Role of the vascular endothelial growth factor (VEGF) in chronic lymphocytic leukemia (CLL): Implications to overcome the apoptotic block

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The only way around is through

Robert Forst
Abstract

Chronic lymphocytic leukemia (CLL) is an incurable disease, which is characterized by an accumulation of monoclonal CD5/CD19/CD23-positive B-lymphocytes, which exhibit a functional apoptotic block. The vascular endothelial growth factor (VEGF) is a potent mitogen with the capacity to induce angiogenesis by stimulation of vascular endothelial cells. It has been suggested that VEGF has an angiogenesis-independent role in hematological diseases. Also, CLL cells could be shown to express and secrete VEGF and to feature VEGF-receptors (VEGF-R).

Despite their apoptotic-resistance *in vivo*, CLL cells die within a few days when taken out of their natural microenvironment and are placed under cell culture conditions, strongly suggesting the bone marrow and peripheral blood to be of critical importance in the prevention of apoptotic CLL cell death *in vivo*. As for example bone marrow stromal cells produce and secrete VEGF, a paracrine feedback loop might be involved in the apoptotic resistance CLL cells feature *in vivo*.

The aim of this investigation was to elucidate the role of VEGF in the apoptotic resistance of CLL cells, especially in the background of a microenvironmental influence and therefore, to discover potential targets for a CLL cell specific therapeutic approach.

In this study it could be demonstrated that CLL cells, but not healthy B-cells express the most common VEGF isoforms and exhibit a phosphorylated VEGF-receptor 2 (VEGF-R2). Phosphorylation was lost with time under cell culture conditions and went along with a loss of the apoptotic resistance. Since addition of rhVEGF increased levels of anti-apoptotic proteins, but did not significantly influence CLL cell survival *in vitro*, it can be concluded that VEGF has pro-survival functions, but requires further components derived from the microenvironment to achieve full apoptotic protection as present *in vivo*. Therefore, CLL cells were cocultured together with the bone marrow-derived stromal cell line HS5, which produced and secreted high levels of VEGF. This resulted in maintenance of the phosphorylated receptor status and a prolonged survival of the CLL cells *in vitro*. Interestingly, healthy
B-cells, which only express low levels of VEGF-R2, did not profit from coculture with HS5 in terms of survival. The essential role of VEGF in HS5-mediated survival-support could be demonstrated, as neutralization of VEGF in CLL/HS5 coculture using a monoclonal VEGF antibody significantly reduced the survival advantage. In this study it could further be demonstrated that paracrine VEGF, derived from bone marrow stromal cells, rather than CLL cell-derived VEGF is essential, as downregulation of VEGF in HS5 cells by siRNA almost completely abolished the coculture-mediated survival support for CLL cells.

As a possible mechanism of VEGF-mediated survival support the activation of signal transducer and activator of transcription (STAT) 3 via tyr705 phosphorylation could be demonstrated. This phosphorylation was induced by addition of recombinant human VEGF to CLL cell monoculture as well as by cocultivation with HS5 cells and was reversible by addition of a VEGF-R inhibitor. The activation of STAT3 could be demonstrated by an upregulation of the known STAT3 targets Bcl\textsubscript{XL} and cyclinD1. The known downstream effector of the VEGF-R2 Akt was neither phosphorylated upon rhVEGF stimulation nor by HS5 coculture.

The VEGF-STAT3 signal transduction pathway can therefore be considered a suitable target for a therapeutic intervention. The tested monoclonal antibody MAb293 and the VEGF-R inhibitor GW 786034 significantly reduced the survival advantage CLL cells gained from HS5 coculture. The selected STAT3-inhibitor was effective in high concentrations after long incubation times with limited selectivity.

In conclusion, we propose that VEGF action is indispensable in a multi-part pro-survival complex involving STAT3 and subsequent expression of pro-survival factors in CLL cells. The VEGF/VEGF-R/STAT3 pathway might therefore be a promising target for selective therapeutic approaches in CLL.
Zusammenfassung


Trotz ihrer Apoptoseresistenz in vivo sterben CLL-Zellen, die aus ihrer Umgebung im Blut oder Knochenmark isoliert werden, innerhalb weniger Tage in vitro ab, weswegen dem natürlichen Mikromilieu der CLL-Zellen eine wichtige Funktion bei der apoptotischen Resistenz zugeschrieben wird. Da Knochenmarkstromazellen etwa VEGF produzieren, könnte eine parakrine VEGF-Rückkopplung an der Verhinderung des Zelltodes der CLL-Zellen in vivo beteiligt sein.

Das Ziel der vorliegenden Arbeit war, die Rolle von VEGF beim apoptotischen Block der CLL-Zellen, insbesondere im Hinblick auf den Einfluss des Mikromilieus zu untersuchen und dadurch potentielle Angriffspunkte für eine zielgerichtete Therapie aufzudecken.


Zusammenfassend kann VEGF als ein essentieller Bestandteil eines überlebensfördernden Zusammenspiels der CLL-Zelle mit ihrem Mikromilieu beschrieben werden, bei dem die Aktivierung von STAT3 eine Rolle spielt. Der VEGF/VEGF-R/STAT3 Signalweg ist demnach ein viel versprechendes Ziel für eine therapeutische Intervention in der CLL.
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<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>°C</td>
<td>grad celcius</td>
</tr>
<tr>
<td>µ</td>
<td>micro</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymbhoblastic leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>acute myelogenous leukemia</td>
</tr>
<tr>
<td>APC</td>
<td>adenomatous polyposis coli</td>
</tr>
<tr>
<td>BAD</td>
<td>(Bcl-2)-associated death promoter homologue</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B-cell lymphoma 2</td>
</tr>
<tr>
<td>BclXL</td>
<td>B-cell lymphoma extra large</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukemia</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned media</td>
</tr>
<tr>
<td>CML</td>
<td>chronic myelogenous leukemia</td>
</tr>
<tr>
<td>CO₂</td>
<td>carbon dioxide</td>
</tr>
<tr>
<td>Ct</td>
<td>threshold cycle</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>double distilled water</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco<code>s Modified Eagle</code>s Medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxid</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>dNTP</td>
<td>deoxynucleotide triphosphate</td>
</tr>
<tr>
<td>DSH</td>
<td>dishevelled</td>
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<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>e.g.</td>
<td>exempli gratia (for example)</td>
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</table>
EC  endothelial cells
ECL  enhanced chemiluminescence
EDTA  ethylenediaminetetraacetic acid
ELISA  Enzyme-linked Immunosorbent Assay
EM  extracellular matrix
et al  et alii (and others)
FACS  fluorescent activated cell sorter
FCS  fetal calf serum
FISH  fluorescent in situ hybridization
FITC  fluoresceinisothiocyanate
FZD  frizzled receptor
g  gram
G-CSF  growth factors like granulocyte colony-stimulating factor
GM-CSF  granulocyte-macrophage colony-stimulating factor
GSK3\(\beta\)  glycogen synthase kinase \(\beta\)
HCL  hairy cell lymphoma
HEPES  4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
HLA  human leucocyte antigen
HSC  hematopoietic stem cell
IC50  half maximal inhibitory concentration
Ig  immunoglobulin
IgV\(_H\)  immunoglobulin heavy chain variable region
IL  interleukin
JAK  Janus kinase
kDa  kilo Dalton
l  litre
LD50  half maximal lethal concentration
Lef-1  lymphoid enhancer factor 1
LRP  LDL receptor related protein
M  molar (mol/litre)
m  mili
MAPK  mitogen activated protein kinase
MBL  monoclonal B-cell lymphocytosis
MCL  mantel cell lymphoma
<table>
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<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tr>
<td>Mcl1</td>
<td>mantel cell lymphoma 1</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>magnesium chloride</td>
</tr>
<tr>
<td>min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>n</td>
<td>nano</td>
</tr>
<tr>
<td>NRP</td>
<td>neuropilin</td>
</tr>
<tr>
<td>p</td>
<td>pico</td>
</tr>
<tr>
<td>p53</td>
<td>proapoptotic protein 53</td>
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<tr>
<td>PAGE</td>
<td>poly acrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PARP</td>
<td>poly (ADP-ribose) polymerase</td>
</tr>
<tr>
<td>PB</td>
<td>peripheral blood</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>platelet-derived growth factor</td>
</tr>
<tr>
<td>pH</td>
<td>negative logarithm of the hydrogen ion concentration</td>
</tr>
<tr>
<td>PI</td>
<td>propidium iodide</td>
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<tr>
<td>Pi3K</td>
<td>phosphatidylinositol-3 kinase</td>
</tr>
<tr>
<td>PLC</td>
<td>phospho lipase C</td>
</tr>
<tr>
<td>PlGF</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>PLL</td>
<td>B-cell prolymphocytic leukemia</td>
</tr>
<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>RB1</td>
<td>retinoblastoma 1</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>rpm</td>
<td>rounds per minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Roswell Park Memorial Park</td>
</tr>
<tr>
<td>RT</td>
<td>room temperature</td>
</tr>
<tr>
<td>RTKs</td>
<td>receptor tyrosine kinases</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>sec</td>
<td>second</td>
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<tr>
<td>SEM (σM)</td>
<td>standard error of the mean</td>
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<td>ser</td>
<td>serine</td>
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</table>
siRNA  short interfering ribo nucelic acid
SMZL  splenic marginal zone lymphoma
STAT  signal transducer and activator of transcription
TCF   T-cell factor
TRIS  Tris hydroxyl methyl aminomethane
tyra  tyrosine
VEGF  vascular endothelial growth factor
VEGF-R vascular endothelial growth factor receptor
w/v   weight per volume
XIAP  X-linked inhibitor of apoptosis
ZAP70 zeta associated protein kinase 70
α     alpha
β     beta
γ     gamma
δ     delta
κ     kappa
μ     mu
σ     standard deviation
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Chapter 1

Introduction

1.1 Chronic lymphocytic leukemia (CLL)

1.1.1 Definition

Chronic lymphocytic leukemia (CLL) is a lymphoproliferative disorder, which is characterized by the accumulation of mature, but immuno-incompetent B-lymphocytes in the bone marrow, peripheral blood, and various organs. CLL is defined by three characteristics which are (i) $< 5,000$ monoclonal B-lymphocytes per $\mu$l blood, (ii) the presence of a clonal population of CD5/CD19/CD23-positive lymphocytes and (iii) less than 55% circulating prolymphocytes (Hallek et al., 2008).

1.1.2 Epidemiology

Chronic lymphocytic leukemia (CLL) affects mainly people of the age of 50 and older and it account for approximately 40% of leukemias in adults older than 65 years. Its incidence increases with age and patients younger than 30 years are very rare. Men are twice as likely to develop CLL as women (Ries LAG, 1999). Prevalence for occurrence of CLL could be encountered in Europe, North America and Australia, whereas the disease is considerably less common in Asian countries such as India, Japan or China (Groves et al., 1995). Since Asians migrating to the USA were shown to maintain their low incidence rates, genetic factors are more likely to be responsible than environmental ones (Groves et al., 1995; Pan et al., 2002; Yanagihara et al.,
1989). Nevertheless, some studies described a correlation of CLL incidence and pesticides or herbicides used in agriculture or exposure to benzene and the rubber industry (Goldin and Slager, 2007; Schnatter et al., 2005), but results are not consistent (Richardson et al., 2005). Furthermore, neither ionizing irradiation nor viral genes could be associated with the prevalence of CLL (Kipps, 1998).

Several families with increased occurrence of the disease have been described over the last 25 years, suggesting a familial predisposition (Cartwright et al., 1987; Goldgar et al., 1994; Pottern et al., 1991). First- and second-degree relatives of patients with CLL have an increased risk of subclinical monoclonal B-cell expansion and lymphoid malignancies including CLL. Furthermore, in successive generations of families with CLL disease onset is frequently seen earlier and often present in a more severe form (Rawstron, 2004; Yuille et al., 1998). Recently, a study demonstrated an 8.5 fold increased risk for case relatives to obtain CLL (Goldin et al., 2009). Nevertheless, no inherited genetic defects, making a member of a CLL family prone to obtain the disease, were identified so far. It is likely that a complex of several aberrant events, rather than one simple genetic defect, is responsible for occurrence of CLL in families.

1.1.3 Etiology and molecular pathogenesis

The etiology of CLL remains largely unclear up to date. Nevertheless, several factors have been associated with disease initiation and progression.

1.1.3.1 Origin of the CLL cell

The determination of the origin of the leukemic CLL cell has been a focus of scientific interest for a long time. The knowledge of the tumor precursor cell in CLL is of high impact to understand the pathogenesis of the disease and to obtain insight into the mechanisms of the transformation process from a healthy B-cell towards a malignant CLL cell.

In general, B-cell development initiates in the bone marrow and in the fetal liver originating from stem cells (HSCs). Starting from HSCs multiple hematopoietic lineages can be generated through a series of intermediate progenitors. B-cells develop from a common lymphoid progenitor. Bone marrow-
derived antigen-inexperienced naïve B-cells move to the germinal center (GC) in the peripheral lymphnodes where they undergo division and proliferation processes. In the GC, the process of immunoglobulin variable region (IgV) somatic hypermutation (SHM) modifies the antibody genes of the B-cells to generate high affinity antibodies. In the progress of normal B-cell development these B-cells with improved antigene-binding are positively selected and differentiate into memory B-cells and plasma cells. GC B-cells also undergo class switching by a somatic DNA recombination mechanism. Disruption of the regulation of B-cell differentiation and activation might result in the occurrence of leukemias or lymphomas. At which step this "oncogenic hit" occurs is not clear up to now.

CLL cells are in general a morphologically homogenous population with a characteristic immunophenotype expressing the antigens CD5, CD19 and CD23 while exhibiting low levels of surface immunoglobulin (Ig) (Caligaris-Cappio and Hamblin, 1999). Though, on the genetic level CLL cases are heterogeneous with rearranged variable genes in the immunoglobulin heavy chain (IgV₇), which can be either somatically hypermutated or unmuted (Chiorazzi and Ferrarini, 2003; Fais et al., 1998; Oscier et al., 1997; Schroeder, Jr. and Dighiero, 1994). Additionally, these genetically-defined subtypes are

Figure 1: Model for the cellular derivation of CLL.
Source: Own illustration
associated with divergent clinical courses. While IgV_H-mutated CLL cases show generally a more benign clinical course, unmutated CLL cases have been demonstrated to have a less favourable prognosis. These facts at first suggested that there might be different tumor precursor cells for the major CLL subtypes originating from distinct stages of B-cell development. Additionally, the presence of somatically mutated antibody genes in CLL patients could be an indication for antigenic selection to be involved in the development of CLL. This was further proofed by the finding of certain IgV_H family members to be more frequently present in CLL patients, independent of their mutational status, than would be expected from their expression in the antibody repertoire in normal B-cells (Fais et al., 1998). In several studies it was proofed that these specific IgV_H gene repertoires in CLL patients are indeed a result of antigen selection rather than simply reflecting the aging process (Potter et al., 2002; Widhopf et al., 2004). A further pointer towards CLL cells being a result of antigen-mediated selection is the fact that unrelated CLL patients can feature almost identical B-cell receptors (BCRs) (Messmer et al., 2004; Murray et al., 2008; Tobin et al., 2003; Tobin et al., 2004; Widhopf et al., 2004). Gene expression profiling (GEP) studies could identify a subset of genes (molecular signature), which allowed differentiation of IgV_H unmutated and IgV_H mutated cases. CLL cases with unmutated IgV_H seem to express high levels of genes which are known to be activated as a result of BCR-mediated stimulation (Rosenwald et al., 2001). A physiological consequence could be antigen-mediated BCR-signaling to have impact on the clinical prognosis of CLL (Muzio et al., 2008).

Another pointer towards CLL cells being derived from antigen-stimulated B-cells is their cell surface phenotype which resembles that of antigen-activated B-cells (Damle et al., 2002). GEP suggested B-cells to be most closely related to a specific B-cell subset of the CD27+ B-cells (Klein et al., 2001). Those cells comprise a heterogeneous pool of B-cells, such as memory B-cells and marginal zone B-cells, which are antigen-experienced. Interestingly, GEP did not identify any correlation between CLL cells and CD5+ cells derived from cord blood, CD27-(naïve), or GC B-cells (Klein et al., 2001; Rosenwald et al., 2001). CD27+ cells comprise up to 40% of B-cells in the peripheral blood (PB) of adults and are mainly found in sites of antigen entry, such as the marginal
zone and the tonsillar subepithelium (Klein et al., 1998). Normal CD27+ cells respond quickly to exogenous antigens by differentiation into antibody-secreting cells (Kindler and Zubler, 1997). These facts provide further evidence for antigen-experienced cells to be the precursor of CLL cells. Additionally, the cytogenetic abnormalities occurring in CLL patients differ markedly from that of other B-cell malignancies and resemble most closely that of hairy cell leukemia (HCL) (Basso et al., 2004). HCL features distinct morphological and phenotypic characteristics in comparison to CLL (Harris et al., 1994), but interestingly, HCL resembles CLL in that its GEP is most closely related to that of the CD27+ cells (Basso et al., 2004).

All the mentioned evidences target on CLL being derived from one common precursor, an antigen-experienced (CD5-CD27+) B-lymphocyte, which are highly presented in the lymphoid organs and the peripheral blood (appr. 40%). As virtually all CD27+ cells are CD5-, the CD5 expression in CLL cells would be a consequence of the activation phenotype of the tumor cell (Wortis et al., 1995). Besides this theory it has recently been suggested that CLL cells might arise from a small population of CD5+ B-cells detected in the lymphoid organs (Dono et al., 2007). With this background, also the relation of monoclonal B-cell lymphocytosis, a benign clonal proliferation of CD5 B-cells, which phenotypically and genetically resemble that of the CLL cell, and CLL remains unclear (Dagklis et al., 2009; Landgren et al., 2009; Rawstron, 2004).

1.1.3.2 Genetic factors

In CLL several frequently occurring chromosomal aberrations have been described. Going along with technique advancement, the numbers of detected chromosomal abnormalities in CLL patients have increased from 50% detected by banding analysis (Carney and Wierda, 2005) to approximately 80% detected by fluorescent in vitro hybridisation (FISH), comparative genomic hybridisation (CGH), single nucleotide polymorphism (SNP) and micorarrays (Pfeifer et al., 2007; Stilgenbauer et al., 2002). Genetic abnormalities in CLL are thought to be acquired after birth through chromosomal instabilities, rather than being inherited. The most common genetic defect in approximately 55% of CLL cases is the deletion of 13q14.1 (Dohner et al., 1999). While at first the tumor suppressor gene RB1 was suggested to be the candidate gene for
this aberration (Liu et al., 1992), this assumption was refuted as an RB1 deletion or mutation is only found in a small percentage of the malignant clone (Dohner et al., 1994). Instead has this region recently been described to encode for two distinct micro RNAs (miRNA), miR15A and miR-16-1 (Calin and Croce, 2006). Up to now, there are no natural targets of those miRNAs. An increase in T cell leukemia 1 (TCL1) and B-cell lymphoma 2 (Bcl2), both anti-apoptotic factors, was observed when these miRNAs where deleted, suggesting those genes to be potential targets of miR15A and miR-16-1 (Calin et al., 2005; Calin and Croce, 2006). As 13q14.1 as a sole abnormality confers a favourable prognosis (Dohner et al., 2000) the exact role of this deletion is not clear up to date. Second prevalent chromosomal defect in CLL is the deletion of 11q22 in approximately 18% of cases (Dohner et al., 1997). As a candidate gene the ATM gene has been described to be present in this region. ATM is a crucial player in cellular response to double strand DNA breaks (Stankovic et al., 2002). Besides deletion, ATM gene mutations are a frequent event in CLL (Bullrich et al., 1999; Stankovic et al., 1999). As also here, deletions in 11q22 are not always accompanied by mutations, it is thought that potentially further genes are involved (Schaffner et al., 1999). 11q22 deletion is typically associated with a poor prognosis and advanced stage disease (Dohner et al., 1997). The third most common chromosomal aberration is trisomy 12 with an incidence of approximately 16% (Oscier, 1994). The crucial segment in this region has yet to be determined. Bands 12q13-q22 include a segment that is found to be duplicated in CLL (Merup et al., 1997) with MDM2, a negative regulator of p53 as a potential oncogene, in this location. However, recently no impact of MDM2 polymorphism in a small cohort (85 patients) of CLL patients could be detected (Lahiri et al., 2007). Several adverse features have been associated with this abnormality such as atypical cell morphology and immunophenotype (Dewald et al., 2003; Matutes et al., 1996). Around 7% of CLL cases exhibit a deletion on the short arm of chromosome 17 which contains the p53 gene and is associated with short survival, rapid disease progression (Dohner et al., 1995a) and drug-resistance (Turgut et al., 2007). The p53 protein arrests cells with damaged DNA and facilitates DNA-repair (Vogelstein et al., 2000). Other than inducing DNA-repair, p53 can also promote apoptosis to abet the destruction of the damaged cells. In this way p53 also
mediates cytotoxicity of many anticancer agents; hence it is not surprising that patients possessing a p53 deletion generally show worse response towards treatment, such as purine analogs or the anti-CD20 antibody rituximab (Byrd et al., 2003; Byrd et al., 2007; Dohner et al., 1995b).

1.1.3.3 The bone marrow microenvironment

Neoplastic CLL cells are characterized by their prolonged survival due to a resistance towards apoptosis in vivo, which entails their accumulation. This feature is completely absent once CLL cells are taken out of their natural microenvironment and put under in vitro culture conditions (Collins et al., 1989), indicating the bone marrow environment to be a crucial supporter of their apoptotic resistance. Since early stages of CLL are characterized by bone marrow infiltration the bone marrow microenvironment can be considered a critical side of nurturing in the disease process. Bidirectional interactions between the malignant CLL cells and the non-transformed bystander cells lead to the establishment by of an abnormal microenvironment favouring the survival of the CLL cells. In turn, this extended survival might create an intracellular milieu which supports the occurrence of unfavourable genetic instabilities. The microenvironment might also represent a niche for the CLL cell to retreat therapeutic interventions.

Several cell types are present in the bone marrow, such as stromal cell, T-cells, follicular dendritic cells (FDCs), BM-derived endothelial cells (BMECs), umbilical vein endothelial cells (HUVECs), monocyte-derived nurse-like-cells and also cells involved in bone homeostatis such as chondroclasts, osteoclasts and osteoblasts. This heterogeneous cell population creates a dynamic microenvironment of direct cell-cell interactions with high concentrations of growth factors and cytokines present.
Several of those accessory cells have been demonstrated to have the capacity of sustaining prolonged viability of the leukemic clone when placed together in *in vitro* culture (Burger et al., 2000; Ghia et al., 2005; Jewell and Yong, 1997; Lagneaux et al., 1998; Panayiotidis et al., 1996a; Pedersen et al., 2002). FDCs stay in contact with CLL cells especially in the early phase of bone marrow involvement as well as in the lymph nodes (Chilosi et al., 1985). It was demonstrated that CLL cell survival support by FDCs involves direct cell contact dependent on CD44-ligation and subsequent upregulation of the anti-apoptotic protein Mcl1 (Pedersen et al., 2002).

The presence of T-cells in a survival supporting *in vitro* coculture system can be mimicked by addition of T-cell-derived cytokines, such as IL4 or soluble CD40L (Buske et al., 1997; Ranheim and Kipps, 1993). CD40 stimulation not only prevents spontaneous apoptosis, but also results in proliferation (Granziero et al., 2001; Patten et al., 2008), activation of CLL cells as well as chemokine production (Ghia et al., 2002b; Yellin et al., 1994). Activated CD40L+ T-cells are present in bone marrow-derived from CLL patients primarily in the so called "proliferation centres" (PC) or pseudofollicles, which are the histological CLL hallmark in lymph nodes. In PCs CLL cells are in close physical contact to
these CD40L+ T-cells providing a CD40L-stimulus (Ghia et al., 2002b). In this background it is of great interest, that lymph node and bone marrow-derived CLL cells themselves might be responsible for the presence of activated T-cells in the PCs, as they were described to express the T-cell attracting chemokines CCL17 and CCL22. Expression of these chemokines on PB-derived CLL cells could be induced upon stimulation with CD40L (Ghia et al., 2002b). Hence, CLL and T-cells in a patient’s bone marrow possess bidirectional interactions regulated by adhesion molecules and chemokines translating into a further production of cytokines by both cell types (Ghia and Caligaris-Cappio, 2000).

Besides T-cells, stromal cells possess a significant influence on CLL cells. Stromal cells are the key regulators of normal B-lymphopoiesis. Despite this knowledge it is up to now not completely resolved, how the precise ligand-receptor interaction between B-cells and stromal cells is controlled. Several adhesion molecules have been implicated, such as selectins, integrins, for example integrin β1-(CD29) and β2-(CD18), immunoglobulins, intracellular adhesion molecules ICAM-1/CD54, ICAM-2/CD102 or ICAM-3/CD50 and the CD44 family of homing receptors (Caligaris-Cappio and Hamblin, 1999; Takeuchi and Katayama, 1993). CLL cells express for example the integrins CD49d/CD11a or CD11b/CD18 which interact with CD54 and CD106 on stromal cells and have been reported to be survival-supportive for CLL cells (Plate et al., 2000). Also other adhesion molecules, such as CD44 are highly expressed in CLL cells and are associated with an aggressive disease progression and adverse prognosis (Eistere et al., 1996).

Besides direct cell-cell contacts also soluble factors, such as cytokines contribute to CLL cell survival. Stromal cells for example produce the interleukins IL-6, IL-7, IL-10, TGF-β, stem cell factor (SCF) and vascular endothelial growth factor (VEGF) therewith exerting a complex regulatory function on CLL cells (Ghia and Caligaris-Cappio, 2000). In general, CLL cells are known to exhibit receptors for many pro-survival cytokines, suggesting a high response potential towards these factors secreted by accessory non-tumorigenic cells in the bone marrow and in the PB. Since CLL cells are also able to produce several of those cytokines themselves, autocrine loops are likely to additionally support CLL cell survival (Kay et al., 2002b).
1.1.3.4 Abberant regulation of intracellular signaling cascades

Possibly reflecting the clinical heterogeneity observed in patients, various aberrantly activated signaling cascades have been associated with the initiation and the course of CLL. A variety of humoral factors and cytokines that play a role in the deregulation of these pathways have been described.

1.1.3.4.1 B-cell receptor (BCR) signaling

A current hypothesis suggests that CLL cells are selected by some sort of antigenic pressure (Johnson et al., 1997) as evidenced by a highly restricted immunoglobulin heavy chain variable region (IgV_H) gene repertoire compared to the normal adult B-cell repertoire (Kipps et al., 1989; Meinhardt et al., 1999). Further the existence of somatic hypermutations of IgV_H genes (Damle et al., 2002) and expression profiling signatures (Klein et al., 2001) support the idea of BCR-mediated stimulation to be involved in the origin of the CLL cell, which was discussed earlier.

The BCR consists of membrane Igs which are associated with a CD79α/CD79b heterodimer (van Noesel et al., 1992). Signal transduction of the BCR leads to activation of non-receptor tyrosine kinases like Src and Syc (Burkhardt et al., 1991; Yamanashi et al., 1991), increased intracellular calcium levels and subsequently to cell division. This defective calcium release has been linked to changes in global tyrosine phosphorylation patterns of cytosolic phosphoproteins.

The typical CLL cell expresses CD19, CD23 and CD5, while expression of CD22, FMC, CD79b and surface immunoglobulins is low or absent (Zomas et al., 1996). CD79b is usually replaced by a truncated form, which is able to inhibit apoptotic signaling (Alfarano et al., 1999; Cragg et al., 2002). CLL cells of patients show a wide heterogeneity in terms of the functional response of their BCRs to stimulation through the surface immunoglobulins (sIg). While some cases are highly sensitive and show effective activation as detected by increased global tyrosine phosphorylation (Lanham et al., 2003), others are completely unresponsive. It is of interest that the BCR unresponsiveness is reversible in vitro and dependent on the surface levels of IgM (Mockridge et al., 2007). High responsiveness could be correlated with unmutated IgV_H status, ZAP70 and CD38 expression (Lanham et al., 2003; Zupo et al., 1996). Nev-
Nevertheless, outcome seems to be highly dependent on time, strength, affinity of the binding and antigen type. It can be speculated that in the cases with more competent BCRs a constant antigenic stimulation might promote cell survival and possibly also cell growth, while in non-responsive cases an ongoing stimulation results in receptor desensitization an anergic state (Stevenson and Caligaris-Cappio, 2004). While CLL cells are in G0/early G1 phase of the cell cycle (Caligaris-Cappio and Hamblin, 1999), they are at the same time apparently phenotypically hyperactivated (Damle et al., 2002). The determination of signaling pathways originating from the BCR have been the focus of intense research. Recently constitutive activation of the mitogen-activated protein kinase (MAPK) could be demonstrated which went along with active NF-AT transcription factor activity. Interestingly, activation of Akt was not seen in this study (Muzio et al., 2008). The combination of active MAPK and NF-AT together with inactive Akt could be correlated with an anergic state in murine B-lymphocytes (Merrell et al., 2006).

1.1.3.4.2 Apoptotic signaling pathways
Apoptosis is the process of programmed cell death and is controlled by a wide range of cell signals, such as growth factors, cytokines, hormones, or toxins. Several apoptotic pathways converge into a common final one, which results in the activation of the family of caspases. Caspases are cysteine proteases with the ability to cleave a variety of substrates in the cell, subsequently resulting in their demise. Numerous pro-death and pro-survival molecules have to be precisely balanced in order to maintain an accurate control of cell death induction and prevention. CLL is a classical example for how dysregulation of the apoptotic pathways can lead to malignancies. In CLL the anti-apoptotic protein Bcl2 is highly upregulated. The Bcl2 family of proteins can be considered a key regulator of programmed cell death (Reed, 1997). Bcl2 is the best characterized member and functions via several mechanisms (Tsujimoto and Shimizu, 2000). It prevents pro-apoptotic proteins from functioning by forming inactivating heterodimers and it can also form channels that stabilize the mitochondrial membrane, therefore impeding the release of apoptosis-inducing factors, such as cytochrome C. Another member of the Bcl2 family of proteins is Mcl1. Mcl1 was recently described to function as a predictive marker in re-
gard to response to chemoimmunotherapy in CLL, where high Mcl1 levels were predictive of a poor response (Kitada et al., 1998). In CLL also the X-linked inhibitor of apoptosis (XIAP), which is a member of the family of inhibitors of apoptosis proteins (IAPs), was described to be present at high levels (Byrd et al., 2002; Schliep et al., 2004). IAPs have a direct negative influence on apoptosis induction through inhibiting caspase activity (Deveraux et al., 1997) and their presents is therefore likely to have strong impact on the ability of a cell to undergo apoptosis.

1.1.3.4.3 **Wnt/β-catennin/Lef-1 signaling pathway**
The Wnt/β-catennin/Lef-1 signaling pathway is known for its crucial role during embryogenesis, while being largely downregulated or even completely shut off in the adult organism. Its aberrant activity has been associated with several cancers such as colon cancer or breast cancer. Wnt-proteins bind to a cell surface receptor complex comprised of a member of the frizzled (FZD) receptor family and its coreceptor LDL receptor related protein 5/6 (LRP5/6), leading to activation of a dishevelled protein family member (DSH). Active DSH inhibits a complex consisting of glycogen synthase kinase 3β (GSK3β), Axin and adenomatous polyposis coli (APC). In its active state, this complex leads to phosphorylation of β-catennin via GSK3β, which is subsequently degraded by the proteasome. Inhibition of the GSK3β/Axin/APC complex prevents β-catennin phosphorylation and degradation. It accumulates in the cytoplasm and translocates into the nucleus, where it binds and activates a member of the T-cell factor (TCF)/lymphoid enhancer binding factor 1 (Lef-1) transcription factor family. This leads to expression of target genes, which are involved in the regulation of cellular processes, such as proliferation and differentiation (Polakis, 2000).

Reasons for aberrant activity of this pathway in cancer are variable and range from reduced presence of natural occurring Wnt-inhibitors to constitutively activating mutations. In CLL, the expression of several Wnt-inhibitors is reduced due to epigenetic silencing (Chim et al., 2008), while Wnt-proteins are significantly overexpressed (Lu et al., 2004) and the final effector of the cascade Lef-1 was described as one of the most overexpressed genes in CLL (Jelinek et al., 2003). It has been demonstrated that this pathways confers a crucial
survival support to CLL cells and that it offers several option for therapeutic interventions (Gehrke I et al., 2009) Wnt-signaling inhibition by for example R-Etodolac (Lu et al., 2004) or the small molecule substances CGP049090 and PKF 115-484 (Gandhirajan RK et al., 2010) could be demonstrated to selectively induce apoptosis in CLL cells.

1.1.4 Clinical aspects

1.1.4.1 Diagnosis

Many patients are diagnosed with CLL without prior symptoms, but rather during a blood test for an unrelated health problem or a routine check up. Usually, CLL symptoms are mainly vague and general. They include weakness, fatigue, weight loss, fever, night sweats and enlarged lymph notes (lymphadenopathy). A further sign of CLL is nausea after eating small meals, which is due to an enlarged spleen (splenomegaly). Most symptoms are a consequence of the severe increase of CLL lymphocytes, which replace normal blood cells, such as healthy functional lymphocytes (leucopenia), erythrocytes (anemia) and platelets (thrombocytopenia). The lack of these blood components results in an increased susceptibility to infections, weakness and excess bruising and bleeding. Also autoimmune effects are frequently observed, such as hematolytic anemia.

Diagnosis of CLL is further based on the revised guidelines of the national cancer institute working group (NCI-WG) (Cheson et al., 1996; Hallek et al., 2008). Several conditions have to be given in order to diagnose CLL. These are the persistence of $>5 \times 10^9$ mature lymphocytes of B-cell origin per litre blood in the absence of other causes. A heavy bone marrow infiltration with consecutive peripheral cytopenia compensates for lymphocyte count in blood. The presence of $<5 \times 10^9$ monoclonal lymphocytes per litre blood without any other clinical symptoms has recently been termed monoclonal B lymphocytosis (MBL) (Marti et al., 2005).

Further, distinct immunophenotypic criteria apply for diagnosis of CLL: The simultaneous existence of B-cell surface molecules, such as CD19, CD20 and CD23, and the T-cell surface marker CD5 must be given. At the same time no further T-cell markers should be detectable. In addition, immunoglobulins
must be light chain restricted, negative for the B-cell antigen FMC7, surface immunoglobulins (sIg) must be low and CD79b expression must be low or completely absent. For diagnosis of CLL special consideration has to be given to several distinct immunophenotypic and/or morphologic patterns to distinguish CLL from other hematologic malignancies with similar clinical and microscopic features, such as mantle cell lymphoma (MCL), hairy cell lymphoma (HCL), B-cell prolymphocytic leukemia (PLL), splenic marginal zone lymphoma (SMZL) or Waldenstrom’s macroglobulinemia (Jaffe et al., 2008).

1.1.4.2 Staging

CLL patients are commonly classified by staging systems to summarize the progression of the cancer. For CLL staging two different systems exist, the Rai system and the Binet system. The former is more often used in the USA, whereas the latter is common in Europe and other parts of the world. Rai staging separates patients into five groups (O-IV) which correspond to three risk groups: low risk (stage 0), intermediate risk (stages I and II) and high risk (stages II and IV). Binet staging focuses on the number of lymphoid tissues which are involved. Enlarged lymph nodes of the neck, underarms, and groin, as well as the spleen, are each considered "one group," whether unilateral (one-sided) or bilateral (on both sides). The stages and their major clinical features are listed in the following tables.

<table>
<thead>
<tr>
<th>clinical feature</th>
<th>Rai stage 0</th>
<th>intermediate risk</th>
<th>high risk</th>
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<tbody>
<tr>
<td>lymphocytosis</td>
<td>x</td>
<td>x</td>
<td>x/ -</td>
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<tr>
<td>lymphadenopathy</td>
<td>x</td>
<td>x</td>
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<td>hepatomegaly</td>
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<tr>
<td>splenomegaly</td>
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<tr>
<td>anemia</td>
<td>x</td>
<td>x</td>
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<tr>
<td>thrombocytopenia</td>
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<td>x</td>
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Table 1: CLL staging after RK Rai (Rai et al., 1975).
Source: Own illustration
1.1.4.3 Prognostic factors

CLL patients show a remarkable clinical diversity. The disease may be characterized by a rather indolent course with good long-term prognosis without the need of a specific therapy or it may take on an accelerated course requiring treatment immediately. Several prognostic factors have been established, which allow to predict time to treatment and overall survival expectancy. The somatic hypermutational status of the rearranged variable regions of the immunoglobulin heavy chain (IgV\textsubscript{H}) has been demonstrated to have substantial prognostic relevance in CLL by separating patients into two different groups (Hamblin et al., 1999). CLL patients who exhibit a mutated IgV\textsubscript{H} gene locus have a considerable better prognosis than those featuring an unmutated IgV\textsubscript{H} gene locus, which generally show a more aggressive disease progression, atypical morphology, adverse cytogenetic features or therapy resistance (Krober et al., 2002; Oscier et al., 2002). The definition of "mutated" or "unmutated" is based on a defined threshold of 98% homology to the most similar germline counterparts (Dighiero, 1998; Hashimoto et al., 1995; Schroeder, Jr. and Dighiero, 1994). Interestingly, the rearrangement of a specific variable-region gene, the V3-21 gene, has been associated with an unfavorable clinical outcome irrespective of the V\textsubscript{H} mutational status (Krober et al., 2002; Tobin et al., 2002).

Other prognostic markers are serum levels of the thymidinkinase (TK), $\beta_2$-microglobulin ($\beta_2$MG) and soluble CD23 (sCD23). All these markers have been described to positively correlate with several parameters such as disease progression, diffuse bone marrow infiltration or rapid doubling time (Keating

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<tr>
<th>clinical feature</th>
<th>Binet stage</th>
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<td></td>
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<tr>
<td>&gt;3 areas of lymphadenopathy</td>
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<tr>
<td>&lt; 3 areas of lymphadenopathy</td>
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<tr>
<td>anemia</td>
<td>x</td>
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<tr>
<td>thrombocytopenia</td>
<td>x</td>
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Table 2: CLL staging after JL Binet (Binet et al., 1981).
Source: Own illustration
et al., 2005; Wierda et al., 2005).

Also the expression of surface markers, such as CD38 and zeta-associated protein (ZAP) 70, has prognostic significance in CLL. Although at first described to correlate with the IgV\textsubscript{H} status (Damle et al., 1999), CD38 was recently described to vary over time (Montillo et al., 2005) and hence, its evaluation should be independent and by its modal expression rather than by a fixed cut-off level. ZAP70 functions as a surrogate marker for the IgV\textsubscript{H} status as the majority of mutated cases are ZAP70 negative, while unmutated cases are ZAP70 positive (Rosenwald et al., 2001).

Furthermore, cytogenetic features are of prognostic value. The most common genetic aberrations in CLL are 13q deletion (55%), 11q deletion (10%-32%), trisomy 12 (11%-18%) and 17p deletion (3%-27%) (Seiler et al., 2006). Whereas patients with 17p deletion, involving p53, have generally the worst outcome (median survival 32 months) going along with resistance towards alkylating drugs and purine analogues (Byrd et al., 2006), patients harboring exclusively a 13q14.1 deletion are considered to have a favorable prognosis (median survival 133 months). An 11q deletion, involving the ATM gene, also predicts poor prognosis as seen by a low median survival of 79 months (Dohner et al., 2000; Seiler et al., 2006). Additionally, del(11q) is associated with male gender, younger age and massive lymphadenopathy (Montillo et al., 2005).
1.1.4.4 Current therapeutic strategies

CLL is a very heterogeneous disease. It progresses slowly in most cases, but can also be aggressive, developing rapidly to advanced disease stages. Therefore, the treatment strategy is highly dependent on prognosis, based on disease stage following Rai and Binet staging systems, and the general composition of prognostic markers of the individual patient. Furthermore, the appearance of symptoms guides the decision for treatment strategy (Eichhorst and Hallek, 2007).

CLL patients in early stages with slowly progressing disease do not initially require treatment. Only upon progress of the disease and the occurrence of life quality reducing effects treatment is indicated (Hallek et al., 2008). It was further demonstrated, that early treatment with alkylating agents does not prolong patient survival, confirming the "watch and wait" strategy at this disease stage (Dighiero, 1997).

In general, CLL treatment focuses on disease control and reduction of symp-

<table>
<thead>
<tr>
<th>prognostic factor</th>
<th>clinical risk</th>
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<tr>
<td>patient gender</td>
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<tr>
<td>clinical stage</td>
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<td></td>
<td>Binet B, C</td>
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<tr>
<td>bone marrow infiltration</td>
<td>non-diffuse</td>
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<tr>
<td>lymphocyte doubling time</td>
<td>&gt;12 months</td>
</tr>
<tr>
<td>genetic abnormalities</td>
<td>none</td>
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<tr>
<td></td>
<td>del 11q, 17p</td>
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<td></td>
<td>del 13q (sole)</td>
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<tr>
<td></td>
<td>p53 mutation</td>
</tr>
<tr>
<td>CD38 expression</td>
<td>&lt;20%</td>
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<tr>
<td>Zap70 expression</td>
<td>low</td>
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<tr>
<td>IgV&lt;sub&gt;h&lt;/sub&gt; gene status</td>
<td>mutated</td>
</tr>
<tr>
<td>serum thymidine kinase</td>
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<tr>
<td>β2-MG</td>
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<tr>
<td>soluble CD23 levels</td>
<td>low</td>
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Table 3: Prognostic factors in CLL.
Source: Own illustration
toms rather than on an outright cure of the disease. The most commonly used therapeutic strategy is conventional chemotherapy. For advanced disease stages, refractory disease or relapsed CLL bone marrow transplantation is an option (Hallek et al., 2008). Recently, also targeted therapies based on the knowledge of the biology of the CLL cell, have been gaining attention and several strategies and compounds are under evaluation. Although the disease still remains incurable, response rates and progression free survival have steadily improved over the last 10 years (Brenner et al., 2008).

1.1.4.4.1 Conventional therapy
Since the 1950´s chlorambucil was the drug of choice for treatment of CLL. This alkylating drug was sufficient in palliation of symptoms, but overall survival was not significantly affected (Sawitsky et al., 1977; Shustik et al., 1988). After introduction of the staging systems of Rai and Binet in the 1970s and 80s, respectively (Binet et al., 1981; Rai et al., 1975), it became clear that in some instances the prognosis for CLL patients can be extremely poor and an improvement in therapy was highly required. At this point purine analogs became available. Up to now, CLL therapy is based on purine analogs alone, fludarabine, pentostatin or cladribine, or their combination with other, mainly alkylating, agents. The first line treatment is a combination of fludarabine and cyclophosphamide. The addition of the monoclonal antibody rituximab (anti-CD20) is up to now not part of the standard therapy regime, but is applied frequently. For patients with insufficient kidney function either of the substance alone is the treatment of choice.

Treatment of relapsed patients is highly dependent on age and comorbidity, as well as the duration of remission and how the disease was initially treated. Long remission duration after an initial potent chemotherapy (fludarabine/cyclophosphamide/rituximab) suggests a repetition of the initial treatment, while after short durations of remission a change in the treatment strategy is indicated. Furthermore, the monoclonal antibody alemtuzumab (anti-CD52) and the hybrid alkylating agent bendamustine are approved for treatment of relapsed or fludarabine-resistant patients.
1.1.4.4.2 Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation is the intravenous infusion of hematopoietic stem cells to re-establish hematopoietic function in patients with damaged or defective bone marrow or immune systems. Prior to stem cell transfusion the patients’ hematopoietic (neoplastic) cell population is eradicated by high dose chemotherapy. Subsequently, healthy hematopoietic stem cells are infused into the patients’ body with the aim of repopulation of the hematopoietic systems. Dependent on whether the infused stem cells originate from the patient himself or from a secondary healthy human leucocyte antigen (HLA)-matching donor, the transplantation is either autologous or allogeneic.

An indication for autologous or allogene stem cell transplantation is therapy-resistant disease, early relapse (within 12 months of complete remission) or 17p-abnormalities.

As a first line treatment stem cell transplantation is only indicated in young high risk patients.

1.1.4.4.3 Novel targeted therapy

Conventional chemotherapy is an unselective therapy, therefore exhibiting high levels of unwanted side effects. Additionally, treatment with cytotoxic drugs is not curative and patients invariably relapse or possess resistance towards this therapy. Hence, the development of treatment strategies aiming on selective targeting of the neoplastic cells is of high interest.

The first targeting agents in CLL therapy were, as mentioned above, rituximab (anti-CD20) and alemtuzumab (anti-CD52). There are several other monoclonal antibodies in clinical development, which are targeting B-cell specific surface antigens, such as lumilixumab (anti-CD23) or ofatumumab (anti-CD20).

Further potential new drugs for CLL therapy are tyrosine kinase inhibitors such as flavpiridol, which is though to act via inhibition of cyclin-kinases and subsequent downregulation of anti-apoptotic proteins, immunomodulating drugs, such as lenalidomid, antisense molecules, such as oblimersen, which targets the mRNA of the anti-apoptotic protein Bcl2 (Pepper et al., 2001) or small molecules, such as the pan-Bcl2-inhibitor obatoclax (O’Brien et al., 2009).
1.2 The vascular endothelial growth factor

In the late 1980’s the vascular endothelial growth factor VEGF was first described as heparin binding angiogenic growth factor with high specificity for endothelial cells (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989). At around the same time, a protein promoting extravasation of proteins from tumor-associated blood vessels was characterized and named vascular permeability factor (VPF) (Senger et al., 1983). It turned out that VEGF and VPF are one and the same as they were derived from a single gene (Keck et al., 1989; Leung et al., 1989; Tischer et al., 1989). The term "vascular endothelial growth factor" has prevailed over "vascular permeability factor" and is commonly accepted.

1.2.1 The VEGF-family

VEGF denotes a family of homodimeric glycoproteins consisting of six members including VEGF-A, placental growth factor (PIGF) (Maglione et al., 1991), VEGF-B (Olofsson et al., 1996a), VEGF-C (Lee et al., 1996), VEGF-D (Achen et al., 1998; Orlandini et al., 1996) and viral homologues of VEGF, termed VEGF-E (Meyer et al., 1999; Ogawa et al., 1998; Wise et al., 1999). All members possess a conserved central core region, the so called VEGF-homology domain, which is a central part of eight invariant cysteine residues essential for assembly of inter-and intramolecular disulfide bonds. Despite the structural similarity, all VEGF family members show distinct tissue distribution and display different biological activities, mainly due to their different abilities to bind to the three VEGF-receptors.

While VEGF-A has strong mitogenic and permeability enhancing activities, PIGF has only weak potential in this regard. However, PIGF has the ability to enhance VEGF-A action (Park et al., 1994). Furthermore, PIGF can form heterodimers with VEGF, which have increased potency to mediate mitogenic stimulation of endothelial cells relative to PIGF alone (Cao et al., 1996). VEGF-B exists in two different isoforms, which are both predominantly expressed in embryonal and adult muscle tissue (myocardium and skeletal muscle) and are co-expressed with VEGF in many tissues, most prominently in the heart (Lagercrantz et al., 1996; Olofsson et al., 1996b). VEGF-C and
VEGF-D are produced as long precursor proteins. After proteolytic processing several variants with different VEGF-receptor binding affinities are created (Joukov et al., 1997; Stacker et al., 1999). In midgestation embryos, VEGF-C is mainly expressed in regions where the lymphatic vessels undergo sprouting from embryonic veins (Kukk et al., 1996). In adult humans VEGF-C is predominantly expressed in heart, placenta, ovary, small intestine, and the thyroid gland (Joukov et al., 1996). During embryogenesis VEGF-D was detected in high amounts in the developing mouse embryo (Stacker et al., 1999) and in human tumors (Achen et al., 2001).

VEGF-A is dimeric, disulfid-bound glycoproteins of 34-42kDa in size and the most common VEGF family member. It is generally referred to as VEGF and is the focus of this work. Due to alternative splicing six major isoforms of VEGF ranging in the sizes from 121 to 206 aminoacids exist. The primary VEGF transcript is derived from a single VEGF gene consisting of 8 exons separated by seven introns. Whereas exons 1 to 5 and exon 8 are conserved domains and present in all isoforms, alternative splicing in exons 6 and 7, which are responsible for heparan and heparin binding abilities, gives rise to the other isoforms.

These isoforms mainly differ in their ability to interact with extracellular matrix components, such as heparan sulfate proteoglycans (Neufeld et al., 1999). VEGF$_{121}$ is freely diffusible (Park et al., 1993), whereas the other isoforms VEGF$_{145}$, VEGF$_{165}$, VEGF$_{183}$, VEGF$_{189}$ and VEGF$_{206}$ have increasing heparin binding ability. VEGF$_{121}$, VEGF$_{145}$, and VEGF$_{165}$ can induce prolifera-
tion of endothelial cells and in vivo (Park et al., 1993; Poltorak et al., 1997; Zhang et al., 1995). VEGF$_{189}$ and VEGF$_{206}$ are sequestered on heparin sulfate proteoglycans of cell surfaces and in the extracellular matrix (EM) and are not secreted. EM-bound VEGF$_{189}$ can be cleaved by proteases such as plasmin to release an active soluble proteolytic fragment of 110 amino acids (VEGF$_{110}$) (Houck et al., 1992; Plouet et al., 1997). Usually, several VEGF isoforms are produced simultaneously by VEGF-producing cells with VEGF$_{121}$ and VEGF$_{165}$ being the predominant variants (Bacic et al., 1995). Furthermore, these two isoforms are also the most active isoforms for binding and activating the VEGF-receptors 1 and 2 (Gerber et al., 1998).

1.2.2 The VEGF-receptors

There are three major VEGF-receptors, which have been described to mediate VEGF-signaling: the 180kDa VEGF-receptor 1 (Fms-like tyrosine kinase 1, Flt-1) (de Vries et al., 1992) the 200kDa VEGF-R2 (kinase insert-domain containing receptor, KDR or Flk-1) (Terman et al., 1991) and the 180kDa VEGF-3 (Fms-like tyrosine kinase 4, Flt-4) (Aprelikova et al., 1992). They belong to the class III receptor tyrosine kinases of the platelet-derived growth factor (PDGF) receptor subfamily (Klagsbrun and D’Amore, 1996).

1.2.2.1 Structure and binding

The VEGF-receptor family is characterized by the presence of seven immunoglobulin homology domains (Ig domains) in their extracellular ligand-binding part and an intracellular tyrosine kinase domain. The kinase domain is split by hydrophilic residues, the so called kinase insert, which is essential for substrate recognition (van der Geer et al., 1994). Intra-and extracellular domains are separated by a single transmembrane region. VEGF-receptors homodimerize upon ligand-binding, which results in transphosphorylation of their intracellular kinase domains and subsequently to activation of signaling pathways.

Besides the VEGF-R1, 2 and 3, additional VEGF-binding receptors have been described. Neuropilin1 (NRP1) and Neuropilin 2 (NRP2), initially discovered as neuronal cell guidance receptors of the semaphorin ligands (He and Tessier-Lavigne, 1997) have the ability to bind VEGF$_{165}$ (Soker et al., 1998) and, in
the case of NRP1, also PlGF (Migdal et al., 1998). As they lack an intracellular catalytic domain, thus not possessing any signal transduction function, they have been described as accessory receptors, which stabilize the VEGF-R/VEGF-complex.

It was shown by domain deletion studies, that ligand-binding is localized to the second Ig domain in VEGF-receptors 1 and 2. In addition, the domains one and three are needed for full affinity-binding of ligand and receptor (Barleon et al., 1997; Cunningham et al., 1997; Fuh et al., 1998). The forth Ig domain plays a role in the process of receptor dimerization upon ligand binding as shown for VEGF-R1 (Barleon et al., 1997). Due to close structural similarity this is also likely to be the case for VEGF-R2. In VEGF-R3 the fifth Ig homology domain of the extracellular part is proteolytically cleaved and the resulting polypeptides remain linked by two disulfide bonds (Pajusola et al., 1994).

To function as a ligand for the VEGF-receptors, two members of the VEGF-family are linked by disulfide bonds in an anti-parallel orientation to form a homodimer. The receptor binding sites are located at the poles of each molecule and consist mainly of hydrophobic residues.

**1.2.2.2 Major expression sites**

The VEGF-R1 and 2 are selectively expressed in embryonic and adult vascular and lymphatic endothelial cells, but have also been identified in several other
tissues in physiological conditions. Whereas the VEGF-R1 was for example found on trophoblasts (Charnock-Jones et al., 1994), circulating monocytes and macrophages (Barleon et al., 1996; Sawano et al., 2001) and hematopoietic stem cells (Gerber et al., 1998; Hattori et al., 2002) the VEGF-R2 was detected in hematopoietic stem cells, megacaryocytes and retinal progenitor cells (Katoh et al., 1995). Also chronic lymphocytic leukemia cells were found to express all three VEGF-receptors (Bairey et al., 2004; Kay et al., 2002a; Kay et al., 2002b). While VEGF-R3 is found in all endothelial cells during embryogenesis, its appearance becomes largely reduced when development progresses and in adult tissues VEGF-R3 is for the most part restricted to lymphatic endothelium (Kaipainen et al., 1995; Kukk et al., 1996). The importance of all three VEGF-receptors during embryogenesis is highlighted by the fact that knock outs of either receptor are embryonic lethal in mice (Dumont et al., 1998; Fong et al., 1999; Shalaby et al., 1995). NRP1 has been described to be abundantly expressed in endothelial cells of developing embryonic vessels (Kitsukawa et al., 1995).

Despite their structural similarity, the three VEGF-receptors possess diverse functions, with VEGF-R1 being mainly involved in vasculogenic and angiogenic activities such as migration and differentiation, while VEGF-R2 is primarily associated with the regulation of survival and proliferation (Gerber et al., 1998). VEGF-R3 is almost exclusively implicated in lymphangiogenesis (Kaipainen et al., 1995). These different functions are for the most parts due to their tissue distribution. Furthermore, since the three VEGF-receptors and the two neuropilins feature distinct ligand specificities, their activity status is also largely dependent on the availability of these ligands.
Table 4: VEGF-receptors and their specific ligands.

<table>
<thead>
<tr>
<th>VEGF-R1</th>
<th>VEGF-R2</th>
<th>VEGF-R3</th>
<th>Neuropilin 1</th>
<th>Neuropilin 2</th>
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<tbody>
<tr>
<td>VEGF165</td>
<td>VEGF121</td>
<td>VEGF C</td>
<td>VEGF165</td>
<td>VEGF165</td>
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<tr>
<td>VEGF145</td>
<td>VEGF145</td>
<td>VEGF D</td>
<td>PIGF</td>
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<tr>
<td>VEGF-B</td>
<td>VEGF165</td>
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<tr>
<td>PIGF</td>
<td>VEGF189</td>
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<td>VEGF205</td>
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<td>VEGF C</td>
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<td>VEGF E</td>
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Source: Own illustration

1.2.3 VEGF/VEGF-R-signaling

The underlying principle of VEGF-mediated modification of cellular processes is the binding to VEGF-receptors and the subsequent activation of distinct intracellular signaling cascades through several auto- and transphosphorylation steps.

In the case of VEGF-R1, ligand binding induces receptor phosphorylation, but results in very little kinase activation. Although VEGF-R1 has a higher affinity for VEGF than VEGF-R2, no mitogenic response is generated (Waltenberger et al., 1994). In contrast, VEGF-R1/VEGF-binding even negatively regulated VEGF function by diverting VEGF from other functional receptors, such as VEGF-R2 (Claesson-Welsh, 2003). The VEGF-R3 is exclusively-stimulated by the VEGF-family members VEGF-C and VEGF-D, which induce transphosphorylation of tyrosine residues in the intracellular kinase domains and subsequent recruitment of signaling molecules and the activation of MAPK and Akt signaling cascades (Robinson and Stringer, 2001; Saharinen and Petrova, 2004). Neither VEGF-R1, nor VEGF-R3 are the focus of this thesis and will therefore not further be discussed.

VEGF-R2-signaling has been associated with the regulation of survival and proliferation processes (Gerber et al., 1998). Signal transduction follows the mentioned principles of receptor tyrosine kinases. The major autophosphory-
lation sites of VEGF-R2 are located in the kinase insert domain (tyr951/996), in the tyrosine kinase catalytic domain (tyr1054/1059) and in the C-terminal domain (tyr1175/1214) (Takahashi et al., 2001). Activation of VEGF-R2 leads to rapid recruitment of adaptor proteins. In the following the major intracellular signal transduction pathways are described.

1.2.3.1 The PLCγ-pathway

One of the best characterized substrates of VEGF-R2 is phospholipase Cγ (PLCγ) (T. Takahashi and M. Shibuya, 1997, Oncogene). PLCγ binds the phospho-tyr1175 residue of VEGF-R2 (Takahashi et al., 2001). Once activated by tyrosine phosphorylation, PLCγ catalyses the hydrolysis of phosphatidylinositol 3,4-bisphosphate (PIP2) to inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG), which in turn either induces calcium (Ca2+) fluxes or activation of members of the protein kinase C (PKC) family (Carpenter and Ji, 1999). This signaling stream has mainly been associated with mitogenic response to VEGF due to increased vasopermeability.

1.2.3.2 The PI3K/Akt-pathway

Phosphatidylinositol 3-kinases (PI3K)/Akt signaling has been described to be essential for survival signaling after VEGF-R stimulation in endothelial cells (Fujio and Walsh, 1999; Gerber et al., 1998). Its abnormal activation has frequently been associated with tumorigenic processes, mainly due to modulation of apoptosis (Fresno Vara et al., 2004).

PI3K contains a catalytic subunit p110α and a regulatory subunit p85. In the absence of an activating signal p85 inhibits the kinase activity of p110α. Upon stimulation of the receptor p110α is released from p85, hence activated and recruited to the plasma membrane (Okkenhaug and Vanhaesebroeck, 2001), where it phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-trisphosphate (PIP3). PIP3 recruits proteins that contain a pleckstrin homology domain, such as the serine-threonine kinase Akt as well as its activating kinase 3-phosphoinositide-dependent kinase 1 (PDK1) to the cellular membrane (Corvera and Czech, 1998). Activated Akt is the predominant and essential mediator for the regulation of growth, proliferation and survival by PI3K. Regulation of survival is mainly controlled by inhibi-
tion of pro-apoptotic pathways by interfering with B-cell lymphoma 2 (Bcl-2)-associated death promoter homologue (BAD) and Caspase 9 (Gerber et al., 1998).

1.2.3.3 The MAPK/ERK-pathway

VEGF stimulation has been shown to induce activation of mitogen-activated protein kinase (MAPK) in endothelial cells leading to gene transcription and proliferation (D’Angelo et al., 1995). Classically, MAPK is activated via the Ras/Raf/MEK cascade (Marshall, 1996). Upon ligand binding and subsequent autophosphorylation of the receptor a signaling cascade is induced in which the SOS protein activates the small G-protein Ras. A cascade of phosphorylation steps involving the serine/threonine kinases Raf and Mek eventually results in MAPK activation. In the case of the VEGF-R2 it could be shown, that MAPK can alternatively be activated independent of Ras via the PLC$\gamma$-pathway (Takahashi et al., 1999).

1.2.3.4 The STAT-pathway

In addition to mentioned well accepted classical intracellular signal transduction pathways induced by VEGF-binding to the VEGF-R2, also the signal transducers and activators of transcription (STAT) proteins have been involved in VEGF-signaling (Bartoli et al., 2000). To date seven mammalian STATs have been identified: STAT1, 2, 3, 4, 5A, 5B and 6 (Ihle, 2001). STAT-activation features phosphorylation at specific tyrosine residues followed by serine-phosphorylation as a result of receptor stimulation with various cytokines, growth factors and hormones (Bowman et al., 2000; Darnell, Jr. et al., 1994; Darnell, Jr., 1997; Ihle, 1996). While STAT activation was initially thought to be exclusively mediated by Janus kinases (JAKs) (Ihle and Kerr, 1995), its JAK-independent activation could also be demonstrated (David et al., 1996; Leaman et al., 1996). In general, tyrosine (tyr) 705 phosphorylation enables STAT to dimerize via reciprocal phosphotyrosine-SH2 domain interaction, which is essential for nuclear translocation of STAT and subsequent transcriptional activity (Darnell, Jr., 1997; Ihle, 1995). The role of phosphorylation at serine (ser) residues (e.g. ser727) within the transcriptional activation domain (TAD)-motif, mediated by MAPK or the mTOR pathway rather than
JAKs (Wen et al., 1995; Yokogami et al., 2000), has been discussed controversially. Whereas it was demonstrated to enhance the DNA-binding affinity of STATs, especially STAT3 (Decker and Kovarik, 2000), it functioned as an inhibitor of transcriptional activity in other studies (Lim and Cao, 1999). Additionally, it was hypothesized that serine phosphorylation allows the integration of signals from multiple pathways, hence providing a degree of modulation of the STAT-mediated gene activation induced by tyrosine phosphorylation (Frank et al., 1997).

Out of the mentioned STAT proteins, mainly STAT3 has been ascribed oncogenic potential as it was shown to be associated with tumor initiation and progression due to facilitation of cellular expansion by transactivating genes encoding pro-survival factors, such as Bcl$_{ax}$ or Bcl2, (Bromberg et al., 1999; Catlett-Falcone et al., 1999; Garcia and Jove, 1998).

![Figure 5: Intracellular signaling cascades downstream of VEGF-R2](Source: Own illustration)
1.2.4 Angiogenesis

The vascular endothelial growth factor (VEGF) is denoted the most important factor in angiogenesis. Angiogenesis is the development of new blood vessels from pre-existing ones. It is a crucial process for vascular development and neovascularisation in physiological and pathophysiological conditions. In the embryo the vast network of arteries, veins and capillaries is produced by vasculogenesis to create the primary network of vascular endothelial cells, which eventually will become mature blood vessels. The pivotal role of VEGF during embryogenesis is clearly demonstrated by studies showing that inactivation of only one VEGF allele results in embryonic lethality due cardiovascular defects (Carmeliet et al., 1996; Ferrara et al., 1996).

In the adult organism angiogenesis is a rare event. It takes places during the female reproductive cycle, during pregnancy and wound healing. Angiogenesis is a highly controlled process. Several positive and negative regulators act in a complex interplay to control angiogenesis.

<table>
<thead>
<tr>
<th>anti-angiogenic factors</th>
<th>pro-angiogenic factors</th>
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<tbody>
<tr>
<td>angiostatin</td>
<td>thrombospondin-1</td>
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<tr>
<td>interferon alpha</td>
<td>placental growth factor</td>
</tr>
<tr>
<td>prolactin 16-kd fragment</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>metallo-proteinase inhibitors</td>
<td>transforming growth factors</td>
</tr>
<tr>
<td>platelet factor 4</td>
<td>angiogenin</td>
</tr>
<tr>
<td>genistein</td>
<td>interleukin-8</td>
</tr>
<tr>
<td>placental prolifern-related protein</td>
<td>hepatocyte growth factor</td>
</tr>
<tr>
<td>transforming growth factor beta</td>
<td>granulocyte colony-stimulating factor</td>
</tr>
<tr>
<td>endostatin</td>
<td>platelet-derived endothelial cell growth factor</td>
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<td></td>
<td>angiopoietin 1</td>
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</tbody>
</table>

Table 5: Anti-and pro-angiogenic factors.
Source: Own illustration

In general inhibitors of angiogenesis are predominant and angiogenesis is "turned off". In the case of tissue injury or oxygen lack pro-angiogenic proteins are released and diffuse into the nearby tissues. Those proteins, such as VEGF, bind to receptors on endothelial cells (ECs) of existing blood vessels, prompt-
ing them to proliferate and produce further pro-angiogenic factors. In addition matrix metalloproteases (MMPs) are produced, which degrade the basement membrane of the existing vessels allowing migration of ECs into the interstitial space towards the angiogenic stimulus. The formation of new blood vessels is supported by integrins inducing the formation of a capillary tube. MMPs "clear the way" by dissolving the tissue in front of the sprouting vessel. New blood vessels are stabilized by the recruitment of small muscle cells (pericytes), which provide structural support.

1.2.5 Angiogenesis-independent physiological roles

Although VEGF is mainly known for its strong pro-angiogenic function, it could be further identified to be implicated in several other physiological processes, such as normal hematopoiesis and hematopoietic stem cell (HSC) differentiation and survival (Gerber et al., 2002). Early and differentiated hematopoietic cells express VEGF and its corresponding receptors, suggesting that autocrine mechanisms are involved in the regulation of hematopoiesis (Janowska-Wieczorek et al., 2001). Several facts further support a crucial role of VEGF in hematopoiesis, as conventional gene knock-out experiments of either VEGF itself (Carmeliet et al., 1996; Ferrara et al., 1996) or the VEGF-R2 (Shalaby et al., 1995) resulted in early embryonic lethality due to impaired hematopoiesis and angiogenesis. In another study, a positive correlation of VEGF-R2 and pluripotent stem cell activity in long-term in vitro cell culture assays could be demonstrated (Kabrun et al., 1997). Furthermore, VEGF-signaling on hematopoietic precursors could be demonstrated to restrict B-lymphoid commitment in vitro and in vivo (Fragoso et al., 2008).

Additional angiogenesis independent roles of VEGF are widely distributed throughout the human body including for example inhibition of dendritic cell maturation by inhibition of NFκB activation (Gabrilovich et al., 1996), recruitment of monocytes (Barleon et al., 1996) and endothelial cell progenitors (Lyden et al., 2001) to the vasculature, increase of osteoclastic bone-resorbing activity (Nakagawa et al., 2000) and osteoclast chemotaxis (Henriksen et al., 2003) or mediation of a direct neuroprotective effect on motor neurons in vitro (Oosthuyse et al., 2001).
1.2.6 VEGF in cancer

1.2.6.1 VEGF in solid tumors

Solid tumors are usually characterized by a growing tumor mass, due to uncontrolled proliferation of the malignant cells. When the tumor size exceeds 1 to 2 mm, the nutrient and oxygen supply by simple diffusion is not sufficient to maintain cell survival anymore and the need of an independent blood supply arises. Hypoxic conditions trigger the production of pro-angiogenic factors, mainly VEGF, which disturbs the balance of pro- and anti-angiogenic factors to induce the development of new blood vessels. The turning point when pro-angiogenic factors outweigh anti-angiogenic factors is commonly referred to as the "angiogenic switch" and displays a critical step in tumorigenesis, allowing the disease to proceed to a more progressive phase (Hanahan and Folkman, 1996). Furthermore, angiogenesis is a critical component of tumor metastasis as it provides an efficient route of exit for tumor cells to leave the primary site and enter the blood stream. VEGF has been demonstrated to be expressed and correlate with bad prognosis or advanced stage disease in several cancers, which are also known for their potential to spread via metastasis, such as breast cancer (Linderholm et al., 2000), lung cancer (Jarzynka et al., 2006) or colorectal cancer (Kuniyasu et al., 2000).

Tumor angiogenesis differs significantly from physiological angiogenesis. While the process of physiological angiogenesis is rapidly initiated and executed following a tight regulation, tumor angiogenesis is characterized by an unorganized progress and a failure of blood vessels to become quiescent. As a consequence, the tumor vasculature develops quite distinct characteristics compared to blood vessels derived from physiological angiogenesis possessing irregular shape, being dilated and tortuous. They can also have dead ends or a leaky structure (Benjamin et al., 1999). Furthermore, tumor vasculature was shown to incorporate cancer cells into vessel walls (McDonald et al., 2000).

Because tumor growth and metastasis depend on new vessel development, interfering with angiogenic signaling is a logical approach for treatment of solid cancers. In the early 1990’s the first studies investigated the effect of blockage of VEGF/VEGF-R signaling as a strategy to inhibit tumor progression (Kim et al., 1993; Millauer et al., 1994). A strong inhibitory effect on tumor
development after blockage of VEGF-signaling in many tumor types could be demonstrated in the following years (Millauer et al., 1996), thereby initiating intense efforts directed at the development of efficient inhibitors of VEGF-production and VEGF-signal transduction for anti-tumor purposes. The first anti-angiogenic agent to enter the clinic in 2004 was the monoclonal anti-VEGF antibody bevacizumab (Avastin®, Genentech Inc, San Francisco, USA). Bevacizumab is currently approved in combination with standard chemotherapy as first and second line treatment in colorectal cancer and as first line treatment in lung cancer in the United States of America (USA) (for further information see: http://www.cancer.gov/cancertopics/factsheet/AvastinFactSheet). In addition, the two broad-spectrum tyrosine kinase inhibitors sorafenib (Nexavar®, Bayer AG, Leverkusen, Germany) and sunitinib (Sutent®, Pfizer, New York, USA), which also target the VEGF-receptors, are approved for clinical use. Both substances are recommended as monotherapy in advanced renal cell carcinoma and sunitinib is also approved for imatinib-resistant gastrointestinal stromal tumor (GIST) patients. Sorafenib was also shown to improve overall survival in liver cancer patients (http://www.onyx-pharm.com/wt/page/pressreleases). Several other anti-VEGF agents are under investigation, such as cediranib (AZD2171, Recentin), vandetanib (ZD6474, Zactima) or vatalanib (PTK787/ZK222584) (Jain et al., 2006).

Besides its role in tumor vascularisation to ensure an appropriate nutrient supply of the tumor, VEGF has also been discussed as an angiogenesis-independent pro-survival factor in several cancers. It has been demonstrated that tumor cells as well as stromal cells express VEGF (Senger and Van De Water, 2000) and the VEGF-R1 and 2, as well as NRP1 (Bachelder et al., 2001; Dias et al., 2001; Masood et al., 2001; Soker et al., 1998). It was subsequently suggested that in those tumors auto- as well as paracrine VEGF-signaling loops might have a direct influence on the tumor cell growth and survival.

1.2.6.2 VEGF in hematologic malignancies

For a long time the involvement of VEGF in hematologic malignancies has been neglected due to the opinion that its role is exclusively limited to angiogenesis. Since in hematologic malignancies no solid growing tumor mass is evident, the problem of tumor vascularisation was though to be negligible. When sev-
eral studies started identifying increased neovascularisation in the bone marrow of patients with hematological cancers a potential involvement of VEGF in the pathophysiology of these so called "liquid tumor" emerged (Aguayo et al., 2000). Several studies could demonstrate increased VEGF/VEGF-R
expression and enhanced neovascularization in the bone marrow of patients with acute myeloid (Fiedler et al., 1997; Ghannadan et al., 2003), acute lymphoblastic (Perez-Atayde et al., 1997) and chronic lymphocytic (Aguayo et al., 2000) leukemias and myelomas (Podar et al., 2001). Furthermore, correlations between status of bone marrow vascularisation or VEGF levels and disease stage, progression or response to treatment have been described in hematologic malignancies (Aguayo et al., 1999; Kini et al., 2000; Salven et al., 2000). Also chronic lymphocytic leukemia cells have been described to express and secrete VEGF (Aguayo et al., 2000; Kay et al., 2002a) and to possess all three VEGF-Rs (Bairey et al., 2004). Studies about the VEGF status in CLL and possible correlation with disease stage, progression or progression free or overall survival are not consistent. For example, VEGF amounts in serum of patients were described to have a positive correlation with disease progression in early CLL (Molica et al., 1999), whereas intracellular VEGF was shown to be negatively correlated with disease progression (Aguayo et al., 2000). In the same study no correlation with Rai/Binet stage was found. In contrast, in another study VEGF and VEGF-R2 correlated positively with each other and were significantly higher in patients of Rai III/IV than Rai stage I/II patients (Gora-Tybor et al., 2005). Also Ferrajoli et al (Clin Canc Res, 2001) found a positive association between VEGF-R2 and shortened survival in CLL patients (Ferrajoli et al., 2001). In accordance, microvessel density was shown to be higher in CLL bone marrow biopsies as an effect of VEGF-induced increased angiogenesis compared to healthy tissue, and to positively correlate with clinical stage (Kini et al., 2000). Intracellular VEGF and the highly up-regulated protein Bcl2 show a negative correlation with each other (Bairey et al., 2001). Most of these data are descriptive and mainly based on the angiogenic function of VEGF. The last mentioned study, correlating VEGF to the potent anti-apoptotic protein Bcl2, predicts an angiogenesis-independent role of VEGF by being involved in CLL cell survival. This assumption could further be proofed by the fact that exogenous VEGF in culture of CLL cells has been
associated with a reduction in both spontaneous and drug induced apoptosis (Lee et al., 2004). Also in myeloid leukemia cells stimulation with recombinant human (rh) VEGF led to phosphorylation of the VEGF-R2 and increased proliferation (Dias et al., 2000). In another study, VEGF induced an increase in mRNA of a number of hematopoietic growth factors like granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), and interleukin-6 (IL-6) in endothelial cells, suggesting that paracrine VEGF pathways may enhance leukemic cell survival (Zhang et al., 2004). Recently, VEGF/VEGF-R2 interaction has been described to interfere with CLL cell extravasation and tissue infiltration processes, as VEGF-signaling activation reduced the expression of the matrix metalloproteinase (MMP) 9 via STAT1-activation, therefore significantly inhibiting CLL cell migration in \textit{in vitro} matrigel studies (Ugarte-Berzal et al., 2010). Schuch et al demonstrated the role of VEGF in promoting leukemia growth in two animal models. The chloroma producing murine myelomonocytic cell line M1 was either injected subcutaneously or administered systemically by intravenous injection. It could be conclusively shown that VEGF promoted leukemia cell growth while addition of a VEGF-antagonist (extracellular soluble portion of NRP1) inhibited leukemia progression. In this context it is highly informative that the used cell line M1 does not show any responsiveness towards VEGF \textit{in vitro}, thereby demonstrating the importance of the microenvironment in the complex pro-survival action of VEGF (Schuch et al., 2002).

In can be concluded that VEGF has pro-survival effects in several hematologic malignancies, including CLL, possibly involving auto-and/or paracrine mechanisms.

\subsection*{1.2.7 Objective}

The objective of this study was to investigate the role of the vascular endothelial growth factor (VEGF) in the apoptotic resistance of CLL cells, thereby potentially identifying targets for novel therapeutic approaches with the aim of overcoming the apoptotic block. In order to achieve that the following aspects had to be addressed:

- What is the influence of VEGF on the apoptotic resistance of CLL cells
and what impact does the bone marrow microenvironment have in this regard?

- How does VEGF exert its anti-apoptotic function and which downstream signaling proteins are involved?

- Is VEGF, its receptor or a downstream player a suitable drug target to efficiently overcome the apoptotic resistance in CLL cells?
Chapter 2

Results

2.1 VEGF status in CLL and healthy B-cells

Primary CLL and healthy B-cells were analysed for VEGF production and secretion. Further the presence of VEGF-receptor 2 (VEGF-R2) and its phosphorylation status was determined. For all experiments CLL cells were extracted from peripheral blood (PB) by Rosette Sep\textsuperscript{®} technique followed by Ficoll density centrifugation. Healthy B-cells were isolated from PB from healthy volunteers by mentioned separation technique or by positive selection using CD19-labeled microbeads. For some experiments peripheral blood mononuclear cells (PBMCs) were used, which were separated from PB by Ficoll density centrifugation without any further treatments.

2.1.1 CLL cells express the major VEGF isoforms to a significantly higher extent than healthy B-cells

The VEGF-expression status in CLL and healthy B-cells was analysed. VEGF exists in several isoforms due to alternative splicing. The most common ones are VEGF\textsubscript{121}, VEGF\textsubscript{165} and VEGF\textsubscript{189}. Their existence was determined in primary CLL samples and healthy volunteer samples by real time PCR using a common forward primer, a common fluorescent probe and isoform specific reverse primers. In addition, a complete VEGF message was amplified. As a housekeeping gene Abl expression was assessed simultaneously.
While all three studied VEGF isoforms were expressed in primary CLL cells to significantly higher extents than in healthy B-cells, the two soluble isoforms VEGF_{121} and VEGF_{165} show higher expression levels as does the heparin-binding isoform VEGF_{189}. Significances comparing CLL (n=25) versus healthy B-cells (n=5) were p=0.0011 for VEGF_{complete}, p=0.0028 for VEGF_{121}, p=0.0004 for VEGF_{165} and p=0.01 for VEGF_{189}. Note, that no difference in VEGF mRNA levels of healthy B-cells was detected between positive (CD19-labeled magnetic beads) and negative selected (Rosette Sep®) healthy B-cells.

### 2.1.2 There is no correlation between VEGF mRNA and ZAP70 or CD38

Several studies showed correlations between the VEGF status in CLL cells and diverse patient characteristics, thereby not achieving consistent results. Furthermore, most of these studies focused on VEGF levels in plasma or serum of peripheral blood from CLL patients, which may not be necessarily derived from CLL cells directly. Therefore, VEGF mRNA levels (complete VEGF message) of 67 patients were determined by real time PCR and correlation analysis of ZAP70 and CD38 status were conducted.
Table 6: Correlation statistics of VEGF mRNA with ZAP70 and CD38 status. Correlation coefficients and their corresponding significances are displayed. Statistics were calculated using SPSS statistical analysis software, version 17.0.

Neither Pearson, Kendall’s Tau nor Spearman’s Rho correlation analyses revealed any significant correlation between VEGF mRNA levels and the analysed parameters.

2.1.3 CLL cells secrete VEGF to a higher extent than healthy B-cells

Primary CLL cells (n=9) and B-cells from healthy volunteers (n=7) were cultured for 24 hours under standard conditions and supernatants were analysed for VEGF content by ELISA. At the same time cell survival was determined to identify VEGF secretion relative to the amount of living cells.

![Figure 7: VEGF levels secreted by CLL and healthy B-cells.](image)

Mann-Whitney-U-test was used to calculate the significance.

CLL cells secreted significantly higher amounts with 198.2 pg +/- 22.1 pg VEGF/10^7 living cells compared to healthy B-cells with 75.5 pg +/- 13.8 pg
VEGF/10^7 living cells (p=0.0003).

2.1.4 **CLL cells exhibit the VEGF-receptor 2 (VEGF-R2)**

Further it was analysed whether CLL cells and healthy B-cells exhibit the VEGF-R2. Freshly isolated primary cells from CLL patients or healthy volunteers were subject to flow cytometry using a VEGF-R2-specific antibody, a FITC-labeled secondary antibody and an appropriate isotype control.

![Figure 8: VEGF-R2 status in CLL and healthy B-cells.](image)

Grey shades represent the isotype control. One representative sample out of five independently carried out experiments is displayed.

VEGF-R2 was detected on an average of 78.8% +/- 5.5% CLL cells (n=5), but only 11.5% +/- 4.2% of healthy B-cells (n=5).

2.1.5 **The VEGF-R2 is constitutively phosphorylated in CLL cells, but not in healthy B-cells**

We analyzed the phosphorylation status of VEGF-R2, which takes place upon VEGF binding, therefore determining its activity. Freshly isolated primary cells were incubated with a VEGF-R2 specific antibody, which only detects the receptor when phosphorylated on tyr951, a major autophosphorylation side in the kinase insert domain of the receptor. Samples were subject to intracellular phospho flow cytometry.
While 89.5% +/- 0.8% of CLL cells (n=5) carried phosphorylated VEGF-R2, only 11.8% +/- 2.2% of healthy B-cells (n=5) were positive for pVEGF-R2. We could further show the functionality of the VEGF-R2 in CLL cells. Stimulation of starved CLL cells (RPMI 1640/3% FCS for at least 6 hours) with recombinant human (rh) VEGF at 100 ng/ml for 1 hour resulted in an additional increase of phosphorylated VEGF-R2-positive CLL cells up to almost 100%. (96.8% +/- 1.2%, n=5). Healthy B-cells also demonstrated a slight increase (17.2% +/- 3.7%, n=5).

2.1.6 Stimulation with rhVEGF induces increased VEGF expression in CLL cells

To further elucidate the existence of a potential VEGF feedback loop mechanism, CLL cells were stimulated with rhVEGF. For that purpose serum-starved
CLL cells (RPMI 1640/3% FCS for at least 6 hours) were incubated with 10 ng/ml, 50 ng/ml and 100 ng/ml rhVEGF for 24 hours. mRNA was isolated, reverse transcribed into cDNA and subject to real time PCR.

![Graph showing VEGF mRNA fold change for different concentrations of rhVEGF](image)

Figure 11: VEGF mRNA levels of rhVEGF-stimulated CLL cells. Fold changes were calculated using the comparative $2^{-\Delta\Delta Ct}$-method with the untreated control as calibrator.

Exogenous stimulation with 50 ng/ml and 100 ng/ml rhVEGF resulted in a moderately increased expression of VEGF mRNA levels in CLL cells (n=7) of 1.49 +/-0.20 fold (p=0.0391) and 3.06 +/-0.81 fold (p=0.0039) compared to untreated control, respectively. 10 ng/ml rhVEGF did not result in significant upregulated VEGF mRNA levels in CLL cells (1.14 +/-0.15 fold). Significances were calculated using non parametric paired t-test (Wilcoxon matched pairs).

### 2.1.7 Secretion of VEGF by CLL cells increases with time in culture

As demonstrated, CLL cells express functional VEGF-R2 (Figure 9) and are capable of expression and secretion of VEGF (Figure 6, Figure 7). VEGF amounts secreted by CLL cells were determined after 24 hours in culture. To elucidate whether this is a steady level, a time course covering 5 days was conducted. CLL cells and healthy peripheral blood mononuclear cells (PBMCs) were cultured in RPMI 1640, supplemented with 3% FCS, supernatant was harvested every 24 hours and analysed for its VEGF content by ELISA. Simultaneously, cells were removed from culture and analysed for survival by flow cytometry (Annexin V-FITC/PI staining).
Figure 12: VEGF levels secreted by CLL and healthy B-cells over 5 days. VEGF amounts were normalized to simultaneously measured amount of living cells.

While all tested CLL samples (n=5) showed increasing levels of secreted VEGF, healthy PBMCs showed a constant VEGF level of 120 pg/10^7 living cells (n=3). The analysed CLL samples showed very heterogeneous responses varying from 2 fold to greater 20 fold increase compared to day 1 (215 pg to 3816 pg/10^7 living cells at day 5).

### 2.1.8 CLL cell-derived VEGF is not sufficient to stimulate the VEGF-R2

With the existence and functionality of VEGF-R2, the ability of CLL cells to express and secrete VEGF and the increase of VEGF secretion over time, it could be speculated that VEGF-signaling is active in CLL cells in vitro. Therefore, VEGF-R2 phosphorylation in CLL cells was analysed over a period of 5 days under culture condition using intracellular phospho flow cytometry.
2.2 Role of VEGF in the apoptotic block of CLL cells

As demonstrated CLL cells are capable of producing and secreting VEGF and also feature the VEGF-R2. VEGF-signaling is a known potent pro-survival stimulus for endothelial cells and other cell types. It has also been implicated in apoptotic resistance of CLL cells, although the underlying functional mechanisms are not well understood up to date. Therefore, the effect of VEGF on CLL cells \textit{in vitro} was analysed.

Despite increasing levels of VEGF in the supernatant of CLL cells (Figure 12) the percentage of pVEGF-R2 positive cells steadily decreased with time. It can be excluded that this reduction was due to reduced survival, hence a lower number of existing total VEGF-R2, as the used gating strategy eliminated dead CLL cells, which show a significant shift in forward/sideward scattergrams upon apoptosis induction due to cell shrinkage and fragmentation. As stimulation with rhVEGF in concentrations similar to those achieved by CLL cells under culture conditions, induced phosphorylation of the VEGF-R2, it can be assumed that CLL cell-derived VEGF is insufficient to stimulate the VEGF-R2 in a monoculture setting.
2.2.1 rhVEGF induces upregulation of anti-apoptotic proteins in CLL cells, but not healthy B-cells

Stimulation of CLL cells with rhVEGF increased the number of phosphorylated, hence active VEGF-R2 in CLL cells almost up to 100% (Figure 10). VEGF/VEGF-R2-signaling is supposed to be involved in apoptotic resistance of the CLL cells and their prolonged survival. To further determine the effect of VEGF stimulation and subsequent activation of VEGF-signaling on survival, CLL and healthy B-cells were stimulated with rhVEGF, lysed and analysed by immunoblotting for protein levels of the anti-apoptotic proteins Mcl1, XIAP and Bcl2.

Figure 14: Anti-apoptotic protein levels in CLL and healthy B-cells after rhVEGF stimulation.

For CLL cells one representative sample out of three independently carried out experiments is displayed.

A concentration-dependent increase of the anti-apoptotic proteins XIAP and Mcl1 could be detected in CLL cells upon rhVEGF stimulation, while Bcl2 levels remained unchanged. In healthy B-cells levels of the analysed anti-apoptotic proteins were unaffected by rhVEGF stimulation. Note the severe overexpression of Bcl2 in CLL cells compared to healthy B-cells.

2.2.2 The anti-apoptotic proteins Mcl1, XIAP and Bcl2 are reduced with time in culture

CLL cells seem to loose the ability for autocrine VEGF stimulation in culture, as demonstrated by a time-dependent reduction of VEGF-R2 phosphorylation.
CLL cells were cultured for 120 hours and harvested every 24 hours (exception 96 hours). Cells were lysed and analysed for protein levels of Mcl1, XIAP and Bcl2. ß-actin served as loading control. At the same time survival was determined by Annexin V-FITC/PI staining and flow cytometry (data not shown). CLL cells showed reduced survival with time as expected, total protein amounts were also reduced, as seen by decreasing amounts of ß-actin. For that reason band intensities were determined densitometrically and values were normalized to ß-actin as input control.

<table>
<thead>
<tr>
<th>hours</th>
<th>Mcl1</th>
<th>Bcl2</th>
<th>XIAP</th>
<th>ß-actin</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.066</td>
<td>0.881</td>
<td>1.066</td>
<td>0.996</td>
</tr>
<tr>
<td>24</td>
<td>1.054</td>
<td>0.779</td>
<td>0.986</td>
<td>0.974</td>
</tr>
<tr>
<td>48</td>
<td>0.998</td>
<td>0.761</td>
<td>0.993</td>
<td>0.974</td>
</tr>
<tr>
<td>72</td>
<td>0.993</td>
<td>0.766</td>
<td>0.993</td>
<td>0.974</td>
</tr>
<tr>
<td>120</td>
<td>0.911</td>
<td>0.766</td>
<td>0.911</td>
<td>0.974</td>
</tr>
</tbody>
</table>

Figure 15: Anti-apoptotic protein levels in CLL cell during a time course of 5 days. To overcome uneven loading, band intensities were normalized to ß-actin. One representative sample of three experiments is shown.

Not surprisingly reduced survival went along with moderate reduction of the anti-apoptotic proteins Mcl1, XIAP and Bcl2. Since those proteins are regulated by a variety of intracellular signaling cascades and their downregulation is strongly involved in apoptosis induction in general, their reduction cannot be assigned to downregulated VEGF-R signaling though.

2.2.3 rhVEGF stimulation does not affect survival of CLL cells

CLL cells were cultured over a time period of 120 hours in RPMI 1640 medium including 20% FCS or RPMI 1640, 20% FCS, supplemented with 100 ng/ml rhVEGF. Medium was exchanged daily.
Addition of rhVEGF at high concentrations of 100 ng/ml did not affect CLL cell survival compared to standard cell culture medium not including rhVEGF. For the most part the survival difference was in the range of +/- 4%.

2.3 Influence of bone marrow (BM) stromal cells on the VEGF status in CLL cells and their survival

Since CLL cells lose their major pathophysiological feature, the resistance towards apoptosis, when taken out of their natural microenvironment and placed into cell culture, the bone marrow microenvironment can be assumed to be of high influence on CLL cell survival \textit{in vivo}. To approximate the \textit{in vivo} micromilieu we used the bone marrow-derived stromal cell line HS5 as a feederlayer for CLL cell culture \textit{in vitro} and analysed the influence on VEGF-mediated survival.

2.3.1 The BM-derived stromal cell line HS5 produces high amounts of VEGF

Stromal cells are a known source for cytokines, chemokines and growth factors. In the following HS5 cells were analysed for VEGF mRNA by real time PCR and secreted VEGF by ELISA after having reached confluency under cell culture conditions.
HS5 exhibited VEGF mRNA levels in considerably higher amounts than healthy B-cells and CLL cells. As shown earlier, CLL cells (n = 9) secreted 198.2 pg +/- 22.1 pg VEGF/10^7 cells and healthy B-cells (n = 7) 75.5 pg +/- 13.8 pg VEGF/10^7 cells (Figure 7). VEGF levels in supernatant of HS5 cell (from two independent batches of HS5) were approximately 20 fold higher than in CLL cells (4067.0 pg +/- 245.4 pg VEGF/10^7 cells).

2.3.2 VEGF expression is significantly increased by co-culture with HS5 in CLL cells, but not healthy B-cells

As demonstrated, HS5 cells produce and secrete high levels of VEGF. Furthermore, the addition of rhVEGF to CLL cells in monoculture led to an increase in VEGF mRNA levels produced by CLL cells. Therefore, the HS5-derived VEGF might act in a paracrine fashion. To test this hypothesis, CLL cells were cultured as monoculture or on a HS5 feederlayer for 24 hours. Also healthy B-cells were cultured on HS5 feederlayer.
Coculture of CLL cells (n=4) on a monolayer of HS5 led to an increased VEGF-expression of 13.6 +/- 0.4 fold in CLL cells. Healthy B-cells (n=3) responded less intensely (2.1 +/- 0.6 fold increase), which excluded HS5 contamination in coculture supernatant as cause for increased VEGF mRNA levels (compare Figure 41).

2.3.3 CLL cells maintain constitutive phosphorylated VEGF-R2 when cocultured with HS5

In monoculture CLL cells lost the constitutive phosphorylation of the VEGF-R2 with time, despite increasing amounts of secreted VEGF in culture supernatant. It was suggested that CLL cell-derived VEGF is not sufficient to stimulate the VEGF-R2. This could be due to inactivation, potentially by cleavage of VEGF in monoculture, or also by a lack of further activating factors. These factors may be microenvironment-derived. Therefore, the phosphorylation status of VEGF-R2 in CLL cells cocultured with HS5 cells was analysed every 24 hours over a time course of 72 hours.
Results

Figure 19: pVEGF-R2 levels of CLL cells cocultured with HS5 over a time course of three days. Grey shades represent the isotype control. One representative sample out of three independently carried out experiments is displayed.

In contrast to CLL cell monoculture, where secreted VEGF did not induce phosphorylation of the VEGF-R2, coculture with HS5 maintained high numbers of approximately 80% pVEGF-R2-positive CLL cells over the analysed period of 72 hours.

2.3.4 Coculture with HS5 supports survival of CLL cells, but not healthy B-cells

CLL cells die quickly when taken out of their natural microenvironment and can generally only be maintained in culture conditions for several days (compare Figure 21). In our experiments CLL cell-derived VEGF was not sufficient to activate the VEGF-R2 and prevent CLL cell death (Figure 13). Also the addition of exogenous rhVEGF (100 ng/ml) did not prolong CLL survival \textit{in vitro} (Figure 16). After observing an increased VEGF expression in CLL cells in coculture with HS5 and a prevention of VEGF-R2 phosphorylation loss with time, it was investigated whether the coculture setting also has impact on CLL cell survival. CLL cells or B-cells from healthy volunteers were seeded at an approximately 10 fold higher density onto a monolayer of HS5 cells. Survival was assessed by Annexin V-FITC/PI staining every 24 hours for 5 days.
Figure 20: Survival advantage of CLL and healthy B-cells cocultured with HS5.
The survival advantage was calculated by subtracting the percentage of living cells in coculture by the percentage of living cells in monoculture.

CLL cell survival was enhanced in HS5 coculture, with a constantly increasing survival advantage over time, which was 16.8% +/- 2.3% at day one (n=13), 22.7% +/- 2.8% at day two (n=12), 33.9% +/- 4.0% at day three (n=10), 53.9% +/- 6.5% (n=3) at day four and 69.7% +/- 7.4% (n=3) at day five compared to monoculture controls. Healthy B-cells (n=3) did not profit from HS5 coculture as their survival remained within a range of +/- 2% during the time course of 5 days compared to monoculture.

To not only show relative numbers Figure 21 displays the absolute survival of three samples analysed for a time period of 5 days.

Figure 21: Absolute survival of CLL cells in mono- or in coculture with HS5 over a time course of five days.
While CLL cells in monoculture presented a steadily decreasing survival \textit{in vitro} (lower three lines), the presence of HS5 cells largely prevented CLL cell from undergoing apoptosis as seen by a constant survival rate of around 80\% (upper three lines). It is important to mention that HS5 cells showed reduced viability after coculture for 120 hours due to increased nutrient needs. Despite this, CLL cell survival in coculture compared to monoculture was more pronounced at later time points.

\subsection*{2.3.5 Neutralization of VEGF by a monoclonal antibody reduces the coculture-mediated survival advantage}

At this point, HS5-mediated CLL cell survival support could not be attributed to VEGF but other factors derived from HS5 cells might be involved. To determine, the importance of VEGF in the prolonged survival of cocultured CLL cells, VEGF was neutralized using a monoclonal antibody (R\&D Systems, MAb 293). This antibody was tested for its VEGF-neutralizing ability by determination of the VEGF-R2 phosphorylation status by intracellular phospho flow cytometry after addition of 1 ng/ml and 10 ng/ml.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure22.png}
\caption{pVEGF-R2 levels upon addition of the VEGF-neutralizing antibody MAb293 (mAb). The pVEGF-R2 status was determined by intracellular phospho flow cytometry. Gray shade represents the isotype control.}
\end{figure}

A significant reduction of the VEGF-R2 phosphorylation status was observed at 10 ng/ml of MAb293 (Figure 22, green peak). That is why this concentration was used in the following experiments.

CLL cells were cultured on HS5 feederlayer with or without addition of the VEGF neutralizing antibody MAb293. Survival was assessed after 24 hours by Annexin V-FITC/PI staining.
In four analysed samples the addition of anti-VEGF-MAb 293 decreased the HS5-mediated survival advantage by an average of 66.3% +/- 4.6% compared to coculture not containing the antibody. This clearly indicates VEGF to be critical in promotion of HS5-mediated CLL cell survival. It has to be mentioned that the used VEGF-antibody did not affect HS5 survival as determined by Annexin V-FITC/PI staining (data not shown).

2.3.6 VEGF-depletion in HS5 by siRNA abolishes the coculture-mediated survival advantage for CLL cells

In addition to blocking VEGF protein in the HS5/CLL coculture supernatant by a neutralizing antibody, expression and secretion of VEGF by HS5 cells was reduced using short interfering RNA (siRNA). 24 hours after transfection of HS5 siRNA was removed and CLL cells were added to confluent HS5 cells, which were either control treated (normal VEGF-expression) or VEGF-depleted by siRNA. CLL monoculture was carried out in parallel. VEGF downregulation upon siRNA treatment was analysed on both, mRNA and protein level by real time PCR and ELISA, respectively.
Figure 24: siRNA-mediated downregulation of VEGF.
Results are displayed relative to control siRNA-treated HS5 set at 1. VEGF mRNA levels (A) were normalized to the calibrator Abl. Levels of secreted VEGF (B) were normalized to the amount of living cells. Mean values of three independent experiments are displayed.

siRNA treatment reduced VEGF mRNA to levels of 0.53 +/- 0.03, 0.52 +/- 0.08 and 0.39 +/- 0.07 after 24, 48 and 72 hours (2^-ΔΔCt-method) relative to siRNA control set at 1 (Figure 24A). The amount of secreted VEGF in the supernatant of HS5 cells was reduced to 0.78 +/- 0.02 at 24 hours, 0.56 +/- 0.05 at 48 hours and 0.60 +/- 0.07 at 72 hours in VEGF siRNA treated HS5 relative to control siRNA treated HS5 set at 1 (Figure 24B).

CLL cell survival and HS5 survival was assessed by Annexin V-FITC/PI staining after 24 hours and 48 hours of coculture with control-treated and VEGF siRNA-treated HS5.

Figure 25: CLL cell survival after cocultivation with control-treated and VEGF siRNA-treated HS5 cells after 24 and 48 hours of coculture.
Survival differences are displayed relative to CLL cell survival in coculture with control-treated HS5 cells. Three independently carried out experiments are displayed.

Survival of CLL cells in coculture with VEGF-knockdown HS5 resembled sur-
vival rates of CLL cells in coculture with the control siRNA transfected HS5 after 24 hours. However, at 48 hours the survival advantage for CLL cells was significantly reduced in knockdown VEGF cocultures (levels of -0.02, -0.53 and 0.43 compared to the control level set at 1. It is important to note that siRNA-treatment did not affect HS5 survival.

![Image](https://via.placeholder.com/150)

**Figure 26:** Survival of siRNA control-treated or VEGF siRNA-treated HS5 cells after three days.
Annexin V-FITC/PI double negative cells are considered dead. One representative sample out of three independently carried out experiments is displayed.

### 2.3.7 Physical separation of CLL cells from HS5 reduces the coculture-mediated survival advantage

Up to this point it could be shown that a coculture with HS5 cells significantly increased the survival of CLL cells and that VEGF is an essential factor in this regard. Since neither rhVEGF stimulation nor CLL cell-derived increased VEGF levels were sufficient to prolong CLL cell survival *in vitro*, it must be concluded that further factors are involved. Besides soluble factors this could also be direct cell-cell contacts. That is why CLL cells were cultured as monoculture or coculture on HS5 feederlayer either physically separated by a transwell or allowing direct cell-cell contact for a time course of 72 hours. Survival was assessed by Annexin V-FITC/PI staining. The survival advantage was calculated by subtracting the percentage of living cells in coculture by the percentage of living cells in monoculture.
The survival advantage for CLL cells (n=4) cultured directly on a HS5 feederlayer was 24.8% +/- 5.3% after 24 hours, 22.1 +/- 0% after 48 hours and 36.4 +/- 8.2 after 72 hours in coculture compared to survival in monoculture. When CLL cells were physically separated by transwell the survival advantage was decreased with 3.2% +/- 4.3% after 24 hours, 7.0% +/- 1.6% after 48 hours and 18.9% +/- 4.9% after 72 hours better survival compared to the corresponding monoculture. It can be concluded, that CLL cells benefit from physical interaction with HS5 stroma cells regarding their survival ability in vitro. To maintain comparable conditions, monoculture controls were also cultured in transwell.

### 2.4 Mechanistical background of VEGF-mediated apoptosis prevention

In study it was demonstrated that VEGF is involved in microenvironment-supported apoptosis resistance of CLL cells, while the mechanistical background is not clear. Knowledge of the events downstream of the VEGF-receptor is of high interest and might possibly reveal targets for specific CLL therapy.
2.4.1 PCR-array suggests an upregulation of STAT3 and downregulation of RB1 and E2F1 upon rhVEGF-stimulation

A PCR array (96 well format) was conducted to detect possible changes in transcription factor levels upon VEGF stimulation in CLL cells. This array contained primer sets for 86 genes coding for transcription factors plus several controls. Real time PCR was carried out using SYBR green fluorescent dye on a 96 well cycler. Starved CLL cells were either-stimulated with rhVEGF for 6 hours or left untreated. cDNA was subject to PCR array. Analysis is based on the $2^{-\Delta\Delta Ct}$-method and was done using a softwaresystem provided by SABioscience.

![Diagram](image)

Figure 28: PCR array results of unstimulated versus rhVEGF-stimulated CLL cells. Two independent experiments were carried out.

The average of two independent experiments revealed an upregulation of the oncogene STAT3 of 8.79 fold +/- 3.11 fold, a 3.68 fold +/- 2.09 fold downregulation of E2F1 and a 5.77 fold +/- 3.67 fold downregulation of the tumor suppressor RB1.
2.4.2 PCR-array suggested downregulation of E2F1 and RB1 is not reproducible by PCR

To confirm the results obtained from the PCR array, CLL cells from 5 patients were stimulated with rhVEGF (100 ng/ml, 6 hours) or left unstimulated. cDNA was prepared and analysed by real time PCR using E2F1 and RB1 specific primers and probes.

![Graph](image)

Figure 29: RB1 and E2F1 mRNA levels of rhVEGF-stimulated CLL cells relative to unstimulated control.
Fold change was calculated based on the $2^{-\Delta\Delta C_{T}}$-method.

RB1 and E2F1 expression was not significantly changed by rhVEGF stimulation (1.04 +/- 0.20 fold and 0.73 +/- 0.04 for RB1 and E2F1, respectively). The $2^{-\Delta\Delta C_{T}}$ value of E2F1 mRNA levels after rhVEGF stimulation compared to untreated control is equivalent to a fold downregulation of 1.37 ($1/0.73$), but does not reach statistical significance as determined by paired student´s t-test (p=0.14).

2.4.3 rhVEGF stimulation does not increase total STAT3 but induces its phosphorylation on tyr705

Based on the array results an upregulation of STAT3 was expected. CLL cells were rhVEGF-stimulated, treated with a combination of rhVEGF and the VEGF-R inhibitor GW 786034 or left untreated. Cell lysates were subject to immunoblotting for total STAT3 levels.

In contrast to the PCR array, total STAT3 protein levels observed upon rhVEGF stimulation were only marginally increased, if at all (Figure 30A). STAT3 has been described by others as a potential downstream effector of the VEGF-R2 in CLL as its nuclear translocation was induced as a consequence
of VEGF-R engagement (Lee et al., 2005). Transcriptional activity of STAT is regulated by its phosphorylation, hence the total protein levels do not necessarily refer to functional and active protein. To further assess this point, STAT3 was investigated for phosphorylation on distinct residues in stimulated and unstimulated CLL cells. CLL cells were starved for 6 hours (3% FCS) followed by rhVEGF stimulation (50 ng/ml) or left untreated for 24 hours with or without simultaneous treatment with the VEGF-R inhibitor GW786034. Cell lysates were prepared and analysed by immunoblotting using antibodies specifically recognizing the serine 727 (ser727) and tyrosine 705 (tyr705) phosphorylation sides of STAT3.

Figure 30: STAT3 and pSTAT3 levels in CLL cells after rhVEGF stimulation or VEGF-R inhibition using GW 786034. (A) Total STAT3 amount. (B) Phosphorylation status of STAT3 on residues ser727 and tyr705. One representative sample out of three (A) and two (B) independent experiments is displayed.

STAT3 ser727 was found to be constitutively phosphorylated, while tyr705 phosphorylation was present only upon rhVEGF stimulation. Tyr705 phosphorylation was either prevented or was reduced again when cells were simultaneously treated with the VEGF-R inhibitor GW786034. STAT3 ser727 phosphorylation remained unchanged upon both, rhVEGF stimulation and VEGF-R inhibitor treatment (Figure 30B).

As a further proof of the results obtained from immunoblotting experiments, the STAT3 phosphorylation status, with and without rhVEGF stimulation, was determined by intracellular phospho flow cytometry. The stimulation process was the same as described above. Instead of cell lysis, cells were fixed,
permeabilized and incubated with directly fluorescently labeled antibodies or the appropriate controls and analysed by intracellular phospho flow cytometry.

Figure 31: STAT3 phosphorylation status after rhVEGF stimulation in CLL cells assessed by flow cytometry. One representative sample out of three independently carried out experiments is displayed.

Immunoblotting results could be confirmed: while phosphorylation of STAT3 on ser727 was present without exogenous VEGF stimulation in about 50% of cells, VEGF stimulation did not further increase this phosphorylation (average 46.9% +/- 0.9). In contrast, tyr705 phosphorylation was detectable only after addition of rhVEGF.

2.4.4 Phosphorylation on tyr705 activates STAT3

It has been described before, that ser727 phosphorylation alone is not sufficient to confer a transcriptional signal and that tyr705 phosphorylation is required for full transcriptional activity (Darnell, Jr., 1997; Ihle, 1995). To assess the activity status of STAT3, protein levels of the known STAT3 target genes cyclinD1 and Bcl_{XL} were analysed by immunoblotting. The experimental set up was as described above.
Both tested STAT3 targets cyclinD1 and Bcl\textsubscript{XL} were increased upon rhVEGF stimulation, while the presence of the VEGF-R inhibitor GW786034 reversed this effect.

2.4.5 Coculture with HS5 provokes tyr705 phosphorylation of STAT3 and its activation in CLL cells

As demonstrated above HS5 cells possess a substantial survival-promoting influence on CLL cells with VEGF being an essential player in this process. It is of interest, whether coculture with HS5 cells also induces STAT3 phosphorylation on tyr705 and subsequent transcriptional activation in CLL cells.

To test this hypothesis, CLL cells were cultured alone or together with HS5 with or without addition of the VEGF-R inhibitor GW 786034 for 24 hours. Lysates from CLL cells and HS5 cells were analysed for protein levels of tyr705 phosphorylated STAT3, the STAT3 target cyclinD1 and the anti-apoptotic proteins Mcl1 and XIAP by immunoblotting. For that purpose membranes were stripped and reincubated twice.
Figure 33: Protein analysis of CLL cells in mono- or coculture with HS5 with and without GW 786034 treatment. DMSO treatment functioned as vehicle control. One representative sample of three independently carried out experiments is displayed.

Similar to external VEGF stimulation using the recombinant protein, STAT3 was clearly phosphorylated on tyr705 upon coculture. To proof VEGF-signaling to be responsible for this phosphorylation, rather than other HS5-derived factors, the VEGF-R inhibitor GW 786034 was added. As GW 786034 clearly reduced the STAT3 phosphorylation again, it can be concluded that VEGF-signaling is responsible for HS5 coculture-mediated STAT3 tyr705 phosphorylation. This was further supported by the upregulation of the STAT3-target cyclinD1 in cocultured CLL cells, which was in turn downregulated when VEGF-signaling was blocked by GW 786034. When comparing STAT3 ser727 levels, it can be seen that GW 786034 downregulates the constitutive phosphorylation in monoculture, while this was not the case when rhVEGF was present (compare Figure 30). Also the anti-apoptotic proteins Mcl1 and XIAP, which were associated with VEGF stimulation, are downregulated upon GW 786034 treatment in both mono- and coculture, while an upregulation in coculture compared to monoculture was only noticed for Mcl1 and not XIAP.
2.4.6 Neither rhVEGF nor coculture with HS5 effectuates phosphorylation of Akt in CLL cells

Several other signaling pathways have been demonstrated to be activated upon VEGF-binding to the VEGF-R. One of them is the Akt-signaling cascade. To test whether Akt is activated upon VEGF stimulation, starved CLL cells were incubated with rhVEGF (100 ng/ml), rhVEGF combined with GW 876034 (50 µM) or the lymphocyte stimulant phorbol 12-myristate 13-acetate (PMA) (100 nM) as a positive control for 6 hours or cultured with or without HS5 feederlayer for 24 hours. Cell lysates were analysed for Akt and phosphorylated (ser473) Akt by immunoblotting using specific antibodies.

![Figure 34: Akt and pAkt protein levels in CLL cells under several conditions: (A) CLL cells stimulated with rhVEGF and/or treated with GW 876034, (B) CLL cells in mono- or coculture with HS5 cells. PMA-stimulation functioned as positive control for Akt phosphorylation. One representative sample of three independently carried out experiments is displayed.](image)

Akt protein was present in CLL cells; at the same time Akt phosphorylated on ser473 was detectable but distinctly lower. While PMA stimulation increased this phosphorylation as expected, neither rhVEGF (A), nor HS5 coculture (B) effectuated increased ser473 phosphorylation of Akt. In contrast, active Akt seemed rather to be downregulated in CLL cells which were cocultured with HS5. This slight downregulation was present in all three tested samples.
2.5 Potential of VEGF as therapeutic target in CLL

CLL cells showed significantly improved *in vitro* survival when placed on an HS5 feederlayer. Since partial blockage of VEGF in this setting using the monoclonal VEGF-neutralizing antibody MAb293 (Figure 23) and an siRNA-mediated downregulation of VEGF-expression and secretion in HS5 cells reduced the coculture-derived survival advantage for CLL cells (Figure 25), an anti-VEGF-based therapeutic approach in CLL seems to be a logical consequence.

For this purpose either VEGF itself or its receptor could serve as a drug target. VEGF exerts its pro-survival effect via activation of intracellular signaling pathways, which in turn transduce the extracellular signal into a survival-supporting response, which is mainly mediated through activation of transcription factors that modulate gene expression in favour to anti-apoptotic survival-promoting proteins. Therefore, a therapeutic intervention at the level of downstream effectors might also be of therapeutic interest.

2.5.1 Anti-VEGF antibody treatment does not alter survival in CLL cell monoculture

CLL cells were treated for 24 hours with the VEGF-neutralizing antibody MAb293 or the commercially available humanized anti-VEGF antibody bevacizumab (Avastin®, Roche) at different concentrations. Survival was assessed by Annexin V-FITC/PI staining.
Figure 35: Survival of CLL cells upon treatment with anti-VEGF MAb293 or bevacizumab. Percentage of surviving cells was normalized to vehicle-treated controls (NaCl 0.9% for bevacizumab and PBS for MAb293).

In a monoculture setting neither MAb293 nor bevacizumab had an influence on CLL cell survival in the tested concentrations. In contrast, in coculture with HS5 cells, where CLL cells remained their apoptotic resistance, MAb293 could be shown to reduce CLL cell survival (Figure 23).

2.5.2 The VEGF-R inhibitor GW 786034 effectively induces apoptosis in CLL cells in mono- and in coculture

An alternative target to prevent VEGF-signaling could be the VEGF-receptor (VEGF-R), rather than VEGF itself. For that purpose the small molecule tyrosine kinase inhibitor GW 786034 was used which has high selectivity towards the VEGF-R. CLL cells in mono- and in coculture with HS5 and healthy PBMCs were treated with 50 \( \mu \text{M} \) of GW 786034 or DMSO vehicle control for 24 hours, followed by assessment of survival by Annexin V-FITC/PI staining.
GW 786034 treatment resulted in decreased survival of CLL cells for both, monoculture (n=6) and coculture with HS5 (n=4), while healthy B-cells (n=4) were not affected. Also in concentrations up to 100 µM primary healthy PBMCs did not show decreased survival (data not shown). Besides the VEGF-R, GW 786034 also has inhibitory function on several other tyrosine kinase receptors such as PDGF (platelet-derived growth factor) and might therefore be a more potent, albeit less specific, inducer of apoptosis compared to monoclonal antibodies, especially in the monoculture setting.

2.5.2.1 VEGF-R inhibitor treatment downregulates anti-apoptotic proteins and activates caspases

To further look into the mechanism of apoptosis induction by GW 786034, protein levels of the anti-apoptotic proteins Mcl1 and XIAP were analysed by immunoblotting in CLL cells upon treatment with varying concentrations for 24 hours.
Both substances led to a concentration-dependent decrease of the anti-apoptotic proteins XIAP and Mcl1. XIAP is a direct inhibitor of caspase activation and therefore it is consequent to investigate the caspase activation upon treatment with the VEGF-R inhibitor. Further, caspase activation leads to cleavage of PARP, which was also analysed. Initiator caspase 9 (cleaved and intact) and effector caspase 3 (cleaved), PARP and cleaved PARP were detected using specific antibodies by immunoblotting.

Caspases 9, 3 and PARP showed a concentration-dependent decrease of the inactive intact forms, while cleaved active forms were increased upon treatment with GW 786034.
2.5.3 The STAT3 inhibitor S3I-201 requires high concentrations to reduce CLL cell survival

CLL cells in mono- (n=5) and in coculture with HS5 (n=2) and PBMCs from healthy volunteers (n=3) were treated with the STAT3 inhibitor IV (S3I-201, Calbiochem) at 10 µM, 100 µM and 150 µM for 24 hours and 48 hours or the appropriate DMSO control. Survival was assessed by Annexin V-FITC/PI staining.

![Graph](image)

Figure 39: Cell survival upon treatment with S3I-201 in mono- and coculture with HS5.
Percentage of surviving cells was normalized to DMSO-treated cells.

At 24 hours CLL cell survival was even at high concentrations of 150 µM only slightly reduced (62.4% +/- 12.2%), while an average of 85.4% +/- 2.7% healthy PBMCs were alive. The substance was most effective in cocultured CLL cells with a survival of 49.7% +/- 8.0% at the highest tested concentration. After 48 hours survival after treatment with 150 µM S3I-201 was 16.4% +/- 6.1% and 46.9% +/- 10.6% in CLL cells and healthy PBMCs, respectively. Also here cocultured CLL cells showed the highest sensitivity towards treatment with a survival rate as low as 6.5% +/- 10.6% at the highest tested
2.5.4 Pan-JAK inhibition by Pyridone 6 does not reduce CLL survival in tested low concentrations

STAT3 activation seems to be an effect of VEGF/VEGF-R-signaling and hence to be involved in the VEGF-mediated survival support of CLL cells. JAKs are the most commonly known mediators of STAT-phosphorylation and subsequent activation. In this study it was not further analysed whether JAK is interconnected between VEGF/VEGF-R-signaling activation and STAT3 phosphorylation. Nevertheless, the potential of JAK inhibition to induce apoptosis in CLL cells and healthy PBMCs was determined.

Cells were treated with several concentration of a Jak inhibitor (Pyridone 6, Calbiochem) for 24 hours and cell survival was analyzed by Annexin V-FITC/PI staining.

Pyridone 6 had a modest effect in reducing CLL cell (n=6) survival after 24 hours at the highest tested concentration (20 µg/ml corresponding to 6.4 µM), while healthy PBMCs (n=3) were completely unaffected, suggesting CLL cell-specificity of the substance. This would justify the use of higher concentrations, which unfortunately could not be carried out in this study due to low concentrated stock solutions (1 mg/ml).
Chapter 3

Discussion

Chronic lymphocytic leukemia (CLL) is up to day still an incurable disease. While it is the most common adult leukemia in western countries treatment is still mainly focusing on minimizing progression rather than on an outright cure. CLL patients show very heterogeneous outcomes with slow progressing disease and no need of treatment for several years, whereas others progress rapidly and require immediate treatment. Possibly reflecting the clinical heterogeneity in CLL patients, CLL research has lately been unravelling an unprecedented flurry of information giving insight into the pathophysiology of the disease. Thereby, a focus is the determination of mechanism underlying the apoptotic block of CLL cells with the aim of a targeted therapy.

This study aimed on obtaining further insight into the pro-survival role of VEGF in CLL cells, especially in the background of their interaction with the bone marrow microenvironment, thus potentially identifying targets for therapeutic interventions.

3.1 VEGF status in CLL cells and its involvement in apoptosis prevention

At first the VEGF status in primary CLL cells and healthy B-cells was assessed. CLL cells express all three major VEGF-isoforms VEGF$^{121}$, VEGF$^{165}$ and VEGF$^{189}$ in higher levels than healthy B-cells. Furthermore, CLL cells secreted significantly more VEGF protein into the supernatant after 24 hours in culture compared to healthy B-cells. This was in accordance with previous
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studies showing significantly higher VEGF protein levels in CLL cells compared to healthy PBMCs (Aguayo et al., 2000; Ihle, 1995). Interestingly, levels of both VEGF mRNA and secreted VEGF protein were quite heterogeneous in different CLL samples, perhaps representing a correlation between VEGF-expression and patient characteristics. Disappointingly, in 67 CLL patients no correlation between VEGF mRNA levels and CD38 or Zap70 status was detected. Possibly, correlation of patient characteristics with VEGF mRNA levels does not reflect the actual impact of VEGF on CLL cells, as mRNA requires several ongoing steps to represent a functional protein. A correlation study with secreted protein might therefore be a more suitable approach. Available studies comparing VEGF levels with patient characteristics and/or disease progression mainly focus on VEGF in serum or plasma. While not being consistent, they show a general trend towards a positive correlation between VEGF and several clinical parameters such as Rai-stage, progression free or overall survival (Aguayo et al., 2000; Ferrajoli et al., 2001; Gora-Tybor et al., 2005; Ihle, 1995; Molica et al., 1999). It has to be kept in mind, that plasma or serum VEGF cannot be exclusively assigned to CLL cells, rather it might be derived from other blood components or be a result of a feedback loop-involving cross talk between CLL cells and their microenvironment in the peripheral blood or the bone marrow.

CLL cells exhibit VEGF-R2, which was found to be constitutively phosphorylated in this study. In contrast to CLL cells the VEGF-R2 was only expressed in low percentages in healthy B-cells. Since stimulation with exogenous rhVEGF further increased VEGF-R2 phosphorylation in CLL cells, it can be concluded that this receptor is biologically functional in CLL cells. In addition, CLL cells showed a concentration-dependent feedback loop response upon stimulation with rhVEGF as seen by increasing VEGF mRNA levels upon stimulation. rhVEGF stimulation further resulted in upregulated protein levels of the anti-apoptotic proteins Mcl1 and XIAP in CLL cells, but not healthy B-cells. Also here the effect was concentration-dependent. Bcl2 levels did not change upon stimulation. The latter result is in accordance with Lee et al, who also did not detect any changes in total Bcl2 levels upon stimulation with rhVEGF in CLL cells (Ihle, 1995; Lee et al., 2004; Molica et al., 1999). The reason for unchanged Bcl2 levels upon VEGF stimulation in CLL cells
is not clear and it can only be speculated that since Bcl2 is already highly upregulated some kind of saturation state is already reached. In acute lymphoblastic leukemia (ALL) the Bcl2 protein was described to be phosphorylated upon VEGF-stimulation, which was also survival-supportive for ALL cells in culture (Wang et al., 2005a). As Bcl2 requires phosphorylation for optimal anti-apoptotic function, the same group studied the effect of rhVEGF on Bcl2 phosphorylation in primary CLL cells and did not see any changes (Wang et al., 2005b). Taken together with our results, it seems that in ALL and CLL cells the crucial anti-apoptotic processes might be differential as ALL cells require Bcl2 activation, while CLL cells seem to be independent on Bcl2.

At this point it stands to reason that CLL cells might be able to maintain their resistance towards apoptosis in vitro, since they are capable of producing VEGF, secreting it into the supernatant, exhibiting functional VEGF-R2 and rhVEGF stimulation resulted in VEGF-R2 phosphorylation and increased levels of anti-apoptotic proteins. Nevertheless, it is widely known that CLL cells can actually not preserve their resistance towards apoptosis when taken out of their natural microenvironment and are placed into cell culture (Collins et al., 1989), which was reproduced in this study. One possible explanation for this discrepancy could be the loss of the ability of CLL cells to produce VEGF with time in culture. To test this hypothesis, CLL cells were cultured for a time course of 5 days and the amount of VEGF present in the supernatant was evaluated. At the same time survival was determined to normalize the VEGF concentration to the amount of living cells. The result contradicted the enunciated hypothesis as VEGF protein per amount of living cells increased, rather than decreased, with time in culture, although to different degrees (range 2-fold to greater 20-fold at day 5 in the tested samples). The VEGF concentration in cell culture supernatant of healthy PBMCs remained unchanged over a period of 5 days. Despite high amounts of VEGF in the supernatant, the percentage of pVEGF-R2 was significantly reduced with time, indicating VEGF derived from CLL cells to be insufficient to stimulate the VEGF-R2. Here, it can be excluded, that the reduced pVEGF-R2 levels are due to reduced overall VEGF-R2 owing to increased cell death, as dead cells can be distinguished from living ones in size and were therefore separated by appropriate gate set up. Possible reasons for reduced VEGF-R2 phosphorylation despite high levels
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of CLL-derived VEGF could be the need of further cofactors either for formation of a VEGF complex capable of binding the receptor or also the need of co-receptors such as NRP1 to properly transduce a signal. Another explanation could be an increased presence of VEGF-R1, which has been suggested to possibly function as a decoy/dummy receptor regulating the availability of VEGF for the signal transducing VEGF-R2. Furthermore, soluble VEGF-receptors might heterodimerize with membrane receptors to form dummy receptors for VEGF that would affect VEGF-signaling (Barleon et al., 1997; Roeckl et al., 1998).

If apoptosis prevention was supported by VEGF-mediated VEGF-R2 stimulation inducing upregulation of the anti-apoptotic proteins Mcl1 and XIAP in CLL cells, then it seems consequent to expect an enhanced *in vitro* survival of CLL cells under addition of rhVEGF. To create an even higher complexity of VEGF-supported survival in CLL cells, in our experiments medium supplemented with rhVEGF did not yield an improved survival of CLL cells *in vitro* over a time course of 5 days with daily refreshment of rhVEGF-supplemented medium. Wang et al observed a reduction of drug-induced apoptosis in CLL cells when pre-treated with rhVEGF, but did not find rhVEGF alone to support CLL cell survival either, though it has to mentioned that the time frame analysed in this study was indicated as "overnight" an is therefore short (Barleon et al., 1997; Wang et al., 2005b). In contrast, Lee et al found rhVEGF to protect primary CLL cells from spontaneous and chlorambucil-induced apoptosis after 24 hours in culture (Lee et al., 2004).

It must be concluded, that the survival support of CLL cells is highly complex and besides VEGF additional stimuli derived from the microenvironment seem to be necessary to restore the central physiological feature of CLL cells to resist apoptosis.
3.2 Influence of the bone marrow-derived stromal cell line HS5 on VEGF-mediated CLL cell survival

The stimulation of CLL cells in vitro with rhVEGF does not reflect the actual situation of the CLL cells as it is present in their natural environment within a patient’s bone marrow or peripheral blood, where CLL cells can physically interact with bystander cells and are exposed to a variety of cytokines, chemokines and humoral factors (Munk-Pedersen and Reed, 2004). Stromal cells are a major component of the bone marrow microenvironment and therefore likely to contribute to the apoptotic resistance of CLL cells in vivo (Burger et al., 2000; Burger and Kipps, 2002; Kay et al., 2007; Lagneaux et al., 1999; Lee et al., 2004).

In this study, the influence of the bone marrow microenvironment on VEGF-mediated CLL cell survival support was analysed. For that purpose the human bone marrow-derived stromal cell line HS5 was utilized. It could be shown that CLL cells can be maintained in cell culture several weeks or even up to several months, when cultured on bone marrow stromal cells as a feederlayer (Kay et al., 2007; Panayiotidis et al., 1996b). HS5 cells comprise for example fibroblasts, adipocytes and endothelial cells, hence representing functionally distinct components of the bone marrow microenvironment (Roecklein and Torok-Storb, 1995; Torok-Storb et al., 1999). They are therefore well suited for creation of an in vivo-like milieu in an in vitro cell culture set up. We found HS5 cells to produce and secret substantially higher VEGF amounts compared to CLL cells. In contrast to monoculture, CLL cells did not show decreased amounts of pVEGF-R2 positive cells, but maintained a constant level of around 80% of cells positive for pVEGF-R2 over three days, suggesting VEGF to be capable of activating VEGF-R2 in HS5/CLL coculture. Furthermore, CLL cells kept in coculture with HS5 cells exhibited a strongly increased VEGF-expression, with mRNA levels being up to almost 14 fold higher than in monoculture. It is of interest that healthy B-cells did not respond with increased VEGF-expression to HS5-coculture. The latter fact also excluded HS5 contamination in the coculture supernatant to be responsible
for increased VEGF mRNA levels in CLL cells. Since HS5 cells secrete a variety of factors other than VEGF and also physically interact with CLL cells, the increased VEGF-expression in CLL cells cannot necessarily be assigned to a paracrine VEGF-loop. Nevertheless, as CLL cells reacted with increased VEGF-expression when-stimulated with exogenous rhVEGF, it can be suggested that VEGF at least theoretically has the potential to act in a feedback loop manner. Going along with constitutive phosphorylation of the VEGF-R2, CLL cells remained their resistance towards apoptosis when cocultured with HS5. The survival advantage CLL cells gained from being kept on an HS5 feederlayer was approximately 17% after 24 hours and continuously increased with time reaching around 34% improved survival after 3 days. While CLL cells in monoculture were dead for the most part at day 5 (average of three tested samples 12.0% +/- 5.9% survival), survival of CLL cells derived from the same patients in coculture with HS5 was 81.7% +/- 1.5%. Interestingly, healthy B-cells did not respond with increased survival as seen by survival rates within a range of +/- 2% over three days compared to monoculture. In a study of Lagneaux et al healthy B-cells did not profit from bone marrow stromal cells going along with our results, while Seiffert et al demonstrated the opposite (Lagneaux et al., 1998; Seiffert et al., 2007). These discrepancies might be explained by the use of either primary bone marrow-stromal cells or the bone marrow-derived stromal cell line HS5 in the different studies, which produce variable qualities and quantities of soluble factors. Furthermore, the purity of the B-cells might be important, since contamination with other PBMCs could contribute to B-cell survival. Interestingly, Kay et al stated that they did not observe a differential survival-supporting capacity of bone biopsy-derived marrow stromal elements from CLL patients compared to those derived from healthy donors (Kay et al., 2007; Lagneaux et al., 1998). This would suggest the marrow components of CLL patients themselves to perhaps be non-tumorigenic and comparable to healthy marrow, therefore, the survival supporting effects might possibly be exclusively mediated by aberrancies in the CLL cells themselves.

At this point the actual impact of VEGF on the HS5-mediated support of CLL cell survival is not clear, as other secreted factors or direct cell-cell contacts could be largely involved. To proof the significance of VEGF at first a mono-
clonal VEGF-neutralizing antibody (MAb293) was used. The capacity of this antibody to reduce VEGF-signaling was assessed by flow cytometric determination of the VEGF-R2 phosphorylation status upon treatment with different concentrations. Addition of this antibody to CLL/HS5 coculture significantly reduced the survival advantage for CLL cells (average 66.3% +/- 4.6% reduction compared to coculture without MAb293) which they gain from coculture. To not only block VEGF externally, but reduce its production, VEGF was downregulated by siRNA in HS5 cells. 24 hours after transfection, transfection complexes were removed to not expose CLL cells, which were added subsequently. VEGF-knockdown was controlled on both the mRNA and the protein level. While at 24 hours no difference of CLL cell survival in coculture with HS5 featuring reduced VEGF-levels compared to CLL cells in coculture with negative control-treated HS5-cells was seen, at 48 hours CLL cells showed a significantly reduced survival when VEGF was downregulated in HS5 cells. The extent of survival-support reduction was very heterogeneous in the three tested samples ranging from a survival which was considerably lower than that in monoculture to an approximately 50% reduced survival advantage compared to CLL cells in coculture with negative control-treated HS5. This different reaction might be due to variable dependencies of CLL cells on a VEGF signal in order to obtain the maximum protection from apoptosis, possibly correlating with certain patient characteristics or patient subtypes. It has to be mentioned, that siRNA treatment did not affect survival of HS5 cells, hence reduced CLL cell survival after 48 hours in coculture cannot be assigned to reduced total levels of HS5.

As a conclusion from these experiments it can be stated that VEGF derived from bone marrow stromal cells is essential for CLL cell survival. This result together with the inability of CLL cells to maintain their own survival in vitro contradicts the hypothesis of an autocrine pro-survival loop in CLL cells. A further proof of this statement could be achieved by analysing the effect of VEGF-downregulation directly in CLL cells. A general problem here is that CLL cells are naturally hard to transfect, showing high rates of occurring cell death upon transfection procedure (Lagneaux et al., 1998; Seiffert et al., 2007). Furthermore, as CLL cells do not possess the ability to resist apoptosis when their natural micromilieu is absent, therefore proposed VEGF-mediated sur-
vival support is not fully functioning, it can be concluded that this experiment in CLL monoculture would probably only be of limited significance. A combined downregulation of VEGF in CLL and HS5 cells in a coculture set up might be an option to further elucidate the significance of VEGF for CLL cell survival but requires a high complexity of experimental set up. Nevertheless, it can be concluded, that both strategies, VEGF-neutralization using a monoclonal antibody and siRNA-mediated VEGF knock down in HS5 cells broadly diminished the coculture-procured survival advantage, therefore clearly identifying VEGF as an indispensable factor in the CLL cell’s micromilieu.

Besides soluble factors, such as cytokines and growth factors, also direct cell-cell contacts have been a focal point of microenvironment-focused CLL research (Ghia et al., 2002a). For example, CLL cell survival support by follicular dendritic cells (FDCs) in the bone marrow requires CD44-ligation-dependent direct cell contact (Pedersen et al., 2002). Also T-cells have been described to be, mostly within the proliferation centers, in close physical contact with CLL cells. Here, CD40 present on the CLL cell is thought to interact with CD40L, provided from T-cells (Ghia et al., 2002b). Also stromal cells interact with CLL cells via receptor-ligand binding. CLL cells express several adhesion molecules. It could be demonstrated that interactions between the integrins CD49d/CD11a or CD11b/CD18 on CLL cells with CD54 and CD106 on stromal cells substantially improve CLL survival in a coculture setting (Plate et al., 2000). Also direct physical contact of β1- and β2-integrins on CLL cells with bone marrow stromal cells (Burger and Kipps, 2002; Lagneaux et al., 1999) or the interaction of CD100 on CLL cells and PlexinB1 on stromal cells (Granziero et al., 2003) entailed improved CLL cell survival. In the present study CLL cells were physically separated by transwell from the HS5 feeder-layer and survival was assessed. The survival advantage CLL cells gain from coculture with HS5 was still present, but reduced when physical contact was prohibited. Interestingly, it seemed that physical separation has a stronger influence on HS5-mediated survival support at short culture periods, as after 24 hours the survival advantage reduction due to physical separation was 87.1% as compared to coculture allowing physical interaction. At 72 hours CLL cells cultured without physical contact to HS5 featured a 48.1% reduced
survival advantage in comparison to CLL cells with direct cell-cell contact to HS5 cells. Hence, it can be suggested that initially, as cytokine concentrations are still low, the direct cell-cell contact is of crucial importance for survival support. With time in culture though a steady increase of the cytokine concentration, including VEGF, gives rise to a micromilieu which has increasing survival-supporting capacity, possibly even being able to compensate for the lack of survival supporting signals through direct physical interactions. This statement though requires a long term study of survival outcome of CLL cells with and without physical separation going along with a determination of cytokine concentrations over time.

To test the influence of soluble factors versus direct physical contacts several groups cultured CLL cells in conditioned medium (CM) derived from HS5 or primary bone marrow cell culture. The survival advantage was always lower as it was achieved in a coculture allowing cell-cell contacts, further proving the need of direct physical interactions between CLL cells and stromal cells for full apoptotic protection. Kay et al further tested whether CM could rescue cells from drug-induced apoptosis and found HS5-CM to effectively protect CLL cells from adaphostin-triggered apoptosis (Granziero et al., 2003; Kay et al., 2007). Adaphostin (NSC 680410) induces cell death through oxidative stress via generation of reactive oxygen species (ROS). Interestingly, it has been suggested that adaphostin-generated ROS might act via downregulation of VEGF in leukemia cells (Avramis et al., 2002; Avramis et al., 2003; Granziero et al., 2003). In the presence of stromal cell-CM, adaphostin had only limited apoptosis-inducing capacity, possibly due to the presence of large amounts of VEGF opposing the VEGF-downregulation by adaphostin-generated ROS. Hence, the protective ability of soluble factors or direct cell contacts against drug-induced apoptosis could be dependent on the mechanism of action of the drug.

From our studies it can be concluded, that VEGF is of severe importance for CLL cell survival, as its inhibition in monoculture, where CLL cells are apparently not exposed to the appropriate and complete survival signaling repertoire including functional VEGF-signaling, does not reduce survival. If CLL cells are cocultured with HS5, they received paracrine VEGF stimuli, resulting in constant VEGF-R2 phosphorylation and a significantly prolonged
in vitro survival, which was in turn largely reduced, when VEGF was neutralized by an antibody. Further, it can be concluded that VEGF-derived from HS5 cells, but not CLL-cells is the essential factor as VEGF downregulation in HS5 cells by siRNA completely abrogated the coculture-mediated survival support. In contrast, high levels of secreted VEGF in the supernatant of CLL cell monoculture were insufficient to prevent in vitro apoptosis of CLL cells. In other words, there is no autocrine pro-survival VEGF-loop in CLL cells. As it was shown in this study that VEGF mRNA is highly upregulated in CLL cells upon coculture with bone marrow stromal cells, it can be hypothesized that an intracellular autocrine (intracrine) VEGF loop, not involving VEGF secretion, might be involved in CLL cell survival.

In general it can be said, that the microenvironment is a highly complex microstructure, which supports CLL cells survival not only by one central feature, but is likely to function via several mechanisms comprised of soluble factors and direct physical cell-cell interactions involving VEGF as an essential component. The influence of these components might further be of variable impact on CLL cell population from individual patients.

3.3 Mechanistical background of VEGF-mediated apoptosis prevention

It was of further interest of this study to obtain insight into the mechanism of VEGF-mediated CLL cell survival support. For that purpose, a PCR array was carried out. mRNA from untreated and rhVEGF-stimulated CLL cells was isolated and subject to PCR-array, which allowed testing of the expression status of 84 different genes, in our case transcription factors, in a 96 well plate format. Several input controls, negative controls and gDNA contamination controls were included. In two independently carried out experiments the potent oncogene STAT3 was upregulated, while the known tumor suppressor RB1 and the E2F family member E2F1 were downregulated. E2F1 is involved in cell cycle control, but has also been described to have the ability to induce apoptosis by a death receptor-dependent mechanism (Phillips et al., 1999). These results suggested a dual mechanism of VEGF-mediated survival support: the upregulation of a potent oncogene (STAT3) and the downregula-
Discussion

Discussion of a tumor suppressor (RB1) and an inhibitor of anti-apoptotic signaling (E2F1). Unfortunately, PCR-array results could not be confirmed by PCR (RB1 and E2F1) and immunoblotting (STAT3), where no significant change in mRNA or protein levels could be detected. Nevertheless is STAT3 a known downstream target of the VEGF-R (Bartoli et al., 2000). As STAT3 requires phosphorylation in order to carry out its function as a transcription factor, which is usually induced by cytokine-mediated receptor tyrosine-kinase activation, the phosphorylation status of STAT3 was investigated. As demonstrated before (Frank et al., 1997), also in our study phosphorylation of the residue serine 727 (ser727) was constitutively present in CLL cells. The kinases responsible for the constitutive serine phosphorylation in CLL have not been identified up to now and could only be characterized as being sensitive to the PKC kinase inhibitor H7 (Frank et al., 1997). In the same study MAP kinase was excluded as responsible kinase. STAT3 can only dimerize when simultaneously phosphorylated on tyrosine 705 (tyr705). As dimerization is essential for STAT3 to translocate into the nucleus, tyr705 phosphorylation is necessary for transcriptional activation of STAT3 (Darnell, Jr., 1997; Ihle and Kerr, 1995). In our studies it could be demonstrated that stimulation of CLL cells with rhVEGF led to phosphorylation of STAT3 on tyr705. As it was demonstrated in this study, rhVEGF stimulation alone does not reflect the actual in vivo situation of the CLLs cell within their natural habitat in the bone marrow. For that reason the STAT3 phosphorylation status in CLL cells upon coculture with HS5 was analysed. And indeed, cultivation of CLL cells on an HS5 feederlayer gave rise to tyr705 phosphorylation. In HS5 coculture other factors besides VEGF could be responsible for STAT3 activation such as IL6, which is secreted by stromal cells and known to activate STAT3 via phosphorylation. When the VEGF-R was blocked by addition of the VEGF-R inhibitor GW 786034 tyr705 phosphorylation was reversed. This was true for both, rhVEGF stimulation and HS5-coculture, thereby clearly demonstrating VEGF signaling to be causative for phosphorylation of STAT3 on the tyr705 residue also in the coculture setting. STAT3 is a highly potent oncogene and has been detected in large number of cancers (Bromberg, 2002). Furthermore, it could be demonstrated, that STAT3 has a direct influence on oncogenic transformation from non-malignant to malignant phenotypes, as for example a dominant
negative form of STAT3 prevented Src-induced transformation of NIH3T3 cells (Turkson et al., 1998), therefore suggesting STAT3 to possibly have a causative role in oncogenesis (Bromberg et al., 1999). Besides CLL, also several other hematologic malignancies have been described to exhibit aberrantly activated STATs, such as acute lymphoblastic leukemia (ALL) (Gouilleux-Gruart et al., 1996) acute myelogenous leukemia (AML) (Chai et al., 1997; Gouilleux-Gruart et al., 1996), and chronic myelogenous leukemia (CML) (Carlesso et al., 1996; Chai et al., 1997).

STATs in general are thought to exert their oncogenic activity through the induction of anti-apoptotic pathways. In multiple myeloma cells for example the anti-apoptotic proteins Bcl\_XL and Mcl1 were upregulated upon IL6-mediated STAT3 activation (Catlett-Falcone et al., 1999; Puthier et al., 1999). In head and neck cancers malignant proliferation through a Bcl\_XL-induced anti-apoptotic mechanism was demonstrated to rely on constitutively active STAT3 (Grandis et al., 2000; Song and Grandis, 2000). Also in our experiments VEGF-mediated tyr705 phosphorylation of STAT3 seemed to entail its activation, as we could observe an increased expression of Bcl\_XL and also of the cyclin-dependent kinase regulator cyclinD1, which is an identified STAT3 target (Bromberg et al., 1999; Sinibaldi et al., 2000). While Bcl\_XL is a known potent pro-apoptotic player and hence, has an obvious impact in VEGF-mediated prevention of survival, the role of the cell cycle regulator cyclinD1 is not clear.

Despite the fact of most circulating CLL cells residing in G0/early G1 phase of the cell cycle, they have been stated to have the capacity to proliferate under appropriate conditions (Stevenson and Caligaris-Cappio, 2004). Further, they exhibit shortened telomeres, indicating that they at one point underwent replication processes (Damle et al., 2004). CyclinD1 levels in CLL cells have been assessed by several groups with variable outcome (Delmer et al., 1995; Paul et al., 2005; Ravandi-Kashani et al., 2000). In general it can be said that cyclinD1 levels were low in CLL cells in the mentioned studies, but nevertheless higher than in healthy counterparts, such as normal peripheral blood B-cells or immunophenotypically matched CD5+/CD19+ cord blood cells (Korz et al., 2002; Paul et al., 2005). It is of further interest that cyclinD1 levels in CLL cells were demonstrated to be associated with distinct patient characteristics. For example, cyclinD1 protein amounts could be correlated to shorter
survival times (Ravandi-Kashani et al., 2000). Contradicting to that were the results of Paul et al who demonstrated an inverse correlation of cyclinD1 mRNA levels and Rai stage, with Rai 0 stage patients featuring the highest cyclinD1 levels (Paul et al., 2005). In the same study a positive correlation between lymphocyte doubling time (LDT) and cyclinD1 levels was described, while no correlation could be found between drug resistance and cyclinD1. In our study cyclinD1 protein levels were low or undetectable. Independent on the base level, cyclinD1 protein was increased in CLL cells upon rhVEGF stimulation and in HS5 coculture. It can be speculated that cyclinD1 levels are higher in CLL cells within their natural environment, where they are in "pro-survival-mode", though the functional basis and relevance of cyclinD1 in the CLL pathophysiology is up to now not clear.

Another common signaling-pathway downstream of the VEGF-R is the Akt-signaling pathway. Akt-activation has frequently been associated with prosurvival signaling following VEGF-R stimulation (Fujio and Walsh, 1999; Gerber et al., 1998). Despite this role, no activation of Akt was detected in our study, neither by rhVEGF stimulatin, nor by coculture with HS5 cells, as assessed by determination of the phosphorylation status at the ser473 residue. Coculture with HS5 seemed to rather have the opposite effect as a slight downregulation of pAkt in CLL cells could be detected when compared to pAkt levels in CLL cells in monoculture. At this point it can only be speculate about cause and implication of a possible pAkt downregulation in cocultured CLL cells. Recently, a crosstalk between the p42/p44 mitogen-activated protein (MAP) kinase/extracellular signal-regulated kinase (ERK) pathway, was described. Growth factor-induced Akt phosphorylation was broadly attenuated when ERK signaling was hyperactivated (Hayashi et al., 2008). Hence, MAPK/ERK signaling in CLL cells might be activated through coculture, possibly even through the VEGF-R (Rousseau et al., 1997) and act in a negative feedback loop mechanism to inhibit Akt. However, this speculation remains to be clarified.
3.4 Potential of VEGF as therapeutic target in CLL

As VEGF has an essential function in the CLL cells’ resistance towards apoptosis, targeting components involved in intracellular signal transduction downstream of the VEGF-R2 seems a consequential approach when aiming on resolving the apoptotic block. Several stages of VEGF-signaling could be possible targets, such as VEGF itself, the VEGF-receptor or also major intracellular signaling molecules. In our study STAT3 activation via phosphorylation on tyr705 was described to occur in CLL cells upon VEGF-R2 stimulation using either recombinant VEGF or in a coculture together with bone marrow stromal cells, hence also targeting STAT3 might be of clinical potential. For that reason, we tested several substances for their ability to induce apoptosis in CLL cells in monoculture and more important, in a survival supporting coculture with bone marrow stromal cells to obtain a microenvironment closer to the actual in vivo situation of the CLL cell. At first two monoclonal antibodies, MAb293 (R&D Systems) and bevacizumab (Avastin®; Roche) were used to neutralize VEGF. Both antibodies were tested for their capacity to reduce the phosphorylation of the VEGF-R2 by flow cytometry and used in an appropriate concentration for further experiments. Neither of the substances induced apoptosis at the tested concentrations in CLL cells in monoculture. Also in other studies treatment of leukemia cells in monoculture with anti-VEGF antibodies (hMAb293 and bevacizumab) in concentrations up to 1 mg/ml did not result in induction of apoptosis in primary CLL cells (Lee et al., 2005). The same was shown in an erythroleukemia cell line (HEL) using the anti-VEGF mMAb 4.6.1 (Santos and Dias, 2004). In this study it was proposed that both internal as well as external VEGF/VEGF-R2 loops exist and that repression of only the external one, as achieved by an anti-VEGF antibody, is not sufficient for effective apoptosis induction. They found the VEGF-R2 to be predominantly located in the nucleus of VEGF-R2-positive HEL cells (human erythroleukemia) and HL-60 cells (human promyelocytic leukemia cells) and detected its shift towards the cell surface when VEGF was blocked externally by an anti-VEGF MAb. Internal inhibition using a VEGF-R2 tyrosine kinase inhibitor only modestly resulted in a shift from a nuclear towards a membrane
associated location. Nevertheless, it can be concluded that both internal and external VEGF loops are necessary for maintenance of active nuclear VEGF-R2. In the same study it was demonstrated that internal and external VEGF loops act via different mechanisms, as external VEGF-signaling inhibition reduced levels of NFκB, whereas internal inhibition resulted in a clear reduction of phosphorylated ERK1/2 and Akt. Going along with our results external inhibition of VEGF alone did not induce apoptosis as compared to internal VEGF-R2 inhibition, which was a highly effective apoptosis inducer in the two tested cell lines. Interestingly, a combined treatment of internal and external inhibitor showed synergistic effects. It can be concluded that a potent survival reduction in VEGF/VEGF-R2-positive cells requires an effective inhibition of VEGF-R2 activity, for which blockage of both internal and external VEGF loops are necessary. That is why we tested the VEGF-R tyrosine kinase inhibitor GW 786034 in primary CLL cells. GW 786034 is an indazolyl pyrimidine, which targets the ATP binding site in the intracellular domain of the receptor and thus prevents homodimerisation induced by VEGF-binding as well as subsequent autophosphorylation of the receptors. At 50 µM GW 786034 efficiently induced apoptosis in CLL cells, supporting the hypothesis of internal VEGF-signaling inhibition to be more potent in reducing CLL cell survival than external inhibition. The rational for using 50 µM was that this concentration was determined to be in the range of the lethal concentration 50 (LC₅₀) of this substance in CLL cells in our group before. GW 786034 was shown to be highly selective towards CLL cells as healthy PBMCs were not significantly affected at concentrations up to 100 µM. Furthermore, GW 786034 possessed effective inhibition of tumor growth in a CLL-like xenograft nude mouse model (Paesler et al., 2010). In this study, we could also show that GW 786034 reduced levels of Mcl1 and XIAP concentration-dependent. This substance is approved for metastatic renal cell carcinoma and is currently being tested in further phase-2 trials for the treatment of soft-tissue sarcoma and ovarian carcinoma. GW 78034 was also effective in hematological malignancies such as multiple myeloma (Podar et al., 2001), which has been demonstrated to exhibit VEGF-R2 and to be capable of producing and secreting VEGF (Kumar et al., 2003). Also other components such as SU11657, a precursor of Sutent® (Pfizer), or the green tea component EGCG are capable of reducing
phosphorylation of the VEGF-R2 (Lee et al., 2004; Sohal et al., 2003) and have proven pro-apoptotic potential in CLL cells (Lee et al., 2004; Lee et al., 2005). Hence, the VEGF-R should in fact be considered a promising target for CLL therapy.

As a major result of this study was the finding that the microenvironment is indispensable for VEGF-mediated CLL cell survival support, it is only of limited significance to test candidate substances for targeted CLL therapy in a CLL monoculture. That is why the monoclonal antibody MAb293 and the VEGF-R tyrosine kinase inhibitor were tested in a coculture setting of CLL cells together with the bone marrow-derived stromal cell line HS5. Interestingly, MAb293 effectively reduced the survival advantage which CLL cells gained from HS5 coculture. As already discussed earlier, it must be concluded that VEGF-signaling is of important survival-supporting capacity as its inhibition in a monoculture setting with limited VEGF-signaling, did not have an influence on CLL survival, but strongly reduces the survival advantages, CLL cells gain from being kept in a coculture with bone marrow stromal cells. Also GW 78034 was capable to overcome the HS5-mediated survival advantage in our studies. As mentioned earlier, by combining the VEGF-R inhibitor with an externally acting anti-VEGF antibody an additive effect might be achieved. A further pointer towards VEGF being a crucial pro-survival factor serving as a promising target for CLL-therapy is the fact that substances which do not have VEGF-specific inhibitory activity were not capable of keeping their CLL cell killing potential upright when VEGF was added. For example, the cytostatic drug chlorambucil (Glaxo Smith Kline), which has for long been the first line treatment for CLL patients, only had reduced potency, when rhVEGF was added to CLL cell monoculture (Lee et al., 2004). Also exposure of CLL cells to rhVEGF prior to incubation with 4-hydroperoxycyclophosphamide (4-HC), the active metabolite of cyclophosphamide (Baxter), which is up to date part of the CLL standard therapy regime, resulted in reduced CLL cell death (Wang et al., 2005b). Cyclophosphamide is an alkylating agent mainly inducing cell death through introduction of double strand breaks in rapidly dividing cells, and is therefore acting in a highly unselective manner. Since those substances also carry a high risk of affecting healthy cells in the patients’ body, a selective strategy is highly wanted. As healthy B-cells cells neither produce VEGF,
exhibit the VEGF-R nor seem to rely on VEGF in any sort (no increased VEGF-expression or survival support upon coculture, no upregulation of anti-apoptotic proteins upon rhVEGF stimulation), targeting the VEGF-signaling pathways seems a promising approach for the development of new CLL therapies with significantly reduced side effects.

From the theoretical point of view, every downstream component which is activated by VEGF-R-activation could serve as potential therapeutic target. In general though, to be considered a "drug target" a "cause and effect" relationship, how its activity relates to the disease is required (Drews, 2000; Gibbs, 2000). For a suitable drug target two important points are ideally required: (i) induction of particular disease phenotype by constitutive activation and (ii) vice versa reversal of this phenotype upon blockage or inhibition. We identified STAT3 as an immediate target of VEGF/VEGF-R-signaling activity. Above mentioned points are given for STAT3 as discussed earlier (Bromberg et al., 1998; Bromberg et al., 1999; Gibbs, 2000; Turkson et al., 1998). Therefore, STAT3-inhibition might be a suitable approach in CLL therapy. For that reason, the STAT3 inhibitor IV (S3I-201, Calbiochem) was used. This compound was discovered through virtual screening as an inhibitor of the Src homology 2 (SH2) dimerization domain of STAT3 resulting in prevention of STAT3 transcriptional activity and subsequent reduction of STAT3 target gene expression. (Siddiquee et al., 2007). In STAT3-active cancers its inhibition might therefore be an effective strategy for a therapeutic intervention. We could demonstrate a concentration-dependent reduction of CLL cell survival by S3I-201, which was more prominent after 48 hours of treatment compared to 24 hours. Used concentrations were admittedly high with up to 150 \( \mu \text{M} \). But since in vitro studies demonstrated an IC\(_{50}\) value for inhibition of DNA-binding activity of 86 \( \mu \text{M} +/- 33 \mu \text{M} \) (Siddiquee et al., 2007), the need of these high concentrations for reduction of survival is not surprising. Three major questions have to be considered when deciding about whether this substance is of potential interest for CLL therapy: (i) is the substance selective towards CLL cells? In this study three healthy PBMC samples were treated with S3I-201 in concentrations up to 150 \( \mu \text{M} \). At 24 hours healthy PBMCs were only marginally effected by the treatment (85.4\% +/- 2.7\% survival) after treatment with 150\( \mu \text{M} \), but also CLL cells did not react with substantial loss of viability.
Discussion

at this time point (62.4% +/- 12.2% survival). After 48 hours of treatment S3I-201 had a significant survival reducing effect on CLL cells (16.4% +/- 6.1% survival). Healthy PBMCs were also affected, but to an articulately smaller extent (46.9% +/- 10.6% survival). (ii) is the substance capable of overcoming the BM microenvironment-mediated survival advantage? S3I-201 seemed to exert an even more potent cytotoxic effect on CLL cells when cultured together with HS5: 6.5% +/- 10.6% surviving cells after treatment with 150 µM for 48 hours in our experiments. It has to be mentioned that these results were generated from only two independent experiments and that it was not tested whether S3I-201 has an effect on HS5 cells. And (iii) can those high concentrations be achieved \textit{in vivo}? Further studies have to be conducted to answer the latter question.

STAT activation is usually induced by Janus kinases (JAKs). JAKs have initially been identified in the process of erythropoietin (EPO)-mediated hematopoiesis, which promotes the conversion of bone marrow cells to red blood cells. With this background it was suggested that JAK might participate in the activation and proliferation of marrow-derived cells, and hence to potentially be of interest for leukemia therapy. Lee et al demonstrated a physical interaction between VEGF-R2 and STAT3 in CLL cells (Lee et al., 2005). The same group also mentioned preliminary data, in which they could not see any response in terms of STAT3 phosphorylation upon JAK-inhibition, suggesting STAT3 phosphorylation in CLL cells to be JAK-independent. Nevertheless, we decided to test the Jak inhibitor Pyridone 6 for its ability to induce CLL cell apoptosis \textit{in vitro}. In the tested concentration range (1 µg/ml - 20 µg/ml) only the highest concentration induced significant reduction of survival compared to healthy PBMCs after 24 hours. Healthy PBMCs were completely unaffected by treatment up to 20 µg/ml, hence an increased concentration would be justified in order to achieve a stronger survival reduction in CLL cells, which was only about 30% lower as the DMSO-treated control. Unfortunately, a limit to our study was the highly diluted stock concentration, which did not allow treatment with higher amounts of the drug. Also here, the above mentioned questions have to be assessed: is the substance also effective in a survival supporting atmosphere (e.g. coculture of CLL cells with HS5 cells) and can effective concentrations be achieved \textit{in vivo}?
Out of the tested substances the VEGF-R tyrosine kinase inhibitor GW 786034 seemed to have the highest potential as a substance for a targeted CLL therapy. Besides the mentioned selectivity towards CLL cells, the ability to overcome HS5-induced survival-support and the \textit{in vivo} efficacy in prevention of tumor growth (CLL-like xenograft nude mouse model), GW 786034 is orally available and showed promising results in metastatic renal cell carcinoma and is tested in further phase-2 trials for the treatment of soft-tissue sarcoma, ovarian carcinoma and multiple myeloma. Furthermore, clinically achievable concentrations have been demonstrated to be 40 $\mu$M and higher after once daily administration of 800 mg (Kumar et al., 2007). Altogether, this substance might be of high potential for an effective and selective CLL treatment.

Up to now, VEGF was commonly accepted as playing a pro-survival role in CLL cells (Lee YK, Blood, 2004, Lee YK, Blood, 2005), but neither the extent of microenvironmental factors, nor any molecular mechanisms in regard to VEGF have been described. We now hypothesize that \textit{in vivo} CLL cells are under the influence of VEGF secreted from non-malignant accessory cells such as bone marrow stromal cells, which turns on a feedback loop stimulating the CLL cells' own VEGF production. High amounts of VEGF stimulate the VEGF receptor, which subsequently activates STAT3 through tyr705 phosphorylation, ultimately leading to expression of target genes involved in counteracting apoptosis. The influence of direct cell-cell interactions and secreted factors other than VEGF in the micromilieu, crucial for full apoptosis protection, are of high interest and will help to further understand the underlying mechanisms of prolonged CLL cell survival. This knowledge could facilitate the development of successful strategies to overcome the apoptotic block in CLL, offering new options for novel therapeutic approaches for a targeted CLL therapy.

\subsection*{3.5 Future directions}

In this study the VEGF-signaling pathway was identified to be essentially involved in the survival of CLL cells within their natural microenvironment, as its inhibition significantly reduced the bone marrow stromal cell-mediated survival advantage. STAT3 activation could be identified as a potential downstream effector, being responsible for the upregulation of proteins involved in
apoptosis prevention. Hence, the VEGF-STAT3 axis might be a suitable target for a selective therapeutic approach in CLL.

In this study several substances have been demonstrated to be of possible interest, such as anti-VEGF antibodies, the small molecule VEGF-R tyrosine kinase inhibitor (GW 786034) and a STAT3 inhibitor (S3I-201). The first two showed effective survival reducing effects in CLL cells when present in a survival-supporting atmosphere such as coculture with the bone marrow stroma-derived cell line HS5, providing the proof-of-concept for the potential clinical use. The STAT3 inhibitor was effective in monoculture, but also affected healthy PBMCs to a certain degree at the highest concentration tested. As this inhibitor appeared to be even more potent towards CLL cells in a coculture together with HS5 cells, it might be possible to reduce concentrations to increase selectivity without substantial loss of effect towards CLL cells. To make a clear statement whether the mentioned substances are potential candidates for clinical use, further pharmacokinetic studies including its bioavailability, especially in the case of S3I-201, are needed. In addition, the selectivity of these substances in regard to interference with physiological roles of STAT3 has to be determined. The latter also applies for VEGF-R inhibition.

In this study the complex interplay between the CLL cells and their bone marrow microenvironment in regard to VEGF/VEGF-R-signaling and the impact on the apoptotic block has been determined. Several parameters were heterogeneous amongst patients in this study, such as VEGF mRNA levels, amount of secreted VEGF in the supernatant after 24 hours and especially its change with time or the response of the CLL cells towards rhVEGF stimulation and/or bone marrow stromal cell coculture in terms of change in VEGF-expression and survival. Therefore, it can be speculated that CLL cells might feature variable dependencies towards VEGF-mediated survival support. This could possibly lead to different sensitivities of the patients towards therapeutic intervention targeting the VEGF-STAT3 axis. Hence, further studies on possible correlations between patients’ characteristics and mentioned parameters might be indicated to potentially identify patient subsets which benefit better from a targeted therapy aiming on blockage of the VEGF-STAT3-pathway.
Chapter 4

Material and methods

4.1 Material

4.1.1 Instruments

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Brand/Model</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose gel chamber</td>
<td>DNA Sub Cell, Bio Rad</td>
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<tr>
<td>Analytical balance</td>
<td>Mettler</td>
</tr>
<tr>
<td>Autoclave</td>
<td>Varioklav Typ 400, Labortechnik GmbH</td>
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<tr>
<td>Capillaries cooling device</td>
<td>Light Cycler Cooling block, Roche</td>
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<tr>
<td>Centrifuge</td>
<td>Laborfuge 400R. Hereaus</td>
</tr>
<tr>
<td></td>
<td>(Rotor: 8172, Hereaus)</td>
</tr>
<tr>
<td>Deep freezer</td>
<td>Labotect -80 °C ilShin® DF8524</td>
</tr>
<tr>
<td>Developer</td>
<td>Curix 60, Agfa</td>
</tr>
<tr>
<td>Flow Cytometer</td>
<td>FACSCanto, BD Bioscience</td>
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<tr>
<td>Gel documenting device</td>
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<tr>
<td>UV/VIS Transluminator</td>
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<td>Camera</td>
<td>Hama</td>
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<tr>
<td>Thermo printer</td>
<td>P91, Mitsubishi</td>
</tr>
<tr>
<td>Screen</td>
<td>Sony</td>
</tr>
<tr>
<td>Hematocytometer</td>
<td>Neubauer, Labortechnik</td>
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<tr>
<td>Heatblock</td>
<td>TB1 Thermoblock, Biometra</td>
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<tr>
<td>Incubator</td>
<td>B 5060 EK/C02, Hereaus</td>
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<tr>
<td></td>
<td>Labotect Incubator C200, BeLoTec</td>
</tr>
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</table>
Material and methods

Lab Scale
TE 153S, Sortius

Laminar flow bench
HLB 2448, Hereaus
SterilGard III Advance, Baker Company

Luminometer
MicroLumatPlus LB 96V, Berthold

MACS® Separator
Miltenyi Biotec

Microcentrifuges
Biofuge fresco, Hereaus
(Rotor: 3325B; Hereaus)
Biofuge pico, Hereaus
(Rotor: 3328, Hereaus)

Microscope
Diavert, Leitz
Axiolab, Carl Zeiss

PAGE System
X-cell SureLock, Invitrogen

pH-meter
pH Level1, inoLab

Photometer
µ Quant, BioTek Instruments, Ultrospec 3000, Pharmacia Biotech

Pipetts
Research, Eppendorf
Multipette stream, Eppendorf
PreCision, Biozym
PreCision Multi, Biozym
Proline, Biohit

Power supply
Power Pac 200, BioRad
Power Pac 1000, BioRad

RT-PCR Systems
LightCycler 2.0 Instruments,
Roche Diagnostics
LightCycler 480, Roche Diagnostics
LightCycler Carousel Centrifuge 2.0,
Roche Diagnostics
Centrifuge 5430, Eppendorf

Rocking plate
Rocky RT-1S, Uniequip

Sealing device
Folio 3602, Severin

Sonication system
Sonoplus HD 2070, Bandelin

Vortex
K-550-GE, Bender & Holbein

Waterbath
GFL-1004, Gesellschaft für Labortechnik

Western Blot System
Xcell II blot module, Invitrogen
4.1.2 Consumables

- Cell culture flasks, 25cm$^2$, 75cm$^2$, 175cm$^2$ (Nunc)
- Cell culture multiwell plates, 6well, 12well (Greiner-Bio-One)
- Centrigugation tubes 15 ml, 50 ml (Greiner-Bio-One)
- FACS tubes (BD Falcon)
- MACS® columns (Miltenyi, Biotec)
- Microcon YM-10 centrifugal filter units (Milipore)
- Micro tubes, 2 ml (Sarstedt)
- Nitrocellulose membrane incl. filter paper (Invitrogen)
- Pipett accessories
  - Tips 10 µl, standard (Eppendorf)
  - Tips 10-100 µl (Sarstedt)
  - Tips 100-1000 µl (Sarstedt)
  - Dualfilter PCRclean, sterile, 10 µl, 200 µl, 1000 µl (Eppendorf)
- Reaction tubes, 1,5 ml (Sarstedt)
- RT-PCR capillaries, 20 µl (Roche Diagnostics)
- Serum tubes, 30 ml (Sarstedt)
- Sterile filters, Sterifix, 0.2 µM (Braun)
- Sterile plastic pipettes, 5 ml, 10 ml, 25 ml (Greiner-Bio-One)
- Syringes, 10 ml, 20 ml (Braun)
- Tissue culture inserts, transwells, 0.4 µM (Greiner-Bio-One)

4.1.3 Chemicals and reagents

- Agarose (Carl Roth)
- Albumin Fraction V (Carl Roth)
- Copper Sulfate Pentahydrate (Carl Roth)
- Dimethylsulfoxid (DMSO) (Carl Roth)
- Dithiotreitol (DTT) (Sigma)
- Ethanol absolute (Carl Roth)
- Ethidium bromide (EtBr) (Serva)
Material and methods

Ethylene glycol tetraacetic acid (EGTA)  Carl Roth
Ethylenediaminetetraacetic acid (EDTA)  Carl Roth
Fetal calf serum (FCS)  BioChrom AG
Ficoll-Hypaque (Lymphoprep)  Axis-Shield
Formaldehyde  Carl Roth
Hydrochloric acid (HCl)  Carl Roth
Methanol  Carl Roth
Milk powder  Carl Roth
NaCl  Carl Roth
PBS tablets  Gibco
Ponceau S  Carl Roth
Phosphatase inhibitor cocktail tablets  Roche
(PhosStop®)
Protease inhibitory cocktail tablets  Roche
(Complete®)
Roti-Blok®  Carl Roth
Roti-Nanoquant®  Carl Roth
TRIS-Base  Carl Roth
Tween20  Serva

4.1.4 Buffer and solutions

4.1.4.1 Substances

<table>
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<th>substance</th>
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<tbody>
<tr>
<td>bevacizumab (Avastin®)</td>
<td>Roche</td>
<td>NaCl</td>
<td>25 mg/ml</td>
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<tr>
<td>GW786034 (Pazopanib)</td>
<td>Glaxo-Smith-Kline</td>
<td>DMSO</td>
<td>10 mM</td>
</tr>
<tr>
<td>phorbol 12-myristate 13-acetate (PMA)</td>
<td>Cell Signaling</td>
<td>DMSO</td>
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<tr>
<td>Pyridone 6</td>
<td>Calbiochem</td>
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<td>1 mg/ml</td>
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<tr>
<td>recombinant human (rh) VEGF</td>
<td>BioMol</td>
<td>ddH2O</td>
<td>100 μg/ml</td>
</tr>
<tr>
<td>STAT3 Inhibitor VI S31 201</td>
<td>Santa Cruz</td>
<td>DMSO</td>
<td>10 mM</td>
</tr>
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Table 7: List of substances used.
Material and methods

4.1.4.2 Buffer and solutions for molecular biology

4.1.4.2.1 siRNA

<table>
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<th>name</th>
<th>target sequence</th>
<th>catalog no.</th>
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<tbody>
<tr>
<td>VEGF_4</td>
<td>5’-ATG CAG ATT ATG CGG ATC AAA</td>
<td>SI00051534</td>
</tr>
<tr>
<td>VEGF_5</td>
<td>5’-AAG AAA GAT AGA GCA AGA CAA</td>
<td>SI02757643</td>
</tr>
<tr>
<td>VEGFA_1</td>
<td>5’-ATA GAG AAT TCT ACA TAC TAA</td>
<td>SI04130748</td>
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<tr>
<td>VEGF_1</td>
<td>5’-CTG GAA TTT GAT ATT CAT TGA</td>
<td>SI00051513</td>
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<tr>
<td>Ctrl_AllStars_2</td>
<td>not disclosed, cell death control</td>
<td>SI04381048</td>
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<tr>
<td>Ctrl_AllStars_1</td>
<td>not disclosed, cell death control</td>
<td>SI03650318</td>
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</table>

Table 8: List of siRNAs and used controls.
* This product is AlexaFlour488 conjugated at the 3’-end. All products are from Qiagen.

siRNA was received lyophilized and reconstituted with RNase-free water using 100 µl per 1 nmol siRNA to obtain a 10 µM solution. This solution was used for further procedures as explained earlier.

4.1.4.2.2 Gel electrophoresis

Ethidium bromide solution

1% (m/V) Ethidiumbromid

Ethidium bromide was dissolved in ddH₂O and stored at 4 °C protected from light.

TAE (TRIS-acetate-EDTA) buffer (50x)

242 g TRIS-base
51.1 ml glacial acetic acid (100%)
100 ml 0.5M EDTA

ddH₂O was added to a final volume of 1000 ml. TAE buffer was diluted 1 in 50 ddH₂O to reach a final concentration of 1x for preparation of agarose gels.
and for use as running buffer.

4.1.4.3 Buffer and solutions for protein biochemistry

Phosphate buffered saline (PBS)
Ten PBS tablets were dissolved in 500 ml ddH$_2$O to obtain a 10x PBS solution. This 10x solution was diluted to 1x with ddH$_2$O when needed. pH was adjusted to 7.6 using HCl or NaOH.

TRIS-buffered saline (TBS)

- 50 mM TRIS-Base
- 150 mM NaCl

Components were dissolved in ddH$_2$O and pH was adjusted to 7.6 using HCl.

Ponceau-S stock solution

- 2% w/v Ponceau S
- 30% w/v Trichloroacetic acid
- 30% w/v Sulfosalicylic acid

Components were dissolved in ddH$_2$O. For use 1 part stock was dissolved with 3 parts ddH$_2$O.

Blocking buffer I (PBS/milk/Roti)

- 5% w/v nonfat dry milk
- 10% Rotiblok®

Components were dissolved in PBS (1x) and filtered. Blocking buffer I was stored at 4 °C for one week.

Blocking buffer II (PBS/milk)

- 5% w/v nonfat dry milk
Material and methods

Milkpowder was dissolved in PBS (1x) and filtered. Blocking buffer II was stored at 4 °C for one week.

Blocking buffer III (TBST/milk)
- 0.1% Tween-20
- 5% w/v nonfat dry milk

Milkpowder was dissolved in TBS (1x), filtered and Tween-20 was added. Blocking buffer III was stored at 4 °C for one week.

Blocking buffer IV (TBST/BSA)
- 0.1% Tween-20
- 5% BSA

BSA was dissolved in TBS (1x), filtered and Tween-20 was added. Blocking buffer IV was stored at 4 °C for one week.

4.1.5 Cell culture reagents and media

4.1.5.1 Reagents

- DMEM + L-glutamine Sigma
- Fetal calf serum Biochrom
- N-2-Hydroxyethylpiperazine-N-ethanesulfonic acid (HEPES) 1 M Gibco
- Penicillin/Streptomycin-solution Biochrom
  - (10.000 µg/ml Streptomyein sulphate,
  10.000 units/ml Penicillin G)
- RPMI 1640-medium + L-glutamine Sigma
- Trypsin-EDTA solution (10x) Sigma
4.1.5.2 Culture medium

Culture medium for HS5
10% v/v FCS
100 U/ml Penicillin
10 μg/ml Streptomycin
10 mM HEPES buffer

Components were dissolved in DMEM including L-glutamine. Medium was stored at 4 °C.

Culture medium for primary material (healthy B-and CLL cells)
20% v/v FCS
100 U/ml Penicillin
10 μg/ml Streptomycin
10 mM HEPES buffer

Components were dissolved in RPMI 1640 including L-glutamine. Medium was stored at 4 °C.

Culture medium for creation of starving conditions (healthy B-and CLL cells)
3% v/v FCS
100 U/ml Penicillin
10 μg/ml Streptomycin
10 mM HEPES buffer

Components were dissolved in RPMI 1640 including L-glutamine. Medium was stored at 4 °C.
4.1.6 Special reagents and kits

LightCycler® FastStart DNA Roche Applied Science
MasterPlus Hybridization Probes

Magnetic Beads, CD19 Miltenyi Biotec
QIAamp® RNA Blood mini Kit Qiagen
Quantikine® VEGF ELISA Kit R&D Systems
Rosette Sep® B-cell enrichment Stem Cell technologies cocktail
RT² First strand Kit SABioscience
RT2 Profiler™ PCR Array SABioscience
RT² qPCR-Grade RNA Isolation Kit SABioscience
SuperScript® III First-Strand Invitrogen
Synthesis System for RT-PCR

4.1.7 Ready-to-use buffers and solutions

ECL Western Blotting detection reagents Amersham (I+II)*
Electrophoresis sample buffer (6x)* Fermentas
Erythrocyte lysis buffer Qiagen
Lymphoprep® Axis Shield* M-PER® lysis buffer Thermo Scientific
NuPage® Antioxidant* Invitrogen
NuPage® LDS sample buffer (4x)* Invitrogen
NuPage® MES Running buffer (20x) Invitrogen
NuPage® Sample reducing agent (10x)* Invitrogen
NuPage® Transfer buffer (20x) Invitrogen
Roti®-Free Stripping buffer (ready-to-use) Carl Roth
Trypanblue solution (0.5%) Biochrom

All buffers and solutions indicated with a star (*) were stored at 4 °C.
4.1.8 Antibodies

4.1.8.1 Antibodies for immunoblotting

Table 9: List of antibodies used for immunoblotting.
Secondary antibodies were from DAKO (mouse P-0447, rabbit P-0448) and horseradish peroxidase (HRP)-labeled.

<table>
<thead>
<tr>
<th>Antibody directed against</th>
<th>Company</th>
<th>Catalogue-no.</th>
<th>Dilution</th>
<th>Incubated in blocking buffer</th>
<th>Secondary antibody against</th>
</tr>
</thead>
<tbody>
<tr>
<td>Akt (pan)</td>
<td>CS</td>
<td>4961</td>
<td>1:1000</td>
<td>IV</td>
<td>rabbit</td>
</tr>
<tr>
<td>Bcl2</td>
<td>BD</td>
<td>619393</td>
<td>1:2000</td>
<td>I</td>
<td>mouse</td>
</tr>
<tr>
<td>Bax</td>
<td>BD</td>
<td>556496</td>
<td>1:2000</td>
<td>I</td>
<td>mouse</td>
</tr>
<tr>
<td>(cl) caspase 3</td>
<td>CS</td>
<td>9681</td>
<td>1:1000</td>
<td>IV</td>
<td>rabbit</td>
</tr>
<tr>
<td>(cl) caspase 9</td>
<td>CS</td>
<td>9531</td>
<td>1:1000</td>
<td>IV</td>
<td>rabbit</td>
</tr>
<tr>
<td>(cl) PARP</td>
<td>BD</td>
<td>5190030117</td>
<td>1:2000</td>
<td>I</td>
<td>mouse</td>
</tr>
<tr>
<td>cyclinD1</td>
<td>BD</td>
<td>554181</td>
<td>1:1000</td>
<td>I</td>
<td>rabbit</td>
</tr>
<tr>
<td>nL-P/XAP</td>
<td>BD</td>
<td>613762</td>
<td>1:1000</td>
<td>I</td>
<td>mouse</td>
</tr>
<tr>
<td>Mcl1</td>
<td>SCBT</td>
<td>20679</td>
<td>1:200</td>
<td>I</td>
<td>rabbit</td>
</tr>
<tr>
<td>pAkt (ser473)</td>
<td>CS</td>
<td>4960</td>
<td>1:1000</td>
<td>IV</td>
<td>rabbit</td>
</tr>
<tr>
<td>pSTAT3 (ser727)</td>
<td>CS</td>
<td>9134</td>
<td>1:1000</td>
<td>IV</td>
<td>rabbit</td>
</tr>
<tr>
<td>pSTAT3 (tyr705)</td>
<td>CS</td>
<td>9134</td>
<td>1:1000</td>
<td>IV</td>
<td>rabbit</td>
</tr>
<tr>
<td>β-actin</td>
<td>BD</td>
<td>612656</td>
<td>1:2000</td>
<td>I</td>
<td>mouse</td>
</tr>
</tbody>
</table>

4.1.8.2 Antibodies for flow cytometry

Table 10: List of antibodies used for flow cytometry.

<table>
<thead>
<tr>
<th>Antibody directed against</th>
<th>Company</th>
<th>Catalogue-no.</th>
<th>Dilution</th>
<th>Fluorochrome/Secondary antibody</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD19</td>
<td>BD</td>
<td>345781</td>
<td>1:10</td>
<td>PerCya5.5</td>
<td>none</td>
</tr>
<tr>
<td>CD23</td>
<td>BD</td>
<td>332790</td>
<td>1:50</td>
<td>APC</td>
<td>none</td>
</tr>
<tr>
<td>CD5</td>
<td>BD</td>
<td>3350306</td>
<td>1:10</td>
<td>PE</td>
<td>fixation &amp; permeabilization</td>
</tr>
<tr>
<td>pSTAT3 (ser727)</td>
<td>BD</td>
<td>556065</td>
<td>20 μl/100</td>
<td>Alexa Fluor 488</td>
<td>fixation &amp; permeabilization</td>
</tr>
<tr>
<td>pSTAT3 (tyr705)</td>
<td>BD</td>
<td>612569</td>
<td>20 μl/100</td>
<td>PE</td>
<td>fixation &amp; permeabilization</td>
</tr>
<tr>
<td>pVEGF-R2 (tyr951)</td>
<td>CS</td>
<td>2476</td>
<td>1:1000</td>
<td>anti-mouse FITC</td>
<td>fixation &amp; permeabilization</td>
</tr>
<tr>
<td>VEGF-R2</td>
<td>CS</td>
<td>2479</td>
<td>1:1000</td>
<td>anti-rabbit FITC</td>
<td>fixation &amp; permeabilization</td>
</tr>
</tbody>
</table>

4.1.8.3 VEGF-neutralizing antibody

The monoclonal anti-VEGF antibody MAb293 from R&D Systems and the commercially available humanized monoclonal anti-VEGF antibody bevacizu-
mab (Avastin®, Roche) were used for neutralization of VEGF bioactivity.

### 4.1.9 Oligonucleotides

<table>
<thead>
<tr>
<th>gene</th>
<th>primer sequence</th>
<th>location</th>
<th>amplicon</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF_forward</td>
<td>5'-CCC TGA ATG AGA TCG AGT ACA TGGT-3'</td>
<td>exon 3</td>
<td></td>
</tr>
<tr>
<td>VEGF_rev1</td>
<td>5'-GCC TGG GCT TGT CAC ATT TT-3'</td>
<td>exon 5/6</td>
<td>254bp</td>
</tr>
<tr>
<td>VEGF_rev5</td>
<td>5'-AGCAAG GCC CAC AGG GAT TT-3'</td>
<td>exon 5/7</td>
<td>254bp</td>
</tr>
<tr>
<td>VEGF_rev10</td>
<td>5'-AAC GCT CCA GGA CTT ATA CCG-3'</td>
<td>exon 6</td>
<td>310bp</td>
</tr>
<tr>
<td>VEGF_revall</td>
<td>5'-ACC GCC TCG GCT TGT CAC-3'</td>
<td>exon 8</td>
<td>267bp</td>
</tr>
<tr>
<td>VEGF_probe</td>
<td>5'-FAM ATC CTG TGT GCC CCT GAT GCG ATG CGG T-TAMRA-3'</td>
<td>exon 3</td>
<td></td>
</tr>
<tr>
<td>E2F1_forward</td>
<td>5'-AGA TGG TTA TGG TGA TCA AAG CC-3'</td>
<td>exon 5</td>
<td></td>
</tr>
<tr>
<td>E2F1_reverse</td>
<td>5'-ATC TGA AAG TTC TCC GAA GAG TCC-3'</td>
<td>exon 5/6</td>
<td>73bp</td>
</tr>
<tr>
<td>E2F1_probe</td>
<td>5'-FAM CTC CTG AGC ACC AGC TCC AAG CC T-TAMRA-3'</td>
<td>exon 5</td>
<td></td>
</tr>
<tr>
<td>RB1_forward</td>
<td>5'-CTT GCA TGG CTC TCA GAT CAG C3-3'</td>
<td>exon 17</td>
<td></td>
</tr>
<tr>
<td>RB1_reverse</td>
<td>5'-AGA GGA CAA GGA GAT TCA AGG TG-3'</td>
<td>exon 17/18</td>
<td>94bp</td>
</tr>
<tr>
<td>RB1_probe</td>
<td>5'-FAM ATT AAA CCA TCA AAG GAC CCA GAA GGA CCA ACT G-TAMRA-3'</td>
<td>exon 17</td>
<td></td>
</tr>
<tr>
<td>ABL_forward</td>
<td>5'-TGGGATAACACTCTAACGATGAACTAAGG-3'</td>
<td>exon 2</td>
<td></td>
</tr>
<tr>
<td>ABL_reverse</td>
<td>5'-GATGATGTGTTGCTGGGACCCA-3'</td>
<td>exon 3</td>
<td>124bp</td>
</tr>
<tr>
<td>ABL_probe</td>
<td>5'-FAM -CCTTTTGGTGGGCTACACCATT- DABCYL-3'</td>
<td>exon 3</td>
<td></td>
</tr>
</tbody>
</table>

Table 11: Primers used for real time PCR.

VEGF primers were adopted from Wellmann et al (Wellmann et al., 2001). Primer and probe stocks are 20 pg/ml (20 µM). All primers were synthesised from TibMolBiol, Berlin.

### 4.1.10 Cell lines

The cell line HS5 was established from human bone marrow stromal cells which were transformed by the human papilloma virus E6/E7 genes (Roecklein and Torok-Storb, 1995; Torok-Storb et al., 1999). HS5 are fibroblastic-appearing cells, which adhere when cultured and form a reticulum of overlapping cells.
HS5 cells were used for coculture experiments. Due to the availability of patient material and the lack of convincing CLL cell lines, all work in this project was carried out using primary cells and no other cell lines were used.

4.1.11 Primary patient material

All CLL patients included in this study had a confirmed diagnosis according to standard criteria (Cheson et al., 1996). Patients were either untreated or had not been treated for at least 3 months prior to blood withdrawal. Patients represented all Binet-stages. All patients provided written informed consent and the study was in accordance with the declaration of Helsinki and approved by the internal review board of the University Hospital Cologne. Peripheral blood from patients or healthy volunteers was taken into EDTA-coated tubes and processed within one day of withdrawal.

4.1.12 Software

BD FACS Diva
Cyflogic 1.2.0 flow cytometry data analysis tool
Graph Pad Prism Software 4.0
Image J, v.1.1.1
Primer3, version 0.4.0
Reference Manager 11
SPSS statistical analysis software, version 17.0.
4.2 Methods

4.2.1 Cells

4.2.1.1 Extraction of CLL cells from whole blood

Peripheral blood from patients was withdrawn into EDTA-coated tubes. Whole blood was incubated with Rosette Sep® B-cell enrichment antibody cocktail at 40 µl/ml blood for 20 minutes. This cocktail contains antibodies directed against surface markers of non-B-cells of the blood (CD2, CD3, CD16, CD36, CD56, CD66b, and glycophorinA). The antibodies crosslink non-B-cells to multiple red blood cells (RBCs), which are pelleted along with free RBCs during the process of subsequently carried out density gradient centrifugation using Ficoll-Hypaque density medium (LymphoPrep®). For this process preincubated blood was diluted with an equal volume of PBS/0.5% FCS and layered carefully on top of Ficoll-Hypaque, followed by centrifugation at 1500 rpm for 20 minutes. Non-labeled B-cells accumulate in the interphase between plasma and the density medium and can easily be isolated. To reduce the amount of contaminating RBCs, isolated cells were incubated with erythrocyte lysis buffer for 10 minutes, followed by two washing steps. Cells were resuspended in RPMI, Pen/Strep, HEPES, 20% FCS. After this process CLL cell purity was usually larger than 95% as assessed by flow cytometry using CD5/CD19 staining.

4.2.1.2 Extraction of healthy B-cells from whole blood

4.2.1.2.1 Positive selection

Positive selection was carried out by MACS® cell separation (Miltenyi Biotec). For this purpose, healthy peripheral blood mononuclear cells (PBMCs) were separated from whole blood of healthy volunteers by Ficoll density gradient centrifugation as mentioned earlier without preincubation with Rossette Sep®. Isolated PBMCs were incubated with magnetically labeled anti-CD19 antibodies. The mixture of CD19-positive labeled B-cells and non labeled cells was pipetted into a MACS® column, which was placed into a permanent magnetic field (MACS® Seperator). Magnetically labeled CD19 positive cells were retained, whereas unlabeled cells were removed from the column by several
washed. Labeled cells were released after removal of the column from the magnet. Isolated cells were dissolved in RPMI, 20% FCS and used for the intended experimental procedure.

4.2.1.2.2 Negative selection
Negative selection of healthy B-cells was carried out using already mentioned RosetteSep® technique. However, instead of whole blood buffy coats established from 500 ml of whole blood obtained from the blood bank of the University Hospital Cologne was used. Those large amounts of blood were required since healthy blood only contains a maximum of approximately 3x10^5 B-cells/ml.

4.2.1.3 Cell culture
All used media contained 100 U/ml Penicillin, 10\( \mu \)g/ml Streptomycin and 10 mM HEPES-buffer if not otherwise indicated. In the following passages only the amount of added FCS is stated.

4.2.1.3.1 Cultivation of cell lines
The bone marrow-derived stromal cell line HS5 was maintained as a stocks of 1x10^7 cells/ml in DMEM, 10% FCS and 10% DMSO at -80 °C. For preparation of cell culture experiments cells were thawed rapidly and washed immediately in medium without DMSO and FCS. After washing, cells were dissolved in 20 ml DMEM, 10% FCS and put in a 75 cm^2 cell culture flask and incubated at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were split every 2\textsuperscript{nd} to 3\textsuperscript{rd} day dependent on cell density. For that purpose adherent HS5 were washed twice with DMEM not containing FCS. Trypsin (10x) was diluted 1 in 10 with FCS-free DMEM and added to HS5 containing culture flask. HS5 were incubated for approximately 10 minutes at 37 °C. After that time HS5 cells could usually be detached by vigorous tapping of the culture flask. HS5 cells were diluted with DMEM, 10% FCS to inactivate trypsin. In the following HS5 cells were pelleted via centrifugation and the pellet was diluted in fresh DMEM, 10% FCS. Cells were usually split 1 in 10. Cells were controlled frequently for viability using light microscopy and used for experiments after reaching confluency.
4.2.1.3.2 Primary cell cultivation
Primary CLL- or healthy B-cells were cultivated in RPMI, 20% FCS. Cells were seeded at 4x10^6/ml and plated in either 6- or 12-well plates dependent on the following experimental procedure. Since CLL-and primary healthy B-cells do no proliferate, actual long term cultivation was not possible. CLL cells usually die within one week after isolation from blood. Therefore, experimental procedures using primary cells were limited to a short time period, which was typically not longer than 48 hours.

4.2.1.3.3 Coculture
HS5 cells were seeded at a density of 3x10^5/ml in DMEM, 10% FCS. After overnight cultivation the supernatant was withdrawn and adherent cells were washed twice with DMEM not containing FCS. Primary CLL cells were isolated from whole blood as described earlier. 4x10^6 CLL cells/ml RPMI, 20% FCS were added to adherent HS5 cells. In any case CLL cells were equivalently seeded in a monoculture setting. CLL cells were removed from HS5 by taking the supernatant of the coculture after a period of time suitable for the experimental procedure subsequently carried out. Extent of HS5 cell contamination was assessed by flow cytometry separating CLL-and HS5 cells by their obvious size difference. Contamination was typically around 1.5%.

![Flow cytometric differentiation of HS5 and CLL cells by cell volume (FSC forward scatter) and granularity (SSC side scatter).](image)

Figure 41: Flow cytometric differentiation of HS5 and CLL cells by cell volume (FSC forward scatter) and granularity (SSC side scatter).
Yellow population represent lymphocytes and red population HS5 cells. Red circle indicates percentage of HS5 contamination in the CLL cell fraction taken from coculture.
4.2.1.3.4 Physical separation of HS5 and CLL by tissue culture inserts

The basic coculture set up was as described above. Tissue culture inserts (transwells) are filters which can be placed into single wells of multiwell plates. They allow cocultivation of two different cell populations, in our case adherent HS5 cells and soluble primary CLL cells, preventing their physical interaction. Both cell types are cultured in the same medium, therefore the exchange of soluble factors can occur. In our experiments transwells had a pore size of 0.4 µM and were used in a 12 well format. The experimental set up is displayed in the following figure.

![Experimental set up of HS5/CLL cell coculture with physical separation using tissue culture inserts.](image)

Figure 42: Experimental set up of HS5/CLL cell coculture with physical separation using tissue culture inserts.
Source: Own illustration

Primary CLL cells were either added directly to HS5 monolayer at 4x10^6/ml or placed into a tissue culture insert. Insert contained 200 µl medium. To maintain CLL cell density the amount of cells was adapted (concentration was maintained). CLL cells were also cultured without HS5 feederlayer. Monoculture controls were treated equally with or without transwell insert.

4.2.2 Molecular biology

4.2.2.1 Extraction of mRNA

RNA was isolated from primary CLL cells using the QIAamp® RNA Blood mini Kit (Qiagen, Hilden, Germany). The procedure was carried out following the manual. In brief, 1x10^7 cells were lysed in 500 µl RLT buffer. Lysed cells were either stored at -20 °C for later use or the subsequent procedure was carried out immediately. Lysates were pre-cleaned from cell debris by centrifugation through a QIAshredder spin column and mixed with equal volumes of 70% ethanol. Samples were pipetted into a QIAamp spin column, where
mRNA was retained in the filter. After three washing steps using buffers RW1 and RPE mRNA was eluted using RNase-free ddH₂O.

### 4.2.2.2 Reverse transcription

Reverse transcription of RNA into cDNA is accomplished by reverse transcriptase, also known as RNA-dependent DNA polymerase. Random hexamers bind RNA and function as a starting point for reverse transcriptase for creation of complementary DNA strands by addition of dNTPs. We used the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen GmbH, Karlsruhe, Germany) as suggested in the manufacturer’s protocoll. Reverse transcription-supermix composition was as follows:

<table>
<thead>
<tr>
<th>supermix-components</th>
<th>amount needed for n=1</th>
</tr>
</thead>
<tbody>
<tr>
<td>random hexameres</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>dNTP-Mix (10 mM)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>10xRT-Buffer</td>
<td>6.0 µl</td>
</tr>
<tr>
<td>MgCl₂ (25 mM)</td>
<td>10.0 µl</td>
</tr>
<tr>
<td>DTT (0.1 M)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>RNaseOUT (40 U/µl)</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>SuperScript™III reverse transcriptase (200 U/µl)</td>
<td>1.5 µl</td>
</tr>
<tr>
<td></td>
<td>30 µl</td>
</tr>
</tbody>
</table>

Table 12: Reverse transcription supermix composition.

30 µl mRNA was added to supermix. Mix was incubated in a PCR cycler set at 25 °C for 10 minutes, 50 °C for 50 minutes, 85 °C for 5 minutes followed by a cooling 4 °C step, which was maintained until manually stopped. cDNA was stored at -20 °C until needed.

### 4.2.2.3 Real time polymerase chain reaction (RT-PCR)

PCR is used for in vitro amplification of specific DNA sequences. Since this reaction is exponential it is possible to gain large amounts out of relatively little amount of starting material. The method relies on thermal cycling. After DNA denaturation (94-96 °C), short oligonucleotides, so called primers, which flank the sequence of interest, bind to the DNA (annealing). The temperature
for this step is dependent on the melting temperature of the oligonucleotides (usually between 55 °C and 65 °C). In the following a DNA-polymerase adds nucleotides complementary to the DNA-single strand to create a DNA-double strand (extension). This takes place at a temperature which is optimal for a particular DNA polymerase to function (usually 72 °C). As PCR progresses, the DNA generated is itself used as a template for replication, setting in motion a chain reaction in which the DNA template is exponentially amplified (Mullis and Faloona, 1987). For real time detection of amplification real time PCR can be performed. Quantification is based on the measurement of fluorescence, which increases proportional to the PCR-product, detected after every cycle in "real time". Quantification can be measured relatively by normalization to a house keeping gene or as absolute measure by simultaneously running a sample of known content for creation of a standard curve. A fluorescent signal can be achieved by addition of DNA-dyes such as SYBR-green. The disadvantage is its unspecificity, since also unspecific amplificates contribute to the fluorescent signal. That is why sequence specific probes based on fluorescence resonance energy transfer (FRET) are widely used. For that purpose two sequence-specific oligonucleotides, which are labeled with different dyes (donor and acceptor) hybridize to the target sequence during the annealing phase, which leads to excitation of the acceptor dye. Fluorescence is measured at the end of the annealing phase. Another method is the use of so called TaqMan or hydrolysation probes. One single sequence specific oligonucleotide is marked with a reporter-dye at one end and a quencher on the other end. When Taqpolymerase carries out its 5´-3´exonuclease function the probe is degraded from the 5´-end while the complementary strand is generated. The resulting physical distance between quencher and reporter causes an increased fluorescent signal, which is measured after the elongation phase. For every run a specific fluorescent threshold is set dependent on the background fluorescence. The cycle when the fluorescence hits this threshold is denominated Ct-value. This value is used for further relative quantification. In our experiments TaqMan probes were used. VEGF primers were adopted from Wellmann et al (Wellmann et al., 2001), whereas Abl, E2F1 and RB1 primers were designed with the help of the software Primer3, which is available online (for sequences see Table 5). The Abl gene was used as house keeping gene. PCR set up
Material and methods

included the following components:

<table>
<thead>
<tr>
<th>PCR reaction components</th>
<th>amount needed for n=1</th>
</tr>
</thead>
<tbody>
<tr>
<td>LightCycler® FastStart DNA MasterPlus Hybridization Probes</td>
<td>4 µl</td>
</tr>
<tr>
<td>primer (20 µM), each</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>hybridization probe (20 µM)</td>
<td>0.5 µl</td>
</tr>
<tr>
<td>uracil-N-glycosylase (1 U/µl)</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>ddH₂O</td>
<td>9.25 µl</td>
</tr>
<tr>
<td></td>
<td>15 µl</td>
</tr>
</tbody>
</table>

Table 13: PCR reaction mix composition.

LightCycler® FastStart DNA MasterPlus Hybridization Probes include all components necessary for carrying out a PCR, such as the appropriate buffer, MgCl₂, dNTPs and a modified Taq-polymerase. Furthermore, BSA is included which is needed for samples not to stick to the glass walls of the capillaries. The exact composition and concentrations are not provided by Roche. Primers were added to a total of 15 µl PCR set up which was carefully pipetted into a light cycler capillary positioned in a cooling block and 5 µl cDNA was added. PCR was carried out using the Roche Light cycler system. PCR programs were as following:

<table>
<thead>
<tr>
<th></th>
<th>denaturation</th>
<th>annealing</th>
<th>elongation</th>
<th>cycle no</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>95 °C, 15sec</td>
<td>55 °C, 15sec</td>
<td>72 °C, 20sec</td>
<td>40</td>
</tr>
<tr>
<td>E2F1</td>
<td>95 °C, 10sec</td>
<td>60 °C, 10sec</td>
<td>72 °C, 15sec</td>
<td>45</td>
</tr>
<tr>
<td>RB1</td>
<td>95 °C, 10sec</td>
<td>60 °C, 10sec</td>
<td>72 °C, 15sec</td>
<td>45</td>
</tr>
<tr>
<td>Abl</td>
<td>95 °C, 10sec</td>
<td>60 °C, 15sec</td>
<td>72 °C, 15sec</td>
<td>45</td>
</tr>
</tbody>
</table>

Table 14: Cycling durations and temperatures for PCR.

Initial denaturation was carried out for 5 minutes at 95 °C prior to cycling for all PCRs.

4.2.2.4 The $2^{-\Delta\Delta C_t}$-method

The $2^{-\Delta\Delta C_t}$ method is a comparative analysis to quantify relative changes in gene expression from real time PCR experiments. In contrast to absolute quantification methods, where the input is determined by means of a copy number by a correlation to a standard curve, relative expression describes
changes in expression levels of a target gene relative to a reference group. The reference is dependent on the experiment or the question asked and can be for example an untreated or healthy sample or the sample at time zero in a time course experiment. The $2^{-\Delta \Delta Ct}$-method requires comparable amplification efficiencies for PCRs of the calibrator, usually a housekeeping gene, and the gene of interest. Efficiencies are calculated by determination of a standard curve. For that purpose PCR has to be performed with a dilution series of the input cDNA. To generate a standard curve, Ct-values (Y-axis) are plotted against the log of template amount or dilution (X-axis). PCR efficiencies are comparable for the case that the slopes are similar. For calculation of relative expression levels the normalized Ct values for both reference and sample of interest, have to be generated:

$$\Delta \text{Ct (sample)} = \text{Ct target} - \text{Ct reference}$$

$$\Delta \text{Ct (calibrator)} = \text{Ct target} - \text{Ct reference}$$

As a next step the $\Delta \Delta \text{Ct}$ value is determined by subtracting the $\Delta \text{Ct}$ value of the calibrator from the $\Delta \text{Ct}$ value of the sample:

$$\Delta \Delta \text{Ct} = \Delta \text{Ct (sample)} - \Delta \text{Ct (calibrator)}$$

$\Delta \Delta \text{Ct}$ can be used to calculate a normalized target gene expression level in the sample of interest by $2^{-\Delta \Delta \text{Ct}}$ with the result of a fold change-value. If PCR efficiencies of the calibrator and the target are not comparable, the error produced can be calculated using the following formular:

$$\text{Error (\%)} = \left[ \frac{2n}{(1+E)n} \right] 100 - 100$$

with $E =$ PCR efficiency, $n =$ cycle number.

4.2.2.5 Agarose gel-electrophoresis

Agarose gel-electrophoresis was used to separate DNA fragments according to their size. Electrophoresis was carried out in a horizontal gel chamber. Agarose
was dissolved in 1xTAE while boiling. Gels contained 1.5% to 2% agarose, dependent on size of DNA fragment to be displayed, and 0.5 µg/ml ethidium bromide (EtBr). EtBr intercalates into DNA and is used for detection of the DNA on the gel using UV-light. Gel-electrophoresis buffer (6x) was added to samples to prevent them from leaking from the wells and further, to mark the running front of the samples during the run. For size determination a 100bp DNA ladder was run in parallel. Agarose gels were run for one hour at 75 V. DNA bands were visualized through UV light (366 nm) exposure, which leads to a fluorescing of EtBr with an orange colour. This fluorescence was detected using a gel documenting device.

4.2.2.6 PCR Array

The PCR array system RT²Profiler® from SABiosciences (Frederick, USA) was used to determine expression levels of 84 transcription factors in a 96-well format. Besides specific gene primers it also provides primer sets detecting five housekeeping genes and three RNA and PCR quality controls. (Cat.No. PAHS-075F, for detailed information about complete gene content, please visit the company’s website). CLL cells were cultured at 4x10⁶ cells/ml RPMI1640, 3% FCS under standard conditions for 6 hours followed by either stimulation with 100 ng/ml rhVEGF or left untreated for one hour. RNA was extracted (RT² qPCR-Grade RNA Isolation Kit) following the recommended instructions. 1 µg RNA was used for reverse transcription into cDNA, preceded by a genomic DNA elimination step (RT² First strand kit). The used master mix contained SYBR Green for fluorescent detection. The array format was optimized for use with the Roche LightCycler® 480. Melting curves proofed specific amplification of a single PCR product. RNA and PCR quality controls were included in the experimental set up. Five different housekeeping genes were used for normalization of cDNA content. For data analysis the SABioscience´s software, based on 2−ΔΔCt-method, offered on the company´s website was used. Expression in untreated sample was compared to VEGF-stimulated sample. 3-fold expression change was considered as upregulation/downregulation threshold. Array experiments were carried out with two different patients under equal conditions.
4.2.2.7 Short interfering RNA (siRNA)

Short interfering RNA (siRNA) was used to knock down VEGF in HS5 cells. siRNAs are short double-stranded RNA molecules of 20 to 25 nucleotides in length and a 2-nucleotide overhang on either end. Those fragments are created by cleavage of dsRNAs through the ribonuclease protein Dicer (Bernstein et al., 2001). These siRNAs are then separated into single strands and integrated into an active RNA-induced silencing complex (RISC), where they function as sequence determinants of the RNAi pathway (Hannon and Rossi, 2004). Integrated siRNAs basepair to sequence homologue mRNA-molecules, thereby initiating their directed cleavage via endonucleases composing RISC. Hence, the cleaved mRNA cannot function as template for translation anymore and its protein expression is reduced (Tuschl, 2003). This principal is widely used for experimental strategies to downregulate the translation of specific proteins. For that purpose a synthetically produced double stranded siRNA molecule targeting the mRNA of interest is introduced into the cell and induces its subsequent downregulation via mentioned mechanism. We used this technique to specifically knock-down the translation of VEGF mRNA into VEGF protein in HS5 cells. For that purpose, HS5 cells were plated at 1x10^5/ml in DMEM, 10% FCS in 12 well plates on the day prior to transfection. Lyophilized siRNA was reconstituted with RNase-free ddH_2O to a final concentration of 10 µM. The amounts of used siRNA and transfection reagent are listed in (Table 9). Four different VEGF-targeting siRNAs (for sequence see Table 2) were used. They were tested for their efficiency to knock down VEGF-expression by PCR and ELISA using concentrations ranging from 1 nM to 100 nM. 50 nM turned out to be most effective and was therefore used for experimental procedures.

<table>
<thead>
<tr>
<th>culture format</th>
<th>volume of medium on cells (µl)</th>
<th>siRNA amount (ng)</th>
<th>volume of HiPerfect reagent (µl)</th>
<th>final siRNA concentration</th>
<th>equivalent volume of 10 µM siRNA stock (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>12 well</td>
<td>1100 µl</td>
<td>750 ng</td>
<td>100 µl</td>
<td>6 µl</td>
<td>50 nM</td>
</tr>
</tbody>
</table>

Table 15: Set up for siRNA experiments.
Calculation is based on approximate values for a double-stranded, 21nt siRNA molecule:

\[ 20 \text{ µM} \times 0.25 \text{ µg/µl, molecular weight} = 14 \text{ µg/nmol}. \]
siRNA (6 µl/ml/well) was diluted with 100 µl DMEM without FCS, HiPerfect transfection reagent was added subsequently (6 µl/ml/well) and the mixture was vortexed. Samples were incubated for 10 minutes at RT to allow formation of complexes and then added drop-wise to HS5 cells. HS5 cells were washed prior to addition of transfection complexes and fresh medium was added.

4.2.3 Protein biochemistry

4.2.3.1 Preparation of cell lysates

To access the protein status of a cell its membranes (cell-, nuclear-, mitochondrial membranes) have to be ruptured. For that purpose cell lysis is carried out. Usually 1x10^7 cells were used. In case of low expected amounts of targeted protein the cell amount was doubled. Cells were pelleted via centrifugation at 6,000 U/minute for 2 minutes. Pellets were dissolved in appropriate amounts of M-PER® mammalian protein extraction reagent containing phosphatase inhibitor (PhosSTOP, Roche, 1 tablet per 20 ml lysis buffer), proteinase inhibitor (Complete, Roche, 1 tablet per 10 ml lysis buffer) and DTT (1:1000). Cells were lysed by constant shaking at 4 °C for 3 hours. Cell debris and DNA were removed by centrifugation at 13,000 rpm for 15 minutes at 4 °C. Lysates were either used immediately or aliquotted and stored at -20 °C for short term storage or -80 °C for long term storage until used. All steps were carried out on ice to prevent degradation of proteins.

4.2.3.2 Protein quantification

For protein quantification Roti®-Nanoquant was used, which is based on the well known procedure described for protein quantification by Bradford (Bradford, 1976). Briefly, the triphenylemethan dye Commassie-Brilliant-Blue G-250 binds in an acidy environment the cationic and hydrophobic side chains of proteins. This results in the formation of a blue complex, which can be quantified photometrically. For quantitative measurements a calibration line was determined from a dilution series of the BSA protein. Protein lysates obtained as mentioned earlier were diluted 1:20 and 1:50. The BSA protein was used at a range between 0 and 250 µg/ml. 50 µl of sample lysate or BSA standard were pipetted into 96well plates and 1x Roti®-Nanoquant (diluted
in ddH$_2$O) was added. Intensity of the blue complex was measured at 590nm with a correction at 450nm using a photometer (µQuant, BioTek). Linearity was determined by calculating the ratio OD$_{590/450}$. All measurements were done in duplicates.

4.2.3.3 SDS Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE is used to separate denatured proteins according to their molecular weight. SDS is an anionic tenside, which covers the proteins natural charge, so that they run towards the positive pole during electrophoresis. LDS (Lithium dodecyl sulphate) can be used equivalently. Furthermore, the secondary and tertiary structure of the proteins is destroyed through disruption of disulfide- and hydrogen-bonds by addition of DTT and heating of the sample. NuPage® LDS sample buffer was added to protein samples (usually 30µg) to obtain a 1 fold concentration. NuPage sample reducing agent, containing DTT was added at 1 µl/10 µl final volume. After incubation at 95 °C for 10 minutes and a brief spin, samples (25 µl) were loaded onto NuPage® 4-12% Bis-Tris gels. Gels were run at 200V for 38 minutes in NuPage® MES running buffer including NuPage® antioxidant. Used electrophoresis chamber was Novex mini-Cell from Invitrogen.

4.2.3.4 Protein transfer (Western Blot)

Western blotting is the transfer of proteins, which were separated by SDS-PAGE, to a membrane by an electric field. The electric field has to be vertical to the SDS gel. Blotting was carried out onto a nitrocellulose membrane using the Western-Blot Module from Invitrogen (XCell II) together with NuPage® transfer buffer at 30V for 1h. To proof blotting efficiency transferred proteins on the membrane were stained with Ponceau-S-Red solution. Ponceau-S visualizes proteins by reversibly binding positively charged aminogroups. Washing with ddH$_2$O or PBS (1x) removes the dye completely.

4.2.3.5 Immunoblotting and development

Proteins can be visualized on the membrane using specific antibodies. To block unspecific binding sides the membranes were preincubated with blocking
solution I (PBS/Milk/Rotiblok) for one hour at room temperature, followed by one washing step with 1x PBS. Primary antibody incubation took place at 4 °C over night in the antibody-specific concentration and blocking buffer. Membranes were washed three times for 10 minutes with PBS (1x) or TBST (1x) according to blocking buffer. Subsequently, membranes were incubated with the appropriate secondary horseradish peroxidise-labeled (HRP) antibody in blocking buffer II for one hour at room temperature, followed by three 10 minute washing steps in PBS (1x). ECL-reagents I and II were mixed in equal amounts. Membranes were incubated in ECL-mix for 2 minutes, briefly freed from excessive liquid and put between two clean clear foils in a light protected film cassette. Kodak X-ray films were exposed to membranes between 10 seconds and 1 hour, dependent on antibody and protein load. Exposed films were developed using an automated photo developing machine (Kodak). Band sizes were estimated by comparison to known sizes of protein ladder. When loading was uneven as seen in variable band intensities for the loading control, bands were densitometrically quantified by image J v.1.1.1 and normalized to input (loading control).

4.2.3.6 Enzyme-linked immunosorbend assay (ELISA)

ELISA is a method for protein detection and quantification based on an enzymatic colour reaction. An antibody is coated to a solid phase, which is usually a 96well plate. When samples are added to the wells proteins specific to the coating antibody are bound and therefore retained in the well during subsequent washing steps. A second enzyme-linked antibody targeting the same antigen at a different epitope as the coating antibody is added. A substrate solution is added and colour develops in proportion to the initially bound amount of the protein of interest. After stopping of the colour development the colour intensity can be quantified densitometrically. For our experiment the VEGF-ELISA kit (DVE00, R&D Systems) was used as suggested by the manufacturers’ instructions.

4.2.3.7 Concentration of supernatants

Supernatants of cell culture were concentrated using Microcon centrifugal filter devices (Milipore) following the manufactures instruction. In brief, the super-
natant was carefully pipetted into the sample reservoir and centrifuged for 30 minutes at maximum speed. After centrifugation the filter vial was removed from the tube and placed upside down into a new tube. The concentrated supernatant was removed from filter by centrifugation for 3 minutes at 1000 g and used for determination of VEGF content by ELISA.

4.2.4 Flow Cytometry

4.2.4.1 Detection of surface markers

To test the purity of CLL cells after isolation using combined Rosette Sep® procedure and subsequent Ficoll-density centrifugation as described earlier, isolated cells were subject to flow cytometric analysis of CLL cell-specific surface markers. For that purpose isolated cells were incubated with anti-CD5-FITC (1:10), CD19-PerCy5.5 (1:10) and CD23-APC (1:50) for 30 minutes. After washing, cells were analysed by flow cytometry (FACS Canto). CD23 and CD19 are classical B-cell markers, while CD5 is as a T-cell marker generally absent on normal B-cells. Possibly during the process of tumorigenesis, CLL cells acquire this surface marker and can therefore easily be distinguished from healthy B-cells. Determined purity of CLL cells in our experiments after isolation from whole blood by Rosette Sep® procedure and Ficoll-density centrifugation was usually larger than 90%.

4.2.4.2 Intracellular phospho flow cytometry

To detect unphosphorylated and phosphorylated proteins, which are not present at the cell surface, intracellular staining techniques are necessary. For that purpose cells need to be permeabilized prior to antibody incubation. If the phosphorylation status of a protein needs to be determined it is useful to fix cells in addition to permeabilization. To ensure rapid fixation of the current phosphorylation status of the cells 4% formaldehyde was added to the cells in culture medium at equal volumes to reach a final concentration of 2% formaldehyde. Cells were incubated for 10 minutes at 37 °C in the incubator following two washing steps with 1x PBS, 0.5% FCS. Subsequently, cell pellets were suspended in 200 µl 1x PBS 0.5% FCS and 100% ice cold methanol was added drop wise while vortexing at low speed to a final concentration of 90%
methanol. Cells were then either stored at -20 °C for later use or incubated at -20 °C for 15 minutes, washed with PBS 0.5% FCS, followed by antibody incubation. Antibodies used for flow cytometry and their dilutions are listed in (Table 4). Cells were incubated with primary antibodies or appropriate isotype control for one hour, followed by two washing steps with 1x PBS, 0.5% FCS and secondary antibody incubation for 30 minutes. Preceding analysis on FACS Canto cells were washed twice with 1x PBS. In the case of directly labeled antibody incubation cells were incubated for one hour followed by two 1x PBS washing steps and subsequent analysis on FACS Canto. Appropriate directly labeled isotype controls were used accordingly.

4.2.4.3 Annexin V-FITC/PI staining

To analyse a cell population for survival, apoptosis and cell death a combined staining with fluorochrome-labeled Annexin V and propidium iodide (PI) can be used. Apoptosis is characterized by several distinct events such as loss of plasma membrane integrity, condensation of cytoplasm and the nucleus or internucleosomal DNA-cleavage. The loss of the plasma membrane integrity is one of the earliest features of apoptosis induction and is characterized by a translocation of phospholipid phosphatidylserine (PS) from the inner to the outer leaflet of the plasma membrane. The now exposed PSs can be bound by Annexin V, which has a high affinity to PSs. Annexin V is bound to a fluorochrome, in this study FITC, and can be detected by flow cytometry. As PS translocation is an early event of apoptosis induction, Annexin V positivity can be assigned a measure for early apoptosis induction. In contrast, permeability for PI can be considered as a sign for damaged or dead cells. Hence, cells which are Annexin V/PI double negative can be considered alive, Annexin V positive/PI negative cells are in early apoptosis, whereas Annexin V/PI double positive cells can be considered dead (compare Figure 23).

Cells to be analysed were washed twice with cold 1x PBS. Approximately 1x10^6 cells were dissolved in 100 µl 1x binding buffer and 2 µl Annexin V-FITC antibody and 2 µl (100 µg/ml) PI solution were added. Binding buffer was prepared by diluting a 10x stock solution with distilled water. Cells were incubated for 20 minutes at room temperature protected from light. 300 µl 1x binding buffer was added and cells were analysed on FACS Canto.
4.2.5 Statistics

4.2.5.1 Standard error

The analysis of measured data was calculated as a mean of a minimum of three independent experiments including the associated standard error of the mean (SEM). The SEM is dependent on the standard deviation, which is a measure of the spread of a distribution. The standard deviation \( s \) is the square root of the variance and can be calculated as follows:

\[
\sigma = \sqrt{\frac{\sum(x_i - m)^2}{n - 1}}
\]

with \( x_i = \) single score, \( m = \) mean and \( n = \) score number.

The standard error of the mean is designated as \( \sigma_m \). It is the standard deviation of the sampling distribution of the mean. The formula for the standard error of the mean is:

\[
\sigma_m = \frac{\sigma}{\sqrt{n}}
\]

with \( s = \) standard deviation, \( n = \) score number.

Standard error of the mean (SEM) represents the accuracy of the mean, whereas the standard deviation rather reflects the variability of single observations.

4.2.5.2 Statistical significances

Statistical significance describes how likely it is that a result occurred by chance in a study population as a sample of a total population. The most commonly used test of significance is the student’s t-test, where the statistics follow a student’s t-distribution if the null hypothesis is true. The student’s t-distribution is a generalized hyperbolic distribution and describes a probability distribution that results from the problem which comes up when the mean of a normally distributed population with a small sample size is being estimated. The null
hypothesis is the assertion that your results are not related and your results are the product of random chance events and is often the reverse of what the experimenter actually believes. The null hypothesis it is put forward to allow the data to contradict it. Student’s t-test can either be unpaired or paired, dependent on whether samples of the different data sets being tested corresponding to each other or are independent. Furthermore, the t-test can be one-tailed or two-tailed. A one-tailed test is used for the case that the expected results are thought to be different in only one direction, e.g. are higher or lower, but not just different so either higher or lower, which means the interest is only on one side of the probability distribution. Two-tailed t-tests are frequently used and consider deviances towards either direction. All statistical calculations done in this work were two-tailed t-tests. Whether they were unpaired or paired is specifically indicated. Significance calculations were carried out using Graph Pad Prism Software 4.0
References


Ehrenwörtliche Erklärung


Köln, den 07. April 2010

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