EXPRESSION AND FUNCTION OF VEGF AND VEGF RECEPTORS IN MALIGNANT B-CELLS FROM PATIENTS WITH CHRONIC LYMPHOCYTIC LEUKAEMIA

Thesis submitted in accordance with the requirements of the University of Liverpool for the Degree of Doctor in Philosophy

by

Andrew T Treweeke

December 2005

Abstract

Vascular endothelial growth factor (VEGF) potently stimulates angiogenesis by inducing proliferation, migration and survival of vascular endothelial cells. Angiogenesis is essential for normal physiological events such as embryonic development and is an important component of solid tumour expansion. Several factors are known to affect VEGF expression including hypoxia, the VEGF transcription factor HIF-1, the tumour suppressor protein p53 and the TNF-family member CD154. Evidence suggests that VEGF also influences the pathogenesis of haematological malignancies such as multiple myeloma, acute lymphocytic leukaemia and chronic lymphocytic leukaemia (CLL). Furthermore, increasing evidence shows that VEGF has an autocrine role in tumour development that requires expression of VEGF receptor(s) by the tumour cells.

The aim of the work presented in this thesis was to establish whether VEGF and VEGF receptors are expressed by CLL cells and to determine what effect such expression might have on CLL-cell function. Specifically, since CLL is a disease characterised by prolonged survival of malignant cells, the role of VEGF in CLL-cell survival was explored.

The results in Chapter 2 show that cultured CLL cells secrete VEGF, and that secretion is increased as HIF-1α becomes stabilised under conditions of hypoxia. However, expression of HIF-1α is not related to the amount of VEGF secreted by these cells, or the presence of a p53 dysfunction mutation. In addition, CD154 stimulation of CLL cells also increased VEGF secretion.

The data presented in Chapter 3, demonstrates that the VEGF secreted by CLL cells is angiogenically active. Thus, CLL-cell conditioned medium (CM) stimulated endothelial-cell proliferation *in vitro* and angiogenesis *in vivo*, effects that were abrogated by pre-incubation of CM with VEGF-neutralising antibodies. The presence of VEGF in lymph nodes and spleen from CLL patients and the elevated microvessel density observed in the lymph nodes, implicate VEGF in the increased angiogenesis that is found in this disease.

Both VEGF-receptor 1 and VEGF-receptor 2 are expressed by CLL cells, and this work is shown in Chapter 4. Moreover, ligation of these receptors by VEGF or the receptor-specific ligands PLGF and VEGF-E, is accompanied by increased expression of Hsp90, thus indicating a possible autocrine role for VEGF in the activation of signalling pathways in CLL cells.

Work presented in Chapter 5 explores the role of VEGF in CLL-cell survival. In culture, exogenous VEGF did not prolong CLL-cell survival nor prevent induction of cell death by fludarabine, ionising radiation or geldanamycin. Neutralising autocrine VEGF and blockade of VEGF receptors did not affect cell survival, but inhibiting VEGF receptor signalling with SU5416 significantly reduced cell viability. Furthermore, CD154-enhanced survival of CLL cells, which involves activation of NF-κB, was reduced by both anti-VEGF neutralising antibody and by SU5416. These results demonstrate that, in CLL cells, autocrine VEGF is an important mediator of the anti-apoptotic effect of CD154 and that such cytoprotection requires combined signalling of both CD40 and VEGF receptors. Taken together, the results presented in this thesis suggest a role for VEGF in the pathogenesis of CLL.

Table of Contents

Acknowledgements

I would like to thank all those members of the Department of Haematology who have helped with this project in some way, and specifically I acknowledge the help of Dr K Till, Dr RJ Harris and Dr M Farahani. Professor JC Cawley generously allowed me to undertake this work. I would like to thank him for this and for his ongoing support of staff education. Professor CH Toh ensured focus and stability during a difficult time; he and all the team have shown great patience with me, for which I'm grateful.

Dr Janet Adamson has been remarkably understanding, patient and firm for what must seem like 24 hours of every day for the past few years. Thank you.

Dr Mirko Zuzel, my supervisor, has been exceptionally helpful in all the time I've known him, but particularly throughout this project. I'd like to thank him for all of the help and advice he has given me over the years.

Finally, the most important person to thank is Dr Haijuan Chen, since it was her idea to study the role of VEGF in CLL. Haijuan's clear plan and her confidence in me, made me believe that I could attempt a higher degree. And here we are, six years later, and she's no longer with us. I hope she would enjoy and be satisfied with this thesis.

Declaration

The work presented in this thesis is my own except for the CAM assay, performed by Dr DC West; molecular biology and endothelial cell proliferation assay performed by Dr HJ Chen; immunohistochemistry on tissue sections performed by Dr KJ Till; NF-κB assay by Dr M Farahani and confocal microscopy by Dr RJ Harris. All the remaining work was performed by myself under the supervision of Dr HJ Chen and Dr M Zuzel.

Publications arising from this work

Chen H, Treweeke AT, West DC, Till KJ, Cawley JC, Zuzel M, Toh CH. In vitro and in vivo production of vascular endothelial growth factor by chronic lymphocytic leukemia cells. Blood. 2000 Nov 1;96(9):3181-7.

Farahani M, Treweeke AT, Toh CH, Till KJ, Harris RJ, Cawley JC, Zuzel M, Chen H. Autocrine VEGF mediates the antiapoptotic effect of CD154 on CLL cells. Leukemia. 2005 Apr;19(4):524-30.

List of Figures and Tables

Figures

Tables

List of Abbreviations

bFGF	basic fibroblast growth factor
BSA	bovine serum albumin
CAM	chick chorioallantoic membrane assay
CLL	chronic lymphocytic leukaemia
CM	conditioned medium
$DiOC_6$	3, 3'-dihexolyloxacarbocyanine iodide
DMSO	dimethyl sulphoxide
ECL	enhanced chemiluminescence
EGCG	epigallocatechin-3-gallate
ELISA	enzyme-linked immunosorbant assay
FACS	fluorescence activated cell sorter
FCS	foetal calf serum
FITC	fluoroscein isothiocyanate
Flk-1	foetal liver kinase-1
Flt-1	fms-like tyrosine kinase-1
GA	geldanamycin
HIF-1	hypoxia-inducible factor-1
HIF-1α	α-subunit of HIF-1
Hsp90	heat-shock protein 90
HUVEC	human umbilical vein endothelial cells
IAPs	inhibitors of apoptosis
IR	ionising radiation
KDR	kinase-insert domain receptor
MAPK	mitogen activated protein kinase
MMP	matrix metalloproteinase
MVD	microvessel density
NF-κB	nuclear factor-κB
NRP-1	neuropilin-1
PAGE	poly-acrylamide gel electrophoresis
PBS	phosphate buffered saline
PFS	progression-free survival

PI	propidium iodide
PLGF	placenta growth factor
PMA	phorbol 12-myristate 13-acetate
poly-HEMA	poly-(2-hydroxyethyl methacrylate)
RT	room temperature
RTK	receptor tyrosine kinase
RT-PCR	reverse transcription-polymerase chain reaction
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
STAT-3	signal transducer and activator of transcription-3
TBS	tris-buffered saline
TBS-T	TBS-Tween
TCA	trichloroacetic acid
TGF	transforming growth factor
TNF	tumour necrosis factor
VPF	vascular permeability factor
VEGF	vascular endothelial growth factor
VEGFR	VEGF-receptor
VEGFR1	VEGF-receptor 1
VEGFR2	VEGF-receptor 2
VEGFR3	VEGF-receptor 3
VHL	von Hippel-Lindau

Chapter 1

General Introduction

1.1 Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a disulphide-linked dimeric protein that potently stimulates angiogenesis by inducing proliferation, migration and survival of vascular endothelial cells. This protein was initially identified as vascular permeability factor (VPF) released by a number of tumours and tumour cell lines. VPF purified from ascites fluid had a molecular weight of 34-42 kDa and was distinct from other known permeability factors (Senger et al, 1983). Subsequently, Ferrara and Henzel (1989) purified a 45 kDa protein from bovine pituitary follicular cells that was mitogenic for vascular endothelial cells, but not for other cell-types, and proposed the name vascular endothelial growth factor. The same mitogenic characteristics as VEGF were also demonstrated for VPF (Connolly et al 1989). Furthermore, when the DNA sequences of both VEGF (Leung et al, 1989) and VPF (Keck et al, 1989) were determined it was found that the two proteins are the same.

1.1.1 *VEGF isoforms*

Early DNA sequencing studies using the human leukaemic cell line HL60 suggested the existence of several molecular species of VEGF (Leung et al, 1989), and this was confirmed by later work using human smooth muscle cells (Tischer et al, 1991). These studies found that the gene for

VEGF (later named VEGF-A), is split among eight exons. The isoforms that arise from this gene by alternative splicing consist of $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$, having 121, 145, 165, 189 and 206 amino acid residues respectively. $VEGF_{121}$ is an acidic polypeptide that lacks the amino acid residues encoded by exons 6 and 7 and cannot therefore bind heparin making it highly soluble, whereas $VEGF_{145}$ lacks exon 7 and most of exon 6. $VEGF_{165}$, the predominant isoform, lacks only exon 6, and is therefore able to partially bind heparin in the extracellular matrix and still be secreted in a soluble form. $VEGF_{189}$ lacks only part of the residues encoded by exon 6, while $VEGF_{206}$ is encoded by all the exons. Both are highly basic, bind to heparin sulphate proteoglycans with high affinity and as such are insoluble.

VEGF-A belongs to a family of proteins that also includes VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (PLGF). VEGF-C and VEGF-D are primarily involved in lymphangiogenesis and act mainly, but not exclusively, through their receptor, VEGF receptor-3 (VEGFR3). VEGF-B binds to VEGF receptor-1 (VEGFR1) and is thought to potentially function in pulmonary vascular remodelling (Mura et al, 2004). VEGF-E is a VEGF homologue derived from the parapoxvirus Orf virus that has VEGF-like properties and binds to VEGF receptor-2 (VEGFR2). It has been suggested that PLGF may play a role in arteriogenesis and is a ligand for VEGFR1. VEGF-A, the isoform studied in this thesis and refered to as VEGF, binds to VEGFR1 with high affinity and to VEGFR2 with about 10-fold lower affinity, This is the principle VEGF isofrom affecting cell function in terms of proliferation, motility and survival.

1.1.2 *Factors affecting expression of VEGF*

Expression of VEGF is induced in conditions that require angiogenesis such as embryonic development and tissue remodelling following various types of injury. Thus, VEGF mRNA is known to be upregulated by growth factors such as EGF, TGF-α, TGF-β, KGF, IGF-1 and PDGF, and inflammatory cytokines such as IL-1α and IL-6 (Neufeld et al, 1999). These agents may play both an autocrine and paracrine role in the induction of VEGF in response to microenvironmental influences such as tissue injury and hypoxia (Ferrara et al, 2003).

One of the factors known to affect the expression VEGF in various cell types is CD40 ligand (CD154). CD154 is a member of the tumor necrosis factor (TNF) family of proteins and is expressed by activated T cells, monocytes, macrophages, dendritic cells and platelets (Banchereau et al, 1994; Schattner EJ, 2000). It is known to be an important microenvironmental survival factor for chronic lymphocytic leukaemia (CLL) cells, and its receptor, CD40, is expressed on B cells and some non-haemic cell types (van Kooten and Banchereau, 1996). The ligation of CD40 by CD154 on endothelial cells, monocytes, fibroblasts and multiple myeloma cells is known to upregulate production of VEGF by these cells (Melter et al, 2000; Cho et al, 2000; Tai et al, 2002).

The best described physiological condition under which stimulation of VEGF occurs is hypoxia. Hypoxia is defined as a reduction of oxygen tension within tissues (Semenza GL, 2000). Under such conditions, there is increased expression of a number of hypoxia-inducible genes including those

for glucose transporter proteins, IGF-2, erythropoietin, transferrin, plasminogen-activator inhibitor 1, nitric oxide synthase 2 and VEGF. The regulation of these genes is controlled by hypoxia-inducible factor-1 (HIF-1). Under hypoxic conditions, HIF-1 induces VEGF transcription and mRNA stabilisation by binding to the hypoxia response element of the VEGF gene (Forsythe et al, 1996). HIF-1 is a heterodimeric protein that consists of an α- and β-subunit (Wang and Semenza, 1995). During hypoxia, HIF-1α rapidly accumulates in the cell nucleus (Wang et al, 1995), where it forms a dimer with HIF-1β and binds to the hypoxia response element of the VEGF gene. Regulation of HIF-1 and its activity is based on the stability of the HIF-1α subunit, which is induced, expressed and stabilised under hypoxic conditions. Under non-hypoxic conditions (normoxia), HIF-1α mRNA is constitutively expressed, but the protein undergoes hydroxylation of proline residues Pro402 and Pro564 (Bruick and McKnight, 2001; Epstein et al, 2001) and is rapidly degraded following binding by the E3 ubiquitin ligase von Hippel-Lindau (VHL) tumour-suppressor protein (Maxwell et al, 1999; Kaelin et al, 2005) with consequent ubiquitination and proteasomal degradation (Salceda and Caro, 1997). Under hypoxic conditions, hydroxylation of HIF-1α is inhibited (Hewitson et al, 2002) and ubiquitination decreases with a concomitant increase in HIF-1α expression (Sutter et al, 2000). It is thought that other factors such as p53 may also affect the stabilisation of HIF-1α. In tumour cells in which p53 function is lost, HIF-1α is constitutively over-expressed under normoxic conditions with an associated increase in VEGF expression (Zhong et al, 1999). Loss of p53 has also been shown to enhance hypoxia-induced HIF-1α expression and cause increased VEGF production

(Ravi et al, 2000). The β-subunit of HIF-1, also known as the aryl hydrocarbon receptor nuclear translocator (ARNT), is not associated solely with HIF-1 and is constitutively expressed since it is stable under both normoxic and hypoxic conditions.

1.2 VEGF Receptors

VEGF binds to two structurally related receptors, namely VEGF receptor-1 (VEGFR1), originally known as fms-like tyrosine kinase-1 (flt-1) and VEGF receptor-2 (VEGFR2), also known as kinase-insert domain receptor (KDR) or foetal liver kinase-1 (flk-1). They are both members of the receptor tyrosine kinase (RTK) family of growth factor receptors and possess an extracellular portion containing seven immunoglobulin-like domains, a single hydrophobic membrane-spanning domain and a cytoplasmic part that consists of a catalytic domain containing RTK-conserved motif that is interrupted by the non-catalytic kinase insert region (Shibuya et al, 1990; Terman et al, 1991; Matthews et al, 1991). A third receptor, VEGF receptor-3 (VEGFR3) that is also a member of the RTK family but is structurally different from both VEGFR1 and VEGFR2. VEGFR3 binds VEGF-C and VEGF-D but not VEGF and is therefore not discussed further.

1.2.1 *VEGFR1*

VEGFR1, the first VEGF receptor to be identified, has a molecular weight of about 180 kDa and the highest affinity for VEGF (kd 10-20 pM; de Vries et al, 1992). VEGF binds to the second and third immunoglobulin domains of this receptor (Davis-Smythe et al, 1996; Wiesmann et al, 1997)

but the exact consequences of this binding remain unclear, since signalling mediated by VEGFR1 appears to be at best weak and in endothelial cells not usually associated with biological effects such as cell proliferation and migration. VEGFR1 is expressed on endothelial cells, haemopoietic stem cells, human trophoblasts, choriocarcinoma cells, renal mesangial cells and vascular smooth muscle cells (Podar and Anderson, 2005). Unlike VEGFR2, VEGFR1 is upregulated by hypoxia (Gerber et al, 1997) and has two other ligands, PLGF and VEGF-B. A soluble, alternatively spliced form of VEGFR1 (sVEGFR1) that consists of six of its extracellular immunoglobulin-like domains, has also been described (Kendall et al, 1993).

In early studies, it was found that stimulation of VEGFR1 with VEGF induces only weak receptor auto-phosphorylation. This led to the proposal that VEGFR1 acts as a decoy receptor (Park et al 1994) by binding VEGF with high affinity and thus reducing availability of this growth factor for stimulation of VEGFR2. Additionally, endothelial cell motility and PI-3 kinase activation by VEGFR2 was inhibited by a repressor sequence from the juxta-membrane domain of VEGFR1 (Gille et al, 2000). However, the weak VEGFR1 response to VEGF exposure may be due to the type of cell studied since the monocyte chemotactic response to VEGF is dependent upon VEGFR1 (Barleon et al, 1996). More recently, VEGFR1 signalling has been linked to the induction of matrix metalloproteinase-9 (Raffi et al, 2003), and the recruitment and survival of haemopoeitic stem cells (Gerber et al, 2002).

1.2.2 *VEGFR2*

VEGFR2 has a molecular weight of about 230 kDa and has a lower affinity for VEGF (kd 75-125 pM) than VEGFR1 (Petrova et al, 1999). In terms of function, VEGFR2 is considered to be the more important of the two VEGF receptors since it is the major mediator of the effects of VEGF (Ferrara et al, 2003). VEGFR2 is expressed on endothelial cells, neuronal cells, osteoblasts, pancreatic duct cells, retinal progenitor cells, megakaryocytes and haemopoietic stem cells (Tammela et al, 2005). Upon ligation, VEGFR2 is dimerised, resulting in auto-phosphorylation and generation of mitogenic, motility and pro-survival signals (Zeng et al, 2001; Rousseau et al, 2000; Gerber et al, 1998b). It is well known that multiple pathways are activated by VEGFR2. Mitogenic signals in endothelial cells include phosphorylation of the Ras GTP-ase-activating protein (Guo et al, 1995), as well as PKC-dependent activation of mitogen activated protein kinase (MAPK) ERK 1,2 (Takahashi et al, 1999). In addition, activation of p38 MAPK by VEGF was shown to be important for VEGF-induced migration of human umbilical vein endothelial cells (HUVEC) (Rousseau et al, 1997). Lastly, the pro-survival effects of VEGF on HUVEC mediated by activation of the PI-3 kinase pathway are also VEGFR2-dependent (Gerber et al, 1998b).

Upon stimulation by VEGF, VEGFRs not only homodimerize but can also form VEGFR1/VEGFR2 heterodimers (Kendall et al, 1996). Moreover, Autiero et al (2003) described amplified receptor phosphorylation by signals generated from VEGF, PLGF or VEGF/PLGF heterodimers upon binding to heterodimeric VEGF receptors, with consequent enhanced angiogenesis. Neuropilin-1 (NRP-1), a receptor that binds semaphorins and is implicated in

neuronal guidance, can also bind $VEGF_{165}$ (but not other VEGF isoforms) (Soker et al, 1998) and PLGF (Migdal et al, 1998). NRP-1 binding of $VEGF_{165}$ enhances chemotaxis mediated by VEGFR2 (Soker et al, 1998). Since VEGF binding to NRP-1 alone does not induce a signal, it has been proposed that NRP-1 presents $VEGF_{165}$ to VEGFR2 such that VEGF-VEGFR2 interactions and signalling are enhanced (Whitaker et al, 2001; Neufeld et al, 2002). Similarly, the formation of a complex between VEGFR2 and the β_3 chain of the $\alpha_v\beta_3$ integrin is thought to participate in the full activation of VEGFR2 following stimulation of endothelial cells with VEGF (Soldi et al, 1999).

1.3 Angiogenesis

Angiogenesis is an important physiological constituent of embryonic development, as well as an essential component of wound healing and inflammation. Angiogenesis defines the process by which new capillaries are formed from pre-existing blood vessels, and involves the migration and proliferation of endothelial cells in response to stimulation by angiogenic growth factors (Carmeliet P, 2003). The first of these factors to be described was basic fibroblast growth factor (bFGF) (Shing et al, 1984). However, numerous other proteins are now known to stimulate angiogenesis, including TGF-α, TGF-β, TNF-α, HGF, matrix metalloproteinases (MMP) and, most importantly, VEGF (Ferrara N, 2004). bFGF, is a mitogenic factor for numerous cell types including endothelial cells and thus stimulates angiogenesis. In contrast, MMPs facilitate angiogenesis by remodelling extracellular matrix during vessel sprouting (Jackson C, 2002). VEGF acts as a potent mitogenic factor for vascular endothelial cells only (Ferrara and

Henzel, 1989; Connolly et al, 1989) and is the predominant regulatory growth factor involved in angiogenesis.

During angiogenesis, VEGF-stimulated endothelial cells become activated and secrete MMPs which degrade the vascular basement membrane and extracellular matrix (Carmeliet P, 2003). Enzymes, and activators of enzymes such as plasminogen, are also released (Pepper et al, 1991). Extracellular matix degradation facilitates the release of heparan-sulphate proteoglycan-bound isoforms of VEGF leading to amplification of endothelial cell stimulation (Pepper et al, 1991). This stimulation induces endothelial-cell proliferation, migration and sprouting of capillaries towards areas of hypoxia. The endothelial cells of these emergent blood vessels are aided in migration by adhesion receptors such as $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins (Brooks PC, 1994; Friedlander et al 1996). This process eventually results in the invasion of the extracellular matrix by sprouting vessels and formation of the capillary lumen. Stabilisation of the newly formed blood vessel requires the cessation of endothelial cell proliferation and recruitment of pericytes to provide structural support (Risau W, 1997). Thus, the formation of new blood vessels is also controlled by factors that inhibit angiogenesis such as angiostatin, plasminogen activator inhibitor, platelet factor-4, endostatin, and the interferons (α, β and γ) (Ferrara N, 2004).

In 1971, Folkman first hypothesised that tumour growth is dependent upon angiogenesis, and it is now well established that tumours require the formation of new blood vessels if they are to expand. Small tumours of about 1-2 mm are able to survive in the absence of their own blood supply by simple diffusion of oxygen and nutrients (Ferrara N, 2004). However, further

tumour expansion requires development of their own blood supply to provide the necessary metabolic support. Hypoxia is a key initiator of this angiogenic switch, in which the balance of pro- and anti-angiogenic factors changes in favour of angiogenesis (Carmeliet and Jain, 2000). This step can occur at any stage of tumour development and is dependent not only on size but also on the tumour-type (Bergers and Benjamin, 2003). During tumour development, new capillaries recruited from the surrounding blood vessels grow towards and infiltrate the avascular tumour mass and thus allow its expansion (Kerbel RS, 2000).

The importance of angiogenesis for tumour development and maintenance is illustrated by the advancement and application of anti-angiogenic treatments for a variety of malignant diseases, as exemplified by the use of the humanized anti-VEGF antibody Bevacizumab (Avastin) in colorectal cancer (Ferrara et al, 2005). Numerous other angiogenic inhibitors have also been used in clinical trials (Kerbal RS, 2000). Recently, the significance of angiogenesis in haematological malignancies has been reported. In the first instance, increased microvessel density in bone marrow of patients with multiple myeloma was described (Vacca et al 1994) and has subsequently been observed in acute lymphocytic leukaemia (ALL) (Perez-Atayde et al, 1997), non-Hodgkins lymphoma (Ribatti et al, 1996) and chronic lymphocytic leukaemia (CLL) (Chen et al, 2000).

1.4 VEGF and cell survival

As discussed in previous sections, VEGF can effectively stimulate the proliferation and migration of endothelial cells as part of the angiogenic

response that is essential for the metabolic support of hypoxic tissues. The third functional response to VEGF that has been described for endothelial cells and other cell types is the enhancement of cell survival.

Generally, cells exist in a state of homeostatsis in which opposing survival and death signals remain in balance preventing premature cell death. The process consisting of a series of events that lead to spontaneous cell death is known as apoptosis (Kerr et al, 1972). In addition to the commitment of cells to apoptosis in response to physiological cues, apoptotic cell death can also be initiated by cytotoxic agents. In both cases, apoptosis is governed by proteins of the Bcl-2 family and mediated by proteases known as caspases. The Bcl-2 family consists of groups of proteins that are either anti-apoptotic or pro-apoptotic in their action. The anti-apoptotic group includes Bcl-2, Bcl-$_{xL}$, A1 and Mcl-1, while the pro-apoptotic group includes, amongst others, BAX and Bak (Cory and Adams, 2002). Dysregulated expression and function of these proteins is responsible for many diseases and particularly for the prolonged survival of malignant cells (Jager et al, 1997; Kondo et al, 2000; Meijerink et al, 1998).

VEGF promotes survival of several cell-types and this pro-survival effect is reported to be due to induction of proteins associated with anti-apoptotic pathways. Gerber et al (1998b) found that VEGF protected endothelial cells from serum starvation by activation of the PI-3 kinase/Akt pathway and confirmed this by using a dominant-negative Akt which blocked VEGF-induced survival. Moreover, use of receptor-specific mutants demonstrated that this survival was regulated through VEGFR2 only. In a second study, the same researchers (Gerber et al, 1998a) found that VEGF

prevented serum-starvation-induced apoptosis in HUVEC by induction of a 5.2-fold increase in Bcl-2 expression and 2.4-fold induction of A1. Furthermore, Bcl-2 over-expression in these cells was sufficient to prevent apoptosis. In a separate study (Nor et al, 1999), VEGF reduced apoptosis in human dermal microvascular endothelial cells (HDMEC) by inducing a dose-dependent increase in Bcl-2 expression, while Bcl-2 over-expressing HDMEC cells implanted into mice showed a 4-fold decrease in cell apoptosis. The enhanced cell survival and induction of Bcl-2 expression was associated with VEGF-mediated angiogenesis.

In addition to Bcl-2 family members, a separate group of proteins known as the inhibitors of apoptosis (IAPs) regulate apoptosis by direct inhibition of effector caspases 3 and 7, and the initiator caspase-9. Examples of IAPs are XIAP and survivin. It has been shown that in HUVEC VEGF upregulates Bcl-2 by 4.2-fold as well as XIAP and survivin by 2.9- and 19.1-fold, respectively (Tran et al, 1999). Of further interest was the observation that bFGF had little effect on XIAP expression but induced a 10-fold increase in survivin. This report of a role for IAPs in VEGF-mediated survival was later supported by the observation that antisense targeting of survivin abolished the anti-apoptotic function of VEGF in TNF-α-induced endothelial cell death (Mesri et al, 2001).

Though VEGF was originally thought to be a mitogenic and survival factor for endothelial cells only, it has become apparent that VEGF can also produce these effects in other cell types. Thus, VEGF induces axonal outgrowth in mouse brain tissue and increases survival of neurons and satellite cells (Sondell et al 1999). Also, cultured dorsal root ganglia were

found to express VEGF and VEGFR2. Further studies have confirmed that VEGF and VEGFR2 are expressed on neuronal tissue, that the levels of both receptors increase under hypoxic conditions, and that inhibitors of VEGFR2 and blockade of endogenous VEGF increases apoptosis of neuronal cells (Ogunshola et al, 2002). The importance of VEGF to the integrity of neuronal tissue was confirmed in a mouse model system for the neurodegenerative disease amyotrophic lateral sclerosis (ALS). In this model low levels of VEGF were associated with motoneuron degeneration (Lambrechts et al, 2003) and administration of VEGF protected the mice against motoneuron loss.

1.5 VEGF and Malignancy

As discussed previously, tumour expansion is dependent upon an improved vascular supply in response to hypoxia. Since VEGF is the principal angiogenic factor, it is not surprising that the role of VEGF in the pathogenesis of malignant disease has been the subject of much research. Initially such work concentrated on solid tumours, but in recent years interest has broadened to include the role of VEGF in haematological malignancies.

1.5.1 *VEGF and solid tumours*

The expression of VEGF mRNA is known to be up-regulated in a large number of tumours including ovarian, kidney, colorectal, gastric, breast and thyroid carcinomas (Ferrara and Davis-Smythe, 1997). VEGF protein has been shown to be secreted by tumours including breast (Yoshiji et al, 1996), lung (Ohta et al, 1996), prostate (Joseph et al, 1997), kidney and bladder

carcinomas (Brown et al, 1993). Moreover, many tumour cells not only produce VEGF but also express VEGF receptors, and VEGF produced by these cells contributes to both angiogenesis and tumour cell survival. The cytoprotective effect of VEGF on tumour cells can therefore be either autocrine or paracrine and depend on the capacity of tumour cells to produce and secrete this growth factor. For example, it has been demonstrated that VEGF plays an essential role in the *in vitro* survival of metastatic breast carcinoma cells via autocrine stimulation (Bachelder et al, 2001). In this study, suppression of VEGF expression potentiated apoptosis, while increased expression under conditions of hypoxia enhanced cell survival. Furthermore, Masood et al (2001) demonstrated that blockade of VEGF or VEGFR2 activity caused a reduction in the viability of cell lines derived from several different tumours.

As described earlier, under hypoxic conditions the VEGF transcription factor HIF-1 is up-regulated by stabilisation of its α-chain, leading to increased expression of VEGF. Under normoxic conditions, HIF-1α degradation is partly regulated by the tumour suppressor protein p53 via promotion of MDM2-mediated ubiquitination (Ravi et al, 2000). In tumours, mutations or loss of p53 enable the malignant cells to express stabilised HIF-1α under normoxic conditions, such that the tumour cells constitutively express this protein. One study demonstrated that over-expression of HIF-1α was present in 13 out of 19 tumour types tested including colon, breast, gastric, lung, skin, ovarian, pancreatic, prostate, and renal carcinomas. Moreover, HIF-1α expression was correlated with aberrant p53 accumulation and cell proliferation (Zhong et al, 1999). Additionally, it was demonstrated

that loss of p53 enhanced hypoxia-induced HIF-1α expression, causing increased levels of VEGF (Ravi et al, 2000). Consequently, elevated HIF-1α expression can be potentially associated with more aggressive disease (Bos et al, 2001).

The importance of the role of VEGF in solid malignancies is illustrated by the development of anti-angiogenic therapies for the treatment of cancers. Examples include an anti-VEGFR2 antibody (Prewett et al, 1999), soluble VEGF receptors (Holash J, 2002) and inhibitors of VEGFR2 signalling (Wood et al, 2000). More recently, the humanized anti-VEGF monoclonal antibody Bevacizumab has been successfully used in trials for the treatment of colorectal cancer (Kabbinnavar et al, 2003; Ferrara et al, 2005).

1.5.2 *VEGF and haematological malignancies*

Increasing evidence suggests that VEGF may play a part not only in solid tumours but also in the survival of cells from haematological malignancies. Early studies indicated that a leukaemic cell line, CMK86, as well as normal haemopoietic stem cells, expressed both VEGF and VEGFR2. VEGF offered these cells protection from apoptosis induced by ionising radiation (Katoh et al, 1995) and chemotherapeutic drugs with a consequent increase in Mcl-1 expression (Katoh et al, 1998). Secreted VEGF is also found in cultures of primary leukaemic cells from mailignancies such as CLL (Chen et al, 2000). Other work suggested that VEGF might play a role in the pathogenesis of leukaemias, since autocrine VEGF was found to induce phosphorylation of VEGF receptors and the release of MMP-9 from myeloid leukaemic cells (Dias et al, 2000). In a follow-up study (Dias et al 2001) it

was found that blocking VEGFR2 delayed, but did not prevent, growth of human myeloid leukaemic cells transplanted into mice. When both human and murine VEGFR2 (but not VEGFR1) were blocked, long term remission was produced, suggesting the presence of both autocrine and paracrine cell-survival loops (Dias et al 2001). Furthermore, blocking VEGFR2 caused decreased cell survival, proliferation and migration.

It was later demonstrated that VEGF induces Bcl-2 expression in HL60 cells and promotes Bcl-2 binding to the molecular chaperone protein, heat-shock protein 90 (Hsp90). VEGF also protected cells from serum starvation and from apoptosis induced by the Hsp90-specific inhibitor geldanamycin (Dias et al, 2002). Recently, exogenous VEGF added to CLL cells in culture was shown to enhance cell survival, while inhibition of VEGF receptor phosphorylation suppressed expression of Bcl-2, XIAP and Mcl-1 (Lee et al, 2003). VEGF also up-regulates Mcl-1 in multiple myeloma cells in a time- and dose-dependent manner (Le Gouill et al, 2004). In more recent work, phosphorylation of Bcl-2 and chemoresistance of acute lymphocytic leukaemia cells were increased by VEGF stimulation (Wang et al, 2005).

. MMP-9, which is important for tumour invasion and angiogenic switch during carcinogenesis (Bergers et al, 2000), was also shown to be produced in response to VEGF in adult T-cell leukaemia (Hayishibara et al, 2002) and CLL (Bauvois et al, 2002). Work from this Department showed that CLL-cell motility is dependent upon autocrine VEGF and $\alpha_4\beta_1$ integrin (Till et al, 2005).

As in solid tumours, angiogenesis in response to VEGF secreted by malignant cells is an important component of leukaemias. This paracrine effect is clinically demonstrable by the presence of elevated microvessel

density (MVD) in the bone marrow of patients with AML (Hussong et al, 2000; Padro et al, 2002) and childhood ALL (Perez-Atayde et al, 1997). Other researchers found elevated MVD in CLL tissues (Chen et al, 2000; Molica et al 2002) and it has been suggested that raised bone marrow MVD in CLL correlates with disease stage (Kini et al, 2000). In contrast, Aguayo et al (2000b) found that MVD was normal in CLL bone marrow despite high plasma VEGF levels. Such contrasting findings can be explained by the relatively low numbers of patients used in these studies or by the differences between the groups of patients selected.

Increased MVD was first reported to be present in bone marrow from patients with multiple myeloma (Vacca et al, 1994), and MVD was subsequently considered to be a significant prognostic indicator in this disease (Rajkumar et al, 2000). Similarly, a study of 88 patients with B-cell non-Hodgkin's lymphoma showed a correlation between increased MVD in lymph nodes and the severity of the disease (Ribatti et al 1996),

As regard the autocrine effects of VEGF, they are dependent on the expression of functional receptors on the cells that produce these growth factors. Katoh et al (1995) first suggested a possible autocrine role for VEGF in haemopoietic stem cells, while other workers reported the presence of both VEGF and VEGFR in haematological tumour cell lines (Bellamy et al, 1999) and myeloid leukaemic cells (Dias et al, 2000). VEGF receptors have also been reported to be present on B-cells from patients with CLL (Ferrajoli et al, 2001; Aguayo et al, 2001) and multiple myeloma cells (Ria et al, 2003).

Various studies have demonstrated the activation of signalling pathways by VEGF in haematological malignancies. In multiple myeloma,

VEGF induced tumour-cell proliferation via a protein kinase C-independent but Raf-1-MEK-ERK protein kinase pathway (Podar et al, 2001). In myeloid leukaemia cells, VEGF was found to affect levels of Bcl-2 through increased expression of Hsp90 via activation of the MAPK pathway (Dias et al, 2002). Furthermore, in the same cell-types, VEGFR2 was reported to be constitutively phosphorylated and located at the nucleus (Santos and Dias, 2004). Autocrine VEGF derived from CD154-stimulated CLL cells causes increased expression and nuclear translocation of NF-κB (Farahani et al, 2005). In these cells, VEGFR ligation induces activation and perinuclear translocation of STAT-3 upon VEGFR endocytosis (Lee at al, 2005).

1.6 VEGF in Chronic Lymphocytic Leukaemia (CLL)

1.6.1 *Current understanding of CLL*

CLL is the commonest form of leukaemia in the West and mainly affects men over the age of 50 years (Call et al, 1994; Diehl et al, 1999). It is characterised by the accumulation of long-lived monoclonal B lymphocytes that are detectable in peripheral blood, and with variable involvement of bone marrow, lymph nodes, liver and spleen. Presentation of the disease varies between patients and ranges from incidental detection of a lymphocytosis in a routine full blood count to investigation for generalised lymphadenopathy. Diagnosis is based on the identification of a persistent lymphocytosis (Robinson et al, 1989) and the presence of smear cells in blood films. The diagnosis is confirmed by immunophenotypic analysis of the malignant CLL cells which express CD5, CD19, CD23, and weak surface immunoglobulin

(displaying light chain restriction to kappa or lambda), CD22 and CD79b (Vilpo et al, 2003).

Marked heterogeneity is found in the clinical course followed by patients. Some remain clinically stable without disease progression with a median survival of 10 years (Binet et al, 1981), while others present with advanced disease that rapidly progresses, is poorly responsive to therapy and has a median survival of 2 years (Binet et al, 1981).

Two staging systems, the Rai system and the Binet system, have been devised for CLL based on tumour load that predict patient survival (Binet et al, 1981; Rai et al, 1975). Neither system is effective at predicting outcome for patients with early stage disease who go on to develop progressive disease (Molica et al, 1999b). Other factors that may act as prognostic indicators are CD38 expression, somatic hypermutation of V_H genes (Caligaris-Cappio and Hamblin, 1999) and functional abnormalities of p53 arising from mutations (Lin et al, 2002).

In progressive, symptomatic disease, treatment is used to improve the patients' symptoms and delay the time to progression (Shustik et al, 1988). This may include an alkylating agent such as chlorambucil or the nucleoside fludarabine, or a combination of both alkylator and nucleoside (Pangalis et al, 2002).

The B-cell type that might be the normal counterpart of the CLL cell has not been clearly established. Initially, CLL was considered to be a malignancy of naïve B cells but this was rejected following the demonstration of V_H gene somatic hypermutation in some cases (Fais et al, 1998). Subsequently, B1 cells were proposed as the CLL cell of origin but gene

array data indicated that B1 cells and CLL cells had dissimilar gene expression patterns (Klein et al, 2001). More recently (Chiorazzi and Ferrarini, 2003), it has been speculated that marginal zone B cells might represent the B-cell population from which CLL evolves. However, uncertainty regarding the nature of the CLL cell of origin still remains.

1.6.2 *The role of VEGF in CLL*

The first report of expression of VEGF mRNA in CLL cells was published in 1996 (Baban et al, 1996). Subsequent work concentrated on detection of plasma and serum VEGF levels as a prognostic indicator for CLL, and this was followed by the detection of increased angiogenesis in CLL tissues. Since it became established that CLL cells express both VEGF protein and VEGF receptors, recent work has concentrated on establishing the mechanisms of VEGF function in CLL cells by using inhibitors that potentially have therapeutic value.

Molica et al (1999) measured serum VEGF levels from 68 CLL patients and 31 healthy individuals and found that VEGF levels were not significantly different between the two groups. However, among patients with Binet stage A (n=41), progression-free survival (PFS) was shorter when VEGF levels were above the median and they concluded that serum levels of VEGF can be useful for predicting the risk of disease progression. In a further study (Molica et al, 2002), the same group studied 81 patients at Binet stage A and found that for patients with serum VEGF levels above the median value, PFS was 24 months compared to 54 months for patients with VEGF levels below the median. In contrast, measurement of cellular levels of VEGF

in 225 CLL patients (Aguayo et al, 2000a) revealed that low levels of VEGF in Rai stages 0 to II and Binet stages A and B correlated with shorter patient survival time. Differences in the findings of these two groups can be attributed to methodology, since Molica et al (2002) used enzyme-linked immunosorbant assay (ELISA) to measure serum VEGF levels of CLL paptients while Aguayo et al (2000a) employed a solid-phase radioimmunoassay to determine cellular VEGF levels in CLL B-cells.

Evidence of increased angiogenesis in tissues from CLL patients has also been investigated with the intention of identifying its suitability as a potential indicator of disease prognosis. Kini et al (2000) quantified the degree of angiogenesis in the bone marrow of CLL patients by measuring the MVD. They found that for CLL patients (n=12) the MVD was significantly higher than controls (n=11) and correlated with disease stage since this elevated MVD suggest that this is a paracrine effect of VEGF derived from infiltrating CLL cells. In contrast, a separate study (Aguayo et al, 2000b) measured MVD in 145 bone marrow biopsies from acute and chronic leukaemias and found that MVD was elevated in every condition except CLL, despite raised plasma VEGF levels. However, further work (Chen et al, 2000; also presented in Chapter 3 of this thesis) established that MVD in lymph nodes from CLL patients (n=3) was higher than that for normal controls (n=3).

The studies mentioned above indicated the presence of high levels of circulating VEGF in CLL patient plasma and provided some evidence of angiogenesis in CLL patient tissues. Baban et al (1996) demonstrated the presence of VEGF mRNA in CLL cells, but this observation was taken a

stage further when CLL cells were shown to secrete angiogenically-active VEGF (Chen et al, 2000). In this study, expression of VEGF was enhanced under hypoxic conditions and abundant VEGF was present in the nodes and spleen of CLL patients. Expression of VEGF and other angiogenic factors by CLL cells was later confirmed by others (Kay et al 2002).

The expression of VEGF receptors is potentially an important limiting factor in CLL-cell responses to VEGF. When expression of VEGFR2 on cells from 216 CLL patients was compared to 31 normal individuals, shortened survival of patients with CLL was shown to be correlated with high levels of VEGFR2, and this was considered to be an indicator of unfavourable prognosis (Ferrajoli et al, 2001). In contrast, expression of VEGFR1 did not correlate with patient survival (Aguayo et al, 2001). The expression of VEGFRs by CLL cells was subsequently confirmed in several other studies (Bairey et al, 2004; Kay et al 2002). Till et al (2005) demonstrated that autocrine VEGF and $\alpha_4\beta_1$ integrin are involved in chemokine-dependent motility on and through endothelium by CLL cells from patients with lymphadenopathy. The CLL cells also failed to cluster and activate $\alpha_L\beta_2$ in response to chemokines unless VEGFR and $\alpha_4\beta_1$ were also ligated.

A possible role for VEGF in the survival of CLL cells has recently been elucidated. Work by Lee et al (2004) suggested that autocrine survival pathways may exist in CLL that are a consequence of VEGF signalling. They found that addition of VEGF to cultured CLL cells had a pro-survival effect, that VEGF receptors are spontaneously phosphorylated and that inhibition of this phosphorylation with epigallocatechin-3-gallate (EGCG) induced cell death. Furthermore, inhibition of the effects of VEGF by EGCG reduced

expression of Bcl-2, XIAP and Mcl-1, and induction of pro-apoptotic events. They concluded that the disruption of VEGF autocrine pathways induced CLL-cell death. Recently, the same researchers demonstrated that the autocrine VEGF survival pathway in CLL is linked to the activation and perinuclear translocation of STAT-3 (Lee et al, 2005). Our own studies described autocrine VEGF as an important mediator of the anti-apoptotic effect of CD154 on CLL cells (Farahani et al, 2005; presented as part of Chapter 5). This cytoprotection required combined signalling by both CD40 and VEGFR, involved NF-κB activation and resulted in increased survivin production. However, addition of exogenous VEGF to cultured cells or blockade of secreted autocrine VEGF in the absence of CD154 had only a small effect on CLL cell survival, indicating that CLL-cell cytoprotection by CD154 involves VEGF production and combined signalling by both CD40 and VEGFR.

Chapter 2

Production and factors affecting production of VEGF by CLL cells

2.1 Introduction

An increase in the abundance of blood vessels and the consequent improvement in the blood supply to tissues is known to be important for the continued growth of malignant tumours. The importance of increased angiogenesis in solid tumours was first described by Folkman et al (1971) and is now widely accepted to be an essential part of the pathogenesis of malignant tumour growth. The potent angiogenic growth factor VEGF is thought to be involved in tumour angiogenesis since elevated production of VEGF has been reported in many different types of tumour, including breast, lung and prostate cancer (Yoshiji et al, 1996; Ohta et al, 1996; Joseph et al 1997).

Recently, elevated serum levels of VEGF have been reported in malignancies which do not form solid tumours, such as leukaemias (Gerber and Ferrara, 2003). At the outset of the present studies, it had already been reported that serum levels of VEGF elevated above the median were associated with poor outcome for CLL patients (Molica et al, 1999), while increased blood vessel density had been observed in bone marrow from patients with B-CLL (Kini et al, 2000). It was, however, not known whether CLL B-cells themselves were able to produce and secrete VEGF or whether the elevated plasma levels and increased bone marrow angiogenesis

reported in these papers were due to VEGF production by other cell-types. Therefore, the initial aim of this study was to determine whether CLL cells produce VEGF and then identify the isoforms produced.

In other cell types, several factors are known to influence the expression of VEGF including hypoxia, HIF-1 expression and cell stimulation by CD154 (CD40 ligand). Numerous reports have shown that VEGF secretion increases under hypoxic conditions (Shweiki et al, 1992), and that hypoxia also stabilises HIF-1α, the α-subunit of the VEGF transcription factor HIF-1. In haemic malignancies such as CLL, expression of HIF-1α by the malignant cells has not been described. However, since results in this Chapter will show that CLL cells produce VEGF and that the level of secretion of VEGF varies between CLL cases, it became important to establish whether this variation could be due to differences in constitutive expression of HIF-1α. Furthermore, in other cell-types, expression of VEGF is negatively regulated by p53 (Blagosklonney et al, 1998), a tumour suppressor protein that promotes degradation of HIF-1α.

Loss of p53 in other cell-types enhances hypoxia-induced expression of HIF-1α with a subsequent increase in VEGF levels (Ravi et al, 2000). In addition, a significant correlation exists between the presence of mutant p53 and HIF-1α over-expression in tumour cells (Zhong et al 1999). Since p53 dysfunction associated with expression of the mutated protein (defined in our Department as Type-A dysfunction) occurs in a proportion of CLL cases (Pettitt et al, 2001) and is considered a poor prognostic indicator, it is possible that the variable production of VEGF in CLL cells under both

normoxic and hypoxic conditions may be due to an influence of p53 mutation on the stability of HIF-1α.

An important microenvironmental stimulus implicated in the pathogenesis of CLL is CD154 (CD40L) expressed on T lymphocytes and a number of other cell types (Banchereau et al, 1994). Ligation of CD40 by CD154 on cells such as endothelial cells, monocytes and fibroblasts, upregulates production of VEGF by these cells (Melter et al, 2000; Cho et al, 2000). Therefore, CD154 had to be considered as a factor that is potentially important in the *in vivo* regulation of VEGF in CLL cells. These questions are addressed in this Chapter.

2.2 Methods

2.2.1 *Patients*

All CLL-cell samples used were from patients with high numbers of circulating malignant cells, as defined morphologically (prolymphocytes <10%) and immunophenotypically ($CD19^+$, $CD5^+$, $CD23^+$ and weak light-chain-restricted surface immunoglobulin). Patients' samples were obtained after informed consent and with the approval of the Liverpool Research Ethics Committee. Typically, white cell counts were $>100x10^9$/L and the B-cell purity of these samples was similar to that which could be attained by either positive or negative depletion of contaminating T cells (>95% CD5+). Some T-cell contamination was presumed to be unavoidable but assumed to have little or no effect on the assays employed. However, it is acknowledged that in terms of disease status, selection of high cell count samples does skew the CLL cases studied such that they may represent a 'worse group'.

2.2.2 *Cell preparation and culture*

Lymphocytes were isolated by density-gradient centrifugation. Heparinised whole blood from CLL patients' was layered onto Lymphoprep (Nycomed), centrifuged at 1600 rpm for 30 minutes, then the mononuclear cells removed and washed in RPMI medium. Finally, the cells were suspended in ice cold RPMI supplemented with 10% FCS and 10% dimethyl sulphoxide (DMSO) prior to freezing at -80^{0}C and subsequent storage in liquid nitrogen.

For cell culture experiments, frozen samples were rapidly thawed at 37°C, slowly resuspended to approximately 10 ml in RPMI medium containing 2mM L-glutamine, centrifuged, then resuspended in the appropriate culture medium prior to use. At this stage, cell viability was measured using trypan blue (Sigma) exclusion and typically found to be >90%. In some preliminary assays, data collected using cryopreserved and fresh CLL cells from the same sample was fully comparable, suggesting that cryopreservation did not adversely affect CLL-cell responses in vitro (data not shown).

2.2.3 *Poly-HEMA-coating of culture surfaces*

Poly-HEMA (Sigma) is a non-toxic, hydrophilic polymer that prevents cell adhesion (Folkman and Moscona, 1978). A stock solution of poly-HEMA (120 mg/ml in 95% ethanol) was diluted to 12 mg/ml in 95% ethanol immediately prior to use. To coat the surfaces of tissue culture plates, a volume of diluted poly-HEMA was added to the wells and allowed to

evaporate to dryness at 37°C. For 24-well plates the volume required to coat a well was 200 µl, while for 96-well plates the volume was 30 µl.

2.2.4 *Preparation of CLL-cell culture supernatants*

Freshly thawed CLL cells were suspended at a density of $8x10^6$ cells/ml in QBSF-51 serum-free medium (Sigma) or RPMI medium (Sigma) supplemented with 1% BSA (Sigma). In duplicate, 250µl of cells ($2x10^6$) was transferred to a 24-well plate and incubated at 37°C in 5% CO_2 in a humidified atmosphere for 24 hours. Following incubation, the medium was removed from the cultures, placed in 1.5 ml eppendorf tubes and centrifuged at 14000 rpm for 30 seconds to remove any CLL cells present. The cell-free medium was then transferred to a clean tube and stored at -20°C until assayed for VEGF.

2.2.5 *VEGF enzyme-linked immunosorbant assay (ELISA)*

Supernatants collected from CLL cells were tested for the presence of VEGF by ELISA. In each case, 200 µl of the collected supernatant was assayed using a commercial human VEGF immunoassay kit (Quantikine, R&D Systems) according to the manufacturer's protocol. With this method, it was found that the minimum amount of VEGF detectable in the cell supernatants was typically less than 5.0 pg/ml.

2.2.6 *Preparation of CLL-cell culture supernatants for Western blotting*

CLL cells were prepared for culture in QBSF-51 as described above (section 2.2.4) and stimulated with 100 ng/ml phorbol 12-myristate 13-acetate

(PMA; Sigma) to enhance VEGF production, since PMA had been show to have this affect in other cell-types (Shih et al, 1999). The supernatants were collected and concentrated by ammonium sulphate precipitation followed by Centricon spin column (Millipore) centrifugation prior to assay. As a control, QBSF-51 that had not been exposed to cells was concentrated in this way.

2.2.7 *Immunocytochemical staining for VEGF*

B-CLL cells ($8x10^4$) cultured for 24 hours with and without PMA (100 ng/mL) were cytocentrifuged at 300 rpm for 3 minutes using a Cytospin 2 (Shandon) and fixed at 4°C with ice-cold acetone for 3 minutes. Fixed slides were washed with phosphate-buffered saline (PBS) and incubated with 3% H_2O_2 in PBS for one hour at room temperature (RT). Cells were then incubated with rabbit polyclonal anti-VEGF antibody (10 µg/ml diluted with 1% BSA/PBS) or with nonspecific rabbit IgG (Santa Cruz Biotechnology) for one hour at RT, followed by incubation with goat anti-rabbit IgG conjugated to biotin and with ExtrAvidin–horseradish peroxidase (both for 30 minutes at RT). Colour was developed by incubating the slides with 3-amino-9-ethylcarbazol at RT for 3 minutes. Slides were counterstained with hematoxylin.

2.2.8 *Detection of VEGF by Western Blotting*

Concentrated supernatant derived from PMA-stimulated cells was added to an equal volume of non-reducing double-strength sample buffer (125mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 0.006% bromophenol blue) and heated at 95°C for 10 minutes. Proteins were separated by

electrophoresis on 10% SDS-polyacrylamide gel under non-reducing conditions, then transferred to an Immobilon membrane (Millipore) and blocked in 5% non-fat powdered milk (Tesco) in TBS containing 0.05% Tween-20 (Sigma). The membrane was then incubated with mouse monoclonal anti-VEGF antibody (R&D Systems), washed in TBS-T and further incubated in horseradish peroxidase-conjugated goat anti-mouse IgG (Affiniti). Following repeated washing in TBS-T, immunoreactive protein bands were visualised with an enhanced chemiluminesence (ECL) detection kit (Amersham) as instructed by the manufacturer and Hyperfilm, high performance chemiluminesence film (Amersham).

2.2.9 *RNA isolation and RT-PCR*

Total RNA was extracted from CLL cells using an RNeasy kit (Qiagen). Single-strand DNA was synthesized from 1 μg total RNA using the First-Strand cDNA Synthesis Kit for RT-PCR (Boehringer Mannheim). Amplification of the cDNA by PCR was performed using the following primers for VEGF: oligo 1, 5'-TCGGGCCTCCGAAACCATGC-3'; and oligo 2, 5'-CCTGGTGAGAGATCTGGTTC-3' (30 cycles, annealing at 56°C). To normalize the product, the actin gene was also amplified using actin primers: oligo 3, 5'-CGCTGCGCTGGTCGTCGACA-39; and oligo 4, 59-GTCACGCACGATTTCCCGCT-3' (30 cycles, annealing at 60°C). The amplified PCR products were subjected to electrophoresis and visualized by ethidium bromide staining. To confirm that the sequences of the products amplified by the PCR reaction represented different VEGF isoforms, the PCR products were cloned into pBlueScript plasmid (Stratagene), and their

sequences were determined by nucleotide sequence analysis using a DNA Sequencing kit (Amersham).

2.2.10 *Slot-blot hybridization.*

Total RNA (2 µg) extracted from unstimulated cells was loaded directly onto a nylon membrane using a slot-blot apparatus (Bio-Rad). Membranes were UV cross-linked and subsequently hybridized with a VEGF cDNA probe labelled with α-^{32}P]-deoxycytidine triphosphate using a random-primed DNA labelling kit (Boehringer Mannheim). For control of the RNA loading of each lane, blots were rehybridized with a radiolabelled actin cDNA probe (Clontech Laboratory). Autoradiography was performed at 27°C using Kodak XAR-5 film (Sigma). The intensity of each band appearing on the film was scanned and analyzed with Phoretix 1D-advanced version 3.1 software.

2.2.11 *Exposure of CLL cells to hypoxia*

To induce hypoxic stimulation, CLL cells were cultured as described previously in 24-well plates and within a specially designed air-tight container (Billups-Rothenberg) that had been constructed with inflow and outflow valves. To achieve the desired oxygen level, the chamber was infused with a pre-analysed air mixture containing 5% CO_2/95% N_2 (BOC) and the cells cultured under such conditions for 24 hours prior to collection of culture supernatants.

2.2.12 *Preparation of cell-line lysates*

In experiments to determine HIF-1α expression in CLL cells, two prostate carcinoma epithelial cell lines that are known to produce VEGF (Chen et al 2000a) were used to act as controls, PC-3 and DU145. Both cell-types were grown to confluence in 6-well plates using RPMI medium supplemented with 10% FCS (Globefarm), 2mM L-glutamine (Gibco) and 100 U/ml penicillin + 100 µg/ml streptomycin (Gibco). Following incubation under normoxic (5% CO_2 in air) or hypoxic conditions for 4 hours, the medium was removed, the cells washed in PBS and 250 µl trypsin-EDTA (Gibco) added to the cells. After 1 min at 37°C, the cells were dislodged and transferred to a 1.5 ml tube, centrifuged at 7000 rpm for 30 seconds, then 200 µl of hot lysis buffer (1% SDS, 10 mM Tris-HCl pH 7.4) added and the samples heated at 95° for 5 minutes. Prior to freezing at -20°C, the cell lysates were sonicated.

2.2.13 *Preparation of CLL-cell lysates*

When preparing lysates to be used for the detection of HIF-1α, it was important that the manipulation of cells was performed rapidly to prevent degradation of HIF-1α. To this end, cell lysates were prepared from no greater than four different CLL cases at any given time. CLL cells ($5x10^6$) were incubated under either hypoxic or normoxic conditions for 4 hours in 24-well plates coated with poly-HEMA (see previously) to prevent adhesion of the cells to the plastic surface. Harvested cells were centrifuged at 7000 rpm for 30 seconds and the cell pellet lysed with 100µl 1% SDS lysis buffer, heated to 95°C for 5 min, then sonicated prior to freezing at -20°C

2.2.14 *Detection of HIF-1α by Western blotting*

Lysate from CLL cells or control cells (PC-3 and DU145) was added to an equal volume of double-strength sample buffer containing 10% β-mercaptoethanol and loaded onto a 7% SDS-polyacrylamide gel prior to separation by electrophoresis under reducing conditions. The procedure for Western Blot has been described previously (section 2.2.8); in this instance, the membrane was probed with monoclonal mouse anti-HIF-1α (ab1; Abcam) diluted 1/500. Following visualisation of immunoreactive bands with ECL, the membrane was incubated at 65°C for 30 minutes in stripping buffer (62.5 mM Tris-HCl, pH 6.8, 100 mM β-mercaptoethanol, 2% SDS), washed in TBS-T, then re-probed with mouse anti-β-actin (Sigma) as a protein loading control.

2.2.15 *Densitometry*

For semi-quantitative protein and mRNA analysis, bands visualised by ECL or auto-radiography were scanned and the intensity of each band appearing on the film measured using Phoretix 1D-advanced version 4.00 software.

2.2.16 *Preparation of culture supernatants from CD154-stimulated CLL cells*

CLL cells ($2x10^6$) were suspended in RPMI medium supplemented with 2 mM L-glutamine and 1% BSA, then placed into a poly-hema-coated 24-well plate. Recombinant human soluble CD154 trimeric protein (200 ng/ml final concentration) and enhancer (1 μg/ml) (Alexis Biochemicals) were added to the cells before incubation at 37°C in 5% CO_2 for 24 hours.

Collection of the cell culture supernatants and measurement of VEGF by ELISA are described previously.

2.3 RESULTS

2.3.1 *Detection of VEGF in CLL-cell culture*

To examine whether CLL cells produce VEGF, freshly thawed cells were resuspended to $8x10^6$ per ml in QBSF-51 serum-free medium and cultured in flat-bottomed 24-well plates, in duplicate, with $2x10^6$ cells per well. At this cell density, microscope viewing revealed that the cells were in close proximity to each other but not overlapping when settled at the bottom of the well. Following an incubation period of up to 72 hours, supernatants were harvested, centrifuged to remove any cells remaining in suspension, and then stored at –20°C for future assay, using a commercially available VEGF ELISA kit.

Preliminary experiments revealed that VEGF first became consistently detectable in culture supernatants after 24 hours incubation; increasing amounts of VEGF were then detected with increasing incubation time. Since the aim of this series of experiments was simply to determine whether VEGF was detectable in CLL-cell cultures, and since 24 hours was the earliest time-point at which VEGF could be consistently detected, in subsequent experiments the supernatants were collected for analysis at 24 hours only.

The production of VEGF by cultured CLL cells from 36 cases of CLL is shown in Figure 2.1A. Detectable levels of VEGF were present in culture supernatants from 33 of these cases but, in contrast, VEGF was not detected

in the 3 remaining cases. Furthermore, the amount of VEGF produced varied between the cases studied, ranging from 5 to 215 pg/ml, with a mean value of 30 pg/ml. This variability was consistent in that high producers were always high and low producers were always low, as illustrated by the small error bars for cases that were studied on more than 2 occasions (n=5). The results from this series of experiments demonstrated, for the first time, that CLL cells in culture produce and secrete VEGF.

In Chapter 5 of this thesis, the role of endogenous VEGF in CLL-cell survival is investigated and for these experiments, alternative culture conditions were determined. To provide an environment that would be suitable for cell survival studies, CLL cells were suspended in RPMI medium containing 1% BSA, which enabled cell survival for 7 days or more. To ensure that CLL cells in this medium also produce VEGF, 24 hour cultures were set up and levels of secreted VEGF measured by ELISA as described previously. This analysis revealed that under these alternative culture conditions, VEGF was detectable in each of the cases tested (n=11; mean= 6 pg/ml) although, compared to results obtained in QBSF-51 culture medium, all the values were at the lower end of the assay range (Figure 2.1B). However, additional work carried out in this Department by other investigators (Slupsky et al, unpublished data) using identical culture conditions and a larger sample of CLL cases confirmed that in all cases the cells produce VEGF. Furthermore, they demonstrated a wide range of VEGF production between cases similar to that shown in Figure 2.1A. Taken together, these data confirmed that RPMI supplemented with 1% BSA, the

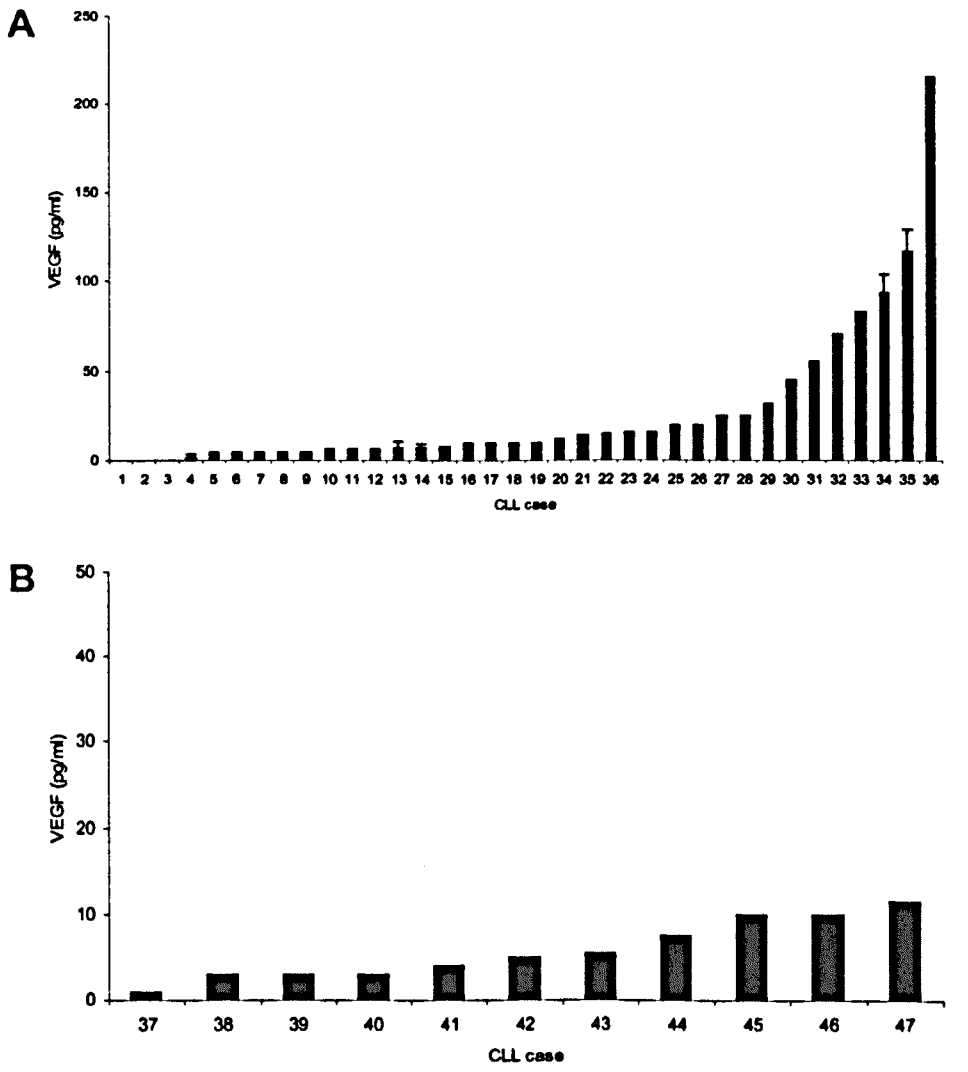


Figure 2.1

Production and secretion of VEGF by cultured CLL cells.

CLL cells were cultured in (A) QBSF-51 serum-free medium (n=36 cases) or (B) RPMI medium containing 1% BSA (n=11) for 24 hours at 37°C in 5% CO_2 prior to collection of medium for VEGF measurement using an ELISA kit. VEGF in the samples was calculated using a reference curve established from serial dilutions of rhVEGF. Individual values are from duplicate samples. Values represent either VEGF levels from single experiments or mean values for up to four separate experiments. Error bars (where shown) represent the SEM calculated from a minimum of three separate experiments. (Note that although low, the VEGF secreted by cells in RPMI medium was still within the range of that secreted by about half of the CLL cases cultured in QBSF-51.)

medium routinely used in our Department, is a suitable medium for studies of VEGF secretion by CLL cells.

2.3.2 *Production of VEGF in CLL-cell cultures is not due to contamination with other cell types*

Since it is known that T cells, macrophages, neutrophils and platelets can all produce VEGF (Freeman et al, 1995; Kolch et al, 1995; Banks et al, 1998; Taichman et al, 1997), it was important to confirm that the VEGF present in cultures of CLL cells was produced by the malignant B-cells and not by other contaminating cell-types. Therefore, cytospins were prepared using cells from several cases of CLL and examined for the presence of VEGF by immuno-cytochemical staining with an anti-VEGF polyclonal antibody (10 μg/ml; Santa Cruz) in conjunction with an ExtrAvidin staining kit (Sigma). Examining a large number of cells from samples with a purity of greater than 95% showed that all cells were uniformly positive for VEGF and that no minority of strongly positive cells was present (data not shown). This finding indicated that the VEGF detected in CLL-cell cultures was produced by the CLL cells themselves and not by a minority population of strongly positive CLL cells or some other contaminating cell-types.

2.3.3 *Expression of VEGF isoforms*

It has long been known that VEGF exists as 5 different isoforms generated by alternative splicing of the VEGF gene. Since there are two soluble forms of VEGF ($VEGF_{121}$ and $VEGF_{165}$), we aimed to identify which of

these two VEGF isoforms were secreted into the medium of cultured CLL cells.

Since the amounts of VEGF detected in medium of CLL cultures described above was relatively low (pg/ml amounts), it was considered necessary to increase the amount of VEGF secreted into the medium to levels detectable by Western blotting. PMA is known to stimulate production of VEGF in other cell-types such as endothelial cells (Shih et al, 1999), so the CLL cells were stimulated with PMA in an attempt to increase VEGF levels in CLL-cell culture medium. It was found that PMA (100 ng/ml) stimulation of CLL cells for 24 hours produced a 5-fold increase in VEGF production (49 ± 59 pg/ml to 266 ± 110 pg/ml), a level at which VEGF was readily detectable in Western blots. Conditioned medium (CM) from 4 different PMA-stimulated CLL cases was collected and concentrated 10-fold prior to loading onto an SDS-PAGE gel. The proteins were then electrophoretically transfered onto an immobilin membrane and probed with anti-VEGF monoclonal antibody (Santa Cruz; 1 μg/ml). This revealed in each of the four CLL cases studied, two immunoreactive bands corresponding to proteins of 28 kDa and 48 kDa. VEGF isoform controls run on the same gel indicated that the bands detected were $VEGF_{121}$ and $VEGF_{165}$ respectively (Figure 2.2A).

To determine whether CLL cells produce other isoforms of VEGF that were not detected by Western blotting, RT-PCR followed by nucleotide sequence analysis of the products was performed using primers recognising all possible VEGF isoforms. It was found that only 2 bands were amplified from the unstimulated CLL cells tested and these were identical in size to the control $VEGF_{121}$ and $VEGF_{165}$ cDNA (Figure 2.2B). Furthermore, nucleotide

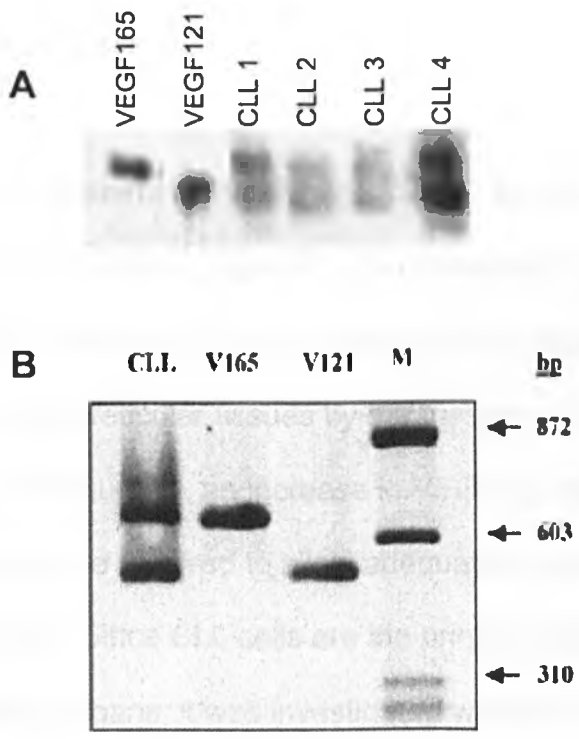


Figure 2.2

Analysis of secreted VEGF isoforms

(A) Western blot analysis of secreted VEGF. CM was collected from cultures of the PMA-stimulated cells and subsequently concentrated 10-fold. CM samples (100 µl), together with control recombinant $VEGF_{165}$ and $VEGF_{121}$, were loaded onto a 10% polyacrylamide gel and electrophoresed under non-reducing conditions. Separated proteins were transferred to a nitrocellulose membrane, and VEGF isoforms detected with anti-VEGF monoclonal antibody were visualized by enhanced chemiluminescence. (B) Analysis of VEGF mRNA isoforms by RT-PCR. First-strand cDNA was transcribed from 1 µg total RNA extracted from unstimulated cells. One tenth of the synthesized cDNA was amplified by PCR using primers recognizing all possible VEGF isoforms. $VEGF_{165}$ and $VEGF_{121}$ cDNAs were included in the PCR reaction as the positive controls.

sequence analysis of the 2 bands confirmed that they were $VEGF_{121}$ and $VEGF_{165}$ (data not shown).

2.3.4 *Effects of hypoxia on VEGF production by CLL cells*

Low oxygen tension (hypoxia), often detected in rapidly expanding tissues, is one of the known mechanisms of VEGF regulation. In CLL, infiltration of lymphoreticular tissues by the malignant B-cells is a feature of the disease. Consequently, an increase in VEGF production and the resultant angiogenesis may be required to allow adequate metabolic support in such expanding tissues. Since CLL cells are the principle cells within potentially hypoxic infiltrated organs, it was investigated whether levels of VEGF mRNA and VEGF protein are upregulated in response to hypoxia in CLL cells. Initially, the effect of hypoxia on six CLL cases was determined by measurement of both mRNA and VEGF protein. CLL cells were cultured for 24 hours in a specially designed airtight chamber with inflow and outflow valves. Cells in 24-well plates were placed into the chamber and exposed to a mixture of 5% CO_2/ 95% N_2. After 24 hours, the culture supernatants were collected and analysed for the presence of VEGF by ELISA, while the cells were harvested for VEGF mRNA determination by slot blot hybridisation. For each of the six cases used, control cells incubated under conditions of normal oxygen tension were also prepared. In each case studied, slot blot hybridisation revealed that hypoxia stimulated an increase in VEGF mRNA expression, ranging from a 1.4 to 3.7-fold increase (Figure 2.3A). Similarly, measurement of VEGF protein revealed that secretion of VEGF increased in each case by up to 7-fold (Figure 2.3B). In a further series of experiments, in

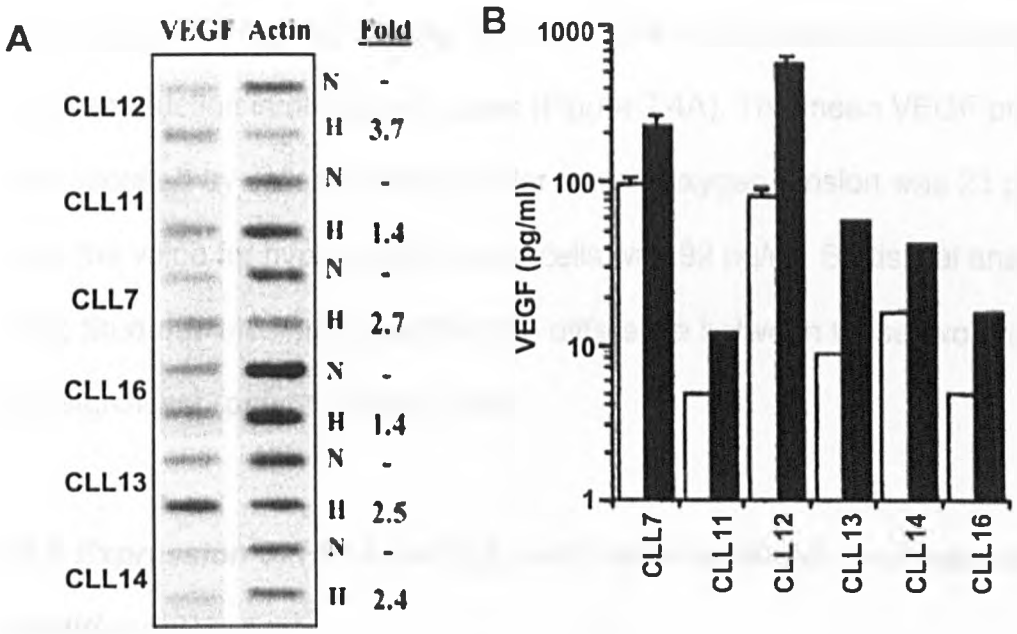


Figure 2.3

VEGF production by CLL cells in response to hypoxia

(A) Cells from the 6 CLL patients were cultured under normoxic and hypoxic conditions. After 24 hours, the cells were subjected to VEGF mRNA determination by slot-blot hybridization. Hypoxia (H) compared with normoxia (N) increased VEGF mRNA expression in all cases. The slot-blot bands were measured densitometrically, and the sample loading was determined by parallel measurement of the actin mRNA in each sample. (B) Culture supernatants from the same cultures used for mRNA determination were also collected and subjected to ELISA. In all cases, higher levels of VEGF protein were detected in the cells cultured under hypoxic conditions (■) than under normoxic conditions (□). Histograms represent the mean of duplicates. Error bars are standard deviations calculated from 6 measurements in 3 separate experiments using the cells of 2 patients.

which a total of 21 CLL cases were studied, it was found that incubation of CLL cells under hypoxic conditions for up to 24 hours induced an increase in VEGF production in all but two cases (Figure 2.4A). The mean VEGF protein level secreted by cells incubated under normal oxygen tension was 23 pg/ml while the value for hypoxia-stimulated cells was 92 pg/ml. Statistical analysis using Students t-test indicated that the difference between these two groups was significant ($p<0.01$; Figure 2.4B).

2.3.5 *Expression of HIF-1α in CLL cells under normoxic and hypoxic conditions*

HIF-1α is rapidly degraded under normoxic conditions, and since the preparation of CLL-cell lysates for experiments in this section was carried out under normal oxygen conditions, it was important to determine whether this would affect detection of HIF-1α. In preliminary experiments, CLL cells were incubated under hypoxic conditions for 4 hours to induce expression of HIF-1α, and then subjected to cell lysis and Western blot analysis. Initially, HIF-1α was not detected so it was speculated that this was due to technical reasons. HIF-1α rapidly degrades under normoxic conditions, so speed of lysate preparation was important. Since relatively large numbers of lysates were prepared in the initial experiments (a time consuming process) the negative findings were possibly due to degradation of HIF-1α during lysate preparation.

Therefore, in further preliminary experiments, the aim was two-fold: firstly to determine whether HIF-1α could be detected under such improved conditions of cell lysis and, secondly, by using suitable control cell lines, to

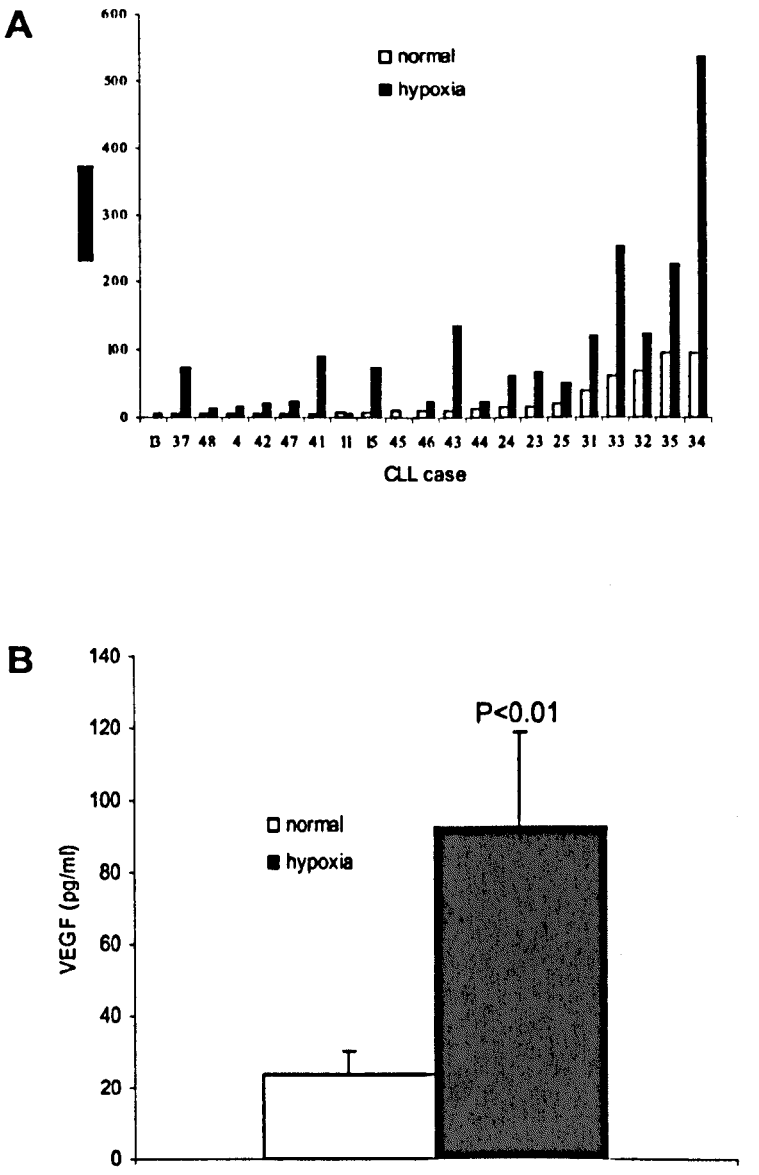


Figure 2.4

Hypoxia induces increased secretion of VEGF by CLL cells

In addition to the CLL cases used in Figure 2.3, cells from a further 21 CLL cases were exposed to hypoxia and the amount of secreted VEGF compared to that produced under normoxic conditions. (A) shows the variability of the response; (B) are the means ± SEM, together with the level of significance of the difference between the 2 groups (Students t-test).

demonstrate that HIF-1α could be induced by the hypoxic environment of the chamber system used. To this end, prostate carcinoma cell lines PC-3 and DU145 that are known to have different HIF-1α expression characteristics were selected as control cells. PC-3 cells constitutively express HIF-1α while DU145 cells express HIF-1α only under hypoxic conditions only (Zhong et al, 1998). Furthermore, work from this Department has previously shown that both of these cell lines produce VEGF *in vitro* (Chen et al, 2000a).Therefore PC-3 and DU145 were considered suitable control cells for the detection of HIF-1α by Western blotting and for its induction by hypoxia.

PC-3 and DU145 cells were grown to confluence in 6-well plates and then incubated under normoxic or hypoxic conditions for 2, 4 and 6 hours. Following incubation, the cells were trypsinized and lysed in boiling 1% SDS lysis buffer. The lysates were sonicated prior to SDS-PAGE, Western blotted with anti-HIF-1α antibody and the immunoreactive bands visualised with ECL. This analysis showed an increase in HIF-1α expression under hypoxia in PC-3 and DU145 cells, reaching a peak after 4 hours incubation. HIF-1α was present in PC-3 cells incubated under both normoxic and hypoxic conditions confirming that PC-3 cells constitutively express HIF-1α (Figure 2.5A). DU145 cells did not express HIF-1α under normal oxygen conditions although this protein was strongly expressed in cells incubated under hypoxic conditions. The presence of several protein bands at around 120kDa on the Western blot (Figure 2.5A) has been observed by others (Agani et al, 2000) and probably represents post-translational modification of HIF-1α (Salceda and Caro, 1997). These results confirmed the suitability of PC-3 and DU145

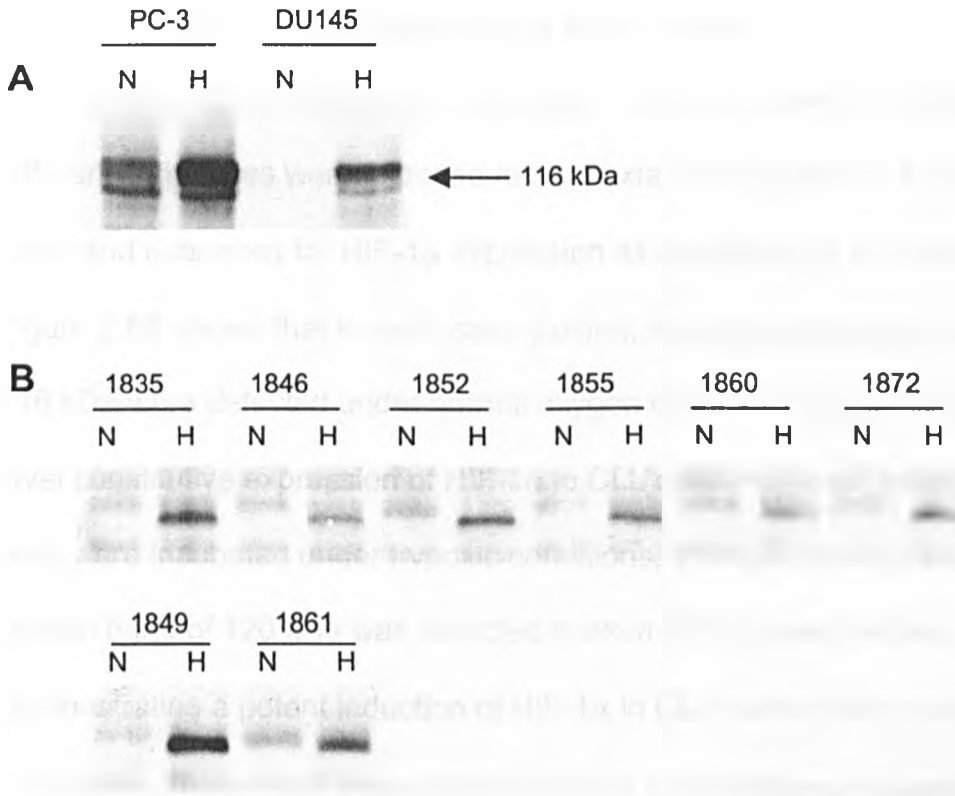


Figure 2.5

Detection of HIF-1α in prostate cell lines and CLL cells under normoxic and hypoxic conditions

(A) Confluent, adherent prostate cell lines were exposed to hypoxic or normoxic conditions for 4 hours and then rapidly detached by treatment with trypsin and lysed as described in the methods. The lysates were subjected to SDS-PAGE and Western blotting with anti HIF-1α antibody. This confirmed the presence of constitutively expressed HIF-1α in PC-3 cells but not in DU145 cells and the induction of HIF-1α expression by hypoxia in both cell lines. (B) shows induction of HIF-1α by hypoxia in each of the 8 CLL cases studied.

cell lines as controls for the detection of changes in the expression of HIF-1α induced by hypoxia in the experimental system used.

Following this validation of the experimental system, cells from 8 different CLL cases were exposed to normoxia and hypoxia for 4 hours, then lysed and examined for HIF-1α expression as described for the cell lines. Figure 2.5B shows that in each case studied, several weak bands of around 116 kDa were detected under normal oxygen conditions suggesting a low-level constitutive expression of HIF-1α in CLL cells. However, when CLL cells were incubated under hypoxic conditions, a single, strongly positive protein band of 120 kDa was detected in each of the cases studied, demonstrating a potent induction of HIF-1α in CLL cells under hypoxic conditions. The level of expression of HIF-1α varied between cases but measurement of β-actin confirmed equal loading of the gel (not shown).

2.3.6 *HIF-1α expression and VEGF production by CLL cells*

Since variability in VEGF expression between CLL cases under both normoxic and hypoxic conditions was observed in this study, it seemed important to determine whether this variation was related to differences in expression HIF-1α. To clarify this relationship, cells from the 8 CLL cases used in the HIF-1α study were cultured for 24 hours under both normoxic and hypoxic conditions, and supernatants collected to determine VEGF production, as described previously. The results of this assay are shown in Figure 2.6A. In 7 of the 8 CLL cases studied, hypoxia induced an increase in VEGF secretion and, as predicted, there was large variability in the response, ranging from 2 to 18-fold increase. To determine whether these differences in

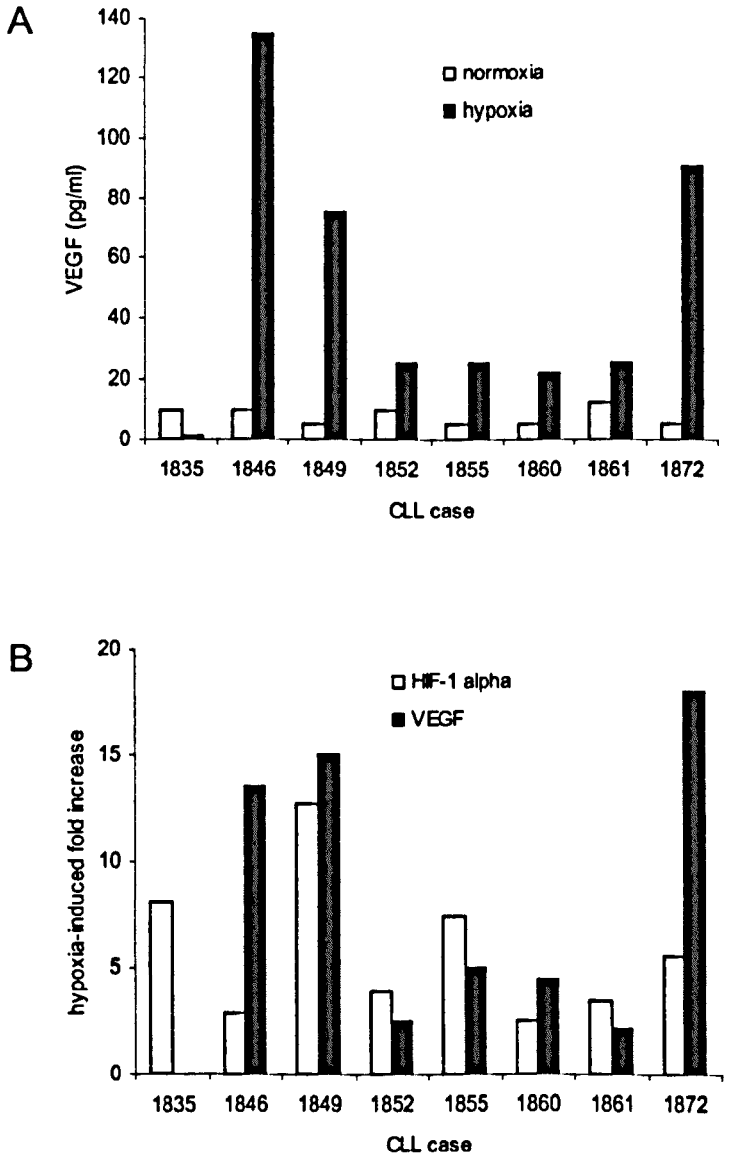


Figure 2.6

Lack of correlation between increases in HIF-1α and VEGF secretion induced by hypoxia

(A) Increases in VEGF production in supernatants from the same cells which were used to estimate the effect of hypoxia on HIF-1α expression (Figure 2.5B). (B) Comparison of the effects of hypoxia on HIF-1α expression and VEGF secretion expressed as a fold-increase relative to normoxia. The apparent lack of correlation suggests that factors other than HIF-1α also contribute to regulation of VEGF production and/or secretion in different CLL-cell clones.

VEGF production were related to HIF-1α expression, a comparison was made between VEGF and HIF-1α levels in CLL cells incubated under hypoxic conditions. For HIF-1α this was done using a comparison of the Western blot protein band intensity determined by densitometry. Since several bands were present at and around 120kDa, and since this might reflect post-translational modification of HIF-1α, densitometry values were measured from an area that corresponded to the region of HIF-1α bands seen in the control cell lines (Figure 2.5A). A direct comparison of the changes in HIF-1α expression and VEGF production under hypoxic conditions (Figure 2.6B) demonstrated considerable variation in the ratios of HIF-1α expression and VEGF production in CLL cells. In case 1849, relatively high HIF-1α expression corresponds with high VEGF production while in two other cases (1846 and 1872), a relatively low increase in HIF-1α expression contrasts with a relatively large increase in VEGF production. In the remaining cases, differences in expression between the two parameters were relatively small. Such variability in the relationship between HIF-1α and VEGF expression in CLL cells under hypoxic conditions suggests that factors other than HIF-1 stability may influence VEGF production.

2.3.7 *The relationship between Type-A p53 dysfunction, VEGF production and HIF-1α expression in CLL cells*

In other cell-types, p53 negatively regulates VEGF expression (Blagosklonny et al, 1998) and mutant p53 is associated with over expression of HIF-1α (Zhong et al, 1999). Therefore, since a sub-group of Type-A p53 dysfunctional CLL patients had been identified within this Department (Pettitt

et al, 2001), a comparison was made between p53 status, VEGF production and HIF-1α expression (Table 2.1). Of the 8 cases used in the HIF-1α study, four had normal p53 and four were Type-A p53 dysfunctional. It was found that wide variability existed within each group with no indication of suppression of VEGF production by normal p53 in comparison to the mutant form. Within the normal p53 group, the mean fold-increase in HIF-1α expression under hypoxic conditions was 4.8 ± 1, while for the Type-A group the mean fold-increase was 6.8 ± 2.2 hinting that, in CLL, p53 dysfunction may play a role in increased expression of HIF-1α. However, the mean fold increase in VEGF production within the p53 Type-A group was 5.5 ± 3.3 while in the p53 normal group, the mean VEGF production increased by 9.6 ± 3.6-fold, which contrasted with the HIF-1α expression data. Clearly, if the true nature of the relationship between p53, HIF-1α expression and VEGF production in CLL cells is to be determined, further investigation is required.

2.3.8 *CD154-induced VEGF production by CLL cells*

As mentioned earlier, raised serum levels of VEGF were found to be associated with poor outcome for some patients with CLL (Molica et al, 1999). Although the above results indicate that hypoxia may be involved in stimulating VEGF production by CLL cells, other *in vivo* stimuli may also be involved. In other cell types, including endothelial cells, monocytes, fibroblasts and multiple myeloma cells, stimulation of CD40 by its ligand, CD154, upregulates production of VEGF. In the present study, this possibility was investigated *in vitro* by culturing CLL cells with a commercial preparation of recombinant CD154 (see Materials and Methods).

CLL case	VEGF	HIF-1α	p53 status
1835	0	8.1	A
1860	4.4	2.5	A
1849	15	12.7	A
1852	2.5	3.9	A
1855	5	7.4	WT
1846	13.5	2.9	WT
1861	2.1	3.4	WT
1872	18	5.6	WT

Table 2.1

Absence of an apparent relationship between VEGF secretion, HIF-1α expression and p53 status of hypoxia-stimulated CLL cells

The effect of hypoxia on VEGF secretion and HIF-1α expression from data in Figure 2.6 is presented together with the p53 status of the cells (A= Type-A p53 mutation; WT= wild-type p53). No apparent influence of p53 status on cell response to hypoxia is evident from the effects on upregulation of either VEGF secretion or HIF-1α stability.

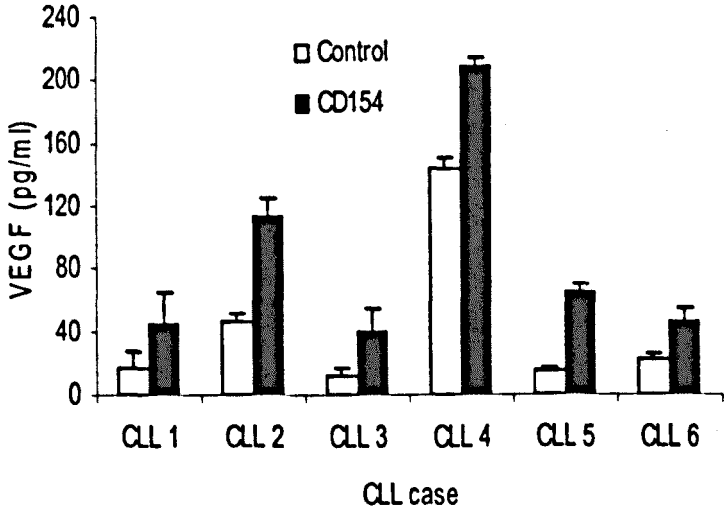


Figure 2.7

Effect of CD154 on VEGF production by CLL cells

VEGF protein content in cell supernatants was determined by ELISA. CLL cells were cultured for 24 hours in duplicate in the absence or presence of CD154 (200 ng/mL). The presented data are means ± SD of at least 3 separate experiments using cells from 6/16 cases. Similar results were obtained in all cases studied, showing a significant increase in VEGF production ($p<0.01$) when cells were cultured in the presence of CD154.

In initial studies, CLL cells were cultured in the presence of 200 ng/ml CD154 for up to 72 hours. These studies revealed that CD154 consistently induced an increase in VEGF secretion, which was first evident at 18 hours and persisted for up to 72 hours. The highest increase relative to the unstimulated control was observed at 24 hours and this incubation time was employed in subsequent experiments. A total of 16 cases were studied and all demonstrated a positive response to CD154 in the form of increased VEGF secretion. Figure 2.7 shows the amounts of VEGF detected at 24 hours in the supernatants of cells from 6 representative CLL cases, with and without CD154. The observed increases in VEGF secretion induced by CD154 were significant ($p<0.05$; $n=16$).

2.4 Discussion

Prior to beginning these studies, some evidence existed to suggest that VEGF might play a role in the pathogenesis of CLL. In an abstract for the 1998 American Society of Haematology (ASH) meeting, Kini et al (1998) described the presence of abnormal angiogenesis in the bone marrow of patients with CLL (this work was formally published after the start of this thesis; Kini et al, 2000) and at the same meeting Molica et al (1998) described elevated serum VEGF levels in CLL patients. Later work (Molica et al, 1999) suggested that for CLL patients, VEGF serum levels were not different from controls, but for patients with Binet stage A VEGF levels above the median predicted disease progression. The source of the serum VEGF and the factor responsible for the angiogenic changes in bone marrow of CLL

patients had not been described. Some evidence suggested that CLL cells themselves might potentially be the source, since they had been shown to express VEGF mRNA (Baban et al, 1996). In the early stages of the project described in this thesis, more evidence emerged of a possible role for VEGF in CLL when VEGF levels in CLL cells were reported to be higher than in controls, and that lower levels of VEGF correlated with shorter survival of CLL patients (Aguayo et al, 2000a).

Against this background, the initial aim of this project was to determine whether VEGF is produced and secreted by CLL cells. Using an ELISA assay, it was found that CLL cells from all but 3 of the 47 different cases studied spontaneously secreted detectable amounts of VEGF into culture medium. The variability in the amount secreted in different cases is not surprising, since work carried out in this Department showed that there is a wide and reproducible heterogeneity of response between different CLL-cell cases to a range of different stimuli (data not shown).

There are five different isoforms of VEGF that have different solubility and heparan sulphate-binding characteristics. Using RT-PCR and Western blotting, it was shown that CLL cells produced and secreted only the soluble forms of VEGF, that is, $VEGF_{121}$ and $VEGF_{165}$, and immunocytochemistry confirmed that secreted VEGF was not derived from contaminating cells known to produce VEGF.

Since it had been shown that bone marrow from CLL patients had evidence of increased angiogenesis (Kini et al, 2000), that infiltration of lymphoreticular tissues is a feature of CLL (Chiorrazi and Ferrarini, 2003) and that expanding tissues require increased blood supply due to hypoxia

(Carmeliet P, 2003), it was important to determine whether hypoxia could stimulate increased secretion of VEGF by CLL cells. The results of *in vitro* studies showed that hypoxia causes increased expression of VEGF mRNA and a significant increase in secretion of VEGF. The response to hypoxia varied between cases and it was speculated that factors that are known to affect the expression of VEGF in other cell types might be responsible for this variation. One such candidate protein was the α-chain of the heterodimeric VEGF transcription factor, HIF-1. HIF-1α is normally stably expressed only under hypoxic conditions (Wang et al, 1995), but in some solid tumours, HIF-1α is constitutively over-expressed (Ravi et al, 2000) with a consequent elevation of VEGF levels. Additionally, HIF-1α is regulated in part by the tumour suppressor protein p53. It has been shown that mutation or loss of p53 can lead to constitutive HIF-1α expression in tumour cells. Since p53 dysfunction associated with expression of mutated p53 (defined as Type-A p53 dysfunction in this Department (Pettitt et al, 2001)) occurs in a proportion of CLL cases, it was hypothesised that variations in expression of secreted VEGF in CLL cases might be due to differences in constitutive and induced expression of HIF-1α, due to altered stabilisation by p53.

Using Western blotting, it was found that HIF-1α expression was weak under normoxic conditions in each of the cases studied. Moreover, a number of protein bands around the molecular weight of HIF-1α were detected. This may be due to poly-ubiquitination of HIF-1α, since this protein undergoes rapid proteasomal degradation under normoxic conditions (Salceda and Caro, 1997). Variability in expression of HIF-1α was observed under hypoxic conditions, but this was not found to be related to expression of VEGF, or to

the p53 mutational status of the cells. This suggested that there was no direct relationship between VEGF expression, HIF-1α stability and p53 mutational status in CLL cells, and if the true nature of this relationship is to be determined, further investigation is required.

It is, of course, possible that the elevated serum levels of VEGF reported in CLL patients may be due to factors other than hypoxia. CD40 ligation by CD154 is implicated in the survival of CLL cells (Wang et al, 1997; Granziero et al, 2000) and is known to stimulate production of VEGF in other cell types (Melter et al, 2000; Cho et al 2000). Work in this Chapter showed that CD154-stimulation of CLL cells causes an increase in VEGF secretion. Thus, this pathophysiologically relevant stimulus for VEGF secretion might potentially be involved in *in vivo* angiogenesis. Furthermore, work in this Department has shown that CD154 is strongly expressed by mononuclear cells and weakly expressed by most CLL cells in infiltrated lymph nodes from CLL patients (data not shown). This indicates that in CLL CD154 might be involved in stimulation of VEGF secretion through both homotypic and heterotypic cell interactions.

Following confirmation of VEGF secretion by CLL cells, it was important to establish whether this protein was angiogenically active and whether it was present at sites of incresed angiogenic activity. Such paracrine effects of CLL-cell derived VEGF are presented in the next Chapter of this thesis.

Chapter 3

Paracrine function of VEGF secreted by CLL cells

3.1 Introduction

Numerous studies have demonstrated that VEGF is a potent angiogenic factor (Podar and Anderson, 2005). In solid tumours, angiogenesis is a pathophysiologically important process that allows metabolic support of rapidly expanding tissues. In some studies, capillaries have been found clustered around VEGF-producing tumour cells (Plate et al, 1992), offering significant support for the key role of VEGF in this process. At the time of the present study, several published reports suggested that angiogenesis may also be involved in the pathogenesis of haemic malignancies such as multiple myeloma (MM) (Vacca et al, 1994) and childhood acute lymphocytic leukaemia (ALL) (Perez-Atayde et al, 1997). Furthermore, increased microvessel density was observed in bone marrow from patients with CLL (Kini et al, 2000). Since the studies presented in Chapter 2 showed that CLL cells have the capacity to produce VEGF, and that VEGF secretion is enhanced under hypoxic conditions, it was decided to explore whether angiogenesis takes place in the expanding lymphoid tissues of CLL patients and determine the potential role of CLL-cell derived VEGF in this process.

3.2 Methods

3.2.1 *Human umbilical vein endothelial cells*

Umbilical cords were generously provided by the maternity unit of Liverpool Women's Hospital (Liverpool, UK). Human umbilical vein endothelial cells (HUVECs) were detached with trypsin using a published protocol (Till et al, 1994). Cells were cultured in 199 medium (Gibco) supplemented with 20% fetal calf serum (Globefarm), 1 pg/mL epidermal growth factor (R&D Systems), 5 U/mL heparin (CP Pharmaceuticals Ltd), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco). HUVECs were used at passage 3.

3.2.2 *Proliferation assay*

HUVECs were plated at a density of $2x10^4$ cells/well in 24-well gelatine (Sigma)-coated plates and grown to approximately 90% confluence. The following day, the medium was removed from the wells, and the cells washed twice with PBS and incubated for a further 24 hours in 199 medium containing 5% FCS without heparin or EGF. Next, the medium was replaced with the fresh medium containing 10% (v/v) concentrated culture supernatant derived from CLL cells (see Chapter 2) or recombinant human (rh) $VEGF_{165}$ protein (R & D Systems). In addition, the culture supernatant was pre-incubated for 1 hour at 22°C with a neutralizing anti-hVEGF monoclonal antibody (1 µg/mL, R & D Systems) before addition to the cultures. After 48 hours, the cells were incubated for 3 hours with ^{3}H-thymidine (0.5 µCi /well), the medium aspirated, and the cells washed twice with ice-cold PBS. Ice-cold 5% trichloroacetic acid (TCA; Sigma) was added to each well (0.5 ml/ well),

and the plates placed at 4°C. Following overnight incubation, the TCA was aspirated, and the cell monolayers washed once with TCA and twice with 95% ethanol. To each dried well, 0.2 mol/L NaOH (0.5 ml /well) was added to dissolve TCA-insoluble materials (37°C, 2 hours with gentle shaking). The samples (0.3 ml) were counted to determine the amount of ^{3}H-thymidine taken up by the cells using a Tri-CARB Liquid Scintillation Analyzer (Packard, England).

3.2.3 *Chick chorioallantoic membrane assay*

The chick chorioallantoic membrane (CAM) assay (West et al, 1985) was used to determine whether the CLL cell–derived culture supernatant had angiogenic activity. To expose CAM, a window was created in the shells of 10-day-old fertilized chicken eggs. Filter paper disks soaked in test culture-supernatant were placed on exposed CAMs. Disks soaked in rhVEGF$_{121}$ (10 ng in 10 ml; R & D Systems) or in control medium were used as positive and negative controls, respectively. The embryos were incubated at 37°C in a humidified egg incubator. CAMs were analyzed after 72 hours using a stereomicroscope. The density of branching blood vessels infiltrating under the disks was scored as follows: 0, negative; 0.5, change in vessel architecture but not directed to the point of sample application; 1, partial spoke-wheel (one third of the circumference exhibits directional angiogenesis); 2, spoke-wheel; 3, strong and full spoke-wheel. For photography, the membranes were fixed in situ with ice-cold 4% paraformaldehyde–PBS that was injected both from above and below the membrane. Membranes were excised, placed on a fresh microscope slide,

and photographed under a Leitz binocular dissecting microscope and indirect fiberoptic illumination. Statistical analysis was performed using the Mann-Whitney *U* test.

3.2.4 *CD34 and VEGF immunohistochemistry*

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. Sections were de-paraffinized, rehydrated, and boiled in 10 mM sodium citrate (pH 6.0) for 10 minutes. Non-specific antibody-binding sites were blocked by incubating the sections in 10% BSA in TBS (20 mM Tris-HCl, pH 7.6, 137 mM NaCl) for 10 min at room temperature (RT). For the detection of VEGF, sections were incubated overnight at 4°C with either rabbit anti-hVEGF antibody (1:200 dilution in TBS) or with non-specific rabbit IgG (Santa Cruz). For blood vessel staining, sections were incubated overnight at 25°C with mouse anti-CD34 monoclonal antibody (Serotec) or with nonspecific mouse IgG (Becton Dickinson). The slides were then incubated with goat anti-rabbit IgG (for VEGF antibody) or rabbit anti-mouse IgG (for CD34 antibody) conjugated to biotin, followed by incubation with ExtrAvidin alkaline phosphatase (both 30 minutes at RT). Colour was developed by incubating the slides with Fast Red TR/Naphthol AS-MX phosphate (Sigma) at RT for 20 minutes. Slides were counterstained with hematoxylin. The finding of 3 or more contiguous $CD34^+$ cells was considered to indicate the presence of a microvessel (Weidner et al, 1993). Such microvessels were counted in 40 high-power (40x) fields. The fields were chosen at random in CLL nodes. In normal nodes, 20 follicular and 20

interfollicular fields in different areas of cortex were scored. Vessel scores were expressed as the mean ± SD per field.

3.3 Results

3.3.1 *CLL-cell-derived VEGF stimulates endothelial cell proliferation*

It is well documented that VEGF induces proliferation of endothelial cells. It was therefore decided to use an endothelial cell proliferation assay to determine whether VEGF secreted by CLL cells is biologically active and, consequently, potentially involved in the increased angiogenesis observed in CLL tissues. Initially, cells from two CLL cases were stimulated with PMA and incubated for 24 hours prior to collection of the VEGF-rich supernatants, which were termed conditioned media (CM). Subsequently, human umbilical vein endothelial cells (HUVEC) were incubated for 48 hours in the presence of CM, and cell proliferation determined by measuring ^{3}H-thymidine uptake by the HUVEC. As a positive control, the proliferative response of HUVEC to rhVEGF was also measured, while baseline proliferation was determined using HUVEC cultured in medium that had not been conditioned by CLL cells. As shown in Figure 3.1, CM from both of the CLL cases used induced HUVEC proliferation that reached a maximum of about 4-fold increase under these experimental conditions. This increase was similar to that observed when 100 ng/ml rhVEGF was used. To confirm that this proliferative effect was caused by the CLL-cell-derived VEGF and not by some other mitogenic factor(s), CM were pre-incubated with an anti-VEGF neutralising antibody (R&D Systems) for 1 hour prior to incubation with the HUVEC. Under this

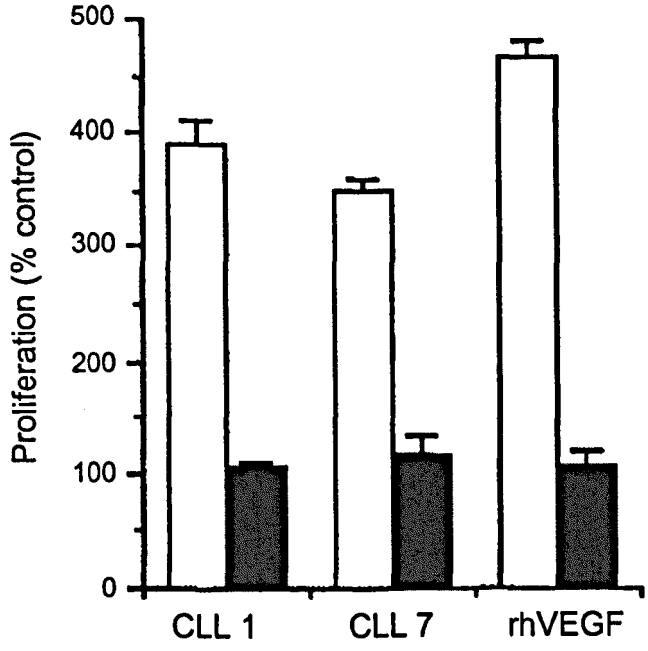


Figure 3.1

Proliferation of endothelial cells in response to CLL-cell–derived CM

HUVECS were cultured in the presence of CM derived from the PMA-stimulated cells of 2 CLL patients (CLL1, CLL7), using rhVEGF as a positive control (□). In the same experiment, the CM was preincubated with a neutralizing anti-VEGF monoclonal antibody for 1 hour at RT before addition to endothelial cell cultures (■). After 48 hours, proliferation was assessed by ^{3}H-thymidine incorporation. Proliferation of cells cultured without addition of CM was considered as 100%; the blocking anti-VEGF antibody had no effect on this proliferation (data not shown).

condition, the CM-induced proliferation of HUVEC was completely abrogated, indicating that the proliferation of HUVEC observed following incubation with CLL-cell-derived CM was indeed mediated by VEGF. This *in vitro* stimulation of endothelial cell proliferation by CLL-cell-derived VEGF underscored its angiogenic potential, since this proliferation is an essential step in the development of new blood microvessels during angiogenesis.

3.3.2 *CLL-cell derived VEGF induces angiogenesis*

Having demonstrated that VEGF from CM induced proliferation of human endothelial cells, it was considered important to determine whether the CLL-cell-derived VEGF present in CM could effectively and demonstrably induce angiogenesis *in vivo*. To determine whether this is indeed the case, we employed the chick chorioallantoic membrane (CAM) assay. CLL-cell conditioned medium from two different CLL patients was prepared as described for the endothelial cell proliferation assay. Filter paper disks soaked in CM were placed on exposed CAM of 10-day-old fertilized chicken eggs and incubated at 37°C in a humidified egg incubator for 72 hours. Disks soaked in 10 ng/ml $VEGF_{121}$ (R&D Systems) or control medium were used as positive and negative controls respectively. The angiogenesis in the CAM was assessed visually using a stereomicroscope and measured by following the semi-quantitative visual assessment protocol described in Materials and Methods. The results show that when disks were soaked in CM, a moderate angiogenic response was observed in which the growth of new blood vessels form a spoke-wheel image with the disc as its hub, indicating directional angiogenesis induced by CLL-cell-derived VEGF (Figure 3.2Ai). When CM

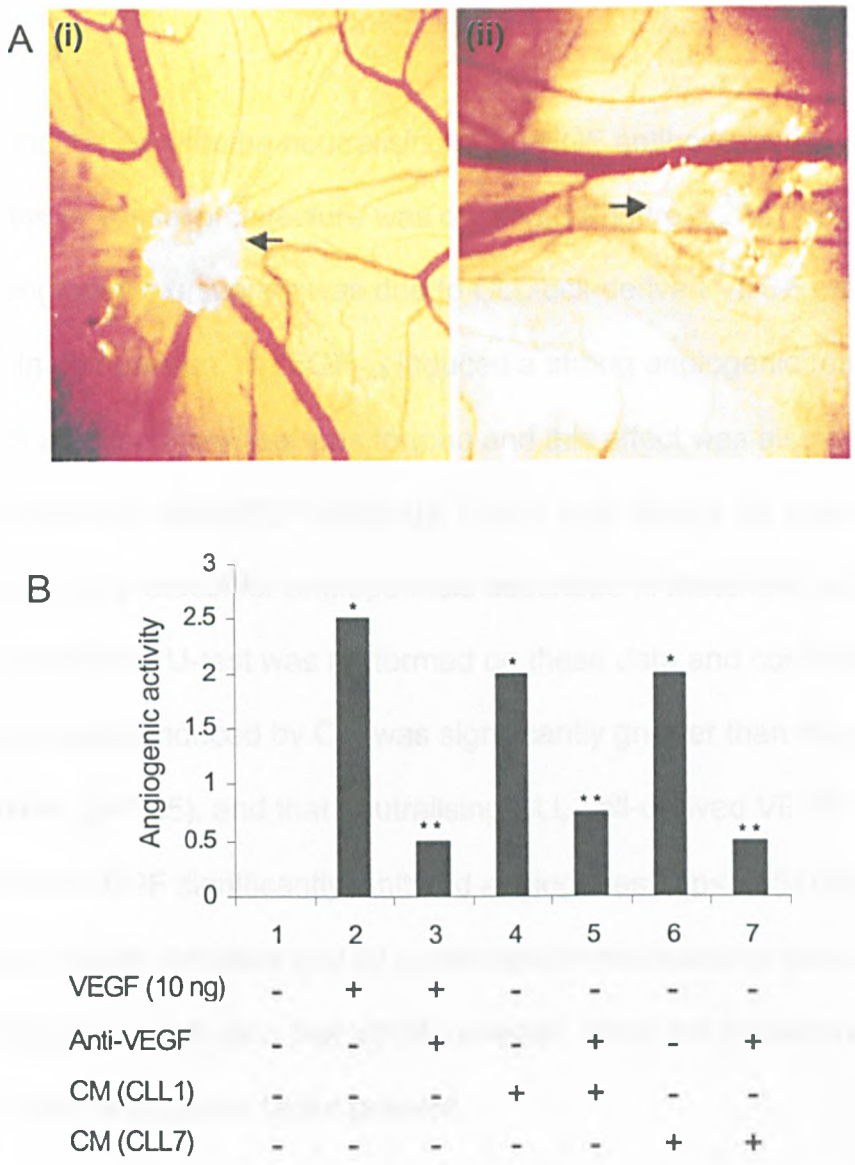


Figure 3.2

Angiogenic responses of CAM to CM derived from CLL cells

(A) Representative angiogenic response and its inhibition by neutralising anti-VEGF antibody. The white area represents the site of application of filters containing either CM (collected from the PMA stimulated cells) alone (i) or the same CM pre-incubated with anti-VEGF for 2 hours at RT (ii). Microvessels radiating from the site of sample application in (i) (Original magnification, x 8), are not formed in the presence of the neutralising antibody (ii) (B) The semi-quantitative data obtained in the angiogenic assay using rhVEGF and CM collected from PMA-stimulated cells from 2 patients. The grading of the angiogenic response is described in Materials and methods. * $P<0.05$ vs control medium and ** $P<0.05$ vs sample without the neutralizing antibody (Mann-Whitney U test). Each bar represents the score of 10 CAM used in 2 separate assays with similar results.

was incubated with the neutralising anti-VEGF antibody, no equivalent change in vessel architecture was observed (Figure 3.2Aii), confirming that the angiogenic response was due to CLL-cell-derived VEGF present in the CM. In comparison, $rhVEGF_{121}$ induced a strong angiogenic response in which a full spoke-wheel was formed and this effect was also completely neutralised by anti-VEGF antibody. Figure 3.2B shows the data derived from the scoring protocol for angiogenesis described in Materials and Methods. A Mann-Whitney U-test was performed on these data and confirmed that the angiogenesis induced by CM was significantly greater than that for control medium ($p<0.05$), and that neutralising CLL-cell-derived VEGF in the CM with anti-VEGF significantly inhibited angiogenesis ($p<0.05$) (Figure 3.2B). These results indicated that CLL-cell-conditioned medium does have angiogenic activity and that VEGF detected within the conditioned medium is the major angiogenic factor present.

3.3.3 *Tissue-phase CLL cells produce VEGF*

Since CLL cells had been shown to produce angiogenically active VEGF when cultured *in vitro*, and in view of the importance of tumour cell-derived VEGF in solid tumour angiogenesis, it was important to confirm that CLL cells that had infiltrated the lymphoreticular tissue of CLL patients produce VEGF. Immunohistochemical staining of formalin-fixed, paraffin-embedded CLL-patient-derived tissues revealed that CLL cells infiltrating lymph nodes and expanded white pulp of the spleen (Figure 3.3A and 3.3C respectively), were uniformly positive for VEGF, although the intensity of

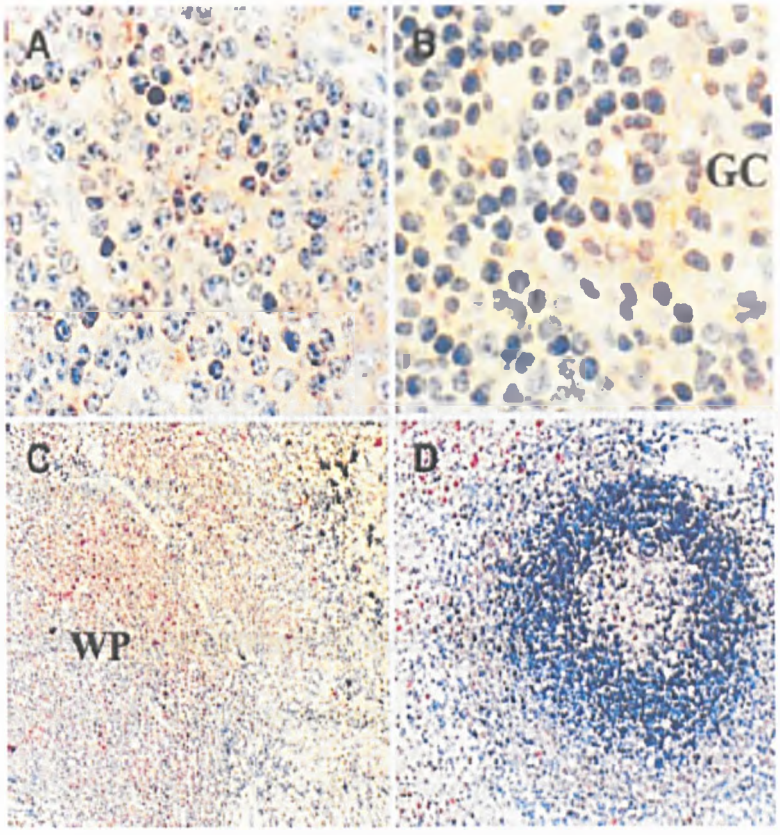


Figure 3.3

Immunohistochemical staining of VEGF

The presence of VEGF protein in CLL nodes (n=3) and spleen (n=2;), together with corresponding normal tissues (n=3), was examined by staining tissue sections with rabbit anti-VEGF polyclonal antibody. Infiltrating cells in CLL lymph node (A) are all positive. Many cells in normal node (B) are also positive, particularly those in the germinal centre (GC) of the follicles. (Original magnification, x 40) CLL cells expanding the white pulp (WP) of the spleen (C) are also uniformly positive. In contrast, in normal spleen (D), the different layers of the white pulp show various degrees of positivity that was strongest in the marginal zone and follicle centres. (Original magnification, x 10)

staining was weaker than that of any neutrophil present. For comparison, the B-cell areas of normal lymph nodes and spleen were also tested for the presence of VEGF. It was found that many cells in the normal node were positive for VEGF, particularly in the germinal centre of the follicles (Figure 3.3B), while in normal spleen (Figure 3.3D) the different layers of white pulp showed various degrees of positivity that were strongest in the marginal zones and follicle centres. The abundance of VEGF protein that is associated with CLL cells within highly infiltrated node and spleen provides support for the pathogenetic importance of *in vivo* production of VEGF by CLL cells.

3.3.4 *Elevated angiogenesis in CLL nodes*

The work in the previous sections had shown that CLL cells produce VEGF both in cultures *in vitro* and in lymphoreticular tissues *in vivo*. In addition, the VEGF derived from CLL-cells *in vitro* induced both proliferation of endothelial cells and *in vivo* angiogenic response as measured by CAM assay. Consequently, the next aim was to search for any evidence that angiogenesis in lymphatic CLL tissues was increased in comparison to normal nodal tissue. To investigate this, blood micro-vessel density was measured in lymph nodes from patients with CLL (n=3) and from normal control lymph nodes (n=3) by immunohistochemical staining of paraffin embedded tissues with the endothelial cell marker CD34. In each tissue section, 40 high-power random fields were examined and the finding of 3 or more contiguous CD34 positive cells was considered to constitute the presence of a micro-vessel. Figure 3.4Ai shows representative CD34 staining of a CLL node which indicates that vascular development accompanies

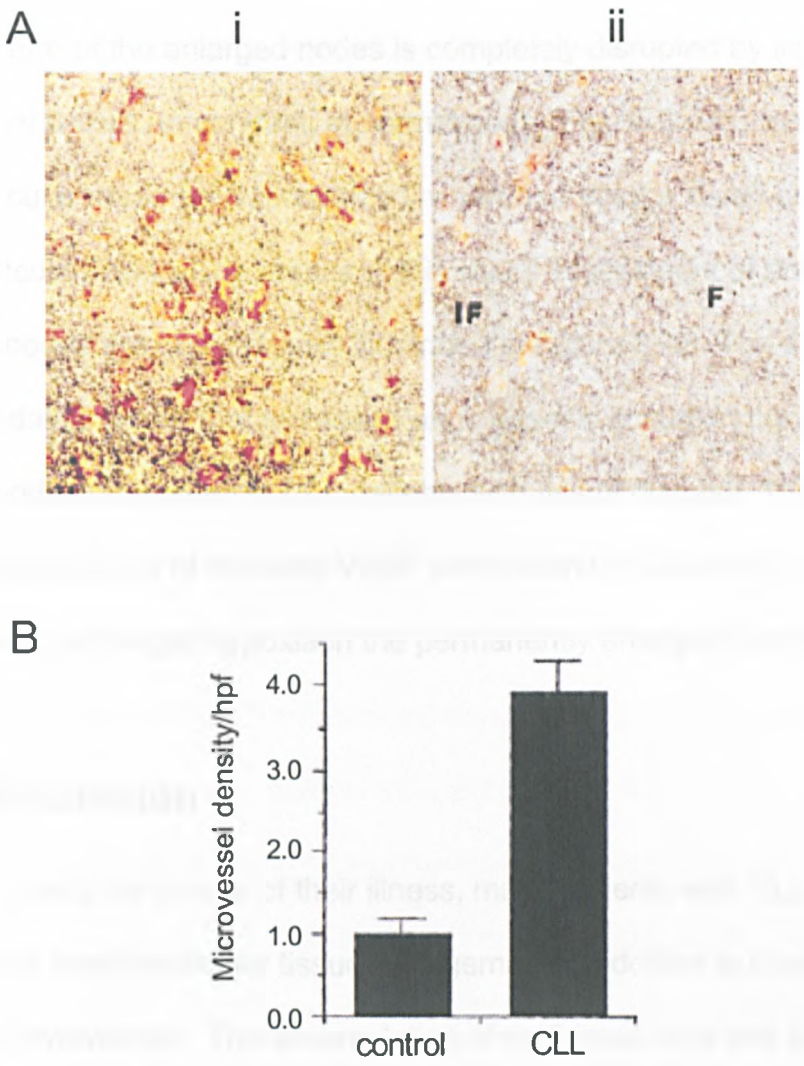


Figure 3.4

Vessel density analysis using CD34 antibody staining

(Ai) Representative CD34 staining of CLL node. (Aii) CD34 staining of normal node where interfollicular (IF) areas are vascularized, and follicular (F) areas display very little vascularization (original magnification, x 10). (B) Quantitative analysis shows the higher vessel density in CLL nodes (n=3) compared with follicular B-cell areas of normal nodes (n=3).

malignant cell infiltration and node enlargement. In CLL, the normal architecture of the enlarged nodes is completely disrupted by infiltrating malignant B cells. In contrast, in normal node (Figure 3.4Aii), only the interfollicular areas are vascularized, whereas follicular B-cell areas display little vascularization. The results of this visual assessment of both CLL and normal nodes are quantitatively illustrated in Figure 3.4B. The microvessel-density data indicate that increased angiogenesis occurs in the enlarged lymph nodes often seen in CLL patients with tissue disease. This is likely to be a consequence of elevated VEGF production by CLL cells in response to increased, prolonged hypoxia in the permanently enlarged lymphatic tissue.

3.4 Discussion

During the course of their illness, many patients with CLL display prominent lymphoreticular tissue enlargement in addition to blood and bone marrow involvement. The accumulation of malignant cells and the consequent expansion of secondary lymphoid tissues demand an increased blood supply. Thus, angiogenesis may be required for the metabolic support of the expanding tissues. In fact, higher blood vessel density has been observed in CLL than in normal bone marrow (Kini et al, 2000). In this Chapter it was demonstrated that neovascularization also takes place in CLL nodes and that VEGF produced by the CLL cells themselves is likely to be the principle angiogenic factor.

The present study initially focused on the effects of the secreted VEGF found in CLL-cell culture supernatants on endothelial-cell proliferation. The quantitative *in vitro* assay demonstrated that CLL-cell–derived supernatants

stimulated HUVEC proliferation, and blocking by a specific anti-VEGF monoclonal antibody showed that the VEGF contained in the conditioned medium was the major mitogenic factor. However, these results do not necessarily mean that the VEGF present in conditioned medium would have the potential to stimulate *in vivo* angiogenesis since this is a complex process involving multiple steps (Carmeliet P, 2003). For this reason, the CAM assay was used to demonstrate the angiogenic effect of the CLL-cell–derived conditioned medium *in vivo*. This assay showed a clear angiogenic response to the conditioned medium and confirmed that VEGF was the predominant angiogenic factor present.

In Chapter 2 it was established that in response to hypoxia, CLL-cell VEGF secretion was significantly increased. Since low oxygen tension is often detected in expanding tissues and enlargement of lymph nodes infiltrated by CLL cells is often observed in CLL patients with tissue disease, it was important to determine whether VEGF is present in infiltrated lymphoid tissues from CLL patients. Immunohistochemical staining of sections from such tissues indicated the *in vivo* relevance of the previous findings by demonstrating abundant VEGF protein associated with CLL cells within highly infiltrated lymph node and spleen. This finding, along with the results of the endothelial cell proliferation assay and the CAM assay, implicated CLL-cell-derived VEGF in angiogenesis within CLL lymphoreticular tissues.

To explore this possibility, immunohistochemistry was again employed, this time to see whether microvessel density in lymph nodes from CLL patients is elevated in comparison to normal controls. This showed that CLL nodes, in which the normal architecture is completely disrupted by the

infiltrating malignant cells, are uniformly vascularized and indicates new blood vessel formation as the node expands. The vessel density in the CLL nodes was comparable to that observed in the interfollicular areas of normal nodes, whereas the follicular B-cell areas of normal nodes displayed a very low vessel density. This is likely to reflect the fact that most follicular B cells undergo apoptosis and that the remaining antigen-selected cells migrate out of the follicle. In contrast, the chronic nature of CLL node enlargement indicates the more permanent nature of the infiltrate requiring neovascularization. Since the overwhelming majority of cells in CLL nodes are malignant B cells, it seemed justified to conclude that the angiogenic stimulus may be provided by the CLL cells themselves.

Having demonstrated that CLL-cell derived VEGF may play a paracrine role in the pathogenesis of CLL through induction of angiogenesis, the question arose of whether VEGF might have a direct effect on CLL cells themselves. In solid tumours, VEGF not only stimulates angiogenesis but, in some instances, also has an autocrine effect on malignant cells. For autocrine responses to VEGF to occur, the cells must express VEGF receptor(s), and at the start of this project it was not known whether these receptors were expressed by CLL cells. The approach taken to determine whether CLL cells express functional VEGF receptors is described in Chapter 4.

Chapter 4

Expression and activation of VEGF receptors on CLL cells

4.1 Introduction

The work in Chapter 2 demonstrated that CLL cells produce and secrete VEGF, while experiments in Chapter 3 showed that the CLL-cell-derived VEGF can induce angiogenesis *in vivo*. Moreover, further experiments suggested a paracrine effect of secreted VEGF, by revealing an increase in micro-vessel density in infiltrated lymph nodes of CLL patients. However, in addition to such paracrine effects, published work increasingly supports the notion of an autocrine role for VEGF in several cell types, including haemopoietic stem cells (Gerber et al. 2002), primary myeloid cells and myeloid cell lines (Santos and Dias, 2004). These studies showed that VEGF produced by the cells binds to and activates VEGF receptors expressed by the cells themselves and in this way acts as an autocrine survival factor. CLL is a disease characterised by the prolonged survival of malignant cells, and for VEGF to contribute to this prolonged survival would require the expression of VEGF receptors by these cells. The aim of the work in this Chapter was to establish whether VEGF receptors are present on the surface of CLL cells and whether VEGF could bind these receptors and trigger receptor signalling.

By activating multiple signalling pathways in cells such as endothelial cells, VEGF is known to have a range of effects including stimulating cell

proliferation, motility and survival. Potentially, several signalling pathways could be activated by VEGF, resulting in gene transcription and activation of these processes. Recent gene expression analysis has demonstrated that VEGF stimulates expression of Hsp90, which in turn may regulate expression of anti-apoptotic proteins such as Bcl-2 and in this way inhibit apoptosis of HL60 leukaemic cells (Dias et al, 2002). Moreover, the association of VEGFR2 with Hsp90 is required for activation of cell motility by VEGF (Rousseau et al, 2000), and VEGF production itself is dependent upon Hsp90-regulated inhibition of HIF-1α degradation by ubiquitination (Mabjeesh et al, 2002). Given that Hsp90 through its role as a protein chaperone may be involved in each of the pathways known to be activated by VEGF, upregulation of Hsp90 expression was chosen as a suitable indicator of a pathophysiologically relevant response of CLL cells to VEGF receptor stimulation.

4.2 Methods

4.2.1 *RNA isolation and RT-PCR*

Total RNA was extracted from CLL cells using an RNeasy kit (Qiagen) according to the manufacturer's instructions. Single-strand DNA was synthesized from 1μg of total RNA using the 1st Strand cDNA Synthesis Kit (Boehringer Mannheim) for RT-PCR. The synthesised cDNA was amplified by PCR using the VEGFR-1 forward primer (X51602; nt:3233-3258): 5'-GATGTCGACGGTATAAATACACATGTGCTT-3' and the reverse primer (nt: 4313-4293): 5'-CTATGGAAGATCTGATTTCTTACAGT-3' (30 cycles, annealing at 57°C) or the VEGFR-2 forward primer (AF035121; nt: 2025-

2046): 5'-CAGATCTACGTTTGAGAACCTC-3' and the reverse primer (nt: 2685-2665): 5'-CTATGGAAGATCTGATTTCTTACAGT-3'(35 cycles, annealing at 56°C). The quality of each cDNA preparation was checked by amplication of the L27 housekeeping gene using the forward primers (BC010026; nt:131-150): 5'-GACGCAAAGCTGTCATCGTG-3' and the reverse primer (nt: 475-456): 5'-GCAGTTTCTGGAAGAACCAC-3' (30 cycles, annealing at 60°C). The amplified PCR products were subjected to electrophoresis and visualized by ethidium bromide staining. To confirm that the sequences of the products amplified by PCR represented VEGFR-1 or VEGFR-2, the PCR products were cloned into pBlueScript plasmid, and their sequences were determined by nucleotide sequence analysis using sequencing kit.

4.2.2 *Slot-blot hybridization*

Total RNA (2 µg) extracted from the malignant cells of 12 CLL patients was loaded directly onto a nylon membrane using a slot-blot apparatus (Bio-Rad). Membranes were UV cross-linked and subsequently hybridized with ^{32}P-deoxycytidine-triphosphate-labelled VEGFR-1 and VEGFR-2 cDNA probes, respectively. For control of the RNA loading of each lane, blots were stripped and rehybridized with radiolabelled GAPDH cDNA probe. Autoradiography was performed at –70°C using Kodak XAR-5 film. The intensity of each band appearing on the film was scanned and analyzed with Phoretics 1D-advanced version 3.1 software.

4.2.3 *Detection of VEGF receptors by FACS (internal staining)*

Freshly thawed CLL cells ($1x10^6$) were fixed in 1% formaldehyde for 30 minutes at room temperature and washed twice in 0.5% Tween in PBS (PBS-T) to permeabilise the cells. The cells were then incubated with either rabbit anti-human VEGFR-1 (C-17) or VEGFR-2 (C-1158) antibodies (both Santa Cruz) or rabbit IgG class control (Santa Cruz) (all at 5 μg/mL in PBS-T) for 20 minutes at RT. After washing in PBS-T, the cells were further incubated with goat anti-rabbit IgG-FITC diluted 1:50 in PBS-T for 20 minutes at RT. Stained cells were analysed by flow cytometry using a Becton Dickinson (BD) FACScan (Becton Dickinson).

4.2.4 *Detection of VEGF receptor-2 by FACS (surface staining)*

Freshly thawed CLL cells were washed in PBS containing 1% BSA (Sigma) and 0.1% sodium azide (Sigma) (PBS/BSA/azide). Next, $5x10^5$ cells were incubated with goat polyclonal anti-human VEGFR2 antibody (R&D Systems; AF357) or goat polyclonal IgG control (R&D Systems; AB108C), both at a concentration of 5 μg/ml, for 20 min at room temperature. The cells were then washed twice in PBS/BSA/azide and resuspended and incubated for a further 20 min at RT in mouse anti-goat-FITC antibody (Sigma; F4891) that had been diluted 1:100 in PBS/BSA/azide. The stained cells were washed twice then analysed by flow cytometery using a BD FACScan.

4.2.5 *VEGF binding assay*

To demonstrate binding of VEGF to CLL cells, we used a human VEGF biotinylated fluorokine kit (R&D Systems; VFVE0). Freshly thawed

CLL cells ($1x10^5$) were treated according to the manufacturer's instructions. Briefly, CLL cells were incubated for 1 hour at 4°C in the presence of biotinylated VEGF or biotinylated control, and then incubated for a further 30 min in the dark with avidin-FITC. Following washing, the presence of VEGF bound to the surface of CLL cells was determined by flow cytometry.

4.2.6 *Detection of Hsp90 by Western blotting*

CLL cells were thawed and prepared as described previously (section 2.2.2) then resuspended at $2x10^7$ cells/ml in RPMI supplemented with 1% BSA. Subsequently, 100µl aliquots of these cells were transferred to a poly-HEMA-coated 96-well plate and stimulated with VEGF, PLGF or VEGF-E for up to 5 hours. Following incubation, the cells were harvested, centrifuged at 7000rpm then resuspended in PBS prior to the addition of an equal volume of boiling 2% SDS lysis buffer (20 mM Tris-HCl, pH 7.4, 2% SDS). The cell lysates were heated for 5 min at 95°C, sonicated and frozen at -20°C. The presence of Hsp90 in the lysates was determined by Western blotting as described previously (section 2.2.8). In this instance, the lysates were separated on a 7% SDS-PAGE gel and the membrane probed with monoclonal mouse anti-Hsp90 antibody (Sigma; H1775) that had been diluted 1:1000 in TBS-T containing 5% non-fat milk powder.

4.2.7 *Hsp90 expression in CLL cells stimulated with growth factors*

For each of the growth factors used, cells were cultured as described in section 4.2.6

4.2.7.1 Stimulation with VEGF

To determine the optimal time for induction of Hsp90 expression in CLL cells following stimulation with VEGF, the cells were incubated with recombinant human $VEGF_{165}$ (100 ng/ml) (R&D Systems; details) for 0.5, 1, 2, 3, 4 and 5 hours at 37°C in 5% CO_2. In subsequent experiments to determine the optimal VEGF concentration necessary to induce changes in Hsp90 expression, cells were incubated for 1 hour in the presence of 1, 10, 50, 100 and 500 ng/ml VEGF.

4.2.7.2 *Stimulation with PLGF*

To determine the most suitable incubation time for studying the effects of the VEGFR1-specific ligand PLGF on induction of Hsp90 expression, CLL cells were incubated with recombinant human PLGF (100 ng/ml) (R&D Systems) for 1, 2 and 4 hours. In experiments to determine the optimal PLGF concentration for induction of Hsp90, CLL cells were incubated for 90 minutes at 37°C in the presence of 1, 10, 50, 100 and 500 ng/ml PLGF.

4.2.7.3 *Stimulation with VEGF-E*

To determine the optimal time for induction of Hsp90 expression in CLL cells following stimulation with VEGF-E, the cells were incubated with recombinant ov-VEGF-E (50 ng/ml) (RELIAH Tech GmbH) for 1, 2 and 4 hours at 37°C in 5% CO_2. In subsequent experiments to determine the optimal VEGF-E concentration necessary to induce changes in Hsp90 expression, cells were incubated for 90 min in the presence of 1, 10, 50, 100 and 500 ng/ml VEGF-E.

4.3 Results

4.3.1 *Detection of mRNA for VEGF receptors by RT-PCR*

As a first step towards determining whether CLL cells express VEGF receptors, it was investigated whether mRNA for both VEGFR1 and VEGFR2, is detectable in non-stimulated CLL cells. Initially, total RNA was extracted from CLL cells that had been purified by positive selection of $CD19^+$ cells using a FACS Sorter (BD). VEGF receptor mRNA was examined by semi-quantitative RT-PCR using specific primers for human VEGFR-1 (1081 bp fragment) or VEGFR-2 (660 bp fragment). As shown in Figure 4.1A, cells from 6 different patients were tested and all expressed mRNA for both of the VEGF receptors. To determine the levels of both VEGFR1 and VEGFR2 mRNA, quantitative slot-blot hybridization was performed on cells from a total of 12 CLL cases. VEGF receptor mRNA expression in HUVEC was used as a control. The levels of VEGF receptor mRNA determined by densitometry are shown in Figure 4.1B. These results confirmed that all cells expressed mRNA for both VEGFR1 and VEGFR2. When the CLL-cell mRNA levels were expressed as a percentage relative to HUVEC mRNA, it was found that the level of mRNA for VEGFR1 was greater than that for VEGFR2. Furthermore, levels of mRNA for each receptor showed wide variation between cases, with a range of 60-90% of control for VEGFR1 and 20-60% for VEGFR2. Since these results demonstrated that CLL cells express mRNA for VEGF receptors, the next series of experiments set out to confirm that CLL cells also express VEGFR1 and VEGFR2 proteins.

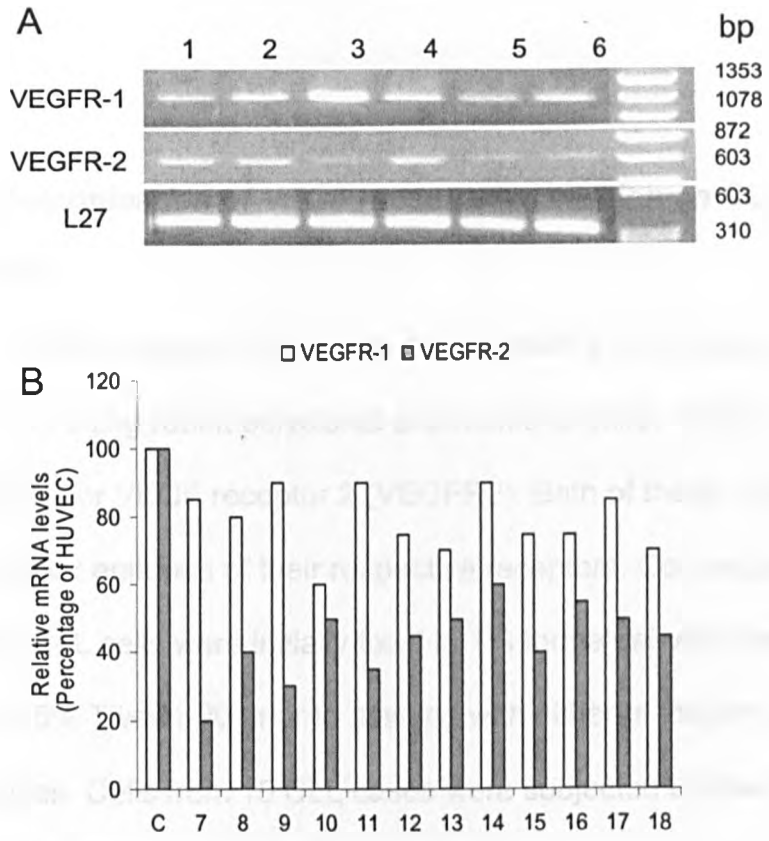


Figure 4.1

RT-PCR and slot-blot analysis of VEGFR1 and VEGFR2 mRNA expression by CLL cells.

(A) For analysis of VEGFR-1 and VEGFR-2 mRNA by RT-PCR, first-strand cDNA was transcribed from 0.5 mg total RNA extracted from the FACS-sorter-purified malignant cells of 6 separate CLL patients (No:1-6). One tenth of the synthesized cDNA was amplified by PCR. Both VEGFR-1 and VEGFR-2 mRNA were readily detectable by this method. Expression of L27 was used as a housekeeping gene control. (B) Quantification of VEGFR-1 and VEGFR-2 mRNA by slot-blot analysis. Total RNA (2 mg) extracted from the Lymphoprep-isolated malignant cells of 12 additional CLL patients was loaded onto duplicate nylon membranes. The membranes were hybridized with [^{32}P]-labelled VEGFR-1 and VEGFR-2 cDNA probes, respectively. Both membranes were stripped and re-hybridised with a GAPDH probe to standardize this procedure. The intensities of the hybridized bands were scanned and analyzed as described in Methods. Levels of VEGFR-1 and VEGFR-2 mRNA in each CLL-cell sample were expressed as a percentage of the levels of these two genes in HUVEC cells (lane C).

4.3.2 *Determination of VEGF receptor expression on CLL cells by flow cytometry*

VEGF receptor expression on CLL cells was analysed by flow cytometry using rabbit polyclonal antibodies to either VEGF receptor 1 (VEGFR1) or VEGF receptor 2 (VEGFR2). Both of these antibodies target intracellular epitopes of their respective receptors. Consequently, freshly thawed CLL cells were initially fixed in 1% formaldehyde, then permeabilized using 0.5% Tween-20 prior to staining with either of the two VEGF receptor antibodies. Cells from 15 CLL cases were subjected to flow cytometric analysis for both VEGFR1 and VEGFR2.

Figure 4.2A shows the levels of expression of VEGFR1 and VEGFR2 by the cells of 15 CLL cases used. In each of the cases studied, the cells expressed both receptors, but there were consistent differences between the levels of expression of VEGFR1 and VEGFR2. As estimated from mean fluorescence, the level of VEGFR2 expression was always higher than that for VEGFR1. VEGF1 expression ranged between 3-7 fluorescence units and that of VEGFR2 between 10-25 units. The levels of expression for each receptor varied between CLL cases but in some instances relatively low VEGFR1 was accompanied by relatively high VEGFR2 while in other cases, high VEGFR1 expression was accompanied by low VEGFR2. A representative plot from the flow cytometry analysis showing mean fluorescence intensity versus cell number for both VEGF receptors is shown in Figure 4.2B.

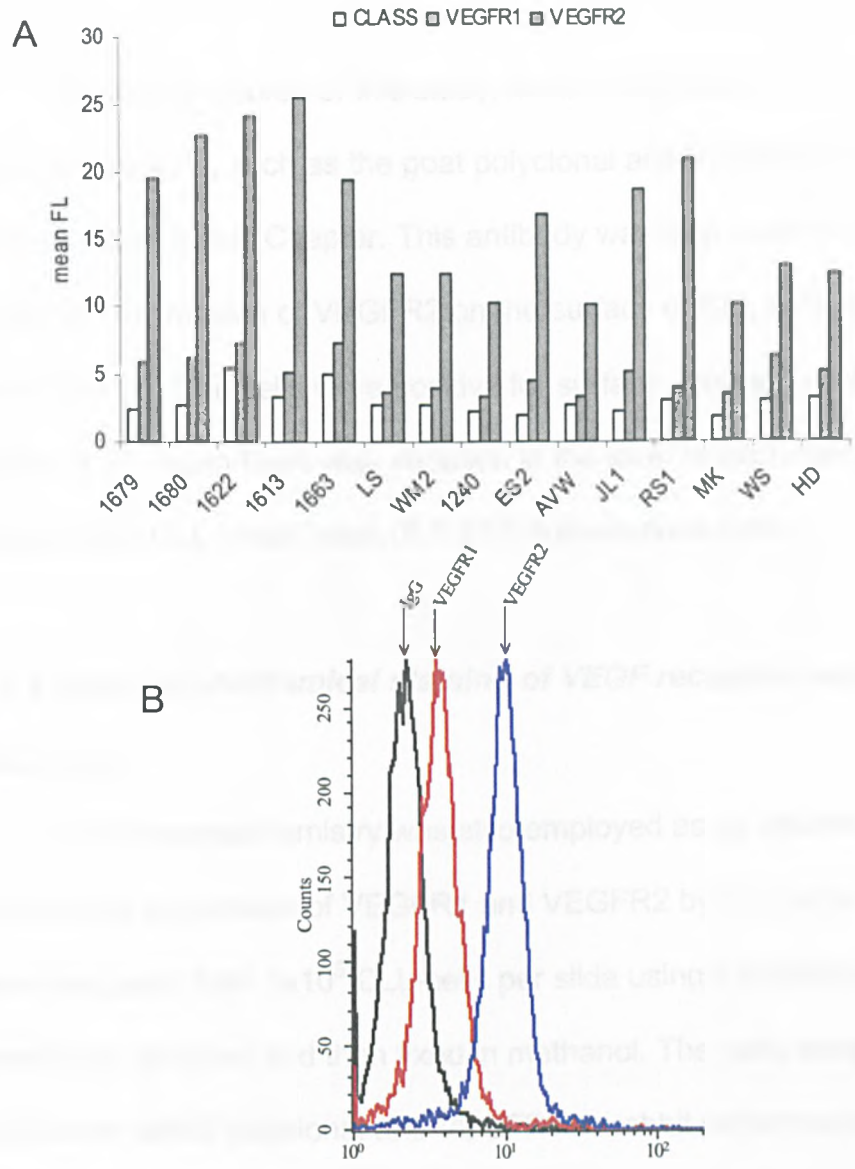


Figure 4.2

Determination of CLL-cell VEGFR1 and VEGFR2 expression by flow cytometry.

Freshly thawed CLL cells were fixed with 1% formaldehyde and permeabilised using 0.5% Tween in PBS. Receptors were detected using polyclonal rabbit antibodies to VEGFR1 (C-17) and VEGFR2 (C-1158) (both against intracellular epitopes) as first layers and goat anti-rabbit-FITC as second layer. Fig 4.2A shows the mean fluorescence (FL) values for cells obtained from 15 CLL cases stained for VEGFR1 and VEGFR2. (Note that VEGFR1 MFI is always lower than that of VEGFR2.) Fig 4.2B is a FACS fluorescence plot from one representative CLL case.

During the course of this study, more antibodies to VEGF receptors became available, such as the goat polyclonal anti-VEGFR2 blocking antibody used in this Chapter. This antibody was also used for flow cytometry to confirm expression of VEGFR2 on the surface of CLL cells. In all cases tested (n=12), CLL cells were positive for surface expression of VEGFR2 (Figure 4.3). Again there was variation in the level of expression of VEGFR2 between the CLL cases used (8.7-21.7 fluorescence units).

4.3.3 *Immunocytochemical staining of VEGF receptors expressed by CLL cells*

Immunocytochemistry was also employed as an alternative method to confirm the expression of VEGFR1 and VEGFR2 by CLL cells. Cytospins were prepared from $1x10^5$ CLL-cells per slide using a Shandon Cytospin 2 centrifuge, air-dried and then fixed in methanol. The cells were first labelled with either rabbit polyclonal anti-VEGFR1 or rabbit polyclonal anti-VEGFR2 and staining then completed using a rabbit ExtrAvidin peroxidase staining kit. Harris haematoxylin was used as a counterstain. Mounted cytospins were visually examined by microscopy and scored by 2 independent examiners for the presence of VEGF receptors using an arbitrary scale from – (negative) to ++ (most strongly positive). Table 4.1 contains the scores for 13 different CLL cases. Generally, VEGFR1 staining was weak, and in 4 of the13 cases VEGFR1 was not detectable, while staining for VEGFR2 was generally stronger and detectable in each case studied.

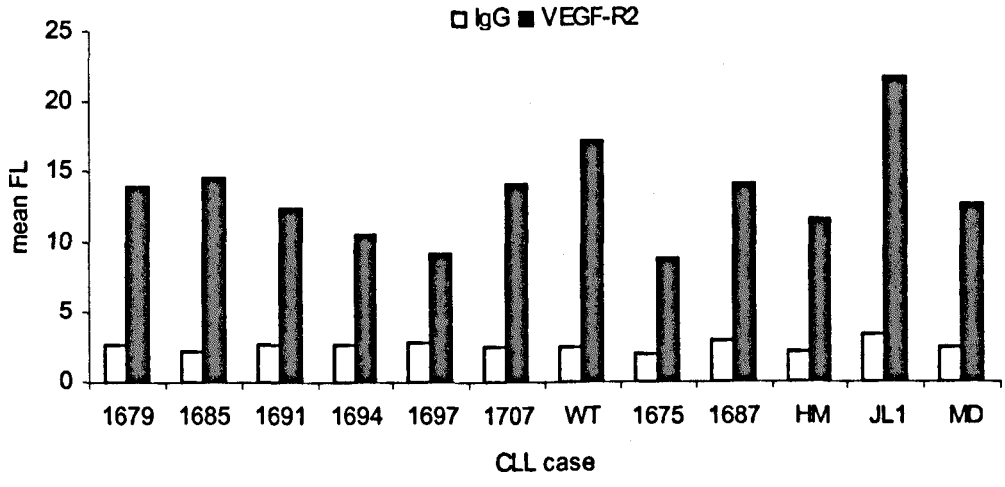


Figure 4.3

Determination of CLL-cell surface expression of VEGFR2 with an antibody against an exracellular epitope.

Freshly thawed CLL cells were stained with goat polyclonal anti-VEGFR2 (R&D Systems; AF357) or goat IgG control as primary antibodies and rabbit anti-goat-FITC as a secondary antibody. The figure shows mean FL values for 12 different CLL cases

	IgG	VEGFR 1	VEGFR 2
1680	-	(+)	(+)
1622	-	(+)	+
1663	-	(+)	+
LS	-	-	(+)
WM2	-	-	+
1240	-	(+)	++
ES2	-	+	++
AVW	-	+	++
JL1	-	(+)	++
RS1	-	(+)	++
MK	-	-	++
WS	-	-	+
HD	-	+	+

Table 4.1

Immunostaining of CLL-cell cytospins for VEGF receptors.
Cytospins were prepared using $1x10^5$ CLL-cells, air-dried, permeabilised then stained using rabbit polyclonal primary antibodies to VEGFR1 and VEGFR2 and an ExtrAvidin peroxidase staining kit (Sigma). Cells were scored during microscope viewing, with a range from ++ for the most positively stained cells, to – for those cells with no staining present. The scores presented in Table 4.1 are from 13 different CLL cases.

From a technical point of view, the immunocytochemical staining was relatively unreliable due to the tendency of CLL cells to break-up during the preparation of the cytospins. However, by preparing duplicate slides it was possible, using this technique, to obtain reproducible estimates of VEGF receptor expression by CLL cells.

Thus, immunocytochemical staining confirmed that CLL cells express VEGF receptors and that the differences in the levels of expression of VEGFR1 and VEGFR2 observed using flow cytometry, were also present when immuno-cytochemistry was used

4.3.4 *Demonstration of VEGF binding to CLL cells by flow cytometry*

In the introduction to this Chapter, it was suggested that VEGF produced by CLL cells could potentially have an autocrine function, since such a role for VEGF has been described in other cell types. Having demonstrated that biologically active VEGF is produced by CLL cells, and that VEGFR1 and VEGFR2 are expressed by these cells, it was next investigated whether VEGF binds to the surface of these cells and whether such binding results in the transduction of intracellular signals generated by VEGF receptors.

To examine whether CLL cells are able to bind VEGF, a commercially available VEGF binding assay kit was employed that had been optimised and validated by the supplier (R&D Systems) using displacement of biotin-labelled VEGF by unlabelled VEGF. Freshly thawed cells were incubated with biotin-labelled VEGF, washed, incubated further with streptavidin-FITC,

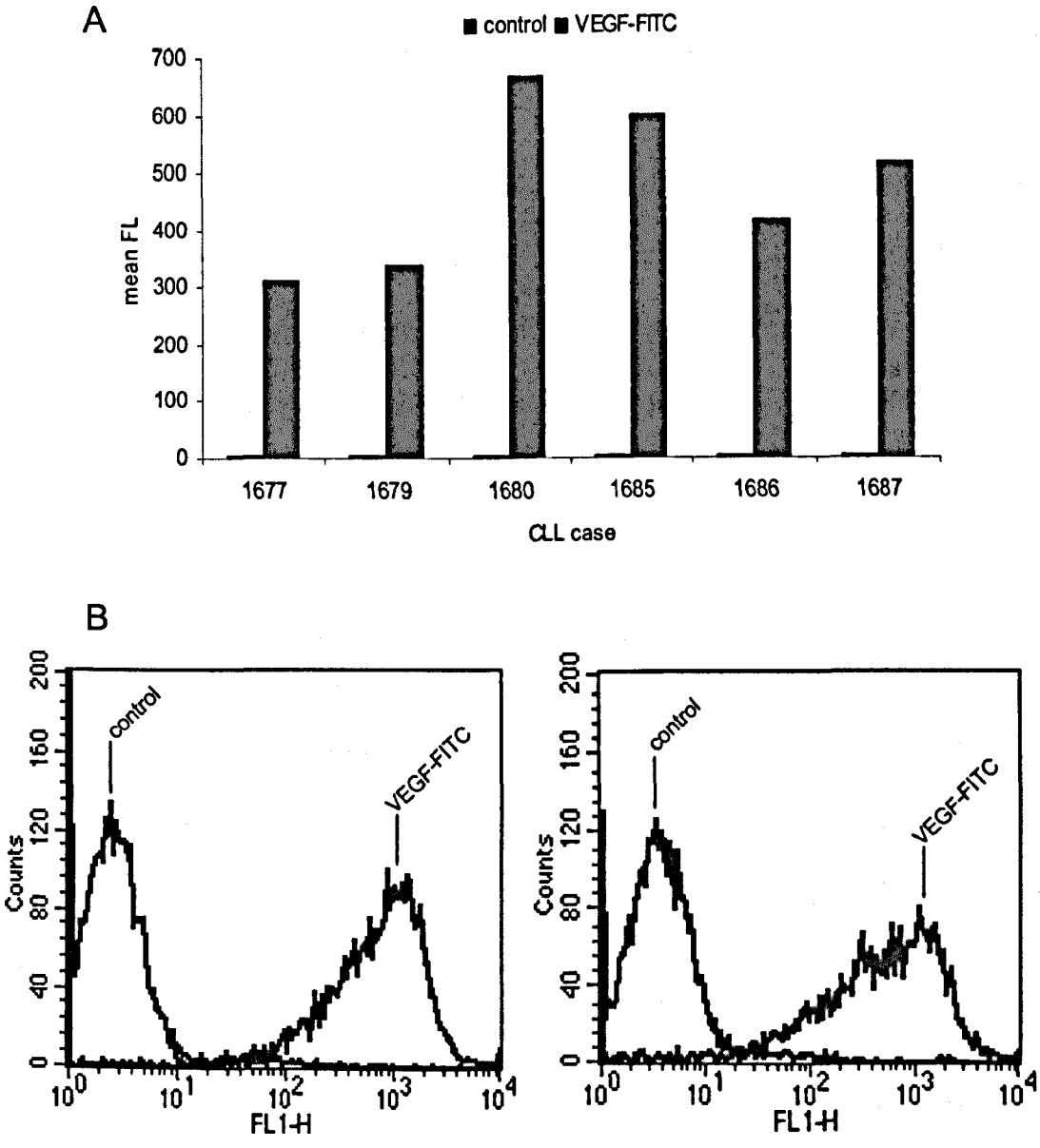


Figure 4.4

VEGF binding to CLL cells.

CLL cells were labelled with biotinylated VEGF followed by streptavidin-FITC using a VEGF ligand binding assay kit (R&D Systems). Binding was then evaluated using flow cytometry. Figure 4.4A shows the mean fluorescence (FL) measured following binding of VEGF to six different CLL-cell clones. FACS histograms from two representative CLL-cases are shown in figure 4.4B.

and the presence of VEGF bound to the surface of the CLL cells determined by flow cytometry. This analysis showed that each of the CLL cases studied (n=6) was strongly positive for VEGF binding (Figure 4.4A) and that the level of binding varied widely between the CLL cases used. The most strongly positive case had a mean fluorescence intensity (MFI) of 667 units, while the least positive case had a MFI value of 312 units. Typical FACS plots for this experiment are shown in Figure 4.4B. Although these results showed that VEGF binds to CLL cells, with this assay it was not possible to determine whether the positive signal was due to VEGF binding to VEGFR1 or VEGFR2 or both.

4.3.5 *VEGF-induced expression of Hsp90*

After showing that VEGF binds to CLL cells, it was important to verify that this binding generates a signal from VEGF receptors. To this end, changes in expression of Hsp90 were measured following stimulation of CLL cells with VEGF.

In order to measure Hsp90-induction, CLL cells in RPMI containing 1% BSA were cultured in poly-HEMA coated 96 well-plates ($2x10^6$/well) at 37°C in 5% CO_2 and stimulated with VEGF for various time intervals. Following this incubation, stimulated cells, as well as unstimulated control cells, were harvested and sonicated in 1% SDS lysis buffer prior to SDS-PAGE and Western blotting.

In preliminary experiments, the optimal incubation time required to induce changes (if any) in Hsp90 expression was determined first, followed by experiments to establish the optimal concentration of VEGF required to

induce these changes. Initially, cells were incubated from 1 to 5 hours with or without 100ng/ml VEGF, a concentration shown to be effective in the endothelial cell proliferation assay (section 3.2.2). Typical Hsp90 responses from two CLL cases together with β-actin loading control are shown in figure 4.5A. In both of these cases, CLL-cell expression of Hsp90 increased following stimulation with VEGF. In comparison to control, this response was strongest after 1 hour incubation, sustained for up to 2 hours but then reduced either slightly (I) or markedly (II) at the later time points. Interestingly, Hsp90 expression in control cells 'spontaneously' increased for one of the cases over the duration of the incubation period (Figure 4.5AII). Since Hsp90 expression in response to VEGF stimulation was already seen at early time points, two further CLL cases were studied in which shorter time points of 30 minutes, 1 hour and 2 hours were employed. Results from these experiments are shown in Figure 4.5B and demonstrate a significant increase in Hsp90 expression after 1 hour incubation with VEGF in both of the CLL cases studied. These blots, analysed by densitometry and corrected for sample loading, are shown in Figure 4.5C. In one of the CLL cases, (Figure 4.5CI) a marked response to VEGF was observed after 30 minutes incubation, and this was sustained for up to 2 hours. In contrast, the second case (Figure 4.5CII) did not respond until the cells had been incubated with VEGF for 1 hour. Therefore, in subsequent experiments involving VEGF-induced expression of Hsp90, CLL cells were stimulated with VEGF for 1 hour prior to harvesting and lysis.

To establish a suitable concentration of VEGF for the induction of Hsp90 expression by CLL cells, the cells were incubated for 1 hour with

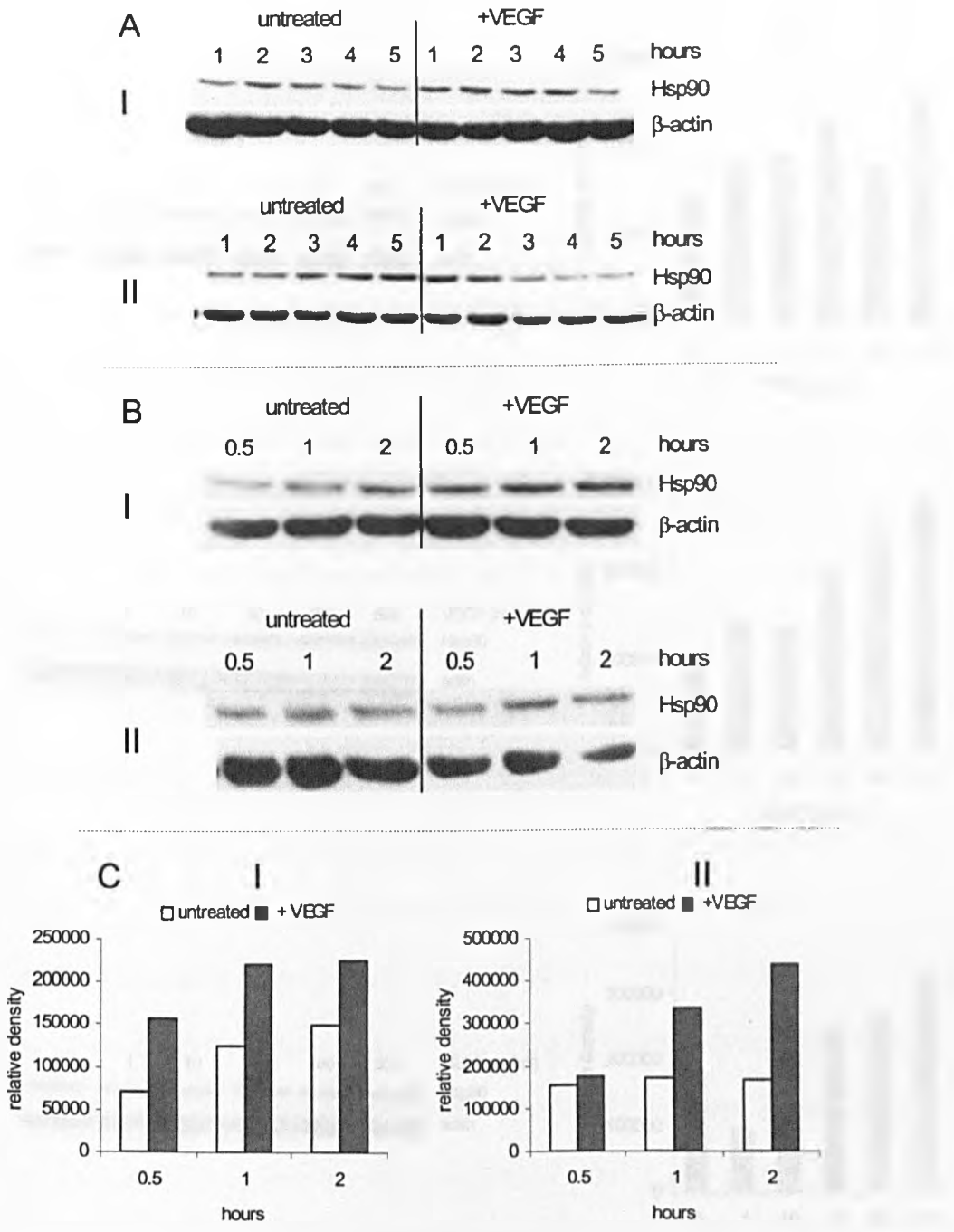


Figure 4.5

Hsp90 expression in VEGF-treated CLL cells.

CLL cells were incubated in RPMI containing 1% BSA at a density of $2x10^6$/well in poly-HEMA coated 96-well plates in the presence or absence of VEGF. Harvested cells were lysed in 1% SDS prior to Western blot analysis. Cells were incubated for up to 5 hours and harvested at 1-hour intervals (A), or for shorter intervals up to 2 hours (B). For the shorter time points, differences between VEGF-treated and untreated cells were measured using densitometry of the Western blots (C). Both 4.5A and 4.5B show two representative cases.

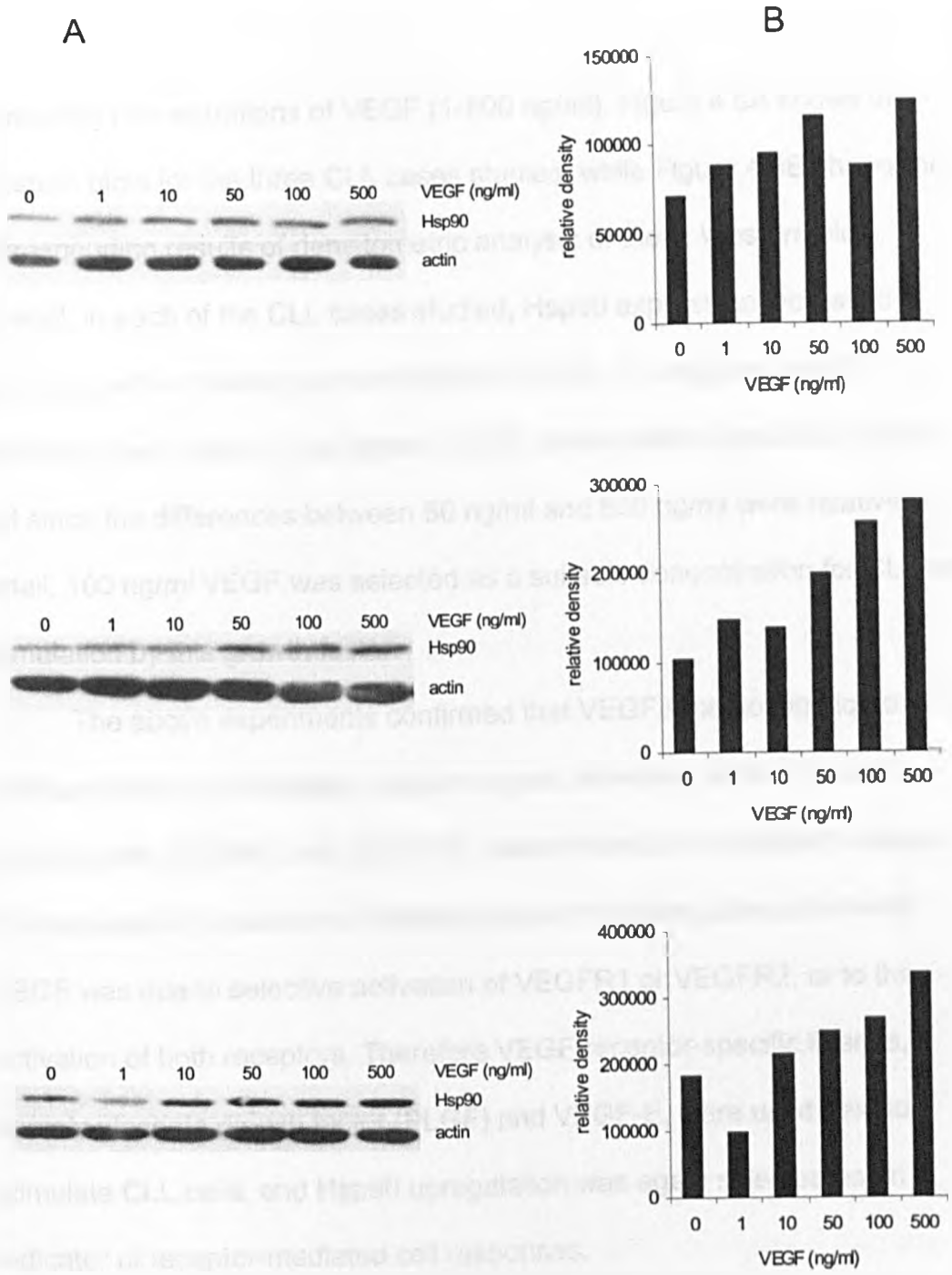


Figure 4.6

Changes in Hsp90 expression in CLL cells in response to a range of VEGF concentrations.

CLL cells were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates in 5% CO_2 for 1 hour at 37°C. For VEGF-treatment, cells were incubated with a range of VEGF concentrations (1-500ng/ml), harvested after 1 hour and lysed in 1% SDS prior to Western blot analysis. The Western blots of three different CLL cases (A) and their densitometry analysis (B) are shown.

increasing concentrations of VEGF (1-500 ng/ml). Figure 4.6A shows the Western blots for the three CLL cases studied, while Figure 4.6B shows the corresponding results of densitometric analysis of these Western blots. Overall, in each of the CLL cases studied, Hsp90 expression increased gradually with increasing concentration of VEGF. The highest Hsp90 expression was seen at the highest VEGF concentration used (500 ng/ml), but since the differences between 50 ng/ml and 500 ng/ml were relatively small, 100 ng/ml VEGF was selected as a suitable concentration for CLL-cell stimulation by this growth factor.

The above experiments confirmed that VEGF receptor ligation on CLL cells generates a biologically relevant signal. However, since CLL cells express both VEGFR1 and VEGFR2, it was important to establish whether the increase in expression of Hsp90 observed following stimulation with VEGF was due to selective activation of VEGFR1 or VEGFR2, or to the activation of both receptors. Therefore VEGF-receptor-specific ligands, namely placenta growth factor (PLGF) and VEGF-E, were used next to stimulate CLL cells, and Hsp90 upregulation was again selected as an indicator of receptor-mediated cell responses.

4.3.6 *Placenta growth factor (PLGF)-induced expression of Hsp90*

PLGF is a receptor-specific ligand for VEGFR1. To determine whether ligation of VEGFR1 expressed on CLL cells could activate signalling pathways, CLL cells were stimulated with PLGF and changes in expression of Hsp90 determined by Western blotting. Briefly, CLL cells ($2x10^6$) were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates in

the presence of PLGF for up to 4 hours. Cells were harvested at 1, 2 and 4 hours and lysed in 1% SDS prior to Western blotting. The initial experiments aimed only to identify the optimal incubation time for inducing a change in Hsp90 expression in CLL cells following stimulation with PLGF. For these experiments, a single concentration of PLGF was selected (100 ng/ml) that was known to induce a signalling response in other cell types (Landgren et al, 1998). It was found that in the two CLL cases used, PLGF induced only a small increase in Hsp90 expression at each time-point used (Figure 4.7A). Densitometry showed a slight increase in expression of Hsp90 after 1-hour incubation and the greatest increase after 2 hours, followed by a reduction in expression after this time (Figure 4.7B). Based on these results, in the next series of experiments CLL cells were stimulated with PLGF for 90 minutes.

To establish the dose-response of CLL cells to PLGF, the cells were incubated with a range of PLGF concentrations (1-500 ng/ml) for 90 minutes, prior to lysis and Western-blot analysis of Hsp90 expression. As shown in figure 4.8A, Hsp90 expression increased in CLL cells with increasing concentrations of PLGF. In both CLL cases studied, the highest Hsp90 expression was observed following stimulation with 100 ng/ml PLGF; this observation was confirmed by analysis of the Western blots by densitometry (Figure 4.8B). However, the response to lower concentrations of PLGF was different in the two cases. In CLL case (I), no clear response was observed at PLGF concentrations below 50 ng/ml. For CLL case (II), there was already a clear response to 1 ng/ml PLGF which was followed by a gradual, incremental increase in Hsp90 expression as PLGF concentration increased.

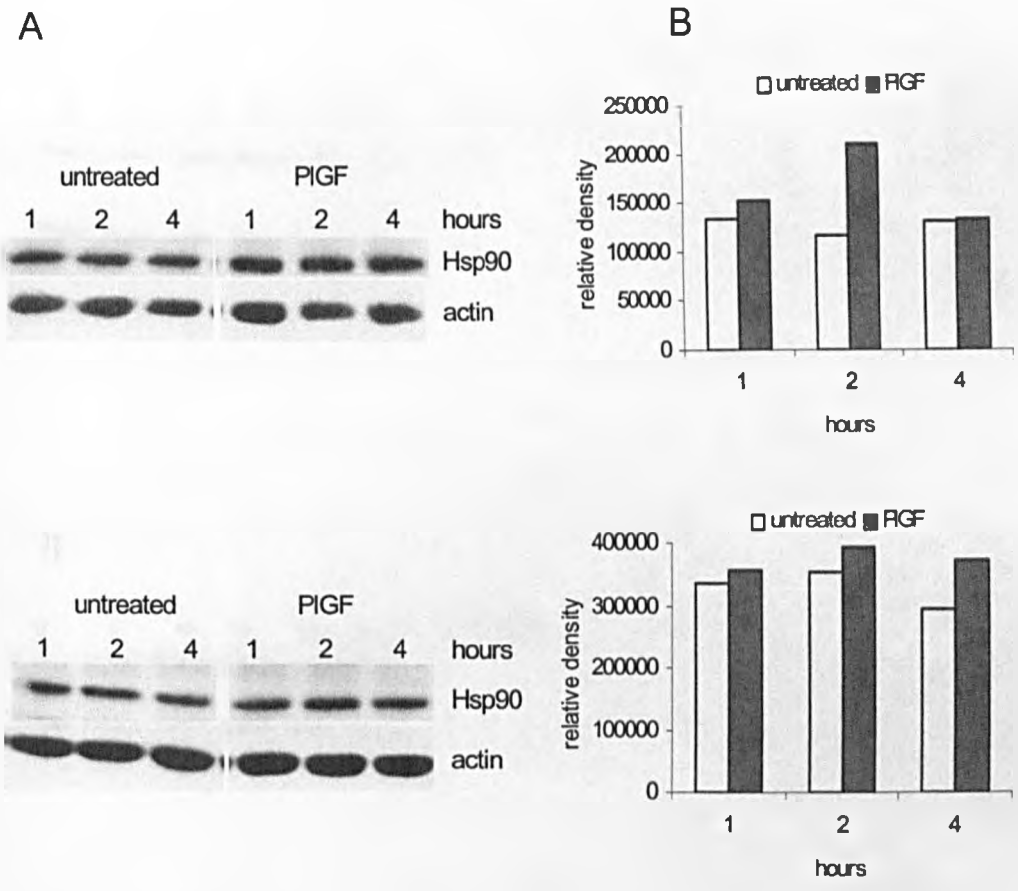


Figure 4.7

Hsp90 expression in PLGF-stimulated CLL cells.

CLL cells ($2x10^6$) were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates in the presence of PLGF (100 ng/ml) for up to 4 hours in 5% CO_2 at 37°C. Cells were harvested at 1, 2 and 4 hours for lysis in 1% SDS prior to Western blotting. (A) A comparison of the response of PLGF-treated and untreated cells from two different CLL cases. (B) Densitometry analysis to determine any differences between treated and untreated cells from both CLL cases. The data presented are from two representative CLL cases.

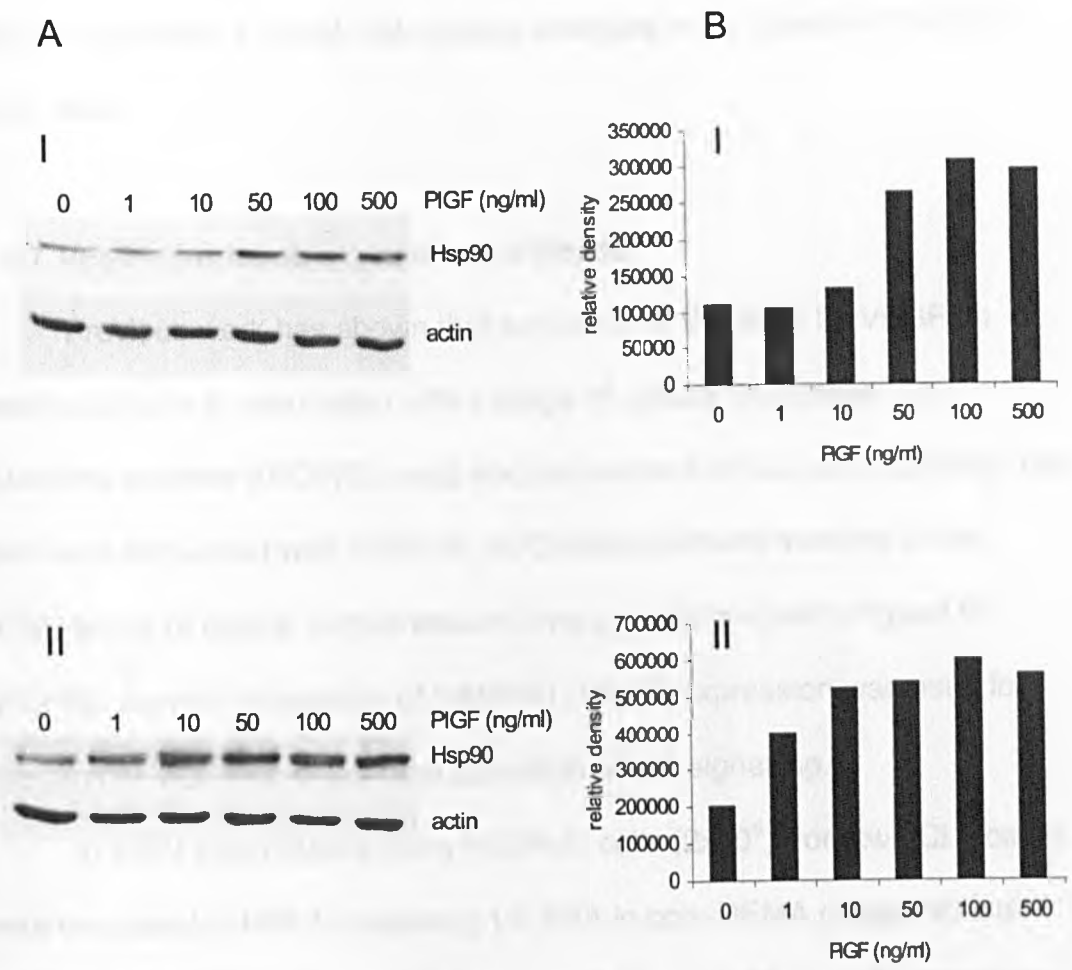


Figure 4.8

Hsp90 expression in CLL cells following stimulation with a range of PIGF concentrations.

CLL cells ($2x10^6$) from 2 different CLL cases were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates, in 5% CO_2 at 37°C in the presence of 1-500 ng/ml PIGF. Cells were harvested after 90 minutes incubation and lysed in 1% SDS prior to Western blotting. (A) Western blots showing Hsp90 expression in CLL cells stimulated with a range of PIGF concentrations. (B) Densitometry analysis of Western blots to quantify changes in Hsp90 expression. (The two representative CLL cases shown are different to those in Figure 4.7)

These results demonstrate that ligation of VEGFR1 expressed on CLL cells can generate a signal that induces changes in expression of Hsp90 by these cells.

4.3.7 *VEGF-E-induced expression of Hsp90*

Previous work has shown that activation of VEGFR2 by VEGF on endothelial cells is associated with a range of cellular responses. To determine whether VEGFR2 could also be involved in CLL-cell signalling, the cells were stimulated with VEGF-E, an Orf-virus-derived member of the VEGF-family of growth factors known to be a receptor-specific ligand for VEGFR2. As with stimulation of VEGFR1, Hsp90 expression was used for detection of VEGFR2-dependent activation of cell signalling.

In initial experiments using VEGF-E, cells ($2x10^6$) from two CLL cases were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates in the presence of VEGF-E (50ng/ml) for up to 4 hours. To determine the optimal period of stimulation with VEGF-E for effective induction of Hsp90 expression, the cells were harvested at 1, 2 and 4 hours and lysed in 1% SDS prior to Western blotting. These experiments revealed small increases in Hsp90 expression in both cases after 1 and 2 hours incubation with VEGF-E (Figure 4.9). In CLL case (I), a further relatively large increase was present after 4 hours incubation, but this was not observed in case (II).

As in the case of stimulation of VEGFR1 by PLGF, a 90 minute incubation time was used for establishing the dose response of CLL cells to VEGF-E. Briefly, CLL cells ($2x10^6$) from two different CLL cases were incubated in the presence of a range of VEGF-E concentrations

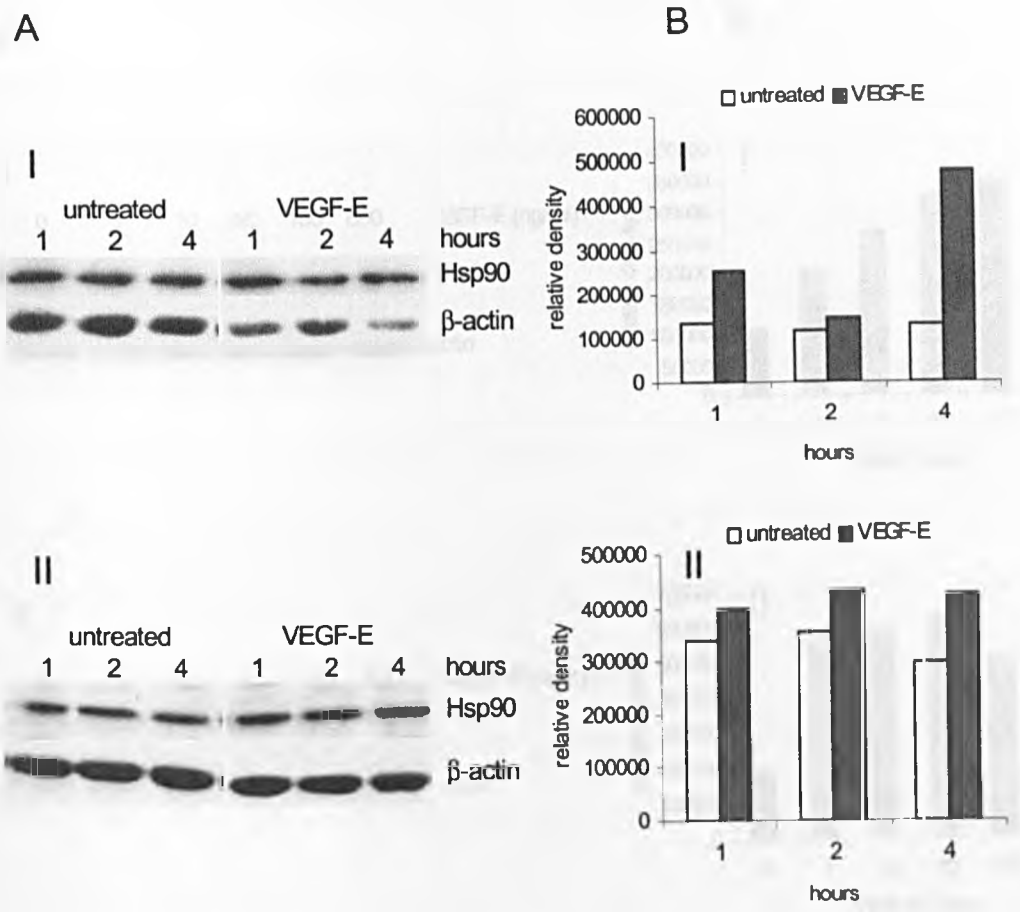


Figure 4.9

Hsp90 expression in VEGF-E-stimulated CLL cells.

CLL cells ($2x10^6$) were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates in the presence of VEGF-E (50ng/ml) for up to 4 hours at 37°C, 5% CO_2. Cells were harvested at 1, 2 and 4 hours for lysis in 1% SDS prior to Western blotting. (A) A comparison of the response of VEGF-E treated and untreated cells for two different CLL cases. (B) Densitometry analysis of the blots in (A), performed to determine differences between treated and untreated cells for both cases. The data shown are from two representative CLL cases.

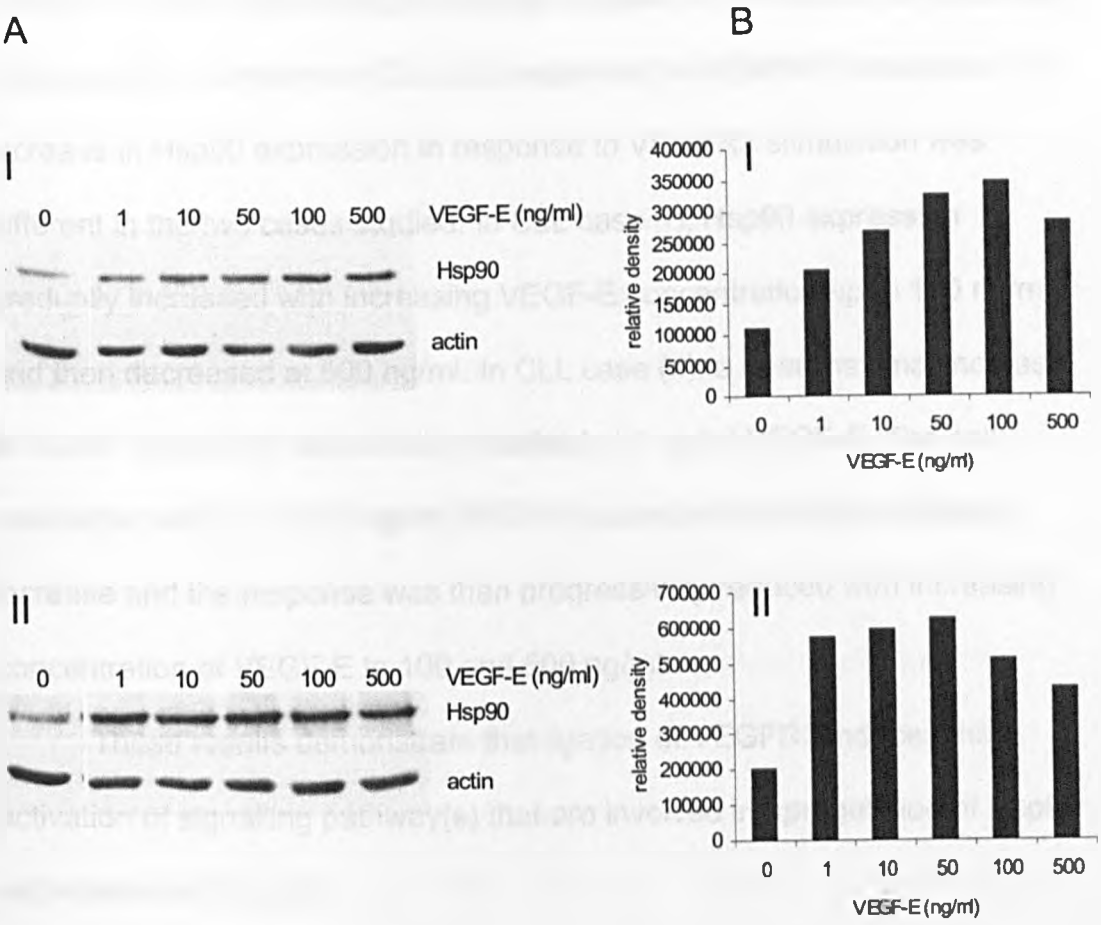


Figure 4.10

Hsp90 expression in CLL cells following stimulation with a range of VEGF-E concentrations.

CLL cells ($2x10^6$) from 2 different CLL cases were incubated in RPMI containing 1% BSA in poly-HEMA coated 96-well plates in the presence of a range of VEGF-E concentrations (1-500ng/ml), at 37°C, 5% CO_2. Cells were harvested after 90 minutes incubation and lysed in 1% SDS prior to Western blotting. (A) Western blots showing Hsp90 expression in CLL cells stimulated with a range of VEGF-E concentrations. (B) Densitometry analysis of the Western blots to quantify changes in Hsp90 expression. (The two representative CLL cases shown are different to those in Figure 4.9)

(1-500ng/ml), harvested after 90 minutes and lysed in 1% SDS prior to Western blotting (Figure 4.10A) and the analysis of the blots by densitometry (Figure 4.10B). Similarly to CLL-cell responses to VEGFR1 stimulation, the increase in Hsp90 expression in response to VEGFR2 stimulation was different in the two cases studied. In CLL case (I), Hsp90 expression gradually increased with increasing VEGF-E concentration up to 100 ng/ml, and then decreased at 500 ng/ml. In CLL case (II), a near maximal increase in Hsp90 expression was already reached at 1 ng/ml VEGF-E. The cell incubation with 10 and 50 ng/ml VEGF-E caused only a slight additional increase and the response was then progressively reduced with increasing concentration of VEGF-E to 100 and 500 ng/ml.

These results demonstrate that ligation of VEGFR2 induces the activation of signalling pathway(s) that are involved in upregulation of Hsp90 expression in CLL cells.

4.4 Discussion

The results of work performed in Chapters 2 and 3 indicated that CLL cells secrete angiogenically-active VEGF. Evidence suggested that VEGF present in nodes of CLL patients may be involved in the angiogenic events identified within this tissue, namely the presence of an increased microvessel density. Such paracrine effects of VEGF have been described in other haematological malignancies (de Bont et al, 2001; Aguayo et al, 2000b). Work with malignant cells had indicated that VEGF may also have an autocrine role in solid tumours (Bachelder et al, 2001; Jackson et al, 2002). Since CLL is a disease characterised by prolonged survival of malignant cells

and VEGF is known to enhance survival of some cell-types, it seemed attractive to explore the possibility that VEGF may support CLL-cell survival through an autocrine mechanism. The first stage in this investigation was to identify whether CLL cells express functional VEGF receptors.

At the outset of this project, it was not known whether CLL cells expressed VEGF receptors. However, during the course of this work, several papers were published indicating that CLL cells do express VEGF receptors (Ferrajoli et al 2001; Kay et al, 2002; Bairey et al, 2004). Moreover, published work (Chen et al, 2000) arising from this thesis, and pre-dating the other publications, mentioned that results of preliminary experiments showed that CLL cells express VEGF receptors and that VEGF may have important autocrine effects on CLL-cell behaviour.

This completed work is now presented in this Chapter and demonstrates that mRNA for both VEGFR1 and VEGFR2 is detectable and constitutively expressed in CLL cells. Quantitative slot-blot hybridisation indicated that the level of mRNA for VEGFR1 was always greater than that for VEGFR2, and that there was wide variation between cases. Expression of VEGF receptors by CLL cells was established by immuno-staining and flow cytometric analysis. Interestingly, and in contrast to mRNA levels, expression of VEGFR2 always appeared higher than that of VEGFR1. This may indicate differences in levels of expression of the two receptors on the cell surface but it would require further work for confirmation since it may simply reflect differences in the binding properties of the two primary antibodies or binding of the FITC-conjugated secondary antibody to the primary antibodies. Although expression of VEGF receptors was confirmed by

immunocytochemical staining of cytospins prepared from CLL cells, this technique was less satisfactory. This was due to the fragile nature of the CLL cells, resulting in poor quality cytospins. The fragile nature of CLL cells is well described and characterised by the presence of smear cells in the blood films of patients with this disease.

For autocrine cell-stimulation to occur, the cells must express functional receptors that bind their ligand and generate signals that stimulate cellular functions. Using a commercially available VEGF ligand-binding assay kit, it was shown that CLL cells do bind VEGF. However, due to the limitations of this assay, it was not possible to confirm that such binding was receptor mediated, since displacement of biotin-labelled VEGF with unlabelled VEGF was not demonstrated. Furthermore, if the binding was specific, it was not possible to determine whether it was due to VEGFR1, or VEGFR2, or both VEGFR1 and VEGFR2.

To confirm that binding and activation of VEGF receptors on the CLL-cell surface had occurred required the demonstration of a cell response to the receptor ligation. As stated previously, VEGF is involved in the motility, proliferation and survival of endothelial cells. The protein chaperone Hsp90 has been implicated in each of the signalling pathways that control these cellular functions activated by VEGF. Furthermore, Hsp90 gene expression is upregulated by VEGF-stimulation of HL60 cells (Dias et al, 2002). Consequently, Hsp90 was selected as a general marker of VEGF-receptor-mediated cellular activation and signalling. In the first instance, Western blotting established that VEGF does upregulate Hsp90 expression in CLL cells in a dose dependent manner, and confirmed that VEGF ligation of

receptor(s) present on the surface of CLL cells activates intracellular signalling pathways. However, since VEGF binds to both VEGFR1 and VEGFR2 (although with different affinity) it was not possible to determine whether one or both of these receptors was responsible for Hsp90 upregulation. This was considered important, since differences in the functions of VEGFR1 and VEGFR2 have been described. VEGFR1 has the higher affinity for VEGF but its role remains uncertain. Though it is known to be involved in monocyte motility (Barleon et al, 1996), induction of MMP-9 (Rafii et al, 2003) and survival of haemopoietic stem cells (Gerber et al, 2002), it has only weak effects on endothelial cell signalling and was proposed to be a decoy receptor (Park et al, 1994). Therefore PLGF, a receptor-specific ligand for VEGFR1 was used to stimulate CLL cells. PLGF-stimulation of CLL cells increased expression of Hsp90 in a dose dependent manner, indicating that ligation of VEGFR1 does activate signalling in CLL cells. Although the identity of all the signalling pathways involved remains unclear, the response is likely to include the activation of the NF-κB pathway (Farahani et al, 2005). VEGFR2 has approximately 10-fold lower affinity for VEGF than VEGFR1 and is the VEGF receptor most strongly implicated in VEGF cellular functions (Rousseau et al, 2000; Gerber et al, 1998b). As expected, the VEGFR2-specific ligand VEGF-E also caused upregulation of Hsp90 expression in CLL cells, suggesting a potential role for both receptors in CLL-cell functions.

Since this study had confirmed that CLL cells secrete VEGF and express functional VEGF receptors, and we had previously proposed a possible autocrine role for VEGF in CLL (Chen et al, 2000), it seemed

important to identify cell responses that would support such a role. As stated previously, CLL is a disease characterised by prolonged survival of the malignant cells. While many survival factors might affect this characteristic of the disease, in the context of this thesis it was appropriate to investigate whether VEGF might be one such factor. Therefore, the effects of VEGF on CLL cell survival are described next in Chapter 5.

Chapter 5

Autocrine function of VEGF with respect to CLL-cell survival

5.1 Introduction

In the previous Chapters of this thesis, it was shown that CLL cells secrete VEGF and express functional VEGF receptors. VEGF secretion and receptor expression has been described in other cell-types in which VEGF acts as an autocrine pro-survival factor (Masood et al, 2001; Santos and Dias, 2004). In the current study, it was presumed that such autocrine pro-survival effects might also exist for CLL cells. Consequently, the experiments performed in this Chapter explore the effects of VEGF on CLL-cell survival.

Two approaches were taken. Firstly, cell survival was measured following the addition of VEGF to CLL cells in culture, with the aim of protecting the cells from spontaneous and drug-induced apoptosis. VEGF is known to protect various cells-types from death induced by factors such as ionising radiation (IR) (Katoh et al, 1995), and the specific inhibitor of Hsp90, geldanamycin (Dias et al, 2002). Furthermore, the angiogenic factor bFGF is known to provide CLL cells with resistance to apoptosis induced by the nucleoside analogue fludarabine (Menzel et al, 1996). Secondly, since autocrine stimulation by VEGF is known to enhance cell survival of some cell-types, it was decided to use inhibition and blocking of VEGF receptors, and neutralisation of secreted VEGF to determine whether such an autocrine survival effect exists for CLL cells. Such potential protective effects of VEGF for CLL cells were investigated for this Chapter.

In Chapter 2, it was demonstrated that stimulation of CLL cells via CD40, a receptor previously implicated in *in vivo* provision of CLL cytoprotection in lymphoid tissues (Schattner EJ, 2000), caused increased secretion of VEGF. The final section of this Chapter explores a possibility that autocrine VEGF is involved in rescue of CLL cells by CD154 and briefly examines a signalling pathway that may be involved in the anti-apoptotic activity of CD154.

5.2 Methods

5.2.1 *Determination of CLL-cell viability*

Determination of CLL-cell viability was based on the analysis of mitochondrial transmembrane potential with 3, 3'dihexyloxacarbocyanine iodide (DiOC6; Calbiochem) and on cell-membrane permeability to propidium iodide (PI) (Sigma), as described previously (Zamzami et al, 1995). Unless stated otherwise, freshly thawed CLL cells were resuspended to $3.3x10^6$ cells/ml in RPMI medium (Sigma) containing 0.1% BSA (Sigma) and cultured in poly-HEMA-coated 96-well plates (100 µl/well) at 37°C in 5% CO_2 for the appropriate incubation period. To determine cell survival, 100 µl of CLL-cell suspension was removed from culture at the indicated time points and incubated with 100 µl of 40nM $DiOC_6$ for 20 minutes at 37°C, prior to incubation on ice for a further 30 minutes with an equal volume of 10 µg/ml PI. Cell viability was determined using by FACS analysis. In this analysis, live cells are $DiOC_6$-bright/PI-dim, early apoptotic cells are $DiOC_6$-dim/PI-dim, and late apoptotic cells are $DiOC_6$-dim/PI-bright.

5.2.2 *Determination of CLL-cell culture conditions*

Freshly thawed CLL cells were resuspended in RPMI medium to a density of $1x10^7$ cells/ml. Aliquots of the cells (1 ml) were transferred to eppendorf tubes and centriguged at 7000g for 20 secs. The supernatant was removed and the cell pellet resuspended in RPMI medium, or RPMI medium supplemented with 0.1% BSA or 1% BSA. The CLL cells were then further diluted in the appropriate medium to produce a range of cell densities ($1.5x10^5$ to $1x10^7$ cells/ml). For each cell density, 100µl of cell suspension was transferred to poly-HEMA-coated 96-well plates followed by the addition of 50µl of medium to each well to give a final culture volume of 150µl. The cells were cultured for up to 4 days at 37°C in 5% CO_2, then harvested and stained with $DiOC_6$/PI to determine cell viability.

5.2.3 *VEGF dose response*

In experiments in which exogenous VEGF was used, freshly thawed CLL cells were resuspended in RPMI medium supplemented with 0.1% BSA at $3.3x10^6$ cells/ml and incubated with 100 ng/ml VEGF. To determine the most suitable VEGF concentration for cell survival experiments, recombinant human VEGF (R&D Systems) was diluted to 1, 10, 50, 100, 250 and 500 ng/ml in RPMI medium containing 0.1% BSA, and 50µl of each dilution (or medium-only control) then added to 100µl of CLL cells in poly-HEMA-coated 96-well plates. The cultures in each well contained $3.3x10^5$ cells in 150µl, and were incubated at 37°C in 5% CO_2 for up to 4 days prior to harvest for the determination of cell viability using $DiOC_6$/PI staining.

5.2.4 *Induction of CLL-cell death*

To determine whether exogenous VEGF could rescue CLL cells from induced cell death, the cells were resuspended in RPMI medium supplemented with 1% BSA and plated in poly-HEMA-coated 96-well plates at a cell of $3.3x10^5$ cells/well, unless otherwise stated. Following the addition of 100 ng/ml recombinant human VEGF (R&D Systems), the cells in the final culture volume of 150 µl were incubated at 37°C in 5% CO_2. The viability of harvested cells was determined by staining with $DiOC_6$/PI.

5.2.4.1 *Fludarabine*

To determine the effects of exogenous VEGF on fludarabine-induced cell death, CLL cells were incubated in the presence of 2 µM fludarabine (Schering), a concentration known to be readily attainable in the plasma of patients treated with this agent (Ross et al, 1993), with or without VEGF (100 ng/ml) for up to 6 days. In further experiments, CLL cells were incubated with a range of VEGF concentrations (0.1-500 ng/ml) in combination with fludarabine (2 µM) for up to 3 days prior to determination of cell viability.

5.2.4.2 *Ionising radiation (IR)*

To determine whether VEGF could rescue CLL cells from IR-induced cell death, cells from each case studied were exposed to 5 Gy IR using a caesium-137 source and cultured in the presence of VEGF (100 ng/ml). Following culture for up to 6 days, the cells were harvested and their viability determined by staining with $DiOC_6$/PI.

5.2.4.3 *Geldanamycin (GA)*

Geldanamycin is a specific inhibitor of Hsp90 and was used in CLL-cell survival experiments with two aims. The first aim was to determine whether GA induces CLL-cell death in culture and to this end, CLL cells were incubated with GA (Sigma) at 0.02, 0.2 and 2 μM for up to 3 days; the viability of harvested cells was determined using $DiOC_6$/PI staining. The second aim was to determine whether VEGF would protect CLL cells from GA-induced killing and for such experiments, CLL cells were incubated with 100 ng/ml VEGF in the presence of 0.2 μM GA since this was the lowest concentration of GA used that was shown to induce CLL-cell death.

5.2.5 *Autocrine VEGF and CLL-cell survival*

In this section, experiments to determine the effects of endogenous VEGF on CLL-cell survival were performed by neutralising VEGF or blocking its binding to VEGF receptors (VEGFR), and by inhibiting tyrosine kinase activity of VEGFR. In all instances, unless stated otherwise, freshly thawed CLL cells were resuspended in RPMI medium containing 0.1% BSA and incubated in poly-HEMA-coated 96-well plates at a cell density of $3.3x10^5$ cells/well for up to 6 days, at 37°C in 5% CO_2. Harvested cells were stained with DiOC6/PI to determine cell viability.

5.2.6 *Neutralising VEGF and blocking VEGF binding*

To neutralise secreted VEGF in CLL-cell cultures, mouse monoclonal anti-VEGF antibody (R&D Systems) or mouse IgG_{2b} isotype control (R&D Systems), both at 1 μg/ml, were added to CLL cells cultured for up to 6 days.

In other experiments, CLL cells were incubated in the presence of high affinity, soluble, recombinant human chimaeric receptors for both VEGFR1 (sVEGFR1/Fc; 300 ng/ml) and VEGFR2 (sVEGFR2/Fc; 200ng/ml) (both R&D Systems, or both in combination, for up to 5 days prior to cell harvest.
To block the binding of VEGF to its receptors, CLL cells were cultured in the presence of antibodies directed to the VEGF-binding site of the VEGF receptors. Goat polyclonal anti-VEGFR1 (1 µg/ml; R&D), goat polyclonal anti-VEGFR2 (1 µg/ml; R&D) or goat IgG (1 µg/ml; R&D) were added to CLL-cell cultures and incubated for up to 4 days prior to cell harvest and measurement of cell viability.

5.2.7 *Inhibition of VEGF receptor-tyrosine kinase activity by SU5416*

The receptor-tyrosine kinase inhibitor SU5416 is an inhibitor of both VEGFR1 and VEGFR2 (Itokawa et al, 2002) and was used to determine the effects of inhibition of both of these receptors on CLL-cell survival. SU5416 (Calbiochem) at a concentration of 10µM or DMSO control (0.2%) was added to CLL-cell cultures and incubated for up to 6 days prior to harvest for determination of cell viability.

5.2.8 *CD154-enhanced CLL-cell survival*

To determine the effects of CD154 on CLL-cell survival, freshly thawed CLL cells were resuspended in RPMI medium supplemented with 0.1% BSA and placed into a poly-HEMA coated 96-well plate at a density of $3.3x10^5$ cells per well. Following the addition of recombinant CD154 (200 ng/ml) and enhancer (1 µg/ml) (Alexis Biochemicals), the cells were

incubated at 37°C in 5% CO_2 for up to 6 days prior to harvest and determination of cell viability. In experiments in which inhibitors or antibodies were used to abrogate the involvement of VEGF in the effects of CD154, CLL cells were incubated with mouse anti-VEGF monoclonal antibody (1 µg/ml; R&D Systems), mouse IgG_{2b} isotypic control antibody (1 µg/ml), SU5416 (10 µM) or DMSO (0.2%) for 1 hour prior to addition of CD154 and enhancer. Treated cells were incubated for up to 6 days, and then harvested to determine cell viability.

5.2.9 *Effect of NF-κB inhibition on CD154-induced VEGF production*

CLL cells were suspended at $1x10^7$/ml in RPMI containing 1% BSA, then 200µl ($2x10^6$) cells were added to each well in a 24-well plate. The cells were pre-incubated for 1 hour with the NF-κB nuclear translocation inhibitor SN50 (18 µM; Calbiochem) or its inactive analogue SN50M (18 µM; Calbiochem). Following addition of CD154 (200 ng/ml) and enhancer (1 µg/ml) (Alexis Biochemicals) and incubation for a further 24 hours, culture supernatants were harvested and the concentration of secreted VEGF determined by VEGF ELISA (R&D Systems), as described in Chapter 2.

5.2.10 *Evaluation of NF-κB activity*

CLL cells ($3.3x10^5$/well) suspended in RPMI supplemented with 1% BSA were transferred to a poly-HEMA-coated 96-well plate and cultured with CD154, CD154 plus anti-VEGF neutralising monoclonal antibody (1 µg/ml) (R&D Systems; MAB292) or with CD154 plus SU5416 (10 µM) for 24 hours. Following incubation at 37°C in 5% CO_2, the cells were harvested and

nuclear extracts prepared using a Nuclear Extract Kit (Active Motif) according to the manufacturer's instructions. Protein concentration was determined by Bradford assay (Bio Rad) and the DNA-binding activity of NF-κB quantified using a TransAM NF-κB p65 transcription factor assay kit (Active Motif), again according to the manufacturer's instructions. The detection limit for this assay was 0.4 ng/ml.

5.2.11 *Confocal microscopy*

To confirm nuclear translocation of NF-κB, cytospins were prepared using a Shandon Cytospin 2 from cells cultured as described above (section 5.2.10), air-dried and fixed in methanol for 10 min at RT. Fixed cells were washed in PBS and blocked for 1 hour at RT with PBS containing human AB, goat and mouse sera (all at 1%). After washing, mouse anti-human p65 monoclonal antibody or non-specific mouse IgG_1 antibody (both Santa Cruz; 2 µg/ml each, diluted in PBS containing 1% human AB serum) was added and cells incubated for 1 hour at RT. Goat anti-mouse-FITC conjugate (Sanata Cruz; diluted 1:10 in 1% human AB serum/PBS) was added after further washing, cells incubated for 1 hour at RT, washed and mounted in Vectashield (Vector Laboratories). The cellular location of NF-kB p65 protein was then analysed by laser confocal microscopy using an Olympus BH2 microscope fitted with a Bio-Rad MR/AG2 Confocal Imaging System.

5.3 Results

5.3.1 *Determination of optimal culture conditions for CLL-cell survival studies*

CLL cells are fragile and extremely sensitive to their culture environment, such that subtle changes in culture conditions can affect the rate of cell death. Thus, factors such as cell adhesion, cell density, and the presence of BSA or FCS are known to affect CLL-cell survival in culture (Pettitt et al, 2001; Moran et al, 2002). Additionally, since CLL-cell viability was measured using flow cytometry, prevention of cell damage during removal from cultures was essential. Therefore, tissue culture plastics used in the survival studies were coated with poly-HEMA to prevent adhesion of the cells to the plastic surface during culture.

Work done by others has shown that cell density is also a critical factor for CLL-cell survival in culture systems. Pettitt et al (2001) found that, under non-adherent conditions, cells cultured at a relatively high density that promotes cell-cell contact survived better than cells cultured at a relatively low density. Additionally, since the presence of FCS in culture media does not prevent CLL-cell death to any greater extent than highly purified BSA (Moran et al, 2002), the latter was employed instead of FCS to avoid addition to the cultures of any growth factors that may be present in the serum. Therefore, initial aim was to determine the optimal cell density and BSA concentration required for the measurement of CLL-cell survival.

To determine the optimal cell density, CLL cells were cultured for up to 4 days in RPMI medium without supplementary BSA at a density of $5x10^4$,

$2.5x10^5$ or $1x10^6$ cells per well. It was found that cells incubated at the highest density were less sensitive to the absence of BSA in the medium than cells at lower densities (Fig 5.1A). In the highest density cultures there was a decrease in cell viability of about 20-30% during the first 24 hours culture period and then the viability remained relatively constant for the remainder of the incubation. Cells cultured at the intermediate density survived less well than those at high density, while those incubated at low density did not survive beyond day 1 of culture. Figure 5.1B shows that the presence of BSA in the medium can compensate for the effects of cell density on cell survival providing that the density of the cells is relatively high ($5x10^4$ to $1x10^6$). However, there is a threshold of cell density (~$1.5x10^4$ cells/well) below which BSA can no longer rescue the cells, even at a concentration approaching that found in serum (Figure 5.1C). Lastly, the effect of albumin concentration on cell survival at an intermediate density ($3.3x10^5$ cells/well), is illustrated in Figure 5.1D. At this density a uniform monolayer of cells that do not overlay each other is formed in 96-well plates as observed by light microscopy. Clearly, in these cultures 1% albumin almost completely inhibited cell death during 3 days of culture while the cell viability continued to decrease if the culture medium containing only 0.1% BSA.

Therefore, in studies of factors involved in *in vitro* cell survival, the optimal culture conditions would be those in which the desired effect is most clearly demonstrable. Thus, to demonstrate the rescue-effect of VEGF, this would be best demonstrated under culture conditions in which the cells show a moderate propensity to die. Consequently, in all future experiments

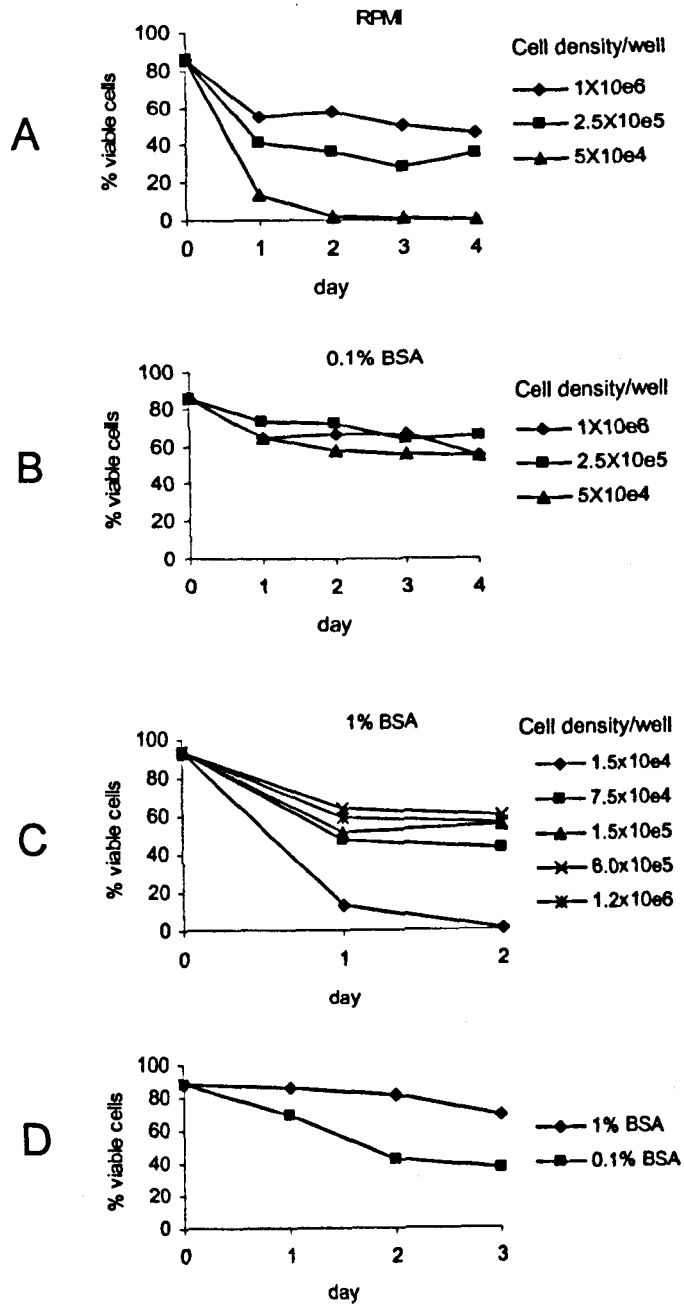


Figure 5.1

Determination of CLL-cell-culture conditions

CLL cells were cultured at cell densities ranging from $1.5x10^4$ to $1.2x10^6$/well for up to 4 days in (A) RPMI medium alone (B) RPMI supplemented with either 0.1% BSA (C) or 1% BSA. (D) Survival of CLL cells cultured at a single density ($3.3x10^5$/well) in RPMI supplemented with 0.1% and 1% BSA. In each instance, the cells were added to poly-HEMA-coated 96-well plates and incubated at 37°C in 5% CO_2. Cell viability at the given intervals was determined by $DiOC_6$/PI staining for flow cytometry.

investigating the effects of VEGF on non-induced CLL-cell death, cells were suspended in RPMI medium supplemented with 0.1% BSA and seeded at a density of $3.3x10^5$ cells per well in poly-HEMA coated flat-bottomed 96-well plates. In all cases, the final volume of the sample was 150μl per well. On the other hand, to assess of the contribution of VEGF to rescue of CLL cells from drug-induced cell death, it would be necessary to use conditions under which cells do not readily die in the absence of the drug. In these experiments, the conditions were identical to those above, with the exception that the BSA concentration was increased to 1% to reduce cell death as illustrated in Figure 5.1D.

5.3.2 Exogenous VEGF and CLL-cell survival

5.3.2.1 *The effect of exogenous VEGF on CLL-cell survival*

Preliminary experiments (not shown) using cells from a small sample of CLL cases indicated that VEGF added to cell cultures does not have a strong survival-enhancing effect on CLL cells. Therefore, in the next phase of the study, a larger sample of CLL cases was used (n=18) to determine the effect of exogenous VEGF on CLL cells undergoing spontaneous cell death. When the cells were incubated for 3 days or more in RPMI supplemented with 0.1% BSA with or without 100 ng/ml VEGF, it was found that in some cases VEGF had a small but consistent pro-survival effect on some of the CLL clones used, particularly at the earliest time points. However, when the viability of all the cases tested was considered (n=18), there was no significant difference between VEGF-treated cells and untreated control cells (figure 5.2A). Characteristically, the decrease in cell viability during

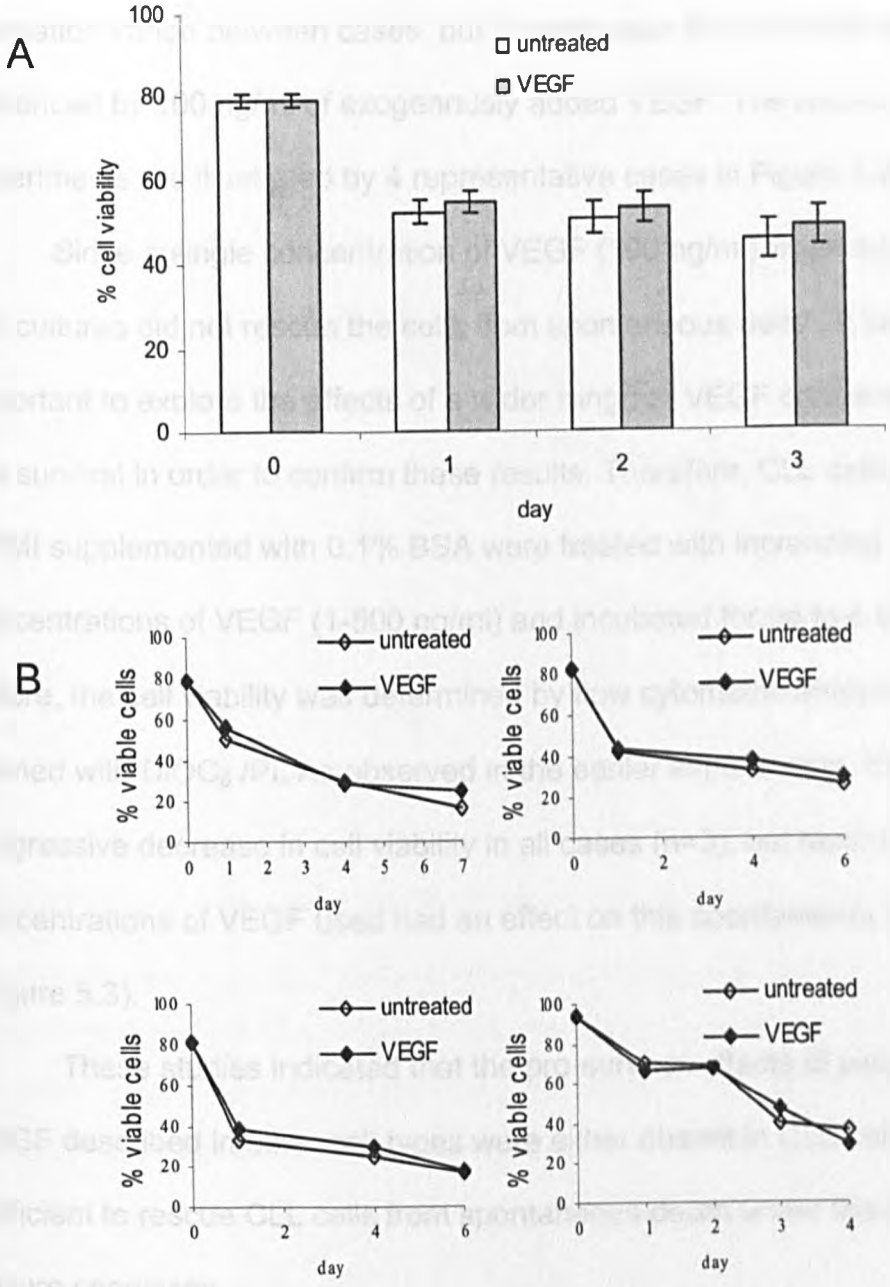


Figure 5.2

The effects of exogenous VEGF on CLL-cell viability

CLL cells ($3.3x10^5$/well) were incubated at 37°C in poly-HEMA-coated 96-well plates in RPMI medium supplemented with 0.1% BSA for up to 6 days with or without VEGF. In each instance, the viability of the harvested cells was determined as in Figure 5.1. (A) Mean viability ± SEM of cells from 18 CLL cases incubated with 100 ng/ml VEGF for up to 3 days. (B) Graphs showing viability of cells from 4 representative CLL cases at different incubation times.

incubation varied between cases, but in each case this decrease was not influenced by 100 ng/ml of exogenously added VEGF. The results of these experiments are illustrated by 4 representative cases in Figure 5.2B.

Since a single concentration of VEGF (100 ng/ml) when added to CLL-cell cultures did not rescue the cells from spontaneous death, it seemed important to explore the effects of a wider range of VEGF concentrations on cell survival in order to confirm these results. Therefore, CLL cells cultured in RPMI supplemented with 0.1% BSA were treated with increasing concentrations of VEGF (1-500 ng/ml) and incubated for up to 4 days. As before, the cell viability was determined by flow cytometric analysis of cells stained with $DiOC_6$ /PI. As observed in the earlier experiments, there was a progressive decrease in cell viability in all cases (n=3), but none of the concentrations of VEGF used had an effect on this spontaneous cell death (Figure 5.3).

These studies indicated that the pro-survival effects of exogenous VEGF described in other cell-types were either absent in CLL cells or not sufficient to rescue CLL cells from spontaneous death under the given *in vitro* culture conditions.

Consequently, a concentration of 100 ng/ml VEGF was selected for all further survival experiments using exogenous VEGF, since at this concentration VEGF had induced an increase in expression of Hsp90 in CLL cells (Chapter 4) and had been shown to induce proliferation of endothelial cells (Chapter 3).

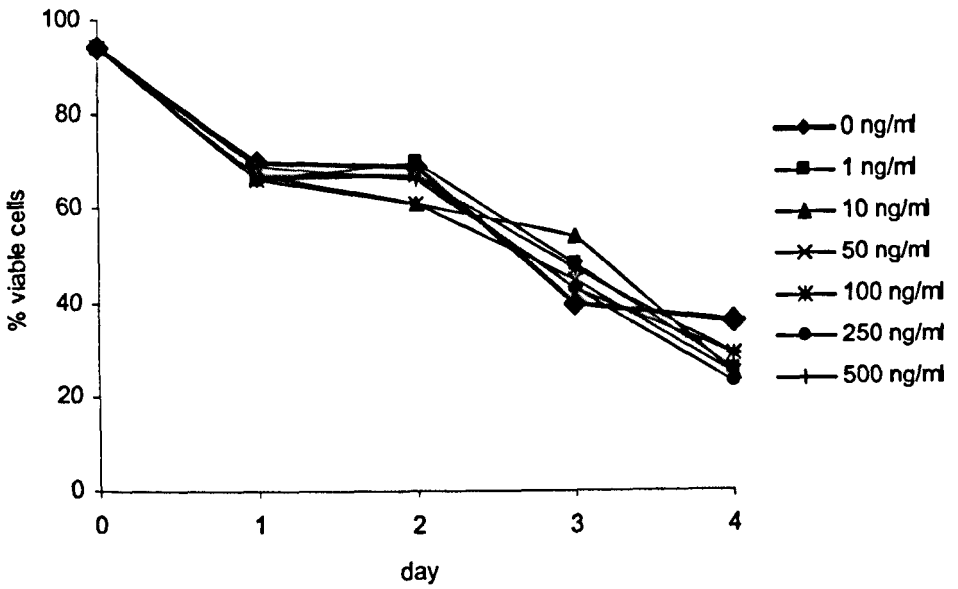


Figure 5.3

The effects of a range of VEGF concentrations on CLL-cell viability

CLL cells were incubated in the presence of a range of VEGF concentrations (1-500 ng/ml) in RPMI medium supplemented with 0.1% BSA for up to 4 days. Harvested cells were stained with $DiOC_6$/PI for determination of viability by flow cytometry.

5.3.2.2 *Effects of VEGF on CLL-cell death induced by different pro-apoptotic treatments*

After demonstrating that exogenous VEGF has no effect on spontaneous death of CLL cells in culture, it was decided to determine whether recombinant VEGF can rescue CLL cells from drug- or IR-induced killing. In these experiments, it seemed important to use culture conditions under which untreated cells remained viable throughout the incubation period so that changes in cell viability could not be attributed to the culture conditions themselves. Therefore CLL cells were suspended in RPMI medium containing 1% BSA, since this concentration of BSA was shown to reduce spontaneous CLL-cell death in culture (Figure 5.1D). In this series of experiments, CLL-cell death was induced using fludarabine, ionising radiation (IR) or geldanamycin (GA) in the presence or absence of VEGF.

5.3.2.3 *The effects of exogenous VEGF on fludarabine-induced cell death*

Previous work has shown that induction of CLL-cell death by fludarabine can be abrogated by the angiogenic growth factor bFGF (Menzel et al, 1996). Therefore, it seemed important to determine whether VEGF could also play a role in the cytoprotection of CLL cells from fludarabine-induced killing. Fludarabine (2 μM) was added to CLL cells ($3.3x10^5$ cells/ well) prior to incubation in RPMI supplemented with 1% BSA for up to 6 days. In some instances, 100ng/ml VEGF was also added to the cultures and where both fludarabine and VEGF were used, they were added to the cells at

the same time. Figure 5.4 shows that fludarabine induces CLL-cell death in culture and, in keeping with observations regarding spontaneous CLL-cell death, the rate of cell death due to fludarabine treatment varied between cases. It was also clear that, in each CLL case studied (n=3), 100 ng/ml VEGF did not protect the cells from fludarabine-induced killing (Figure 5.4).

Because it was possible that exogenous VEGF did not provide cytoprotection to CLL cells due to the use of an unsuitable concentration of VEGF, fludarabine (2 μM) treated cells were incubated with a wide range of VEGF concentrations (0.1-500ng/ml) for up to 3 days. Figure 5.5 shows that at each of the concentrations used, VEGF did not protect the CLL cells from fludarabine-induced killing. It was therefore concluded that exogenous VEGF alone cannot protect CLL cells from killing by this drug.

5.3.2.4 *The effects of exogenous VEGF on IR-induced cell death*

In some cell types, the presence of VEGF can protect the cells against IR-induced cell death (Katoh et al, 1995). Exposure to IR induces cell death by a different mechanism to that employed by fludarabine. It is well established that IR induces cell apoptosis by p53-dependent pathway, whereas fludarabine-induced killing depends only partly on functional p53 (Pettitt AR, 2003). Therefore, it seemed fitting to also investigate the effects of exogenous VEGF on IR-induced killing of CLL cells. CLL cells, suspended in RPMI containing 1% BSA, were exposed to 5 Gy ionising radiation prior to incubation with or without 100 ng/ml VEGF. The effect of IR on cell viability was assessed daily for up to 6 days. This confirmed that IR induces death of

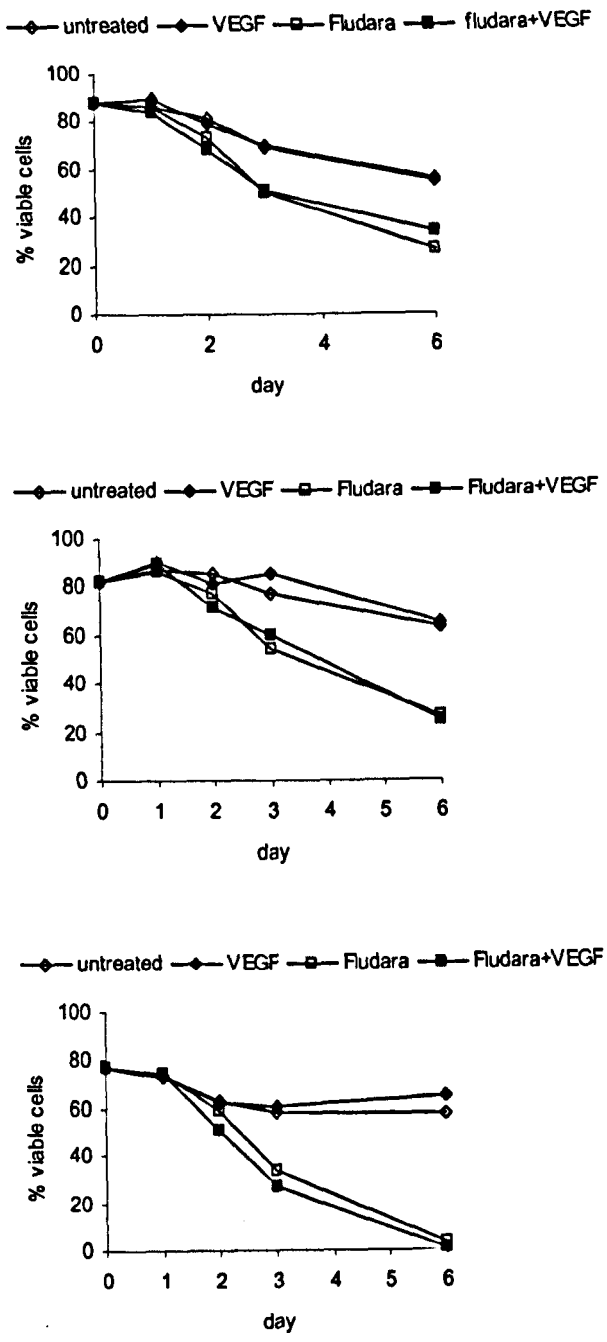


Figure 5.4

The effects of VEGF on fludarabine-induced CLL-cell death

The cells from 3 CLL cases suspended in RPMI medium containing 1% BSA were placed in poly-HEMA-coated 96-well plates ($3.3x10^5$ cells/well) and incubated in 5% CO_2 at 37°C for up to 6 days. VEGF (100 ng/ml), fludarabine (Fludara, 2 μM) or both in combination were added to the cells immediately prior to incubation. Cell viability was determined as in Figure 5.1.

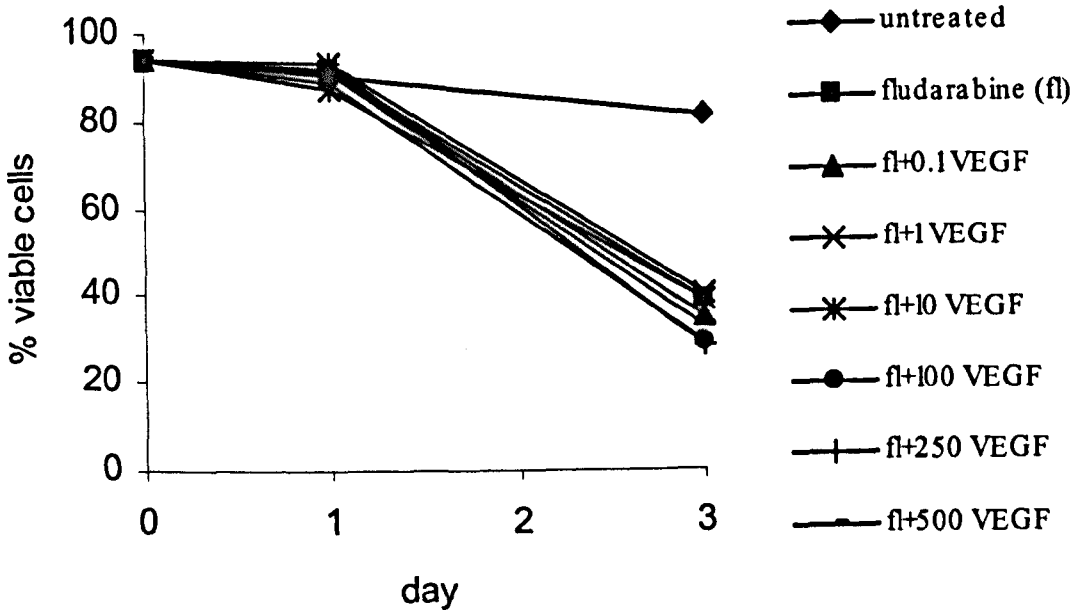


Figure 5.5

The effects of a range of VEGF concentrations on fludarabine-induced CLL-cell death

CLL cells suspended in RPMI containing 1% BSA were placed in a 96-well plate ($3.3x10^5$ cells/well) in the presence of 2 μM fludarabine and VEGF (0.1-500 ng/ml). Following incubation at 37°C in 5% CO_2 for 3 days, the harvested cells were stained with $DiOC_6$/PI to determine viability. The graph shows the results from a single experiment.

CLL cells, but the presence of exogenous VEGF did not prevent IR induced cell killing (Figure 5.6).

It was therefore concluded that, in contrast to observations made with some other cell-types, exogenous VEGF alone cannot protect CLL cells from IR-induced death.

5.3.2.5 *The effects of exogenous VEGF on geldanamycin-induced CLL-cell death*

Geldanamycin is a specific inhibitor of the molecular chaperone heat-shock protein 90 (Hsp90). In myeloid leukaemias and myeloid cell lines, incubation of cells with GA induces cell death, an effect that is abrogated by incubation with VEGF (Dias et al 2002). Therefore, since experiments in Chapter 4 showed that VEGF stimulates an increase in expression of Hsp90 in CLL cells, it seemed important to establish whether GA inhibition of Hsp90 in CLL cells would induce cell death, and whether CLL cells could be rescued from such killing by VEGF.

Since GA had not previously been used in this Department, initial experiments were carried out to establish the optimal concentration of GA for induction of CLL-cell death. Cells from three CLL cases were incubated in RPMI containing 1% BSA, with 0.02, 0.2 or 2μM GA for up to 3 days. This showed that GA induces CLL-cell death at concentrations of 0.2 and 2μM but not at 0.02μM (Figure 5.7). In one case, the increase in cell death was observed at day 1, while in the remaining 2 cases GA did not affect CLL cell survival until day 2. However, once the viability began to fall, it continued to decrease at a relatively constant rate throughout the period of observation.

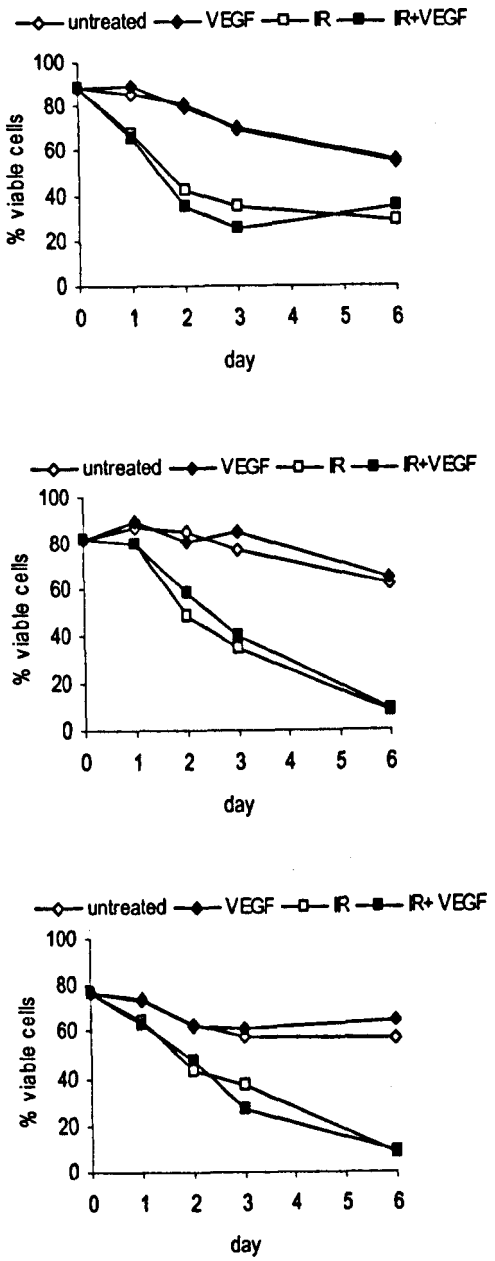


Figure 5.6

The effect of VEGF on IR-induced CLL-cell death

CLL cells suspended in RPMI medium containing 1% BSA were exposed to 5 Gy ionising radiation and then transferred to poly-HEMA-coated 96-well plates ($3.3x10^5$ cells/well). VEGF (100 ng/ml) or control medium was added to the cells prior to incubation at 37°C in 5% CO_2 for up to 6 days. The viability of harvested cells was assessed by $DiOC_6$/PI staining and flow cytometry. The graphs show the results of a single experiment with cells from 3 CLL cases.

For further experiments 0.2 μM of GA was considered the most suitable concentration, since increasing the concentration to 2μM had no greater effect (Figure 5.7).

To determine the effects of VEGF on GA-induced killing, cells from 6 CLL cases were suspended in RPMI containing 1% BSA and exposed to 0.2 μM GA with or without 100ng/ml VEGF for up to 3 days in culture. Harvested cells were stained with $DiOC_6$/PI to determine their viability. In each of the six cases studied, GA induced significant CLL-cell death by day 2 that continued to increase up to the end of the culture period. Furthermore, it was found that the presence of VEGF in culture did not prevent this killing. Representative survival plots for three CLL cases are shown in Figure 5.8. These results contrast with the findings of Dias et al (2002), who found that in primary myeloid cells and cell lines the presence of VEGF in culture produced cytoprotection from the effects of GA.

It was therefore concluded that, as in the case of fludarabine- and IR-induced cell death, exogenous VEGF alone does not offer primary CLL cells protection from death that has been induced by the inhibition of Hsp90 with the specific inhibitor geldanamycin.

5.3.3 Endogenous VEGF and CLL-cell survival

In previous Chapters of this thesis, it was established that CLL cells secrete VEGF and express VEGF receptors. Therefore, to explore the cell-rescuing effect of autocrine VEGF, endogenous VEGF was either neutralised

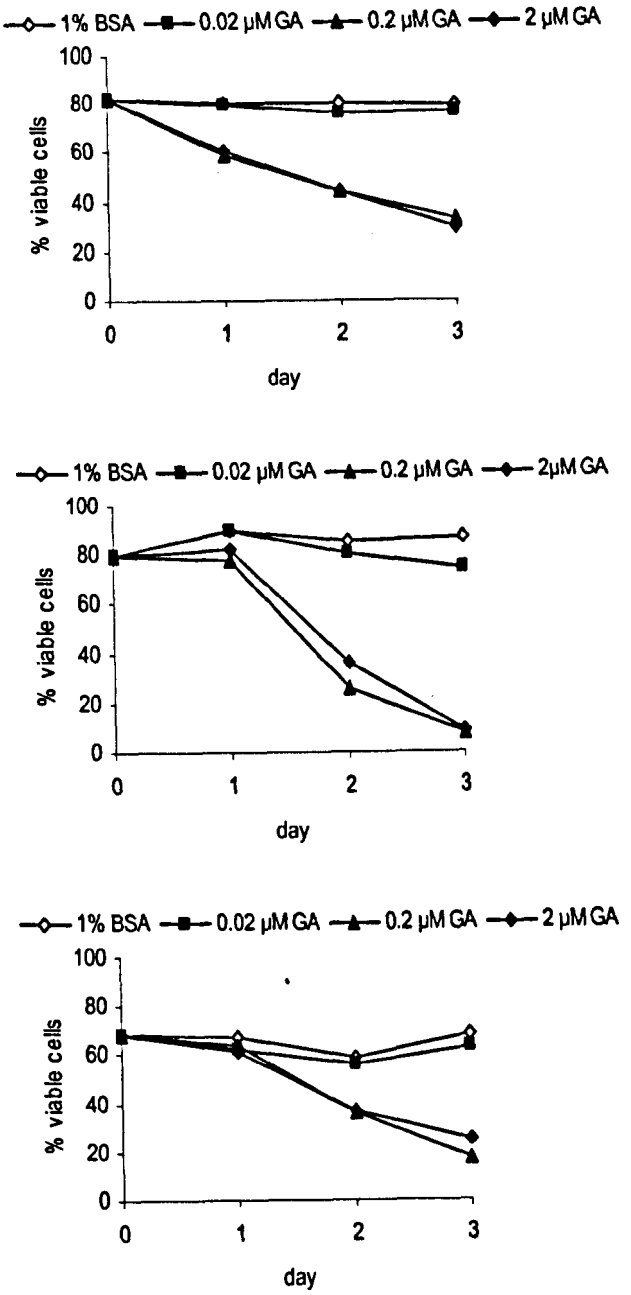


Figure 5.7

The effects of geldanamycin on CLL-cell survival

CLL cells suspended in RPMI containing 1% BSA were incubated in poly-HEMA-coated 96-well plates ($3.3x10^5$ cells/well) at 37°C and 5% CO_2 for up to 3 days in the presence of 0.02, 0.2 or 2 µM geldanamycin. Harvested cells were stained with $DiOC_6$/PI to determine cell viability. The Figure shows the response to GA of cells from 3 CLL cases.

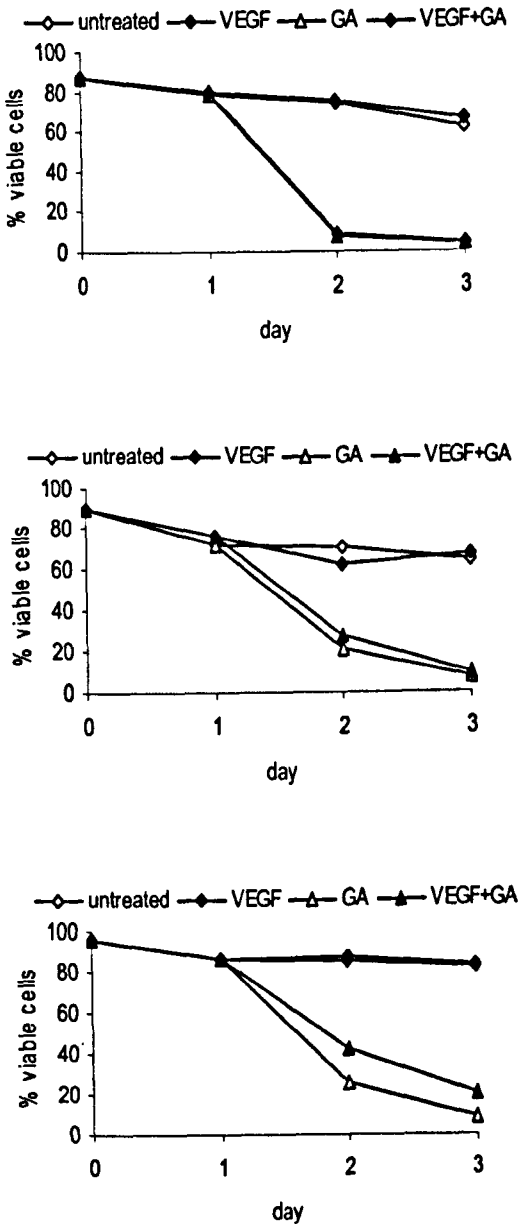


Figure 5.8

The effect of VEGF on GA-induced CLL-cell death

CLL cells ($3.3x10^5$ cells/well) in RPMI containing 1% BSA were cultured in poly-HEMA-coated 96-well plates at 37°C and 5% CO_2. Immediately prior to incubation, VEGF (100 ng/ml) was added to the cells. After 90 minutes, GA (0.2 µM) was added and the cells further incubated for up to 3 days. Cell viability was determined by staining with $DiOC_6$/PI and analysis by flow cytometry. The graphs show 3 representative results of a single experiment involving 6 CLL cases.

or VEGF receptors were inhibited prior to the measurement of cell survival in the absence of exogenous VEGF.

In all experiments to determine the effects of autocrine VEGF on CLL cell survival, the cells were cultured in RPMI containing 0.1% BSA. Since CLL cells undergo gradual, spontaneous death at this BSA concentration (Figure 5.1D), it was presumed that any effects on cell viability of blocking VEGF or its receptors would be demonstrable under these conditions.

5.3.3.1 *The effect of neutralising secreted VEGF on CLL-cell survival*

To determine the effects of neutralising secreted VEGF on CLL-cell survival, monoclonal anti-VEGF antibody or mouse IgG_{2b} control (both 1μg/ml) was added to CLL cells ($3.3x10^5$ /well) cultured in 0.1% BSA in RPMI for up to 6 days (final culture volume was 150μl). At appropriate intervals, cells were harvested, stained with $DiOC_6$/PI and analysed by flow cytometry to determine cell viability. Figure 5.9 shows that the viability of cells incubated for 4 days with VEGF-neutralising antibody was not significantly different to that of control cells.

To confirm these results, high affinity, recombinant human soluble chimaeric receptors to both VEGFR1 and VEGFR2 (sVEGFR1/Fc and sVEGFR2/Fc) were used to neutralise secreted VEGF. CLL cells were cultured for up to 5 days in the presence of excess sVEGFR1/Fc (300ng/ml) or sVEGFR2/Fc (200ng/ml) singly and in combination. The results of these experiments showed that in each CLL case studied (n=3), neither sVEGFR1/Fc nor sVEGFR2/Fc, when used alone or in combination, had any effect on CLL-cell survival (Figure 5.10).

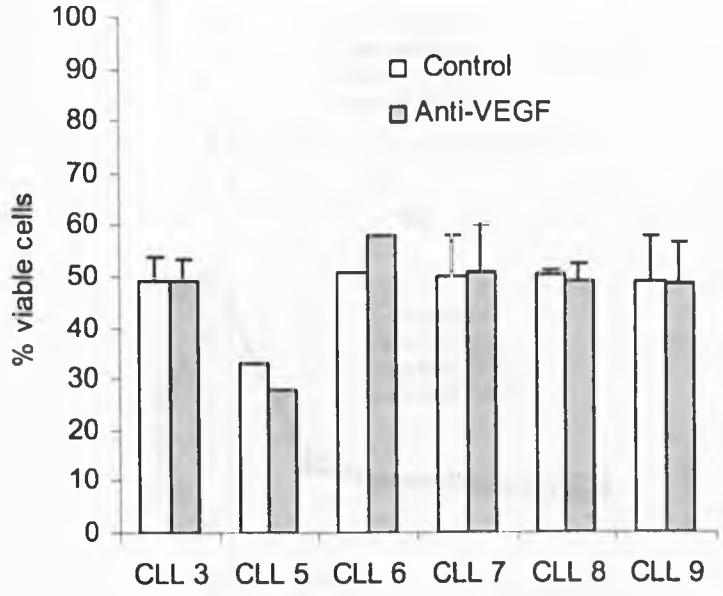


Figure 5.9

Effect of secreted endogenous VEGF on CLL-cell survival.

CLL cells were cultured in RPMI medium containing 0.1% and the viability of harvested cells measured as in Figure 5.1. Where no error bars are shown, the histograms are the means of two independent measurements; all the other data were obtained from three or more repeated measurements. Cells from six patients were cultured for up to 6 days in the presence of monoclonal anti-VEGF-neutralising antibody (1 μg/ml) to neutralise secreted VEGF. Mouse IgG_{2b} served as a control. Histograms show mean values ± SD for cells harvested at day 4 in each case. The differences between the viability of control versus anti-VEGF-treated cells were not significant. Similar results were obtained after 6 days incubation.

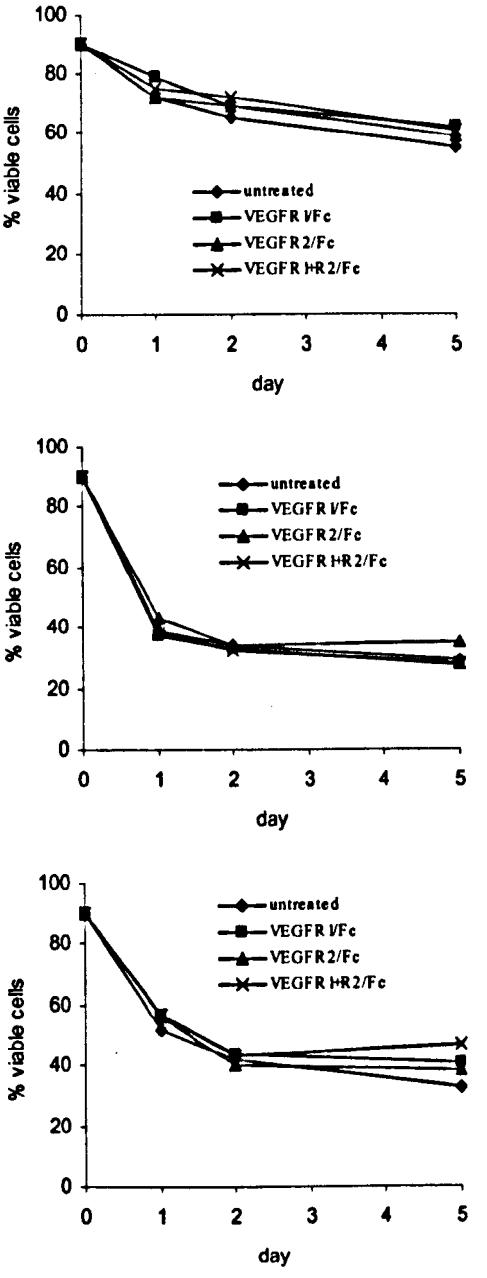


Figure 5.10

The effects on CLL-cell survival of neutralising secreted VEGF with soluble VEGF receptors

CLL cells ($3.3x10^5$/well) were cultured as in Figure 5.2 for up to 5 days. Secreted VEGF was neutralised using excess sVEGFR1/Fc (300 ng/ml) or sVEGFR2/Fc (200 ng/ml), or both in combination. The viability of harvested cells was determined by flow cytometry as previously. The Figure shows the survival of cells from 3 CLL cases studied in a single experiment.

5.3.3.2 *The effects of blocking endogenous VEGF binding to VEGF receptors*

In this series of experiments, goat polyclonal anti-VEGFR1 and anti-VEGFR2 antibodies that block VEGF interaction with these receptors were used. These antibodies or goat IgG control (all used at a concentration of 1μg/ml) were added to CLL cells cultured for up to 4 days in RPMI containing 0.1% BSA. Cell viability was determined by FACS analysis of DiOC6/PI-stained cells that had been harvested from cultures at various time points. Figure 5.11A shows representative results of experiments in which CLL cells were incubated with VEGF receptor blocking. Neither anti-VEGFR1 nor anti-VEGFR2 affected the survival of the CLL-cells over this incubation period. Figure 5.11B shows that when all CLL cases studied were considered (n=5), the receptor-blocking antibodies did not affect CLL cell survival. Similarly, in a separate experiment, combined use of anti-VEGFR1 and anti-VEGFR2 also had no effect on the survival of CLL cells cultured for up to 6 days (data not shown).

Together, the results of the above experiments in which attempts were made to neutralise either VEGF or its receptor(s) indicated that secreted VEGF may not affect CLL cell survival. This observation is in agreement with recent studies showing that neutralising secreted VEGF with antibodies had little or no effect on the survival of myeloid cells (Santos and Dias, 2004) or haemopoeitic stem cells (Gerber et al, 2002) although in both studies, pharmacological inhibition of VEGF receptors decreased the viability of these cells. This led to the proposal that the pro-survival effect of VEGF involves

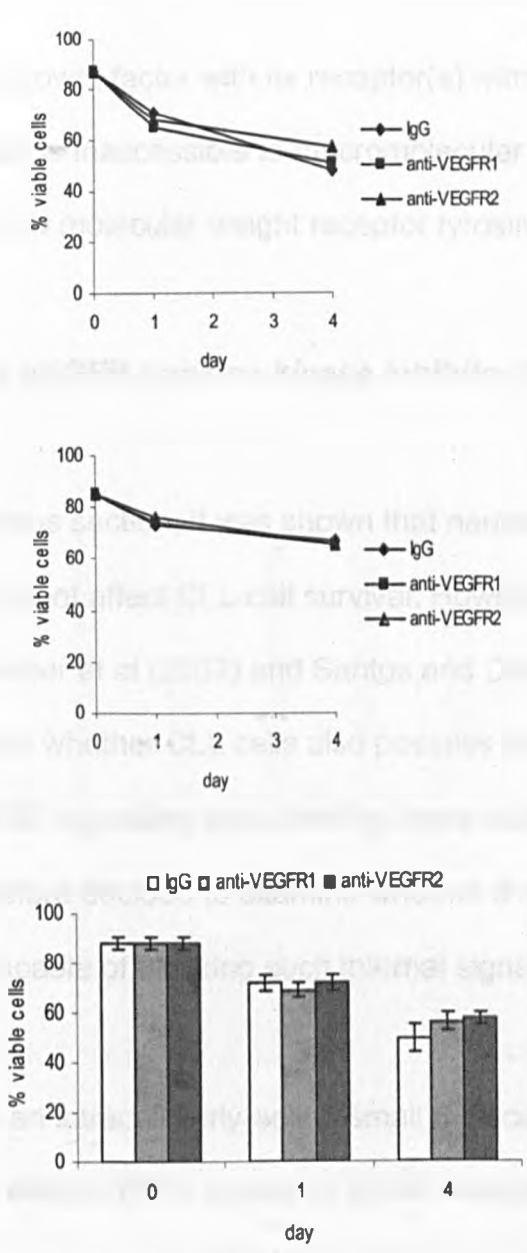


Figure 5.11

The effects on CLL-cell viability of VEGFR-blocking antibodies

CLL cells in RPMI supplemented with 0.1% BSA in poly-HEMA coated 96-well plates were incubated for up to 4 days with goat polyclonal antibodies to either VEGFR1 or VEGFR2. The antibodies were added to the cells prior to incubation and the viability of harvested cells determined at given time points as before. (A) shows the results obtained with cells from two representative CLL cases, while (B) shows the mean viability of cells from 5 CLL cases ± SEM.

interaction of this growth factor with its receptor(s) within an intracellular compartment which is inaccessible to macromolecular neutralising agents, but accessible to low molecular weight receptor tyrosine-kinase inhibitors.

5.3.3.3 *Effects of VEGFR tyrosine-kinase inhibitor SU5416 on CLL-cell survival*

In the previous section, it was shown that neutralising the effects of secreted VEGF did not affect CLL-cell survival. However, following the publications of Gerber et al (2002) and Santos and Dias (2004) it seemed important to assess whether CLL cells also possess the internal autocrine mechanism of VEGF signalling described by these authors in other cell-types. It was therefore decided to examine whether a receptor tyrosine-kinase inhibitor capable of blocking such internal signalling would affect CLL-cell survival.

SU5416 is an intracellularly-acting small molecule that inhibits the receptor tyrosine kinase (RTK) activity of VEGF receptors 1 and 2 (Itokawa et al, 2002). In previous studies, SU5416 concentrations from 0.5-10 μM were shown to effectively inhibit VEGF receptor signalling in other cell types (Itokawa et al, 2002; Smolich et al, 2001). Therefore, CLL cells were cultured in RPMI containing 0.1% BSA, for up to 6 days in the presence of 10 μM SU5416. DMSO alone at 0.2% (the concentration equivalent to that in SU5416 dilutions) was employed as the control. Figure 5.12 shows the viability of CLL cells harvested after four days from control cultures and from cultures treated with SU5416. In all cases, treatment with SU5416 reduced cell viability. In four of the six cases, the experiments were repeated on three

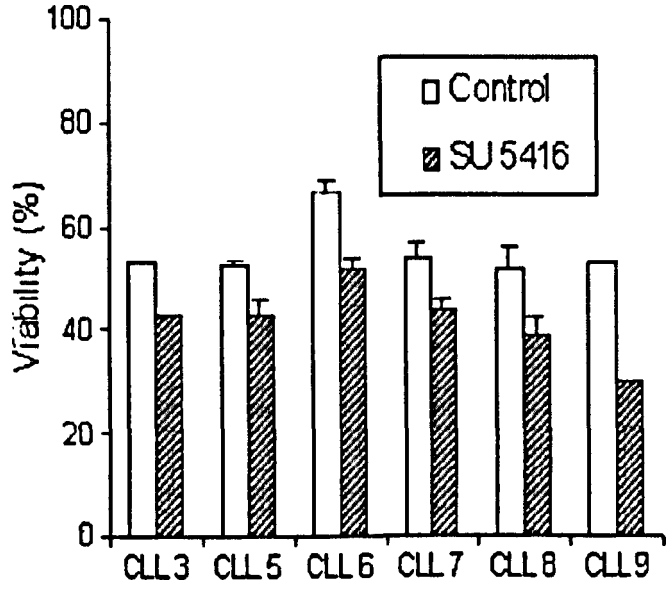


Figure 5.12

Effect of SU5416 on CLL-cell survival

CLL cells were cultured in RPMI medium containing 0.1% BSA in poly-HEMA-coated wells ($3.3x10^5$ /well) and the viability of harvested cells was determined by $DiOC_6$/PI staining and flow cytometry as previously. Where no error bars are shown, the histograms are the means of two independent measurements; all the other data were obtained from three or more repeated measurements. CLL cells from six patients were cultured with or without the receptor-tyrosine kinase inhibitor SU5416 (10 μM) for up to 6 days. Histograms represent the mean values ± SD for cells harvested at day 4. The viabilities of the control and SU5416-treated cells were significantly different ($P<0.01$). Similar results were obtained at day 6.

or more separate occasions showing that this reduction was highly reproducible and significant ($p<0.001$) when compared to control cells.

Thus, the reduction of cell viability in the presence of SU5416, together with the absence of such a response to agents that block secreted VEGF, indicates the presence in CLL cells of internal signalling by autocrine VEGF that may be important for the survival of these cells.

5.3.3.4 *The role of autocrine VEGF in the cytoprotection of CLL cells by CD154*

In Chapter 2, it was shown that CLL cells produce VEGF and that this production is increased upon ligation of CD40 by CD154. Since CD154 is an important regulator of CLL-cell survival, and since VEGF is known to promote survival in other cell types, the possibility that autocrine VEGF might participate in the cytoprotection of CLL cells stimulated with CD154 was next examined.

The cytoprotective effect of CD154 on CLL cells is known to vary between CLL cases; some cases exhibit enhanced survival while a proportion shows no response (Schattner ER, 2000). Therefore, in initial studies, CLL cases that demonstrated enhanced cell survival when cultured in the presence of CD154 were identified. In accordance with previous studies, it was found that only a proportion of the CLL cases (12/16) exhibited a clear pro-survival response to CD40 ligation (data not shown). Therefore, in subsequent experiments within this section, CLL cases were selected from this sub-group of CD154-responders.

The approach taken to determine whether VEGF was involved in the pro-survival cell response to CD154 was the same as that taken when investigating the effects of autocrine VEGF earlier in this thesis. Cells from 10 CD154-responding CLL cases were cultured for up to 6 days in RPMI containing 0.1% BSA with or without CD154, and in the presence or absence of either neutralising anti-VEGF antibody (1 μg/ml) or the VEGFR inhibitor SU5416 (10 μM). Control antibody or DMSO were used as required. Figure 5.13 shows that CD154 alone enhanced CLL-cell survival in culture, while the presence of anti-VEGF or SU5416 reduced the viability of CD154-treated cells near to the levels observed in cultures of untreated cells. These results strongly suggest that the anti-apoptotic effect of CD154 is at least in part mediated by VEGF.

In earlier experiments, the use of the RTK inhibitor SU5416 demonstrated the presence of weak autocrine, VEGFR-mediated, pro-survival signalling in CLL cells. However, neither secreted VEGF nor exogenous VEGF could protect CLL cells from either spontaneous or induced cell death in culture. In contrast, the pro-survival effects of CD154 were shown to be inhibited by anti-VEGF antibody and therefore mediated by secreted VEGF. Since the effects of secreted VEGF also depend on VEGF-receptor activation, the overall effects of both internal and external VEGF are inhibited by SU5416. Importantly, these experiments show that CD154 mediated-cytoprotection depends almost exclusively on secretion and action of VEGF. Furthermore, in the absence of CD154, VEGF alone has only minimal cytoprotective effects and acts through internal signalling. Therefore, it seems that for full CLL-cell cytoprotection to occur, cooperation of signals

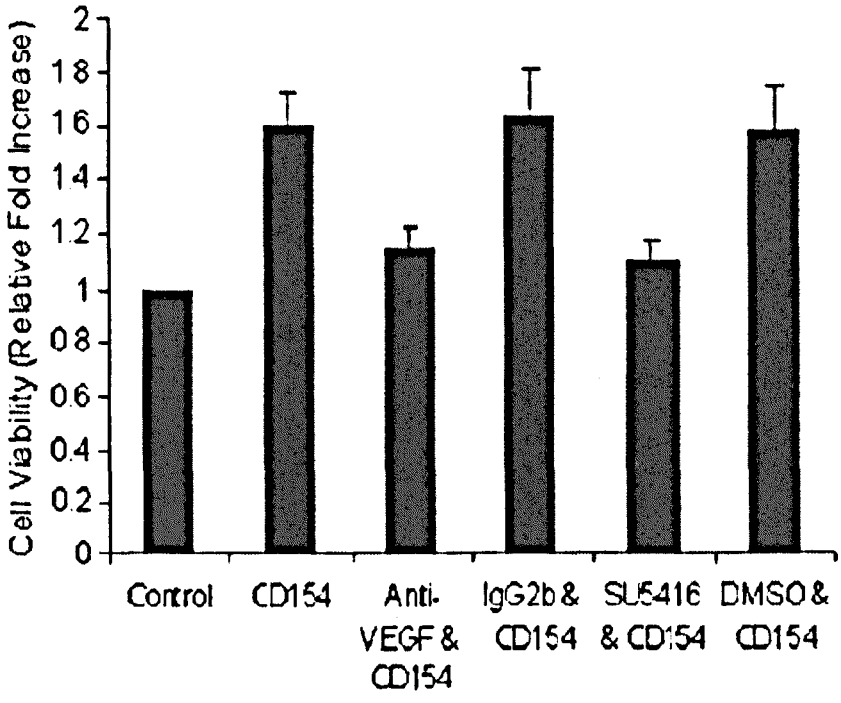


Figure 5.13

Role of VEGF in the anti-apoptotic activity of CD154.

CLL cells were incubated with either the anti- VEGF monoclonal antibody (1µg/ml), IgG_{2b} as isotype control, SU5416 (10 µM) or 0.2% DMSO (diluent for the SU5416) for 1 hour prior to the addition of CD154 (200 ng/ml). Cell viability was measured at day 4 of culture using $DiOC_6$/PI staining for FACS analysis. Owing to the considerable variation in the viability of the control at day 4, the data are shown as values relative to the control. The Figure shows that the increase in cell viability induced by CD154 was greatly reduced by anti-VEGF or SU5416 ($P<0.05$). The results represent the mean ± SD of eight experiments involving eight different CLL-cell clones.

derived from CD40 and VEGF receptors is required. Previous studies have shown that NF-κB signalling may be a key point of convergence of the signals originating from these receptors (Furman et al, 2000; Karin and Lin, 2002; Kim et al, 2001). Therefore preliminary studies of the role of NF-κB in CLL-cell cytoprotection by dual signalling of VEGFR and CD40 were carried out.

5.3.3.5 *NF-κB activation is involved in the production and action of VEGF during CD154-dependent CLL-cell rescue*

The anti-apoptotic activity of CD154 in CLL cells and other cell types is known to be mediated by NF-κB. Therefore, the next series of experiments aimed to determine whether NF-κB is involved in stimulating VEGF production in response to CD154 ligation, and whether VEGF secreted in response to NF-κB activation contributes to the pro-survival effect of CD154 via NF-κB activity.

The NF-κB/Rel family consists of five subunits, that is, p65/RelA, p50, p52, c-Rel and RelB. The NF-κB components known to be activated in CD154-stimulated cells are p65, p50 and c-Rel (Furman et al, 2000). Therefore, in the present study, nuclear translocation of p65 was measured to assess the activation by CD154 of NF-κB in CLL cells. To inhibit NF-κB, SN50 peptide, a cell permeable inhibitor of nuclear translocation of NF-κB, was employed. CLL cells were cultured in the presence or absence of CD154 (200 ng/ml), either with or without SN50 peptide, anti-VEGF neutralising antibody (1 μg/ml), or SU5416 (10 μM) for 24 hours, all with appropriate controls. In cultures in which NF-κB involvement in VEGF production was

studied, cells were treated with SN50 peptide or SN50M inactive peptide control. Figure 5.14 shows that in all three cases studied, SN50 peptide reduced CD154-stimulated VEGF secretion indicating involvement of NF-κB in the stimulation of VEGF production. In studies investigating the contribution of VEGF to NF-kB activation, nuclear extracts were prepared from harvested cells and the concentration of p65 in these extracts estimated using an ELISA kit. Figure 5.15A shows the presence of constitutive activation of NF-κB in each of the cases tested (n=3). Addition of CD154 to the cultures significantly increased the level of activation ($p<0.05$), an increase that was reduced by incubation with anti-VEGF ($p<0.05$) and completely abolished with SU5416 ($p<0.05$), demonstrating a role for VEGF in CD154-activation of NF-κB.

To confirm these results, the cells were also analysed for nuclear translocation of NF-κB using confocal microscopy. Cytospins were prepared from untreated CLL cells or cells treated with CD154 alone, with CD154 plus SU5416, or with CD154 plus anti-VEGF antibody for 24 hours, and then stained using anti-p65 antibody (Figure 5.15B). In untreated cells, p65 positivity was localised mainly outside the nucleus (I). In contrast, in cells cultured with CD154, p65 was found mainly in the nucleus (II). This nuclear translocation was virtually eradicated by SU5416 (III) or anti-VEGF (IV). These results confirmed that VEGF signalling is involved in the nuclear transport of NF-κB in CD154-stimulated CLL cells.

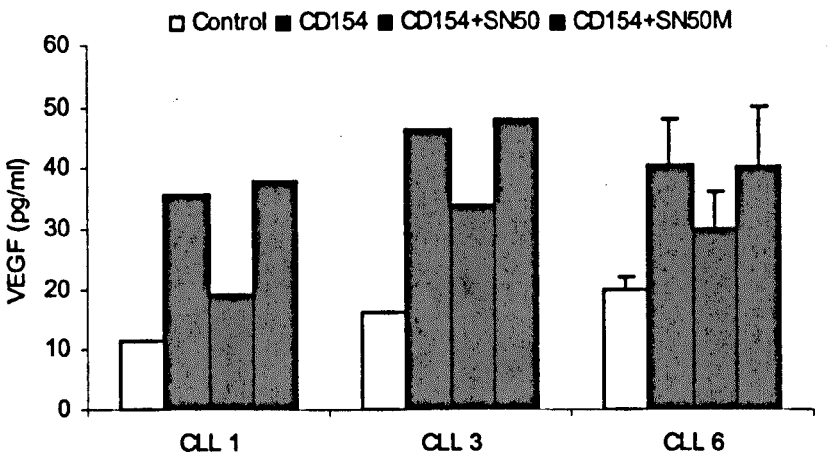


Figure 5.14

NF-κB is involved in CD154-induced VEGF production.

CLL cells were pre-incubated for 1 hour with the NF-κB nuclear translocation inhibitor SN50 (18 μM) or with its inactive analogue SN50M (18 μM) and then stimulated with CD154 (200 ng/mL) plus enhancer (1 μg/ml) for 24 hours. The data are from 3 representative cases. SN50 significantly inhibited CD154-induced VEGF production ($P<0.05$), while the control peptide SN50M had no effect. In cases 1 and 3 the histograms represent the mean of duplicate determinations. For case 6, the histogram represents the mean ± SD of 3 separate experiments, each performed in duplicate. Similar results were obtained with an additional 4 cases.

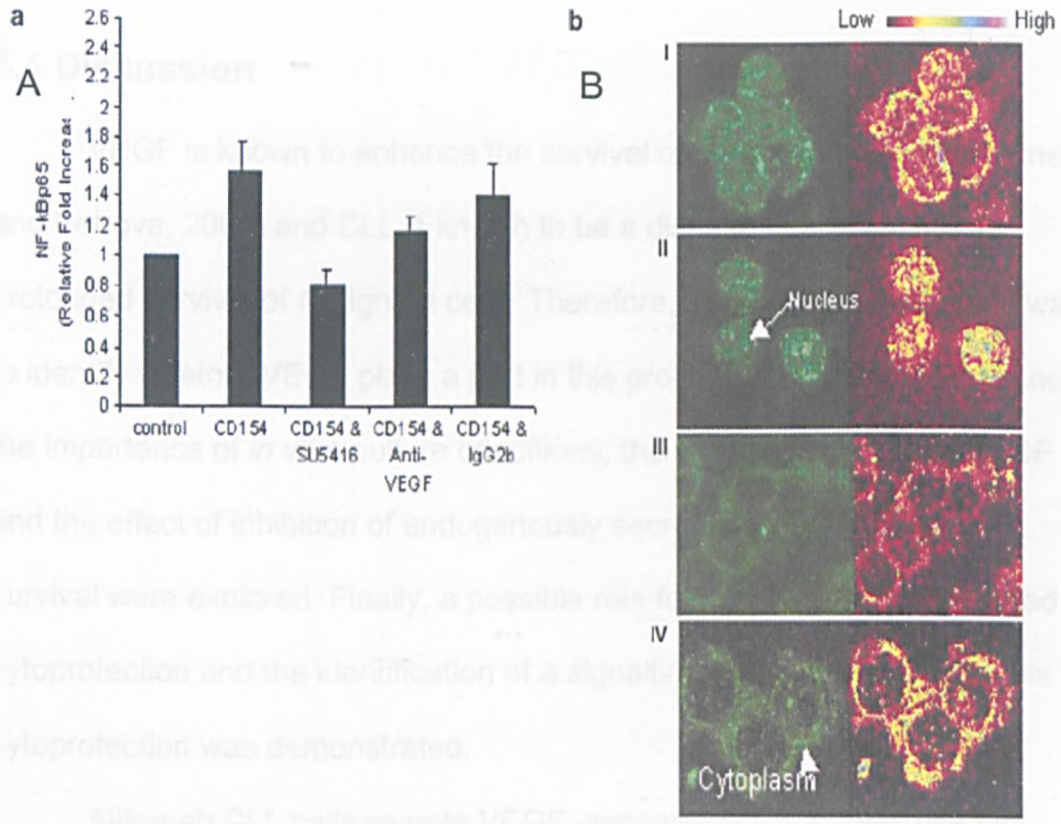


Figure 5.15

Role of VEGF in CD154-induced NF-κB activation in CLL cells.

(A) CLL cells from three separate cases were incubated with CD154 (200 ng/ml) in the absence or presence of SU5416 (10 μM), anti-VEGF antibody (1 μg/ml) or IgG_{2b} (1 μg/ml). After 24 hours, nuclear extracts were prepared and examined for the activation of NF-κB using the TransAM NF-κB p65 assay kit. (B) CLL cells were incubated for 24 hours in the absence (I) or presence (II) of CD154, or CD154 plus SU5416 (III) or CD154 plus the anti-VEGF antibody (IV) and stained with anti-p65 antibody. Fluorescent staining was analysed by confocal microscopy as described in Materials and methods. Note the translocation of NF-κB to the nucleus in (II) and the inhibition of this translocation in (III) and (IV). Green fluorescence in the first columns shows endogenous p65. The second column is presented in false colour to show the signal intensity in the nucleus compared with the cytoplasm. Results shown are representative of three separate experiments.

5.4 Discussion

VEGF is known to enhance the survival of other cell types (Kakkainen and Petrova, 2000) and CLL is known to be a disease characterised by prolonged survival of malignant cells. Therefore, in this Chapter, the aim was to identify whether VEGF plays a part in this prolonged survival. To this end, the importance of *in vitro* culture conditions, the effect of exogenous VEGF and the effect of inhibition of endogenously secreted VEGF on CLL-cell survival were explored. Finally, a possible role for VEGF in CD154-induced cytoprotection and the identification of a signalling pathway involved in this cytoprotection was demonstrated.

Although CLL cells secrete VEGF, exogenous VEGF was used in some experiments, since it was not certain that the amount of VEGF secreted would be sufficient to protect the cells from pro-apoptotic signals, particularly during the early stages of culture. However, exogenous VEGF, used over a wide range of concentrations, did not protect cultured CLL cells from spontaneous cell death. This indicated that the pro-survival effects of VEGF described for other cell types were not present in CLL cells, or were too weak to rescue the cells from apoptosis under the culture conditions used. In experiments in which VEGF was used to rescue CLL cells from induced cell death, three different factors, whose pro-apoptotic effects were known to be abrogated by angiogenic growth factors, were used. CLL cell death induced by fludarabine, a nucleoside used in the treatment of CLL, is known to be inhibited by bFGF (Menzel et al, 1996), while the effect of exposure of endothelial cells to ionising radiation is ameliorated by VEGF

(Katoh et al, 1995). In addition, myeloid leukaemia cells treated with geldanamycin are rescued from apoptosis by VEGF (Dias et al, 2002). VEGF treatment of CLL cells exposed to these factors did not prevent cell death, indicating that pro-survival signals generated by VEGF in CLL cells were not sufficient to overcome induction of cell death by these factors.

Having determined that exogenous VEGF did not affect the survival of CLL cells, attention turned to possible autocrine effects of VEGF on CLL cells. It has been known for some time that VEGF prevents the *in vitro* apoptosis of endothelial cells induced by serum starvation (Gerber et al, 1998b) and induces the expression of several anti-apoptotic proteins in these cells (Gerber et al, 1998a; Tran et al, 1999). It has recently been reported that inhibition of VEGFR signalling in CLL cells by a green tea component epigallocatechin (EGCG) shortens *in vitro* survival of these cells (Lee et al, 2004). Furthermore, a potential contribution of VEGF to the pro-survival effect of CD154 on CLL cells is suggested by work in Chapter 2, which shows that CD154 enhances the production of VEGF by CLL cells.

In the present Chapter, it was found that blocking either VEGF itself or its receptors greatly reduces the survival-promoting effect of CD154 on CLL cells. However, in the absence of CD154, blocking the effects of autocrine VEGF with an anti-VEGF antibody, anti-VEGFR antibodies or soluble VEGF receptors had no significant impact on cell survival. In contrast, the VEGF receptor inhibitor (SU5416) did shorten CLL-cell survival, suggesting the presence of an internal autocrine loop that is not inhibited by externally acting blocking agents. Such internal signalling has recently been described in malignant myeloid cells and haematopoietic progenitor cells (Santos and

Dias, 2004; Gerber et al, 2002). Since neither CD154 without VEGF nor VEGF alone effectively rescue CLL cells from apoptosis, it is proposed that full cytoprotection requires cooperation of signals provided by these factors. However, since blocking VEGF and inhibiting VEGF receptors did not completely reverse the cytoprotection provided by CD154, it is possible that some other factors induced by this agent, such as bFGF (Reinders et al, 2003) might also contribute to this cytoprotection.

Since previous reports suggested that the increase in CLL-cell survival induced by CD154 largely depends on activation of NF-κB, (Furman et al, 2000; Karin and Lin, 2002) the contribution of VEGF to this activation was next examined. It was first shown that CD40 engagement by CD154 upregulates production of VEGF, and that this production is at least partially dependent upon NF-κB activation. Previous studies have also implicated NF-κB in the upregulation of VEGF production in various other cell types such as human microvascular endothelial cells (Yoshida et al, 1997), prostate cancer cells (Huang et al, 2001) and breast cancer cell lines (Shibata et al, 2002). In addition, it has been reported that NF-κB decoy oligonucleotides block the induction of VEGF in UV-irradiated skin cells (Abeyama et al, 2000), and that in human macrophages CD154 stimulation results in strong NF-kB-dependent VEGF induction (Kiriakidis et al, 2003).

After showing that VEGF production involves NF-kB, it was important to determine whether the effects of this growth factor also involve activation of the NF-kB pathway. The effect of VEGF on the NF-kB pathway is largely dependent on cellular context. VEGF induces NF-kB activity in bovine retinal endothelial cells (Marumo et al, 1999) and human vascular endothelial cells

(Kim et al, 2001), but inhibits NF-kB activation in haematopoietic progenitor cells (Oyami et al, 1998; Dikov et al, 2001). Culturing CLL cells in the presence of CD154 resulted in a sustained increase in the activation of NF-kB, which was abolished by either anti-VEGF antibody or SU5416. These results were confirmed by confocal microscopy showing nuclear translocation of NF-kB in response to CD154 and prevention of this translocation by inhibition of VEGF or its receptors. Taken together, these results indicate that, in CLL cells, NF-kB activation by CD154 is largely mediated by autocrine VEGF. However, since activation of the NF-kB pathway by VEGF alone was not sufficient to enhance CLL cell survival, the results presented here indicate that other signalling pathway(s) must also be involved in cytoprotection by CD154. This conclusion is in accord with gene-expression analyses showing the cooperation of multiple signalling pathways in CD40-regulated gene expression in B-lymphocytes (Dadgostar et al, 2002)

Chapter 6

General Discussion

The importance of angiogenesis to tumour expansion was first described by Folkman (1971). Since then, the large number of studies concerning angiogenesis in malignancy has largely concentrated on solid tumours. The identification of angiogenic factors, firstly bFGF (Shing et al, 1984) then, more importantly for this thesis, VEGF/VPF (Ferrara and Henzel, 1989; Senger et al, 1983), demonstrated these growth factors affect multiple cellular functions. Later research concentrated on clarifying the mechanisms by which these cellular affects are produced.

VEGF is the predominant angiogenic factor, and since angiogenesis is important for tumour growth, interest began to be directed towards identifying a possible role for VEGF in the pathogenesis of different malignancies. Early focus on solid tumours was later joined by interest in the role of VEGF in haematological malignancies. These studies found evidence of increased angiogenesis in the bone marrow of patients with multiple myeloma (Vacca et al, 1994) and childhood ALL (Perez-Atayde et al, 1997), as well as in the lymph nodes of patients with non-Hodgkins lymphoma (Ribatti et al, 1996).

Regarding CLL, initial studies attempted to utilise levels of serum VEGF and bone marrow angiogenesis as indicators of disease progression and prognosis. Elevated serum levels of VEGF had been reported in this disease (Molica et al, 1998; Aguayo et al, 2000a), though one of these authors later suggested that the levels were not different from normal (Molica et al, 1999). Likewise, in studies involving analysis of bone marrow,

angiogenesis was reported to be elevated (Kini et al, 2000) or normal (Aguayo et al, 2000b).

It was against this background that work on this thesis began. The work proposed differed from that published, in that it aimed to determine not just the expression, but also the function of VEGF and VEGF receptors in malignant CLL cells.

Although during the early stages of this project others reported that CLL cells contain VEGF (Aguayo et al, 2000a), secretion of this growth factor had not been shown. Using both *in vivo* and *in vitro* assays, the current work demonstrated secretion of angiogenically active VEGF by CLL cells, increased secretion under hypoxic conditions and confirmed elevated angiogenesis in CLL-patient tissues. This was the first direct evidence implicating CLL-cell derived VEGF in the pathogenesis of CLL (Chen et al, 2000).

These studies also revealed a large variation in the levels of VEGF secreted by malignant cells from different CLL cases. This was not surprising because all ongoing studies in this Department, and published work in general, show a pronounced heterogeneity in the response of different CLL clones to different stimuli. While it was never the aim of this thesis to assign prognostic importance to VEGF production, attempts were made, in a relatively small number of CLL cases, to correlate VEGF secretion with somatic hypermutation of V_H genes, CD38 expression, patient lymphadenopathy and Type-A p53 dysfunction. In this analysis, no correlation was found; typically both high and low producers of VEGF were present in each group (data not shown). Consequently, it was decided not to

pursue associations between levels of VEGF production and clinical or laboratory findings for CLL patients, while at the same time acknowledging that this might have been of some interest to a broader readership.

In Chapter 2 of this thesis, attempts to demonstrate a relationship between Type-A p53 dysfunction, HIF-1α expression and VEGF expression are presented. Such a relationship could not be demonstrated despite p53 mutation being known to cause increased expression of HIF-1α and, consequently, increased expression of VEGF in solid tumours (Zhong et al, 1999; Ravi et al, 2000). Type-A p53 dysfunction in CLL is known to be caused by p53 mutation, but the precise mutation(s) in each case is unknown. It is possible that the p53 mutation of the Type-A patients used in this study might not affect HIF-1α expression to the same extent as other p53 mutations. The identification of CLL patients with this p53 dysfunction is an ongoing process within this Department, providing a convenient, expanding pool of CLL patients to select from. However, the identification of a group of CLL patients with p53 mutation that would affect HIF-1α regulation and VEGF expression was beyond the scope of this thesis.

In the first publication based on work presented in this thesis (Chen et al, 2000), it was stated in the discussion that VEGF receptors are expressed by CLL cells, although no data were provided, and that autocrine VEGF responses may be important for CLL-cell functions. Concentrating on the effect of VEGF on CLL-cell survival seemed appropriate since the role of VEGF in endothelial-cell survival was well described (Karkkainen and Petrova, 2000), and malignant CLL cells are characterised by their prolonged survival *in vivo*. Work in this Department (Pettitt et al, 2001; Moran et al,

2002) had established a model culture system for CLL cells on which the survival studies could be based. These studies had shown that CLL-cell survival *in vitro* is sensitive to changes in cell density and the presence of BSA in culture medium. By manipulating these variables, it was demonstrated that CLL cells could be made either more or less susceptible to apoptosis. By adapting this model system, it was possible to study the effects of VEGF on both spontaneous and drug-induced CLL cell death.

The earliest experiments provided no evidence for VEGF-enhanced survival of CLL cells *in vitro*, by either endogenous or exogenously added VEGF. Since VEGF had been shown to rescue other cell-types from induced apoptosis (Katoh et al, 1995; Katoh et al, 1998) and since this work could not be replicated here, it became necessary to confirm that the VEGF receptors identified on CLL cells were functional. Potentially, there are many markers for VEGF receptor activation. The most obvious option is to look for phosphorylation of VEGF receptors, and recent work using myeloid leukaemia cells (Santos and Dias, 2004) and CLL cells (Lee et al, 2004) has demonstrated that autocrine VEGF does induce such phosphorylation. However, Hsp90 was selected since changes in its expression would reflect not just receptor activation, but also activation of the downstream signalling pathways of VEGFR1 and/or VEGFR2. Hsp90 therefore appeared to be a good marker for activation of both VEGF receptors. The dose-dependent increases in Hsp90 expression following ligation of VEGFR1 and VEGFR2 provided evidence to suggest that both receptors were functional, and that the absence of a pro-survival response of CLL cells to VEGF was not due to ineffective or redundant VEGF receptors. Interestingly, the increased

expression of Hsp90 detected following cell stimulation with PLGF indicated a role for VEGFR1 in CLL-cell function and that this receptor was not simply a decoy as had previously been suggested (Park et al, 1994).

When identifying factors that affect VEGF expression in CLL cells, it was found that stimulation with CD154, the ligand for CD40, caused increased secretion of VEGF. This had previously been shown in other cell types and played a crucial role in a mitogenic response of endothelial cells to CD40 ligation (Melter et al, 2000). Other work has shown that CD154 inhibits spontaneous and induced death of CLL cells (Cho et al, 2000; Tai et al, 2002). In the present study, after confirming the cytoprotective effect of CD154 in cells from a proportion of CLL cases, it was important to determine whether this cytoprotection was due to autocrine VEGF. In the absence of CD154, external VEGF receptor blockade and VEGF-neutralisation did not affect cell survival. However, by use of an internally acting VEGF receptor inhibitor, SU5416, it was possible to demonstrate that VEGF acts through an internal autocrine loop to provide a pro-survival effect on CLL cells. This was similar to the autocrine effect of VEGF previously described in myeloid leukaemic cells (Santos and Dias, 2004). In contrast, it was also shown that CD154-induced CLL-cell survival was reduced by both externally acting anti-VEGF neutralising antibody and by inhibiting VEGF-receptor signalling with SU5416. It was therefore concluded that secreted VEGF does play a role in CLL-cell survival. However, since neither exogenous VEGF nor CD154 alone prolonged CLL-cell survival, cytoprotection provided by CD154 clearly requires combined signalling by both CD40 and VEGF receptors.

Current work in this Department is aimed at establishing which signalling pathways are involved in this cooperative cell rescue by CD154 and VEGF, and in the precise role of these signals in transcription of pro-apoptotic and anti-apoptotic genes that regulate CLL-cell survival. Furthermore, due to the interest generated by the work presented in this thesis, attempts are currently underway to establish a Phase II clinical trial for the treatment of CLL patients using Avastin, a humanized anti-VEGF antibody.

References

Abeyama K, Eng W, Jester JV, Vink AA, Edelbaum D, Cockerell CJ, Bergstresser PR, Takashima A. A role for NF-kappaB-dependent gene transactivation in sunburn. J Clin Invest. 2000 Jun;105(12):1751-9.

Agani FH, Pichiule P, Chavez JC, LaManna JC. The role of mitochondria in the regulation of hypoxia-inducible factor 1 expression during hypoxia. J Biol Chem. 2000 Nov 17;275(46):35863-7.

Aguayo A, Kantarjian H, Manshouri T, Gidel C, Estey E, Thomas D, Koller C, Estrov Z, O'Brien S, Keating M, Freireich E, Albitar M. Angiogenesis in acute and chronic leukemias and myelodysplastic syndromes. Blood. 2000b Sep 15;96(6):2240-5.

Aguayo A, Manshouri T, O'Brien S, Keating M, Beran M, Koller C, Kantarjian H, Rogers A, Albitar M. Clinical relevance of Flt1 and Tie1 angiogenesis receptors expression in B-cell chronic lymphocytic leukemia (CLL). Leuk Res. 2001 Apr;25(4):279-85.

Aguayo A, O'Brien S, Keating M, Manshouri T, Gidel C, Barlogie B, Beran M, Koller C, Kantarjian H, Albitar M. Clinical relevance of intracellular vascular endothelial growth factor levels in B-cell chronic lymphocytic leukemia. Blood. 2000a Jul 15;96(2):768-70.

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P. Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. Nat Med. 2003 Jul;9(7):936-43.

Baban DF, Murray JA, Earl HM, Kerr DJ, Seymour LW. Quantittative analysis of vascular endothelial growth factor expression in chronic lymphocytic leukaemia. Int. J. Oncol. 1996 Jan 8(1):29-34.

Bachelder RE, Crago A, Chung J, Wendt MA, Shaw LM, Robinson G, Mercurio AM. Vascular endothelial growth factor is an autocrine survival factor for neuropilin-expressing breast carcinoma cells. Cancer Res. 2001 Aug 1;61(15):5736-40.

Bairey O, Boycov O, Kaganovsky E, Zimra Y, Shaklai M, Rabizadeh E. All three receptors for vascular endothelial growth factor (VEGF) are expressed on B-chronic lymphocytic leukemia (CLL) cells. Leuk Res. 2004 Mar;28(3):243-8.

Banchereau J, Bazan F, Blanchard D, Briere F, Galizzi JP, van Kooten C, Liu YJ, Rousset F, Saeland S. The CD40 antigen and its ligand.Annu Rev Immunol. 1994;12:881-922. Review.

Banks RE, Forbes MA, Kinsey SE, Stanley A, Ingham E, Walters C, Selby PJ. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. Br J Cancer. 1998 Mar;77(6):956-64.

Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D. Migration of human monocytes in response to vascular endothelial growth factor (VEGF) is mediated via the VEGF receptor flt-1.Blood. 1996 Apr 15;87(8):3336-43.

Bauvois B, Dumont J, Mathiot C, Kolb JP. Production of matrix metalloproteinase-9 in early stage B-CLL: suppression by interferons. Leukemia. 2002 May;16(5):791-8.

Bellamy WT, Richter L, Frutiger Y, Grogan TM. Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. Cancer Res. 1999 Feb 1;59(3):728-33.

Bergers G, Benjamin LE. Tumorigenesis and the angiogenic switch. Nat Rev Cancer. 2003 Jun;3(6):401-10. Review.

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D. Matrix metalloproteinase-9 triggers

the angiogenic switch during carcinogenesis. Nat Cell Biol. 2000 Oct;2(10):737-44.

Binet JL, Auquier A, Dighiero G, Chastang C, Piguet H, Goasguen J, Vaugier G, Potron G, Colona P, Oberling F, Thomas M, Tchernia G, Jacquillat C, Boivin P, Lesty C, Duault MT, Monconduit M, Belabbes S, Gremy F. A new prognostic classification of chronic lymphocytic leukemia derived from a multivariate survival analysis. Cancer. 1981 Jul 1;48(1):198-206.

Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, Neckers L. p53 inhibits hypoxia-inducible factor-stimulated transcription. J Biol Chem. 1998 May 15;273(20):11995-8.

Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, Pinedo HM, Abeloff MD, Simons JW, van Diest PJ, van der Wall E. Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst. 2001 Feb 21;93(4):309-14.

Brooks PC, Clark RA, Cheresh DA. Requirement of vascular integrin alpha v beta 3 for angiogenesis. Science. 1994 Apr 22;264(5158):569-71.

Brown LF, Berse B, Jackman RW, Tognazzi K, Manseau EJ, Dvorak HF, Senger DR. Increased expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in kidney and bladder carcinomas. Am J Pathol. 1993 Nov;143(5):1255-62.

Bruick RK, McKnight SL. A conserved family of prolyl-4-hydroxylases that modify HIF. Science. 2001 Nov 9;294(5545):1337-40.

Caligaris-Cappio F, Hamblin TJ. B-cell chronic lymphocytic leukemia: a bird of a different feather. J Clin Oncol. 1999 Jan;17(1):399-408. Review.

Call TG, Phyliky RL, Noel P, Habermann TM, Beard CM, O'Fallon WM, Kurland LT. Incidence of chronic lymphocytic leukemia in Olmsted County, Minnesota, 1935 through 1989, with emphasis on changes in initial stage at diagnosis. Mayo Clin Proc. 1994 Apr;69(4):323-8.

Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature. 2000 Sep 14;407(6801):249-57. Review.

Carmeliet P. Angiogenesis in health and disease. Nat Med. 2003 Jun;9(6):653-60. Review.

Chen H, Treweeke AT, West DC, Till KJ, Cawley JC, Zuzel M, Toh CH. In vitro and in vivo production of vascular endothelial growth factor by chronic lymphocytic leukemia cells. Blood. 2000 Nov 1;96(9):3181-7.

Chen HJ, Treweeke AT, Ke YQ, West DC, Toh CH. Angiogenically active vascular endothelial growth factor is over-expressed in malignant human and rat prostate carcinoma cells. Br J Cancer. 2000a May;82(10):1694-701.

Chiorazzi N, Ferrarini M. B cell chronic lymphocytic leukemia: lessons learned from studies of the B cell antigen receptor. Annu Rev Immunol. 2003;21:841-94. Review.

Cho CS, Cho ML, Min SY, Kim WU, Min DJ, Lee SS, Park SH, Choe J, Kim HY. CD40 engagement on synovial fibroblast up-regulates production of vascular endothelial growth factor. J Immunol. 2000 May 15;164(10):5055-61.

Connolly DT, Heuvelman DM, Nelson R, Olander JV, Eppley BL, Delfino JJ, Siegel NR, Leimgruber RM, Feder J. Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. J Clin Invest. 1989 Nov;84(5):1470-8.

Cory S, Adams JM. The Bcl2 family: regulators of the cellular life-or-death switch. Nat Rev Cancer. 2002 Sep;2(9):647-56. Review.

Dadgostar H, Zarnegar B, Hoffmann A, Qin XF, Truong U, Rao G, Baltimore D, Cheng G. Cooperation of multiple signaling pathways in CD40-regulated gene expression in B lymphocytes. Proc Natl Acad Sci U S A. 2002 Feb 5;99(3):1497-502.

Davis-Smyth T, Chen H, Park J, Presta LG, Ferrara N. The second immunoglobulin-like domain of the VEGF tyrosine kinase receptor Flt-1

determines ligand binding and may initiate a signal transduction cascade. EMBO J. 1996 Sep 16;15(18):4919-27.

de Bont ES, Rosati S, Jacobs S, Kamps WA, Vellenga E. Increased bone marrow vascularization in patients with acute myeloid leukaemia: a possible role for vascular endothelial growth factor. Br J Haematol. 2001 May;113(2):296-304.

de Vries C, Escobedo JA, Ueno H, Houck K, Ferrara N, Williams LT. The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor. Science. 1992 Feb 21;255(5047):989-91.

Dias S, Hattori K, Heissig B, Zhu Z, Wu Y, Witte L, Hicklin DJ, Tateno M, Bohlen P, Moore MA, Rafii S. Inhibition of both paracrine and autocrine VEGF/ VEGFR-2 signaling pathways is essential to induce long-term remission of xenotransplanted human leukemias. Proc Natl Acad Sci U S A. 2001 Sep 11;98(19):10857-62.

Dias S, Hattori K, Zhu Z, Heissig B, Choy M, Lane W, Wu Y, Chadburn A, Hyjek E, Gill M, Hicklin DJ, Witte L, Moore MA, Rafii S. Autocrine stimulation of VEGFR-2 activates human leukemic cell growth and migration. J Clin Invest. 2000 Aug;106(4):511-21.

Dias S, Shmelkov SV, Lam G, Rafii S. VEGF(165) promotes survival of leukemic cells by Hsp90-mediated induction of Bcl-2 expression and apoptosis inhibition. Blood. 2002 Apr 1;99(7):2532-40.

Diehl LF, Karnell LH, Menck HR. The American College of Surgeons Commission on Cancer and the American Cancer Society. The National Cancer Data Base report on age, gender, treatment, and outcomes of patients with chronic lymphocytic leukemia. Cancer. 1999 Dec 15;86(12):2684-92.

Dikov MM, Oyama T, Cheng P, Takahashi T, Takahashi K, Sepetavec T, Edwards B, Adachi Y, Nadaf S, Daniel T, Gabrilovich DI, Carbone DP. Vascular endothelial growth factor effects on nuclear factor-kappaB activation in hematopoietic progenitor cells. Cancer Res. 2001 Mar 1;61(5):2015-21.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ. C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. Cell. 2001 Oct 5;107(1):43-54.

Fais F, Ghiotto F, Hashimoto S, Sellars B, Valetto A, Allen SL, Schulman P, Vinciguerra VP, Rai K, Rassenti LZ, Kipps TJ, Dighiero G, Schroeder HW Jr, Ferrarini M, Chiorazzi N. Chronic lymphocytic leukemia B cells express

restricted sets of mutated and unmutated antigen receptors. J Clin Invest. 1998 Oct 15;102(8):1515-25.

Farahani M, Treweeke AT, Toh CH, Till KJ, Harris RJ, Cawley JC, Zuzel M, Chen H. Autocrine VEGF mediates the antiapoptotic effect of CD154 on CLL cells. Leukemia. 2005 Apr;19(4):524-30.

Ferrajoli A, Manshouri T, Estrov Z, Keating MJ, O'Brien S, Lerner S, Beran M, Kantarjian HM, Freireich EJ, Albitar M. High levels of vascular endothelial growth factor receptor-2 correlate with shortened survival in chronic lymphocytic leukemia. Clin Cancer Res. 2001 Apr;7(4):795-9.

Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev. 1997 Feb;18(1):4-25. Review.

Ferrara N, Gerber HP, LeCouter J. The biology of VEGF and its receptors. Nat Med. 2003 Jun;9(6):669-76. Review. PMID: 12778165

Ferrara N, Henzel WJ. Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. Biochem Biophys Res Commun. 1989 Jun 15;161(2):851-8.

Ferrara N, Hillan KJ, Novotny W. Bevacizumab (Avastin), a humanized anti-VEGF monoclonal antibody for cancer therapy. Biochem Biophys Res Commun. 2005 Jul 29;333(2):328-35.

Ferrara N. Vascular endothelial growth factor as a target for anticancer therapy. Oncologist. 2004;9 Suppl 1:2-10. Review.

Folkman J, Moscona A. Role of cell shape in growth control. Nature. 1978 Jun 1;273(5661):345-9.

Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med. 1971 Nov 18;285(21):1182-6. Review.

Forsythe JA, Jiang BH, Iyer NV, Agani F, Leung SW, Koos RD, Semenza GL. Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1. Mol Cell Biol. 1996 Sep;16(9):4604-13.

Freeman MR, Schneck FX, Gagnon ML, Corless C, Soker S, Niknejad K, Peoples GE, Klagsbrun M. Peripheral blood T lymphocytes and lymphocytes infiltrating human cancers express vascular endothelial growth factor: a potential role for T cells in angiogenesis. Cancer Res. 1995 Sep 15;55(18):4140-5.

Friedlander M, Theesfeld CL, Sugita M, Fruttiger M, Thomas MA, Chang S, Cheresh DA. Involvement of integrins alpha v beta 3 and alpha v beta 5 in ocular neovascular diseases. Proc Natl Acad Sci U S A. 1996 Sep 3;93(18):9764-9.

Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukaemia B cells. J Immunol. 2000 Feb 15;164(4):2200-6.

Gerber HP, Condorelli F, Park J, Ferrara N. Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. J Biol Chem. 1997 Sep 19;272(38):23659-67.

Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. J Biol Chem. 1998 May 22;273(21):13313-6.

Gerber HP, Ferrara N. The role of VEGF in normal and neoplastic hematopoiesis. J Mol Med. 2003 Jan;81(1):20-31. Review.

Gerber HP, Malik AK, Solar GP, Sherman D, Liang XH, Meng G, Hong K, Marsters JC, Ferrara N. VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism. Nature. 2002 Jun 27;417(6892):954-8.

Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V, Ferrara N. Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. J Biol Chem. 1998 Nov 13;273(46):30336-43.

Gille H, Kowalski J, Yu L, Chen H, Pisabarro MT, Davis-Smyth T, Ferrara N. A repressor sequence in the juxtamembrane domain of Flt-1 (VEGFR-1) constitutively inhibits vascular endothelial growth factor-dependent phosphatidylinositol 3'-kinase activation and endothelial cell migration. EMBO J. 2000 Aug 1;19(15):4064-73.

Granziero L, Ghia P, Circosta P, Gottardi D, Strola G, Geuna M, Montagna L, Piccoli P, Chilosi M, Caligaris-Cappio F. Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. Blood. 2001 May 1;97(9):2777-83.

Guo D, Jia Q, Song HY, Warren RS, Donner DB. Vascular endothelial cell growth factor promotes tyrosine phosphorylation of mediators of signal transduction that contain SH2 domains. Association with endothelial cell proliferation. J Biol Chem. 1995 Mar 24;270(12):6729-33.

Hayashibara T, Yamada Y, Onimaru Y, Tsutsumi C, Nakayama S, Mori N, Miyanishi T, Kamihira S, Tomonaga M, Maita T. Matrix metalloproteinase-9 and vascular endothelial growth factor: a possible link in adult T-cell leukaemia cell invasion. Br J Haematol. 2002 Jan;116(1):94-102.

Hewitson KS, McNeill LA, Riordan MV, Tian YM, Bullock AN, Welford RW, Elkins JM, Oldham NJ, Bhattacharya S, Gleadle JM, Ratcliffe PJ, Pugh CW, Schofield CJ. Hypoxia-inducible factor (HIF) asparagine hydroxylase is

identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. J Biol Chem. 2002 Jul 19;277(29):26351-5.

Holash J, Davis S, Papadopoulos N, Croll SD, Ho L, Russell M, Boland P, Leidich R, Hylton D, Burova E, Ioffe E, Huang T, Radziejewski C, Bailey K, Fandl JP, Daly T, Wiegand SJ, Yancopoulos GD, Rudge JS. VEGF-Trap: a VEGF blocker with potent antitumor effects. Proc Natl Acad Sci U S A. 2002 Aug 20;99(17):11393-8.

Huang S, Pettaway CA, Uehara H, Bucana CD, Fidler IJ. Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. Oncogene. 2001 Jul 12;20(31):4188-97.

Hussong JW, Rodgers GM, Shami PJ. Evidence of increased angiogenesis in patients with acute myeloid leukemia. Blood. 2000 Jan 1;95(1):309-13.

Itokawa T, Nokihara H, Nishioka Y, Sone S, Iwamoto Y, Yamada Y, Cherrington J, McMahon G, Shibuya M, Kuwano M, Ono M. Antiangiogenic effect by SU5416 is partly attributable to inhibition of Flt-1 receptor signaling. Mol Cancer Ther. 2002 Mar;1(5):295-302.

Jackson C. Matrix metalloproteinases and angiogenesis. Curr Opin Nephrol Hypertens. 2002 May;11(3):295-9. Review.

Jackson MW, Roberts JS, Heckford SE, Ricciardelli C, Stahl J, Choong C, Horsfall DJ, Tilley WD. A potential autocrine role for vascular endothelial growth factor in prostate cancer. Cancer Res. 2002 Feb 1;62(3):854-9.

Jager R, Herzer U, Schenkel J, Weiher H. Overexpression of Bcl-2 inhibits alveolar cell apoptosis during involution and accelerates c-myc-induced tumorigenesis of the mammary gland in transgenic mice. Oncogene. 1997 Oct 9;15(15):1787-95.

Joseph IB, Isaacs JT. Potentiation of the antiangiogenic ability of linomide by androgen ablation involves down-regulation of vascular endothelial growth factor in human androgen-responsive prostatic cancers. Cancer Res. 1997 Mar 15;57(6):1054-7.

Kabbinavar F, Hurwitz HI, Fehrenbacher L, Meropol NJ, Novotny WF, Lieberman G, Griffing S, Bergsland E. Phase II, randomized trial comparing bevacizumab plus fluorouracil (FU)/leucovorin (LV) with FU/LV alone in patients with metastatic colorectal cancer. J Clin Oncol. 2003 Jan 1;21(1):60-5.

Kaelin WG. Proline hydroxylation and gene expression. Annu Rev Biochem. 2005;74:115-28.

Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol. 2002 Mar;3(3):221-7. Review.

Karkkainen MJ, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. Oncogene. 2000 Nov 20;19(49):5598-605. Review.

Katoh O, Takahashi T, Oguri T, Kuramoto K, Mihara K, Kobayashi M, Hirata S, Watanabe H. Vascular endothelial growth factor inhibits apoptotic death in hematopoietic cells after exposure to chemotherapeutic drugs by inducing MCL1 acting as an antiapoptotic factor. Cancer Res. 1998 Dec 1;58(23):5565-9.

Katoh O, Tauchi H, Kawaishi K, Kimura A, Satow Y. Expression of the vascular endothelial growth factor (VEGF) receptor gene, KDR, in hematopoietic cells and inhibitory effect of VEGF on apoptotic cell death caused by ionizing radiation. Cancer Res. 1995 Dec 1;55(23):5687-92.

Kay NE, Bone ND, Tschumper RC, Howell KH, Geyer SM, Dewald GW, Hanson CA, Jelinek DF. B-CLL cells are capable of synthesis and secretion of both pro- and anti-angiogenic molecules. Leukemia. 2002 May;16(5):911-9.

Keck PJ, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT. Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science. 1989 Dec 8;246(4935):1309-12.

Kendall RL, Thomas KA. Inhibition of vascular endothelial cell growth factor activity by an endogenously encoded soluble receptor. Proc Natl Acad Sci U S A. 1993 Nov 15;90(22):10705-9.

Kendall RL, Wang G, Thomas KA. Identification of a natural soluble form of the vascular endothelial growth factor receptor, FLT-1, and its heterodimerization with KDR. Biochem Biophys Res Commun. 1996 Sep 13;226(2):324-8.

Kerbel RS. Tumor angiogenesis: past, present and the near future. Carcinogenesis. 2000 Mar;21(3):505-15. Review.

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer. 1972 Aug;26(4):239-57. Review.

Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. J Biol Chem. 2001 Mar 9;276(10):7614-20

Kini AR, Kay NE, Peterson LC. Increased bone marrow angiogenesis in B cell chronic lymphocytic leukemia. Leukemia. 2000 Aug;14(8):1414-8.

Kini AR, Peterson LC, Kay NE. Evidence for abnormal angiogenesis in the bone marrow of patients with B-cell chronic lymphocytic leukaemia (CLL) [abstract]. Blood. 1998;92:2947.

Kiriakidis S, Andreakos E, Monaco C, Foxwell B, Feldmann M, Paleolog E. VEGF expression in human macrophages is NF-kappaB-dependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2. J Cell Sci. 2003 Feb 15;116(Pt 4):665-74.

Klein U, Tu Y, Stolovitzky GA, Mattioli M, Cattoretti G, Husson H, Freedman A, Inghirami G, Cro L, Baldini L, Neri A, Califano A, Dalla-Favera R. Gene expression profiling of B cell chronic lymphocytic leukemia reveals a homogeneous phenotype related to memory B cells. J Exp Med. 2001 Dec 3;194(11):1625-38.

Kolch W, Martiny-Baron G, Kieser A, Marme D. Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis. Breast Cancer Res Treat. 1995;36(2):139-55. Review.

Kondo S, Shinomura Y, Miyazaki Y, Kiyohara T, Tsutsui S, Kitamura S, Nagasawa Y, Nakahara M, Kanayama S, Matsuzawa Y. Mutations of the bak gene in human gastric and colorectal cancers. Cancer Res. 2000 Aug 15;60(16):4328-30.

Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F, Marklund SL, Wyns S, Thijs V, Andersson J, van Marion I, Al-Chalabi A, Bornes S, Musson R, Hansen V, Beckman L, Adolfsson R, Pall HS, Prats H, Vermeire S, Rutgeerts P, Katayama S, Awata T, Leigh N, Lang-Lazdunski L, Dewerchin M, Shaw C, Moons L, Vlietinck R, Morrison KE, Robberecht W, Van Broeckhoven C, Collen D, Andersen PM, Carmeliet P. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet. 2003 Aug;34(4):383-94.

Landgren E, Schiller P, Cao Y, Claesson-Welsh L. Placenta growth factor stimulates MAP kinase and mitogenicity but not phospholipase C-gamma and migration of endothelial cells expressing Flt 1. Oncogene. 1998 Jan 22;16(3):359-67.

Le Gouill S, Podar K, Amiot M, Hideshima T, Chauhan D, Ishitsuka K, Kumar S, Raje N, Richardson PG, Harousseau JL, Anderson KC. VEGF induces Mcl-1 up-regulation and protects multiple myeloma cells against apoptosis. Blood. 2004 Nov 1;104(9):2886-92.

Lee YK, Bone ND, Strege AK, Shanafelt TD, Jelinek DF, Kay NE. VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. Blood. 2004 Aug 1;104(3):788-94

Lee YK, Bone ND, Strege AK, Shanafelt TD, Jelinek DF, Kay NE. VEGF receptor phosphorylation status and apoptosis is modulated by a green tea component, epigallocatechin-3-gallate (EGCG), in B-cell chronic lymphocytic leukemia. Blood. 2004 Aug 1;104(3):788-94.

Lee YK, Shanafelt TD, Bone ND, Strege AK, Jelinek DF, Kay NE. VEGF receptors on chronic lymphocytic leukemia (CLL) B cells interact with STAT 1 and 3: implication for apoptosis resistance. Leukemia. 2005 Apr;19(4):513-23.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989 Dec 8;246(4935):1306-9.

Lin K, Sherrington PD, Dennis M, Matrai Z, Cawley JC, Pettitt AR. Relationship between p53 dysfunction, CD38 expression, and IgV(H) mutation in chronic lymphocytic leukemia. Blood. 2002 Aug 15;100(4):1404-9. Erratum in: Blood 2002 Oct 1;100(7):2291.

Mabjeesh NJ, Post DE, Willard MT, Kaur B, Van Meir EG, Simons JW, Zhong H. Geldanamycin induces degradation of hypoxia-inducible factor 1alpha protein via the proteosome pathway in prostate cancer cells. Cancer Res. 2002 May 1;62(9):2478-82.

Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. Diabetes. 1999 May;48(5):1131-7.

Masood R, Cai J, Zheng T, Smith DL, Hinton DR, Gill PS. Vascular endothelial growth factor (VEGF) is an autocrine growth factor for VEGF receptor-positive human tumors. Blood. 2001 Sep 15;98(6):1904-13.

Matthews W, Jordan CT, Gavin M, Jenkins NA, Copeland NG, Lemischka IR. A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit. Proc Natl Acad Sci U S A. 1991 Oct 15;88(20):9026-30.

Maxwell PH, Wiesener MS, Chang GW, Clifford SC, Vaux EC, Cockman ME, Wykoff CC, Pugh CW, Maher ER, Ratcliffe PJ. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature. 1999 May 20;399(6733):271-5.

Meijerink JP, Mensink EJ, Wang K, Sedlak TW, Sloetjes AW, de Witte T, Waksman G, Korsmeyer SJ. Hematopoietic malignancies demonstrate loss-of-function mutations of BAX. Blood. 1998 Apr 15;91(8):2991-7.

Melter M, Reinders ME, Sho M, Pal S, Geehan C, Denton MD, Mukhopadhyay D, Briscoe DM. Ligation of CD40 induces the expression of

vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis in vivo. Blood. 2000 Dec 1;96(12):3801-8.

Menzel T, Rahman Z, Calleja E, White K, Wilson EL, Wieder R, Gabrilove J. Elevated intracellular level of basic fibroblast growth factor correlates with stage of chronic lymphocytic leukemia and is associated with resistance to fludarabine. Blood. 1996 Feb 1;87(3):1056-63.

Mesri M, Morales-Ruiz M, Ackermann EJ, Bennett CF, Pober JS, Sessa WC, Altieri DC. Suppression of vascular endothelial growth factor-mediated endothelial cell protection by survivin targeting. Am J Pathol. 2001 May;158(5):1757-65.

Migdal M, Huppertz B, Tessler S, Comforti A, Shibuya M, Reich R, Baumann H, Neufeld G. Neuropilin-1 is a placenta growth factor-2 receptor. J Biol Chem. 1998 Aug 28;273(35):22272-8.

Molica A, Vitelli G, Levato D, Gandolfo GM, Liso V. Clinico-prognostic implications of increased levels of serum vascular endothelial growth factor in B-CLL chronic lymphocytic leukemia [abstract]. Blood. 1998;92:410.

Molica S, Levato D, Dattilo A. Natural history of early chronic lymphocytic leukemia. A single institution study with emphasis on the impact of disease-progression on overall survival. Haematologica. 1999 Dec;84(12):1094-9.

Molica S, Vacca A, Ribatti D, Cuneo A, Cavazzini F, Levato D, Vitelli G, Tucci L, Roccaro AM, Dammacco F. Prognostic value of enhanced bone marrow angiogenesis in early B-cell chronic lymphocytic leukemia. Blood. 2002 Nov 1;100(9):3344-51.

Molica S, Vitelli G, Levato D, Gandolfo GM, Liso V. Increased serum levels of vascular endothelial growth factor predict risk of progression in early B-cell chronic lymphocytic leukaemia. Br J Haematol. 1999 Dec;107(3):605-10.

Molica S, Vitelli G, Levato D, Ricciotti A, Digiesi G. Clinicoprognostic implications of increased serum levels of vascular endothelial growth factor and basic fibroblastic growth factor in early B-cell chronic lymphocytic leukaemia. Br J Cancer. 2002 Jan 7;86(1):31-5.

Moran EC, Kamiguti AS, Cawley JC, Pettitt AR. Cytoprotective antioxidant activity of serum albumin and autocrine catalase in chronic lymphocytic leukaemia. Br J Haematol. 2002 Feb;116(2):316-28.

Mura M, dos Santos CC, Stewart D, Liu M. Vascular endothelial growth factor and related molecules in acute lung injury.J Appl Physiol. 2004 Nov;97(5):1605-17. Review.

Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors. FASEB J. 1999 Jan;13(1):9-22. Review.

Neufeld G, Cohen T, Shraga N, Lange T, Kessler O, Herzog Y. The neuropilins: multifunctional semaphorin and VEGF receptors that modulate axon guidance and angiogenesis. Trends Cardiovasc Med. 2002 Jan;12(1):13-9. Review.

Nor JE, Christensen J, Mooney DJ, Polverini PJ. Vascular endothelial growth factor (VEGF)-mediated angiogenesis is associated with enhanced endothelial cell survival and induction of Bcl-2 expression. Am J Pathol. 1999 Feb;154(2):375-84.

Ogunshola OO, Antic A, Donoghue MJ, Fan SY, Kim H, Stewart WB, Madri JA, Ment LR. Paracrine and autocrine functions of neuronal vascular endothelial growth factor (VEGF) in the central nervous system. J Biol Chem. 2002 Mar 29;277(13):11410-5.

Ohta Y, Endo Y, Tanaka M, Shimizu J, Oda M, Hayashi Y, Watanabe Y, Sasaki T. Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. Clin Cancer Res. 1996 Aug;2(8):1411-6.

Oyama T, Ran S, Ishida T, Nadaf S, Kerr L, Carbone DP, Gabrilovich DI. Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. J Immunol. 1998 Feb 1;160(3):1224-32.

Padro T, Bieker R, Ruiz S, Steins M, Retzlaff S, Burger H, Buchner T, Kessler T, Herrera F, Kienast J, Muller-Tidow C, Serve H, Berdel WE, Mesters RM. Overexpression of vascular endothelial growth factor (VEGF) and its cellular receptor KDR (VEGFR-2) in the bone marrow of patients with acute myeloid leukemia. Leukemia. 2002 Jul;16(7):1302-10.

Pangalis GA, Vassilakopoulos TP, Dimopoulou MN, Siakantaris MP, Kontopidou FN, Angelopoulou MK. B-chronic lymphocytic leukemia: practical aspects. Hematol Oncol. 2002 Sep;20(3):103-46. Review.

Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, in vitro and in vivo, and high affinity binding to Flt-1 but not to Flk-1/KDR. J Biol Chem. 1994 Oct 14;269(41):25646-54.

Pepper MS, Ferrara N, Orci L, Montesano R. Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells. Biochem Biophys Res Commun. 1991 Dec 16;181(2):902-6.

Pepper MS. Extracellular proteolysis and angiogenesis. Thromb Haemost. 2001 Jul;86(1):346-55. Review.

Perez-Atayde AR, Sallan SE, Tedrow U, Connors S, Allred E, Folkman J. Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. Am J Pathol. 1997 Mar;150(3):815-21.

Petrova TV, Makinen T, Alitalo K. Signaling via vascular endothelial growth factor receptors. Exp Cell Res. 1999 Nov 25;253(1):117-30. Review.

Pettitt AR, Moran EC, Cawley JC. Homotypic interactions protect chronic lymphocytic leukaemia cells from spontaneous death in vitro. Leuk Res. 2001 Nov;25(11):1003-12.

Pettitt AR, Sherrington PD, Stewart G, Cawley JC, Taylor AM, Stankovic T. p53 dysfunction in B-cell chronic lymphocytic leukemia: inactivation of ATM as an alternative to TP53 mutation. Blood. 2001 Aug 1;98(3):814-22.

Pettitt AR. Mechanism of action of purine analogues in chronic lymphocytic leukaemia. Br J Haematol. 2003 Jun;121(5):692-702. Review.

Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. Nature. 1992 Oct 29;359(6398):845-8.

Podar K, Anderson KC. The pathophysiologic role of VEGF in hematologic malignancies: therapeutic implications. Blood. 2005 Feb 15;105(4):1383-95. Review.

Podar K, Tai YT, Davies FE, Lentzsch S, Sattler M, Hideshima T, Lin BK, Gupta D, Shima Y, Chauhan D, Mitsiades C, Raje N, Richardson P, Anderson KC. Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. Blood. 2001 Jul 15;98(2):428-35.

Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, Overholser J, Hooper A, Pytowski B, Witte L, Bohlen P, Hicklin DJ. Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. Cancer Res. 1999 Oct 15;59(20):5209-18.

Rafii S, Avecilla S, Shmelkov S, Shido K, Tejada R, Moore MA, Heissig B, Hattori K. Angiogenic factors reconstitute hematopoiesis by recruiting stem cells from bone marrow microenvironment. Ann N Y Acad Sci. 2003 May;996:49-60.

Rai KR, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. Blood. 1975 Aug;46(2):219-34.

Rajkumar SV, Leong T, Roche PC, Fonseca R, Dispenzieri A, Lacy MQ, Lust JA, Witzig TE, Kyle RA, Gertz MA, Greipp PR. Prognostic value of bone

marrow angiogenesis in multiple myeloma. Clin Cancer Res. 2000 Aug;6(8):3111-6.

Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, Zeng Q, Dillehay LE, Madan A, Semenza GL, Bedi A. Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1alpha. Genes Dev. 2000 Jan 1;14(1):34-44.

Reinders ME, Sho M, Robertson SW, Geehan CS, Briscoe DM. Proangiogenic function of CD40 ligand-CD40 interactions. J Immunol. 2003 Aug 1;171(3):1534-41.

Ribatti D, Vacca A, Nico B, Fanelli M, Roncali L, Dammacco F. Angiogenesis spectrum in the stroma of B-cell non-Hodgkin's lymphomas. An immunohistochemical and ultrastructural study. Eur J Haematol. 1996 Jan-Feb;56(1-2):45-53.

Risau W. Mechanisms of angiogenesis. Nature. 1997 Apr 17;386(6626):671-4. Review.

Robinson D, Lackie P, Aber V, Catovsky D. Morphometric analysis of chronic B-cell leukemias--an aid to the classification of lymphoid cell types. Leuk Res. 1989;13(5):357-65.

Ross SR, McTavish D, Faulds D. Fludarabine. A review of its pharmacological properties and therapeutic potential in malignancy. Drugs. 1993 May;45(5):737-59. Review.

Rousseau S, Houle F, Kotanides H, Witte L, Waltenberger J, Landry J, Huot J. Vascular endothelial growth factor (VEGF)-driven actin-based motility is mediated by VEGFR2 and requires concerted activation of stress-activated protein kinase 2 (SAPK2/p38) and geldanamycin-sensitive phosphorylation of focal adhesion kinase. J Biol Chem. 2000 Apr 7;275(14):10661-72.

Rousseau S, Houle F, Landry J, Huot J. p38 MAP kinase activation by vascular endothelial growth factor mediates actin reorganization and cell migration in human endothelial cells. Oncogene. 1997 Oct;15(18):2169-77.

Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem. 1997 Sep 5;272(36):22642-7.

Salceda S, Caro J. Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem. 1997 Sep 5;272(36):22642-7.

Santos SC, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. Blood. 2004 May 15;103(10):3883-9

Schattner EJ. CD40 ligand in CLL pathogenesis and therapy. Leuk Lymphoma. 2000 May;37(5-6):461-72. Review.

Semenza GL. HIF-1: mediator of physiological and pathophysiological responses to hypoxia. J Appl Physiol. 2000 Apr;88(4):1474-80. Review.

Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Dvorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983 Feb 25;219(4587):983-5.

Senger DR, Ledbetter SR, Claffey KP, Papadopoulos-Sergiou A, Peruzzi CA, Detmar M. Stimulation of endothelial cell migration by vascular permeability factor/vascular endothelial growth factor through cooperative mechanisms involving the alphavbeta3 integrin, osteopontin, and thrombin. Am J Pathol. 1996 Jul;149(1):293-305.

Shibata A, Nagaya T, Imai T, Funahashi H, Nakao A, Seo H. Inhibition of NF-kappaB activity decreases the VEGF mRNA expression in MDA-MB-231 breast cancer cells. Breast Cancer Res Treat. 2002 Jun;73(3):237-43.

Shibuya M, Yamaguchi S, Yamane A, Ikeda T, Tojo A, Matsushime H, Sato M. Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family. Oncogene. 1990 Apr;5(4):519-24.

Shih SC, Mullen A, Abrams K, Mukhopadhyay D, Claffey KP. Role of protein kinase C isoforms in phorbol ester-induced vascular endothelial growth factor expression in human glioblastoma cells. J Biol Chem. 1999 May 28;274(22):15407-14.

Shing Y, Folkman J, Sullivan R, Butterfield C, Murray J, Klagsbrun M. Heparin affinity: purification of a tumor-derived capillary endothelial cell growth factor. Science. 1984 Mar 23;223(4642):1296-9.

Shustik C, Mick R, Silver R, Sawitsky A, Rai K, Shapiro L. Treatment of early chronic lymphocytic leukemia: intermittent chlorambucil versus observation. Hematol Oncol. 1988 Jan-Mar;6(1):7-12.

Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature. 1992 Oct 29;359(6398):843-5.

Smolich BD, Yuen HA, West KA, Giles FJ, Albitar M, Cherrington JM. The antiangiogenic protein kinase inhibitors SU5416 and SU6668 inhibit the SCF

receptor (c-kit) in a human myeloid leukemia cell line and in acute myeloid leukemia blasts. Blood. 2001 Mar 1;97(5):1413-21.

Soker S, Takashima S, Miao HQ, Neufeld G, Klagsbrun M. Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor.Cell. 1998 Mar 20;92(6):735-45.

Soldi R, Mitola S, Strasly M, Defilippi P, Tarone G, Bussolino F. Role of alphavbeta3 integrin in the activation of vascular endothelial growth factor receptor-2. EMBO J. 1999 Feb 15;18(4):882-92.

Sondell M, Lundborg G, Kanje M. Vascular endothelial growth factor has neurotrophic activity and stimulates axonal outgrowth, enhancing cell survival and Schwann cell proliferation in the peripheral nervous system. J Neurosci. 1999 Jul 15;19(14):5731-40.

Sutter CH, Laughner E, Semenza GL. Hypoxia-inducible factor 1alpha protein expression is controlled by oxygen-regulated ubiquitination that is disrupted by deletions and missense mutations. Proc Natl Acad Sci U S A. 2000 Apr 25;97(9):4748-53.

Tai YT, Podar K, Gupta D, Lin B, Young G, Akiyama M, Anderson KC. CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells. Blood. 2002 Feb 15;99(4):1419-27.

Taichman NS, Young S, Cruchley AT, Taylor P, Paleolog E. Human neutrophils secrete vascular endothelial growth factor. J Leukoc Biol. 1997 Sep;62(3):397-400.

Takahashi T, Ueno H, Shibuya M. VEGF activates protein kinase C-dependent, but Ras-independent Raf-MEK-MAP kinase pathway for DNA synthesis in primary endothelial cells. Oncogene. 1999 Apr 1;18(13):2221-30.

Terman BI, Carrion ME, Kovacs E, Rasmussen BA, Eddy RL, Shows TB. Identification of a new endothelial cell growth factor receptor tyrosine kinase. Oncogene. 1991 Sep;6(9):1677-83.

Till KJ, Cawley JC. Accessory cells mediate hairy-cell proliferation by mechanism(s) involving both adhesion and TNF alpha secretion. Br J Haematol. 1994 Aug;87(4):687-94.

Till KJ, Spiller DG, Harris RJ, Chen H, Zuzel M, Cawley JC. CLL, but not normal, B cells are dependent on autocrine VEGF and alpha4beta1 integrin for chemokine-induced motility on and through endothelium. Blood. 2005 Jun 15;105(12):4813-9.

Tischer E, Mitchell R, Hartman T, Silva M, Gospodarowicz D, Fiddes JC,Abraham JA. The human gene for vascular endothelial growth factor.

Multiple protein forms are encoded through alternative exon splicing. J Biol Chem. 1991 Jun 25;266(18):11947-54.

Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG, Kerbel RS. Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. Biochem Biophys Res Commun. 1999 Nov 2;264(3):781-8.

Vacca A, Ribatti D, Roncali L, Ranieri G, Serio G, Silvestris F, Dammacco F. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol. 1994 Jul;87(3):503-8.

Van Kooten C, Banchereau J. CD40-CD40 ligand: a multifunctional receptor-ligand pair. Adv Immunol. 1996;61:1-77. Review.

Vilpo J, Tobin G, Hulkkonen J, Hurme M, Thunberg U, Sundstrom C, Vilpo L, Rosenquist R. Surface antigen expression and correlation with variable heavy-chain gene mutation status in chronic lymphocytic leukemia. Eur J Haematol. 2003 Jan;70(1):53-9.

Wang D, Freeman GJ, Levine H, Ritz J, Robertson MJ. Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. Br J Haematol. 1997 May;97(2):409-17.

Wang GL, Jiang BH, Rue EA, Semenza GL. Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension. Proc Natl Acad Sci U S A. 1995 Jun 6;92(12):5510-4.

Wang GL, Semenza GL. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem. 1995 Jan 20;270(3):1230-7.

Wang L, Chen L, Benincosa J, Fortney J, Gibson LF. VEGF-induced phosphorylation of Bcl-2 influences B lineage leukemic cell response to apoptotic stimuli. Leukemia. 2005 Mar;19(3):344-53.

Weidner N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. Am J Pathol. 1993 Aug;143(2):401-9.

West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic acid. Science. 1985 Jun 14;228(4705):1324-6.

Whitaker GB, Limberg BJ, Rosenbaum JS. Vascular endothelial growth factor receptor-2 and neuropilin-1 form a receptor complex that is responsible for the differential signaling potency of VEGF(165) and VEGF(121). J Biol Chem. 2001 Jul 6;276(27):25520-31.

Wiesmann C, Fuh G, Christinger HW, Eigenbrot C, Wells JA, de Vos AM. Crystal structure at 1.7 A resolution of VEGF in complex with domain 2 of the Flt-1 receptor. Cell. 1997 Nov 28;91(5):695-704.

Wood JM, Bold G, Buchdunger E, Cozens R, Ferrari S, Frei J, Hofmann F, Mestan J, Mett H, O'Reilly T, Persohn E, Rosel J, Schnell C, Stover D, Theuer A, Towbin H, Wenger F, Woods-Cook K, Menrad A, Siemeister G, Schirner M, Thierauch KH, Schneider MR, Drevs J, Martiny-Baron G, Totzke F. PTK787/ZK 222584, a novel and potent inhibitor of vascular endothelial growth factor receptor tyrosine kinases, impairs vascular endothelial growth factor-induced responses and tumor growth after oral administration. Cancer Res. 2000 Apr 15;60(8):2178-89.

Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H, Kuwano M. Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. Mol Cell Biol. 1997 Jul;17(7):4015-23.

Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res. 1996 May 1;56(9):2013-6.

Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX, Kroemer G. Reduction in mitochondrial potential constitutes an early

irreversible step of programmed lymphocyte death in vivo. J Exp Med. 1995 May 1;181(5):1661-72.

Zeng H, Sanyal S, Mukhopadhyay D. Tyrosine residues 951 and 1059 of vascular endothelial growth factor receptor-2 (KDR) are essential for vascular permeability factor/vascular endothelial growth factor-induced endothelium migration and proliferation, respectively. J Biol Chem. 2001 Aug 31;276(35):32714-9.

Zhong H, Agani F, Baccala AA, Laughner E, Rioseco-Camacho N, Isaacs WB, Simons JW, Semenza GL. Increased expression of hypoxia inducible factor-1alpha in rat and human prostate cancer. Cancer Res. 1998 Dec 1;58(23):5280-4.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW. Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res. 1999 Nov 15;59(22):5830-5.

Appendix

Papers arising from this work

This section contains the two published papers arising from the work presented in this thesis, and are given in PDF format.

In vitro and in vivo production of vascular endothelial growth factor by chronic lymphocytic leukemia cells

Haijuan Chen, Andy T. Treweeke, Dave C. West, Kathleen J. Till, John C. Cawley, Mirko Zuzel, and Cheng H. Toh

Expansion of primary solid tumors and their malignant dissemination are angiogenesis-dependent. Vascular endothelial growth factor (VEGF) is the key factor playing a pivotal role in solid tumor-induced angiogenesis. Recent studies indicate that angiogenesis may also be involved in the pathogenesis of certain hemic malignancies, including B-cell chronic lymphocytic leukemia (B-CLL). Mechanisms underlying angiogenesis in B-CLL and the role of VEGF in this process are incompletely understood. In this study, it was examined whether angiogenically functional VEGF is produced by B-CLL cells. Immunohistochemical staining with antibodies against VEGF and CD34, an endothelial cell marker, demonstrated the presence of VEGF protein and abundant blood vessels in infiltrated lymphoreticular tissues. Low levels of VEGF were detected by ELISA in the culture media of unstimulated cells; this was enhanced up to 7-fold by hypoxic stimulation. SDS-PAGE and Western blot analysis of the concentrated culture media showed 2 isoforms of VEGF protein with molecular weights of 28 and 42 kd, respectively. RNA hybridization showed that these cells expressed VEGF mRNA. Reverse transcription–polymerase chain reaction, combined with nucleotide sequence analysis, revealed that the predominantly expressed isoforms were VEGF121 and VEGF165. Moreover, ^{3}H-thymidine incorporation and an in vivo angiogenic assay demonstrated that the VEGF produced by CLL cells can induce angiogenesis by stimulating endothelial cell proliferation. In conclusion, this study shows that B-CLL cells produce VEGF and demonstrates the angiogenic effects of this growth factor, which may be relevant for the tissue phase of the disease. (Blood. 2000;96: 3181-3187)

Introduction

Angiogenesis is the process in which new microvessels form from preexisting ones.[1] It has been well established that the expansion and dissemination of primary solid tumors are angiogenesis-dependent[2,3] and that vascular endothelial growth factor (VEGF) is one of the most potent factors involved in this process.[4] Various solid tumors, including breast, lung, colon, and prostate carcinomas, secrete VEGF,[5-8] and capillaries are found clustered along VEGF-producing tumor cells.[9] Tumor angiogenesis and subsequent tumor growth are inhibited in vivo by antibodies directed against VEGF,[10] by soluble VEGF receptors,[11] and by expression of dominant-negative VEGF receptors.[12] These properties have made the study of VEGF function important for the understanding of the angiogenesis associated with solid tumors.

Recent studies have suggested that angiogenesis may also be involved in the pathogenesis of certain hemic malignancies. A study[13] of 88 patients with B-cell non-Hodgkin lymphoma showed an increase in microvessel density in lymph nodes that correlated with the severity of the disease. Another[14] of childhood acute lymphocytic leukemia revealed an increased microvessel density in the patient's bone marrow compared with normal tissue. Increases in bone marrow microvessel density were also observed in a series of patients diagnosed with multiple myeloma and hairy-cell leukemia.[15,16]

B-cell chronic lymphocytic leukemia (B-CLL), the most common adult leukemia in Western Europe and North America, is characterized by a persistent lymphocytosis in the peripheral blood and by progressive infiltration of lymphoreticular tissue by malignant cells.[17] In fact, increased vessel density has been observed in bone marrow from patients with B-CLL.[18] The current study explores whether angiogenesis takes place in the expanding secondary lymphoid tissues of the disease and the role of VEGF in this process.

Although VEGF protein has been detected in serum from patients with B-CLL,[19] whether the CLL cells themselves make VEGF protein remains controversial. By using Northern blot analysis and quantitative reverse transcription–polymerase chain reaction (RT-PCR) techniques, one group[20] detected VEGF mRNA in the peripheral lymphocytes of 6 patients with B-CLL. However, another group[21] could not detect any VEGF mRNA in B-CLL cells using an in situ hybridization technique.

In the current study, we show that B-CLL cells express VEGF mRNA and produce VEGF protein. Our functional studies reveal that the VEGF produced by B-CLL cells stimulates endothelial cell proliferation and in vivo angiogenesis. Furthermore, VEGF secretion by B-CLL cells was enhanced by hypoxic stimulation, a fundamental stimulus for neovascularization in tumor microenvironments.[22]

Patients, materials, and methods

Patients

All patients had typical CLL as defined morphologically (prolymphocytes less than 10%) and immunophenotypically (CD19^{+}, CD5^{+}, CD23^{+}, and

From the Departments of Haematology and Immunology, University of Liverpool, Liverpool, United Kingdom.

Submitted January 24, 2000; accepted June 29, 2000.

Supported in part by the Leukemia Research Fund and the North West Cancer Research Fund.

Reprints: Haijuan Chen, Department of Haematology, University of Liverpool, PO Box 147, Liverpool L69 3BX, United Kingdom; e-mail: hjchen@liverpool.ac.uk.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 U.S.C. section 1734.

weak light-chain–restricted surface immunoglobulin). Clinical details are shown in Table 1. The table shows that the patients constituted a mixed group with regard to stage and treatment. Approximately half had early-stage disease (Rai, 0 and 1; Binet, A), and only 2 patients (CLL17, CLL18) displayed a clearly progressive course. Three of the patients (CLL11, CLL15, CLL16) had newly diagnosed disease and therefore the progressiveness of their cases is as yet unknown. All materials were obtained with informed consent.

Cell preparation and culture

B-CLL cells and hypoxia treatment. Peripheral blood (PB) from 18 patients with high count (white blood cell count greater than 100×10^9/L) was drawn into heparin-containing tubes (20 U/mL). PB lymphocytes were isolated by Ficoll-Hypaque (Lymphoprep, Oslo, Norway) density-gradient centrifugation and cryopreserved.

T cells and monocytes were always less than 5%, and, of the remaining cells, more than 95% were $CD5^+$ CLL cells. To ensure that cryopreservation did not affect VEGF production, in 3 patients both fresh and cryopreseved cells were studied. In all cases, unstimulated and stimulated VEGF production by fresh and cryopreserved cells was fully comparable. To recover cells from liquid nitrogen, they were rapidly thawed in a 37°C water bath and slowly resuspended in 10 mL QBSF-51 serum-free medium (Sigma-Aldrich, Dorset, UK). After centrifugation (1600 rpm for 5 minutes), the cell pellets were resuspended in the same medium before use.

For hypoxic stimulation, B-CLL cells were cultured for 24 hours in a specially designed air-tight chamber (Billups-Rothenberg, Del Mar, CA) that was prewarmed at 37°C overnight. The chamber, constructed with inflow and outflow valves, was infused with a preanalyzed air mixture containing 5% CO_2/95% N_2 (BOC, Surrey, UK) to achieve the desired oxygen level.[23] To determine their viability, the cells were incubated with 5 μg/mL propidium iodide for 20 minutes at 4°C and then analyzed by flow cytometry. Propidium iodide stains dead cells but not live cells.

Human umbilical vein endothelial cells. Umbilical cords were generously provided through the maternity unit of Liverpool Women's Hospital (Liverpool, UK). Human umbilical vein endothelial cells (HUVECs) were detached with trypsin using a published protocol.[24] Cells were cultured in 199 medium (Gibco Life Technologies, Paisley, UK) supplemented with 20% fetal calf serum, 1 pg/mL epidermal growth factor, 5 U/mL heparin, 100 U/mL penicillin, and 100 μg/mL streptomycin. HUVECs were used at passage 3.

Preparation of conditioned medium. Unless stated otherwise, the conditioned medium (CM) was the culture supernatant harvested from B-CLL cells. The cells were cultured in QBSF-51 at a density of 2×10^6/well in 24-well plates for 24 hours under either normoxic or hypoxic conditions. CM was centrifuged at 1600 rpm for 10 minutes and stored at −20°C until assayed. To test whether CLL-cell–derived CM contained angiogenic factor(s), the CM harvested from the cells stimulated with phorbol 12-myristate 13-acetate (PMA, 100 ng/mL) was concentrated by ammonium sulfate precipitation and a Centricon (Millipore, Hertfordshire, UK) spin column before assay. As a control, QBSF-51 medium not exposed to cells (referred as the control medium) was concentrated in a similar fashion.

Table 1. Patient characteristics

Patient	Stage at diagnosis		Stage at study		Prior treatment
	Binet	Rai	Binet	Rai	
CLL1	A	I	A	0	CLB + pred
CLL2	C	IV	C	IV	CLB + pred; flu
CLL3	A	0	A	0	CLB
CLL4	A	I	A	I	CLB + pred
CLL5	A	I	A	I	CLB + pred
CLL6	C	IV	C	IV	CLB; CHOP; etoposide
CLL7	C	IV	C	IV	CLB; cyclo + pred
CLL8	A	I	A	I	None
CLL9	A	0	A	0	CLB; flu
CLL10	C	III	C	III	None
CLL11	A	0	A	0	None
CLL12	B	II	B	II	None
CLL13	A	0	A	0	None
CLL14	A	I	A	I	None
CLL15	C	III	C	III	None
CLL16	A	0	A	0	None
*CLL17	B	II	C	IV	CLB; CHOP; flu
*CLL18	A	I	B	II	CLB + flu
†CLL19	A	I	A	I	None
‡CLL20	B	II	B	II	pred; CLB + pred; cyclo + vincristine + pred
‡CLL21	A	I	A	I	CLB + pred

CLB indicates chlorambucil; CHOP, cyclophosphamide + doxorubicin + vincristine + prednisolone; pred, prednisolone; flu, fludarabine; cyclo, cyclophosphamide.
*Both PB cells and node were studied.
†Only node examined.
‡Only spleen examined.

Tissues

CLL nodes (n = 3) were diagnostic samples taken from the neck; 2 of the 3 nodes were from patients (CLL17, CLL18) whose PB CLL cells were also studied for VEGF production. Clinical details of all the patients from whom this material was obtained are given in Table 1. Splenectomy material (CLL20, CLL21) had been removed for treatment of autoimmune hemolytic anemia secondary to CLL. Normal nodes (n = 3) were obtained from axillary clearances for breast cancer and were macroscopically and microscopically normal. "Normal" spleen was tissue removed for the treatment of idiopathic thrombocytopenic purpura (n = 2) or because of surgical trauma during laparotomy (n = 1). All tissues were formalin-fixed and paraffin-embedded.

Immunostaining

CD34 and VEGF immunohistochemistry. Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissues. Briefly, sections were deparaffinized, rehydrated, and boiled in 10 mmol/L sodium citrate (pH 6.0) for 10 minutes. Nonspecific antibody-binding sites were blocked by incubating the sections in 10% bovine serum albumin in TBS (20 mmol/L Tris/HCl [pH 7.6], 137 mmol/L NaCl) for 10 minutes at room temperature (RT). For the detection of VEGF, sections were incubated overnight at 4°C with either rabbit anti-hVEGF antibody (1:200 dilution in TBS) or with nonspecific rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA). For blood vessel staining, sections were incubated overnight at 25°C with mouse anti-hCD34 mAb (Serotec, Oxford, UK; 1:10 dilution in TBS) or with nonspecific mouse IgG (Becton Dickinson, UK). The slides were then incubated with goat antirabbit IgG (for VEGF Ab) or rabbit antimouse IgG (for CD34 mAb) conjugated to biotin, followed by incubation with ExtrAvidin alkaline phosphatase (both 30 minutes at RT). Color was developed by incubating slides with Fast Red TR/Naphthol AS-MX phosphate (Sigma) at RT for 20 minutes. Slides were counterstained with hematoxylin.

The finding of 3 or more contiguous $CD34^+$ cells was considered as a microvessel.[25] Such microvessels were counted in 40 high-power (40×) fields. The fields were chosen at random in CLL nodes. In normal nodes, 20 follicular and 20 interfollicular fields in different areas of cortex were scored. Vessel scores were expressed as the mean ± SD per field.

Immunocytochemical staining for VEGF. B-CLL cells (8×10^4) cultured for 24 hours with and without PMA (100 ng/mL) were cytocentrifuged at 300 rpm for 3 minutes using a Cytospin 2 (Shandon, Pittsburgh, PA) and fixed at 4°C with ice-cold acetone for 3 minutes. Fixed slides were washed with phosphate-buffered saline (PBS) and incubated with 3% H_2O_2 in PBS for one hour at RT. Cells were then incubated with polyclonal rabbit anti-hVEGF antibody (10 μg/mL diluted with 1% BSA/PBS) or with nonspecific rabbit IgG (Santa Cruz Biotechnology) for one hour at RT, followed by incubation with goat antirabbit IgG conjugated to biotin and with ExtrAvidin–horseradish peroxidase (both for 30 minutes at RT). Color was developed by incubating the slides with 3-amino-9-ethylcarbazol at RT for 3 minutes. Slides were counterstained with hematoxylin.

Enzyme-linked immunosorbent assay. The CM (200 μL) harvested from CLL cells cultured under hypoxic or normoxic conditions was tested for the presence of soluble VEGF protein by a commercial human VEGF immunoassay kit (Quantikine) according to the manufacturer's protocol (R & D Systems, Abingdon, UK). With this method, we found that the minimum detectable dose of VEGF contained in CM was typically less than 5.0 pg/mL.

Western blot analysis. The concentrated CM collected from PMA-stimulated cells was fractionated by electrophoresis on 10% SDS polyacrylamide gels under nonreducing conditions and electrotransferred to nitrocellulose membranes. After blocking with 5% nonfat powdered milk in TBS containing 0.1% Tween-20 (TBS-T), the membranes were incubated for one hour at RT with anti-hVEGF mAb (2 μg/mL; R & D Systems). After washing with TBS-T, the membranes were incubated for one hour at RT with horseradish peroxidase–conjugated goat antimouse IgG (Affiniti, Exeter). After an extensive rinsing with TBS-T, immunoreactive protein bands were visualized with a chemiluminescence-based procedure using the ECL detection kit according to the instructions of the manufacturer (Amersham, Little Chalfont, UK).

RNA isolation and reverse transcription–polymerase chain reaction. Total RNA was extracted from CLL cells using an RNeasy kit (Qiagen, Hilden, Germany). Single-strand DNA was synthesized from 1 μg total RNA using the First-Strand cDNA Synthesis Kit for RT-PCR (Boehringer Mannheim, Mannheim, Germany). Amplification of the cDNA by PCR was performed using the following primers: oligo 1, 5′-TCGGGCCTC-CGAAACCATGC-3′; and oligo 2, 5′-CCTGGTGAGAGATCTGGTTC-3′ (30 cycles, annealing at 56°C). To normalize the product, the actin gene was also amplified using actin primers: oligo 3, 5′-CGCTGCGCTGGTCGTC-GACA-3′; and oligo 4, 5′-GTCACGCACGATTTCCCGCT-3′ (30 cycles, annealing at 60°C). The amplified PCR products were subjected to electrophoresis and visualized by ethidium bromide staining. To confirm that the sequences of the products amplified by the PCR reaction represented different VEGF isoforms, the PCR products were cloned into pBlueScript plasmid (Stratagene, Cambridge, UK), and their sequences were determined by nucleotide sequence analysis using a DNA Sequencing kit (Amersham).

Slot-blot hybridization. Total RNA (2 μg) extracted from unstimulated cells was loaded directly onto a nylon membrane using a slot-blot apparatus (Bio-Rad, Hercules, CA). Membranes were UV cross-linked and subsequently hybridized with a VEGF cDNA probe labeled with α-^{32}P]-deoxycytidine triphosphate using a random-primed DNA labeling kit (Boehringer Mannheim). For control of the RNA loading of each lane, blots were rehybridized with a radiolabeled glyceraldehyde 3-phosphate dehydrogenase cDNA probe (Clontech Laboratory, Hampshire, UK). Autoradiography was performed at −70°C using Kodak XAR-5 film (Sigma). The intensity of each band appearing on the film was scanned and analyzed with Phoretix 1D-advanced version 3.1 software.

Proliferation assay. HUVECS were plated at a density of 2×10^4/well in 24-well gelatin-coated plates and grown to approximately 90% confluence in the growth medium. The following day, the medium was removed from the wells, and the cells were washed twice with PBS and incubated for another 24 hours in 199 medium containing 5% fetal calf serum without heparin or EGF. Then the medium was replaced with the fresh medium containing 10% (vol/vol) concentrated CM derived from CLL cells or rhVEGF 165 protein (R & D Systems). In addition, the CM was preincubated for 1 hour at 22°C with a neutralizing anti-hVEGF MoAb (1 μg/mL, R & D Systems) before addition to the cultures. After 48 hours, the cells were incubated for 3 hours with ^{3}H-thymidine (0.5 μCi/well), the medium was aspirated, and the cells were washed twice with ice-cold PBS. Ice-cold 5% TCA was added to each well (0.5 mL/well), and the plates were placed at 4°C. After overnight incubation, TCA was aspirated, and cell monolayers were washed once with TCA and twice with 95% ethanol. To each dried well, 0.2 mol/L NaOH (0.5 mL/well) was then added to dissolve TCA-insoluble materials (37°C, 2 hours with gentle shaking). The samples (0.3 mL) were counted to determine the amount of ^{3}H-thymidine taken up by the cells using a Tri-CARB Liquid Scintillation Analyzer (Packard, England).

Chick chorioallantoic membrane assay. The chick chorioallantoic membrane (CAM) assay[26] was used to determine whether the CLL cell–derived CM had angiogenic activity. To expose CAM, a window was created in the shells of 10-day-old fertilized chicken eggs. Filter paper disks soaked in test CM were placed on exposed CAMs. Disks soaked in rhVEGF 121 (10 ng in 10 μL; R & D Systems) or in control medium were used as positive and negative controls, respectively. The embryos were incubated at 37°C in a humidified egg incubator. CAMs were analyzed after 72 hours using a stereomicroscope. The density of branching blood vessels infiltrating under the disks was scored as follows: 0, negative; 0.5, change in vessel architecture but not directed to the point of sample application; 1, partial spoke-wheel (one third of the circumference exhibits directional angiogenesis); 2, spoke-wheel; 3, strong and full spoke-wheel. For photography, the membranes were fixed in situ with ice-cold 4% paraformaldehyde–PBS that was injected both from above and below the membrane. Membranes were excised, placed on a fresh microscope slide, and photographed under a Leitz binocular dissecting microscope and indirect fiberoptic illumination. Statistical analysis was performed using the Mann-Whitney *U* test.

Results

High blood vessel density is observed in lymph nodes infiltrated by B-CLL cells. Blood vessel density in lymph nodes from patients with B-CLL (n = 3) and healthy control donors (n = 3) was examined by using CD34 as an endothelial cell marker. In CLL, the normal architecture of the enlarged nodes is completely effaced by infiltrating CLL cells. Figure 1, panel Ai shows representative CD34 staining of a CLL node, indicating that vascular development accompanies the malignant cell infiltration and node enlargement. In contrast, in normal node (Figure 1Aii), only the interfollicular areas are vascularized, whereas follicular B-cell areas display little vascularization. This is quantitatively illustrated in Figure 1, panel B. These results indicate that, as proposed before for CLL bone marrow,[18] angiogenesis may be required to supply oxygen and nutrients to the enlarged nodes often seen in CLL patients with tissue disease.

Both circulating and tissue-phase CLL cells produce VEGF protein. In view of the importance of tumor cell–derived VEGF in

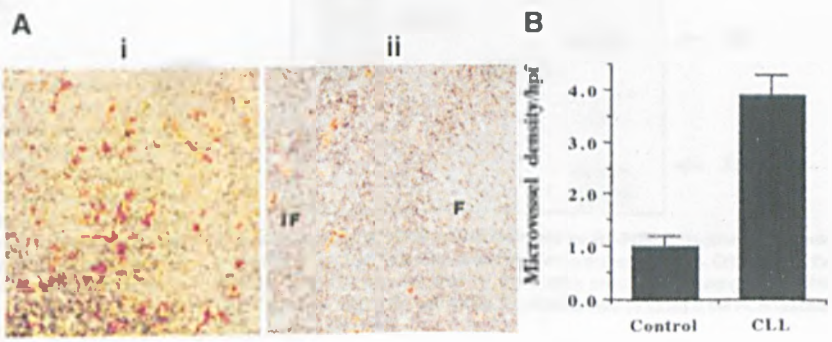


Figure 1. Vessel density analysis using CD34 mAb staining. (Ai) Representative CD34 staining of CLL node. (Aii) CD34 staining of normal node where interfollicular (IF) areas are vascularized, and follicular (F) areas display very little vascularization. Original magnification, × 10. (B) Quantitative analysis shows the higher vessel density in CLL nodes (n = 3) compared with follicular B-cell areas of normal nodes (n = 3).

solid tumor angiogenesis,[27] we next investigated the possible production of VEGF protein by B-CLL cells. After 24-hour incubation at 37°C, the CM was collected and examined for VEGF protein using an enzyme-linked immunosorbent assay (ELISA). Secreted VEGF was detected in all cases as demonstrated in Figure 2, panel A. This result shows that all tested B-CLL cells are capable of constitutively synthesizing and secreting variable amounts of VEGF (5-116 pg/8 × 10^6 cells/mL). Because of the relatively small number of patients studied and because of their heterogeneity with regard to disease stage and duration, no attempt was made to correlate levels of VEGF production with clinical parameters.

Because PMA is known to stimulate VEGF production in other cell types,[28] we examined whether basal VEGF secretion by CLL cells could be increased by phorbol ester stimulation. PMA increased secreted VEGF levels from 49 ± 59 pg/mL to 266 ± 110 pg/mL (n = 7). Therefore, PMA stimulation was used to obtain enough CLL-cell–derived VEGF to be readily detectable by Western blot analysis (after 10-fold concentration). As shown in Figure 2, panel B, Western blotting using an anti-hVEGF mAb revealed 2 bands with molecular weights of 28 and 42 kd. These are the only known secreted isoforms of the cytokine encoded by the VEGF121 and 165 mRNA species demonstrated below (by RT-PCR) in unstimulated CLL cells.

We then tested whether CLL cells that have infiltrated lymphoreticular tissues produce VEGF protein. Immunohistochemical staining (Figure 3) showed that CLL cells infiltrating lymph node (A) and spleen (C) are positive for VEGF protein. CLL cells were uniformly positive, and the intensity of staining was weaker than that of any neutrophils present but stronger than that of the unstimulated PB CLL cells as described below. Figure 3 also shows positive staining of B-cell areas of normal node (B) and spleen (D).

VEGF production in CLL samples is not due to contamination by non-CLL cells. It has been shown that many hemic-cell types (eg, T lymphocytes,[29] monocytes,[30] and platelets[31]) contain VEGF protein. Therefore, it was important to verify that the observed production of VEGF protein in CLL samples was not due to contamination with other cell types. To address this, we used immunocytochemical staining to examine the VEGF protein positivity of the cells in the CLL samples used. The purity of CLL cells in all samples was greater than 95%. The staining showed that all unstimulated cells stained uniformly weakly for VEGF protein and that no minority population of strongly positive cells was observed. After stimulation with PMA, all CLL cells showed a substantial increase in VEGF positivity. These results (data not shown) indicated that the VEGF protein detected in the CLL cell cultures was produced by the CLL cells themselves.

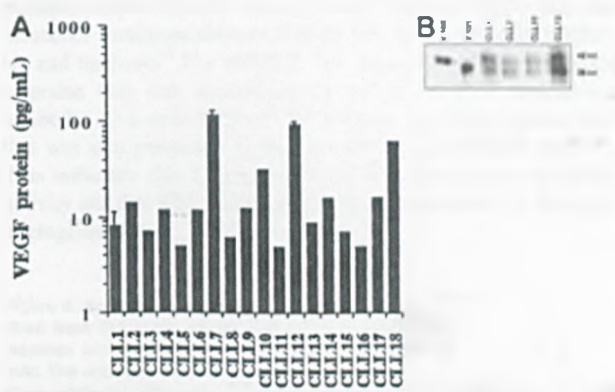


Figure 2. Detection of VEGF protein. (A) ELISA measurement of secreted VEGF. Levels of VEGF in the CM collected from unstimulated CLL cells were measured using a commercial ELISA kit. The amount of VEGF protein in the samples was calculated using a reference curve established from serial dilutions of rhVEGF protein. Individual histograms represent the means of duplicates. Error bars are standard deviations calculated from 6 measurements in 3 separate experiments using the cells of 3 different patients. (B) Western blot analysis of secreted VEGF. CM was collected from cultures of the PMA-stimulated cells and subsequently concentrated by 10-fold. Each sample (100 μL) was loaded onto a 10% polyacrylamide gel and electrophoresed under nonreducing conditions. Separated proteins were transferred to a nitrocellulose membrane, and VEGF protein was detected with anti-hVEGF mAb and visualized by enhanced chemiluminescence.

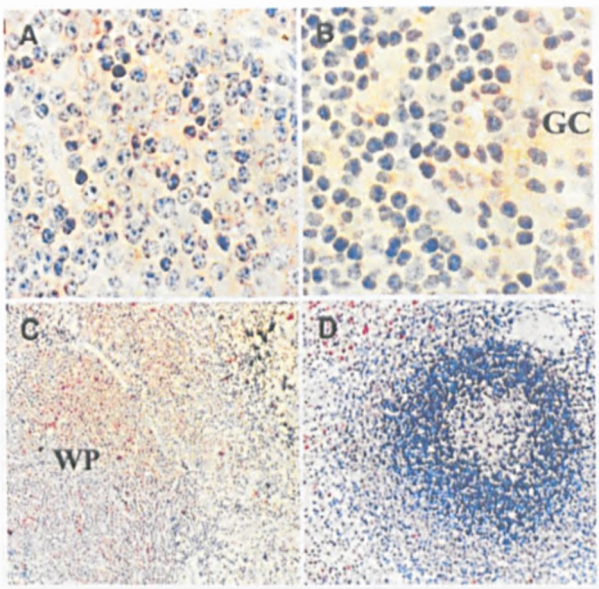


Figure 3. Immunohistochemical staining of VEGF protein. The presence of VEGF protein in CLL nodes (n = 3; CLL17 shown) and spleen (n = 2; CLL20 shown), together with corresponding normal tissues (n = 3), was examined using anti-VEGF antibody staining. Infiltrating cells in CLL lymph node (A) are all positive. Many cells in normal node (B) are also positive, particularly those in the germinal center (GC) of the follicles. (Original magnification, × 40.) CLL cells expanding the white pulp (WP) of the spleen (C) are also uniformly positive. In contrast, in normal spleen (D), the different layers of the white pulp show various degrees of positivity that was stronger in the marginal zone and follicle centers. (Original magnification, × 10.)

B-CLL cells express 2 smaller VEGF transcripts. There are 5 different VEGF mRNA isoforms generated from the VEGF gene by alternative splicing mechanisms.[32-34] To investigate whether isoforms additional to the 2 detected by Western blotting are expressed in unstimulated CLL cells, both RT-PCR and nucleotide sequence analysis were performed. As shown in Figure 4, only 2 bands were amplified from CLL cells, and they were identical in

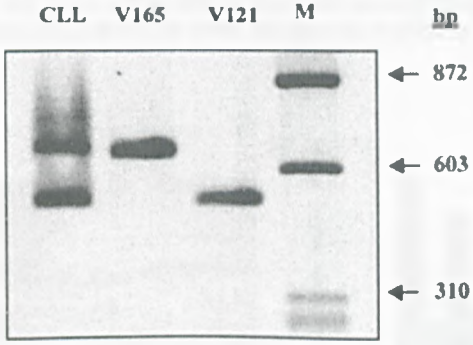


Figure 4. Analysis of VEGF mRNA isoforms by RT-PCR. First-strand cDNA was transcribed from 1 μg total RNA extracted from unstimulated cells. One tenth of the synthesized cDNA was amplified by PCR using primers recognizing all possible VEGF isoforms. VEGF 165 and VEGF 121 cDNAs were included in the PCR reaction as the positive controls.

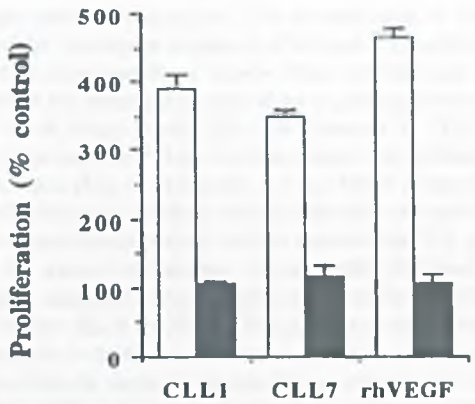


Figure 5. Proliferation of endothelial cells in response to CLL-cell–derived CM. HUVECS were cultured in the presence of CM derived from the PMA-stimulated cells of 2 CLL patients (CLL1, CLL7), using rhVEGF protein as a positive control (□). In the same experiment, the CM was preincubated with a neutralizing anti-hVEGF mAb for 1 hour at RT before addition to endothelial cell cultures (■). After 48 hours, proliferation was assessed by ^{3}H-thymidine incorporation. Proliferation of cells cultured in the medium without CM was considered as 100%; blocking anti-hVEGF mAb had no effect on this proliferation.

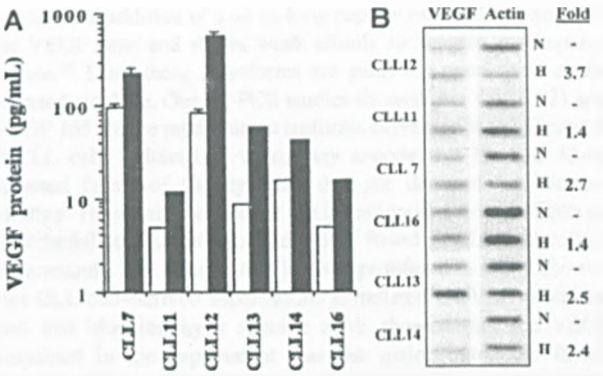


Figure 7. VEGF production by CLL cells in response to hypoxic stimulation. (A) CLL cells from 6 patients were cultured under normoxic and hypoxic conditions. After 24 hours, CM was collected and subjected to ELISA. Higher levels of VEGF protein were detected in the cells cultured under hypoxic conditions (■) than in those grown under normoxic conditions (□). Histograms represent the mean of duplicates. Error bars are standard deviations calculated from 6 measurements in 3 separate experiments using the cells of 2 patients. (B) Cells from the same patients used for ELISA were also subjected to cellular VEGF mRNA determination by slot-blot hybridization. Hypoxia (H) compared with normoxia (N) increased VEGF mRNA expression in all cases. The bands were measured densitometrically, and the sample loading was determined by parallel measurement of the actin mRNA in each sample.

size to control VEGF 121 and 165 cDNA. Furthermore, nucleotide sequence analysis confirmed that the top band was VEGF 165 and the bottom band was VEGF 121 (data not shown).

VEGF produced by CLL cells stimulates proliferation of endothelial cells. To examine whether the VEGF secreted by CLL cells stimulates endothelial cell proliferation, HUVECS were incubated for 48 hours with CM from CLL cell cultures, and cell proliferation was assessed by ^{3}H-thymidine uptake. As shown in Figure 5, CLL-cell–derived CM increased endothelial cell proliferation by 3- to 4-fold. The addition of an anti-hVEGF neutralizing mAb completely blocked this growth-stimulatory effect. This demonstrates that the mitogenic effect of CLL-cell–derived CM is principally mediated by VEGF.

VEGF produced by CLL cells enhances angiogenesis. To test whether CLL-cell–secreted VEGF affects in vivo angiogenesis, the CAM assay was used with angiogenesis by rhVEGF 121 as a positive control. Results were assessed visually (Figure 6A) and measured semiquantitatively (Figure 6B), as described in "Materials and methods." The rhVEGF 121 induced a strong angiogenic response that was neutralized by an anti-hVEGF neutralizing antibody. CLL-cell–derived CM induced moderate angiogenesis that was also prevented by the neutralizing anti-hVEGF antibody. This indicates that CLL-cell–derived CM does have angiogenic activity and that VEGF contained in these supernatants is the major angiogenic factor.

Hypoxia enhances the levels of VEGF mRNA and protein in CLL cells. One known mechanism of VEGF gene regulation is the low oxygen tension (hypoxia) often detected in rapidly expanding tissues.[22] An increase in VEGF production and resultant angiogenesis in response to hypoxia are presumably required to allow adequate metabolic support of expanding tissues, including infiltrated CLL lymphoreticular tissues. Because CLL cells are the principle cells in these organs, we investigated whether they respond to hypoxia by up-regulating their levels of VEGF mRNA and protein. After 24 hours, the percentage of viable cells cultured under normoxic and hypoxic conditions was 79% ± 6% and 86% ± 3%, respectively (n = 3; viability determined by propidium iodide staining and FACS analysis). The CM collected from the cultured cells was subjected to ELISA assay. Results demonstrated that hypoxia elevated VEGF protein levels to 7-fold in all patients with CLL (n = 6; Figure 7A). In addition, hypoxia also increased the level of VEGF mRNA in each sample as determined by slot-blot hybridization (Figure 7B).

Discussion

During the course of their illness, many patients with CLL display prominent lymphoreticular tissue enlargement in addition to blood

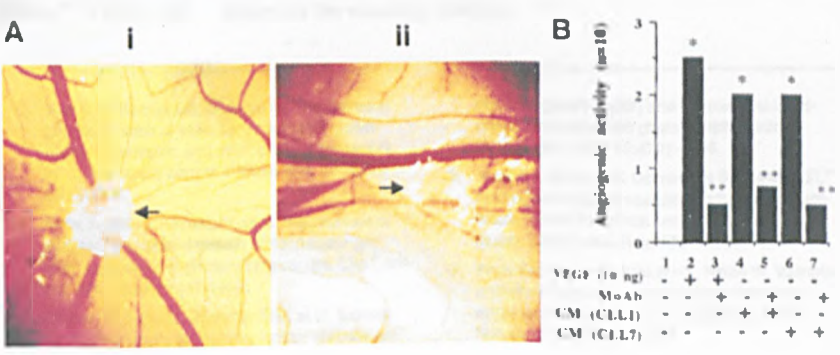


Figure 6. Angiogenic responses of CAM to CM derived from CLL cells. (A) Representative angiogenic response and its inhibition by a blocking anti-hVEGF mAb. The white area represents the site of application of filters containing either CM (collected from the PMA-stimulated cells) alone (i) or the same CM preincubated with anti-hVEGF MoAb for 2 hours at RT (ii). Microvessels radiating from the site of sample application in (i) are not formed in the presence of the blocking mAb (ii). (Original magnification, × 8.) (B) The semiquantitative data obtained by using rhVEGF and CM collected from PMA-stimulated cells from 2 patients. The grading of the angiogenic response is described in "Materials and methods." *$P < .05$ vs control and $P < .05$ vs sample without the neutralizing antibody (Mann-Whitney U test). Each bar represents the score of 10 CAM used in 2 separate assays with similar results.

and bone marrow involvement. The accumulation of malignant cells and the consequent expansion of secondary lymphoid tissues demand an increased blood supply. Thus, angiogenesis may be required for the metabolic support of the expanding tissues. In fact, higher blood vessel density has been observed in CLL than in normal bone marrow.[18] Here, we demonstrate that neovascularization also takes place in CLL nodes and that VEGF produced by the CLL cells themselves is likely to be the principle angiogenic factor.

Our immunohistochemical studies showed that CLL nodes, in which the normal architecture is completely disrupted by the infiltrating malignant cells, are uniformly vascularized. This indicates that new blood vessels are formed as the node expands. The vessel density in the CLL nodes was comparable to that observed in the interfollicular areas of normal nodes, whereas the follicular B-cell areas of normal nodes displayed a very low vessel density. This is likely to reflect the fact that most follicular B cells undergo apoptosis and that the remaining antigen-selected cells migrate out of the follicle. In contrast, the chronic nature of CLL node enlargement indicates the more permanent nature of the infiltrate requiring neovascularization. Because the overwhelming majority of cells in CLL nodes are malignant B cells, we postulated that the angiogenic stimulus may be provided by the CLL cells themselves.

In considering possible angiogenic factors, we chose to focus on VEGF. VEGF is a multifunctional protein that, on binding to its receptors on endothelial cells, affects vascular permeability,[35-37] cell proliferation,[38,39] migration,[40] and survival,[41,42] all of which are required for angiogenesis. Among the variety of cytokines with angiogenic or endothelial cell–activating properties, or both, VEGF is considered the most predominant, direct, and selective.[5-12,43,44] Therefore, we first examined the possible production of VEGF protein by B-CLL cells. We found, by ELISA and immunocytochemical staining, that B-CLL cells already constitutively produce measurable amounts of VEGF under in vitro culture conditions and that this production was increased by PMA stimulation. Moreover, because low oxygen tension is often detected in expanding tissues[22] and enlargement of lymph node infiltrated by CLL cells is often clinically observed in patients with tissue disease, we have examined whether hypoxia up-regulates VEGF expression by PB CLL cells. Our observation revealed that the rate of VEGF secretion in these cells was indeed increased up to 7-fold in response to hypoxic stimulation. The in vivo relevance of these observations was supported by our demonstration of abundant VEGF protein associated with CLL cells within highly infiltrated lymph node and spleen.

Five human VEGF mRNA species encoding VEGF isoforms of 121-, 145-, 165-, 189- and, 206-aa peptides are produced by alternative splicing of the VEGF pre-mRNA.[32-34] An important biologic property that distinguishes the different VEGF isoforms is their heparin- and heparan-sulfate–binding ability. VEGF 121 lacks the amino acids encoded by exons 6 and 7 of the VEGF gene[32] and does not bind to heparin or extracellular matrix.[45] VEGF 165 contains the addition of a 44 aa-long peptide encoded by exon 7 of the VEGF gene and shows weak affinity to heparin and heparan sulfate.[45] Thus, these 2 isoforms are generally considered as the secreted cytokine. Our RT-PCR studies showed that VEGF 121 and VEGF 165 are the predominant isoforms expressed in unstimulated B-CLL cells cultured in vitro; they encode the 28- and 42-kd secreted forms of the cytokine that we detected by Western blotting. Therefore, we focused our investigations on the effects on endothelial cells of the secreted VEGF found in CLL-cell culture supernatants. The quantitative in vitro proliferation assay showed that CLL-cell–derived supernatants stimulated HUVEC proliferation and blocking by a specific mAb showed that the VEGF contained in the supernatant was the major mitogenic factor. However, these results do not necessarily mean that the supernatant would have the potential to stimulate in vivo angiogenesis because the latter is a complex process involving multiple in vivo steps.[3] For this reason, we also used the CAM assay to demonstrate the angiogenic effect of the CLL-cell–derived supernatants in vivo. The results of both assays implicate CLL-cell–secreted VEGF in angiogenesis within lymphoreticular tissues such as node.

It is known that a wide range of malignant cells produce VEGF.[46] Although the role of this cytokine in the angiogenesis-dependent growth of solid tumors is well established,[47] its importance for the survival of different types of leukemic cells is less clear. In CLL, high blood vessel density in bone marrow has recently been noted and related to high levels of bFGF.[18] Very recently, VEGF has been measured in the serum of patients with CLL[48] and has also been demonstrated in the cells themselves.[49] In these studies, low cellular and high serum levels of VEGF were related to an adverse prognosis.[48,49]

In conclusion, we demonstrate here, using several different techniques, that CLL cells produce and secrete VEGF. We also establish that the predominantly expressed mRNA isoforms are VEGF 121 and VEGF 165, which encode the secreted 28- and 42-kd protein isoforms of the cytokine. Furthermore, we show that VEGF is produced by both circulating and tissue-phase CLL cells, and we provide direct evidence of the angiogenic effects of CLL-cell–derived VEGF. We propose that this angiogenic effect is likely to be important for the expansion of lymphoreticular tissues in CLL. However, because VEGF has multiple biologic effects and because we have shown that CLL cells express VEGF receptors (unpublished data), the cytokine may also have important autocrine effects on different aspects of CLL-cell behavior, such as transmigration from blood to tissues and prolonged CLL-cell survival.

Acknowledgments

The authors thank Dr Roy Bicknell and ZENECA Pharmaceuticals for providing the VEGF 121 and VEGF 165 cDNAs and Peter Baker for the scanning analysis.

References

1. Folkman J, D'Amore PA. Blood vessel formation: what is its molecular basis? Cell. 1996;87:1153-1155.
2. Folkman J. Clinical applications of research on angiogenesis. N Engl J Med. 1995;333:1757-1763.
3. Folkman J. Tumor angiogenesis. In: Mendelssohn J, Howely PM, Israel MA, Liotta LA, eds. The Molecular Basis of Cancer. Philadephia: WB Saunders; 1995:206-235.
4. Folkman J, Klagsburn M. Angiogenesis factors. Science. 1987;235:442-447.
5. Yoshiji H, Gomez DE, Shibuya M, Thorgeirsson UP. Expression of vascular endothelial growth factor, its receptor, and other angiogenic factors in human breast cancer. Cancer Res. 1996;56:2013-2016.
6. Ohta Y, Endo Y, Tanaka M, et al. Significance of vascular endothelial growth factor messenger RNA expression in primary lung cancer. Clin Cancer Res. 1996;2:1411-1416.
7. Brown LF, Berse B, Jackman RW, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in adenocarcinomas of the gastrointestinal tract. Cancer Res. 1993;53:4727-4735.
8. Joseph I, Nelson JB, Denmeade SR, Isaacs JT. Androgens regulate vascular endothelial growth factor content in normal and malignant prostatic tissue. Clin Cancer Res. 1997;3:2507-2511.
9. Plate KH, Breier G, Weich HA, Risau W. Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. Nature. 1992;359:845-848.

10. Kim JK, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumor growth in vivo. Nature. 1993; 362:841-844.
11. Kendall RL, Thomas KA. Inhibition of vascular endothelial-cell growth-factor activity by an endogenously encoded soluble receptor. Proc Nat Acad Sci U S A. 1993;90:10705-10709.
12. Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A. Glioblastoma growth inhibited in-vivo by a dominant-negative Flk-1 mutant. Nature. 1994; 367:576-579.
13. Ribatti D, Vacca RD, Nico B, Fanelli M, Roncali L, Dammacco F. Angiogenesis spectrum in the stroma of B-cell non-Hodgkin's lymphoma: an immunohistochemical and ultrastructural study. Eur J Haematol. 1996;56:45-53.
14. Oerez-Atayde AR, Sallan SE, Tedrow U, Connors S, Allred E, Folkman J. Spectrum of tumor angiogenesis in the bone marrow of children with acute lymphoblastic leukemia. Am J Pathol. 1997;150: 815-821.
15. Vacca A, Ribatti D, Roncali L, et al. Bone marrow angiogenesis and progression in multiple myeloma. Br J Haematol. 1994;87:503-508.
16. Kini AR, Tallman MS, Peterson LC. Abnormal angiogenesis in the bone marrow of patients with hairy cell leukemia (HCL) [abstract]. Lab Invest. 1999;79:819.
17. Cheson B, Bennet JM, Grever M, et al. National institute-sponsored working group guidelines for chronic lymphocytic leukemia: revised guidelines for diagnosis and treatment. Blood. 1996;87: 4990-4997.
18. Kini AR, Peterson LC, Kay NE. Evidence for abnormal angiogenesis in the bone marrow of patients with B-cell chronic lymphocytic leukemia (CLL) [abstract]. Blood. 1998;92:2947.
19. Molica A, Vitelli G, Levato D, Gandolfo GM, Liso V. Clinico-prognostic implications of increased levels of serum vascular endothelial growth factor in B-CLL chronic lymphocytic leukemia [abstract]. Blood. 1998;92:410.
20. Baban DF, Murray JA, Earl HM, Kerr DJ, Seymour LW. Quantitative analysis of vascular endothelial growth factor expression in chronic lymphocytic leukemia. Int J Oncol. 1996;8:29-34.
21. Foss HD, Araujo I, Demel G, Klotzbach H, Hummel M, Stein H. Expression of vascular endothelial growth factor in lymphomas and Castleman's disease. J Pathol. 1997;183:44-50.
22. Shweiki D, Itin A, Soffer D, Keshet E. Vascular endothelial growth factor induced by hypoxia, a mediator hypoxia-initiated angiogenesis. Nature. 1992;359:843-845.
23. Mazure NM, Chen EY, Laderoute KR, Giaccia AJ. Induction of vascular endothelial growth factor by hypoxia is modulated by a phosphatidylinositol 3-kinase/Akt signaling pathway in Ha-*ras*-transformed cells through a hypoxia inducible factor-1 transcriptional element. Blood. 1997;90: 3322-3331.
24. Till KJ, Cawley JC. Accessory cells mediate hairy-cell proliferation by mechanism(s) involving both adhesion and TNF-α secretion. Br J Haematol. 1994;87:687-694.
25. Weider N, Carroll PR, Flax J, Blumenfeld W, Folkman J. Tumor angiogenesis correlates with metastasis in invasive prostate cancer. Am J Pathol. 1993;143:401-409.
26. West DC, Hampson IN, Arnold F, Kumar S. Angiogenesis induced by degradation products of hyaluronic-acid. Science. 1985;228:1324-1326.
27. Grunstein J, Roberts WG, Mathieu-Costello O, Hanahan D, Johnson RS. Tumor-derived expression of vascular endothelial growth factor is a critical factor in tumor expansion and vascular function. Cancer Res. 1999;59:1592-1598.
28. Shih SC, Mullen A, Abrams K, Mukhopadhyay D, Claffey KP. Role of protein kinase C isoforms in phorbol ester-induced vascular endothelial growth factor expression in human glioblastoma cells. J Biol Chem. 1999;274:15407-15414.
29. Freeman MR, Schneck FX, Granon ML, et al. Peripheral blood T lymphocytes and lymphocytes infiltrating human cancer express vascular endothelial growth factor: a potential role for T cells in angiogenesis. Cancer Res. 1995;55:4140-4145.
30. Kolch W, Martiny-Baron G, Kieser A, Marme D. Regulation of the expression of the VEGF/VPS and its receptors: role in tumor angiogenesis. Breast Cancer Res Treat. 1995;36:139-155.
31. Banks RE, Forbes MA, Kinsey SE, et al. Release of the angiogenic cytokine vascular endothelial growth factor (VEGF) from platelets: significance for VEGF measurements and cancer biology. Br J Cancer. 1998;77:956-964.
32. Tischer E, Mitcher R, Hartman T, et al. The human gene for vascular endothelial growth factor, multiple protein forms are encoded through alternative exon splicing. J Biol Chem. 1991;266: 11947-11954.
33. Houck KA, Ferrara N, Winer J, Cachianes G, Li B, Leung DW. The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA. Mol Endocrinol. 1991;5:1806-1814.
34. Poltorak Z, Cohen T, Sivan R, et al. VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix. J Biol Chem. 1997;272:7151-7158.
35. Connolly DT, Olander JV, Heuvelman D, et al. Human vascular permeability factor: isolation from U937 cells. J Biol Chem. 1989;254:20017-20024.
36. Senger DR, Galli SJ, Dvorak AM, Perruzzi CA, Harvey VS, Devorak HF. Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid. Science. 1983;219:983-985.
37. Keck PJ, Hauser SD, Krivi G, et al. Vascular permeability factor, an endothelial cell mitogen related to platelet-derived growth factor. Science. 1989;246:1309-1312.
38. Conn G, Bayne M, Soderman L, et al. Amino acid and cDNA sequence of a vascular endothelial cell mitogen homologous to platelet-derived growth factor. Proc Nat Acad Sci U S A. 1990;87:2628-2632.
39. Leung DW, Cachianes G, Kuang WJ, Doeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science. 1989; 246:1306-1309.
40. Abedi H, Zachary I. Vascular endothelial growth factor stimulates tyrosine phosphorylation and recruitment to new focal adhesions of focal adhesion kinase and paxillin in endothelial cells. J Biol Chem. 1997;272:15442-15451.
41. Alon T, Hemo I, Itin A, Peer J, Stone J, Keshet E. Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity. Nat Med. 1995;1:1024-1028.
42. Benjamin LE, Keshet E. Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. Proc Nat Acad Sci U S A. 1997;94:8761-8766.
43. Campbell CL, Savarese DMF, Quesenberry P, Savarese TM. Expression of multiple angiogeneic cytokines in cultured normal human prostate epithelial cells: predominance of vascular endothelial growth factor. Int J Cancer. 1999;80:868-874.
44. Deroanne CF, Hajitou A, Calberg-betty CM, Nusgens BV, Lapiere CM. Angiogenesis by fibroblast growth factor 4 is mediated through an autocrine up-regulation of vascular endothelial growth factor expression. Cancer Res. 1997;57:5590-5597.
45. Park JE, Keller GA, Ferrara N. Vascular endothelial growth factor (VEGF) isoforms-differential deposition into the subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF. Mol Biol Cell. 1993;4:1317-1326.
46. Neufeld G, Cohen T, Gengrinovitch S, Poltorak Z. Vascular endothelial growth factor (VEGF) and its receptors [review]. FASEB J. 1999;13:9-22.
47. Hanahan D, Folkman J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis [review]. Cell. 1996;86:353-364.
48. Molica S, Vitelli G, Levato D, Gandolfo GM, Liso V. Increased serum levels of vascular endothelial growth factor predict risk of progression in early B-cell chronic lymphocytic leukaemia. Br J Haematol. 1999;107:605-610.
49. Aguayo A, Kantarjian H, Manshouri T, et al. Low levels of vascular endothelial growth factor (VEGF) in cells of patients with chronic lymphoid leukemia (CLL) correlate with survival, particularly in patients with low β2-microglobulin (β2M) levels [abstract]. Blood. 1998;92:921.

Leukemia (2005), 1–7

www.nature.com/leu

Autocrine VEGF mediates the antiapoptotic effect of CD154 on CLL cells

M Farahani[1], AT Treweeke[1], CH Toh[1], KJ Till[1], RJ Harris[1], JC Cawley[1], M Zuzel[1] and H Chen[1]

[1]*Department of Haematology, University of Liverpool, Royal Liverpool University Hospital, Liverpool, UK*

CD154 is an important regulator of chronic lymphocytic leukaemia (CLL)-cell survival. In CLL, high serum levels of VEGF are a feature of advanced disease, and we and others have previously shown that CLL cells produce and secrete this growth factor. Since CD154 stimulates VEGF production in other cell types, and VEGF is known to promote cell survival, we examined whether the cytoprotection of CLL cells by CD154 involves VEGF. We report for the first time that treatment of CLL cells with CD154 results in increased VEGF production and demonstrate involvement of NF-κB in this process. Moreover, we show that CD154-induced CLL-cell survival is reduced by anti-VEGF-neutralising antibody and by inhibiting VEGF receptor (VEGFR) signalling with SU5416. However, addition of exogenous VEGF alone or blocking secreted autocrine VEGF had little or no effect on CLL-cell survival. We therefore conclude that CLL-cell cytoprotection in the presence of CD154 requires combined signalling by both CD40 and VEGFR. This combined signalling and resulting cytoprotection were shown to involve NF-κB activation and increased survivin production. In conclusion, our findings identify autocrine VEGF as an important mediator of the antiapoptotic effect of CD40 ligation, and thus provide new insights into CLL-cell rescue by CD154 in lymphoreticular tissues.
Leukemia advance online publication, 27 January 2005;
doi:10.1038/sj.leu.2403631
Keywords: CD154; VEGF; CLL

Introduction

Chronic lymphocytic leukaemia (CLL) is characterised by the accumulation of long-lived, mature neoplastic B-lymphocytes in blood and tissues.[1,2] It is believed that prolonged *in vivo* survival of malignant cells contributes to their clonal expansion.[3,4] The fact that the malignant cells, despite progressively accumulating *in vivo*, rapidly undergo apoptosis when cultured *in vitro* implies that microenvironmental factors are likely to play a prominent role in prolonging the lifespan of CLL cells.

VEGF is one factor that could potentially be important for the pathogenesis and prognosis of CLL. CLL cells themselves produce VEGF,[5,6] and high levels of this growth factor in serum,[7] as well as high expression of VEGF receptor (VEGFR)-2 on the malignant cells,[8] correlate with shortened patient survival. Moreover, in a previous study,[5] we observed increased vascularisation in enlarged lymph nodes of CLL patients and proposed that this was caused by paracrine stimulation of endothelial cells by CLL-cell-derived VEGF. Increased microvessel density has also been reported in the bone marrow of patients with CLL.[9] We suggested that increased angiogenesis might play an important role in the survival of tissue-phase CLL cells by providing metabolic support.[5]

In addition to its role in angiogenesis, VEGF has been found to be an autocrine survival factor for some cell types including solid tumour and endothelial cells,[10–12] as well as CLL cells.[13] VEGF, like many other growth factors, often achieves its effects in cooperation with other stimuli.[14] An important microenvironmental factor affecting CLL-cell survival is CD40 ligand (CD154) expressed on activated T cells, monocytes, macrophages, dendritic cells and platelets.[15,16] Its receptor CD40 is expressed by mature B-lymphocytes including CLL cells, and also by some nonhaemic cell types.[17] Stimulation of CD40 on endothelial cells, monocytes, fibroblasts and multiple myeloma cells upregulates production of VEGF by these cells.[18–20] Furthermore, neutralisation of autocrine VEGF by anti-VEGF antibody (Ab) completely abolished the mitogenic effect of CD154 on endothelial cells.[18] In CLL cells, ligation of CD40 by CD154 is known to inhibit spontaneous and drug-induced cell death.[21–24] However, whether this effect also requires cell stimulation by the secreted autocrine VEGF is not known.

In the present study, we show that the previously observed promotion of CLL-cell survival by CD154 does indeed depend on autocrine VEGF production, and that both VEGFR and CD40 engagement are essential for this effect. We also show that VEGF provides a significant contribution to CD154-induced NF-κB activation and upregulation of survivin, one of the NF-κB target genes.

Materials and methods

Patients

All patients had typical CLL with high numbers of circulating malignant cells as defined morphologically (prolymphocytes <10%) and immunophenotypically ($CD19^+$, $CD5^+$, $CD23^+$ and weak light-chain-restricted surface immunoglobulin). Patients' samples were obtained after informed consent and with the approval of the Liverpool Research Ethics Committee. Relevant clinical and laboratory data of the patients studied are included as Supplementary information accompanying the paper.

Materials

Human VEGF Quantikine Colorimetric Sandwich ELISA (enzyme-linked immunosorbent assays) kit, anti-human VEGF-neutralising monoclonal antibody (mAb) (MAB293), recombinant human VEGF165 and PLGF were all from R & D Systems (Abingdon, UK). Anti-human p65 mAb was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The CD154 kit (ALX-850-064) containing recombinant human soluble CD154 trimeric protein (rhsCD154) and enhancer was from Alexis Biochemicals (San Diego, CA, USA). TransAM NF-κB p65 assay kit and nuclear extract kit were from Active Motif North America (Carlsbad, CA, USA). The NF-κB translocation inhibitor SN50, its control peptide (SN50M) and the VEGFR tyrosine kinase inhibitor SU5416 were from Calbiochem (Nottingham, UK). Recombinant human VEGF-E was from RELIA Tech GmbH (Braunschweig, Germany). Human Total Survivin ELISA kit was

Correspondence: Dr M Farahani, Department of Haematology, Royal Liverpool University Hospital, Daulby Street, Liverpool L69 3GA, UK; Fax: +44 151 706 4311; E-mail: Mosavar@liv.ac.uk
Received 8 September 2004; accepted 12 November 2004

Leukemia (2005), 1–7

www.nature.com/leu

Autocrine VEGF mediates the antiapoptotic effect of CD154 on CLL cells

M Farahani[1], AT Treweeke[1], CH Toh[1], KJ Till[1], RJ Harris[1], JC Cawley[1], M Zuzel[1] and H Chen[1]

[1]*Department of Haematology, University of Liverpool, Royal Liverpool University Hospital, Liverpool, UK*

CD154 is an important regulator of chronic lymphocytic leukaemia (CLL)-cell survival. In CLL, high serum levels of VEGF are a feature of advanced disease, and we and others have previously shown that CLL cells produce and secrete this growth factor. Since CD154 stimulates VEGF production in other cell types, and VEGF is known to promote cell survival, we examined whether the cytoprotection of CLL cells by CD154 involves VEGF. We report for the first time that treatment of CLL cells with CD154 results in increased VEGF production and demonstrate involvement of NF-κB in this process. Moreover, we show that CD154-induced CLL-cell survival is reduced by anti-VEGF-neutralising antibody and by inhibiting VEGF receptor (VEGFR) signalling with SU5416. However, addition of exogenous VEGF alone or blocking secreted autocrine VEGF had little or no effect on CLL-cell survival. We therefore conclude that CLL-cell cytoprotection in the presence of CD154 requires combined signalling by both CD40 and VEGFR. This combined signalling and resulting cytoprotection were shown to involve NF-κB activation and increased survivin production. In conclusion, our findings identify autocrine VEGF as an important mediator of the antiapoptotic effect of CD40 ligation, and thus provide new insights into CLL-cell rescue by CD154 in lymphoreticular tissues.

Leukemia advance online publication, 27 January 2005;
doi:10.1038/sj.leu.2403631
Keywords: CD154; VEGF; CLL

Introduction

Chronic lymphocytic leukaemia (CLL) is characterised by the accumulation of long-lived, mature neoplastic B-lymphocytes in blood and tissues.[1,2] It is believed that prolonged *in vivo* survival of malignant cells contributes to their clonal expansion.[3,4] The fact that the malignant cells, despite progressively accumulating *in vivo*, rapidly undergo apoptosis when cultured *in vitro* implies that microenvironmental factors are likely to play a prominent role in prolonging the lifespan of CLL cells.

VEGF is one factor that could potentially be important for the pathogenesis and prognosis of CLL. CLL cells themselves produce VEGF,[5,6] and high levels of this growth factor in serum,[7] as well as high expression of VEGF receptor (VEGFR)-2 on the malignant cells,[8] correlate with shortened patient survival. Moreover, in a previous study,[5] we observed increased vascularisation in enlarged lymph nodes of CLL patients and proposed that this was caused by paracrine stimulation of endothelial cells by CLL-cell-derived VEGF. Increased microvessel density has also been reported in the bone marrow of patients with CLL.[9] We suggested that increased angiogenesis might play an important role in the survival of tissue-phase CLL cells by providing metabolic support.[5]

In addition to its role in angiogenesis, VEGF has been found to be an autocrine survival factor for some cell types including solid tumour and endothelial cells,[10–12] as well as CLL cells.[13] VEGF, like many other growth factors, often achieves its effects in cooperation with other stimuli.[14] An important microenvironmental factor affecting CLL-cell survival is CD40 ligand (CD154) expressed on activated T cells, monocytes, macrophages, dendritic cells and platelets.[15,16] Its receptor CD40 is expressed by mature B-lymphocytes including CLL cells, and also by some nonhaemic cell types.[17] Stimulation of CD40 on endothelial cells, monocytes, fibroblasts and multiple myeloma cells upregulates production of VEGF by these cells.[18–20] Furthermore, neutralisation of autocrine VEGF by anti-VEGF antibody (Ab) completely abolished the mitogenic effect of CD154 on endothelial cells.[18] In CLL cells, ligation of CD40 by CD154 is known to inhibit spontaneous and drug-induced cell death.[21–24] However, whether this effect also requires cell stimulation by the secreted autocrine VEGF is not known.

In the present study, we show that the previously observed promotion of CLL-cell survival by CD154 does indeed depend on autocrine VEGF production, and that both VEGFR and CD40 engagement are essential for this effect. We also show that VEGF provides a significant contribution to CD154-induced NF-κB activation and upregulation of survivin, one of the NF-κB target genes.

Materials and methods

Patients

All patients had typical CLL with high numbers of circulating malignant cells as defined morphologically (prolymphocytes $<10\%$) and immunophenotypically ($CD19^+$, $CD5^+$, $CD23^+$ and weak light-chain-restricted surface immunoglobulin). Patients' samples were obtained after informed consent and with the approval of the Liverpool Research Ethics Committee. Relevant clinical and laboratory data of the patients studied are included as Supplementary information accompanying the paper.

Materials

Human VEGF Quantikine Colorimetric Sandwich ELISA (enzyme-linked immunosorbent assays) kit, anti-human VEGF-neutralising monoclonal antibody (mAb) (MAB293), recombinant human VEGF165 and PLGF were all from R & D Systems (Abingdon, UK). Anti-human p65 mAb was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The CD154 kit (ALX-850-064) containing recombinant human soluble CD154 trimeric protein (rhsCD154) and enhancer was from Alexis Biochemicals (San Diego, CA, USA). TransAM NF-κB p65 assay kit and nuclear extract kit were from Active Motif North America (Carlsbad, CA, USA). The NF-κB translocation inhibitor SN50, its control peptide (SN50M) and the VEGFR tyrosine kinase inhibitor SU5416 were from Calbiochem (Nottingham, UK). Recombinant human VEGF-E was from RELIA Tech GmbH (Braunschweig, Germany). Human Total Survivin ELISA kit was

Correspondence: Dr M Farahani, Department of Haematology, Royal Liverpool University Hospital, Daulby Street, Liverpool L69 3GA, UK; Fax: +44 151 706 4311; E-mail: Mosavar@liv.ac.uk
Received 8 September 2004; accepted 12 November 2004

SPOTLIGHT

from Assay Designs Inc. (Ann Arbor, MI, USA). Ficoll–Hypaque (Lymphoprep) was from Nycomed (Birmingham, UK). RPMI-1640 medium, Kodak XAR-5 film and poly(2-hydroxyethyl methacrylate) (polyHEMA) were from Sigma-Aldrich (Poole, UK). Goat anti-mouse-fluorescein isothiocyanate (FITC) conjugate was from BD Biosciences (San Diego, CA, USA).

Cell preparation and culture

Lymphocytes were isolated from heparinised PB by Ficoll–Hypaque density-gradient centrifugation and cryopreserved. Purified cells were always >95% CD19 positive. Frozen samples were rapidly thawed at 37°C, slowly resuspended in RPMI-1640 medium containing 1% BSA, centrifuged, then resuspended in culture medium and incubated at 37°C for at least 2 h prior to use.

Measurement of cell death

Determination of CLL-cell viability was based on the analysis of mitochondrial transmembrane potential with 3,3′dihexyloxacarbocyanine iodine ($DiOC_6$) and on cell-membrane permeability to propidium iodide (PI), as described previously.[25] Freshly thawed CLL cells were resuspended to 3×10^6/ml in RPMI-1640 medium containing 0.1% BSA and cultured in polyHEMA-coated 96-well plates (100 μl/well) at 37°C in 5% CO_2 for up to 5 days. To determine cell survival, 100 μl of CLL-cell suspension was removed from culture at the indicated time points and incubated with 100 μl of 40 nM $DiOC_6$ for 20 min at 37°C, and then incubated on ice for a further 30 min with an equal volume of 10 μg/ml PI. Cell viability was determined by flow cytometry using a FACScan.

Enzyme-linked immunosorbent assays

Secreted VEGF was measured using a human VEGF ELISA kit with a detection limit of 5.0 pg/ml. Survivin protein in total cell lysates was measured using a human Total Survivin ELISA kit with a detection limit of 3.6 pg/ml.

Evaluation of NF-κB activity

Cells were cultured with or without CD154 alone, with CD154 plus anti-VEGF-neutralising mAb or with CD154 plus SU5416 for 24 h. Nuclear extracts were prepared and their protein concentration determined by a Bradford-based assay (Bio-Rad). The DNA-binding activity of NF-κB was quantified using a TransAM NF-κB p65 transcription factor assay kit with a detection limit of 0.4 ng/ml.

Confocal microscopy

To confirm nuclear translocation of NF-κB, cytospins were prepared from cultured cells (300 r.p.m. for 3 min; Shandon Cytospin 2, Pittsburgh, PA, USA), air-dried and fixed in methanol for 10 min at RT. Fixed slides were washed in PBS and blocked for 1 h at RT with PBS containing human Ab, goat and mouse sera (all at 1%). After washing, mouse anti-human p65 mAb or nonspecific mouse IgG_1 mAb (2 μg/ml each diluted in PBS containing 1% human AB serum) was added and cells incubated for 1 h at RT. Goat anti-mouse-FITC conjugate (diluted 1:10 in 1% human AB serum/PBS) was added after further washing, cells incubated for 1 h at RT, washed and mounted in Vectashield. The cellular location of NF-κB p65 protein was then analysed by laser confocal microscopy using an Olympus BH2 microscope fitted with a Bio-Rad MR/AG2 Confocal Imaging System.

Results

CD154 stimulates VEGF production by CLL cells through a process involving NF-κB activation

Since it has been reported that CD154 enhances VEGF secretion by a number of cell types,[18–20] we first examined whether CD154 enhances VEGF production by CLL cells.

Using RT-PCR, we initially found that treatment of CLL cells with 200 ng/ml soluble trimeric CD154 for 6 h resulted in a two-fold increase in VEGF mRNA expression (data not shown). We then assessed the effect of CD154 on CLL-cell production of VEGF protein. When cells from different CLL cases were cultured with 200 ng/ml CD154, this consistently caused an increase in VEGF secretion, which was first evident at 18 h, and persisted for up to 72 h. The highest relative increase compared with unstimulated cells was at 24 h and this incubation time was used in subsequent studies. Figure 1a shows the amounts of VEGF measured at 24 h in the supernatants of six different CLL-cell clones cultured with or without CD154. The clear upregulation of VEGF secretion by CD154 seen in this figure was significant in all CLL cases tested ($n=16$, $P<0.05$).

Since NF-κB is a major pathway activated by CD40 engagement,[23] we next examined whether activation of NF-κB is required for the CD154-stimulated VEGF production. CLL cells were cultured in the presence or absence of CD154 alone, with CD154 plus the NF-κB inhibitor SN50[26] or with CD154 plus SN50M, an inactive analogue of SN50. SN50 is a cell-permeable inhibitor of nuclear translocation of NF-κB derived from the nuclear localisation sequence of its p50 component. As shown in Figure 1b, induction of VEGF by CD154 was significantly reduced by SN50 ($P<0.01$), but not by SN50M. This reduction was not due to nonspecific cytotoxicity of this reagent because, at the concentration used (18 μM), SN50 alone had no effect on the viability of CLL cells (data not shown).

Taken together, these results show that the upregulation of VEGF production by CLL cells in response to CD154 involves NF-κB activation.

VEGF production mediates the cytoprotective effect of CD154 on CLL cells

Having demonstrated that CD154 upregulates VEGF production by CLL cells, we next investigated whether VEGF plays a role in the cytoprotective effect of CD40 engagement.

Since it has been reported that cytoprotection by CD154 varies between CLL cases,[21,27] we first examined 16 CLL clones for the antiapoptotic effect of CD40–CD154 interaction. In accordance with published data,[21,27] only a proportion of the CLL clones (12 of 16 cases examined) showed a clear positive survival response, although all expressed VEGFR-1 and VEGFR-2 at both mRNA and protein levels (data not shown).

To determine whether VEGF is involved in this response, 10 CLL clones from this responder group were cultured for up to 6 days with or without CD154 ± inhibitors of VEGF or VEGFRs. In eight of the 10 CLL clones examined, a neutralising VEGF

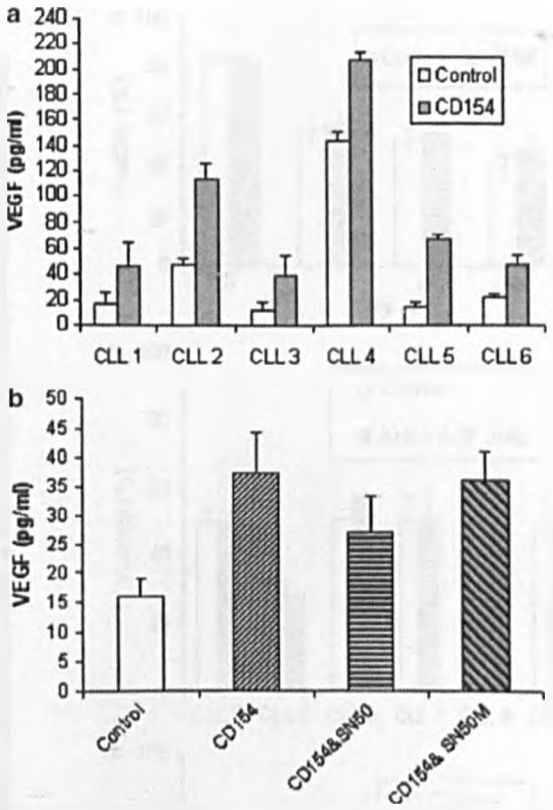


Figure 1 Effect of CD154 on VEGF production by CLL cells. VEGF protein content in all supernatants was determined by ELISA. (a) CLL cells from six individual patients were cultured for 24 h in duplicate in the absence or presence of CD154 (200 ng/ml). Data are presented as means ± s.d. of at least three separate experiments. Similar results were obtained in 10 additional cases. CD154 significantly induced VEGF production in all cases studied ($P<0.01$). (b) CLL cells were preincubated for 1 h with the NF-κB inhibitor SN50 (18 μM) or with its inactive analogue SN50M (18 μM), and then stimulated with CD154 (200 ng/ml) for 24 h. SN50 significantly inhibited CD154-induced VEGF production ($P<0.05$), while SN50M had no effect. The data are means ± s.d. of seven CLL cases.

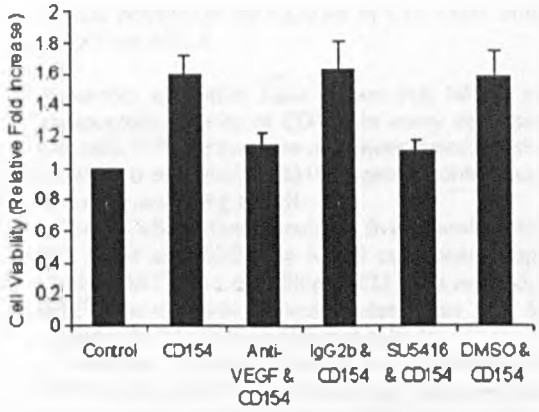


Figure 2 Role of VEGF in the antiapoptotic activity of CD154. CLL cells were incubated with anti-human VEGF mAb (1 μg/ml), IgG 2b as isotype control, SU5416 (10 μM) or DMSO (0.2% diluent for the SU5416) for 1 h prior to the addition of CD154 (200 ng/ml) as indicated in the figure. Cell viability was measured at day 4 of culture using $DiOC_6$/PI staining followed by FACS analysis. Owing to the considerable variation in the viability of the control at day 4, the data are shown as relative viabilities compared with the control. The figure shows that the increase in cell viability induced by CD154 was greatly reduced by anti-VEGF or SU5416 ($P<0.05$). The results show the mean ± s.d. of eight experiments involving eight different CLL-cell clones. The concentration of anti-VEGF mAb (1 μg/ml) was sufficient to inhibit all secreted VEGF measured in our study (20–220 pg/ml, see Figure 1) since, according to the supplier, 0.04–0.08 μg/ml of this mAb causes half-maximal inhibition of the effects of HUVEC stimulation by 10 ng/ml of rhVEGF.

mAb or 10 μM SU5416 (an inhibitor of both VEGFRs[28]) significantly reduced the prosurvival effect of CD154 (Figure 2).

Taken together, these results demonstrate that VEGF mediated the antiapoptotic effect of CD154 in the 80% of cases in which such an effect was observed.

Increased secretion of VEGF alone is insufficient for CLL-cell rescue

Having established that CD154 induces NF-κB-dependent VEGF production by CLL cells and that secreted VEGF is involved in the prosurvival effects of CD40 ligation, we next examined how VEGF contributes to this cell rescue by CD154.

We first examined whether the addition of VEGF alone produces the same rescue effect as that observed with CD154. To eliminate potential effects of adhesion and cytokines on cell survival, CLL cells were cultured in polyHEMA-coated 96-well plates (3.3×10^5 cells/100 μl/well) in serum-free medium (RPMI-1640 plus 0.1% BSA). During 3-day culture under these conditions, exogenous VEGF (100 ng/ml) had no effect on spontaneous cell death (Figure 3a). Furthermore, a range of different concentrations of VEGF (1–500 ng/ml) had no effect on CLL-cell survival during culture for up to 7 days (data not shown). This suggests that the rescue effect of VEGF secreted in response to CD154 engagement is achieved through signalling that requires simultaneous engagement of both VEGFR(s) and CD40.

Basal autocrine VEGF provides some CLL-cell rescue through internal signalling

Since CLL cells constitutively produce VEGF in culture,[5] we next investigated any potential prosurvival effects of this autocrine VEGF. The effects of VEGF were blocked in two ways: by neutralising secreted VEGF with anti-VEGF mAb or by inhibiting VEGFR signalling.

Treatment with anti-VEGF mAb had no significant effect on the survival of cultured CLL cells (Figure 3b). However, the VEGFR inhibitor SU5416 at a concentration of 10 μM caused a modest but significant decrease in cell survival (Figure 3c, $P<0.001$). Similar results were observed using 5 μM SU1498, another VEGFR inhibitor. We also treated cells with a combination of SU5416 and anti-VEGF mAb, but this did not have an any more pronounced effect on CLL-cell survival than SU5416 alone (data not shown).

Thus, although blocking secreted VEGF had no effect on CLL-cell viability, the receptor tyrosine kinase inhibitors acting internally did shorten cell survival. This indicates the presence in cultured CLL cells of VEGFR-dependent signals for survival that cannot be blocked by neutralisation of secreted VEGF. Such divergent effects of external cytokine blockade versus receptor-signalling inhibition have been observed for other cell types.[29,12]

SPOTLIGHT

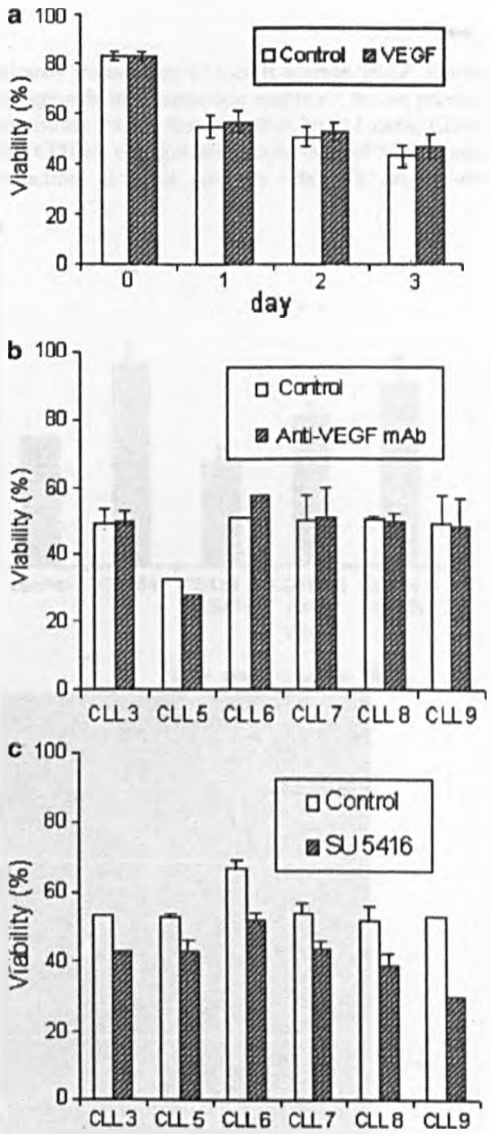


Figure 3 Effect of VEGF on CLL-cell survival. CLL cells were cultured in RPMI-1640 medium containing 0.1% BSA in polyHEMA-coated wells. Viability of harvested cells was measured by $DiOC_6$/PI staining and flow cytometry. In (b and c), when no error bars are shown, the histograms are the means of two independent measurements, whereas all the other data were obtained from three or more repeated measurements. (a) CLL cells from seven separate individuals were cultured with or without 100 ng/ml VEGF for up to 3 days and the mean levels of cell survival were determined at the indicated time points. The data are shown as mean ± s.d. The differences between levels of survival of control versus VEGF-treated cells were not significant. (b) Cells from six separate patients were cultured for up to 6 days in the presence of monoclonal anti-VEGF-neutralising mAb (1 μg/ml) to abrogate the effects of endogenous VEGF; mouse IgG2b served as a control. Histograms represent mean values ± s.d. for cells harvested at day 4 from each case studied. The differences between levels of viability for the control versus the anti-VEGF-treated cells were not significant. Similar results were obtained after 6 days. (c) CLL cells from the same six individuals in (b) were cultured with or without the VEGFR tyrosine kinase inhibitor SU5416 (10 μM) for up to 6 days. Histograms represent the mean values ± s.d. for cells harvested at day 4. The viabilities of the control and SU5416-treated cells were significantly different ($P<0.01$). Similar results were obtained at day 6.

NF-κB activation by CD154 in CLL cells involves autocrine VEGF

A number of studies have shown that NF-κB mediates the antiapoptotic activity of CD154 in many cell types including CLL cells.[23,30] We therefore next investigated whether the VEGF secreted in response to CD154 ligation contributes to CLL-cell rescue by activating NF-κB.

The NF-κB/Rel family includes five subunits: p65/RelA, p50, p52, c-Rel and RelB. The NF-κB components reported to be involved in CD154 signalling in CLL cells are p65, p50 and c-Rel,[23] whereas only p50/p65 heterodimer was found to be activated by VEGF in endothelial cells.[31]

Therefore, in order to assess the contribution of VEGF to CD154-induced NF-κB activation, we measured p65 in nuclear extracts of CLL cells stimulated with CD154 in the presence or absence of VEGF blockade. Figure 4a shows that NF-κB was already activated in CLL cells and that CD154 increased this activation in each of the three cases tested. This increase was significantly reduced ($P<0.05$) or completely abolished by including anti-VEGF mAb or SU5416 in the cultures (Figure 4a).

The role of VEGF in NF-κB activation in CLL cells stimulated by CD154 was confirmed by confocal microscopy. Cytospins made from untreated cells or from CLL cells cultured with CD154 alone, with CD154 plus SU5416, or with CD154 plus anti-VEGF mAb were stained with anti-p65 mAb (Figure 4b). In untreated cells, p65 positivity was localised mainly outside the nucleus (I). In contrast, after 24 h culture with CD154, p65 was found largely in the nucleus (II). This nuclear translocation was almost completely abolished by SU5416 (III) or anti-VEGF mAb (IV). These data confirm that VEGF signalling is involved in the nuclear transport of NF-κB in CLL cells stimulated with CD154.

To determine which of the two VEGF receptors expressed by CLL cells has the capacity to mediate this NF-κB activation, we stimulated the cells with the receptor-specific ligands PLGF and VEGF-E. Both PLGF and VEGF-E are members of the VEGF protein family. PLGF binds to VEGFR-1 with high affinity but fails to bind to VEGFR-2, whereas VEGF-E binds specifically to VEGFR-2.[32,33] Figure 4c shows that both these ligands increased NF-κB activation in CLL cells, demonstrating that both VEGFR-1 and -2 can potentially be involved in VEGF signalling. However, since VEGF alone could not effectively protect cells from apoptosis (Figure 3), NF-κB activation by VEGF alone does not explain the cytoprotection observed upon CD40 engagement. Rather a dual signal from both CD40 and VEGFRs is required.

VEGF is involved in the upregulation of survivin by CD154

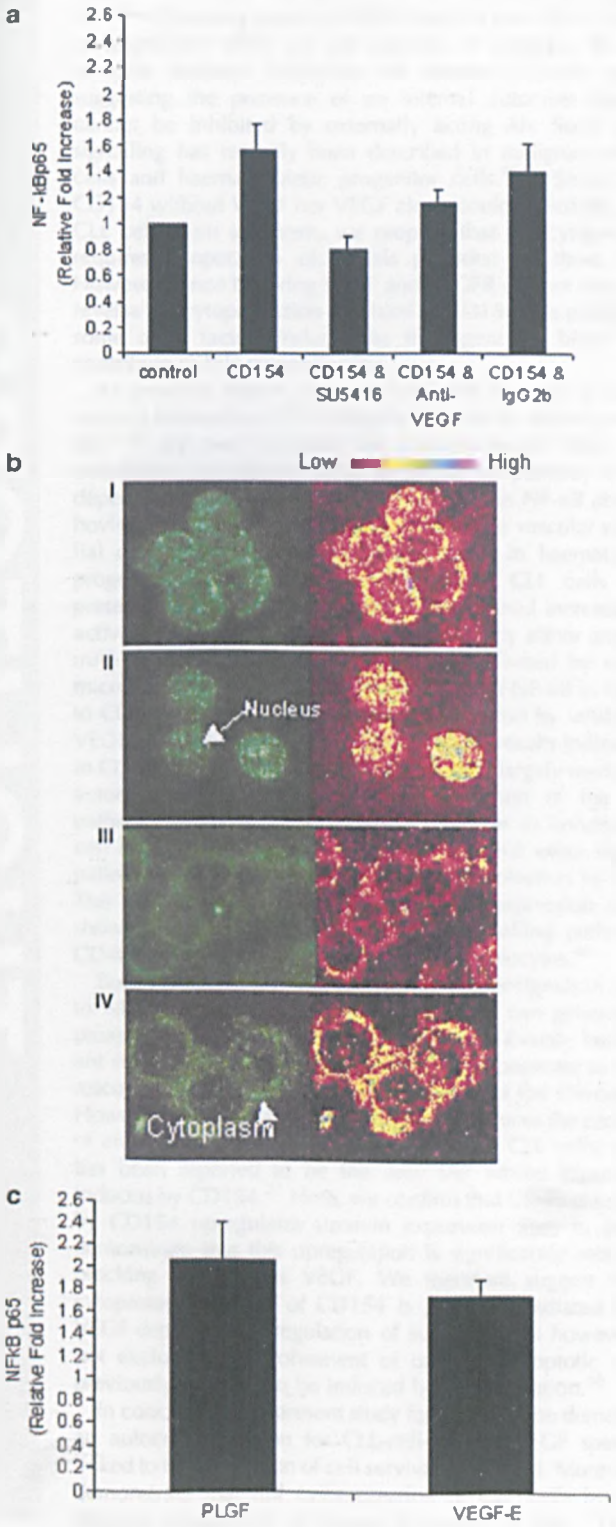
Since a previous report implicated an increase in the production of the antiapoptotic protein survivin in the rescue of CLL cells by CD40 ligation,[27] we next examined to what extent VEGF contributes to this increased survivin production.

When CLL cells were cultured for periods of 24, 48 and 72 h in the presence or absence of CD154 (200 ng/ml), we found that the relative increase in survivin production was highest at 72 h (data not shown). This incubation period was then used in subsequent studies. Figure 5 shows that blocking VEGF receptor signalling with SU5416 significantly reduced the survivin expression induced by CD154 ($P<0.05$). Therefore, the survivin production implicated in cytoprotection by CD154[27] is at least partially dependent on VEGFR signalling.

Discussion

We have previously shown that CLL cells secrete VEGF, which stimulates angiogenesis in a paracrine manner.[5] In the present study, we demonstrate for the first time that, in CLL cells, CD40 engagement by CD154 upregulates production of VEGF, and that this production at least partially depends on NF-κB activation. This is in accord with a number of previous studies implicating NF-κB in the upregulation of VEGF production in various other cell types. Thus, in human microvascular endothelial cells, NF-κB was found to be involved in the upregulation of VEGF mRNA and in the VEGF-dependent development of vessel-like structures in response to TNF-α.[34] Also, involvement of NF-κB in VEGF expression was found in prostate cancer cells and in breast cancer cell lines.[35,36] In addition, it has been reported that NF-κB decoy oligonucleotides block the induction of VEGF in UV-irradiated skin cells[37] and that, in human macrophages, CD154 stimulation results in strong NF-κB-dependent VEGF induction.[38]

We then examined the contribution of VEGF to the well-known prosurvival effect of CD154 on CLL cells.[23,27] This seemed important because a number of studies have shown that VEGF can support survival of different cell types. For example, VEGF prevents the *in vitro* apoptosis of endothelial cells induced by serum starvation,[39] and induces the expression of several antiapoptotic proteins in these cells.[40,41] Moreover, an internal



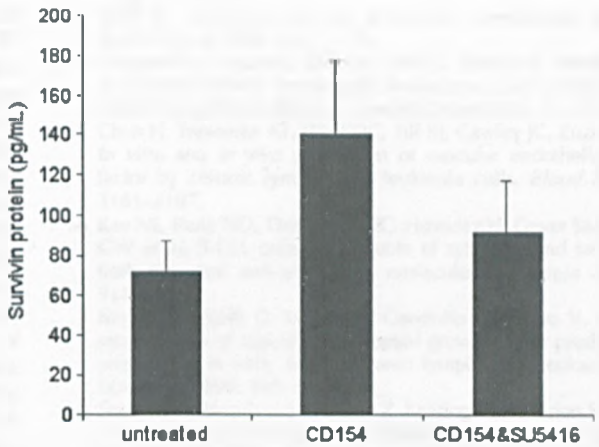


Figure 5 Effect of VEGFR inhibition on CD154-enhanced survivin expression. CLL cells (8×10^6/ml) were treated with CD154 (200 ng/ml) or CD154 plus SU5416 (10 μM) for 72 h, and survivin protein was measured by ELISA. The results show the mean ± s.d. of six individual cases. Both the increase in survivin in the presence of CD154 and the subsequent reduction by SU5416 were statistically significant ($P<0.05$).

Figure 4 Role of VEGF in CD154-induced NF-κB activation in CLL cells. (a) CLL cells from three separate cases were incubated with CD154 (200 ng/ml) in the absence or presence of SU5416 (10 μM), anti-VEGF mAb (1 μg/ml) or IgG 2b (1 μg/ml) as a control for the anti-VEGF. After 24 h, nuclear extracts were prepared and examined for the activation of NF-κB using the TransAM NF-κB p65 assay kit. (b) CLL cells were incubated for 24 h in the absence (I) or presence (II) of CD154, or CD154 plus SU5416 (III) or CD154 plus anti-VEGF mAb (IV) and stained with anti-p65 mAb. Fluorescent staining was analysed by confocal microscopy as described in Materials and methods. Note the translocation of activity to the nucleus in (II) and the inhibition of this translocation in (III) and (IV). Green fluorescence in the first columns shows endogenous p65. The second column is presented in false colour to show the signal intensity in the nucleus compared with the cytoplasm. Results shown are representative of three separate experiments. (c) CLL cells were incubated with VEGF receptor-specific ligands PLGF (VEGFR-1 specific, 100 ng/ml) and VEGF-E (VEGFR-2 specific, 50 ng/ml). The NF-κB activity was measured by the same method as in (a) ($n=3$).

SPOTLIGHT

18 Melter M, Reinders ME, Sho M, Pal S, Geehan C, Denton MD *et al.* Ligation of CD40 induces the expression of vascular endothelial growth factor by endothelial cells and monocytes and promotes angiogenesis *in vivo. Blood* 2000; **96**: 3801–3808.
19 Cho CS, Cho ML, Min SY, Kim WU, Min DJ, Lee SS *et al.* CD40 engagement on synovial fibroblast up-regulates production of vascular endothelial growth factor. *J Immunol* 2000; **164**: 5055–5061.
20 Tai YT, Podar K, Gupta D, Lin B, Young G, Akiyama M *et al.* CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells. *Blood* 2002; **99**: 1419–1427.
21 Wang D, Freeman GJ, Levine H, Ritz J, Robertson MJ. Role of the CD40 and CD95 (APO-1/Fas) antigens in the apoptosis of human B-cell malignancies. *Br J Haematol* 1997; **97**: 409–417.
22 Romano MF, Lamberti A, Tassone P, Alfinito F, Costantini S, Chiurazzi F *et al.* Triggering of CD40 antigen inhibits fludarabine-induced apoptosis in B chronic lymphocytic leukemia cells. *Blood* 1998; **92**: 990–995.
23 Furman RR, Asgary Z, Mascarenhas JO, Liou HC, Schattner EJ. Modulation of NF-kappa B activity and apoptosis in chronic lymphocytic leukemia B cells. *J Immunol* 2000; **164**: 2200–2206.
24 Grdisa M. Influence of CD40 ligation on survival and apoptosis of B-CLL cells *in vitro. Leukemia Res* 2003; **27**: 951–956.
25 Zamzami N, Marchetti P, Castedo M, Zanin C, Vayssiere JL, Petit PX *et al.* Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo. J Exp Med* 1995; **181**: 1661–1672.
26 Lin YZ, Yao SY, Veach RA, Torgerson TR, Hawiger J. Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J Biol Chem* 1995; **270**: 14255–14258.
27 Granziero L, Ghia P, Circosta P, Gottardi D, Strola G, Geuna M *et al.* Survivin is expressed on CD40 stimulation and interfaces proliferation and apoptosis in B-cell chronic lymphocytic leukemia. *Blood* 2001; **97**: 2777–2783.
28 Itokawa T, Nokihara H, Nishioka Y, Sone S, Iwamoto Y, Yamada Y *et al.* Antiangiogenic effect by SU5416 is partly attributable to inhibition of Flt-1 receptor signaling. *Mol Cancer Ther* 2002; **1**: 295–302.
29 Santos SC, Dias S. Internal and external autocrine VEGF/KDR loops regulate survival of subsets of acute leukemia through distinct signaling pathways. *Blood* 2004; **103**: 3883–3889.
30 Karin M, Lin A. NF-kappaB at the crossroads of life and death. *Nat Immunol* 2002; **3**: 221–227.
31 Kim I, Moon SO, Kim SH, Kim HJ, Koh YS, Koh GY. Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells. *J Biol Chem* 2001; **276**: 7614–7620.
32 Park JE, Chen HH, Winer J, Houck KA, Ferrara N. Placenta growth factor. Potentiation of vascular endothelial growth factor bioactivity, *in vitro* and *in vivo*, and high affinity binding to Flt-1 but not to Flk-1/KDR. *J Biol Chem* 1994; **269**: 25646–25654.
33 Meyer M, Clauss M, Lepple-Wienhues A, Waltenberger J, Augustin HG, Ziche M *et al.* A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* 1999; **18**: 363–374.
34 Yoshida S, Ono M, Shono T, Izumi H, Ishibashi T, Suzuki H *et al.* Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. *Mol Cell Biol* 1997; **17**: 4015–4023.
35 Huang S, Pettaway CA, Uehara H, Bucana CD, Fidler IJ. Blockade of NF-kappaB activity in human prostate cancer cells is associated with suppression of angiogenesis, invasion, and metastasis. *Oncogene* 2001; **20**: 4188–4197.
36 Shibata A, Nagaya T, Imai T, Funahashi H, Nakao A, Seo H. Inhibition of NF-kappaB activity decreases the VEGF mRNA expression in MDA-MB-231 breast cancer cells. *Breast Cancer Res Treat* 2002; **73**: 237–243.
37 Abeyama K, Eng W, Jester JV, Vink AA, Edelbaum D, Cockerell CJ *et al.* A role for NF-kappaB-dependent gene transactivation in sunburn. *J Clin Invest* 2000; **105**: 1751–1759.
38 Kiriakidis S, Andreakos E, Monaco C, Foxwell B, Feldmann M, Paleolog E. VEGF expression in human macrophages is NF-kappaB-dependent: studies using adenoviruses expressing the endogenous NF-kappaB inhibitor IkappaBalpha and a kinase-defective form of the IkappaB kinase 2. *J Cell Sci* 2003; **116**: 665–674.
39 Gerber HP, McMurtrey A, Kowalski J, Yan M, Keyt BA, Dixit V *et al.* Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 1998; **273**: 30336–30343.
40 Gerber HP, Dixit V, Ferrara N. Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells. *J Biol Chem* 1998; **273**: 13313–13316.
41 Tran J, Rak J, Sheehan C, Saibil SD, LaCasse E, Korneluk RG *et al.* Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells. *Biochem Biophys Res Commun* 1999; **264**: 781–788.
42 Reinders ME, Sho M, Robertson SW, Geehan CS, Briscoe DM. Proangiogenic function of CD40 ligand–CD40 interactions. *J Immunol* 2003; **171**: 1534–1541.
43 Marumo T, Schini-Kerth VB, Busse R. Vascular endothelial growth factor activates nuclear factor-kappaB and induces monocyte chemoattractant protein-1 in bovine retinal endothelial cells. *Diabetes* 1999; **48**: 1131–1137.
44 Oyama T, Ran S, Ishida T, Nadaf S, Kerr L, Carbone DP *et al.* Vascular endothelial growth factor affects dendritic cell maturation through the inhibition of nuclear factor-kappa B activation in hemopoietic progenitor cells. *J Immunol* 1998; **160**: 1224–1232.
45 Dikov MM, Oyama T, Cheng P, Takahashi T, Takahashi K, Sepetavec T *et al.* Vascular endothelial growth factor effects on nuclear factor-kappaB activation in hematopoietic progenitor cells. *Cancer Res* 2001; **61**: 2015–2021.
46 Dadgostar H, Zarnegar B, Hoffmann A, Qin XF, Truong U, Rao G *et al.* Cooperation of multiple signaling pathways in CD40-regulated gene expression in B lymphocytes. *Proc Natl Acad Sci USA* 2002; **99**: 1497–1502.
47 Dallman C, Johnson PW, Packham G. Differential regulation of cell survival by CD40. *Apoptosis* 2003; **8**: 45–53.
48 Kroll J, Waltenberger J. The vascular endothelial growth factor receptor KDR activates multiple signal transduction pathways in porcine aortic endothelial cells. *J Biol Chem* 1997; **272**: 32521–32527.
49 Aramoto H, Breslin JW, Pappas PJ, Hobson RW, Duran WN. Vascular endothelial growth factor stimulates differential signaling pathways in *in vivo* microcirculation. *Am J Physiol Heart Circ Physiol* 2004; **287**: H1590–H1598.
50 Kitada S, Zapata JM, Andreeff M, Reed JC. Bryostatin and CD40-ligand enhance apoptosis resistance and induce expression of cell survival genes in B-cell chronic lymphocytic leukaemia. *Br J Haematol* 1999; **106**: 995–1004.

SPOTLIGHT