13S25 and the tumor suppressor retinoblastoma RB1 gene. Crossen (1997) suspected that RB1 may be lost in CLL cells. Further studies using restriction fragment length polymorphism (RFLP), single strand conformation polymorphism (SSCP) and fluorescence *in situ* hybridization (FISH) confirmed that CLL cells do express RB1 and this tumor suppressor gene may not be involved in the pathological changes observed in CLL (Crossen, 1997; Juliusson & Merup, 1998; Reed, 1998). Recent efforts are focused on identification of other tumor suppressor, or pro-apoptotic gene loss in this area (Reed, 1998). Some have already named this gene as DBM (deleted in B cell malignancy) (Zwiebel & Cheson, 1998).

Another common chromosome abnormality trisomy 12 accounts for 10-30% of B cell CLL (O'Brien et al., 1995) and one-third of CLL patients with chromosome abnormalities (Juliusson & Merup, 1998). Polymerase chain reaction (PCR) and FISH identified a higher rate of trisomy 12 than traditional cytogenetic analysis (Juliusson & Merup, 1998). Recent studies confirmed that trisomy 12 is not the result of triplication of one homolog chromosome, but the duplication of one homolog chromosome (Crossen,

1997). Since most of CLL patients with trisomy 12 show poor prognosis, rapid disease progression and atypical morphology of CLL cells, it is believed that this chromosome may encode an oncogene, an anti-apoptotic gene, or a cell cycle-related gene (Reed, 1998). At present no CLL-related gene has been reported on this chromosome (Crossen, 1997). The appearance of trisomy 12 accompanied by deletion or translocation of chromosome 13q14 is rare (Crossen, 1997; Hamblin & Oscier, 1997; Juliusson & Merup, 1998; Reed, 1998), so the two chromosome abnormalities may reflect two different pathologic pathways of the disease (Hamblin & Oscier, 1997).

A number of CLL patients also have translocations involving chromosome 14, such as t[11;14], t[14;18], and t[14;19] (Crossen, 1997; Hamblin & Oscier, 1997; Reed, 1998). Chromosome 14 encodes the immunoglobulin heavy chain (IgH) (Reed, 1998). Translocation of chromosome 11q14 and IgH locus 14q32 involves the BCL-1 (PRAD-1) gene that encodes cyclin D1 (Reed, 1998), an important factor in the cell cycle. In normal cells, the cyclin D1 gene is only transiently activated in G1 and cyclin D1 interacts with cyclin-dependent kinases (CDK) to cause the phosphorylation of the RB gene product. The phosphorylation of RB protein releases the transcription factor E2F and moves the cell into cell cycle (Reed, 1998). It is known that cells involved in the BCL-1 gene translocation represent a more aggressive disease than CLL-mantle cell lymphoma (MCL). The MCL cells are negative for CD23 compared with CD23-positive CLL cells (Crossen, 1997; DiGiuseppe & Borowitz, 1998; Reed, 1998). This translocation also causes the constitutive activation of the cyclin D1 gene by transcriptional enhancer elements in the IgH gene (Reed, 1998). Cyclin D2 mRNA level was also found to be higher in CLL patient cells. It was mapped on chromosome 12p13 and the high

expression of cyclin D2 may contribute to the longer survival of CLL cells (Delmer, et al., 1995). $p27^{Kip}$, an inhibitor of the cell cycle, was also found to be more expressed in CLL patient cells, especially patients with poor prognosis (Vrhovac, et al., 1998).

The translocation of chromosome 14 and 18 also causes the activation of the Bcl-2 gene (on 18q21) by IgH enhancer elements (Reed, 1998). Bcl-2 is an apoptosis-suppressing gene. This translocation is more common in non-Hodgkins lymphoma (Crossen, 1997; Reed, 1998), a disease with similarities to CLL (Reed, 1998). The translocation of Bcl-2 gene in CLL is between chromosome 18 and chromosome 2 or 22, which encodes immunoglobin light chains (Crossen, 1997). Bcl-2 overexpression increases cell survival of CLL cells and lymphoma cells (Reed, 1998). Translocation between chromosome 14 and 19 causes the juxtaposition of Bcl-3 gene and the IgH locus (Crossen, 1997). Bcl-3 is a member of IκB family, which inhibits transcription factor NFκB (Baeuerle & Baltimore, 1996).

P53 is a tumor-suppressor gene located on chromosome 17p13. The product of p53 gene promotes apoptosis or gene repair (Milner, 1997; Evan & Littlewood, 1998; Kirsch & Kastan, 1998). Mutations of p53 in CLL patients have recently been shown to correlate with advanced stages of the disease and drug resistance (Zwiebel & Cheson, 1998).

V.3 ***Abnormal Immune Function in CLL*:** B-cell CLL patients manifest many immunologic abnormalities (Bartik et al., 1998; Pritsch et al., 1998). Low levels of surface immunoglobulin (SIg) have been reported in malignant B cells as compared with normal B-lymphocytes (Hamblin & Oscier, 1997; Bartik et al., 1998; Pritsch et al., 1998). In addition, malignant B cells exhibit poor response to exogenous stimuli, such as

Epstein-Barr virus (EBV), B-cell receptor ligation, and other mitogenic stimuli (Pritsch et al., 1998). This may in part be responsible for the dysfunction of the immune system of CLL patients (Bartik et al., 1998). CLL patients also show a higher incidence of autoimmune phenomenon, reflected mainly as hemolytic anemia (Pritsch et al., 1998). Although higher expression of CD5 surface antigen, which is normally expressed on T cells, may contribute to autoimmune phenomenon, this hypothesis still remains controversial (Hamblin & Oscier, 1997). Furthermore, CLL cells express high levels of surface antigen CD23 (Hamblin & Oscier, 1997). Reed (1998) speculated that CLL might be caused by a defect in differentiation.

V.4 ***Signaling Defects in CLL***: It is established that most of the CLL cells stay in G0 of the cell cycle and also live longer than normal lymphocytes (Hamblin & Oscier, 1997; Reed, 1998). This may cause gradual accumulation of CLL cells and lymphocytosis observed in the blood, bone marrow and other organs of patients (Hamblin & Oscier, 1997). A small number of CLL cells are also found in G1 of the cell cycle. These cells can be stimulated by mitogens and cytokines to divide and secrete immunoglobulin (Hamblin & Oscier, 1997). Based on these observations, Lankester et al. (1995) divided CLL cells into two types, non-responsive and responsive cells. These investigators reported that although the expression of B-cell receptor (BCR) in responsive and non-responsive CLL cells is not altered, non-responsive cells exhibit significant reduction in the protein tyrosine kinase activity related to BCR ligation (Lankester et al., 1995). The non-responsive cells have also been shown to exhibit defective association of BCR with adaptor proteins Shc and Grb-2 (Lankester et al., 1995). Furthermore, the level of protein tyrosine kinase Syk, an important kinase of BCR-mediated cell signaling (Pani &

Siminovitch, 1997), is also found to be lower in non-responsive cells. A defect in the calcium response was also observed when CLL cells were stimulated (Michel et al., 1993; Lankester et al., 1996). On the other hand, expression of the Src family T-cell receptor kinase Lck and/or Fgr was found to be elevated in CLL cells (Abts et al., 1991; Knethen et al., 1997; Majolini et al., 1998). It is reasonable to speculate that higher expression of Src family tyrosine kinases may contribute to the transformation of CLL cells. Frank et al (1997) reported that signal transducer and activator of transcription (STAT) family members STAT1 and STAT3 are constitutively phosphorylated on serine residues in CLL cells. Interleukin-10 can also cause STAT1 and STAT3 serine phosphorylation, resulting in differentiation but not apoptosis (Jurlander et al., 1997). High expression of cytokine genes on CLL cells suggests their role in the long survival of CLL cells via an autocrine or paracrine pathway (Hamblin & Oscier, 1997).

The defect in B cell development may also contribute to CLL. B cell development requires cell-cell and cell-matrix interactions in bone marrow and lymph nodes (De Rossi et al., 1994; Lagneaux et al., 1998). It has been reported that the adhesion properties and cell behavior of CLL cells can be modified *in vitro* (Lagneaux et al., 1998), but the role of cell adhesion in CLL pathogenesis requires further investigation.

V.5 ***Defective Programmed Cell-Death Pathways in CLL:*** Apoptosis (programmed cell-death) is an essential and evolutionary conserved mode of cell-death important for morphogenesis during embryonic development, tissue homeostasis in adult organisms, host defense, and suppression of oncogenesis (Kerr et al., 1972; Thompson, 1995; Jacobson et al., 1997). In general this form of death process is morphologically different

from necrosis (Kerr et al, 1972; Jacobson et al, 1997). Cells undergoing apoptosis shrink. the plasma membrane forms blebs. the nucleus condenses and nuclear DNA is degraded into oligonucleosomal fragments (Thompson. 1995). Recently, CLL cells have been shown to exhibit defects in cell-death pathways (Adams & Cory. 1998; Green & Reed. 1998; Reed. 1998). These studies suggest that apoptosis may be one of the mechanism(s) responsible for gradual accumulation of CLL cells in patients. Better understanding of the signaling events that trigger or contribute to apoptosis in CLL cells may help in identification of cellular targets that could modulate the response of CLL cells to chemo- and radiotherapy.

In recent years. major efforts have focused on identification of novel molecules that positively or negatively influence cell survival. Genetic and molecular studies of Caenorhabditis elegans (C. elegans) indicated that the apoptosis-regulating nematode genes. ced-3. ced-4. and ced-9. are highly conserved among other species (Hortvitz et al.. 1994; Vaux. 1997). The mammalian homologues of ced-3 gene are the ICE family of cysteine proteases; the closest mammalian homologue being caspase-3 or CPP32 (Alnemri et al.. 1996). The Bcl-2 family of proteins that include both repressors and promoters of apoptosis are identified as mammalian homologues of the ced-9 gene (Vaux et al.. 1992; Hengartner et al.. 1994). A recently discovered human protein. apoptosis inducing factor 1 (Apaf-1). is a CED-4 homologue and participates in the activation of the mammalian CED-3 homologue (Zou et al.. 1997). Apoptosis pathways in C. elegans involve CED-3 and CED-4 proteins as inducers of cell death during the development of the worm (Yuan & Horvitz. 1990; Hengartner et al., 1992). CED-9 has been shown to prevent cell death by inhibiting CED-4 (Shaham & Horvitz. 1996). Based on current

literature, two distinctly different pathways of caspase activation and apoptosis can be delineated in mammals. In the first pathway, ligation of death receptors such as Fas (the cell surface polypeptide also known as CD95 or Apo-1) or the type 1 tumor necrosis factor-α (TNF-α) induces binding of adapter molecules, which in turn recruit pro-caspases-2, 8, and 10 to the membrane-associated signaling complex. This results in proximity-induced activation of some of these caspases. Activation of these upstream caspases is sufficient to directly cleave/activate precursors of effector caspases such as caspase-3, -6, and -7. In the second pathway, various forms of cellular stress triggers mitochondrial release of cytochrome c, which interacts with cytosolic docking protein Apaf-1, leading to the activation of pro-caspase-9. Activated caspase-9 proteolytically activates effector caspases, caspase-3 and possibly caspase-7. Additionally, the caspases can also be activated by granzyme B, a major serine protease in cytotoxic lymphocyte granules (Shi et al., 1992). Once the effector caspases are activated, these enzymes cleave a number of cellular polypeptides leading to disassembly of key structural components of the nucleus and cytoskeleton; inhibition of DNA repair, replication and transcription; and activation of endonucleases that irreversibly damage the genome (Guchelaar et al., 1997; Meyn et al., 1997).

Role of mitochondria: Recently, mitochondria have been shown to play an important role in inducing apoptosis by releasing holocytochrome c (referred to hereafter as cytochrome c) (Liu et al., 1996) and apoptosis-inducing factor (AIF), an apoptotic protease (Kroemer et al., 1997). Apocytochrome c is translocated to mitochondria by cytoplasmic ribosomes where it binds covalently to a heme group to form holocytochrome c. Cytochrome c is localized to the intermembrane space and to the

surface of the inner mitochondrial membrane. Several studies demonstrated the release of cytochrome c in the cytosol of cells in response to a variety of DNA damaging agents and this signaled the cell to undergo apoptosis (Liu et al., 1996; Kharbanda et al. 1997; Kharbanda et al., 2000). Using immunoblotting and electron microscopy, it was demonstrated that exposure of U937 cells to ionizing radiation (IR), cisplatin, methylmethanesulphonate (MMS) and 1-ß-D-arabinofuranosylcytosine (Ara-c) cause accumulation of cytochrome c in the cytosol (Kharbanda et al., 1997). Cytochrome c is released into the cytoplasm in concert with the induction of caspase-3, cleavage of poly (ADP-ribose) polymerase (PARP) into characteristic apoptotic fragments, and induction of internucleosomal DNA fragmentation. The finding that IR-induced cytochrome c release remains unaffected by overexpression of p35, a cysteine protease inhibitor (Bump et al., 1995), suggests that cytochrome c release is upstream to activation of cysteine proteases. By contrast, cytochrome c was not an obligatory step in Dex- and Fas-induced apoptosis in certain cell types (Chauhan et al., 1997). Thus, release of cytochrome c appears to be induced by only certain forms and cellular stress. Cai et al. (1998) summarized that some chemical agents and drugs such as staurosporine, etoposide, Ara-C, paclitaxel, antinomycin D, hydrogen peroxide, cisplatin, *in vitro* treatment of cells with ultraviolet light and ionizing radiation, and IL-3 withdrawal in many cell types (HL 60, U937, Jurkat, FL 5.12, CEM), can induce cytochrome c release from mitochondria. Chauhan et al. (1997) reported that dexamethasone (Dex), anti-Fas monoclonal antibody (mAb) will not induce cytochrome c release from mitochondria, but can still activate caspase-3 and induce apoptosis in myeloma cells. Further studies in cell-free systems demonstrated that the addition of cytochrome c, dATP, or ATP to cytosolic preparations

from growing cells activates caspase-3, an enzyme responsible for the cleavage of PARP (Kaufmann et al., 1993) and protein kinase C-delta (PKCδ) (Emoto et al., 1995). Cytochrome c also induced DNA fragmentation in isolated nuclei incubated with cytosolic lysates (Liu et al., 1996). The finding that intact cells undergo apoptosis following release of cytochrome c into the cytosol has further supported a critical role of cytochrome c release in cell-death pathways. The release of cytochrome c is independent of the mitochondrial permeability transition (MPT) but the accompanying cellular redox change can also trigger the MPT. Since AIF is released by changes in MPT (Kroemer et al., 1997), the two separate pathways may provide redundancy that ensures effective execution of the cell death program.

The mechanisms underlying cytochrome c-dependent regulation of apoptosis are largely unknown. The available evidence indicates that cytochrome c interaction with Apaf-1 results in the activation of procaspase-9 (Li et al., 1997; Zou et al., 1997; Srinivasula et al., 1998). The mechanism of Apaf-1 action is unclear but may involve clustering of procaspase-9 molecules, thereby facilitating autoprocessing of adjacent zymogens (Srinivasula et al., 1998). The crystal structures of the caspase-recruitment domain (CARD) of Apaf-1 by itself and in complex with the prodomain of caspase-9 revealed that the molecule of Apaf-1 interacts with the molecule of procaspase-9 through two highly charged and complementary surfaces formed by non-conserved residues (Qin et al., 1999). Apaf-1 requires dATP/ATP hydrolysis to interact with cytochrome c, self-associate, and bind to procaspase-9. A recent study using highly purified cytochrome c, recombinant Apaf-1, and procaspase-9 indicated that Apaf-1 binds and hydrolyzes ATP or dATP to ADP or dADP, respectively (Zou et al., 1999). The hydrolysis of ATP/dATP

and the binding of cytochrome c promote Apaf-1 oligomerization, forming a large multimeric Apaf-1-cytochrome c complex that by itself is sufficient to recruit and activate procaspase-9 (Hu et al., 1999). Once activated, caspase-9 dissociates from the complex and becomes available to cleave and activate downstream caspases, such as caspase-3. Recent studies demonstrated that homozygous embryonic fibroblasts from Apaf-1$^{-/-}$ mice exhibit reduced response to various apoptotic stimuli (Cecconi et al., 1998; Yoshida et al., 1998). Moreover, the absence of the Apaf-1 protein prevents the activation of caspase-3 *in vivo* and the phenotype can be correlated with a defect in apoptosis. Caspase-9 and its cofactor Apaf-1 are essential downstream components of p53 in Myc-induced apoptosis (Soengas et al., 1999). Like p53 null cells, mouse embryo fibroblast cells deficient in Apaf-1 and caspase-9, but expressing c-Myc, are resistant to apoptotic stimuli that mimic conditions in developing tumors. Inactivation of Apaf-1 or caspase-9 substitutes for the p53 loss and promotes the oncogenic transformation of Myc-expressing cells. Taken together, these studies indicate that Apaf-1 is a critical component of cytochrome c-mediated apoptosis and may play a crucial role in controlling tumor development.

Role of Bcl-2 family members: The Bcl-2 family of proteins includes both repressors (Bcl-2, Bcl-x_L, Bcl-w, Bfl-1, Brag-1, Mch-1, and A1) and promoters (Bax, Bad, Bak, Bid, Bik, and Bcl-x_S) of apoptosis (Thompson, 1995; Yang et al., 1996). The variable amounts of Bcl-2 homology (BH) regions (BH1, BH2, BH3, and BH4) in Bcl-2 family members determine their capacity to interact with each other or with other unrelated proteins (Vaux et al., 1992; Boise et al., 1993; Oltvai et al., 1993; Hengartner & Horvitz, 1994; Sato et al., 1994; Yin et al., 1994; Yang et al., 1995). The ratio of pro- and anti-apoptotic members of the Bcl-2 family determines, in part, the response to death

signals. The presence of a carboxy-terminal transmembrane (TM) domain in most, but not all, e.g., Bid and Bad, influences their subcellular distribution (Monaghan et al., 1992; Krajewski et al., 1993; de Jong et al., 1994). While many proteins encoded by the Bcl-2 gene family predominantly localize to mitochondria (Baffy et al., 1993), Bcl-2 is also found in the nuclear membrane and endoplasmic reticulum (Lam et al., 1994; Ryan et al., 1994).

Growing evidence suggests that anti-apoptotic members of the Bcl-2 family can prevent or delay apoptosis induced by a variety of stresses, including radiation, anti-tumor drugs, viral agents, heat shock, oxygen free radicals, and depletion of trophic factors (Kharbanda et al., 1997; Kim et al., 1997; Kluck et al., 1997; Yang et al., 1997). It is believed that hydrophobic C-terminus of Bcl-2 and Bcl-x_L help in anchoring these proteins to the outer membranes of mitochondria, where they form a cation-selective ion channel, block the release of cytochrome c, and attract cytosolic adaptor molecules. The predicted three-dimensional structure of Bcl-x_L supports an arrangement of α-helices that mimics the membrane-translocation domain of bacterial toxins, such as the diphtheria toxin, and a membrane pore-forming domain N-terminal to the transmembrane domain (Parker & Pattus, 1993; Muchmore et al., 1996). Other pro-apoptotic family members, such as Bad and Bax, interfere with the activity of Bcl-2 or Bcl-x_L by binding to them and forming non-functional heterodimers (Manfredini et al., 1998). The interaction of Bcl-x_L with caspase-9 and Apaf-1 results in the inhibition of caspase-9 activation (Pan et al., 1998). Clem et al. (1998) reported direct interaction between Bcl-x_L and caspases. The caspase-mediated cleavage of Bcl-x_L loop domain converts Bcl-x_L from a protective to a lethal protein (Clem et al., 1998). Recent studies demonstrated that overexpression of

Bcl-2 and Bcl-x_L prevents mitochondrial release of both cytochrome c and AIF, and activation of caspase-3 (Kharbanda et al., 1997). It was also observed that cytochrome c interacts with Bcl-x_L, but not Bcl-x_S, and addition of excess Bcl-x_S inhibits the binding of cytochrome c to Bcl-x_L (Kharbanda et al., 1997). These studies suggest Bcl-2 and Bcl-x_L act upstream of cytochrome c, and their anti-apoptotic effects may, in part, be mediated by interfering with the cytochrome c-triggered cell death cascade.

CLL cells have recently been shown express high levels of Bcl-2 (Adams & Cory, 1998; Green & Reed, 1998). This may be one of the mechanisms responsible for reduced cell-death in CLL (Reed, 1998). The complicated actions of all the Bcl-2 members in CLL are not fully known (Hamblin & Oscier, 1997). To date it is not clear whether the proportion of anti-apoptotic and pro-apoptotic Bcl-2 family protein expression in CLL cells is different from normal cells, or there is hypomethylation of Bcl-2 gene in CLL cells to make this gene more active (Hanada et al., 1993; O'Brien et al., 1995). Translocation-caused Bcl-2 overexpression only accounts for 10% of CLL patients (O'Brien et al., 1995) or fewer (Reed, 1998), but the overall overexpression of Bcl-2 protein is much higher (O'Brien et al., 1995). Douglas et al. (1997) reported that CLL cells are resistant to transforming growth factor (TGF)-β-induced apoptosis. CLL cells have been shown to express caspases but the role of caspases on CLL cell behavior remains unclear (Reed, 1998).

Regulation of Bcl-2 gene family by phosphorylation: Studies have shown that partnering between Bcl-2 family proteins may be dependent on the phosphorylation state of the different members. The function of the pro-apoptotic molecule Bad is regulated by phosphorylation of two sites, serine-112 (Ser-112) and serine-136 (Ser-136) (Zha et al.,

1996). Phosphorylation at either site results in loss of the ability of Bad to heterodimerize with the survival proteins Bcl-x_L or Bcl-2. Phosphorylated Bad binds to 14-3-3 and is sequestered in the cytoplasm. Phosphorylation of Bad at Ser-136 is mediated by the serine/threonine protein kinase Akt-1/PKB, which is downstream of phosphatidylinositol 3-kinase (PI 3-kinase) (Datta et al., 1997). Fang et al. (1999) reported that activated Ras and Raf stimulate selective phosphorylation of Bad at Ser-112. A recent study demonstrated that p21-activated kinase 1 (PAK-1) phosphorylates Bad *in vitro* and *in vivo* on Ser-112 and Ser-136 (Schurmann et al., 2000).

The loop regions of Bcl-2 and Bcl-x_L contain several phosphorylation sites, and phosphorylation in this region modulates the anti-apoptotic effect of these proteins (Ito et al., 1997; Maundrell et al., 1997). This phosphorylation interferes with the dimerization of Bcl-2 and Bcl-x_L with Bax, thus driving the cells toward death (Haldar et al., 1995; Haldar et al., 1996). Phosphorylation of Bcl-2 protein is necessary for its anti-apoptotic effect in some models (May et al., 1994; Poommipanit et al., 1997). Since deletion of the "variable region", which lies between BH4 and BH3, potentiates the protective effect of Bcl-2, Bcl-2 phosphorylation may be involved in disabling its anti-apoptotic function. The apoptotic responses to many drugs, including paclitaxel, vincristine vinblastine, nocodazole, and dolastatin, have been shown to be mediated by Bcl-2/Bcl-x_L phosphorylation (Ling et al., 1998; Ruvolo et al., 1998; Scatena et al., 1998; Rodi et al., 1999; Srivastava et al., 1999). Taken together, these studies highlight the importance of Bcl-2/ Bcl-x_L in apoptosis.

JNK/SAPK phosphorylates Bcl-2/Bcl-x_L: The kinases involved in Bcl-2 phosphorylation include paclitaxel-activated Raf-1 (Blagosklonny et al., 1997);

bryostatin-1-induced mitochondrion-localized PKCα (Ruvolo et al., 1998), and paclitaxel or vincristine-induced PKA (Srivastava et al., 1998). Recently, a novel role for c-jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) in apoptosis was uncovered (Maundrell et al., 1997; Srivastava et al., 1999; Kharbanda et al., 2000). JNK was shown to phosphorylate Bcl-2 and Bcl-x_L (Maundrell et al., 1997; Poruchynsky et al., 1998; Srivastava et al., 1999; Kharbanda et al., 2000). IR exposure of U937 cells was shown to induce translocation of JNK to mitochondria. JNK associates with Bcl-x_L and phosphorylates this protein on threonine-47 (T-47) and threonine-115 (T-115) *in vitro* and *in vivo* (Kharbanda et al., 2000). In contrast to wild-type Bcl-x_L, a mutant Bcl-x_L with the two threonines substituted by alanines (A-47, -115) was found to be a more potent inhibitor of IR-induced apoptosis (Kharbanda et al., 2000). In addition, inhibition of JNK1 activity, either by overexpression of mitogen-activated protein kinase phosphatase 1 (MKP-1) or by a dominant negative SAPK/ERK kinase 1 (SEK-1), block Ser-70 phosphorylation of Bcl-2 and apoptosis in parallel (Srivastava et al., 1999). The translocation of JNK to mitochondria may be functionally important for interactions with Bcl-x_L in the apoptotic response to genotoxic stress. The JNK-mediated phosphorylation may be responsible for disabling the anti-apoptotic function of Bcl-2 and Bcl-x_L.

JNKs are a family of p54/p46 serine-threonine kinases encoded by three genes generating 10 protein kinase polypeptides (Derijard et al., 1995; Woodgett et al., 1996). These kinases are related to, but distinct from, the mitogen-activated protein kinases (MAPKs) and are regulated by SEK-1 (MKK4) (Kyriakis et al., 1994; Nishina et al., 1998). JNK signaling pathways have been implicated in cell proliferation and apoptosis, but its function seems to be dependent on the cell type and inducing signal. JNK-

mediated phosphorylation of c-Jun stimulates the transactivating potency of AP-1 (e.g., c-Jun/c-Fos; c-Jun/ATF-2), thereby increasing the expression of AP-1 target genes (Kyriakis et al., 1994; Kallunki et al., 1994; Gupta et al., 1995). The activation of JNK1 is an early response of many cells exposed to diverse stimuli including DNA damage, heat shock, interleukin-1 (IL-1), TNF-α, paclitaxel, and Fas ligation (Macgregor et al., 1990; Hibi et al., 1993; Derijard et al., 1994; Sluss et al., 1994; Adler et al., 1995; Osborn et al., 1996; Smith et al., 1997; Potapova et al., 1997). Some antineoplastic drugs, such as mafosfamide, mitomycin C, N-hydroxyethyl-N-chloroethylnitrosourea, and treosulfan, fail to induce JNK (Fritz & Kaina, 1999). The observation that defects in JNK signaling (e.g., in c-abl deficient fibroblasts) leads to a state of relative resistance to genotoxic stress supports the likelihood that abnormalities in JNK expression and/or function may play a role in the development of clinical drug resistance.

V.6 ***Present Treatment of CLL:*** The traditional drug used for many years for CLL treatment is the nitrogen mustard alkylating agent chlorambucil (CLB), with or without combination with prednisone (Begleiter et al., 1996a). CLB enters the cells through passive diffusion and is generally thought to cross-link with DNA to cause inhibition of DNA replication (Begleiter et al., 1994; Begleiter et al., 1996a). CLB can also bind to RNA, proteins and the membranes of cells, and thus affect protein synthesis, enzyme activities and signal transduction of CLL cells (Begleiter et al., 1996a). Since CLL cells do divide little and most of them stay in G0, CLB may also affect other pathways involved in cell cycle control (Hambin & Oscier, 1997; Cory & Adams, 1998). CLB has also been shown to induce apoptosis of CLL cells (Begleiter et al., 1994). The clinical response rate to CLB is from 38-87% (O'Brien et al., 1995), and the survival of patients

has changed little since its inception (O'Brien et al., 1995). This drug rarely causes complete remission of the disease and clinical drug resistance is reported (O'Brien et al., 1995; Begleiter et al., 1996a). The mechanism of drug resistance is not clear but may involve the induction of glutathione S-transferase (GST) activity to metabolize the drug, increased DNA repair, and the induction of the multiple drug resistance (MDR) gene (O'Brien et al., 1995; Begleiter et al., 1994; Begleiter et al., 1996a; Byrd et al., 1998). The combination of CLB with prednisone is used to treat complicating autoimmune phenomena (Byrd et al., 1998).

Nucleoside analogues, such as fludarabine and 2-chorodeoxyadenosine (2-CDA), were introduced into clinical trials in the mid-80s and the most promising one is fludarabine. Fludarabine is a purine analogue (O'Brien et al., 1995). It enters the cells by carrier-mediated transport and is then changed to F-ara-adenine triphosphate (F-ara-ATP). F-ara-ATP is not easily metabolized, stays in the cells to inhibit ribonucleotide reductase and DNA polymerase, and promote apoptosis (Byrd et al., 1998; O'Brien et al., 1995). In normal cells, it is more potent for $CD4^+$ cells than for $CD8^+$ cells. This may explain the autoimmune phenomenon observed in fludarabine-treated patients (Pritsch et al., 1998). Compared with CLB, fludarabine gives a higher complete remission rate and disease-free survival (Byrd et al., 1998). Some patients are refractory to fludarabine treatment and myelosupression and hemolytic anemia is also observed in some fludarabine-treated patients (Byrd et al., 1998). The combination of purine analogue and alkylator yields a synergic effect (Johnston et al., 1994), but it is too early to draw a conclusion about the benefit of this combination.

Recently, some biological agents, such as PKC inhibitor UCN-01, protein kinase activator bryostatin, topoisomerase I inhibitors, CDK inhibitors and anti-CD52 antibody Campath-1 H have also been tried both *in vitro* and *in vivo* (Byrd et al., 1998). None of these therapies are curative. In addition, *de novo* drug resistance or the development of cellular resistance during drug therapy are major obstacles encountered to cure this disease. Newer modalities of therapy such as allogenic bone marrow transplantation may have the potential of cure (Flinn & Vogelsang, 1998). However, most of the patients are not eligible due to advanced age, and the unavailability of suitable donors limits the potential of this modality to younger patients. Therefore, the search for new therapies to treat CLL patients remains an elusive goal.

MATERIAL AND METHODS

VI.1 ***Antibodies and Reagents:*** The protein assay kit was bought from Pierce Chemical Company (Rockford. IL). Mouse anti-phosphotyrosine (4G10). mouse anti-Src. mouse anti-PLC-γ. rabbit anti-p85. rabbit anti-MAP kinase and rabbit anti-Lyn antibodies were purchased from Upstate Biotechnology (Lake Placid. NY). goat anti-Akt. goat-anti-CPP32. goat and rabbit anti-stress-activated protein kinase (SAPK). rabbit anti-focal adhesion kinase (FAK). rabbit anti-Src antibodies. donkey anti-goat IgG and donkey anti-mouse IgG conjugated fluorescein isothiocyanate (FITC). and donky anti-goat IgG conjugated with horseradish peroxidase (HRP) were from Santa Cruz Biotechnology. Inc. (Santa Cruz. CA). mouse anti-Grb-2. anti-Shc and anti-paxillin antibodies were from Transduction Laboratories (Lexington. KY). goat anti-mouse IgG1 and goat anti-rabbit IgG1 conjugated with Texas Red were from Southern Biotechnology Associates. Inc. (Birmingham. AL). Goat anti-mouse IgG conjugated HRP and goat anti-rabbit IgG conjugated with HRP were from Bio-Rad Laboratories Ltd. (Mississauga. ONT). Rabbit anti-cytochrome c. rabbit anti-Bcl-x_L antibodies and purified c-Jun protein were kindly provided by Dr. S. Kharbanda (Dana-Farber Cancer Institute. Boston. MA). Mitochondria-specific probe MitoTracker™ Green FM was purchased from Molecular Probes. Inc. (Eugene. OR). Protein A Sepharose CL-4B beads were purchased from Amersham-Pharmacia Biotech Inc. (Baie d'Urfe'. Quebec), p-nitrophenyl phosphate (p-NPP) and poly-amino acid mixtures [Poly (Glu. Tyr) 4:1. Poly (Glu. Tyr) 1:1 and Poly (Glu. Ala. Tyr) 6:3:1] were from Sigma-Aldrich Canada Ltd. (Oakville. ONT). sodium

dodecyl sulfate (SDS) and tris (hydroxymetyl) aminomethane (Tris) were from Bio-Rad Laboratories Ltd. (Mississauga, ONT), acrylamide and acrylamide/Bis were from Fisher Scientific Ltd. (Nepean, ONT). 4,6-diamidino-2-phenylindole (DAPI), Mowiol 40-88, 1,4 diazabicyclo[2,2,2]octane (Dabco), fludarabine, genistein and other chemicals and reagents were from Sigma-Aldrich Canada Ltd. Adenosine 5' triphosphate (ATP, 100 μM solution) was purchased from Amersham Pharmacia Biotech Inc. Phosphorus-^{32}-ATP (γ-ATP) was purchased from NEN™ Life Science Products, Inc. (Boston, MA).

VI.2 ***Cell Lines and Lymphocytes from Normal Donors and CLL Patients:*** Human CLL cell line WSU-CLL was kindly provided by Wayne State University School of Medicine (Detroit, MI). This cell line is a B-CLL line established from the peripheral blood of a 66-year-old male with CLL without EBV infection or growth factor stimulation. The cell line grows in liquid culture, soft agar and SCID mice. WSU-CLL cells were maintained in RPMI 1640 containing 10% fetal calf serum (FCS) (Gibco BRL Products, Life Technologies, Gaithersburg, MD), 100 units/ml penicillin G and 200 μg/ml streptomycin (Sigma-Aldrich Canada Ltd., Oakville, ONT) at a density of 2×10^5 /ml at 37°C with 5% CO_2. Human monoblastoid cell line U937 and its stable Bcl-x_L-transfected counterpart U937/Bcl-x_L (provided by Dr. S. Kharbanda, Dana Farber Cancer Institute, Boston, MA) were maintained under conditions described above for the WSU-CLL cells. Human acute myloid leukemia cell line HL60 was from American Type Culture Collection (ATCC, Rockville MD) and was maintained in the same condition as U937. Blood samples from normal donors and CLL patients were collected according to the guidelines of the University of Manitoba. Lymphocytes were collected by the following method: heparinized blood was centrifuged at 250 g for 10 min. The plasma

layer was aspirated and the buffy coat was then aspirated and resuspended in 5 ml phosphate buffer saline (PBS), pH 7.2. The cells were then layered onto 5 ml Ficoll-Hypaque (Lymphoprep Nyegaard, Oslo) and centrifuged for 30 min at 800 g. The lymphocytes at the interphase were aspirated and resuspended in 5 ml solution containing 10 mM Tris-HCl, 140 mM NH_4Cl, pH 7.4. Incubating the cell suspension at 37°C for 5 min destroyed red blood cells. Finally, the lymphocytes were pelleted in PBS and counted by a Coulter Counter. Normal lymphocytes are total peripheral blood lymphocytes (PBL) of the mixture of total B and T cells. For culturing the cells, the cells were suspended in RPMI 1640 containing 10% FCS at the density of 5×10^5/ml and cultured at 37°C in 5% CO_2 with 100% humidity.

VI.3 ***Preparation of Sodium Orthovanadate:*** To make sodium orthovanadate active and functional as a phosphotyrosine phosphatase inhibitor, it was prepared according to the protocol from the manufacture (Sigma-Aldrich Canada). Briefly, sodium orthovanadate was diluted with water and adjusted to a pH of 10 by sodium hydroxide and then boiled until the yellow color disappeared. The pH was readjusted to 10 and the solution was boiled until it became clear. The stock solution (100 mM) was aliquoted and stored at –20°C.

VI.4 ***Vanadate Treatment of Cells and Preparation of Cell Lysates:*** Freshly prepared normal lymphocytes, patient lymphocytes and sub-confluent cell lines were put into fresh media at a concentration of 2×10^5/ml and different concentrations of sodium orthovanadate were added. The cells were cultured at 37°C in 5% CO_2 with 100% humidity and collected at various time points. For immunoprecipitation, western blotting and *in vitro* kinase assays, treated and control cells collected at different time points were

washed twice with cold PBS, pH 7.4 and the cell pellet was resuspended in a buffer containing 50 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, 10 μg/ml NaF, 1 mM DTT and 1% NP-40 (lysis buffer). The cells were incubated on ice and mixed every 5 min. After 30-min incubation on ice, the cell lysates were passaged through a 23 G needle 5 times and centrifuged at 10,500 g for 15 min at 4°C. The cell lysates for studies aimed at analyzing total protein tyrosine kinase and phosphatase activity were prepared by suspending the cells in 50 mM Tris-HCl, pH 7.4 and sonicating the cells at 4°C. The supernatant collected after centrifugation was stored at -80°C.

To study the release of cytochrome c from mitochondria, cytosolic fractions were prepared according to Kharbanda et al. (1997) and Yang et al. (1997). The vanadate-treated and untreated cells at different time points (from 6 to 48 hr) were suspended in cold hypotonic buffer containing 20 mM HEPES, pH 7.5, 250 mM sucrose, 10 μg/ml pepstatin A, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM PMSF, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 10 mM KCl, and 1.5 mM $MgCl_2$. After 30-min incubation on ice, cells were homogenized by douncing (2 times) with a Wheaton dounce tissue grinder (Sigma-Aldrich). The cell homogenate was centrifuged at 800 g for 5 min at 4°C. The supernatant was then centrifuged again at 10,000 g for 30 min at 4°C. The soluble cytosolic fraction was obtained by further ultracentrifugation at 50,000g at 4°C for 30 min and collection of the supernatant.

VI.5 ***Immunoprecipitation and Western Blotting:*** Protein assays were performed according to the manufactur's protocol (BCA Protein Assay Kit, Pierce, USA). Briefly, BSA standard and cell extracts were diluted in the same buffer to make the testing

condition equal and bicinchoninic acid (BCA) reagents were added and incubated with the testing sample for 30 min at 37°C. The absorption of the samples was tested at 562 nm and the protein amount was calculated and obtained by Sigma plot. Immunoprcipitation was done according to Sambrook, et al. (1989) and the protocols from Transduction Laboratories. Briefly, 500 μg of cell extract equilibrated in 500 μl lysis buffer was mixed with 2 μg antibody. To test the non-specific binding, a control immunoglobulin or serum matching the isotype of the antibody was also added in a separate tube. After overnight incubation at 4°C, the antigen-antibody complexes were recovered on protein A-sepharose beads. For another checking of the non-specific binding of the cell lysates to the beads, the cell lysate was also incubated with beads alone for 2 hr. The beads collected after 2-hr incubation were washed twice with lysis buffer and once with 0.5 M $LiCl_2$ in 0.1 M Tris-HCl, pH 7.4. For western blotting after immunoprecipitation or direct western blooting, 30 μl 1x sample buffer (62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 100 mM DTT, 1 mM sodium orthovanadate, 0.02% bromophenol blue) was added onto the beads or certain amount (10 μg protein for mini gel and 30 μg for large gel) of cell extract and the mixtures were immediately boiled for 5 min. The supernatant collected after centrifugation (5 min at 10,000 g) was subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The concentration and the type of the gel used were dependent on the molecular weight of the protein of interest. Most proteins were resolved by regular SDS-PAGE (Sambrook et al., 1989), but caspase 3 and cytochrome c were resolved in tricine SDS-PAGE (Schagger and Von Jagow, 1987). The proteins were transferred onto the nitrocellulose membrane (Bio-Rad) at 4°C for 8 hr in a wet system according to the instrument manufacture (Amersham-Phamacia). After

protein transfer, the membrane was stained with ponceau S to double check the equal loading of protein and equal transfer. The membrane was washed with Tris buffer saline-tween-20 (TBST) (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1% Tween 20) for 5 min and then blocked with 2% bovine serum albumin (BSA) in TBST for for 2 hr. The membrane was then washed 2 times (10 min each) with TBST, and incubated overnight with appropriate primary antibody overnight in the cold room. After two 30-min washes with TBST the membrane was incubated for 1 hr (at room temperature) with appropriate secondary antibody conjugated with HRP. The membrane was washed thoroughly in Tris buffer saline (TBS) buffer and proteins were visualized by enhanced chemiluminescence (ECL, Amersham-Phamacia) with ECL specific Hyperfilm (Amersham-Phamacia).

VI.6 ***Protein Tyrosine Phosphatase Assay:*** The experimental method was used according to the previously reported (Kremerskothen and Barnekow, 1993). Briefly, 30 μg of sonicated cell lysate in 50 mM Tris-HCl; pH 7.5 was transferred to a 96-well plate (total volume 100 μl per well) in triplicate. To each well, 100 μl of 10 mM p-NPP in 50 mM Tris-HCl, pH 7.5 was added (the final concentration of p-NPP was 5 μM). The release of p-nitrophenyl (yellow color) by tyrosine phosphatases in the cell lysate was measured at 405 nm using a Titertek Multiskan® MCC/340 ELISA reader (LabSystems, Finland).

VI.7 ***In Vitro Kinase Assay:*** Kinase assay was done according to the protocol from Transduction Laboratories. The total kinase activity in the sample was detertermined by incubating 25 μg of sonicated cell lysate with 0.1-100 μg/ml poly-amino acid mixture, 1 μCi γ-ATP, 50 μM ATP in 50 μM Tris-HCl in a total volume of 20 μl. The incubations were conducted for 15 min at 30°C and reactions stopped by the addition of 5x SDS

sample buffer (5 μl). To quantify the kinase reaction, a fraction of the reaction mixture was spotted onto filter papers. After air-drying, the filter paper was washed in 1% phosphoric acid buffer for 40 min with the change of wash every 10 min. The radioactivity in air-dried filters was counted by a Beckman LS 9800 liquid scintillation counter (Beckman® Instruments Inc., Irvine, CA). Another fraction of the reaction mixture was also analyzed by SDS-PAGE and radioactivity in the band of interest was analyzed by autoradiography with Kodak film.

To determine the kinase activity of Akt and SAPK, 500 μg of cell lysate was used for immunoprecipitation as described above. The beads with immune complexes were washed 2 times with lysis buffer and once with 0.5 mM $LiCl_2$ in 0.1 mM Tris-HCl, pH 7.4. The beads were finally washed three times with kinase buffer (20 mM HEPES-KOH, pH 7.5, 10 mM $MgCl_2$, 0.1 mM sodium orthovanadate and 2 mM DTT) and the appropriate substrate (total histone for Akt and c-Jun protein for SAPK) was added together with 50 μM ATP and 5 μCi γ-ATP in 30 μl kinase buffer. The mixture was incubated at 30°C for 15 min and reaction was stopped by the addition of 5x SDS sample buffer. After the removal of beads by centrifugation at 10,000 g for 5 min, the supernatant was analyzed by either regular SDS-PAGE or tricine SDS-PAGE. The incorporation of radioactivity in the substrate was determined by a phosphoimager (Molecular Dynamics, Sunnyvale, CA).

The phosphotyrosine associated PI 3-kinase activity was determined by *in vitro* kinase assay performed on anti-phosphotyrosine immune complexes on protein A Sepharose beads. The protocol was from Dr Saxena's laboratory in Manitoba Institute of Cell Biology. Briefly, 500 μg protein was used to immunoprecipitate phosphotyrosine-

containing proteins using 2 μg anti-phosphotyrosine antibody 4G10 (Upstate Biotechnology). The immune-complexes on beads were washed 2 times with lysis buffer and 3 times with 10 mM Tris-HCl, pH 7.4. 10 μg of phosphatidylinositol (PI) was then added in a total volume of 10 μl. After 10-min incubation on ice, 40 μl of kinase buffer (30 mM HEPES-KOH, pH 7.4, 30 mM $MgCl_2$, 50 μM ATP, 10 μCi γ-ATP) was added to start the reaction. The incubations were performed at room temperature for 15 min and the reaction was stopped by the addition of 100 μl HCl followed by chloroform: methanol (50:50, v/v). The mixture was vortexed for 30 s and layers were separated by centrifugation (10000 g for 5 min at 4°C). The lower organic layer was removed carefully and stored at −20°C. TLC plate was prepared by drying at 80°C for 1 hr. Lipids (PI) were then separated on TLC plate by spotting the organic layer fraction (20 μl) on plates and air-dry. The plate was put in a glass container with an organic buffer containing 50% chloroform, 40% methanol, 3% ammonium hydroxide. The plate was taken out when the buffer moved to the top end of the plate and air-dried. The radioactivity was visualized by exposing the Kodak film at −80°C and autoradiography.

VI.8 ***Cell Growth and Clonogenic Assay:*** The method was used according to the previously reported (Kennedy et al., 1997; Uckun et al., 1998) and the protocol from Dr Johnston's laboratory on Manitoba Institute of Cell Biology. The viable and dead cells were counted at 40-hr vanadate treatment with trypan blue dye exclusion under a light microscope (Nikon-TMS, Japan). About 30% of cell died in 10 μM vanadate-teated cells. The viable cells (250-5,000) from vanadate-treated and control experiments were transferred to 12-well plates containing fresh media with 15% FCS and 0.3% agar. After the solidification of agar on ice, the plates were kept in an incubator for 10-14 days and

the cell colonies were counted under a light microscope. A colony is defined here as a cell colony with more than 20 cells. The percentage of the cell clones formed out of the cells originally put in the well at the beginning of the experiment was calculated.

VI.9 ***Apoptosis Assay:*** The method was adopted from the previous reports (Uckun et al., 1995; Uckun et al., 1998). Cells (1×10^7) collected at each time point were washed with cold PBS and resuspended in 500 µl buffer containing 5 mM Tris-HCl, pH 7.4, 0.5% Triton-100, 5 mM EDTA. After 20-min incubation on ice, the supernatant was collected by centrifugation at 1000 g, RNAase A added (final concentration of 50 µg/ml), and mixture was incubated at 37°C for 2 hr. Proteinase K was then added to a final concentration of 20 ng/ml and the supernatant was incubated at 65°C for 15 min followed by overnight incubation at 37°C. After overnight incubation, 1/10 volume (50 µl) of sodium acetate, pH 7.4 was added. After thorough mixing an equal volume (550 µl) of chloroform: isoamyl alcohol: phenol (24:1:25) was added. The layers were separated by centrifugation at 10,000 g for 20 min. The aqueous layer was transferred to a 2-ml tube and 2.5 volume (around 1.3 ml) of absolute ethanol was added. The mixture was kept at -80°C for 15 min and then centrifuged at 10,000g for 20 min at 4°C. The pellet was drained, air dried and resuspended in 50 µl TE (10 mM Tris-HCl, pH 7.4, 1 mM EDTA). All the DNA samples collected were then run on a 2% agarose gel with 0.5 µg/ml ethidium bromide and DNA was observed under ultraviolet light.

VI.10 ***Immunofluorescence Staining:*** The protocol was from Dr Mai's laboratory in Manitoba Institute of Cell Biology. Cells at desired time points were washed with PBS and collected onto glass slides by centrifugation. The slides were fixed with 3.7% formaldehyde in PBS for 10 min, washed twice with PBS, and cells permeabilized with

0.2% Triton-100 in deionized water for 12 min. The cells were washed 3 times with PBS prior to blocking with 2% BSA in PBS for 5 min. The cells were then incubated for 45 min with the anti-phosphotyrosine antibody 4G10 (5 μg/ml). The slides were washed three times with PBS and blocked again with 2% FCS for 5 min. The cells were finally incubated with Texas Red-conjugated goat anti-mouse IgG1 (1:100 dilution). In a few studies, cells were incubated with MitoTraker, a mitochondria specific probe at concentrations of 1000 nM. After incubation for 30 min, the slides were washed 3 times with PBS and incubated with DAPI (1 μg/ml) for 5 min to get nuclear staining. Finally, anti-bleach was added and cover slips were put onto the slides. Anti-bleach was prepared by mixing 6 g glycerol, 2.4 g Mowiol 40-88, and 6 ml deionized water. The mixture was stirred for 2 hr at room temperature and 1-2 hr at 50°C and then aliquoted. 2.5% Dabco was added before use. The immunofluorescence was analyzed with a Zeiss Axiophot microscope (Carl Zeiss Canada, Don Mills, ONT).

RESULTS

VII.1 ***WSU-CLL Cell Line is Sensitive to Vanadate Treatment:*** To study the role of tyrosine phosphorylation on CLL cell death. CLL cell line WSU-CLL and three other leukemia cell lines were first treated with different concentrations of vanadate and the viable cell numbers were checked at different time points with the trypan blue dye exclusion method. As shown in figure 1A. WSU-CLL cells were most sensitive to vanadate treatment compared to HL60 (acute myloid leukemia). U937 (monoblastoid leukemia) and even $Bclx_L$-transfected U937 cells. Further studies from 3 separate experiments shown in figure 1B confirmed that cell growth of WSU-CLL cells was more inhibited as the time of incubation and the dose of vanadate were increased. At 48 hr. the number of viable cells decreased to about 50% in vanadate (10 μM) -treated cells. This indicates that WSU-CLL cells are easily killed by vanadate treatment. The dead cells were about 40% of the total number of cells after 10 μM vanadate treatment for 48 hr (data not shown).

VII.2 ***Vanadate Inhibits Colony Formation of WSU-CLL Cells:*** To further explore the role of vanadate on WSU-CLL cells. The cytotoxicity of vanadate was determined by soft agar colony formation assay as described in Material and Methods. The experiment was repeated three times. WSU-CLL cells were treated with vanadate (10 μM) or fludarabine (as a positive control. Johnston et al.. 1994). Viable WSU-CLL cells were cultured for 14 days in semi-solid media and the cell colonies are counted under microscope. As shown in Fig. 2. vanadate and fludarabine treatment of WSU-CLL cells dramatically inhibited

colony formation. In vanadate-treated cells, the colony formation was inhibited by 68% compared with the non-treated cells. The co-treatment of cells with vanadate and fludarabine did not produce a synergistic effect on the inhibition of colony formation.

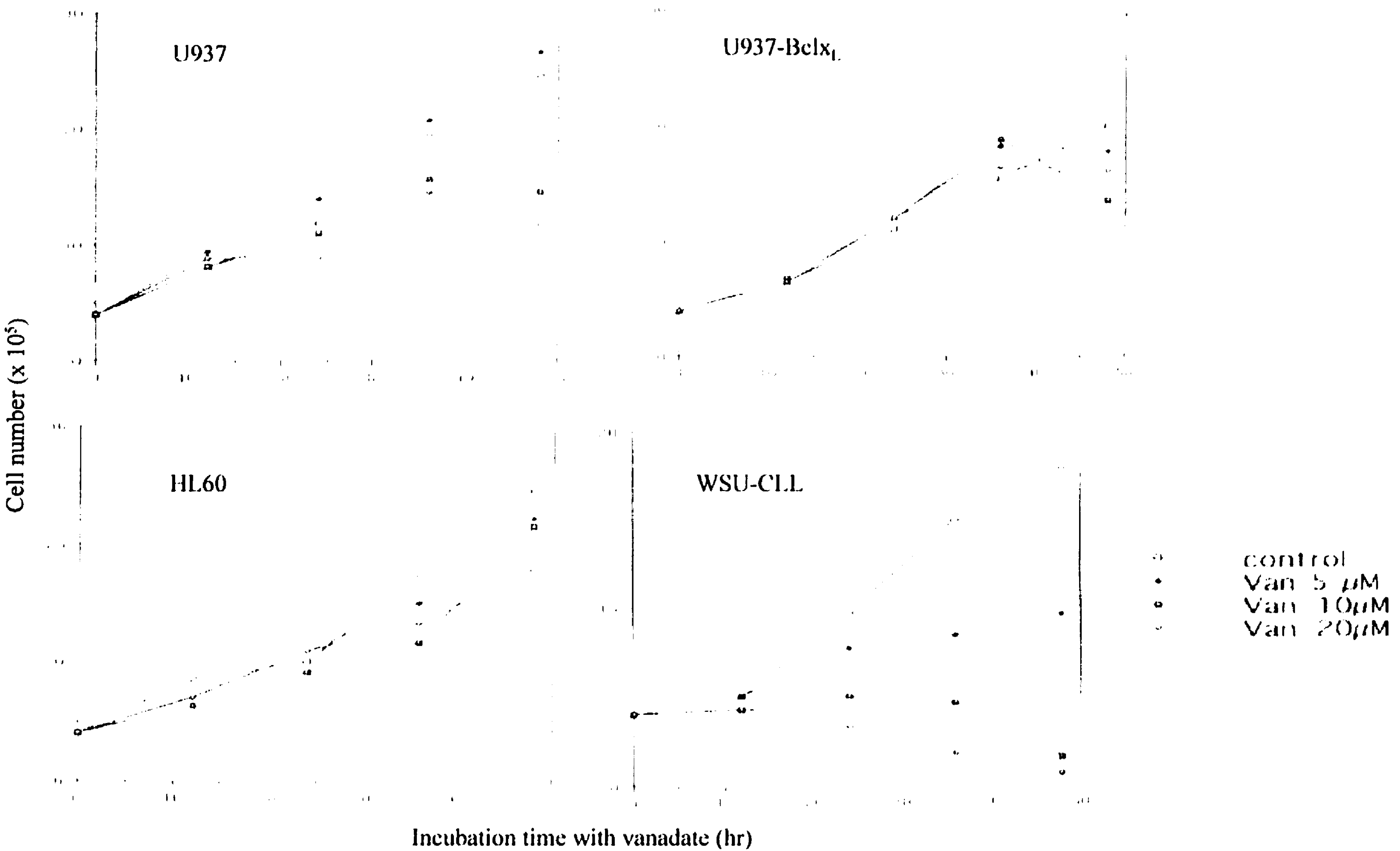


Fig. 1A Vanadate treatment on the growth of different leukemia cell lines. Cells were incubated with different concentration of vanadate and the cell number were counted at different time points with trypan blue dye exclusion method.

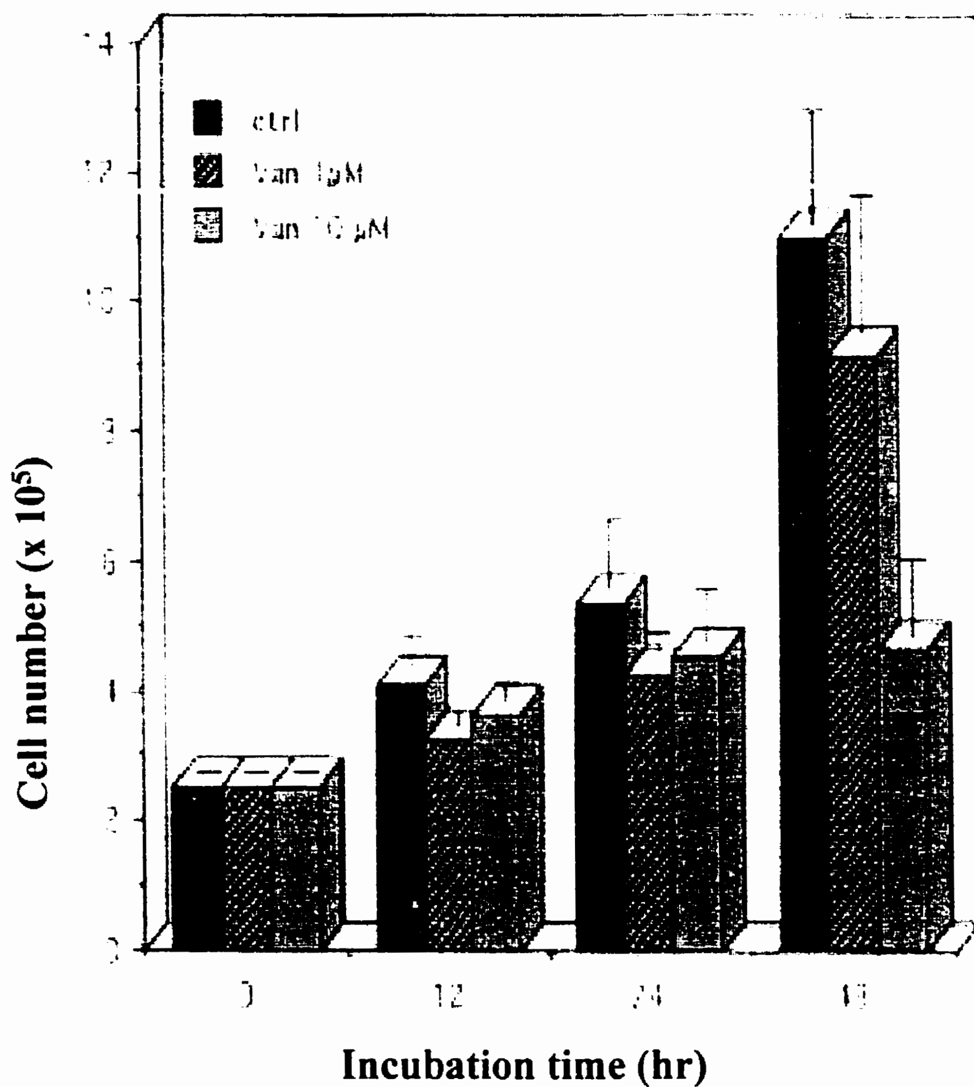


Fig. 1B Vanadate inhibits WSU-CLL cell growth. WSU-CLL cells were incubated with different concentrations of vanadate (Van) and cell number were counted by trypan blue dye exclusion. The results were from three separate experiments and expressed as means±SE.

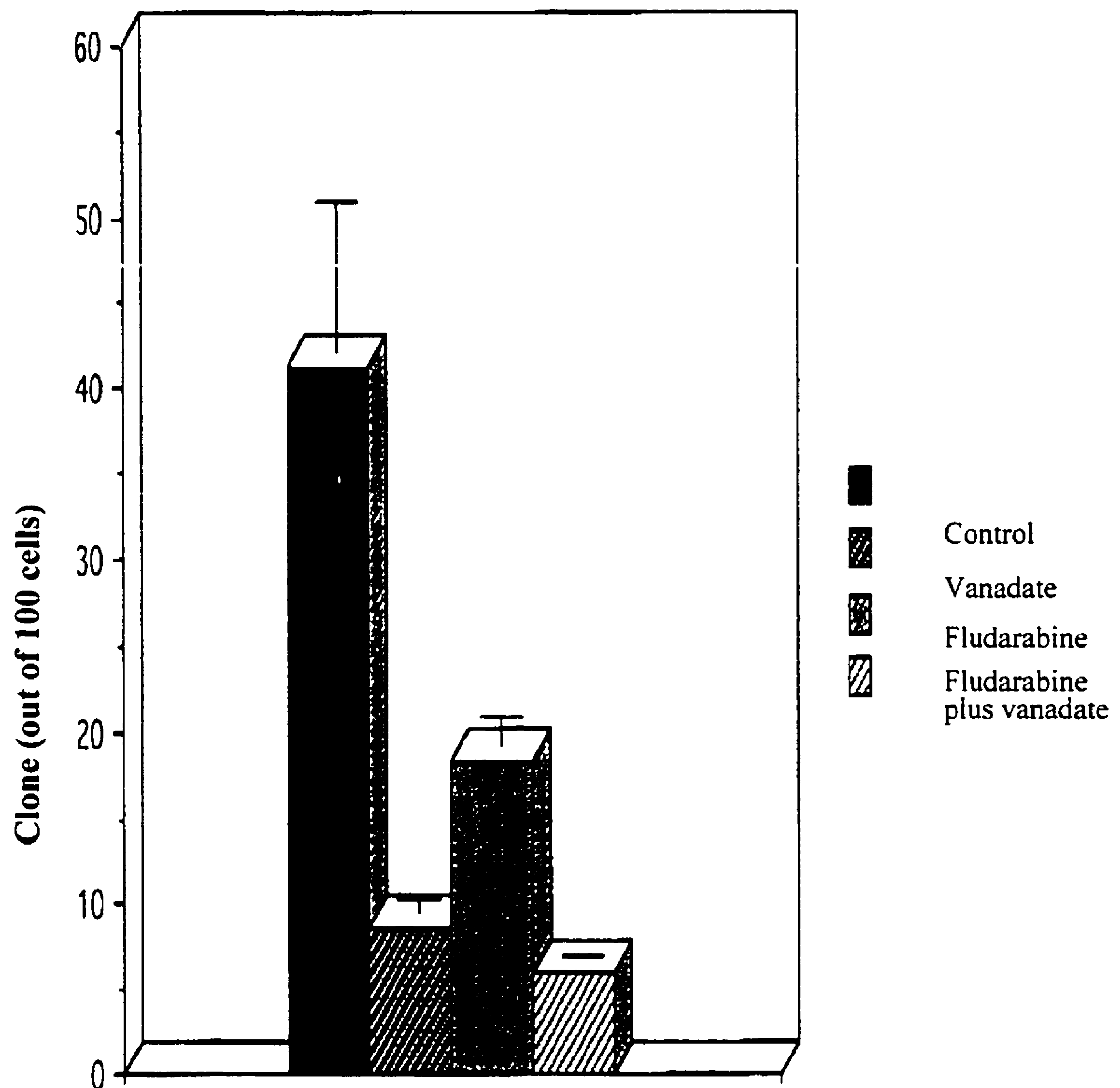


Fig. 2 Vanadate inhibits colony formation of WSU-CLL cells. The cytotoxicity of vanadate was determined by soft agar clonogenic assay as described in the Material and Method section. Cells were treated with vanadate (10 μM) and fludarabine (2 μM) alone or in combination. Viable cells (250-5000 cells per well of 6-well culture plates) were cultured for 14 days in semi-solid media and the cell colonies (maximum 150-300 colonies in untreated well of 500 cells originally put) were counted under light microscope. The values are from 3 standard experiments and expressed as means±SE.

VII. 3. ***Vanadate-induced Growth Inhibition was Accompanied by Increased Tyrosine Phosphorylation.*** To examine whether protein tyrosine phosphorylation can be modulated by vanadate treatment. Tyrosine phosphorylation was checked by western blotting with phosphotyrosine specific antibody. As shown in Fig. 3, incubation of WSU-CLL cells with increasing concentrations of vanadate greatly increased the tyrosine phosphorylation of several proteins. These include proteins of apparent *Mr* 150-170, 120-130, 97-100, 80-85, 70-72, 55-60, 40-45, and 32-35 kDa. Since 1 μM vanadate caused a minimal effect on protein tyrosine phosphorylation, 10 μM and 100 μM vanadate was used in subsequent experiments.

VII.4. ***Vanadate-induced Increase in Tyrosine Phosphorylation is Through the Inhibition of Tyrosine Phosphatase Activity, but not the Increase of Total Tyrosine Kinase Activity.*** To confirm whether vanadate-induced tyrosine phosphorylation in WSU-CLL cells is specifically due to inhibition of protein tyrosine phosphatase activity, cells were treated with vanadate (10 and 100 μM) for 40 hr and protein tyrosine phosphatase activity was measured. As shown in Fig. 4A, vanadate treatment dramatically inhibited (55% after 10 μM and 70% after 100 μM) the protein tyrosine phosphatase activity in WSU-CLL cells in parallel with increased tyrosine phosphorylation shown in Fig. 3.

The total tyrosine kinase activity was also measured in the cell lysate following 48-hr vanadate (10 μM) treatment. As shown in Fig. 4B, vanadate treatment enhanced the total phosphotyrosine kinase activity by only 9%. Because this experiment was only done once, statistics was not done in this experiment.

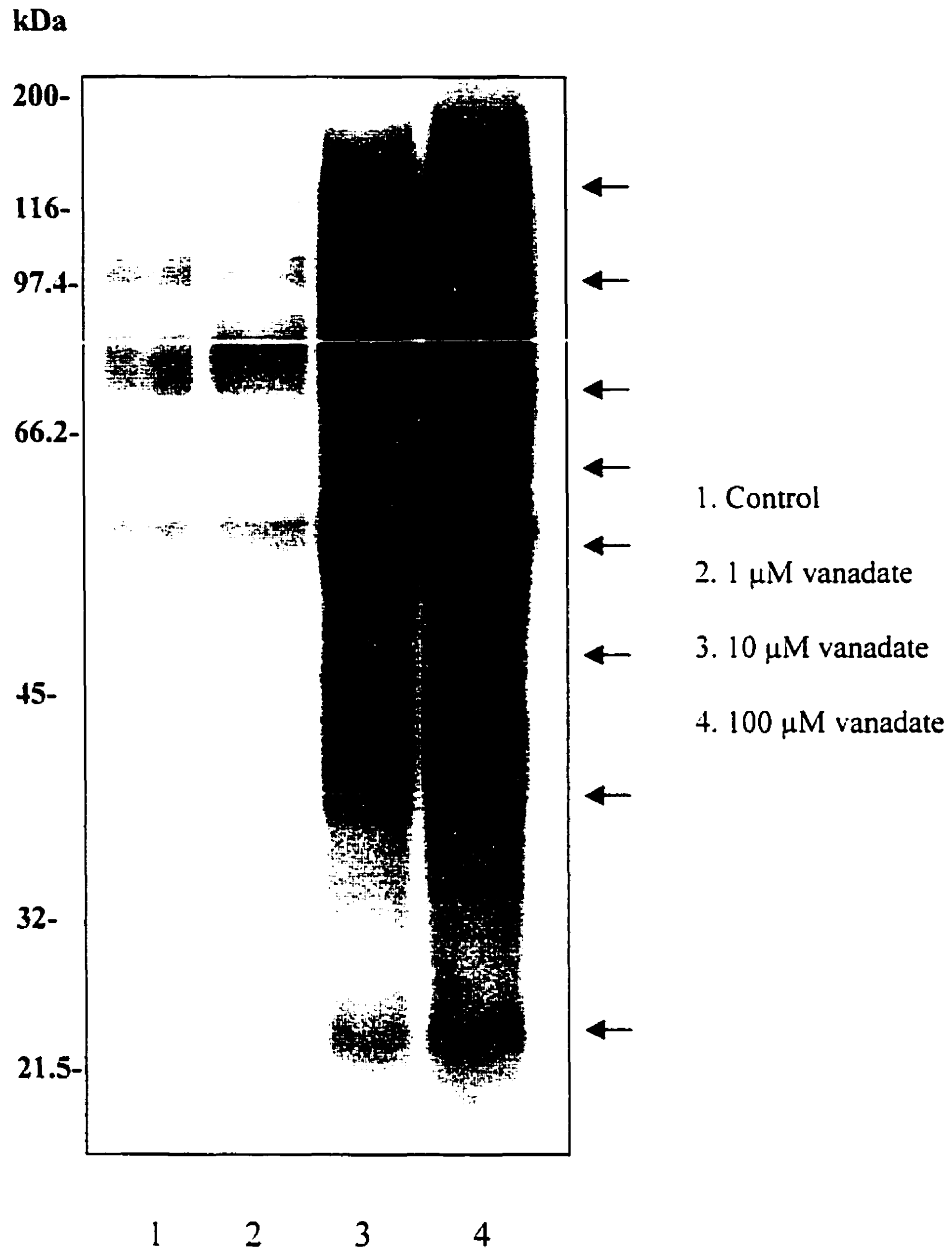


Fig. 3 Vanadate induces tyrosine phosphorylation in WSU-CLL cell line. WSU-CLL cells were treated with 1 μM (lane 2), 10 μM (lane 3) and 100 μM (lane 4) vanadate and cell lyastes were resolved on 10% SDS-PAGE, transferred onto nitrocellulose membrane, stained with ponceau S to confirm equal loading of protein and then immunoblotted with anti-phosphotyrosine antibody 4G10. The position of the tyrosine phosphorylated proteins exhibiting changes and molecular weight are indicated. Similar experiment was done 3 times.

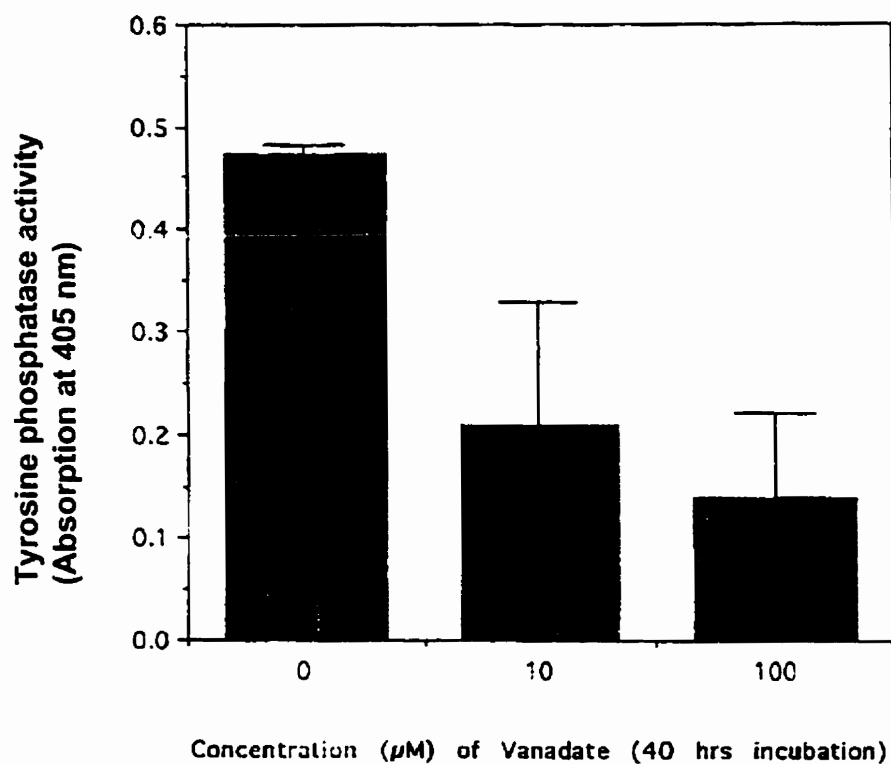


Fig. 4A Vanadate-induced tyrosine phosphorylation in WSU-CLL cells is specifically due to inhibition of protein tyrosine phosphatase activity. WSU-CLL cells were treated with 10 and 100 μM vanadate and cells harvested 40 hrs after treatment. 30 μg of sonicated cell lysate in 50 mM Tris-HCl: pH 7.5 was used for protein tyrosine phosphatase assays using p-NPP as a substrate. The release of p-nitrophenyl (yellow color) by tyrosine phosphatases in the cell lysates was measured at 405 nm using an ELISA reader. The values are means±SE of the results of three standard experiments.

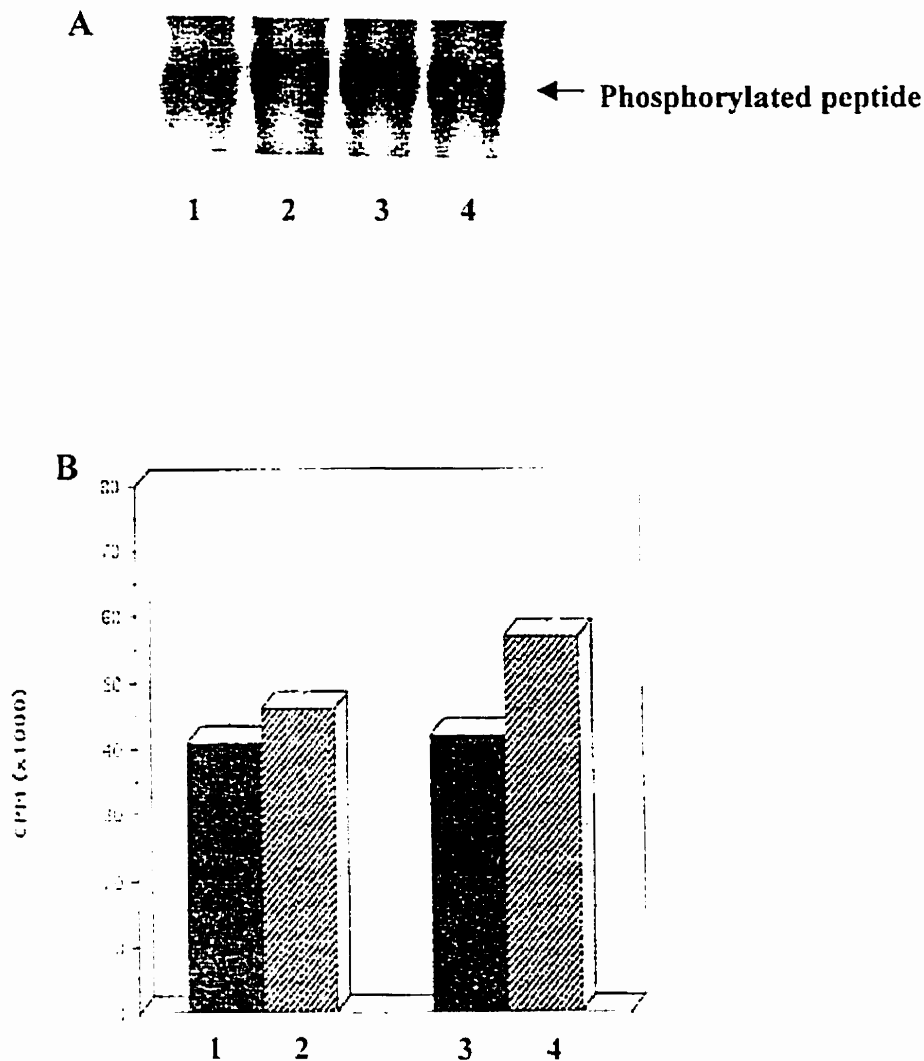


Fig. 4B Effect of vanadate treatment on total tyrosine kinase activity in WSU-CLL cells. WSU-CLL cells were treated with 10 μM vanadate and cells were harvested after 48 hrs. The total tyrosine kinase activity in control (lane 1 and 3) and vanadate treated (lane 2 and 4) cells was measured as described in the Material and Methods section. The reactions were terminated at 15 (lane 1 and 2) and 30 min (lane 3 and 4) after the addition of kinase buffer. A fraction of the reaction mixture was analysed by SDS-PAGE and autoradiography (A). To quantify the kinase reaction, reaction mixture was also spotted onto filter papers and the radioactivity in filter papers (triplicate) was measured by a scintillation counter (B). The result is from one standard experiment after preliminary titration of the substrate amount and reaction time.

VII. 5 ***DNA Fragmentation Appeared in WSU-CLL Cells Following Treatment with Vanadate:*** To determine if the decrease in cell viability and the increase of tyrosine phosphorylation and cell death of WSU-CLL cells was through programmed cell-death (apoptosis), vanadate-treated WSU-CLL cells were stained with DAPI and anti-phosphotyrosine antibody and examined through fluorescence microscopy. As shown in Fig. 5A, the dead cells exhibited the characteristics of apoptotic cells: cell membrane blebbing, nuclear condensation and apoptotic body formation. The vanadate-induced apoptosis in WSU-CLL cells is accompanied by high levels of tyrosine phosphorylation in these cells as confirmed with the co-staining with anti-phosphotyrosine antibody. The effect of vanadate on apoptosis was further confirmed by apoptosis assay. As shown in Fig. 5B, even though total amount of DNA in each lane was not titrated, vanadate and fludarabine (as a positive control) treatment definitely induced the DNA ladder formation in WSU-CLL cells.

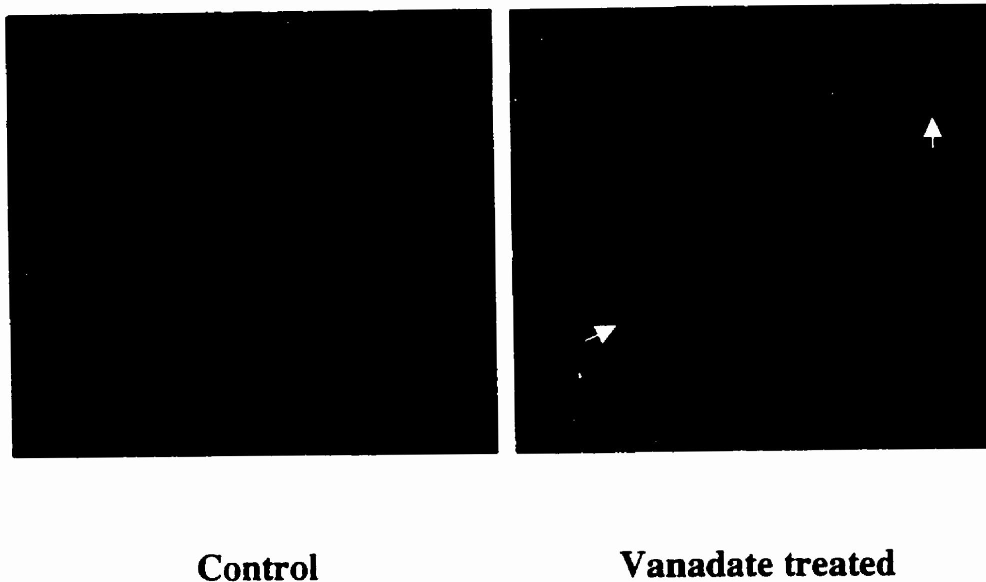
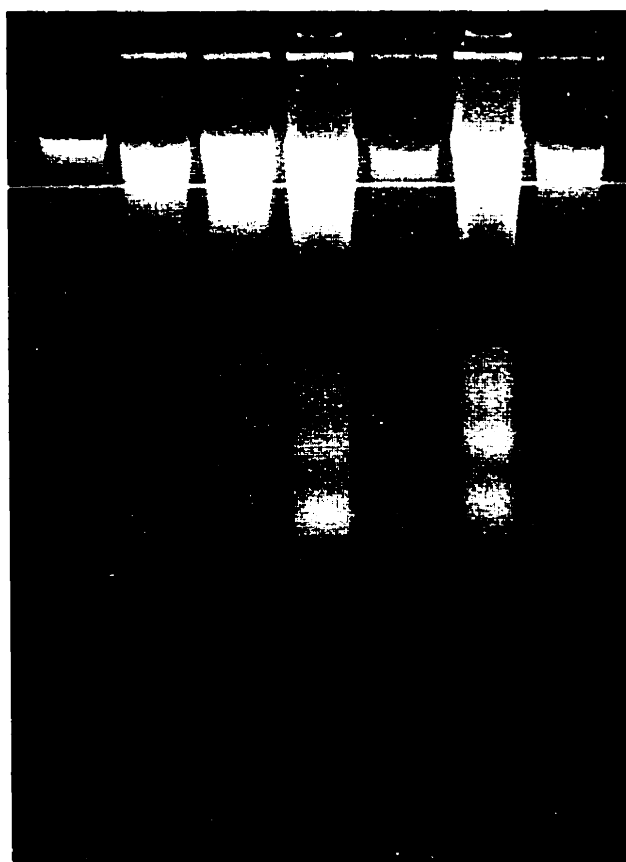


Fig. 5A Vanadate-induced apoptosis in WSU- CLL cells is accompanied by increased protein tyrosine phosphorylation. Cells were treated with 10 μM vanadate and harvested after 24 hr. After washing the cells with PBS, cells were immobolized on slides, fixed and incubated with anti-phosphotyrosine antibody 4G10 followd by Texas Red-conjugated secondary antibody. Nuclei were stained with 4,6-daimidino-2-phenylindole (DAPI). The slides were visualized using a Zeiss Axiophot fluorescence microscope (Carl Zeiss Canada Ltd.) coupled to a high sensitivity Photometrics Series 200 CCD camera and image analyzer (Power Macintosh 8100/100 computer and IPLab Spectrum™ software version 3.1 from Scanalytics). The pictures were taken at an equal exposure time. The arrows indicate the characteristics of some apoptotic cells (cell membrane blebbing, nuclear condensation and fragmentation).

Vanadate	-	+	-	+	+	-	+
Fludarabine	-	-	+	+	-	+	+



1 2 3 4 5 6 7

Fig. 5B Vanadate-induced apoptosis in WSU-CLL cells. 1x10^7 WSU-CLL cells treated with media alone (lane 1), 10 μM vanadate (lane 2 and 5), 2 μM fludarabine (lane 3), 4 μM fludarabine (lane 6), 10 μM vanadate plus 2 μM fludarabine (lane 4), 10 μM vanadate plus 4 μM fludarabine lane 7) were harvested after 48 hr. DNA extraction and apoptosis assay was porformed as described in Materials and Methods section. The amount of DNA in each lane was not titrated.

VII.6 ***Vanadate-induced Apoptosis is Mediated by Activation of Caspase-3 in WSU-CLL Cells:*** Proteins of the caspase family are involved in various forms of apoptosis. To investigate whether vanadate-induced apoptosis was mediated by activation of caspase cascade, we monitored the activation of procaspase CPP32 (caspase-3). As shown in Fig. 6, following treatment of WSU-CLL cells with vanadate, cleaved caspase-3 fragments (*Mr* 20,000 and 17,000) began to appear 12-hr after vanadate treatment. At 48-hr treatment, more cleavage/activation of caspase-3 was evident even though not all caspase-3 was cleaved.

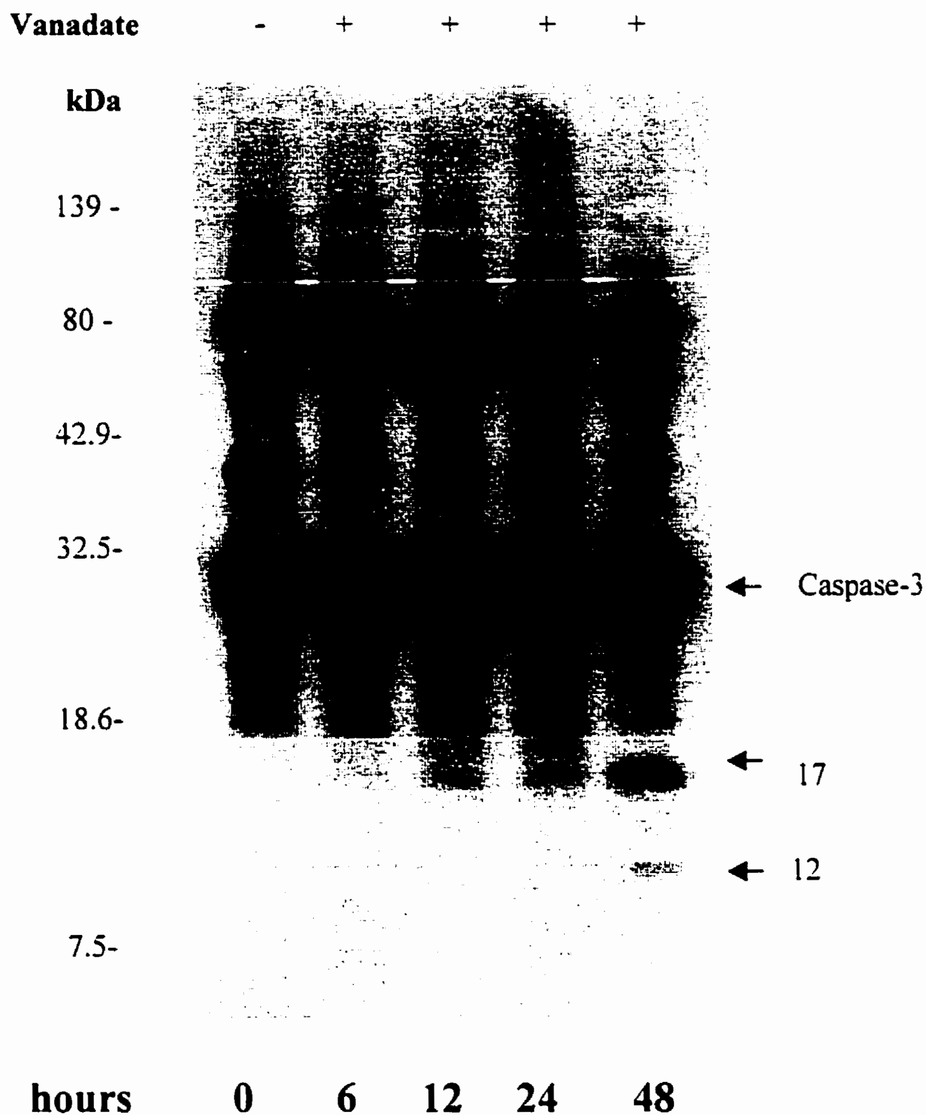


Fig. 6 Vanadate-induced apoptosis in WSU-CLL cells is mediated by activation of caspase-3. Cells treated with 10 μM vanadate were harvested at indicated time points. Proteins (10 μg per lane) from total cell lysates of untreated and vanadate-treated cells were separated by 10% tricine SDS-PAGE, transferred to nitrocellulose, stained with ponseau S to confirm the equal loading and analyzed by immunoblotting with anti-caspase-3 antibody. Arrows indicate the position of total caspase-3 and cleaved 17 kDa and 12 kDa fragments.

V.7 ***Vanadate-induced Apoptosis in WSU-CLL Cells is Accompanied by Release of Cytochrome c from Mitochondria:*** The activation of the effector caspase-3 is catalyzed by the initiator caspase-9. Activation of pro-caspase-9 requires the release of cytochrome c into the cytoplasm, where it forms a complex with procaspase-9, Apaf-1, and dATP. We assessed whether vanadate induced the release of cytochrome c in WSU-CLL cells. The results shown in Fig. 7 demonstrate minimal levels of cytochrome c in the cytoplasm of untreated WSU-CLL cells. Following treatment with vanadate for 24 hr, a dramatic increase in cytoplasmic cytochrome c was detected in these cells.

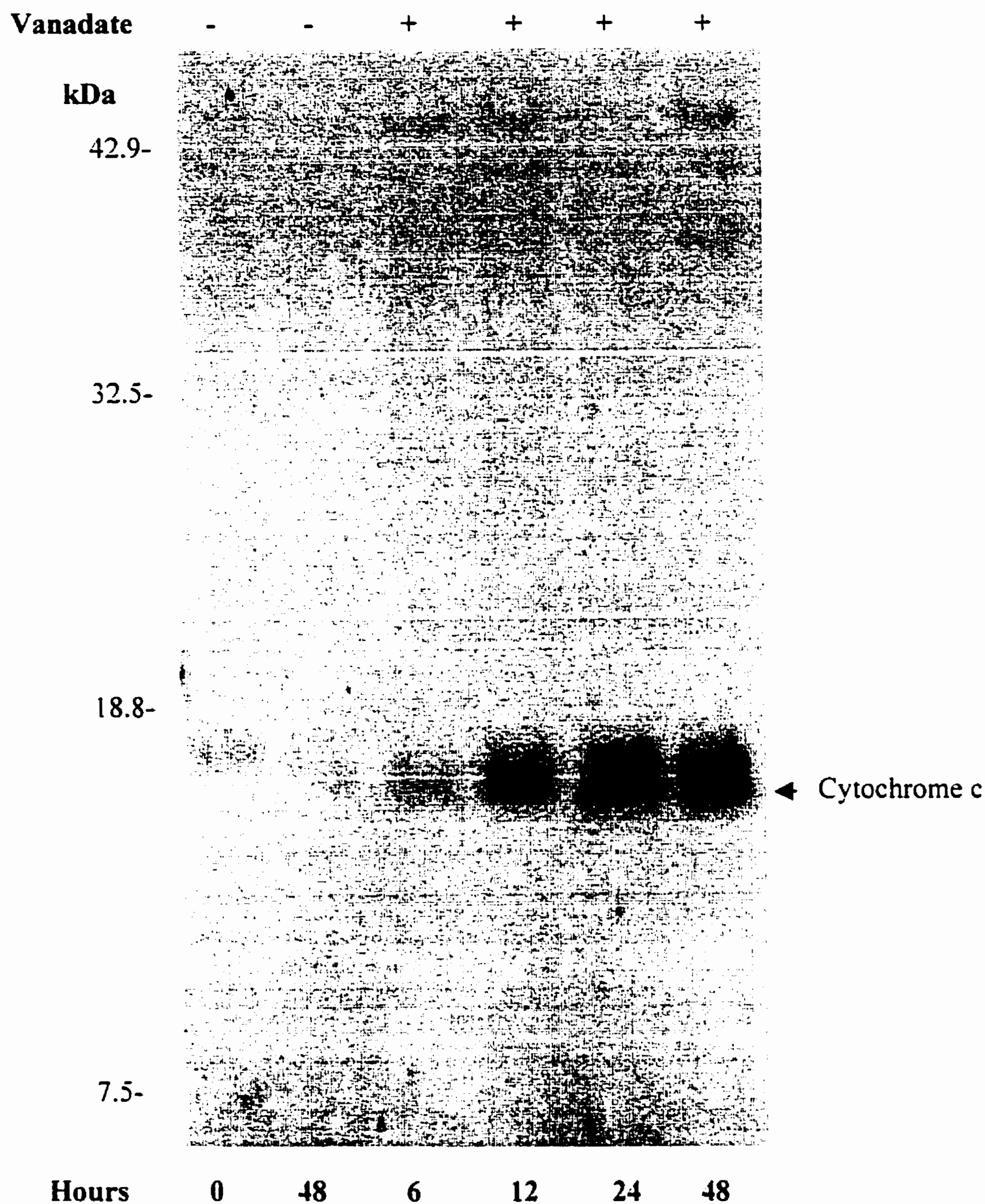


Fig. 7 Vanadate-induced apoptosis in WSU-CLL cells is accompanied by release of cytochrome c from mitochondria. WSU-CLL cells were treated with 10 μM vanadate and harvested at indicated time points. Proteins (10 μg per lane) from cytosolic fraction (prepared as indicated in Material and Methods section) were separated by 10% tricine SDS-PAGE, transferred onto nitrocellulose membrane, stained with ponseau S to confirm the equal loading, and blotted with anti-cytochrome c antibody. Similar results were also obtained from other two experiments.

VII. 8 ***Vanadate Induces the Activation of JNK/SAPK in WSU-CLL Cells:*** Recently, a novel role for JNK/SAPK in apoptosis was described (Maundrell et al., 1997; Srivastava et al., 1999; Kharbanda et al., 2000). JNK was shown to phosphorylate Bcl-2 and Bcl-x_L (Maundrell et al., 1997; Poruchynsky et al., 1998; Srivastava et al., 1999; Kharbanda et al., 2000) and this posttranslational modification disables the anti-apoptotic function of these proteins. Since Bcl-2 members and JNK/SAPK are involved in the regulation of cytochrome c release (Kharbanda et al, 1997), we monitored the vanadate-induced activation of JNK/SAPK in WSU-CLL cells. As shown in Fig. 8, vanadate treatment enhanced the activity of JNK/SAPK, as measured by binding/protein kinase assay. The increase in JNK/SAPK activity was observed after 12-hr vanadate treatment.

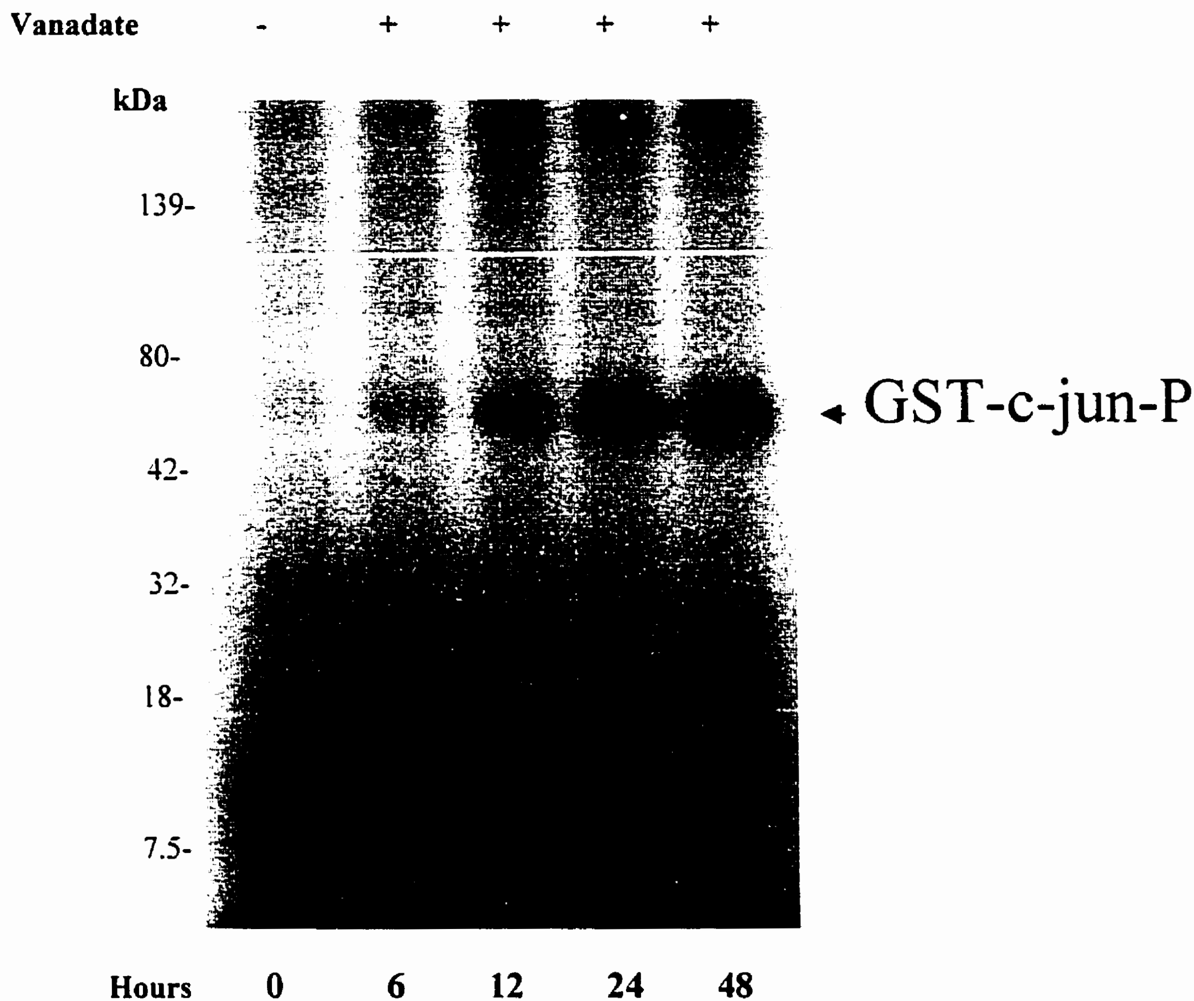


Fig. 8 Vanadate induces the JNK/SAPK activition in WSU-CLL cells. Cells were treated with 10 μM vanadate at indicated time points and then harvested. Whole cell lysate (500 μg protein in each sample) was incubated with rabbit anti-SAPK antibody (2 μg) overnight at 4 °C and the immunocomplexes were collected with protein A sepharose. Rabbit serum was used to check the non-specific binding (data not shown). The kinase activity was determined by using GST-c-jun as a substrate. The kinase reaction mixture was resolved in 10% tricine SDS-PAGE and the acrylamide gel was dryed and exposed to Kodak X-ray film. GST-c-jun-P, phsphorylated GST-c-jun. This figure represents 1 of 3 separate experiments.

VII. 9 ***PI 3-Kinase Activity and Akt Kinase Activity was Decreased After Vanadate Treatment in WSU-CLL Cells:*** We next determined whether cell survival signals are decreased in vanadate-treated WSU-CLL cells. PI 3-kinase and Akt kinase activity were measured at different time points following vanadate treatment. As shown in Fig. 9A. Akt kinase activity, as measured by the phosphorylation of histone H1, decreased as early as 6-hr after vanadate treatment and little activity was observed after 12-hr treatment with vanadate. A similar trend was observed when anti-phosphotyrosine precipitable PI 3-kinase activity was measured by *in vitro* kinase assays using phosphatidylinositol (PI) as a substrate (Fig. 9B).

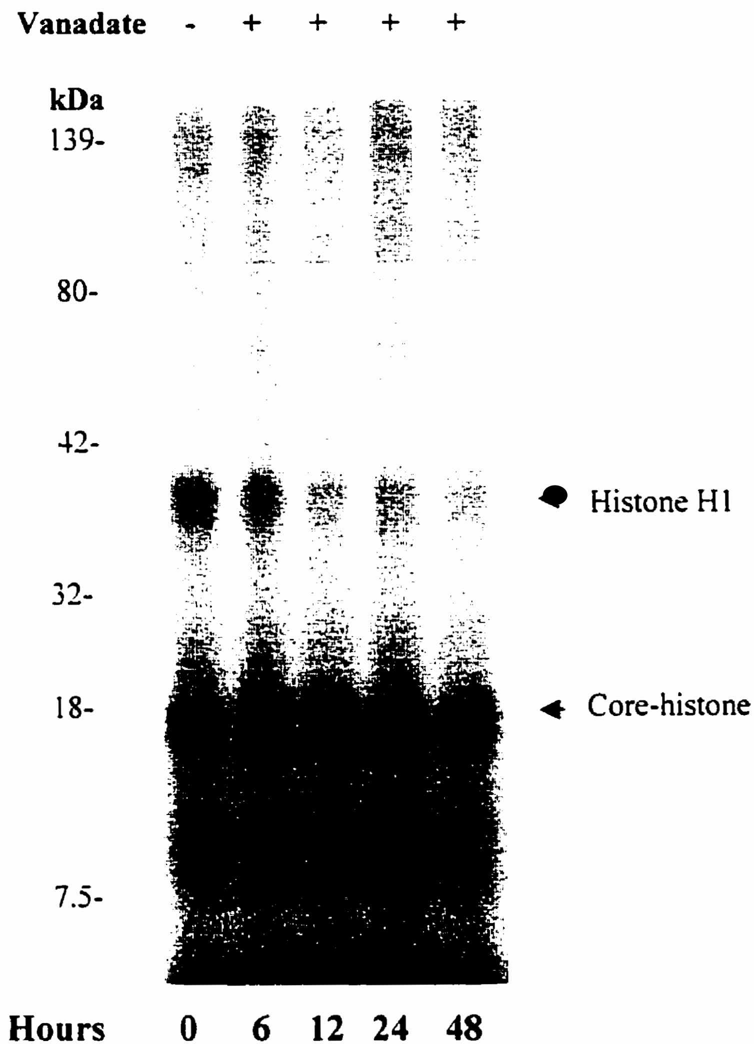


Fig. 9A Vanadate treatment decreases the activity of Akt in WSU-CLL cells. Cells treated with 10 µM vanadate were harvested at indicated time points. Akt was immunoprecitated from cell lysates (500 µg per sample) and activity determined by using total histone as substrate as described in the Meterials and Methods section. The proteins were separated by 10% tricine SDS-PAGE and phosphorylation of linker histone H1 was quantitated by autoradiography. Core histone was a mixture of histone H2A, H2B, H3 and H4 (Tordera et al., 1993).

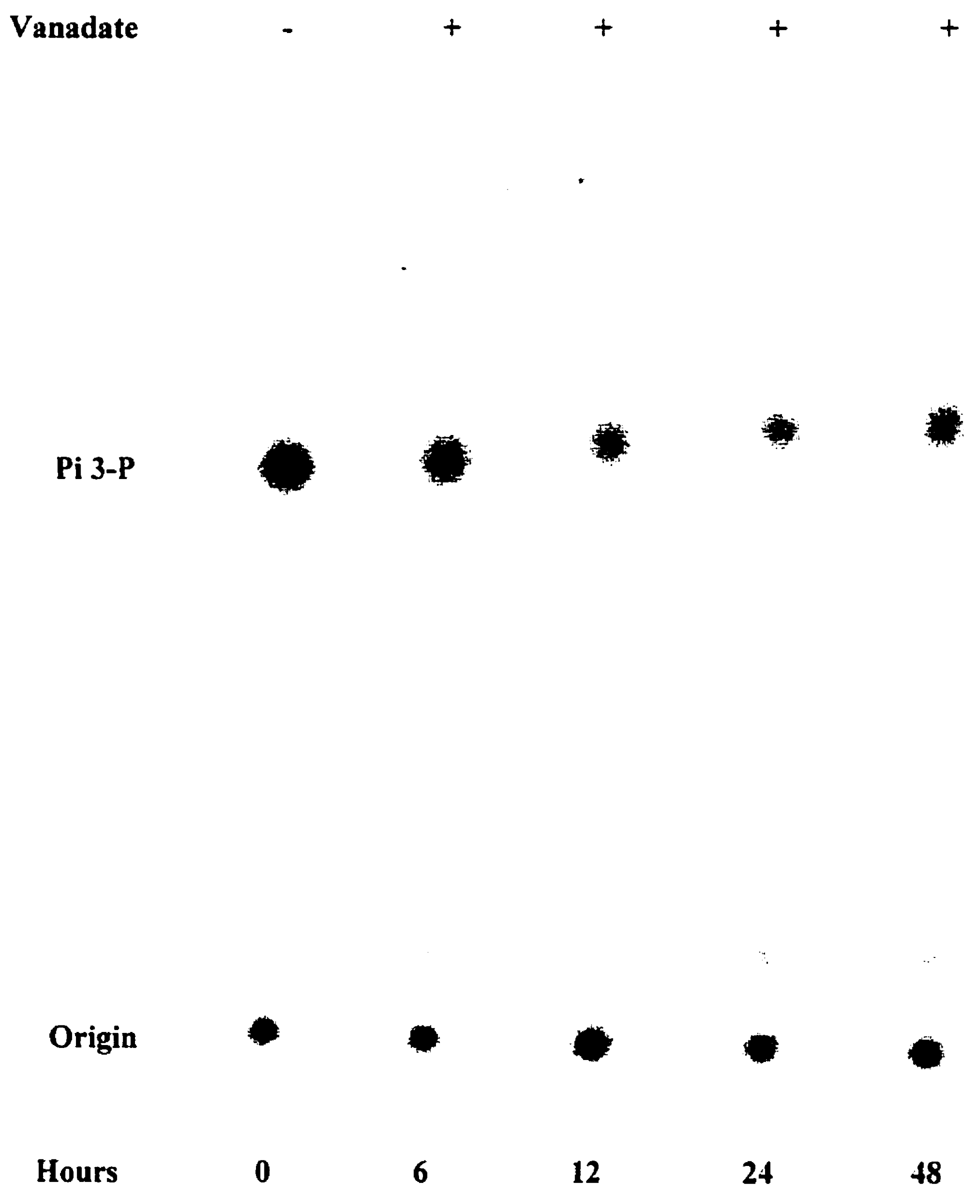


Fig. 9B Vanadate treatment attenuates the anti-phosphotyrosine precipitable PI 3-kinase activity in WSU-CLL cells. Cells treated with 10 μM vanadate were harvested at indicated time points. PI 3-kinase activity in anti-phosphotyrosine immunoprecipitates of vanadate-treated and control WSU-CLL cells was measured by *in vitro* kinase assays using phosphatidylinositol (PI) as a substrate. The position of PI 3-P, phosphorylated product of PI 3-kinase and origin of TLC separation is shown.

VII.10 ***Different Protein Tyrosine Phosphorylation in WSU-CLL Cells and Lymphocytes from CLL Patients:*** To compare the protein tyrosine phosphorylation in B-CLL patient cells, WSU-CLL cells and normal lymphocytes, cell lysates from WSU-CLL cells, two CLL Rai 0 patients and normal lymphocytes from a healthy volunteer were analyzed by immunoblotting using phosphotyrosine specific antibody 4G10. Although BCA protein assay and ponceau S staining were done to control the equal protein loading, non-specific loading control, tested by probing with a house-keeping protein antibody, was not done in this experiment. As shown in Fig. 10, both WSU-CLL and patient cells exhibited much lower levels of protein tyrosine phosphorylation as compared to normal lymphocytes. A major change in tyrosine phosphorylation of proteins of apparent *Mr* 150-170, 120-130, 97-100, 80-85, 70-72, 55-60, 40-45, 32-35, and 24 kDa was observed. The pattern of protein tyrosine phosphorylation among normal lymphocytes, WSU-CLL cells, and patient cells (most of them are CD5 positive lymphocytes) are different. The two patients have different CLB sensitivity, as expressed by CLB IC_{50}, the concentration (μM) of CLB required to kill 50% of CLL cell *in vitro* (Silber, et al. 1994; Begleiter et al., 1996b). The CLB IC_{50} data of the CLL patients tested in this paper were previously obtained by Dr. Begleiter's lab in the Manitoba Institute of Cell Biology and was used with his permission. The cells from patient L (CLB IC_{50} =4.0) exhibited higher tyrosine phosphorylation as compared to cells from patient H with relatively lower CLB sensitivity (IC_{50}=8.1), although both patients were in Rai 0 stage and in a CLB sensitive category (Silber et al, 1994). This result indicates that the pattern and extent of tyrosine phosphorylation of CLL cells are different from normal lymphocytes, and different CLL patient cells may have different tyrosine phosphorylation status that related to the clinical

condition of the disease. Due to sample limitation, this experiment was only done once and needs to be repeated.

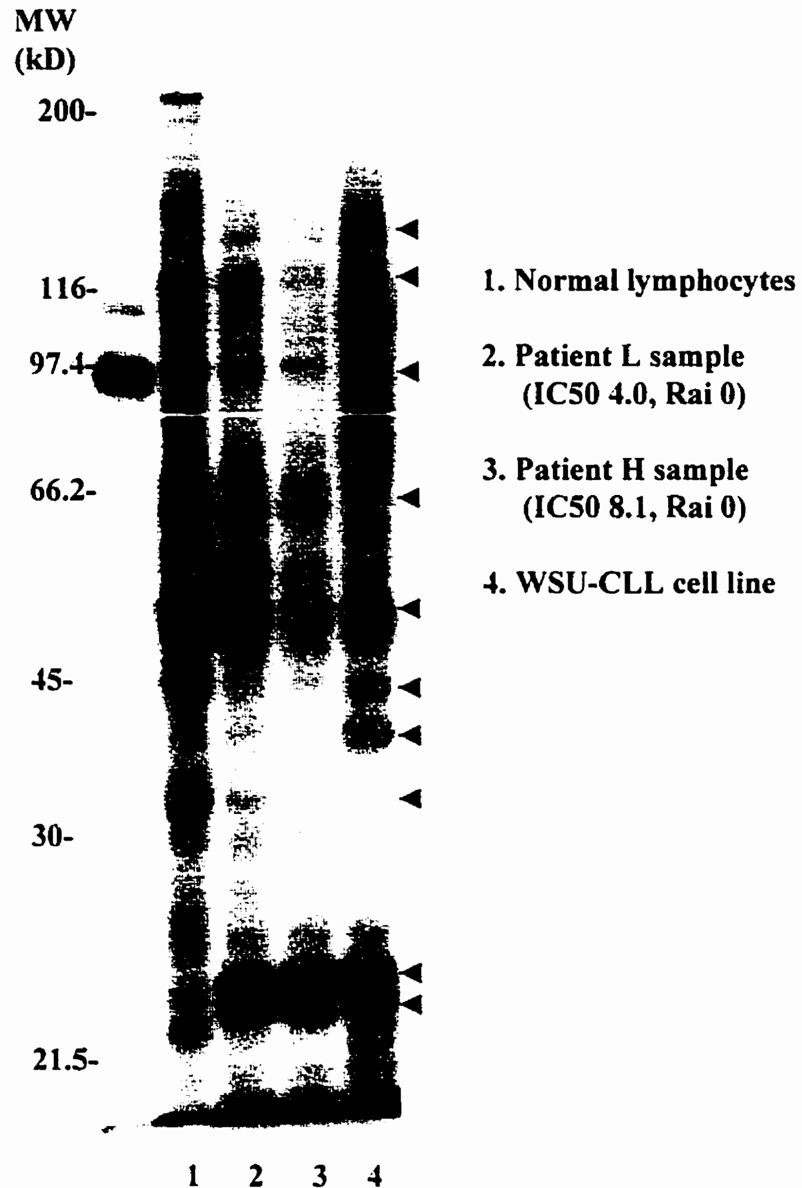


Fig.10 Tyrosine phosphorylation of normal lymphocytes, CLL patient cells and the WSU-CLL cell line. Proteins (30 μg) from whole cell lysates of lymphocytes from a normal volunteer (lane 1), two Rai 0 patients (lane 2 and 3), and WSU-CLL cell line (lane 4) were resolved on 10% SDS-PAGE, transferred to nitrocellulose membrane, stained with ponceau S to double-check equal loading and then immunoblotted with anti-phosphotyrosine antibody 4G10. The molecular weight is also indicated on the left side and the major changes in tyrosine phosphorylated proteins are indicated on the right side.

VII.11 ***CLL Patient Cells Have Different Sensitivities to Vanadate:*** To confirm whether CLL patient cells also respond to vanadate treatment, we analyzed protein tyrosine phosphorylation in lymphocytes of 4 more patients with different CLB IC_{50} and Rai stages. The lymphocytes of these four patients had been studied *in vitro* previously in the laboratory of Dr Begleiter to determine the sensitivity of the cells to chorambucil and the IC_{50} data of these patients were kindly provided for my study. These studies are quoted in my results (4 patients). The IC_{50} in the patients ranged from 4.5 to 28.7. I am indebted to Dr Begleiter for permission to use these cells and the results obtained in his laboratory. These patients are all in a CLB sensitive category according to Silber, et al. (1994). As shown in Fig. 11. without any *in vitro* treatment, the cells from patients with the lowest CLB sensitivity (IC_{50}=28.7) had very low levels of tyrosine phosphorylation as compared to cells from patients with greater CLB sensitivity (IC_{50} from 4.5 to 10) and normal lymphocytes. Although *in vitro* culture (48 hr) was found to increase tyrosine phosphorylation in all CLL cells, the cells with lower CLB sensitivity exhibited lower levels of tyrosine phosphorylation. Furthermore, exposure of cells to vanadate failed to fully induce the tyrosine phosphorylation in cells with lower CLB sensitivity. Interestingly, no correlation was found between the clinical Rai stage and level of tyrosine phosphorylation.

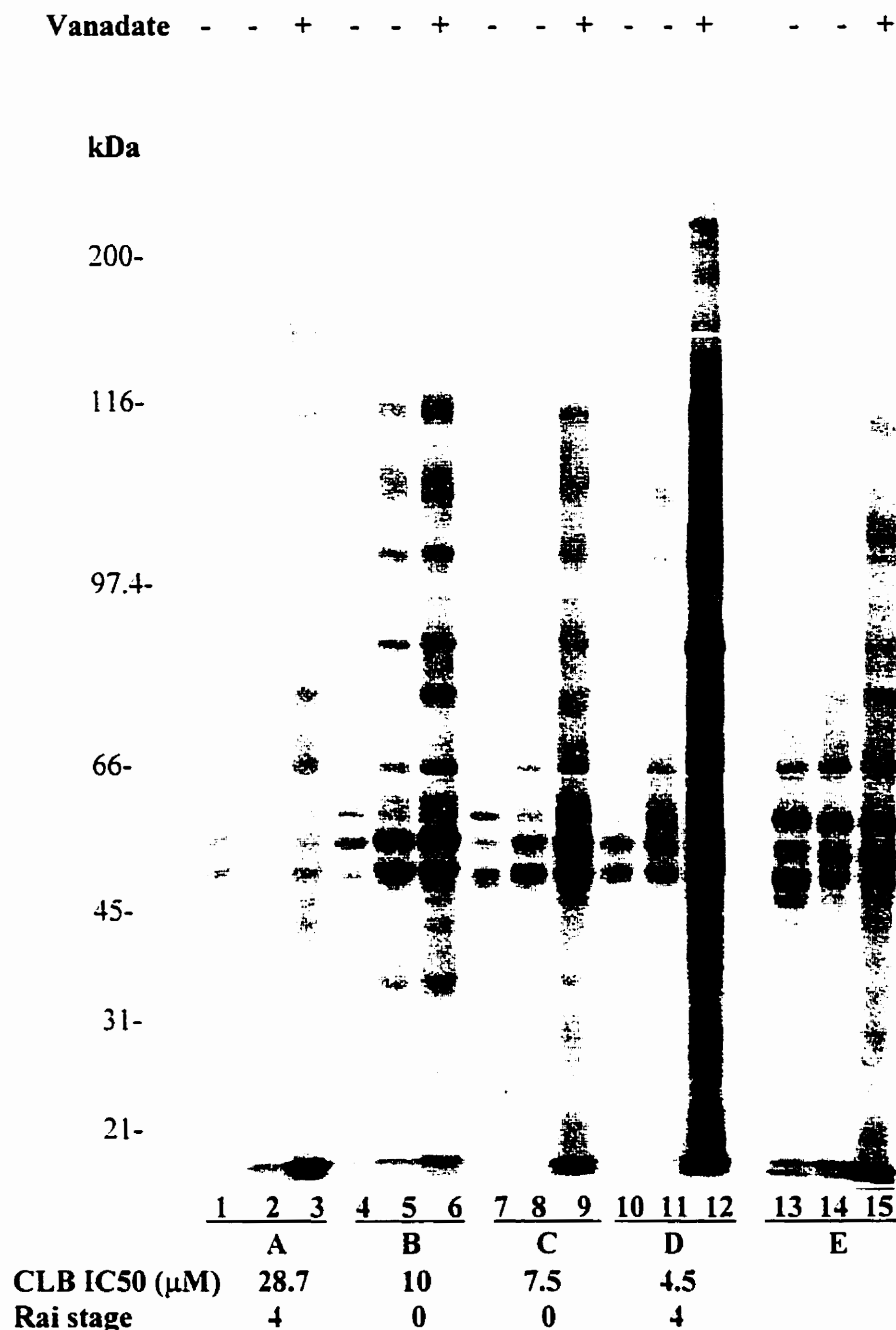
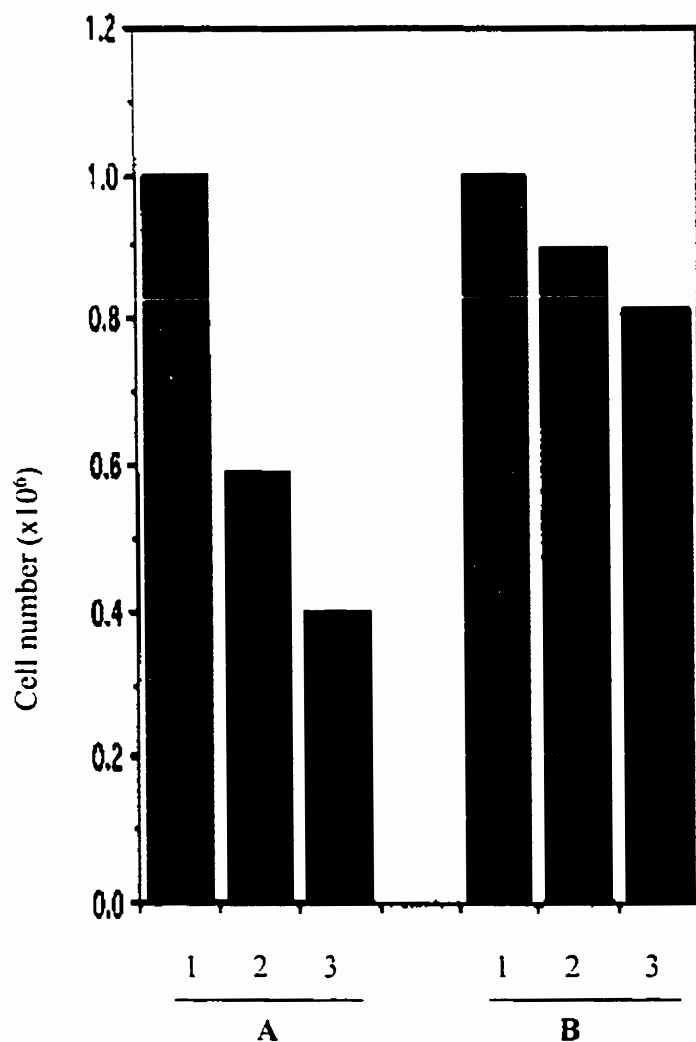


Fig. 11 Comparison of protein tyrosine phosphorylation in lymphocytes of normal volunteer and CLL patients with different degrees of resistance to CLB. Untreated fresh lymphocytes (lanes 1, 4, 7, 10 and 13) from CLL patients (A, B, C, D) and a normal volunteer (E) were cultured for 48 hr in the absence (lanes 2, 5, 8, 11, and 14) or presence of 50 μM vanadate (lanes 3, 6, 9, 12, and 15). 30 μg lysates of lymphocytes were run on SDS-PAGE, transferred to nitrocellulose membrane, stained with ponseau Sto double-check the equal loading and then blotted with anti-phosphotyrosine antibody. The results were observed by ECL. The experiment was only done once and normal lymphocytes were control cells.

VII.12 ***Effect of Vanadate on the Survival of Lymphocytes of CLL Patients in Vitro:***

The effect of vanadate on cell-death of CLL patient cells was also examined. It is known that the lymphocytes from CLL patients die of spontaneous cell-death when cultured in vitro (Collins, et al., 1989). The lymphocytes collected from patient L and patient H shown in Fig.10 were cultured *in vitro* with or without vanadate (50 μM). The viability of cells was checked at 48 hr by trypan blue dye exclusion under light microscopy. Due to sample limitation, this experiment was only done once. As shown in Fig. 12, the cells with higher CLB sensitivity exhibited about 40% spontaneous apoptosis at 48 hr compared to only 11% in cells from CLB resistant cells. Vanadate treatment enhanced the cell-death in both patient samples but was less in cells with a relatively higher CLB IC_{50} (patient H).

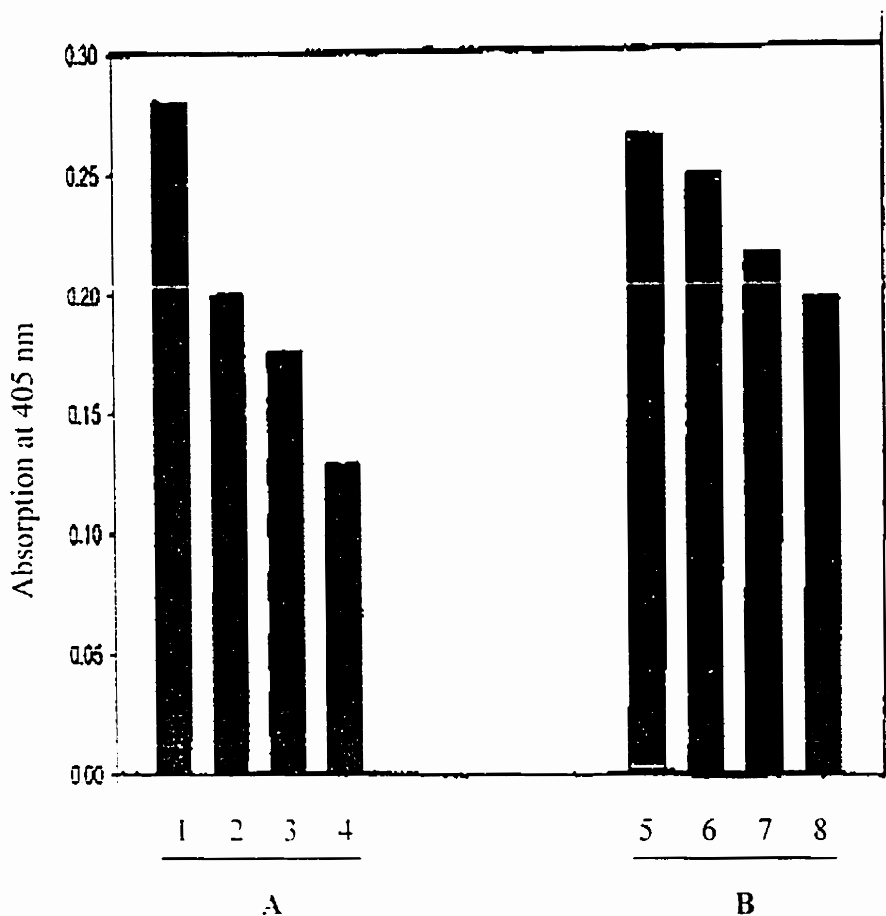


A. Patient L sample (IC50 4.0. Rai 0) B. Patient H sample (IC50 8.1. Rai 0)

1. Time 0 2. 48 hr control 3. 48 hr Vanadate

Fig. 12 Effect of vanadate on the survival of lymphocytes of CLL patients *in vitro*. The newly-separated lymphocytes from two CLL patients were cultured *in vitro with* (column 3 and 6) and without (column 2 and 5) vanadate (50 μM). The viable cells were counted at 48 hr by trypan blue dye exclusion under light microscopy. Due to sample limitation, this experiment was only done once.

VII.13 ***Protein Tyrosine Phosphatase Activity was also Decreased in CLL Patient Cells After Vanadate Treatment:*** The effect of vanadate on protein tyrosine phosphatase activity was monitored in lymphocytes of the two Rai stage 0 patients shown in Fig 10, patient H (CLB IC_{50}=4.0) and patient L (CLB IC_{50}=8.1). The two patient samples have some differences in protein tyrosine phosphorylation (Fig. 10). Cells treated with vanadate (50 μM) were harvested at 12 and 36 hr and protein tyrosine phosphatase activity was measured. As shown in Fig. 13, although protein tyrosine phosphatase activity was inhibited in both samples, some differences were observed in the extent of vanadate inhibitable protein tyrosine phosphatase activity. While vanadate treatment markedly inhibited protein tyrosine phosphatase activity in cells with higher CLB sensitivity, a smaller decrease in phosphatase activity was observed in cells with relatively lower CLB sensitivity. Due to sample limitation, this experiment was only done once.



A. Patient L sample (IC50-4.0. Rai 0) B. Patient H sample (IC50-8.1. Rai 0)

1.5. Time 0 2.6. 36 hr control 3.7. 12 hr vanadate 4.8. 36 hr vanadate

Fig. 13. Different protein tyrosine phosphatase activity in CLL patient lymphocytes with different CLB sensitivity. Freshly prepared lymphocytes from two CLL patients were treated with 50 μM vanadate and cells harvested at 12 (lane 3 and 7) and 36 hrs (lane 4 and 8). Cells without any treatment were also harvested at time 0 (lane 1 and 5) and 36 hrs (lane 2 and 6). 30 μg of sonicated lysate in 50 mM Tris-HCl. pH7.5 was used for protein tyrosine phosphatase assay using p-NPP as a substrate. The release of p-nitrophenyl (yellow color) was measured at 405 nm using an ELISA reader. The samples were tested in triplicate and the results are the means ±SE of absorption values. Due to sample limitation. only one experiment was done.

DISCUSSION

CLL is a malignant disorder characterized by the proliferation and accumulation of mature looking, though dysfunctional lymphocytes (Foon et al, 1990; Tefferi & Phyliky, 1992). The malignant lymphocytes infiltrate the bone marrow, lymph nodes, liver and spleen, resulting in enlargement of these organs and in bone marrow failure (Hoffman et al., 1991; O'Brien et al., 1995; Zwiebel & Cheson, 1998). Better understanding of specific defects that contribute to the clonal expansion of these neoplasms may lead to improved treatment of this disease. Growing evidence suggests that programmed cell-death (apoptosis) plays a key role in the selection and differentiation of lymphoid cells (Smith et al., 1989; Nieman et al., 1991). Since CLL lymphocytes accumulate (Reed, 1998) rather than proliferate (O'Brien et al., 1995), this disease may involve defects in apoptosis. Consistent with this notion, defective apoptosis has been shown to contribute to the onset of several malignancies, including those of the hematopoietic B-cell system (Williams, 1991; Vaux et al., 1988). CLL cells are more resistant to chemotherapy-induced cytotoxicity and apoptosis compared with many other cancers (Zwiebel and Cheson, 1998). This may be related to enhanced Bcl-2 function (Pepper, et al., 1999), p53 mutation, or other genetic changes (Begleiter, et al., 1996a).

Studies on the transduction of cell surface signals from growth factors, cytokines, hormones, and cell adhesion molecules have shown that protein tyrosine phosphorylation events are essential for proliferation and differentiation. Under physiological conditions,

the protein tyrosine phosphorylation levels in a cell are maintained by protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPs). The relative abundance of distinct PTKs in both T- and B-lymphocytes from mouse, rat and human leukemic cell lines suggests their involvement in cellular activation (Harrison et al, 1984; Earp et al, 1984). Studies have shown that CLL cells also overexpress Src family kinases (Abts et al., 1991; Majolini et al., 1998). BCR aggregation by antigens activates several Src-family kinases such as Lyn, Fyn, Lck and Fgr. These kinases then activate non-receptor kinases such as Syk and ZAP-70 and cause downstream activation of several genes through PI 3-kinase/Akt pathway or Ras/MAP kinase pathway, leading to cell differentiation, proliferation or apoptosis (Tamir & Cambier, 1998). Recently, PTK activation has been shown to provide an early and requisite signal for Fas-induced apoptosis in Jurkat (human T cell leukemia), U937 (human histiocystic lymphoma), and K562 (human myelogenous leukemia) cell lines (Eischen et al, 1994). In the light of these findings, we evaluated the regulatory role of protein tyrosine phosphorylation in B-CLL apoptosis.

To understand the role of protein-tyrosine phosphorylation on CLL cells, we use sodium orthovanadate (vanadate) as an agent and check its effect on CLL cells. Sodium orthovanadate (Na_3VO_4) is a common tyrosine phosphatase inhibitor in biological research (Gordon, 1991). It will form vanadate (VO_4^{3-}) in solution (Gordon, 1991). Interest in vanadate began more than twenty years ago and it has been proved that vanadate has widespread effects to cells (Huyer et al., 1997; Simons, 1979). Before molecular biology methods were widely used in biological research, it had already been found that vanadate had some inhibitory effect on Na^+-K^+ ATPase and other ATPases

(Simos, 1979). Now it has been found that vanadate has a lot of biological effects, one of which being inhibition of protein tyrosine phosphatase activity and enhancement of tyrosine phosphorylation in cells (Huyer et al., 1997). Vanadate has very similar spacial structure with phosphate (PO_4^{3-}), so it can mimic phosphate as the substrate of protein tyrosine phosphatases. This will generate inhibitory effects of protein tyrosine phosphatase activity by blocking phosphate hydrolysis (Huyer et al., 1997) because the basic function of protein tyrosine phosphatases is taking the phosphate out from the phosphorylated substrates and leading to dephosphorylation of the cells (Denu et al., 1996; Fauman & Saper, 1996; Zhang, 1997). Tyrosine phosphorylation in cells is maintained by a balance between tyrosine phosphatases and kinases (Zhang, 1997). Inhibition of tyrosine phosphatase will relatively enhance the effects of tyrosine protein kinases and result in enhanced tyrosine phosphorylation. Recent studies demonstrated that vanadate/H2O2 (pervanadate) induces insulin secretion (Kadota et al., 1987; Molero et al., 1998; Ruff et al., 1997) and reverse the multi-drug resistance of lymphoblastic leukemia cells (Colin et al., 1994).

The cell growth of WSU-CLL cells and other two leukemia cell lines (U937 and HL60) was first checked (Fig. 1A and 1B). Cell growth of WSU-CLL cells was more easily to be inhibited by vanadate treatment compared with other cell lines. The growth inhibition was also accompanied by some cell death. This result shows that WSU-CLL cells are very sensitive to vanadate treatment. To further prove the effect of vanadate on the cell growth of WSU-CLL cells, clonogenic assay was done. Fludarabine was used as a positive control in these studies because this drug induces apoptosis in CLL cells (Byrd et al., 1998; O'Brien et al., 1995). The studies showed that vanadate dramatically

inhibited colony formation of WSU-CLL cells (Fig. 2) and suggested that vanadate has cytotoxic effect on WSU-CLL cells.

To explore the mechanism of growth inhibition and cell death of WSU-CLL cells after vanadate treatment, tyrosine phosphorylation was then checked and proved that the cell growth inhibition and death was accompanied by enhanced tyrosine phosphorylation (Fig.3), and this enhanced tyrosine phosphorylation was caused by the dramatic inhibition of protein tyrosine phosphatase activity, not much enhancement of total protein kinase activity (Fig 4A and 4B). Further study needs to be done to check which specific proteins are tyrosine phosphorylated and which tyrosine phasphatases are inhibited. More experiments are also needed to confirm the result of total kinase activity after vanadate treatment. Other studies have shown that vanadate (Elberg et al., 1997) or pervanadate (Fantus et al., 1989) mimics insulin action through activation of non-receptor protein kinase (Elberg et al., 1997; Fantus et al., 1989) or insulin receptor protein kinase (Fantus et al., 1989) in rat adipocytes. Since we observed that the total protein tyrosine kinase activity in vanadate-treated CLL cells remain unaltered, the effect of vanadate on tyrosine phosphorylation in CLL cells may mainly be due to the unbalance between the inhibited protein phosphatase and unaltered tyrosine kinases.

We then checked whether vanadate could induce apoptosis and its relationship with enhanced tyrosine phosphorylation. In other studies, vanadate was shown to induce or enhance apoptosis in many cell lines. Schieven et al. (1995) treated human B and T cell lines with bis(maltolato)oxavanadium(IV) (BMLOV) and found that there was a similar pattern of tyrosine phosphorylation in BMLOV-treated cells and B cells after antigen receptor stimulation. The author found that BMLOV selectively induces

apoptosis in B lymphoma and myeloid leukemia cell lines, but not T cell leukemia or colon cancer cell lines. In promyelocytic leukemia cell line HL60, Chang and Yung (1996) found that vanadate enhanced the apoptosis induced by sodium butyrate (BuONa), an agent that can induce cell differentiation and inhibit cell growth. Heat shock induced apoptosis of HL60 cells was also enhanced by vanadate (He & Fox, 1997). Uckun et al. (1992) proved that ionizing radiation-induced apoptosis of human fetal liver pro-B cell line FL112 was also enhanced by vanadate, and some unidentified proteins were phosphorylated. Sit et al. (1997) found that M-phase cells were induced into apoptosis after 30-min treatment with 200 μM vanadyl. Fas ligation (Ruiz-Ruiz et al., 1997) or surface antigen CD43 ligation (Brown et al., 1996) of human T-lymphoblastoid cell lines causes tyrosine phosphorylation of the cells accompanied by the apoptosis of the cells. This effect becomes more apparent after vanadate-related componds are added (Brown et al., 1996; Ruiz-Ruiz et al., 1997). Cisplatin is a commonly used drug in cancer therapy, and cisplatin-induced apoptosis has been proven to be accompanied by increased tyrosine phosphorylation (Sanchez-Perez et al., 1998; Shishodia et al., 1997). Enhanced tyrosine phosphorylation by vanadate increased TNF and nitric oxide (NO) production and apoptosis (Shishodia et al., 1997). Recently, Dawson (2000) found that a vanadate compound, Bis(maltolato)oxovanadium (IV) (BMOV) also induced apoptosis of a neuron cell line, E7CH.

Through immunofluorescence staining with antiphosphotyrosine antibody and DNA staining with DAPI (Fig. 5A), we did find that vanadate induced apoptosis (cell membrane blebbing, nuclear condensation and DNA fragmentation). This apoptosis was accompanied by enhanced tyrosine phosphorylation. We also found that WSU-CLL cells

did not go to apoptosis at the same time. As shown in Fig 5, at 24 hr treatment with vanadate, only some of the cells went into apoptosis. This may be related to the growth phase and the tyrosine phosphorylation status of the cells. Fig. 5A showed that even WSU-CLL cells were treated at the same time with the same concentration of vanadate, the tyrosine phosphorylation status is different among the cells. Apoptosis assay (DNA laddering) shown in Fig 5B proved that vanadate could induce DNA fragmentation in WSU-CLL cells. More experiments needs to be done to have an equal amount of DNA loading in the apoptosis assay for each condition of the treatment of WSU-CLL cells.

We then checked the pathways involved in vanadate-induced apoptosis in WSU-CLL cells. Activation of caspase family of proteases has been detected in numerous cell systems and appears to function as a common pathway through which apoptotic mechanisms may operate (Kerr et al., 1972; Thompson, 1995; Jacobson et al., 1997). Caspases are synthesized as precursors (pro-caspases) and are converted into mature enzymes by apoptotic signals (Li et al., 1997; Zou et al., 1997; Srinivasula et al., 1998). Based on current literature, two distinctly different pathways of caspase activation and apoptosis can be delineated in mammals. In the first pathway, ligation of death receptors such as Fas (the cell surface polypeptide also known as CD95 or Apo-1) or the type 1 TNF-α induces binding of adapter molecules, which in turn recruit pro-caspases-2, 8, and 10 to membrane associated signaling complex. This results in proximity-induced activation of some of these caspases. Activation of these upstream caspases is sufficient to directly cleave/activate precursors of effector caspases such as caspase-3, -6, and -7. A special case in this pathway is in cytotoxic cell killing in which granzyme B, a protease released by granules from cytotoxic cells, can directly activate ICE/CED-3 family

protease CPP32 (caspase-3) (Martin et al., 1996). In the second pathway, various forms of cellular stress triggers mitochondrial release of cytochrome c, which interacts with cytosolic docking protein Apaf-1, leading to the activation of pro-caspase-9. Activated caspase-9 proteolytically activates effector caspases, caspase-3 and possibly caspase-7. Once the effector caspases are activated, these enzymes cleave a number of cellular polypeptides leading to disassembly of key structural components of the nucleus and cytoskeleton; inhibition of DNA repair, replication and transcription; and activation of endonucleases that irreversibly damage the genome (Guchelaar et al., 1997; Meyn et al., 1997). Hehner et al. (1999) found that pervanadate could induce Jurkat T cell line into apoptosis through activation of caspase-3, caspase-8 and cleavage of poly(ADP ribose) polymerase (PARP). CPP32 exists in the cytosolic fraction as an inactive 32-kDa precursor that is proteolytically cleaved in apoptotic cells to active 17-kDa and 12-kDa subunits. The involvement of the activation of CPP32 was checked in vanadate-induced apoptotic pathways in this investigation.

In our experiment, treatment of WSU-CLL cells with vanadate activated CPP32 as evident from cleaved caspase-3 fragments (M_r 20,000 and 17,000) (Fig 6). These studies suggested that vanadate-induced apoptosis in WSU-CLL cells correlates with a selective activation of caspase cascade. At 48 hr vanadate treatment, not all caspase-3 were cleaved (activated). This may be related to the growth phase and tyrosine phosphorylation status of the individual WSU cells because not all cells were highly phosphorylated accompanied by apoptotic cell death. More caspases need to be checked.

Growing evidence suggests that the activation of the effector caspase-3 is catalyzed by the initiator caspase-9 (Li et al., 1997; Zou et al., 1997; Srinivasula et al.,

1998). Activation of pro-caspase-9 requires the release of cytochrome c into the cytoplasm, where it forms a complex with procaspase-9, Apaf-1, and dATP (Hu et al., 1999; Zou et al., 1999). Consistent with these findings, a variety of apoptotic stimuli have been shown to induce the release of cytochrome c from the mitochondrial intermembrane space (Liu et al., 1996; Kharbanda et al, 1997; Kharbanda et al., 2000). Cytochrome c release from mitochondria was found from pervanadate-induced apoptosis in Jurkat T cells (Hehner et al., 1999).

In our study, WSU-CLL cells were exposed to vanadate to determine whether cytosolic cytochrome c levels change during vanadate-induced initiation of apoptosis. The levels of cytochrome c in the cytosol increased dramatically at 24 hr after vanadate treatment (Fig. 7). These studies suggest the involvement of cytochrome c in vanadate-induced apoptotic pathways in WSU-CLL cells. Direct testing of mitochondria function needs to be done to prove the malfunction of mitochondria after vanadate treatment.

The initial signal that triggers mitochondrial changes in response to apoptotic stimuli is presently not known. A lot of studies have found that whether the apoptosis is induced by TNF receptor or Fas receptor ligation (Guo et al., 1998a; Guo et al., 1998b; Okamoto et al., 1996; Suhara et al., 1998), ionization or UV stimulation (Verheij et al., 1996), hyperoxide stress (Suhara et al., 1998), or treatment with cisplatin (Sanchez-Perez, Murguia & Perona, 1997), stress activated protein kinase (SAPK) or c-jun N-terminal kinase (JNK) is activated. This activation is enhanced by vanadate (Guo et al., 1998a; Guo et al., 1998b; Okamoto et al., 1996; Sanchez-Perez et al., 1997; Suhara et al., 1998;). Recently, a novel role for JNK/SAPK in apoptosis was uncovered (Maundrell et al., 1997; Srivastava et al., 1999; Kharbanda et al., 2000). JNK was shown to translocate to

mitochondria and phosphorylate Bcl-2 and Bcl-x_L (Maundrell et al., 1997; Poruchynsky et al., 1998; Srivastava et al., 1999; Kharbanda et al., 2000). This posttranslational modification disables the anti-apoptotic function of these proteins (Kharbanda et al., 2000). Since CLL cells overexpress Bcl-2 (Adams & Cory, 1998; Reed, 1998) and Bcl-2 members are involved in the regulation of cytochrome c release (Kharbanda et al, 1997), activation of JNK will attenuate anti-apoptotic function of Bcl-2 in CLL cells.

The kinase activity of JNK/SAPK in vanadate-induced apoptosis in WSU-CLL cells was assessed in our study. Vanadate treatment enhanced the activity of JNK/SAPK in WSU-CLL cells (Fig. 8). These findings provide a potential link between JNK/SAPK activation and mitochondrial release of cytochrome c in vanadate treated WSU-CLL cells. We did not test the relation between JNK/SAPK activity and Bcl-2 family protein function in this project.

We next determined whether cell survival signals are decreased in vanadate-treated WSU-CLL cells. PI 3-kinases, responsible for the formation of novel 3-phosphorylated phosphoinositides (3-PPI), are now recognized as critical components of signal transduction cascades initiated by receptor tyrosine kinases, non-receptor tyrosine kinases, cytokine receptors, integrins, chemokines, and oncogene products (Downes & Carter, 1991; Soltoff et al., 1992; Stephens et al., 1993; Varticovski et al., 1994). Activation of PI 3-kinase commonly involves localization of the enzyme to the plasma membrane (Stephens et al., 1993; Varticovski et al., 1994) where it phosphorylates PI, PI 4-phosphate (PI 4-P), and PI 4,5-bisphosphate (PI 4,5-P_2) to produce PI 3-phosphate (PI 3-P), PI 3,4-bisphosphate (PI 3,4-P_2), and PI 3,4,5-trisphosphate (PI 3,4,5-P_3), respectively. Unlike the products of PI kinases in the classical PI cycle (PI 4-P and PI 4,5

–P2), 3-PPI are not the substrates of any known phospholipases, and may themselves act as second messengers (Downes & Carter, 1991; Soltoff et al., 1992). PI 3-kinase and its products are now recognized as one of the most promising regulators of apoptotic pathways (Burgerling & Coffer, 1995; Datta et al., 1997; Cardone et al., 1998). Studies have shown that direct binding of 3-phosphorylated lipids to pleckstrin homology (PH) domain of Akt, a serine threonine kinase, results in the induction of activating phosphorylation at residues Thr308 and Ser473 by upstream kinases such as phosphoinositide-dependent protein kinase-1 (PDK-1) (Burgerling & Coffer, 1995; Datta et al., 1997; Cardone et al., 1998). Activated Akt has been shown to protect cells from apoptosis by phosphorylating/deactivating Bcl-2 family member Bad (Datta et al., 1997) and the protease caspase 9 (Cardone et al., 1998). Recent studies by Brunet et al (1999) implicate Akt mediated phosphorylation of transcription factor FKHRL-1 to the suppression of pro-apoptotic genes, such as the Fas ligand gene, thereby highlighting potent effect of PI 3-kinase/Akt pathways on cell survival.

In WSU-CLL cells, vanadate treatment significantly attenuated the activities of PI 3-kinase and Akt in parallel (Figures 9A and 9B). These studies suggest that the effect of vanadate on WSU-CLL cell apoptosis may be mediated, in part, by downregulating the cell survival signals. The relationship between reduced PI 3-kinase/Akt function and Bcl-2 family function needs to be studied.

Taken together, vanadate-induced protein tyrosine phosphorylation seems to trigger apoptosis in WSU-CLL cells at several levels involving cytochrome c release from mitochondria, activation of caspases, and removal of cell survival signals.

To compare the result from WSU-CLL cell line with CLL patient cells, we then did some preliminary studies with limited patient samples. We first compared the levels and patterns of tyrosine phosphorylation in WSU-CLL cells, two Rai 0 patients and a normal healthy volunteer. The cells from both the WSU cell line and the patients exhibited lower levels of protein tyrosine phosphorylation as compared to normal lymphocytes, and the patterns of protein tyrosine phosphorylation of CLL cells are also different from normal lymphocytes (Fig. 10). The result may partly be due to the lymphocyte subtype differences since most WSU-CLL cells and patient cells are CD5 positive cells, and normal lymphocytes include many subtypes of lymphocytes. Earlier studies showed lack of tyrosine phosphorylation in non-proliferating CLL cells with reduced expression of Syk (Lankester et al., 1996) and antigen receptors (Hambin & Oscier, 1997). Pure CD5 positive cells were not available to us. Since tyrosine phosphorylation is important in lymphocyte signaling, including cell differentiation, proliferation and clonal selection (Weiss & Littman, 1994; Lavin et al., 1996), defects in tyrosine phosphorylation may contribute to the clonal expansion of these neoplasms. More experiment needs to be repeated to compare the protein tyrosine phosphorylation status of pure CD5 positive cells and patient cells.

B-CLL apoptosis may occur spontaneously (Collins et al, 1989), or may be induced by stimuli such as a calcium ionophore, specific antibodies binding to membrane associated IgM, steroids (McConkey et al, 1991), purine nucleosides (fludarabine and 2-CDA), and the alkylating agents. Cytokines (IL-10, IL-5) can induce or prevent (IL-4, interferon γ and α) apoptosis of B-CLL cells, suggesting that these molecules modulate this process *in vivo*. Further studies on four patients confirmed that when cells were

maintained for 48 hr *in vitro*, an increase in tyrosine phosphorylation was observed compared with normal lymphocytes (Fig.11). This correlated with the occurrence of spontaneous cell death of the patient cells in *in vitro* culture (Fig. 12). The viability of the two patient cells was decreased after 48-hr *in vitro* culture without treatment or with vanadate (50 μM). The cells with lower CLB IC_{50} exhibited about 40% spontaneous apoptosis at 48 hr compared to only 11% in cells with relatively higher CLB IC_{50}. Both patients were clinical Rai 0 stage and belong to the CLB-sensitive group. These studies indicate that protein tyrosine phosphorylation may play an important role in the process of CLL cell apoptosis. These results do not correlate with the clinical Rai stage of these patients, but may have some relevance to CLB IC_{50}. More experiments are needed to confirm the result of patient cells and compare with normal CD5-positive cells.

We then checked protein tyrosine phosphatase activity on CLL patient cells after vanadate treatment. In two patient samples, vanadate (50 μM) also inhibited total protein tyrosine phosphatase activity, although some differences existed between these two samples. While 50 μM vanadate treatment markedly inhibited protein tyrosine phosphatase activity cells with lower CLB IC_{50}s, a smaller decrease in phosphatase activity was observed in cells with higher CLB IC_{50} (Fig. 13). These differences may be due to higher protein tyrosine phosphatase activity in cells with a higher CLB IC_{50}, or the existence of heterogeneity of the disease since both patients are categorized as CLB sensitive. More clinical samples are needed to confirm the clinical sample results, and compare the tyrosine phosphatase status of normal CD5 positive cells and patient cells. It will be also be very interesting to have a deeper study to see which specific phosphatase is inhibited in CLL patient cells after vanadate treatment.

In summary, we have observed the protein tyrosine phosphorylation and apoptosis in CLL cell line WSU-CLL and CLL patient cells and explored the mechanism of vanadate-induced apoptosis. The WSU-CLL cell line is a good model for studying CLL although there are differences between WSU-CLL cells and patient cells due to the heterogeneity of clinical CLL. Protein tyrosine phosphorylation has been shown to be very important in CLL cell apoptosis, and it is possible that there is a defect in protein tyrosine phosphorylation signaling pathways in CLL. This may contribute to the defect in apoptosis and gradual accumulation of cells in CLL patients. Vanadate was shown to induce WSU-CLL apoptosis and enhance clinical CLL cell death. The pathways involved in this apoptotic cell death include inhibiting protein tyrosine phosphatases and cell survival signals such as PI 3-kinase and Akt kinase, as well as activation of the apoptotic cascade including SAPK activation, caspase-3 (CPP32) cleavage, and cytochrome c release from mitochondria. Due to the limited number of patient samples in this study, and the heterogeneity of CLL, more patient samples are needed to extend the conclusion of this interesting observation to clinical CLL.

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