

***In Vitro Regulation of
Growth, Differentiation and
Survival of
Leukemic CD5+ B Cells***

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Declaration

I certify that this thesis has not already been submitted for any degree and is not being submitted as part of candidature for any degree.

I also certify that this thesis has been written by me and that any help that I received in preparing this thesis, and all sources used, have been acknowledged.

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Abstract

B cell chronic lymphocytic leukemia (B-CLL) is a hematologic neoplasm characterised by the proliferation and accumulation of sIgM⁺/D⁺ B cells that fail to progress to the final stages of B cell development. The malignant cells in B-CLL also express the pan-T cell antigen CD5, suggesting that CLL is a malignancy of the CD5⁺ subset of B cells. Additional characteristics of the malignant clone include a low proliferative index, enhanced *in vivo* survival and constitutive expression of the anti-apoptosis oncoprotein bcl-2. The behaviour of leukemic CD5⁺ B cells *in vitro* contrasts their arrested *in vivo* state. That is, despite the majority of cells being arrested in the G₀ phase of the cell cycle, the leukemic B cells are not irreversibly frozen as they can be induced to differentiate to Ig-secreting cells under appropriate *in vitro* conditions. Furthermore, leukemic CD5⁺ B cells rapidly undergo death by apoptosis following *in vitro* culture. This thesis describes the requirements for *in vitro* activation of leukemic CD5⁺ B cells, the characterisation of the events involved in apoptosis of these cells as well as the identification of various growth factors capable of modulating these events.

Stimulation of unfractionated peripheral blood lymphocytes (PBLs) from three patients with B-CLL with the phorbol ester PMA and the mitogens PHA and PWM resulted in significant increases in cell proliferation, RNA synthesis and IgM secretion when compared to unstimulated cell populations. PMA was the most potent inducer of IgM secretion and this occurred irrespective of the presence of residual T cells. PMA-induced proliferation and RNA synthesis were also independent of T cells. However, in the presence of T cells, these parameters of cellular activation were enhanced during *in vitro* culture. Thus, the inductive ability of PMA on leukemic CD5⁺ B cells was independent of T cells. In

contrast, activation and differentiation of the leukemic CD5⁺ B cells into IgM-secreting cells following culture with mitogens did not occur in the absence of T cells. Interestingly, co-stimulation of leukemic CD5⁺ B cells with PMA and anti-Ig induced cellular responses that exceeded those induced by either activator alone. Thus, leukemic CD5⁺ B cells from patients with B-CLL can be activated *in vitro* and differentiate in response to stimulation via both T cell-dependent and T cell-independent mechanisms.

Apoptotic cell death was characterised in purified leukemic CD5⁺ B cells obtained from six B-CLL patients. All leukemic CD5⁺ B cell populations entered an apoptotic pathway *in vitro* as evidenced by a reduction in cell size, loss of cell viability and fragmentation of DNA into multimers of ~180 base pairs. Following 24 hours of *in vitro* culture 24.0±16% of DNA was fragmented. After 8 days, the majority of DNA was fragmented, and fewer than 10% of cultured cells were viable. Examination of bcl-2 expression in the malignant B cells by flow cytometry revealed a unimodal pattern of expression in >85% of cells from each B-CLL patient prior to culture. During *in vitro* culture, bcl-2 expression became bimodal such that the B cells displayed a bcl-2_{high} and bcl-2_{low} phenotype. The level of expression by the bcl-2_{high} cells was similar to that observed prior to *in vitro* culture, indicating that bcl-2 is down-regulated in apoptosing cells. Interestingly, despite this down-regulation, the overall number of cells positive for bcl-2 remained constant. This suggests that the enhanced survival of leukemic CD5⁺ B cells *in vivo* is mediated by the sustained expression of bcl-2 and that additional mechanisms exist capable of overriding the protective effect of bcl-2 when bcl-2 is present at reduced levels.

Leukemic B cell apoptosis has previously been reported to be delayed or prevented by IL-4, IFN- γ and IFN- α . These results were confirmed in this study where it was found that culture of leukemic CD5⁺ B cells with IL-4 or IFN- γ enhanced cell viability and delayed apoptosis in 6/6 and 5/6 populations of leukemic B cells, respectively. This function was also found to be shared by IL-2, IL-6, IL-13 and TNF- α as these cytokines enhanced cell viability and delayed apoptosis in some of the cell populations examined at a level similar to that observed for IL-4 and IFN- γ . These cytokines may mediate their effect via the expression of bcl-2 as culture in the presence of IL-2, IL-4, IL-6, IL-13, IFN- γ or TNF- α resulted in a higher percentage of cells displaying the bcl-2_{high} phenotype, compared to unstimulated cells. Taken together, these results suggest that autocrine and/or paracrine growth loops may play a role in the pathogenesis of B-CLL and that cytokines that prevent apoptosis *in vitro* may be targets for treatment of this B cell malignancy.

Publications arising from work presented in this thesis

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Abbreviations

AET	2-aminoethyisothiuronium bromide
Ag	antigen
AML	acute myeloid leukemia
BCIP	bromochloroindolphenyl
B-CLL	B cell chronic lymphocytic leukemia
bp	base pairs
BSA	bovine serum albumin
BSF-MP6	B cell stimulatory factor derived from a T cell hybridoma
C	constant
cIg	cytoplasmic Ig
°C	degrees Cecius
CD	cluster of differentiation
CD40L	ligand for CD40
CLL	chronic lymphocytic leukemia
CD	cluster of differentiation
CIAA	chloroform isoamylalcohol
CPM	counts per minute
ELISA	enzyme-linked immunosorbant assay
EBV	Epstein Barr virus
FACS	fluorescence activated cell sorter
F(ab') ₂ anti-μ	F(ab') ₂ fragments of rabbit polyclonal Ig specific for human μ chains of IgM
FBS	fetal bovine serum
FcR	Fc receptor
FITC	fluorescein isothyocyanate
FSC	forward angle light scatter
H	Ig heavy chain

HCL	hairy cell leukemia
HIGM-1	hyper IgM immunodeficiency syndrome
IFN	interferon
Ig	immunoglobulin
IgA	immunoglobulin A
IgD	immunoglobulin D
IgE	immunoglobulin E
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
kDa	kilodalton
L	Ig light chain
LMW-BCGF	low molecular weight B cell growth factor
LPS	lipopolysaccharide
mAb	monoclonal antibody
MFI	mean fluorescence intensity
mins	minutes
μL	microlitre
mL	millilitre
NBT	nitroblue tetrazolium
NK	natural killer
nm	nanometre
pNPP	p-nitrophenyl phosphate
PBL	peripheral blood lymphocytes
PBS	phosphate buffered saline
PBS-BSA	0.1% BSA prepared in PBS, containing 0.1% sodium azide
PE	phycoerythrin
PHA	phytohemagglutinin

PKC	protein kinase C
PLL	prolymphocytic leukemia
PMA	phorbol myristic acetate
PMA _{low}	a suboptimal, submitogenic concentration of PMA
PMA _{opt}	optimal concentration of PMA
PWM	pokeweed mitogen
rpm	revolutions per minute
SAC	<i>Staphylococcus aureus</i> Cowan I
SCID	severe combined immunodeficiency
s.d.	standard deviation
SDS	sodium dodecyl sulfate electrophoresis
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
s.e.m.	standard error of the mean
sIg	surface immunoglobulin
SRBC	sheep red blood cells
SSC	side (90°) light scatter
ssDNA	single stranded DNA
TBS	tris buffered saline
TBS-T	tris buffered saline containing 0.05% Tween
TGF	transforming growth factor
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor receptor
TPA	tetradodecanoyl phorbol acetate
U	units
V	variable
XID	x-linked immunodeficiency