Role of Bcl-2 family proteins in mediating CD40-induced drug resistance in Chronic lymphocytic leukaemia (CLL)

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Abstract

Despite the advent of novel therapeutic agents, fludarabine-based chemo-immunotherapy still forms the backbone of modern treatment regimens for fit patients with CLL. Even with the high efficacy of these regimens, a significant proportion of patients respond poorly to therapy. Although TP53 deletion/mutation is strongly associated with fludarabine resistance, it does not account for all patients displaying this resistance phenotype. Understanding the mechanisms underlying fludarabine resistance in patients without TP53 defects is therefore a major priority. In this study, the role of the leukemic microenvironment in mediating such resistance resistance was investigated by focusing on the cytoprotective effect of CD40 stimulation as a result of engagement of CD40 on CLL cells by CD154 on activated T cells which is known to occur at sites of lymph node involvement. This study indicated that in-vitro treatment of resting CLL cells with fludarabine resulted in the consistent up-regulation of Puma and a concomitant increase in cell death. Reciprocal immunoprecipitation experiments using antibodies to Puma or Bcl-2 followed by Western blotting showed that these two Bcl-2 family members form a complex in resting CLL cells. Taken together, the above findings suggest that CD40 stimulation up-regulates Bcl-XL and Mcl-1 which bind to Puma, preventing its activation of mitochondrial apoptosis and subsequently inhibiting fludarabine-induced killing. Therefore, the study revealed a potential mechanism responsible for CD40-mediated resistance to fludarabine in CLL.
Keyword: Bcl-2 family proteins, CD40, drug, Chronic lymphocytic leukaemia (CLL).
في المختبر لخلايا CLL، تحدث في المواقع من تورط العقدة الليمفاوية. أشارت هذه الدراسة إلى أن العلاج المتبوع بالنشاط الغربي أن هذين الفردتين من Bcl-2- عائلة-2، يعملان بـ Puma و Bcl-XL، وينظم Bcl-XL الفردتين من تنشيط موت الخلايا المبرمج الميكرونيتريلا وبالنسبة لحمض الفنتلي موت الخلايا الليمفاوية. لذلك، كشفت الدراسة عن آلية محتملة مسؤولة عن

الكلمات المفتاحية: بروتينات عائلة-2، Bcl-2، علاج، أيبك، الليمفاوي المزمن (CLL).
1. Introduction

Chronic lymphocytic leukaemia (CLL) is the most common blood cancer, accounting for approximately 30% of all leukaemias. Considerable morbidity and mortality is associated with this disease, and it remains incurable by conventional treatments. Patients with progressive forms of CLL will typically be subjected to several rounds of therapy and relapse prior to succumbing to the suppression of bone marrow and immune organ function that accompanies this disease. Development of therapy resistance by the malignant clone in CLL accounts for relapse of the disease and requirement for changed and more severe treatments. Recent studies of clonal evolution imply development of therapy resistance through selection of clones bearing an appropriate mutation (Woyach & Johnson et al. 2015). However, this is not the whole of the story of treatment resistance. It is well known that microenvironment can provide protection signals to CLL cells exposed to cytotoxic stimuli, but the mechanism(s) involved have not been completely characterised. This thesis examines the role of CD40 ligation in providing CLL cells with protection against fludarabine (Kater et al. 2004).

According to current estimates, CLL is the most common leukaemia, with highest incidence rates in Europe and North America (Dores et al. 2007). Approximately 2,400 new cases of CLL are reported each year in the UK (Cramer & Hallek et al. 2011) and more than 15,000 in the USA (Siegel et al. 2014). CLL is considered as a disease of advanced age, with median age at diagnosis between 67 and 72 years, although sporadically there are CLL patients diagnosed in their 30s or 40s. Males are nearly twice more likely to develop CLL. Inherited genetic predisposition to CLL has been documented, although is not yet fully understood (Neuland et al. 1983; Yuille et al. 2000).

Clinical presentation

CLL is a lymphoproliferative and neoplastic disease characterized by a clonal accumulation of B lymphocytes within the blood, bone marrow, lymph nodes, liver, and spleen (Hallek & Pflug et al 2010; Schlette et al 2010). CLL is very heterogeneous when it comes to the clinical course, with some patients having slow
and asymptomatic disease and living for many years without any treatment, and others developing an aggressive disease requiring treatment relatively early (Dighiero 2005). When diagnosed, most patients are asymptomatic or present with only minor symptoms. The diagnosis is thus determined by blood counts (presence of ≥ 5x10^3 clonal B lymphocytes per µl of peripheral blood), microscopic evaluation of the blood smear, and flow cell immunoassay of circulating lymphocytes. Clinical features of CLL may include lymph node enlargement, anemia and / or thrombocytopenia, bone marrow failure, an enlarged liver and / or an enlarged spleen, and may include weight loss, shortness of breath, fatigue, and recurrent infections (Hallek & Pflug 2010). Despite recent advances in CLL management, it is still an incurable disease.

**The role of apoptosis in the pathogenesis of CLL**

CLL is a disease characterized by an abnormality in the death of cancer cells. The cell death network in mammals consists of many distinct functional units, including apoptosis, autophagy and necrosis (Leist & Jäättelä 2001). Apoptosis of the first type of these modules has been studied extensively, and is known to be of prime importance to the health of multicellular organisms, and it is widely known that disturbances in the intracellular apoptosis process can lead to conditions such as cancer where from it is recognized that evading cell death is the main distinguishing feature (Hanahan & Weinberg 2000).

Apoptosis is a very complex process of removing damaged or redundant cells and is necessary for tissue homeostasis. There are two main pathways to apoptosis, the death receptor pathway (external) and the mitochondrial pathway (intrinsic), both of which are regulated by the concerted action of various pro- and anti-apoptotic proteins. The mitochondrial pathway of apoptosis is precisely controlled by a large group of proteins belonging to the Bcl-2 family (Chipuk et al. 2010; Czabotar et al. 2014a; Youle & Strasser 2008). The main event during the intrinsic pathway of apoptosis is the mitochondrial outer membrane permeabilisation (MOMP), which allows the release of proteins localised between the outer and inner mitochondrial membranes, such as cytochrome c or Smac, into the cytosol. Upon release, these proteins cooperate with cytosolic factors, leading to the formation of a multiprotein complex called the apoptosome, resulting in activation of caspases and execution of apoptosis.
Control over the integrity of the mitochondrial outer membrane is executed by proteins of the Bcl-2 family, which can be further divided into three subfamilies: anti-apoptotic (e.g. Bcl-2, Bcl-XL, Mcl-1 and Bcl-w), pro-apoptotic effectors (Bax and Bak), and BH3-only proteins (e.g. Bid, Puma, Bim and Bad), which form a complex network of interactions (Chipuk et al. 2010; Cory & Adams 2002; Czabotar et al. 2014a; Danial 2007; Skommer, Wlodkowic & Deptala 2007). Briefly, Bax and Bak become activated upon association with the BH3 activators, which induces a conformational change in Bax/Bak, leading to their oligomerisation and formation of pores within the outer mitochondrial membrane. The BH3 activator proteins are antagonised by the anti-apoptotic Bcl-2 family members, which are counteracted by the second group of BH3 proteins, referred to as sensitisers.

**Bcl-2 family of proteins**

It has been firmly established that the pro-apoptotic multi-domain Bcl-2 family members Bax and Bak mediate permeabilisation of the outer mitochondrial membrane although other proteins of the mitochondrial membrane, such as VDAC (the voltage-dependent anion channel), may also participate in this process (Kuwana et al. 2002). When cells encounter stress signals, either internal or external, Bax and Bak undergo conformational changes to form homo-oligomers, which then insert into the membrane to form pores. It has been shown that Bax has to first translocate from the cytosol to the mitochondrial membrane, while Bak is constitutively associated with the mitochondrial membrane (Chipuk et al. 2008; Kuwana et al. 2002).

In a direct activation model, when inactive cytosolic Bax is in contact with a group of BH3-only proteins (e.g. tBid, Bim, Puma), the latter directly induces conformational changes in Bax and prompts it to translocate to the outer mitochondrial membrane (Zhu et al. 2013). This is then followed by Bax oligomerisation driven by the bound BH3-only proteins (also known as activators). In an indirect activation model, in healthy cells the BH3-only proteins are antagonised by the anti-apoptotic Bcl-2 family members, which are counteracted by the second group of BH3-only proteins (sensitizers), such as Bad, Bik, Hrk, Noxa and Bmf (Santidrián et al. 2010). Binding of the “sensitizers” liberates the “activators” that engage Bax and Bak (Willis et al. 2010).
The indirect activation model is based on observations that apoptosis can proceed in the absence of direct activators Bim and tBid (Terrones et al. 2008), and supported by the observed apoptosis of Bim/Bid/Puma triple-deficient lymphoid cells in response to glucocorticoid treatment (Yu et al. 2001).

p53 (known as tumor protein and p53 cell tumor antigen), which is an important tumor suppressor gene that works to prevent the growth and survival of malignant cells. These p53 activities reflect its role as a transcription factor that regulates the expression of many genes in response to different stress stimuli. Classically, when DNA is damaged, p53 stimulates the shutdown of the cell cycle allowing time to repair the DNA and enhance cell survival (Mohr et al. 2011). But p53 can also cause cell death programs such as apoptosis or autophagy.

Aberrant apoptosis signals have been widely reported in CLL. The importance of the Bcl-2 family of proteins in the pathogenesis of CLL has been demonstrated, with overexpression of Bcl-2 being one of the hallmarks of the disease (Buggins et al. 2010). Another anti-apoptotic Bcl-2 protein, Mcl-1, as well as IAP proteins (survivin, CIAP1, CIAP2, and XIAP) are also up-regulated in CLL patients (Chiorazzi, Rai & Ferrarini 2005; Grzybowska-Izydorczyk et al. 2010). Moreover, decreased expression of pro-apoptotic molecules such as Smac has been observed in CLL (Grzybowska-Izydorczyk et al. 2010).

Mechanisms of CD40L-mediated protection from drug-induced cell death in CLL

CD40 ligation can save CLL cells from apoptosis, and is an important survival signal for CLL cells within the lymph node microenvironment. Anti-apoptosis action is associated with enhanced expression of intracellular apoptosis-regulating proteins for example Bcl-2, Bcl-XL, Mcl-1 and A1/Bf1-1 (Ghia et al. 2001) and the IAP family member survivin (Granziero et al. 2001).

The effect of CD40 activation on the expression of Bcl-2 family members appears to be dependent on the experimental design. Some authors have reported decreased expression of Bcl-2 Willimott et al. 2007) and increased expression of pro-apoptotic proteins only (such as Bid) after CD40 activation (Kater et al. 2004). Although CD40 stimulation alters the overall profile of the Bcl-2 family to support cell survival, single
proteins may exhibit a non-intuitive pattern of expression. The different results can also be explained by the different culture systems used, i.e. administration of soluble recombinant CD40L (Schattner 2000) versus co-culture with fibroblasts expressing human CD40L with or without interleukins (Willimott et al. 2007) (e.g. IL-4, IL-2, IL-10 or IL-21).

Management of CLL
Chemotherapy is not usually recommended in early and stable disease, and is only given to patients with more progressive or advanced CLL (Robak, Jamroziak & Robak 2009). For a long time, treatments with a chlorambucil alkylating agent were the standard regimen due to its low toxicity, low cost, and ease of oral delivery. Purine analogues such as fludarabine, pentostatin or cladribine are another widely used class of cytostatic drugs, with fludarabine being a spinal improvement in patient treatment outcomes for CLL. A series of clinical trials also demonstrated the benefits of using fludarabine in combination therapy to treat CLL. The LRF CLL4 pathway found that fludarabine was more effective when used with the alkylating agent cyclophosphamide. Complete and overall response rates were better with fludarabine plus cyclophosphamide than with fludarabine alone (complete response rate 38% versus 15%, respectively; overall response rate 94% versus 80%, respectively) (Catovsky et al. 2007). It also found that survival without progression at 5 years was significantly better with fludarabine plus cyclophosphamide (36%) compared to fludarabine or chlorambucil alone (10%). Moreover, a recent CLL8 trial demonstrated the benefits of using the CD20 monoclonal antibody rituximab in combination with fludarabine and cyclophosphamide (Hallek & Pflug 2011). The use of this chemotherapy immunotherapy was effective in prolonging the progressive-free survival and overall survival of patients with symptoms of CLL and helped set a new standard of treatment for physically fit patients (Catovsky et al. 2007).

Fludarabine (9-beta-D-arabinofuranosyl-2-fluoroadenine 5'-phosphate) is a watersoluble synthetic fluorinated purine nucleoside analogue of the antiviral agent
vidarabine (ara-A) (Ricci et al. 2009). On infusion into the bloodstream fludarabine phosphate undergoes rapid dephosphorylation to the respective nucleoside F-ara-A.

There is extreme variability in the therapeutic response to CLL treatment, and most patients eventually develop drug resistance. The microenvironment provides important survival and proliferative signals to CLL cells, and such signals can be provided by CD40L (CD154)-expressing T-cells engaging their corresponding receptor (CD40) on CLL cells. These signals are thought to contribute to drug resistance by inducing the expression of anti-apoptotic Bcl-2 family proteins, culminating in resistance to apoptosis. The fate of CLL cells following drug exposure is likely governed by specific interactions between pro-apoptotic Bcl-2 family proteins induced by drug treatment and anti-apoptotic Bcl-2 proteins induced by CD40L. Therefore, this research aims to address the following research questions:

1) How do drugs that induce p53-dependent (fludarabine) or p53-independent (dexamethasone) apoptosis affect the levels of Bcl-2 family proteins in CLL cells?

2) How does stimulation of CLL cells with CD40L affect the levels of Bcl-2 family proteins?

3) How do pro- and anti-apoptotic Bcl-2 family proteins interact in CLL cells, and how are these interactions influenced by drug treatment and CD40L?

4) How do interactions between pro- and anti-apoptotic Bcl-2 family proteins influence the fate of CLL cells following drug treatment?

5) Can this knowledge be used to overcome drug resistance?

2. Methodology and Materials

Methods

Flow cytometry is routinely used to assess cell viability and to determine cellular immunophenotyping using fluorescence-labelled monoclonal antibodies against surface marker to identify subsets of cells of interest (Craig & Foon 2008). Cell viability assays are based on morphological changes (FSC/SSC), identification of plasma membrane permeability and phosphatidylserine exposure in dying cells with
fluorescent probes such as propidium iodide (PI) and detection of caspase activation, loss of mitochondrial membrane potential, or fractional DNA content (sub-G1), again using fluorescent probes (Wlodkowic, Skommer & Darzynkiewicz 2009). Immunophenotyping is performed with the use of fluorescently-labelled antibodies that recognise specific cell surface proteins (lineage markers), for example CD3 for T lymphocytes and CD19 for B lymphocytes or CD4 and CD8 for helper and cytotoxic T lymphocytes, respectively.

The BD fluorescence-activated cell sorting (FACS) Calibur™ was used for all experiments in my thesis.

Peripheral blood (PB) samples were taken from patients previously diagnosed with CLL after giving informed consent and with the approval from the Liverpool Research Ethics Committee (LREC). Heparinised blood samples were slowly layered on top of Lymphoprep TM (Axis-Shield, Kimbolton, U.K) and centrifuged at 800 g for 30 min at room temperature. The mononuclear layer of cells were carefully collected prior to washing and resuspending in ice-cold RPMI-1640 containing 10% v/v FCS, after which an equal volume of ice-cold RPMI-1640 plus 10% v/v FCS and 20% v/v DMSO was gradually added on ice. One mL aliquots of the final cell suspension were then placed in cryotubes housed in polystyrene holders and stored at -80°C for one week to freeze gradually before being transferred into liquid nitrogen for long-term storage in the University of Liverpool Leukaemia Biobank. For the majority of experiments, cells were not purified further and only CLL cases with white blood cell counts greater than 50x10^9/L were employed to ensure there was minimal contamination from non-malignant cells.

3 x 10^6 CLL cells were seeded on the respective monolayers prepared earlier and co-cultured with parental or CD40L (CD154)-expressing fibroblasts in RPMI-1640 (supplemented with 10% v/v FCS, 2mM L-glutamine, and 100units/mL penicillin and 100µg/mL streptomycin) in a 37°C incubator with 5% CO^2.

6x10^5 CLL cells were collected at the end of incubation and centrifuged at 550g for 5 min at 4°C and washed once with RPMI-1640 medium and again with phosphate buffered saline (PBS) comprised of 137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4,
1.47mM KH2PO4 (pH 7.4). CLL cells were suspended in 500µL of PBS and incubated with 1µg/mL propidium iodide (PI) in the dark for 10 min. Cellular viability was assessed by measuring the number of PI bright (dead) cells in a total of 10,000 gated events on the FACS. PI is a DNA-binding fluorochrome and commonly used for identifying dead cells whereas live cells with an intact plasma membrane exclude PI. Dead cells take up the fluorochrome as they have lost their membrane integrity and therefore fluoresce bright red.

Western blotting is used for detection of specific proteins, endogenous or ectopically expressed, in a mixture of proteins, in a qualitative and quantitative manner. For quantitative analysis, the expression level relative to a control sample, or a purified protein sample of known concentration, is used. Western blotting can be used to compare the level of expression of proteins of interest, monitor protein phosphorylation and changes in molecular weight of particular protein in cells with or without drug treatment.

Immunoprecipitation was used to detect protein-protein interactions in CLL cells cultured under standard or co-culture conditions. The two main uses were to identify whether two known Bcl-2 family proteins interact with one another, for example to investigate interactions between anti-apoptotic and pro-apoptotic proteins.

siRNA is used as a method to down-regulate the expression of target genes in order to establish the link between gene identity and function. It can be used to test function of newly discovered genes. In addition, siRNA is used in pathway analysis whereby disrupting one gene could affect the expression and/or activities of other genes in the same pathway. Finally, siRNA can be used to study gene redundancy (Katome et al. 2003).

**Materials**

**Reagents, cytotoxic agents and Antibodies**

Cryotubes were from Nuncbrand (Fisher Scientific, Loughborough, U.K). Dimethyl sulfoxide (DMSO), trypsin, Dulbeccos Modified Eagles Medium (DMEM), trypan blue, phosphatase and protease inhibitor cocktails, propidium iodide (PI), 2-mercaptoethanol and bovine serum albumin (BSA) were from Sigma-Aldrich (Exeter,
U.K). Roswell Park Memorial Institute (RPMI) 1640 and fetal calf serum (FCS) were from Biosera (Ringmer, U.K). ECL Western blotting kit was from Millipore (Watford, U.K). NIH 3T3 mouse parental and CD40L-expressing fibroblasts were a kind gift from Professor Gerry Cohen (University of Liverpool, U.K). SDS-PAGE gel stacking buffer, resolving buffer, transfer buffer, and tetramethylethylenediamine (TEMED) were from Geneflow (Staffordshire, U.K). SDS-PAGE protein bench ladder was from Invitrogen, U.K. Human B cell Nucleofector kit was from (Lonza, U.K). Puma/BBC3 specific siRNA and non-specific siRNA were from Dharmacon/Thermo Scientific (via Abgene Ltd, Kent, UK). FITC Mouse anti-human CD40L and FITC Mouse IgG isotype control were from B.D. Pharmingen (Oxford, UK). All secondary antibodies were purchased from Santa Cruz Biotechnology (Heidelberg, Germany).

Patients samples
All samples were obtained with informed consent and with the approval of the Liverpool Research Ethics Committee. The diagnosis of CLL was based on standard morphological, and immunophenotypic criteria, as described elsewhere (Melarangi et al 2012).

Statistical analysis
Statistical analysis was performed to compare the effects of fludarabine and dexamethasone on expression of Bcl-2 family proteins in CLL cells cultured under different conditions, and the effects of Puma expression on fludarabine-induced CLL-cell death. All data analysed were of paired measurement and presented as mean ± standard deviation (SD). To compare the effects between any two different conditions in each experiment, the paired t-test was performed using the Graph Pad Prism 5 software (GraphPad Software, San Diego, CA, USA). The α level of < 0.05 (P-value) in 2-sided tests was set to accept any difference with the statistical significance. With no statistical analysis applied.
3. Characterisation of the effects of fludarabine and dexamethasone on CLL cells cultured under standard and coculture conditions

The fact that CLL cells live for a long time in vivo but undergo apoptosis rapidly in vitro (Collins et al. 1989; Coscia et al. 2011) clearly demonstrates that they retain the ability to carry out apoptosis, and that their prolonged survival in vivo requires micro-environmental factors at sites of tissue injury including bone marrow and lymph nodes. As previously described, the interaction of CLL cells with T cells in the bone marrow and lymph nodes of patients plays an important role in the expansion and survival of malignant cells. One of the most important reactions mediating these effects is the stimulation of CD40 on CLL cells by CD40L on T cells. CD40 stimulation has been shown to protect CLL cells from spontaneous and drug-induced apoptosis in vitro (Kitada et al. 1999; Zhuang et al. 2014), suggesting its involvement in mediating drug resistance in vivo.

CLL cells were stained with propidium iodide (PI) according to the procedure described in section 2.1.2.5. Flow cytometry was carried out following this staining using a BD FACS Calibur, and percentage cell apoptosis was calculated. Western blotting was used to detect Bcl-2 proteins. Whole cell lysates were prepared by solubilising cell pellets from CLL cell samples with lysis buffer (10 mM Tris-HCl (pH 7.4), 5 mM MgCl₂, 100 mM NaCl, 1% Triton X-100 and a protease inhibitor cocktail from Sigma-Aldrich (Exeter, U.K). Cells lysates along with the Benchmaker™ Pre-sained protein ladder (life technologies catalogr no: 10748-010) were then sonicated using a tip sonicator set to maximum, and then centrifuged at 13,000rcf for 15min. A protein concentration of the supernatant was determined by Bio DC™ protein assay (Biorad) according to the manufacturer’s instructions (Lowry et al.,1951, Peterson,1979). A volume containing 10µg of protein was then mixed with an equal volume of double strength Laemelli sample buffer (10 mM Tris-HCl (pH 6.7), 1% sodium dodecyl sulphate (SDS), 2-mercaptoethanol and bromophenol blue), and then heated at 95°C for 5 min to fully denature the proteins.
Samples were then applied to a SDS-PAGE gel made up of a 5% acrylamide stacking gel and 15% acrylamide resolving gel to achieve good resolution of lower molecular weight proteins. Separated proteins were transferred to Immobilon-PVDF membranes (Millipore, Fisher Scientific UK Ltd, Loughborough, U.K.) by electrotransfer for 1h at 400mA in chilled transfer buffer. Membranes was washed in TBS-T (150mM NaCl, 25 mM Tris pH 7.5, 0.1% Tween 20) for 15 minutes to remove any residual transfer buffer, and then blocked in blocking buffer (TBS-T supplemented with 5% dry milk) for 45min. Membranes were briefly washed in TBS-T for 15 mins, and probed with primary antibodies targeting Bcl2 proteins diluted at 1:1000 in blocking buffer overnight at 4°C with gentle agitation. The membranes were washed 3 times for 15mins with TBS-T to remove unbound primary antibody, and then exposed to horse radish peroxidase (HRP)-conjugated secondary antibody diluted at 1:10,000 in blocking buffer for 1h at room temperature. Unbound secondary antibody was washed an additional 3 times for 15 mins with TBS-T. Specific proteins were detected using enhanced chemiluminescence (ECL) reagents (Millipore), and reactive bands were visualised using a Fujifilm LAS-1000 chemiluminescence imaging system (Fujifilm, Tokyo, Japan). Quantitative analysis of signals corresponding to the protein band of interest was carried out by densitometry for quantification of data, the images were further analysed on the same instrument using 2D densitometry AIDA image software package (Fujifilm).

**Results**

Spontaneous cell death is moderately reduced in CLL cells when co-cultured with parental fibroblasts and significantly reduced when co-cultured with CD40L (CD154)-expressing fibroblasts as compared with standard culture conditions (p=0.01). Compared to standard culture conditions, CLL cells are significantly protected from dexamethasone- or fludarabine-induced cell death when co-cultured with parental fibroblasts (p values of 0.02 and 0.05, respectively) and even more protected when co-cultured with CD40L-expressing fibroblasts (P=0.002 in both cases).

The expression of different pro-apoptotic and anti-apoptotic Bcl-2 family of proteins followed a characteristic overall pattern when CLL cells were cultured under different
conditions and exposed to different cytotoxic drugs, although there was some variation between individual cases. Fludarabine treatment consistently resulted in the increased expression of Puma in CLL cells cultured under all conditions. Dexamethasone treatment consistently resulted in the increased expression of Bim in CLL cells cultured under all conditions. Levels of Bak and Bax were largely unaffected by different culture conditions and treatment with dexamethasone or fludarabine. Levels of Mcl-1 were reduced by treatment with dexamethasone or fludarabine under standard culture conditions and increased by co-culturing CLL cells with fibroblasts expressing CD40L.

Bcl-xL levels were elevated after co-culture with both parental cells and fibroblasts expressing CD40L and further increased under these conditions by dexamethasone or fludarabine treatment. Levels of Bcl-2 were reduced by co-culture of CLL cells with parental cells and even more so following by co-culture with CD40L-expressing fibroblasts. However, levels were unaffected by treatment with dexamethasone or fludarabine irrespective of the culture condition used.

4. Functional study of Puma in fludarabine-induced death of CLL cells under standard conditions

A consistent finding within the previous section was that Puma is upregulated by fludarabine treatment of CLL cells regardless of culture condition. So far it is assumed that induced expression of Puma results in CLL cell apoptosis because of established studies on the mechanism of fludarabine-induced cytotoxicity in other cell systems) (Zhang, Li & Xu 2013). In order to understand the way in which co-culture of CLL cells with parental and CD40L-expressing fibroblasts provides cytoprotection, it was important to establish whether Puma is required for fludarabine-induced cytotoxicity of CLL cells cultured under standard conditions.

Pooled Puma siRNA (cat#L-004380-00-0005, Dharmacon/Thermo Scientific /GE Healthcare, Little Chalfont, Bucks, UK,) was transfected into CLL cells using nucleofection. 1 x 10^7 CLL cells were resuspended in 100μl transfection solution V from the human B cell nucleofector kit (Amaxa AG/Lonza). 0.5nmol of either Puma
siRNA duplexes or non-specific control siRNA (cat#-001810-02-05, Dharmaco/Thermo Scientific/GE Healthcare,) were added, and CLL cells were electroporated using a Nucleofector apparatus (Amara AG/Lonza, Cologne, Germany) set to program X-01. Following electroporation, cells were mixed with 0.9 ml of pre-warmed medium, and then cultured overnight at 37°C. The cells were subsequently incubated at a density of 5 x 10^6 cells/ml, and cultured with or without fludarabine (10μg/ml) for a further 48h before harvesting for analysis of cell death by flow cytometry and Puma protein expression by Western blot.

**Results**

Western blotting analysis of untreated and fludarabine-treated CLL cells showed that Puma-specific siRNA but not control siRNA prevented the up-regulation of Puma protein by fludarabine. Drug-specific killing was significantly reduced in the Puma siRNA-transfected cells compared with un-transfected cells or control siRNA-transfected cells.

5. **Characterisation of Puma-interacting proteins in CLL cells treated with fludarabine**

Data in the previous section show that Puma is a pivotal mediator of killing induced by fludarabine in CLL cells cultured under standard conditions. Immunoprecipitation is a technique that is used for the purification of an antigen from a mixture of antigens by using the basic precipitation principle. Generally, an antibody specific to that concerned antigen is used for this purpose. The antibody usually attached to an agarose resin which act as a supporting bead. The antigen may arise from various sources such as tissues or cells, translated proteins and metabolically labelled cells. After the pre-immobilization of the specific polyclonal or monoclonal antibody in the insoluble solid support, incubation is done with the cell lysate that contains the required antigen. Sometimes mild agitation is required for binding of the target antigen with the specific antibody. The immune complex thus formed is immobilized and collected followed by elution from the insoluble support for subsequent analysis.
CLL cells were first washed in ice-cold PBS. The cell pellet from this washing step was lysed with 200μl IP lysis buffer (10mM HEPES (pH7.4), 150mM NaCl, 2mM EDTA, 1% CHAPS (Sigma, UK) and 1/100 dilutions of protease and phosphatase inhibitor cocktails from Sigma-Aldrich (Exeter, U.K). Cells suspended in this lysis were then agitated on a rotor mixer at 4°C for 1-2h. Samples were centrifuged at 13,000rcf for 15secs at 4°C, and pellets were discarded. 30μl of both protein A sepharose and protein G sepharose beads that were pre-rinsed in lysis buffer were added to each sample, which were then rotor-mixed for 1h at 4°C (preclearing). Samples were pulse centrifuged at 10,000rcf, and a protein concentration of the precleared supernatant was measured by DC protein assay (Biorad) according to the manufacturer’s instructions. 100μg of protein/sample was used for immunoprecipitation with 2μg/sample of antibody targeting either Puma, Bcl-2, Mcl-1, or Bcl-XL. The antibody/cell lysate mixture was incubated overnight at 4°C, and then with 30μl of protein A sepharose beads that were pre-rinsed in lysis buffer. The samples were further mixed for 1h at 4°C on a rotor mixer, and then pulse centrifuged. Beads were washed in IP wash buffer (10mM HEPES (pH7.4), 150mM NaCl, 2mM EDTA, 0.2% CHAPS) 3 times, and the final pellet resuspended in 30μl total Laemmli sample buffer and prepared for PAGE.

**Results**

Having shown that fludarabine treatment up-regulates Puma in CLL cells, and that Puma contributes to fludarabine-induced killing of these cells under standard culture conditions. Puma was shown associates with Bcl-2 and Mcl-1 in fludarabine-treated CLL cells cultured under standard conditions. When CLL cells are subjected to co-culture conditions Puma co-associated also with Bcl- XL. It seemed that when CLL cells were cultured under standard conditions, the level of induced Puma exceeded the levels of Bcl-2 and Mcl-1. Conversely, when CLL cells were co-cultured with parental or CD40L-expressing fibroblasts then the levels of Bcl- XL and perhaps also Mcl-1 exceeded the level of Puma. These results therefore suggest that the excess of Bcl- XL and Mcl-1 induced under co-culture conditions was sufficient to provide fludarabine-treated CLL cells with protection against apoptosis.
6. Discussion

The heterogeneous response of CLL patients to therapy suggest that there are multiple mechanisms involved, yet it is likely that the Bcl-2 family of proteins play an essential role in determining the sensitivity of CLL cells to therapeutic agents because of the instrumental role this family of proteins plays in regulating the mitochondrial death pathway. CLL cells rapidly undergo apoptosis in-vitro and this strongly suggests that these cells receive important pro-survival signals from their microenvironment. Within the microenvironment pro-survival and proliferative signals can be provided by accessory cells such as stromal cells, dendritic cells, nurse-like cells and T-cells. The latter express CD40L (also known as CD154) on their surface that engages its corresponding receptor on the surface of CLL cells. This stimulation has been shown to protect CLL cells from spontaneous and drug-induced apoptosis. Others have also shown that such stimulation can cause increased expression of anti-apoptotic members Bcl-2 family proteins (Hussein et al. 2009; Willimott et al. 2007).

The purine analogue fludarabine is a widely used class of cytotoxic drugs that has been established as a backbone of chemotherapy for CLL. However, most patients eventually become resistant to the chemotherapy, a situation that is frequently associated with deletion/mutation of the TP53 tumour suppressor gene. In keeping with their p53-independent mechanism of action, glucocorticoids (GCs) such as dexamethasone, either alone or in combination with other agents, have emerged as a useful and important treatment option for patients with fludarabine-refractory or TP53-defective CLL (Steele et al. 2008; Zenz et al. 2010) However, as with chemotherapy, response to glucocorticoids is variable.

Both parental and CD40L-expressing fibroblasts inhibited the spontaneous apoptosis of CLL cells as previously reported. Under standard conditions, dexamethasone and fludarabine caused a significant increase in cell death following 48h incubation in primary CLL cells from 6 different CLL patients. However, co-culturing of CLL cells on parental, and to a greater extent CD40L-expressing fibroblasts markedly attenuated spontaneous, fludarabine- and dexamethasone-induced apoptosis, confirming previous reports showing that CLL cells become resistant to drug-induced apoptosis when they
are stimulated by the microenvironmental factors (de Totero et al. 2003; Kater et al. 2004).

It has already been shown that CLL cells isolated from the peripheral blood differ in levels of expression of Bcl-2 family of proteins to those extracted from the lymph node, but the effect of therapeutic agents on these levels of expression was still unknown. The data presented in this study demonstrated that culturing of CLL cells on CD40L-expressing fibroblasts increased the expression of Mcl-1 and Bcl-XL and this increase was associated with insensitiveness to fludarabine or dexamethasone treatment. Culturing CLL cells on parental fibroblasts was sufficient enough to cause an increase in Bcl-XL expression suggesting its expression can also be upregulated by a CD40-independent mechanism. Treatment with fludarabine induced the expression of Puma in CLL cells under standard and co-culture conditions but failed to cause apoptosis in cells co-cultured with CD40L-expressing fibroblasts. This demonstrated that regulation of apoptosis is far more complex than at the expression levels of Bcl-2 family of proteins. Each anti-apoptotic member has a preference as to which pro-apoptotic member it binds and this can be cell-type specific (Willimott et al. 2007; Youle & Strasser 2008). The increase in other anti-apoptotic proteins such as Mcl-1, and Bcl-XL induced by CD40 stimulation may shift the balance in favour of survival by changing protein-protein interaction pattern with corresponding binding partners (Edwards et al. 2013).

The data presented in this study clearly showed that fludarabine up-regulated the expression of Puma and that knockdown of Puma by siRNA resulted in a reduction in cell death induced by fludarabine, thus demonstrating that Puma is required for fludarabine-induced cell death in CLL cells. This result is, to my knowledge, the first demonstration that Puma is critically involved in mediating cell killing by fludarabine in CLL cells.

The undisputable importance of Puma in the regulation of cell death is underscored by its ability to interact with all anti-apoptotic Bcl-2 family proteins. I have detected two interacting partners of Puma, Bcl-2 and Mcl-1, in un-stimulated CLL cells. I have also detected additional Puma-interacting protein Bcl-XL in CLL cells co-cultured with CD40L-expressing fibroblasts. Given that the overexpression of Bcl-2 is one of the
hallmarks of CLL (Buggins & Pepper 2010; Kitada et al. 1998) and that high Mcl-1 expression is associated with a poor disease prognosis understanding the characteristics of these interactions is important as it may provide a rational basis in selecting targets for intervention in cancer. Puma expression is p53-inducible (Jeffers et al. 2003; Mackus et al. 2005; Ren et al. 2010), and increases rapidly upon treatment with chemotherapeutic agents such as fludarabine that are used in the first-line treatment of CLL. The present study confirmed that Puma expression increases upon fludarabine treatment in primary CLL cells. This study also detected the interactions of Puma with both Bcl-2 and Mcl-1 in fludarabine-treated CLL cells, indicating that Puma may bind to these two anti-apoptotic members of Bcl-2 family of proteins to release Bax and/or Bak activating mitochondrial apoptosis pathway. Considering that fludarabine induced a similar level of CLL-cell death in all samples, and taking into account previous reports on the additive effect of Bcl-2 inhibitors to cytotoxicity by fludarabine in CLL cells, it is likely that Bcl-2 inhibitors will increase cytotoxic efficacy of fludarabine (Campas et al. 2006; Kang & Reynolds 2009).

Puma is a pro-apoptotic BH3-only protein which can be activated by cellular stresses including DNA damage, endoplasmic reticulum stress and growth factor deprivation (Wang & Kaufman 2014). Understanding how this protein functions on a molecular level within CLL cells could provide insight into the effectiveness of therapeutic agents, such as fludarabine, which stimulate its expression. This understanding is particularly important with respect to mechanisms of drug resistance mediated by microenvironment-derived pro-survival signals.

This experiment also showed that Mcl-1 associates with Puma in co-cultured CLL cells. It is likely that induced Mcl-1 also interacts with Puma because immunoprecipitation of the former quantitatively removed the latter from lysates of fludarabine-treated CLL cells. However, technical problems render this conclusion inconclusive. The quality of Western blots of Mcl-1 was poor and bands corresponding to Mcl-1 in molecular weight seemed to migrate close to the Ig heavy chain in immunoprecipitated samples. This made interpretation difficult, particularly because upregulated Mcl-1 expression was not clearly observed in the CD40-stimulated CLL cells.
Nevertheless, the experiments potentially provide an explanation for CD40-mediated drug resistance as mimicked by the co-culture conditions. Co-culture induces the expression of Bcl-XL and Mcl-1 in sufficient quantities to bind and inhibit the apoptotic function of the upregulated Puma. This then rescues the cells from fludarabine-induced apoptosis. This in turn presents a potential avenue of therapeutic intervention. Specific targeting of either Bcl-XL or Mcl-1 in co-cultured CLL cells should release sequestered Puma and cause cell death. This notion is supported by studies showing that compounds such as (Bcl-XL inhibitor) and (Mcl-1 inhibitor) can effectively restore cell death in co-culture models (Choudhary et al. 2015).

7. Conclusion

In conclusion, this work sought to explore in greater detail the resistance mechanism in CD40-stimulated CLL cells in response to drug-induced, p53-dependent apoptosis. Microenvironmental stimuli such as CD40 stimulation shift the balance of cellular fate in favour of survival by up-regulating anti-apoptotic members of Bcl-2 family of proteins including Bcl-XL and Mcl-1. It is shown that these proteins can bind to pro-apoptotic members of the Bcl-2 family of proteins and prevent apoptosis induction. This study is the first to demonstrate a possible mechanism of fludarabine resistance in CLL cells where CD40 stimulation up-regulated expression of Mcl-1 and Bcl-XL which sequestered and inhibited apoptotic activity of fludarabine-induced Puma in CLL cells. This inhibition might be relevant in the survival and expansion of the malignant clone in these cells. Future studies would be to clarify the importance of the interacting partners of Puma in mediating fludarabine resistance using siRNAs specific to Bcl-XL and/or Mcl-1 in CD40-stimulated CLL cells. Furthermore, it would be very interesting to examine if fludarabine-resistant CLL cells can be induced to undergo apoptosis when treated in combination with selective Bcl-XL or Mcl-1 inhibitors. The findings from these studies would be very important in helping design less toxic individualised therapies in CLL.
References


'Puma is an essential mediator of p53-dependent and -independent apoptotic pathways', *Cancer Cell*, vol. 4, no. 4, pp. 321-328.


Schattner, E.J. (2000) 'CD40 ligand in CLL pathogenesis and therapy', *Leukemia & Lymphoma*, vol. 37, no. 5-6, pp. 461-472.


