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AUTOCRINE AND PARACRINE GROWTH MECHANISMS IN HUMAN B LYMPHOCYTES

by

Clare Louise Bradshaw

This thesis is submitted in part fulfilment of the degree of Doctor of Philosophy at the University of Glasgow

Division of Biochemistry and Molecular Biology
Institute of Biomedical and Life Sciences
Faculty of Science
University of Glasgow

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To my Mum and Dad,
and the loving memory of my Grandma
SYNOPSIS

The SMS-SB cell line is an acute lymphoblastic leukaemia cell line with pre-B lymphocyte-like characteristics. Once removed from the patient, the cells could be grown in the absence of all exogenous mitogens, and were density-dependent for growth thus demonstrating the synthesis and secretion of an autocrine growth factor. Previous studies have found that this autocrine factor (SB-AF) can rescue SMS-SB cells from death, but have not been able to identify a cytokine able to substitute for its activity.

The original aim of this thesis was to identify and characterise SB-AF and determine its anti-apoptotic mechanism. SB-AF was found to sustain the expression levels of the anti-apoptotic protein Bcl-2 and promote cell cycle progression. In addition, SB-AF seems to be a multi-component factor, whose anti-apoptotic activity results from the synergistic action of all its components. During these investigations, SMS-SB cells were found to express high levels of Bcl-2, which is unusual for lymphocytes at the pre-B developmental stage.

In addition to the autocrine factor, previous work has demonstrated that soluble CD23 (sCD23) can act in a paracrine fashion to rescue low cell density cultures of SMS-SB cells from apoptotic death. This pleiotropic cytokine is a cleavage product of the 45kDa type II transmembrane CD23 antigen. As SMS-SB cells do not express the known receptors for CD23, namely, CD21, CD11b, and CD11c, the anti-apoptotic signals of sCD23 are mediated by a novel CD23 receptor.

The work in this thesis shows that unlike sCD23, 45kDa membrane-associated CD23 does not seem to elicit the same response in SMS-SB cells, thus suggesting that only the soluble forms of CD23 can mediate anti-apoptotic effects via the novel receptor. However, the monolayer cell system used to present 45kDa CD23 appeared to be affecting SMS-SB cultures possibly influencing their response to CD23.

Investigations were also undertaken to determine whether the novel receptor for CD23 was unique to the SMS-SB cell line. Preliminary data obtained from BIAcore surface
plasmon resonance technology, demonstrate that the leukaemic pre-B cell lines Blin-1 and Nalm-6, and the mature B cell line 1E8, are able to bind sCD23 in the absence of the known CD23 receptors. Therefore, in addition to SMS-SB these cell lines also express a novel CD23 receptor. Further studies are required to determine whether all the cell lines express the same molecular receptor species, and whether signalling via these receptor(s) can prevent the apoptosis of Blin-1, Nalm-6 and 1E8 cells.

The discovery of a novel CD23 binding receptor on a mature B cell line implies that, in addition to potentially having a role in precursor B cell development, sCD23 may also influence the growth and survival of more mature cell types away from the bone marrow microenvironment. It will therefore be interesting to investigate the role of CD23, and its novel receptor, throughout all stages of B cell development, and ultimately in all haematopoietic cells.
TABLE OF CONTENTS

Synopsis ii
List of Figures x
List of Tables xi
Acknowledgements xii
Abbreviations xiii

CHAPTER 1: INTRODUCTION 1
1.1 THE IMMUNE SYSTEM 2
1.2 B CELL DEVELOPMENT 3
1.3 REGULATION OF PRECURSOR B CELL DEVELOPMENT BY STROMAL CELLS 6
   1.3.1 Role of cell-cell contacts 7
   1.3.2 Role of cytokines 9
1.4 AUTOCRINE GROWTH REGULATION 12
1.5 CD23-ADHESION MOLECULE AND CYTOKINE 15
   1.5.1 Discovery and general distribution of CD23 15
   1.5.2 Structure of CD23 15
   1.5.3 Murine CD23 19
   1.5.4 Regulation of CD23 expression 19
   1.5.5a CD21 21
   1.5.5b CD11b/CD11c 22
   1.5.6 Function of membrane CD23 with respect to B cells 23
   1.5.7 Function of sCD23 with respect to B cells 26
   1.5.8 CD23 and bone marrow haematopoiesis 29
   1.5.9 CD23 and disease 30
1.6 APOPTOSIS/PROGRAMMED CELL DEATH 31
   1.6.1 The morphology of apoptosis 32
   1.6.2 The role of apoptosis in B cell development 33
   1.6.3 The basic apoptotic machinery 34
2.1.6 Antibodies
2.1.7 CD23 cytokine
2.1.8 Plasmids
2.19 cDNA probes for Northern blotting

2.2 METHODS

2.2.1 Cell culture
2.2.1a Culture of Human B lymphocytes
2.2.1b Culture of non-B cell lines
2.2.1c Frozen cell stocks
2.2.1d Detection of mycoplasma
2.2.1e Determination of cell number and viability of B cell lines
2.2.1f Preparation of Conditioned medium (CM)
2.2.2 Transfection of mammalian cells
2.2.2a Transfection of human B cell suspension cultures
2.2.2b Transfection of non-human cell lines
2.2.3 Flow cytometry
2.2.3a Analysis of cell cycle and intracellular proteins
2.2.3b Cell phenotyping
2.2.4 Flow cytometric cell sorting
2.2.5 Conditioned medium manipulation
2.2.5a CM activity assay (recovery of LCD SMS-SB cells from apoptosis)
2.2.5b Enzymatic and heat treatment of conditioned medium
2.2.5c Lentil Lectin Chromatography
2.2.5d G25 Chromatography
2.2.6 Proliferation assay
2.2.7 Isolation and preparation of DNA
2.2.7a Small scale preparation of DNA (mini-preps)
2.2.7b Large scale preparation of DNA (maxi-preps)
2.2.8 Basic manipulation of DNA
2.2.8a Restriction endonuclease DNA digestion
2.2.8b Agarose gel electrophoresis
2.2.8c DNA fragment purification
2.2.8d Ligation of plasmid vector and insert DNA
2.2.9  Transformation of DNA into E. coli
2.2.9a Preparation of competent cells (rubidium chloride method)
2.2.9b Transformation of competent cells
2.2.9c Analysis of transformants
2.2.10 Polymerase Chain Reaction (PCR)
2.2.10a Oligonucleotide design and preparation
2.2.10b PCR reaction
2.2.10c Blunt ended cloning of 37kDa and 25kDa CD23 forms
2.2.11 Nucleotide sequence analysis
2.2.12 RNA manipulation
2.2.12a SMS-SB cell stimulation
2.2.12b Isolation of RNA from SMS-SB cells
2.2.12c Northern blotting
2.2.12d Radiolabelling of cDNA probes
2.2.12e Hybridisation with $^{32}$P-labelled cDNA
2.2.13 BIAcore analysis of the novel receptor for CD23
2.2.13a Preparation of cell membrane extracts
2.2.13b Surface plasmon resonance measurements
2.2.14 Immunocytochemistry
2.2.15 Western blotting
2.2.15a Preparation of cell extracts
2.2.15b SDS-PAGE electrophoresis and Western blotting
2.2.15c ECL detection system

CHAPTER 3: RESULTS

Studies of an anti-apoptotic autocrine factor produced by SMS-SB cells

3.1 INTRODUCTION

3.2 RESULTS

3.2.1 SMS-SB cells produce an autocrine growth factor

3.2.2 SMS-SB cells express Bcl-2
3.2.3 SMS-SB autocrine factor sustains Bcl-2 expression and promotes cell cycle progression

3.2.4 SMS-SB cells express a number of Bcl-2 family members

3.2.5 SMS-SB autocrine factor has an essential protein component that is heat stable to 90°C

3.2.6 SMS-SB autocrine factor is a multi-component factor

3.3 DISCUSSION

CHAPTER 4: RESULTS

Analysis of the effects of 45kDa membrane-associated CD23 on SMS-SB cells

4.1 INTRODUCTION

4.2 RESULTS

4.2.1 The effect of 45kDa CD23a transiently expressed in SMS-SB cells

4.2.2 Generation and cloning of CD23 constructs

4.2.2a PCR amplification of the CD23 constructs

4.2.2b Cloning the CD23 constructs

4.2.2c Sequence analysis of the CD23 constructs

4.2.3 Stable transfection of 45kDa CD23a into SMS-SB cells

4.2.4 The effect of 45kDa CD23a when presented to SMS-SB cells from monolayer cell cultures

4.2.4a Generation of the CD23a expressing ‘feeder’ cells

4.2.4b SMS-SB cells bind to CD23a COS7 cells

4.2.4c 45kDa CD23a on COS7 cells does not promote SMS-SB cell proliferation at low cell density

4.2.4d 45kDa CD23a on COS7 cells does not specifically induce changes in the expression of the intermediate early genes c-fos, c-jun, or c-myc in SMS-SB cells

4.3 DISCUSSION
### List of Figures

1.1  A schematic representation of Ag-independent B cell development  
     
1.2  Linear model of the CD23 antigen  
     
1.3  Members of the Bcl-2 family  

3.1  $[^3]H$-Thymidine incorporation by SMS-SB cells cultures at various 
     densities in the presence and absence of conditioned medium  
3.2  Flow cytometric analysis of Bcl-2 expression in SMS-SB cells by 
     specific antibody staining  
3.3  Flow cytometric analysis of Bcl-2 expression and cell cycle status 
     in SMS-SB cells  
3.4  Effect of conditioned medium on Bcl-2 expression and cell cycle 
     status of intermediate density SMS-SB cultures  
3.5  Effect of conditioned medium on Bcl-2 expression and cell cycle 
     status in low cell density cultures  
3.6  Flow cytometric histogram analysis of Bcl-2 expression in low 
     cell density SMS-SB cultures in the presence and absence of 
     conditioned medium  
3.7  Western blot analysis of specific Bcl-2 family members in 
     SMS-SB cells  
3.8  The effects of enzymatic and heat treatment on conditioned 
     medium activity  
3.9  Lentil Lectin Chromatography  
3.10 G25 Gel Filtration Chromatography  

4.1  Photomicrographs of SMS-SB cells transiently-transfected 
     with CD23a  
4.2  Flow cytometric analysis of CD23a transfected SMS-SB cells  
4.3  Diagramatic representation of the different CD23 forms and 
     the oligonucleotide primers designed for their PCR generation  

X
4.4 Gel electrophoresis showing the PCR amplification of the CD23 forms

4.5 Gel electrophoresis showing the CD23 forms excised from their cloning vectors

4.6 The nucleotide sequence and the deduced amino acids of the cloned CD23 constructs

4.7 Analysis of 45kDa CD23a-transfected COS7 cells

4.8 Analysis of 45kDa CD23a expression in transfected COS7 cells by immunocytochemistry

4.9 Effect of 45kDa CD23a on $[^3]H$-thymidine incorporation by SMS-SB cells at various densities

4.10 Northern blot analysis of the expression of c-fos, c-jun and c-myc in SMS-SB cells, upon stimulation with CD23a expressed on COS7 cells

5.1 Flow cytometric analysis of Blin-1 cells

5.2 Flow cytometric analysis of 207 cells

5.3 BIAcore analysis of SMS-SB cells

5.4 BIAcore analysis of Blin-1, Nalm-6 and 1E8 cell lines

List of tables

Table 1 B cell autocrine factors

Table 2 Statistical analysis of the effect of conditioned media on cell cycle status in low cell density cultures

Table 3 Flow cytometric analysis of various B cell lines

Table 4 Binding of various B cell lines to 45kDa CD23a expressed on COS7 cells

Table 5 Binding responses and kinetics of BIAcore interaction
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DECLARATION

The work described in this thesis was performed personally unless otherwise stated.
<table>
<thead>
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<tr>
<td>Ab</td>
<td>antibody</td>
</tr>
<tr>
<td>Ag</td>
<td>antigen</td>
</tr>
<tr>
<td>AIF</td>
<td>apoptosis inducing factor</td>
</tr>
<tr>
<td>ALL</td>
<td>acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>Apaf</td>
<td>apoptotic protease activating factor</td>
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<tr>
<td>B-ALL</td>
<td>B cell acute lymphoblastic leukaemia</td>
</tr>
<tr>
<td>BCGF</td>
<td>B cell growth factor</td>
</tr>
<tr>
<td>B-CLL</td>
<td>B cell chronic lymphocytic leukaemia</td>
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<tr>
<td>BH</td>
<td>Bcl-2 homology domain</td>
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<tr>
<td>BM</td>
<td>bone marrow</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>°C</td>
<td>degrees celsius</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CD23NR</td>
<td>novel receptor for CD23</td>
</tr>
<tr>
<td>CLL</td>
<td>chronic lymphocytic leukaemia</td>
</tr>
<tr>
<td>CM</td>
<td>conditioned medium</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>dH₂O</td>
<td>distilled water</td>
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<tr>
<td>EBV</td>
<td>Epstein-Barr Virus</td>
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<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>ECM</td>
<td>extracellular matrix</td>
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<tr>
<td>FcR</td>
<td>receptor for the Fc region of the Ig molecule</td>
</tr>
<tr>
<td>FCS</td>
<td>foetal calf serum</td>
</tr>
<tr>
<td>FDC</td>
<td>follicular dendritic cell</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein-isothiocyanate</td>
</tr>
<tr>
<td>g</td>
<td>relative centrifugal force</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>HCl</td>
<td>hydrochloric acid</td>
</tr>
<tr>
<td>HRP</td>
<td>horse-radish peroxidase</td>
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HSC  haematopoietic stem cell
Igμ  heavy chain
ICD  intermediate cell density
IEG  immediate early genes
IFN  interferon
Ig  immunoglobulin
IgE-IC  IgE immune complex
IL  interleukin
kₐ  association constant
kb  kilobase
kₐd  dissociation constant
Kₐp  equilibrium constant
kDa  kilodalton
LB  Luria-Bertani
LCD  low cell density
LTBMC  long-term bone marrow cultures
NCD  normal cell density
OGP  n-Octyl-β-D-glucopyranoside
PARP  poly (adenosine diphosphate-ribose) polymerase
PBS  phosphate buffered saline
PCD  programmed cell death
PCR  Polymerase Chain Reaction
PDGF  platelet-derived growth factor
PFHMII  Protein-Free Hybridoma Medium-II
Ph¹  Philadelphia chromosome
PI  propidium iodide
PI3-K  phosphatidylinositol-3-kinase
PKC  protein kinase C
Rb  retinoblastoma
RNA  ribonucleic acid
RU  resonance units
SB-AF  SMS-SB cell autocrine growth factor
sCD23  soluble CD23
<table>
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<th>Full Form</th>
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<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>SSV</td>
<td>Simian Sarcoma Virus</td>
</tr>
<tr>
<td>TdT</td>
<td>terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>[^3\text{H}]-TdR</td>
<td>tritiated thymidine</td>
</tr>
<tr>
<td>TGF-(\beta)</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TNF</td>
<td>tumour-necrosis factor</td>
</tr>
<tr>
<td>VnR</td>
<td>vitronectin receptor</td>
</tr>
<tr>
<td>v/v</td>
<td>volume by volume</td>
</tr>
<tr>
<td>w/v</td>
<td>weight by volume</td>
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\(\Delta \Psi_m\) mitochondrial transmembrane potential
CHAPTER 1

INTRODUCTION
1.1 THE IMMUNE SYSTEM

The immune system is a highly complex adaptable defence system that has evolved in vertebrates to protect them from invading pathogenic microorganisms. It generates an enormous variety of cells and molecules that specifically recognise non-self molecules and co-operate to efficiently eliminate the foreign particle. There are two main types of immune response; innate (or non-adaptive) and adaptive. The former is present prior to infection with a foreign particle and is mainly mediated by phagocytic cells, such as monocytes and macrophages, and the complement serum proteins. The opsonisation (coating) of microorganisms with complement aids in the non-specific recognition, internalisation and elimination of the infectious agent by the phagocytes. In comparison, the adaptive immune response is induced/stimulated by exposure to foreign substances, is highly specific, and improves with each successive encounter with the same foreign.

Central to all adaptive immune responses are the T and B lymphocytes. T cells are the mediators of cellular immunity and play both cytotoxic and helper roles in the destruction of an infected cell. They are activated by the specific recognition of foreign antigen on antigen presenting cells. B cells are activated by the binding of specific antigen to their membrane immunoglobulin (Ig) receptor, and by receiving signals from activated T cells in the form of secreted cytokines and cell-cell contacts. If all these signals are received simultaneously, the B cell can develop into a plasma cell which secretes antibodies, the mediators of humoral immunity.

Antibodies are a soluble form of the B cell antigen receptor and thus bind to specific antigens. The effects of antibodies include the neutralisation of soluble antigens, the activation of the complement system, the opsonisation of particles to enhance the efficiency of phagocytosis, and antibody-dependent cell-mediated cytotoxicity (ADCC). In addition to producing antibodies, B cells play another important roles in the immune response by acting as antigen presenting cells, and as memory B cells they are the basis of immunological memory.
This thesis is an investigation into the mechanisms of growth and survival of the pre-B acute lymphoblastic leukaemia cell line, SMS-SB, by autocrine and CD23-mediated paracrine mechanisms. These concepts will therefore be discussed in this introduction, namely; the regulation of precursor B cell development, the function of the CD23 molecule and cytokine, the control of programmed cell death, and the contribution of deregulated cell death to malignancies. Finally, the characteristics of the SMS-SB cell line will be discussed.

1.2 B CELL DEVELOPMENT

B lymphocytes, like other haematopoietic cells, are continuously produced in the intramedullary cavity of the bone marrow (BM) of adult mammals. They are descendants of pluripotent haematopoietic stem cells (HSC), which express CD34, but lack all known lineage-specific markers (Andrews et al., 1990). The HSC is believed to initially differentiate into either a lymphoid or myeloid stem cell, an idea supported by Galy and colleagues, who demonstrated that a CD34+lineage-/B220-/CD10+ bone marrow cell could only give rise to T, B, natural killer, and dendritic cells but not to cells of either the erythroid or myeloid lineages (Galy et al., 1995). In the BM microenvironment, haematopoietic cell development occurs on a meshwork of stromal cells, which include fibroblasts, adipocytes, macrophages, and endothelial cells. This structure is essential for haematopoiesis through the production of cytokines and direct cell-cell contact with the developing cells (reviewed by LeBein, 1998).

Development within the BM is antigen-independent and proceeds with a series of immunoglobulin (Ig) gene re-arrangements. During this process (termed VDJ recombination), the genes encoding the antibody variable regions are assembled from gene segments encoding V (variable), D (diversity), and J (joining) regions for the Ig heavy chain, and from V and J regions for the Ig light chain. The complex process of VDJ recombination will not be discussed here in detail, but has recently been reviewed by Grawunder et al., 1998, and Papavasilious et al., 1997. All subsequent development is antigen-dependent and occurs in the secondary lymphoid organs, such as the lymph node, where immature IgM+ cells develop into mature IgM+/IgD+ B cells,
which may be activated to become Ab-producing plasma cells or memory B cells (reviewed by Liu et al., 1997b). Only precursor B cell, and therefore antigen-independent B cell development will be discussed here in detail.

Over the last 13 years, two major phenotypic models of murine antigen-independent B cell development have emerged. The first, formulated by Osmond and colleagues, was based on the sequential expression of B-lineage-related molecules, including various CD antigens (reviewed in Osmond et al., 1994). In the second model, Melchers and Rolink compartmentalised developmental stages based on the changing status of Ig-gene rearrangements (reviewed in Melchers et al., 1995; ten Boekel et al., 1995). Recently, these researchers have recognised the consistency between their two models and have combined their findings to produce a unified model of B cell development in the bone marrow (Osmond et al., 1998). In addition, the development of new techniques and reagents has demonstrated, that contradictory to previous findings (reviewed by Billips et al., 1995a), human B cell development is very similar to that in the mouse (Ghia et al., 1998; LeBien, 1998).

The first identifiable stage of B cell development is the pro-B cell (see figure 1.1). Based on the expression of terminal deoxynucleotidyl transferase (TdT), an enzyme which adds nucleotides at the VDJ joint sites, and tyrosine phosphatase B220 (CD45RA), these cells have been subdivided into early pro-B (TdT− B220+), intermediate pro-B (TdT+ B220+), and late pro-B (TdT+ B220−) (Osmond et al., 1994). Examination of the Ig genes of these precursor cells revealed them to be either in the germline configuration, or as having DJH rearrangements. The latter population of cells were designated as pre-BI cells by Melchers and Rolink and correspond to the late pro-B cell of the Osmond model.

The next developmental stage, the pre-B (Osmond), or the pre-BII cell (Melchers and Rolink), was initially identified by Raff and colleagues as a cell displaying cytoplasmic IgHμ, but no light chains or surface IgM (Raff et al., 1976). These cells can be sub-divided into large mitotically active cells and their progeny, small non-dividing pre-B cells. Both of these cells have fully rearranged VDJ heavy chain genes, with the small pre-B cell additionally exhibiting VJ light chain rearrangements but no
Figure 1.1 A schematic representation of Ag-independent B cell development.
The surface expression of IgM. At the large pre-B cell stage, the rearranged heavy chain is found at the cell surface covalently associated with surrogate light chain (ΨL) proteins. These proteins are encoded by the VpreB and λ5 (mouse), or 14.1 (human) genes during the pro-B stage of development. The Hμ and ΨL chains associate with Igα and Igβ to form the pre-B receptor complex (reviewed by Lassoued et al., 1996). The importance of this receptor is demonstrated in λ5-deficient mice, who are unable to generate pre-BII cells (Kitamura et al., 1992). Signalling through the receptor complex is believed to select cells with appropriately rearranged heavy chain genes, prevent further heavy chain rearrangement, and promote the subsequent differentiation of the pre-B cell (Karasuyama et al., 1997).

The final B cell stage to be identified within the BM is that of the immature B cell, characterised by the surface expression of IgM. These cells exit the BM and migrate to the periphery where they develop into IgM^IgD^ mature B cells.

### 1.3 REGULATION OF PRECURSOR B CELL DEVELOPMENT BY STROMAL CELLS

As previously mentioned, B lymphopoiesis is regulated by the stromal cells of the bone marrow via cell-cell contacts and the production of cytokines. As these complex interactions are difficult to study in vivo, long-term bone marrow cultures (LTBMC) have been developed as a way of mimicking the bone marrow environment. A LTBMC system for B lymphocytes was initially developed by Whitlock and Witte in 1982. Since then, a number of stromal cell lines have been obtained from LTBMC and have been extensively used to analyse the regulation of haematopoietic development (reviewed by Deryugina and Muller-Sieburg, 1993). These lines have been found to exhibit heterogeneity of functions with respect to the stage of B cell development they can support. For example, the S17 line can only support the maturation of pro-B to pre-B cells, whereas the S10 cell line supports pre-B to immature B cell maturation, but cannot regulate earlier stages of differentiation (Henderson et al., 1990). Witte and colleagues demonstrated functional differences with regard to interleukin-7 (IL-7)
production in primary cultured stromal cells, thus supporting the notion that the bone marrow may consist of distinct populations of stromal cells (Witte et al., 1993). Even though the stromal cell lines used in vitro may not accurately reflect the bone marrow microenvironment, they have helped to identify a number of important regulatory factors involved in precursor B cell development.

1.3.1 Role of cell-cell contacts

Developing B lymphocyte precursors have been found in intimate contact with the stromal cells in the bone marrow (Jacobsen et al., 1990). In vitro studies have revealed that these direct cell-cell or cell-extracellular matrix (ECM) interactions are required for both murine and human B lymphopoiesis to occur (Kierney et al., 1987; Villablanca et al., 1990). These interactions are responsible for the localisation of precursors in the bone marrow and, like cytokine receptors, play an important role in the regulation of progenitor proliferation. Adhesion within the bone marrow is developmentally regulated, since interactions decline during the progressive stages of human B cell maturation (Ryan et al., 1990).

The integrin family of adhesion receptors are believed to have a role in precursor B cell development. This family are responsible for adhesion to ECM components (e.g., fibronectin, collagen and laminin), and to cell surface expressed Cell Adhesion Molecules (CAM's) (reviewed by Springer, 1990). Direct cell contact is mediated by the β1 integrin family member VLA-4 (α4β1) expressed on the surface of pre-B cells and VCAM-1 (vascular CAM), expressed on stromal cells (Miyake et al., 1991a). Antibodies to either of these two molecules suppress B cell lymphopoiesis in some culture systems (Miyake et al., 1991b; Ryan et al., 1992). VLA-4 also binds fibronectin at a site distinct from the VCAM-1 binding site, further demonstrating how VLA-4 may mediate adhesions within the bone marrow (Elcés et al., 1990).

In vivo studies have identified a role for the β1 integrins in the retention of progenitors in the BM and for the homing of progenitors to the BM after BM transplantation (Williams et al., 1991; Papayannopoulou et al., 1995). However, the β1 integrins are expressed on many cell types and so cannot account for the exclusive progenitor-
marrow interactions. Another receptor(s) must be responsible for the specific interactions of HSC and their immediate progeny with components of the bone marrow. A recent study by Zannettino et al., 1998, has identified the sialomucin CD164 as a potential candidate receptor for mediating this specific adhesion as a number of splice variants of CD164 were found to exist, some of which are not expressed on haematopoietic cells (Zannettino et al., 1998).

In addition to retaining precursor B cells within the bone marrow, adhesion receptors also transmit signals that affect proliferation and survival of the precursors. For example, Borghesi and colleagues have demonstrated that direct contact with stromal cells prevents the spontaneous cell death of pre-B lymphocytes. Interestingly, antibodies to VCAM-1 did not interfere with the protective effect of the stromal cells, demonstrating that this pair of adhesion molecules does not participate in the delivery of survival signals to these B lymphocytes (Borghesi et al., 1997). However, a recent study has demonstrated the importance of the VLA-4/VCAM-1 interaction for the survival of HSC and B cell precursors up to the pre-B1 stage of development (Wang et al., 1998).

The effect of stromal cells on precursor cell proliferation seems to be dependent on the presence of cytokines. When integrins are engaged on haematopoietic progenitors cultured under physiological concentrations of cytokines, precursor proliferation is inhibited (Hurley et al., 1995, 1997). In contrast, when progenitors are cultured with pharmacological concentrations of cytokines, engagement of integrins results in the recruitment of cells into the cell cycle (Levesque et al., 1996).

In addition to receiving signals from stromal cells, B cell precursors can also signal the stromal cells through cell contact to release cytokines such as IL-6 (Jarvis and LeBein, 1995), and IL-7 (Stephan et al., 1998). The B cell precursors therefore are able to influence their own fate by controlling cytokine production from the stromal cells.

Although the exact role of cell-cell and cell-ECM interactions occurring within the bone marrow is not known, they are however important to precursor B cell
development. Understanding how precursor B cells modulate and are affected by adhesions has relevance to the spread of leukaemic cells out of the bone marrow. Aberrant adhesive interactions caused by decreased function or expression of adhesion receptors may underlie the premature mobilisation of progenitors into the blood. In addition, these defects may participate in the deregulated proliferation and differentiation seen in leukaemic transformation.

1.3.2 Role of cytokines

A considerable amount of research has been performed to assess the role of the IL-7 cytokine in B cell development. IL-7 was initially identified as a 25kDa soluble growth factor capable of stimulating the proliferation of murine B cell precursors in vitro (Namen et al., 1988a), and was the first cytokine to be identified and cloned from a stromal cell line (Namen et al., 1988b). The importance of IL-7 was initially demonstrated by the absence of pre-B cells in mice injected with antibodies to either IL-7 or its receptor (IL-7R) (Grabstein et al., 1993; Sudo et al., 1993). Subsequent studies, using IL-7 (von Freeden-Jeffry et al., 1995), and IL-7R gene deficient mice (Peschon et al., 1994), confirmed that IL-7 mediated signals were essential for the pro-B to pre-B transition. The administration of IL-7 to normal mice has been shown to increase the number of pre-B cells, which subsequently differentiate into mature sIgM+/sIgD+ B cells (Morrissey et al., 1991).

In addition to inducing proliferation, Corcoran and colleagues have demonstrated that IL-7 is also involved in B lymphocyte differentiation. The researchers found that a mutation (Tyr449→Phe), in the α-chain component of the IL-7 receptor which prevented its binding to phosphatidylinositol-3-kinase (PI3-K), abrogated precursor B cell proliferation, but still permitted the rearrangement and expression of cytoplasmic Hμ chain. This demonstrated that the IL-7/IL-7R interaction actively regulates Ig gene rearrangement, and does not simply enable proliferation of cells undergoing rearrangement (Corcoran et al., 1996). In support of these initial findings, the researchers have subsequently reported impaired Ig H chain gene rearrangements in mice lacking the IL-7 receptor (Corcoran et al., 1998). A recent study by Stephan and colleagues has demonstrated that the gradual reduction of B cell lymphopoiesis in
ageing mice is related to the impaired release, rather than production, of IL-7 from BM stromal cells. They also found that IL-7 was not continually expressed, and contact between the lymphoid and the stromal cells was required for proliferation in both young and old animals. Based on these findings, the researchers suggest that B cells can influence their own fate by signalling the stromal cells through cell contact to release IL-7 into the local microenvironment. Stromal cells may therefore regulate B lymphopoiesis by limiting the amount of IL-7 available to developing precursors (Stephan et al., 1998).

Although IL-7 can support the pro-B to pre-B transition, it is not required for the subsequent maturation to sIgM⁺ immature B cells (Cumano et al., 1990). Late pre-BII and immature B cells do not detectably express the IL-7R, thus losing their responsiveness to IL-7 (Henderson et al., 1992). However, it has been demonstrated that the presence of IL-7 at the earlier pre-BI stage, inhibits any further maturation by preventing Ig light chain rearrangement, and thus subsequent IgM expression. Subsequent removal of IL-7 allows maturation to continue (Rolink et al., 1993). It was recently shown that IL-7-induced inhibition of maturation can be overcome by the expression of a transgenic B cell receptor in B cell precursors (Melamed et al., 1997). The inhibitory effect of IL-7 also explains why later stages of differentiation within the bone marrow are not dependent on stromal cell contact, the cells that actually produce the IL-7 cytokine (Ryan et al., 1990).

In comparison to the mouse, IL-7 has been found to be non-essential for human B cell development (Prieyl and LeBien, 1996). However, IL-7 has been shown to down-regulate the expression of RAG-1/RAG-2 and TdT, which are involved in the recombination and formation of Ig chains, suggesting that this cytokine may modulate Ig receptor diversification (Billips et al., 1995b). Although not essential to the development of an individual cell, receptor diversification is important for the development of a highly effective immune response.

A number of synergistic factors, such as stem cell factor (Funk et al., 1993), flt3-ligand (Hirayama et al., 1995; Namikawa et al., 1996), and insulin-like growth factor-1 (IGF-1) (Landreth et al., 1992), have also been found to act on B cell precursors, but
only in combination with IL-7. Other factors, such as pre-B cell stimulating factor (PBSF), can stimulate pre-B cell proliferation, both by itself and in synergy with IL-7 (Nagesawa et al., 1994). Recent studies have demonstrated that IL-7 can form a heterodimeric complex with a 30kDa cofactor that selectively stimulates the proliferation and differentiation of pre-pro-B cells (i.e., early pro-B cells), and ‘primed’ them to proliferate in response to monomeric IL-7 (McKenna et al., 1998; Lai et al., 1998). In pro-B cell cultures, IL-7 was found to exist almost entirely in the heterodimeric complex (termed pre-pro-B cell stimulating factor (PPBSF)), whereas in pre-B cell cultures it was found in monomeric form. The exact role and importance of this complex in vivo has yet to be determined but the development of pro-B cells in IL-7 gene deficient mice (von Freeden-Jeffry et al., 1995), suggests that PPBSF either is non-essential to development or compensatory mechanisms exist (Lai et al., 1998).

In contrast to the reported positive effects, some cytokines produced from stromal cells have been found to negatively modulate lymphopoiesis, for example: IL-1α (Hirayama et al., 1994; Fauteux et al., 1996), IL-3 (Hirayama et al., 1994), IL-4 (Pandrau et al., 1992), and IFNs α/β (Wang et al., 1995). The inhibitory effect mediated by TGF-β has recently been found to be due, at least in part, to its ability to down-regulate IL-7 secretion from stromal cells (Tang et al., 1997). In addition to inhibiting B cell lymphopoiesis, IL-1α, IL-4 and TNF-α have been found to simultaneously induce the release of myeloid growth factors, demonstrating how the balance between lymphopoiesis and myelopoiesis in the BM might be regulated (Ryan et al., 1994a).

In addition to the local effects of cytokines, systemic hormones have also been implicated as negative regulators of B cell development within the BM. A reduction in pre-B cell levels have been observed during pregnancy, suggesting a role for sex steroids in the negative regulation of B cell development (Medina et al., 1993). Subsequent studies have demonstrated that oestrogen is the hormone responsible for this negative regulation (Medina et al., 1994; Smithson et al., 1995).

Overall the array of positive and negative factors, presented by the BM microenvironment in soluble, membrane-bound or matrix-bound form act in concert
to determine the progression of precursor B cells through their sequential stages of
development.

1.4 AUTOCRINE GROWTH REGULATION

In addition to receiving growth regulatory signals in a paracrine fashion from bone
marrow stroma or other cell types, B lymphocytes also respond to autocrine growth
stimulation, whereby cells both release and respond to a growth promoting factor. This
mechanism of self regulation is believed to be an important factor involved in
tumorigenic transformation, as constitutive secretion of autocrine factors may allow
cells to escape normal paracrine control (Sporn and Roberts, 1985; Lang and Burgess,
1990).

A number of cytokines, some of which are known to be involved in paracrine control
of growth and differentiation of B lymphocytes, have been identified as autocrine
factors (summarised in table 1). The majority of studies have been carried out with
either EBV-transformed, myeloma, or chronic lymphocytic leukaemia (CLL) B cells.
The latter is a malignancy of relatively mature B cells and is characterised by the
accumulation of slowly dividing B lymphocytes in the peripheral blood (reviewed by
Dighiero and Binet, 1996). At present, very little evidence exists to demonstrate the
role of autocrine factors in early B cell development.

In addition to acting independently, some cytokines have been found to act
synergistically as autocrine growth factors. For example, it was reported that IL-1, IL-
6, TNF-α and TNF-β are all required for the continued growth of immortalised human
B cell lines (Abken et al., 1992). With this in mind, the identification of autocrine
factors can be very difficult.

B cell growth factors have also been found to be produced by normal B cells,
demonstrating that autocrine growth regulation is not a property specific to malignant
transformation (Gordon et al., 1985; Jurgensen et al., 1986; Muraguchi et al., 1986;
Gordon and Cairns, 1991). However, cellular activation of normal B lymphocytes is
Table 1.1 B cell autocrine factors

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Cell type</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1</td>
<td>-EBV-transformed</td>
<td>Scala et al., 1984</td>
</tr>
<tr>
<td></td>
<td>-ALL</td>
<td>Mori et al., 1994</td>
</tr>
<tr>
<td>IL-5</td>
<td>-EBV-transformed</td>
<td>Baumann and Paul, 1992</td>
</tr>
<tr>
<td>IL-6</td>
<td>-EBV-transformed</td>
<td>Yokoi et al., 1990</td>
</tr>
<tr>
<td></td>
<td>-myeloma cell lines</td>
<td>Tosato et al., 1990</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Kawano et al., 1988</td>
</tr>
<tr>
<td>IL-7</td>
<td>-EBV-transformed</td>
<td>Benjamin et al., 1994</td>
</tr>
<tr>
<td></td>
<td>-B-CLL</td>
<td>Frishman et al., 1993</td>
</tr>
<tr>
<td>IL-8</td>
<td>-B-CLL</td>
<td>Francia di Celle et al., 1996</td>
</tr>
<tr>
<td>IL-10</td>
<td>-EBV-transformed</td>
<td>Burdin et al., 1993, 1997</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Beatty et al., 1997</td>
</tr>
<tr>
<td>TNF-α</td>
<td>-B-CLL</td>
<td>Cordingley et al., 1988</td>
</tr>
<tr>
<td></td>
<td>-normal human splenic B cells</td>
<td>Boussiotis et al., 1994</td>
</tr>
<tr>
<td>Lymphotoxin (TNF-β)</td>
<td>-EBV-transformed</td>
<td>Estrov et al., 1993</td>
</tr>
<tr>
<td></td>
<td>-B-CLL</td>
<td>Kuhnburg et al., 1998</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>-EBV-transformed</td>
<td>Pike et al., 1991</td>
</tr>
<tr>
<td>sCD23</td>
<td>-EBV-transformed</td>
<td>Swendeman &amp; Thorley-Lawson, 1987</td>
</tr>
<tr>
<td></td>
<td>-normal receptor-stimulated B cells</td>
<td></td>
</tr>
<tr>
<td>GM-CSF</td>
<td>-B-ALL (Ph^1 positive)</td>
<td>Estrov et al., 1996</td>
</tr>
<tr>
<td>PDGF</td>
<td>-SSV-transformed</td>
<td>Huang et al., 1984</td>
</tr>
</tbody>
</table>
required for the production of cytokines, including autocrine factors, to take place (reviewed by Pistoia, 1997). The importance of autocrine growth regulation in normal B cell development is not known, but it is deemed unlikely that this mechanism plays a predominant role, since B cells are largely influenced by stromal cells and subsequently by T cells during their development (Pistoia, 1997). B cell autocrine factors may therefore play an auxiliary role in the regulation of B cell development.

Although identified in various malignant cell types, the importance of autocrine growth factors in tumorigenic transformation has been questioned. A number of researchers have reported that autocrine stimulation was in fact inversely proportional to the degree of tumorigenicity (Gordon et al., 1985; Swendeman and Thorley-Lawson, 1987; Abken et al., 1992). In addition, Young and colleagues demonstrated that although the over-expression of IL-7 in pre-B cells caused autocrine growth stimulation, the cells did not acquire a tumorigenic phenotype (Young et al., 1991). Autocrine factor production is therefore not the sole requirement for the transformation of B cells.

Although not the main factor causing transformation, the current view is that autocrine loops may still contribute to leukaemogenesis. A recent study found that TNF-β was expressed in leukaemic B cells, but not in B cells from normal donors, and that antisense TNF-β oligonucleotides were able to inhibit B-CLL proliferation (Kulmburg et al., 1998). A number of studies have also demonstrated that although autocrine factors may not be directly involved in proliferation, they may be important, if not essential, for the survival of malignant cells (Francia di Celle et al., 1996; Kulmburg et al., 1998).

In addition to autocrine factors providing a positive survival or proliferative signal to B cells, autocrine growth inhibitors, such as TGF-β (Kehrl et al., 1986), have also been identified. The down-regulation of these factors could lead to a loss of negative regulation, allowing continued cell growth, and thus may play a role in the development of malignancies (Gordon and Cairns, 1991). TGF-β has been found to be over-expressed in B-CLL and as such, is believed to contribute to the slow proliferative characteristic of this leukaemia (Lotz et al., 1994).
1.5 CD23 -ADHESION MOLECULE AND CYTOKINE

CD23 is a 45kDa cell surface antigen which is believed to have multiple functions in B lymphocytes and in the effector cells of IgE-mediated immune responses (reviewed by Bonnefoy et al., 1997). With respect to B cell functions, it is proposed to have roles in the regulation of cell growth and development, IgE regulation, antigen presentation, cell adhesion, and the prevention of apoptosis. After considering the structure and distribution of CD23, these B cell associated functions will be discussed.

1.5.1 Discovery and general distribution of CD23

Historically, the CD23 molecule was originally identified as an IgE receptor on human B cells (Gonzalez-Molina and Spiegelberg, 1976), then independently described as a cell surface marker expressed on Epstein-Barr-Virus (EBV) transformed B cells (Kinter and Sugden, 1981), and as a B cell activation antigen (Blast 2) (Thorley-Lawson et al., 1985). Subsequent studies confirmed this antigen as the low-affinity receptor for IgE (FceRII) (Bonnefoy et al., 1987; Yukawa et al., 1987). In addition to B cells, CD23 is also expressed on various other human cells including T cells, monocytes, macrophages, eosinophils, basophils, neutrophils, follicular dendritic cells, epidermal Langerhans cells and platelets (Delespesse et al., 1991). Within the mouse, CD23 expression is restricted to mainly B lymphocytes and follicular dendritic cells (Rao et al., 1987; Maeda et al., 1992). Although initial studies focused on its ability to bind IgE, recent findings have identified other ligands for CD23, indicating that CD23 has numerous roles, as a cytokine and as an adhesion molecule involved in cell-cell interaction (reviewed by Bonnefoy et al., 1997).

1.5.2 Structure of CD23

The CD23 gene is a single gene located on chromosome 19 (Suter et al., 1987; Wendel-Hansen et al., 1990). The gene encodes two isoforms (named ‘a’ and ‘b’), which differ only at the cytoplasmic amino-terminus where 7 amino acids of CD23a are replaced by 6 different amino acids in CD23b, and are generated by the use of
alternative initiation sites (Yokota et al., 1988). In contrast to other Fc receptors, CD23 does not belong to the immunoglobulin superfamily but is a type II transmembrane glycoprotein with a short N-terminal intracytoplasmic tail (23 residues), a single transmembrane domain (20 residues), and a large C terminal extracellular region (277 residues). It displays sequence homology to the C-type lectins, a family of proteins that bind carbohydrates in a calcium-dependent fashion (Drickamer, 1988). The region homologous to C-type lectins spans from cysteine 163 to 282 and contains four highly conserved (positions 191, 259, 273, 282), and two partially conserved cysteines (positions 163 and 174) (Kikutani et al., 1986a; Ludin et al., 1987) (see figure 1.2). The presence of this lectin domain, the inverted membrane orientation, and the presence of several other conserved amino acids, classifies CD23 as a member of a superfamily of type II integral membrane proteins including CD72, the asialoglycoprotein receptor-1, and the selectins (Delespesse et al., 1992).

Mutational analysis of the lectin region revealed that it comprises the IgE binding site (Bettler et al., 1989b, 1992). The binding of IgE to CD23 is calcium-dependent (Richards and Katz, 1990), and can be inhibited by fucose-1-phosphate (Delespesse et al., 1992). In addition, the interaction also appears to involve protein-protein interactions as deglycosylation of IgE does not influence binding (Vercelli et al., 1989a). The cytokine effects of CD23 however are mediated by an epitope distinct from the IgE binding site (Mossalayi et al., 1992).

In addition to the lectin homology domain, CD23 bears a triplet of amino acids DGR (Asp, Gly, Arg), at the carboxyl terminus, which in the reverse orientation, i.e. RGD, is a common recognition site for the integrin receptors (see figure 1.2). The importance of this motif, if any, to CD23 function remains to be determined.

Another feature of CD23 is the presence of 3 short consensus repeats of 21 amino acids each, located between the N-glycosylation site and the lectin domain. This repetitive region contains 5 heptadic repeats of leucines (or isoleucines) forming a ‘leucine zipper’ motif. Analysis revealed that this region in CD23 and other members of the C-type lectin superfamily, adopt an α-helical coiled-coil structure, representing a ‘stalk’ separating the lectin heads from the membrane. It was believed that this
Figure 1.2  Linear model of the CD23 antigen.

aa = amino acids, C = conserved/partially conserved cysteine, ▼ = N-glycosylation,  ■ = short consensus repeat (21 aa), ▲ = sites of cleavage into sCD23. Adapted from Delespesse et al., 1992.
structure would mediate the formation of protein dimers or trimers at the cell surface (Beavil et al., 1992). In support of this, more recent studies have found that when subjected to protein-protein chemical cross-linking, CD23 forms trimers on the cell surface (Beavil et al., 1995).

Similar to other Fc receptors, soluble forms of CD23 (sCD23) are released into the extracellular fluids. Five major sCD23 fragments, apparent molecular masses 37, 33, 29, 25 and 16kDa, have been identified, all derived from cleavage within the stalk region of 45kDa CD23 (see figure 1.2). The soluble components are first released as the 37kDa oligomeric form, which is subsequently cleaved to the other forms, with the 25kDa form being the most stable (Letellier et al., 1989). The soluble forms of CD23 contain the complete lectin domain and thus retain the ability to bind IgE - 'IgE-binding factors' (Sarfati et al., 1987, 1988). Although able to bind IgE, the 16kDa fragment binds with lower affinity than the other fragments, a property believed to due to the monomeric nature of this soluble form (Bettler et al., 1989a). In addition to IgE-associated activities, sCD23 has been shown to exhibit pleiotropic cytokine activities (reviewed by Delespesse et al., 1992).

The rate of cleavage of CD23 is reduced by IgE and anti-CD23 antibodies, and is increased after the use of agents that prevent CD23 glycosylation, indicating that the carbohydrate chain of CD23 exerts a stabilising effect (Delespesse et al., 1989). The specific proteases involved in CD23 shedding have as yet not been identified. Previous studies have suggested an autocatalytic mechanism (Letellier et al., 1990), although CD23 does not resemble any known proteases. A recent study by Marolewski and colleagues, has demonstrated that in several human cell types, the initial release of CD23 from the cell surface is mediated by a membrane-bound metalloprotease that can be blocked by specific protease inhibitors. The researchers suggest that the previous proposal of autoproteolysis is a result of the co-purification of a protease with CD23, and speculate that a family of metalloproteases will mediate the cleavage of all the sCD23 fragments (Marolewski et al., 1998).

With regard to the oligomerisation, Beavil and colleagues (1995), demonstrated that after chemical cross-linking, soluble CD23 exists as hexamers in solution. As only the
37kDa and 33kDa fragments possess any of the α-helical coiled-coil required for oligomerisation, the researchers suggest that the lectin heads in the 25 and 16kDa forms could self-associate independently of the stalk, thus allowing oligomerisation of these fragments. A recent study has demonstrated that 25kDa sCD23 exists in human serum as a trimer complex, which allows efficient interactions with both IgE and sCD21, suggesting that this trimeric form of sCD23 is the form exhibiting biological activities (Fremeaux-Bacchi et al., 1998). These data, in addition to the previous demonstration of trimeric membrane bound CD23 (Beavil et al., 1995), are in agreement with a model proposed by Sutton and Gould, in which two of the three lectin heads of CD23 are bound to an IgE molecule, thus leaving one of the heads to associate with other ligands such as CD21 (Sutton and Gould, 1993). An oligomeric form of CD23 therefore binds to IgE leading to an effective interaction.

1.5.3 Murine CD23

In contrast to the human form, 49kDa murine CD23 has 4 (instead of 3) consensus repeats, 2 (instead of 1) N-glycosylation sites, and no DGR motif (Better et al., 1989a; Gollnick et al., 1990). Initial studies reported 2 murine CD23 isoforms, one that was equivalent to the human ‘a’ isoform, and another that was unrelated to the amino terminus of the human ‘b’ form (Richards et al., 1991). However, controversy surrounds the existence of murine CD23 isoforms as subsequent studies have disputed the existence of a second form (Conrad et al., 1993), while others have reported an isoform with homology to human subtype ‘b’ at the amino terminus (Kondo et al., 1994). Like the human form, murine CD23 has been found to require oligomerisation for efficient binding to IgE, although it is not known whether they exist as dimers or trimers (Dierks et al., 1993).

1.5.4 Regulation of CD23 expression

CD23 is rapidly superinduced in B cells transformed with EBV and, as such, was one of the ways in which the CD23 antigen was first identified (Kintner and Sugden, 1981). Subsequent studies have determined that the EBV nuclear antigen (EBNA-2) and the latent membrane protein (LMP-1) induce the CD23a and CD23b membrane
isoforms respectively, synergistically leading to high levels of membrane CD23 on the cells (Wang et al., 1990).

In untransformed B cells, IL-4 is known to be a major regulator of CD23 expression. This cytokine induces the expression of the ‘a’ isoform of human CD23, expressed exclusively on peripheral blood B cells, and of the ‘b’ isoform, expressed on B cells and a number of other human cells including T cells and monocytes (Yokota et al., 1988). Induction of CD23 expression by IL-4 also occurs in the mouse (Hudak et al., 1987). IL-13 has also been found to induce human B cell CD23 expression independently of IL-4, with no synergistic effect between these two cytokines (Punnonen et al., 1993).

In the B cell lineage, CD23 is expressed on normal mature IgM⁺ IgD⁺ B cells, but is lost following isotype switch and differentiation into Ig-secreting cells (Kikutani et al., 1986b; Waldschmidt et al., 1988; Kehry and Hudak, 1989). With respect to precursor B cells, CD23 can be induced on malignant pre-B cells from acute lymphoblastic leukaemia (ALL) patients by IL-4 (Law et al., 1991), and by the ligation of CD40 on normal pre-B cells (Saeland et al., 1993). This latter effect has also been demonstrated with murine B cells using CD40 ligand in conjunction with IL-4 (Maliszewski et al., 1993).

In addition to cytokine regulation, CD23 expression in vitro is reported to be regulated by its ligand, IgE, which prevents the proteolytic cleavage of CD23, thereby stabilising its surface expression (Lee et al., 1987; Kawabe et al., 1988). Recent studies by Kisselgof and Oettgen have demonstrated this effect in vivo. IgE-deficient mice were found to have a defect in CD23 expression on B cells compared to wild-type animals, that could be restored by the injection of IgE. The researchers suggest that IgE-mediated up-regulation of CD23 may be important in enhancing immune responses by participating in the uptake of IgE-antigen complexes, thus facilitating antigen presentation by B cells (discussed in section 1.5.6). In addition, they also speculate that if the mechanism whereby IgE regulates CD23 expression in vivo is the same as its anti-proteolytic effect in vitro, it is possible that IgE binding to CD23 mediates additional effects via regulating the release of sCD23 (Kisselgof and Oettgen, 1998).
Soluble CD23 is reported, albeit controversially, to have various biological activities (see section 1.5.7).

1.5.5 Ligands for CD23 other than IgE

In addition to IgE binding, a number of other non-IgE-related activities have been described for CD23/sCD23 (described in sections 1.5.6 and 1.5.7), leading researchers to investigate whether CD23 could interact with ligands other than IgE. The initial demonstration of the existence of an alternative ligand for CD23 was made in 1992 by Pochon and colleagues by the use of recombinant full-length CD23 incorporated into fluorescent liposomes (Pochon et al., 1992). Since then, a number of CD23 ligands have been identified namely CD21, CD11b and CD11c (see below).

1.5.5a CD21

Human CD21 is a 145kDa membrane glycoprotein expressed on various cell types including B cells, a subpopulation of T cells, and follicular dendritic cells. It has been identified as a receptor for the gp350/220 envelope glycoprotein of EBV (Tanner et al., 1987), interferon-α (Delcayre et al., 1991), and as the complement receptor-2 (CR2) (Weis et al., 1984). On the membrane of B cells, CD21 is present in part within a molecular complex, in association with CD19, Leu 13 and TAPA-1 (Matsumoto et al., 1991; Bradbury et al., 1992). Co-ligation of this complex with surface IgM decreases the amount of antigen required to trigger B cell activation (reviewed by Fearon and Carter, 1995; Carroll, 1998).

Structurally, the CD21 protein is composed of an extracellular domain of 15 to 16 short consensus repeats of 60 to 75 amino acids (SCRs), followed by a transmembrane domain and an intracytoplasmic domain of 24 and 34 amino acids, respectively (Weis et al., 1988). Studies using inhibitory anti-CD21 antibodies as well as the binding of CD23 liposomes to recombinant CD21 transfected cells revealed that CD21 was an alternative ligand for CD23 (Aubry et al., 1992). Subsequently, Aubry and colleagues determined the sites of interaction of CD23 on CD21 by the use of CD21 mutants bearing deletions in the SCRs. An interaction site was mapped to SCRs 5-8, which was found to involve a lectin interaction, as tunicamycin treatment inhibited the
binding of CD23-containing liposomes to this region. In addition, SCRs 1-2 were also found to be involved in binding, but via a protein-protein interaction with CD23 (Aubry et al., 1994).

A soluble form of CD21 (sCD21) has been identified in human serum and in culture supernatants of human lymphocyte cell lines (Myones et al., 1987; Ling et al., 1991; Huemer et al., 1993). The soluble 135kDa protein that is cleaved and shed from human B and T lymphocytes corresponds to the extracellular portion of the CD21 molecule (Fremeaux-Bacchi et al., 1996). Recent studies have shown that sCD21 retains the ligand binding properties of the membrane form, and as such, exists in normal plasma in a complex with trimeric sCD23, and thus regulates some sCD23 activities (see section 1.5.7) (Fremeaux-Bacchi et al., 1998).

Overall, the interaction of CD23/sCD23 and CD21/sCD21 is believed to contribute to a number of important events within the immune system including, the regulation of IgE production, and the formation of homotypic and heterotypic cell adhesions (see section 1.5.6).

1.5.5b CD11b/CD11c

CD11b and CD11c represent the α chains of the β2 integrin adhesion molecules which participate in many cell-cell and cell-matrix interactions. These α chains (17kDa and 15kDa respectively), exist as heterodimers with a common β subunit, CD18, (95kDa), to form the Mac-1 (CD11b-CD18), and p150,95 (CD11c-CD18) glycoprotein receptors (Kurzinger et al., 1982; Corbi et al., 1987, 1988). The third member of this integrin family is CD11a-CD18, known as the leucocyte function-associated antigen-1 (LFA-1) (Saachez-Madrid et al., 1983; Larson et al., 1989). LFA-1 is expressed on virtually all leucocytes, whereas Mac-1 and p150,95 are expressed on myeloid cells and on activated but not resting lymphocytes, where they can also act as receptors for complement proteins (Anderson and Springer, 1987). The final member of this family, CD11d-CD18, is expressed on subsets of peripheral blood leukocytes (Van der Vieren et al., 1995).
The importance of the β2 integrin family is signified by the clinical syndrome known as leucocyte adhesion deficiency (LAD) (Anderson and Springer, 1987). The primary defect in LAD is in the β subunit, which is required for the correct processing and expression of the α subunits (Kishimoto et al., 1987). LAD patients have recurrent bacterial infections which are sometimes fatal, as circulating neutrophils and monocytes are unable to bind and migrate into infected tissue.

In a series of experiments performed by Lecoanet-Henchoz and colleagues, CD23 was found to bind to CD11b and CD11c on human monocytes. CD23 containing liposomes bound to COS7 cells transfected with cDNA encoding CD11b-CD18 or CD11c-CD18, but not CD11a-CD18. CD23 binding to CD11d-CD18 has not been investigated. The interaction between CD23 and CD11b/c appears to involve both lectin and protein-protein interactions, as IgE, which binds to the lectin domain of CD23, partially inhibits the binding of CD23 to monocytes (Lecoanet-Henchoz et al., 1995). The binding of these β2 integrin components is thus reminiscent of that observed between CD23 and CD21 (Aubry et al., 1994). Although CD23 includes a DGR triplet of amino acids, that in the reverse orientation is a common recognition site for integrin receptors, this was found not to be involved in the interaction between CD23 and CD11b/c (Lecoanet-Henchoz et al., 1995). Upon binding to monocytes via CD11b/c, CD23 causes a marked increase in nitric oxide levels and the release of proinflammatory cytokines including, IL-1α, IL-1β, IL-6, IFN-γ and TNF-α (Lecoanet-Henchoz et al., 1995). This biological activity of CD23 has also been found within the mouse, mediated via CD11b on murine monocyte cells (Lecoanet-Henchoz et al., 1997), further demonstrating a role for CD23 as a proinflammatory mediator. By mediating this activity, CD23 is believed to have a significant role in inflammation, supported by the presence of elevated levels of CD23 in various inflammatory diseases (Bonnefoy et al., 1996) (discussed further in section 1.5.9).

1.5.6 Function of membrane CD23 with respect to B cells

CD23 has been postulated to play a role in IgE regulation ever since the finding that IL-4 and IL-13 upregulate CD23 expression as well as IgE production from B cells (Defrance et al., 1987; Punnonen et al., 1993). In addition, factors which block IL-4
induced IgE synthesis, such as interferon-γ (Pene et al., 1988), also inhibit CD23 induction by IL-4 on B cells (Defrance et al., 1987). The importance of CD23, and its soluble fragments, in in vitro IgE synthesis, was confirmed by the demonstration that anti-CD23 antibodies were able to inhibit IL-4 induced IgE production (Sarfati and Delespesse, 1988; Pene et al., 1988; Bonnefoy et al., 1990). Subsequently, the same result was obtained in vivo with studies in the rat system (Flores-Romo et al., 1993). As physical interactions between B and T cells are known to be required for IgE production (Vercelli et al., 1989b), these results suggested that CD23 somehow contributes to the B cell/T cell interaction necessary for IgE production. Conjugate formation between these two cell types is now known to involve interactions between CD23 and CD21, as anti-CD21 antibodies, like anti-CD23 antibodies, were found to decrease T-B conjugate formation (Aubry et al., 1992, 1993). More importantly, engagement of CD21 on B cells by either anti-CD21 antibody or sCD23, resulted in an increase of IL-4 induced IgE production (Aubry et al., 1992), thus suggesting that the IgE-promoting effects of CD23 are probably mediated through CD21. Based on this observation, and the reported induction of CD23 on T cells by IL-4 and allergen (Prinz et al., 1990), Bonnefoy and colleagues suggest that in allergic individuals, T cell-associated CD23 interacts with CD21 on B cells to cause an increase in IgE production. Therefore, in the absence of allergen and IL-4 as in normal individuals, the T-B interaction does not take place and the resting B cell is not induced to differentiate into an IgE producing cell (Bonnefoy et al., 1997).

Increased IgE production via triggering of CD21 has been observed in both T cell-dependent and independent systems (Henchoz et al., 1994), suggesting that heterotypic T-B (Aubry et al., 1993), and also homotypic B-B (Bjorck et al., 1993) interactions can occur through CD23/CD21 pairing. The latter effect is believed to be important for the exchange of autocrine factors during the later stages of B cell activation and differentiation (Bjorck and Paulie, 1993).

The CD23 expressed on B cells regulates two important B cell functions namely, IgE-dependent antigen presentation to T cells, and B cell differentiation into Ig-producing cells. The role of CD23 in antigen presentation is related to its physical association with MHC class II antigens on the surface of B cells which present antigen to T cells.
(Bonnefoy et al., 1988). Flores-Romo et al., 1990, demonstrated that occupancy of CD23, by either anti-CD23 antibodies or IgE, prevented B cells from stimulating allogeneic T cells. Further studies demonstrated that the CD23/CD21 interaction, resulting in T/B conjugate formation, is required for antigen presentation and suggest that this interaction provides a co-stimulatory signal to the T cell (Grosjean et al., 1994).

Antigen presentation by resting B cells is mediated by CD23a, as this isoform is expressed exclusively on these cells and has been found to mediate efficient endocytosis of IgE immune complexes (IgE-IC) (Yokota et al., 1992). Specific IgE antibodies and CD23 on B cells may amplify the T cell response to the corresponding antigen by expanding the pool of Ag-specific T cells involved (Delespesse et al., 1992).

The B cell presenting antigen via the use of CD23 is not antigen specific as the B cell antigen receptor is not involved. It was found that cross-linking of B cell CD23 by anti-CD23 antibodies or IgE-IC prevented the activation and differentiation of these cells, thus preventing B cell differentiation and the production of antibodies with unrelated specificities (Luo et al., 1991). This effect suggests that by using CD23, IgE down-regulates its own production by preventing the recruitment of new IgE producing cells from the sIgM+/sIgD+/CD23+ B cells (Delespesse et al., 1992). The inhibitory effect reported by Luo and colleagues was not observed in the absence of IL-4 driven B cell stimulation (Luo et al., 1991). This cytokine induces the expression of both CD23 isoforms, predominately isoform ‘b’ (Yokota et al., 1988), suggesting that the inhibitory signal is delivered to the B cell after engagement of this inducible ‘b’ isoform (Delespesse et al., 1992). This theory would explain why the cross-linking of CD23 on IL-4 stimulated mouse B cells has no effect on their differentiation into IgE-secreting cells, as these do not seem to express the equivalent of the human ‘b’ isoform of CD23 (Delespesse et al., 1992).

In contrast to these reports of inhibitory signals, some researchers have demonstrated that CD23 may deliver growth-promoting signals to human and mouse B cells. Using phorbol ester-pre-activated human B cells, Gordon and colleagues demonstrated that
anti-CD23 antibodies could promote cell cycle progression and DNA synthesis (Gordon et al., 1986). However, this growth-promoting effect was only mediated by a small number of CD23 monoclonal antibodies all directed against the same epitope. In addition, the anti-CD23 antibody used by Luo et al. (1991), to demonstrate the inhibitory effects of CD23 ligation, failed to increase the proliferation of cells under the experimental conditions previously used by Gordon and colleagues (Delespessse, unpublished observations in Luo et al., 1991). In light of these results, it was suggested that anti-CD23 antibodies may deliver either positive or negative signals to B cells, depending on their epitope specificity and also perhaps upon the activation state of the cell (Luo et al., 1991).

1.5.7 Function of sCD23 with respect to B cells

In addition to being able to bind to IgE and thus regulate IgE synthesis, soluble fragments of CD23 exhibit multiple functions that are IgE-independent, resulting in sCD23 being described as a pleiotropic cytokine (Gordon et al., 1989). With respect to IgE, however, human sCD23 is capable of up-regulating the in vitro synthesis of IgE in the presence of IL-4 (Pene et al., 1989; Aubry et al., 1992; Delespessse et al., 1992; Saxon et al., 1990). This activity has been shown to be restricted to fragments >29kDa, with the 16kDa fragment actually inhibiting IgE synthesis in vitro (Sarfati et al., 1992). In comparison, this IgE-dependent activity of human sCD23 has not been detected for the murine counterpart, highlighting a major difference between these two species (Bartlett and Conrad, 1992). A recent study has shown that human sCD23-induced IgE synthesis can be inhibited by sCD21, suggesting that when complexed with sCD21, sCD23 is unable to bind to the membrane CD21 receptor and elicit its effects (Fremeaux-Bacchi et al., 1998). This result therefore demonstrates that in addition to the membrane associated forms of these antigens (discussed previously), interactions between the soluble forms of CD21 and CD23 also mediate important biological effects.

A number of researchers have studied the B cell growth factor (BCGF) effects exhibited by sCD23. Swendeman and Thorley-Lawson (1987), were the first to demonstrate that affinity purified sCD23, made of 25 and 12kDa proteins, possessed
growth promoting activity, and acts as an autocrine factor for EBV-immortalised and receptor-stimulated B lymphocytes. In support of this observation, a number of other studies have demonstrated the BCGF activity exhibited by sCD23 (Gordon et al., 1988; Armitage and Goff, 1988; Delespesse et al., 1989). However, by using recombinant sCD23 derived from cDNA constructs, Uchibayashi and colleagues demonstrated that the 25kDa fragment does not possess any BCGF activity. In light of this, the researchers suggested that the BCGF activity of sCD23 is mediated by the 12kDa protein present in the affinity purified sCD23, which had been used previously by researchers (Uchibayashi et al., 1989). In support of this, the co-purified 12kDa protein, but not the 25kDa fragment alone, has been found to display growth promoting properties (Luo et al., 1989). Subsequent research by Cairns and Gordon (1990), found that intact 45kDa CD23, purified from cell lysate material, was consistently mitogenic for normal and transformed B cells. They also demonstrated that although on occasion sCD23 exhibited BCGF activity, sCD23 preparations were highly variable in their biological activity, making its full characterisation problematic. They suggest that the BCGF activity may be associated with an unstable cleavage product such as the 37kDa isoform, which initially exhibits the activity of the 45kDa form but is rapidly degraded into the non-stimulatory stable 25kDa fragment (Cairns and Gordon, 1990). As sCD23 BCGF activity has only been observed for substantially activated B cells, previous reports of no activity (Uchibayashi et al., 1989), may be due, in part, to inappropriate pre-activation of the B cells (Cairns and Gordon, 1990). While these discrepancies exist, and the identity of the soluble isoform responsible for cell growth stimulation is unknown, the role of sCD23 in B cell growth remains controversial.

Interestingly, sCD23 has been identified as a cytokine able to deliver survival signals to germinal centre B cells. Germinal centres have two anatomical compartments: 1) the dark zone which is populated by rapidly proliferating activated B cells called centroblasts and 2) a light zone which contains the progeny of centroblasts, centrocytes. Within the 'basal' light zone, many centrocytes undergo programmed cell death/apoptosis (discussed in section 1.6), by failing to undergo high affinity interactions with antigen held on the follicular dendritic cells (FDC) which also populate the this zone (MacLennan et al., 1992). Ever since FDC in the 'apical' region
of the light zone, but not the basal region, were found to express high levels of CD23. CD23 has been speculated to be involved in the subsequent survival of FDC-Ag selected B cells (Gordon et al., 1989). This idea was confirmed by Liu and colleagues (1991a), who demonstrated that in synergy with IL-1α, recombinant 25kDa sCD23 promotes the survival of freshly isolated germinal centre B cells placed into culture, and promotes plasmacytoid differentiation. Previous studies by these researchers demonstrated that death could be prevented, without any subsequent plasmacytoid differentiation, by activating the cells via their antigen receptor and CD40 (Liu et al., 1989). In summation, these results lead to a model for germinal centre B cell development in which centrocytes initially rescued from apoptosis through re-encounter with antigen, subsequently develop into plasma or memory B cells by receiving signals mediated by sCD23 and IL-1α or CD40, respectively (Liu et al., 1991a).

Studies by Bonnefoy and colleagues (1993), have demonstrated that sCD23 mediated rescue of germinal centre B cells from apoptosis can be mimicked by anti-CD21 antibodies. These antibodies were also found to promote plasmacytoid differentiation and up-regulate the expression of the anti-apoptotic proto-oncogene bel-2 in the B cells. These results therefore suggest that FDC-associated CD23 affects B cell development in germinal centres by acting through CD21 (Bonnefoy et al., 1993). As yet this survival mechanism has not been demonstrated in vivo, and it is unknown whether this function is actually mediated by the soluble or membrane form of CD23 on FDC.

In further support for sCD23 pleiotropic cytokine activity, this protein has proposed functions outwith the B cell compartment, namely in the proliferation of T cells (Armitage et al., 1989; Bertho et al., 1991), and for the growth and differentiation of both early human myeloid and thymocyte precursors (Mossalayi et al., 1990a, 1990b). However, it remains to be demonstrated if these effects of CD23, in addition to the ones described previously for B cells, represent true in vivo functions and whether they are actually mediated by the soluble or membrane bound form of CD23.
In an attempt to evaluate the in vivo roles of CD23, CD23-deficient mice have been generated. Fujiiwara and colleagues (1994), demonstrated that these mice have normal lymphocyte differentiation and can mount normal antibody responses. These results suggested that previously reported in vitro activities were not representative of in vivo functions. However, the researchers suggest that these discrepancies may be explained by the differences between the human and murine CD23 molecules, namely in the differences in cellular distribution, structure, and the inability of murine sCD23 to mediate any of the activities ascribed to human sCD23 (Bartlett and Conrad, 1992). The results do however confirm the participation of CD23 in antigen presentation, as the deficient mice did not display IgE-dependent enhancement of antibody responses (Fujiwara et al., 1994).

1.5.8 CD23 and bone marrow haematopoiesis

CD23 has been ascribed a role in Ag-independent phase of haematopoiesis within the bone marrow due to the ability of sCD23 to promote the differentiation of human myeloid precursors from haematopoietic stem cells (Mossalayi et al., 1990a). Subsequent studies by Fourcade and colleagues, 1992, demonstrated that CD23 is expressed and secreted as a soluble form in freshly isolated BM cells, and stromal cells derived from long-term BM-cultures (LTBMC). The researchers found that addition of anti-CD23 antibody to LTBMC significantly reduced haematopoiesis, further confirming the ability of CD23 to regulate development (Fourcade et al., 1992).

CD23 has also been shown to be involved in mediating adhesion within the bone marrow. Human myeloma cells, the malignant counterpart of plasma cells, have been found to attach via their CD21 antigen to BM stromal cell CD23 (Huang et al., 1995; Ishikawa et al., 1998), demonstrating another role for this adhesion pair.

Although BM-derived CD23 has yet to be ascribed a direct role in human precursor B cell development, it is possible that either the membrane-bound or soluble form, or indeed both, may play a contributory role.
1.5.9 The role of CD23 in disease

Although the precise in vivo roles of CD23 are unconfirmed, the biological importance of CD23 is implicated by its involvement in various pathological conditions.

Normal healthy individuals have less than 5ng/ml of sCD23 in serum (Yanagihara et al., 1990; Delespesse et al., 1992). In atopic individuals, where there is an overproduction of IgE antibodies, sCD23 serum levels are elevated and are correlated with those of IgE (Delespesse et al., 1992). Recent studies have demonstrated that Der pI, a major allergen of the house dust mite, is able to actively cleave CD23 from the surface of B cells, demonstrating how an allergen can disrupt the regulation of IgE synthesis mediated by CD23, leading to an excess of IgE in an immune response (Hewitt et al., 1995; Schulz et al., 1997; Shakib et al., 1998).

There are elevated levels of CD23-positive B cells (Kumagai et al., 1989), and sCD23 in the serum and more importantly, in the synovial fluid of rheumatoid arthritis (RA) sufferers (Delespesse et al., 1991; Hellen et al., 1991; Bansal et al., 1993). In murine models of arthritis, injection of anti-CD23 antibodies has been found to ameliorate the symptoms of the disease (Plater-Zyberk et al., 1995). A mechanism of action for anti-CD23 antibody treatment is believed to be the blocking of the interaction between CD23 and CD11b/c on monocytes (Bonnefoy et al., 1996). As previously discussed in section 1.5.5b, this interaction induces the release of proinflammatory cytokines by monocytes (Lecoanet-Henchoz et al., 1995), demonstrating how increased levels of CD23 may mediate inappropriate inflammatory reactions. In the inflamed synovium, macrophages have also been found to express CD23, which if ligated by IgE immune complexes or anti-CD23 antibodies, also triggers the release of proinflammatory mediators (Dugas et al., 1995). Simultaneous expression of CD23 and CD11b/c may also allow stimulatory homotypic interactions to take place, and may explain why, once located to the joints, macrophages can maintain and exacerbate inflammatory responses (Bonnefoy et al., 1996).

Compared to normal individuals and patients with other B cell lymphoproliferative disorders, levels of sCD23 are significantly elevated, up to 500-fold, in the serum of
patients with B cell chronic lymphocytic leukaemia (B-CLL) (Sarfati et al., 1988). This malignancy is characterised by the accumulation of slowly dividing mature B lymphocytes in the peripheral blood (reviewed by Dighiero and Binet, 1996). Elevated sCD23 levels correlate with the clinical stage of the disease (Sarfati et al., 1988), and has recently been shown to help predict disease progression (Knauf et al., 1997). The elevated levels of CD23 in B-CLL arise from both the increased numbers of CD23-positive B cells, and also from the over-expression of CD23 on the surface of these neoplastic cells (Sarfati et al., 1990). In contrast to normal B cells that exclusively express CD23 isoform ‘a’, freshly isolated B-CLL cells express both the CD23a and CD23b isoforms (Fournier et al., 1991). Subsequent studies have shown that CD23a and CD23b are abnormally regulated in B-CLL cells by cytokines such as IL-2, IFN-α, IFN-γ and IL-4, and are believed to contribute to the survival and proliferation, respectively, of B-CLL cells (Fourrier et al., 1995).

1.6 APOPTOSIS/PROGRAMMED CELL DEATH

Cell death is fundamental for the development and homeostatic maintenance of multicellular organisms, in processes such as the sculpting of limbs, controlling cell numbers, and the elimination of abnormal, misplaced, non-functional, or harmful cells. Deregulation of cell death contributes to the pathogenesis of diseases, such as lymphomas and leukaemias, and is therefore an essential area of scientific study.

The importance of ‘normal cell death’ was initially highlighted in 1972 by Kerr and colleagues. They described a distinct set of morphological features that categorised dying cells into one of two categories, either necrosis or apoptosis. The former has often been referred to as accidental cell death as it results from acute cellular injury, which is typified by rapid cell swelling and lysis. In contrast, apoptotic cell death is characterised by controlled autodigestion of the cell, reflecting the operation of an active or programmed mechanism of death (Kerr et al., 1972; Wyllie et al., 1980). Previously called shrinkage necrosis on morphological grounds (Kerr, 1965), the word apoptosis is derived from a Greek term describing the process of leaves falling off
trees in the autumn, and was chosen to suggest that cell loss is desirable for the survival of the host (Kerr et al., 1972).

The terms apoptosis and programmed cell death (PCD) have often been used interchangeably causing much confusion within the literature. These terms are now however considered non-synonymous. PCD is a functional term describing a cell death that is normal part of life, while apoptosis is purely a descriptive term that represents a type of cell death with distinctive morphological characteristics (Martin et al., 1994).

1.6.1 The morphology of apoptosis

During apoptosis a distinct sequence of morphological events has been characterised (Kerr et al., 1972; Wyllie et al., 1980). In the early stages, the nucleus shrinks and its chromatin becomes condensed into compact masses along the nuclear membrane. Parallel to these nuclear changes, the cytoplasm compacts resulting in the crowding of organelles, which characteristically retain their form. As a result of overall cell shrinkage the plasma membrane becomes ruffled, a process called zetosis, or rapid blebbing (Cohen et al., 1992b). At this stage the cell often breaks up into membrane bound fragments called apoptotic bodies which retain their osmotic balance. These apoptotic bodies undergo rapid phagocytosis by either neighbouring cells acting as semi-professional phagocytes, or by cells of the macrophage lineage. This rapid and efficient removal of apoptotic cells occurs before there is any leakage of cellular components, thus preventing the induction of an immune response, a feature that clearly distinguishes apoptosis from necrosis.

In addition to the obvious morphological events, the chromatin changes during apoptosis are accompanied by the internucleosomal cleavage of DNA. This process was originally believed to be the biochemical hallmark of apoptosis, and can be visualised upon gel electrophoresis as multiples of 200bp oligonucleosome fragments - the DNA ladder (Wyllie, 1980). However, this degradation of DNA is now believed to be a late apoptotic event, as evidence has arisen for the cleavage of DNA into larger 300 and/or 50kb fragments due to the severing of DNA from its sites of attachment at the nuclear matrix (Oberhammer et al., 1993; Brown et al., 1993). In addition,
apoptosis has been observed in the absence of a DNA ladder, and, as such internucleosomal cleavage cannot be a major criterion for identifying apoptotic cells (Cohen et al., 1992a; Oberhammer et al., 1993; Ormerod et al., 1994). Programmed cell death has also been found to occur in the absence of a nucleus, suggesting that events such as nuclear condensation and nuclear fragmentation are not essential for the process, thus implicating the role of a cytoplasmic initiator (Jacobson et al., 1994).

1.6.2 The role of apoptosis in B cell development

During development, the majority of B cells produced from haematopoietic stem cells undergo apoptosis, never participating in a specific immune response. This process begins in the bone marrow when precursor B cells fail to productively rearrange their immunoglobulin genes. It is estimated that around 75% of B cells are eliminated during the transition from the pro-B to pre-B cell stage (Osmond et al., 1992). Cells with abortive rearrangements die by apoptosis and are engulfed by macrophages residing in the bone marrow (Osmond et al., 1994; Lu and Osmond, 1997).

The next stage of B cell development susceptible to apoptosis is the immature B cell, which characteristically expresses surface IgM. Engagement of the antigen receptor at this developmental stage results in clonal deletion, the mechanisms of which appear to include both the induction of unresponsiveness (anergy), and apoptosis (reviewed by Rajewsky, 1996).

Mature (IgM⁺/IgD⁺) B cells are also susceptible to apoptosis. During an immune response, these cells undergo affinity maturation, to generate antibodies with high affinity for antigen, via the processes of somatic hypermutation and antigen-driven selection (reviewed by Liu et al., 1997b). Somatic hypermutation occurs in activated B cells (centroblasts) within the dark zone of the germinal centre. The progeny of these cells, the centrocytes, are then positively selected based on the affinity of their modified antigen receptor to antigen retained on follicular dendritic cells. Centrocytes that bind with low affinity do not receive positive survival signals, and thus undergo apoptosis. As previously mentioned in section 1.5.7, the signals mediating centrocyte survival are delivered via the antigen receptor and CD40 (Liu et al., 1989), and also by
soluble CD23 and IL-1α (Liu et al., 1991a). The former signal is believed to induce differentiation into a memory B cell, whereas the latter promotes plasmacytoid differentiation (Liu et al., 1991a). As somatic hypermutation may also generate autoreactive mutants, a negative selection mechanism also occurs within the germinal centre (reviewed by Liu et al., 1997).

1.6.3 The basic apoptotic machinery

The potential to undergo apoptosis is an inherent property of all nucleated cells, each constitutively expressing the protein components required to execute the death pathway, without the need for additional protein synthesis (Jacobson et al., 1997). Evidence for this has come from the use of the drug staurosporine, a bacterial product that inhibits many protein kinases. When used in the presence of the protein synthesis inhibitor cycloheximide, staurosporine rapidly induces PCD in all the cells that can be dissociated from a 13 day mouse embryo (Ishizaki et al., 1995), and in the cultures of a variety of neonatal and adult rodent organs (Weil et al., 1996). In addition, the nucleus is not required for PCD in cells that normally have one, as anucleate cytoplasts also undergo PCD upon treatment with staurosporine (Jacobson et al., 1994). Therefore, with the exception of red blood cells, all mammalian cells are capable of undergoing PCD. In those cases where inhibitors of RNA or protein synthesis have been shown to inhibit PCD, it is believed that synthesis is required to activate rather than execute the pathway (Weil et al., 1996).

The molecular basis of programmed cell death and its control have in recent years been the focus of much attention. Considerable progress has been made from the genetic analysis of Caenorhabditis elegans which identified three genes, ced-3, ced-4 and ced-9, that are pivotal to PCD in the nematode (Ellis and Horvitz, 1986). Loss of function mutations determined that ced-3 and ced-4 are both essential for cell death to occur (Ellis and Horvitz, 1986), whereas ced-9 protects cells from undergoing apoptosis (Hengartner et al., 1992). Subsequently, the order in which these cell death genes act in the nematode was delineated, namely that ced-9 functions upstream of ced-4, and ced-4 acts upstream of ced-3 (Shaham and Horvitz, 1996). Further studies demonstrated that Ced-4 interacts with Ced-3 and promotes its activation, whereas
Ced-9 binds to Ced-4 preventing its interaction with, and therefore activation of Ced-3 (Chinnaiyan et al., 1997, Wu et al., 1997). Ced-4 therefore plays a central role in the cell death pathway, physically linking the regulators of apoptosis to the effectors (Chinnaiyan et al., 1997, Wu et al., 1997).

The importance of these *C. elegans* studies was only really appreciated upon the identification of mammalian homologues to the *C. elegans* cell death genes that regulate mammalian cell death. Ced-3 is homologous to the caspase family (Yuan et al., 1993), Ced-4 to mammalian Apaf-1 (Zou et al., 1997), and Ced-9 is related to the Bcl-2 family of apoptotic regulators (Hengartner and Horvitz, 1994).

### 1.6.4 The Bcl-2 family of apoptotic regulators

#### 1.6.4a Bcl-2

Bcl-2 is the mammalian homologue of the nematode regulator of apoptosis Ced-9 (Hengartner and Horvitz, 1994). Importantly, Bcl-2 is able to suppress apoptosis in *ced-9* mutants (Vaux et al., 1992; Hengartner and Horvitz, 1994), demonstrating the conserved nature of the death pathway regulated by Bcl-2. Bcl-2 was first identified in follicular B cell lymphomas where a t(14:18) chromosomal translocation puts the gene under the powerful transcriptional regulatory elements of the immunoglobulin heavy chain gene, resulting in Bcl-2 over-expression (Tsujimoto et al., 1984; Bakhshi et al., 1985; Tsujimoto and Croce, 1986; Cleary et al., 1986). The discovery that bcl-2, unlike oncogenes previously studied, functions by preventing cell death, rather than affecting proliferation, established bcl-2 as a new class of oncogene (Hockenbery et al., 1990; Nunez et al., 1990; Korsmeyer, 1992). Investigations therefore began into the role of bcl-2 in neoplasia, enforced by its initial discovery in follicular lymphoma. Researchers found that deregulated expression of bcl-2 as a single agent was not sufficient for tumorigenesis (Tsujimoto, 1989; Nunez et al., 1989). However, bcl-2 was found to complement and even to synergise with the transforming effects of the c-myc oncogene in B cell precursors by increasing the frequency and shorting the latency of tumour induction in transgenic mice (Nunez et al., 1989; Strasser et al., 1990), thus confirming the importance of bcl-2 in neoplasia.
The initial observations of the ability of Bcl-2 to enhance survival was that Bcl-2 over-expression increased the viability of certain cytokine-dependent cells upon cytokine withdrawal. In IL-3-dependent pro-B and pro-myeloid cell lines, Bcl-2 promoted cell survival in the absence of IL-3 and maintained the cells in the G0 phase of the cell cycle (Vaux et al., 1988; Nunez et al., 1990). Bcl-2 has since been shown to protect a variety of different cell types from apoptosis both in vitro and in vivo, that are induced by diverse cytotoxic stimuli including γ-irradiation and chemotherapeutic drugs. These discoveries led to the important realisation that Bcl-2 blocks a final common effector pathway that leads to apoptotic cell death.

The bcl-2 gene encodes two proteins, Bcl-2α and Bcl-2β, which differ only in their carboxy-terminal tails due to an alternative splicing mechanism (Tsujimoto and Croce, 1986). The presence of a C-terminal hydrophobic domain (which is only possessed by Bcl-2α), serves as integral membrane anchor (Chen-Levy et al., 1990; Nguyen et al., 1993). Various studies have localised this 25-26kDa protein to the mitochondrial membrane (Hockenbery et al., 1990; Nguyen et al., 1993), suggesting a role for this organelle in apoptosis. Other researchers have demonstrated that the protein also resides in the endoplasmic reticulum and the nuclear membrane (Krajewski et al., 1993; Lithgow et al., 1994). Membrane anchor deletion mutants have however demonstrated that Bcl-2 does not require membrane attachment for its anti-apoptotic activity, thereby suggesting a ‘soluble’ activity in the cytoplasm (Nguyen et al., 1994; Borner et al., 1994).

Although involved in apoptosis, evidence exists that suggests that Bcl-2 regulation is not universal. Firstly, Bcl-2 is unable to prevent cell death in some circumstances, such as the apoptosis mediated by the CD95 (Fas/Apo-1) receptor (Strasser et al., 1995; Scaffidi et al., 1998). Secondly, Bcl-2 is undetectable in some cell types, such as the neurons in the central nervous system (Merry et al., 1994), and thirdly, Bcl-2-deficient mice are able to develop relatively normally (Veis et al., 1993). This evidence suggested to researchers that Bcl-2-independent mechanisms existed, prompting a search for other mammalian proteins like Bcl-2 that could regulate apoptosis.
1.6.4b Bcl-2 family members

Bcl-2 is now just one of an ever expanding family of apoptotic regulators, the Bcl-2 family. The family is unusual in that it contains both inhibitors and promoters of apoptosis. The inhibitors include mammalian Bel-xL (Boise et al., 1993), Bel-w (Gibson et al., 1996), Mcl-1 (Kozopas et al., 1993; Reynolds et al., 1994), A1 (Lin et al., 1993), adenovirus E1B19kD (White et al., 1992), Epstein Barr Virus BHRF1 (Henderson et al., 1993), African Swine Fever Virus LMWS-HL (Neilan et al., 1993), and Human Herpes Virus 8 KSBcl-2 (Cheng et al., 1997b). The inducers of apoptosis include Bax (Oltvai et al., 1993), Bel-xS, an alternative splice variant of the Bel-x gene (Boise et al., 1993), Bad (Yang et al., 1995), Bak (Farrow et al., 1995; Kiefer et al., 1995; Chittenden et al., 1995b), Bik/Nbk (Boyd et al., 1995; Han et al., 1996), Bid (Wang et al., 1996a), Harakiri (Hrk) (Inohara et al., 1997), Bok (Iltu et al., 1997), Bim (O'Connor et al., 1998), and Blk (Hegde et al., 1998).

The ability of the opposing members of the Bcl-2 family to regulate apoptosis was found to be governed by their ability to form homo and heterodimers. Indeed, some of the family members such as Bax, were identified through co-immunoprecipitation with Bcl-2 (Oltvai et al., 1993; Yin et al., 1994). Bax was found to accelerate death and when over-expressed could override Bcl-2 repressor activity. Based on this activity, it was believed that the inherent protein ratio of Bcl-2:Bax, and therefore pro-apoptotic:anti-apoptotic protein ratios, were important in determining susceptibility to apoptosis (Oltvai et al., 1993; Oltvai and Korsmeyer, 1994). This idea was confirmed by demonstrations of heterodimer formation between other Bcl-2 family members. For example, pro-apoptotic Bax was found to interact with anti-apoptotic Bel-xL, Mcl-1 and A1 (Sedlak et al., 1995; Sato et al., 1994), and pro-apoptotic Bak could interact with Bel-xL and Bel-2 (Chittenden et al., 1995a).

1.6.4c Bcl-2 homology domains involved in the regulation of apoptosis

All Bcl-2-related proteins share homology in one to four regions designated as the Bcl-2 homology domains (BH1-BH4) (figure 1.3). Mutational analysis revealed that key BH domains in each Bcl-2 family member are required for their apoptotic activity and for their ability to form homo and heterodimers (reviewed in detail by Kelekar and Thompson, 1998).
The BH1 and BH2 domains are essential for the survival functions of Bcl-2 and Bcl-xL, and for their dimerisation with the death agonists Bax and Bak (Yin et al., 1994). However, heterodimerisation is not required for pro-survival function (Cheng et al., 1996). In contrast, the death agonists rely on their BH3 domain alone for their pro-apoptotic activity which is only exerted through heterodimerisation with the anti-apoptotic proteins (Chittenden et al., 1995a; Zha et al., 1996a; Cosulich et al., 1997). Recently however, Bax has been shown to have additional mechanisms of inducing death based on the presence of the BH1 and BH2 domains (Antonsson et al., 1997) (see section 1.6.7).

The three-dimensional structure of Bcl-xL has provided additional insight into the physical basis of heterodimerisation and further clarified the BH domains involved. Bcl-xL has been found to comprise of two central hydrophobic α-helices (α5 and α6), surrounded by five amphipathic helices (see figure 1.3). These helices are arranged such that the BH1, 2 and 3 homology domains form an elongated hydrophobic cleft (Muchmore et al., 1996). Further studies investigated the Bcl-xL:Bak complex, demonstrating that the BH3 domain of Bak forms an amphipathic α-helix which binds to the hydrophobic pocket of Bcl-xL via hydrophobic and electrostatic interactions (Sattler et al., 1997). This interaction therefore explains why although only BH1 and 2 domains are required for Bcl-2/Bcl-xL anti-apoptotic function (Yin et al., 1994), the additional BH3 domain is required for cleft formation allowing interaction with the BH3 death domains of the apoptotic induces (Sattler et al., 1997).

Although the importance of the BH1, 2 and 3 domains is well established, the role of the fourth N-terminal α-helical domain BH4, has until recently been uncertain. The domain is only possessed by Bcl-2, Bcl-xL and Bcl-w, and is essential for their survival activity, but not for their homodimerisation, or heterodimerisation with Bax (Borner et al., 1994; Lee et al., 1996; Huang et al., 1998). Recent findings have demonstrated that the BH4 domain is essential for interactions with other components of the apoptotic machinery, namely Ced-4/Apaf-1 (Huang et al., 1998) (see section 1.6.6). This implied that other anti-apoptotic members of the Bcl-2 family not possessing this domain cannot utilise this mechanism of death regulation (Huang et al., 1998).
**Pro-survival**

Bcl-2/Bcl-x\textsubscript{L}
Bcl-w/Mcl-1
A1

**Pro-apoptosis**

Bax/Bak/Bok
Bik(Nbk)/Hrk/Bim
Bad/Bid

*Figure 1.3 Members of the Bcl-2 family.* BH1 to BH4 are conserved sequence motifs within each family member. Of the pro-survival proteins Mcl-1 and A1 lack BH4. \(\alpha_1\) to \(\alpha_6\) depict regions corresponding to \(\alpha\)-helices in the tertiary structure of Bcl-x\textsubscript{L}. Modified from Adams & Cory, 1998.
1.6.4d Genetic ordering of the agonists and antagonists

Although mutational analysis demonstrated the importance of Bcl-2 family protein-protein interactions in regulating apoptosis, it proved inconclusive in determining whether the death agonists or antagonists were dominant in regulating apoptosis. In 1997, Knudson and Korsmeyer found that in bcl-2-deficient mice, which have reduced lymphocyte populations, the elimination of bax reverses the deficient phenotype, demonstrating that bax was functioning in the absence of bcl-2. In addition, they demonstrated that bcl-2 could prevent apoptosis in bax-deficient mice, and thus concluded that Bcl-2 and Bax function independently of each other (Knudson and Korsmeyer, 1997). In support of these findings, recent studies have shown that members of the Bcl-2 family, including Bcl-2 and Bax are able to form channels in lipid bilayers in vitro suggesting a mechanism for apoptotic regulation independent of dimerisation (Schendel et al., 1997; Minn et al., 1997; Antonsson et al., 1997).

1.6.4e Role of the Bcl-2 family in B Lymphocytes

As mentioned previously, cell death plays a pivotal role in the selection of B cell populations during B cell development. Bcl-2 was a clear candidate for the regulation of this process as over-expression of the protein prevented the death of B cells cultured in vitro (Nunez et al., 1990; Strasser et al., 1991), and its expression is diminished or absent in germinal centres, a site associated with excessive cell death (Hockenbery et al., 1991). In 1994, Merino and colleagues demonstrated developmental regulation of Bcl-2 in B lymphocytes, namely that Bcl-2 is highly expressed in pro-B and mature B cells, but it is down-regulated in pre-B cells undergoing Ig gene rearrangement, and in immature B cells within the germinal centre. These findings demonstrated that Bcl-2 expression correlates with the susceptibility of B cells to apoptosis: during selection they express low levels of Bcl-2, whereas after maturation, the levels increase, rendering the cells non-susceptible to apoptosis (Merino et al., 1994). In contrast to Bcl-2 expression, Bcl-xL is highly expressed in pre-B, but is down-regulated in pro-B and mature B cells (Grillot et al., 1996). This reciprocal pattern of Bcl-2/Bcl-xL expression suggested that the two proteins differ in their anti-apoptotic capacity at developmental stages, which is likely to be critical for lymphocyte development.
The use of gene deficient animals has revealed stage specific roles for Bcl-2 and Bcl-xL in lymphocyte development. Bcl-2-deficient mice develop normally, with successful lymphocyte development and differentiation. However, at 4 to 8 weeks of age, the lymphoid organs undergo massive cell death and involution, showing a failure to maintain homeostasis in both B and T cell populations, while other haematopoietic lineages remained unaffected (Veis et al., 1993; Nakayama et al., 1994; Kamada et al., 1995). The loss of Bcl-2 function does not seem to impair B cell development, but profoundly affects the survival of normal mature lymphocytes (Kamada et al., 1995). Other studies have demonstrated that Bcl-2 is essential for B cell selection within germinal centres (Liu et al., 1991b), and for the maintenance of B cell memory (Nunez et al., 1991), and so are consistent with the idea that Bcl-2 is required for the long-term maintenance of the immune system.

In contrast to Bcl-2, Bcl-xL-deficient mice exhibit massive cell death of haematopoietic cells in the embryonic liver, and die around embryonic day 13 (Motoyama et al., 1995). To further study the effects of this deficiency, chimaeric mice were generated which had lymphocytes derived from Bcl-xL-deficient embryonic stem cells. Motoyama and colleagues found that as the cells were capable of differentiation, the dramatic reduction in mature B cell numbers was directly related to the reduction in the number of immature (namely pre-B) cells. In addition, the lifespan of immature cells cultured in vitro was greatly reduced compared to those derived from wild-type mice. These data suggest that the gene-targeted deletion of Bcl-xL in B cells, severely impairs development beyond the pro-B stage, suggesting that Bcl-xL expression (probably in pre-B cells), is crucial for B cell development (Motoyama et al., 1995). Recent studies confirm these ideas and suggest that Bcl-xL in pre-B cells is important for regulating survival during initial light chain rearrangements (Fang et al., 1996; Behrens and Mueller, 1997). In addition to its role in pre-B cells, Fang and colleagues demonstrated that Bcl-xL is important in the clonal deletion of autoreactive immature cells. Bcl-xL over-expressing B cells are protected from developmental arrest and editing, thus allowing immature B cells that bind self antigen to survive. In contrast, Bcl-2 has no effect on developmental arrest. The researchers suggested that Bcl-xL protects cells at an early phase of the immature B cell stage, within which, antigen receptor engagement can initiate new Ig
rearrangement. Bcl-xL levels then fall in immature B cells allowing Ag receptor mediated death to proceed (Fang et al., 1998).

With respect to the pro-apoptotic members of the Bcl-2 family, Bax-deficient mice appear healthy but exhibit lymphoid hyperplasia, with mature B cell numbers being 1.8-fold greater than in wild-type mice (Knudson et al., 1995). Bax has also been found to be responsible for the lymphoid death in Bcl-2-deficient mice (Knudson and Korsmeyer, 1997). These results suggest that normal cell death during B cell development depends on Bax. This result is further supported by the recent finding that a loss of function mutation in Bax contributes to haematopoietic malignancies (Meijerink et al., 1998).

1.6.4f Regulation of the Bcl-2 family through post-translational mechanisms
Post-translational modifications of the Bcl-2 family have been found to regulate their ability to control apoptosis. The best understood mechanism involves the phosphorylation of the distant Bcl-2 family member Bad (reviewed by Franke and Cantley, 1997). In response to the survival factor IL-3, Bad is rapidly phosphorylated on two serine residues causing it to bind to a 14-3-3 protein. Bad is thereby sequestered in the cytosol and is no longer able to interact with Bcl-xL and inhibit its pro-survival function. The released Bcl-xL is then able to resume its activity and inhibit apoptosis (Zha et al., 1996b; Franke and Cantley, 1997).

Phosphorylation is also important in the regulation of Bcl-2, as it leads to a loss of its anti-apoptotic function (Halder et al., 1995). This can be induced in acute leukaemia and prostate cancer cell lines by the use of anti-cancer drugs such as taxol (Halder et al., 1996; 1997). The three dimensional structure of Bcl-xL revealed an unstructured 60-residue flexible loop within the protein and predicted a similar structure within Bcl-2 (Muchmore et al., 1996). Deletion of this loop was found to increase the anti-apoptotic activity of the proteins and, in addition, prevented protein phosphorylation, demonstrating the importance of this region in post-translational modifications (Chang et al., 1997).
Evidence also exists for positive Bcl-2 regulation by phosphorylation. Using a growth factor dependent myeloid cell line, Ito and colleagues demonstrated that a serine mutant (residue 70), was unable to be phosphorylated by IL-3, and when over-expressed was unable to support cell survival after IL-3 withdrawal, suggesting that phosphorylation is required for the anti-apoptotic activity of Bcl-2 in some cell types (Ito et al., 1997).

1.6.5 The Caspase family of apoptotic executioners

The term 'caspases' denotes a family of cysteine proteases which cleave their substrates after aspartic acid residues (Alnemri et al., 1996). The founding member of the family, interleukin-1β-converting enzyme (ICE or caspase-1), was initially identified due to homology with the C. elegans gene ced-3 (Yuan et al., 1993). Thirteen caspases have now been identified, which are subdivided into two main groups; those that seem to play a role in inflammation (caspases-1, 4, 5, 11, 12 and 13), and those that are largely involved in apoptosis. All caspases exist in cells as inactive precursors (zymogens) that are cleaved at aspartic acid residues to generate active proteases. Activated caspases then cleave a number of important cellular proteins (e.g. PARP, nuclear lamins, DNA-dependent kinases, PKC and Rb), other caspases, and some members of the Bcl-2 family, thus leading to irreversible cell death (Cohen, 1997; Cheng et al., 1997a; Clem et al., 1998).

Since caspases are cleaved at aspartic acid residues, some caspases can sequentially activate others thus establishing a hierarchy of caspases. Such a model was proposed by Fraser and Evan in 1996, in which some caspases (such as caspase 8, 9 and 10) act as initiator proteases to activate the effector caspases such as caspase-3 and 7. Determining the precise sequence of caspase activation is difficult, as expression and activation of the proteases seems to be cell type specific (Cohen, 1997).

Caspase-3 (also known as CPP32, Yama or Apopain), represents the closest mammalian homologue to Ced-3 (Fernandes-Alnemri et al., 1994), and has been identified as a key effector caspase, being partially or totally responsible for the cleavage of many key proteins (Nicholson et al., 1995; Tewari et al., 1995; Wang et
In addition, caspase-3 activates a downstream apoptotic effector DFF (DNA Fragmentation Factor), which induces DNA fragmentation, a hallmark of apoptosis (Liu et al., 1997a). The activation, and therefore the activity, of caspase-3 (and Ced-3), can be blocked by anti-apoptotic proteins such as Bcl-2, demonstrating that the inhibitors of mammalian apoptosis act, like their nematode counterpart, upstream of the death effectors (Shimizu et al., 1996; Chinnaiyan et al., 1996, 1997). Interestingly, activated caspase-3 can cleave Bcl-2 and Bcl-xL, converting these anti-apoptotic proteins into Bax-like proteins that promote death, thereby further enhancing the apoptotic demise of the cell (Cheng et al., 1997a; Clem et al., 1998).

The activation of 'effector' caspase-3 has been demonstrated in a number of apoptosis transduction pathways which involve other activator caspases. The tumour necrosis factor-1 (TNF-1) receptor and the CD95-Fas/Apo receptor, utilise the adapter molecule FADD (Fas-associating protein with death domain) to transduce their death signals into the cell (Boldin et al., 1995; Chinnaiyan et al., 1995). FADD recruits caspase-8 (FLICE/MACH) to the membrane bringing about its activation, which subsequently activates caspase-3 resulting in apoptotic demise of the cell. Within this system caspase-8 is therefore the apical caspase (Boldin et al., 1996; Muzio et al., 1996; 1997). Another pathway for caspase-3 activation involves cytochrome C, which in mammalian cells is released from the mitochondria during apoptosis (Liu et al., 1996). Released cytochrome C (Apaf-2), then induces the dATP-dependent formation of the Apaf (Apoptosis protease activating factor) protein complex that subsequently activates pro-caspase-3 (Liu et al., 1996). The complex consists of the Ced-4 homologue Apaf-1, and caspase-9 (Apaf-3), which represents the upstream activating caspase in this pathway of caspase-3 activation (Li et al., 1997; Zou et al., 1997).

Although an essential component of the apoptotic system, the exact contribution of caspase-3 is unclear as requirement for this effector is tissue-specific and can even be stimulus-specific within the same cell type, suggesting that the apoptotic machinery used to implement death is more complex than originally envisaged (Woo et al., 1998). This idea supports previous work which demonstrated that Bax was capable of inducing cell death independently of known caspases, suggesting an alternative pathway to death (Xiang et al., 1996).
1.6.6 Apaf-1: linking of the regulators and activators of apoptosis

Apaf-1 (Apoptotic protease activating factor 1), is the mammalian homologue of Ced-4 in *C. elegans* (Zou *et al.*, 1997). Within the nematode system, Ced-4 was found to interact with and activate Ced-3 and members of the mammalian caspase family, thereby promoting apoptosis. In addition, Ced-9 and the mammalian homologue Bcl-x₁ are able to interact with and inhibit Ced-4 death activity and binding to Ced-3 (Chinnaiyan *et al.*, 1997; Wu *et al.*, 1997).

In the presence of cytochrome C (Apaf-2) and dATP, Apaf-1 has been found to bind to caspase-9 (Apaf-3) via its caspase recruitment domain (CARD) (Hofmann *et al.*, 1997), creating an Apaf complex which leads to the activation of caspase-3 (Li *et al.*, 1997; Pan *et al.*, 1998). In line with the Ced-4 nematode model, Apaf-1 also complexes with Bcl-x₁, suggesting a potential mechanism by which anti-apoptotic proteins may prevent Apaf-1 from interacting with and activating the caspases (Hu *et al.*, 1998; Pan *et al.*, 1998).

Apaf-1-deficient mice have recently demonstrated the *in vivo* importance of Apaf-1 in apoptosis. These mice exhibit severe craniofacial malformation, brain overgrowth, persistence of interdigital webs, and cannot survive beyond embryonic day 16.5 (Yoshida *et al.*, 1998; Cecconi *et al.*, 1998). In addition, these mice did not respond to various apoptotic stimuli and could not activate caspase-3 (Yoshida *et al.*, 1998; Cecconi *et al.*, 1998). Caspase-3-deficient mice also have alterations in brain development suggesting that the Apaf-1/caspase-3 pathway of apoptosis activation is important in these tissues (Kuida *et al.*, 1996). In addition, caspase-9-deficient mice are unable to perform caspase-3-mediated death, demonstrating the importance of the entire Apaf complex in the activation of apoptosis (Kuida *et al.*, 1998).

Apaf-1 is however, not essential for all apoptotic pathways. Apaf-1 is dispensable for the CD95-Fas/Apo signalling pathway that is not subject to Bcl-2 regulation (Scaffidi *et al.*, 1998; Yoshida *et al.*, 1998). However, it remains to be seen if this apoptotic component is involved in CD95 signalling that is subject to Bcl-2 family regulation. In addition, because mammals possess multiple caspase and Bcl-2 homologues, it is
envisaged that Apaf-1 will also have relatives that potentially may mediate other pathways of apoptosis.

### 1.6.7 Bcl-2 family proteins and mitochondria

The multi-functional capability of the Bcl-2 family to regulate apoptosis has already been highlighted in that these proteins are able to dimerise with other members of the Bcl-2 family, and can also bind to non-homologous proteins such as Apaf-1. A third regulatory mechanism has recently come to light, that centres on the regulation of various mitochondrial events during apoptosis. The events associated with mitochondria during apoptosis such as, changes in electron transport and the alteration of cellular reduction-oxidation (redox) potential, will not be discussed here, but have recently been reviewed by Green and Reed (1998), and Reed et al. (1998).

The most compelling evidence for the role of mitochondria in the execution of apoptosis is the release of the pro-apoptotic proteins cytochrome C and AIF (Apoptosis Inducing Factor). The latter appears to directly activate members of the caspase family (Susin et al., 1996), whereas cytochrome C, as already discussed, is the component in the Apaf complex responsible for the activation of caspase-3 (Liu et al., 1996; Li et al., 1997). Several research groups have demonstrated that the over-expression of Bcl-2 can prevent apoptosis by blocking the release of cytochrome C from mitochondria (Kluck et al., 1997; Yang et al., 1997). Conversely, Bax was found to induce directly cytochrome C release, suggesting a way in which the opposing members of the Bcl-2 family may act (Jurgensmeier et al., 1998). The mechanism behind cytochrome C release was initially believed to be due to the loss of mitochondrial transmembrane potential ($\Delta\Psi_{m}$), that is mediated by the opening of the mitochondrial permeability transition (PT) pore (reviewed by Green and Reed, 1998). However, a recent study by Bossy-Wetzel and colleagues, demonstrated that cytochrome C is released before any reduction in $\Delta\Psi_{m}$, suggesting that the PT pore is not involved. Based on this and a number of other observations, the researchers proposed a model of apoptosis involving mitochondria. The model suggests that apoptotic signals induce the release of cytochrome C from mitochondria, activating the caspases through the Apaf complex. The activated caspases then act upon
mitochondria to induce permeability transition and a reduction in $\Delta \Psi_m$, which triggers the release of AIF and the generation of reactive oxygen species (ROS) (Bossy-Wetzel et al., 1998). A recent study has demonstrated that cytochrome C itself is not detrimental to cells, and that Bcl-2 must have important functions downstream of the mitochondria, since Bcl-2 could inhibit Bax-induced death, but could not prevent the release of cytochrome C from the mitochondria (Rosse et al., 1998).

A clue to how the Bcl-2 proteins exert their mitochondrial effects has come from the structural analysis of the proteins, which demonstrated their striking similarity to the pore-forming domains of certain bacterial toxins that act as channels for either ions or proteins (Muchmore et al., 1996). There is now direct evidence that Bcl-2, Bcl-xL, and Bax have ion channel activity when incorporated into synthetic lipid membranes (Schendel et al., 1997; Minn et al., 1997; Antonsson et al., 1997). Significantly, Bcl-2 is able to interfere with the ability of Bax to form channels, suggesting a further mechanism for the antagonism displayed between these two proteins (Antonsson et al., 1997). Bax is believed to be able to form a large channel in the outer mitochondrial membrane, allowing the release of cytochrome C without changes to $\Delta \Psi_m$ (Jurgensmeier et al., 1998). In contrast, the small conductance channels formed by Bcl-2 and Bcl-xL seem to have a role in regulating the membrane potential and volume homeostasis of mitochondria (Vander Heiden et al., 1997).

### 1.6.8 Involvement of the Bcl-2 family and other apoptotic regulatory genes in cancer.

Normal lymphoid development and homeostasis can be disrupted by mutations that interfere with the normal proliferative or cell death process, leading to the development of lymphomas and leukaemias. As discussed, the Bcl-2 family is one of the main regulators of apoptosis. Deregulation of these genes, i.e., the loss of function of pro-apoptotic genes, or gain of function of anti-apoptotic genes, is therefore of significance to the development of cancers. In addition to this family, other proto-oncogenes traditionally associated with proliferation, have now been found to also have roles in regulating apoptosis (reviewed by King and Cidlowski, 1998), thus widening the contribution of the genes in malignancies. Some of these genes are able
to cooperate with the effects of deregulated \textit{bcl-2}, further enhancing the survival and growth advantage of the cancer. The contribution of some Bcl-2 family members, p53, and c-myc to malignancies will now be briefly discussed.

1.6.8a Bcl-2
Deregulation of \textit{bcl-2} expression, leading to elevated levels of Bcl-2 mRNA and protein, has been reported in various leukaemias and lymphomas (reviewed by Kusenda, 1998). In addition to the classical t(14;18) chromosomal translocation associated with this gene (Tsujimoto \textit{et al.}, 1984), Bcl-2 over-expression has also been associated with amplification of the gene (Pettersson \textit{et al.}, 1992; Monni \textit{et al.}, 1997). However, Bcl-2 on its own is considered to be insufficient to elicit transformation, supported by the finding that t(14;18) translocations are regularly generated in normal individuals and require additional oncogenic ‘hits’ to establish a malignant phenotype (Limpens \textit{et al.}, 1995). In addition to its anti-apoptotic function, Bcl-2 has also been found to restrain cell cycle entry, and as such may in part be responsible for the low oncogenic potential of Bcl-2 (O’Reilly \textit{et al.}, 1996; Huang \textit{et al.}, 1997).

1.6.8b Bax
Investigations by Yin and colleagues have demonstrated that the pro-apoptotic protein Bax acts \textit{in vivo} as a tumour suppressor, and that the loss of this molecule promotes tumorigeneisis (Yin \textit{et al.}, 1997). Bax mutations have been identified in a number of human haematopoietic malignancies, most commonly in the acute lymphoblastic leukaemia subset (Meijerink \textit{et al.}, 1995, 1998). These mutations, including nucleotide insertions/deletions and single amino acid substitutions, demonstrated altered patterns of protein dimerisation and a loss of death promoting activity, thus supporting the role of Bax as a tumour suppressor (Meijerink \textit{et al.}, 1998).

1.6.8c p53
Mutations in the tumour suppressor gene \textit{p53} are the most frequent abnormality in human cancer, implying that loss of this gene function represents a fundamentally important step in the pathogenesis of cancer. Wild-type p53 plays a pivotal role in both cell cycle arrest (stages G1 and G2), and in the induction of apoptosis under certain conditions of DNA damage and cellular stress (reviewed by Levine, 1997).
Inactivation or loss of p53 therefore allows cells that have sustained DNA damage to both survive and replicate, which has two devastating effects. Firstly, the cells accumulate further mutations that contribute to malignancy, and secondly, the cells can become resistant to anti-cancer drugs and ionising radiation treatments. The ability of p53 to induce cell cycle arrest has been ascribed to its ability to induce expression of the cellular gene WAF1 that encodes the G1 cyclin-dependent kinase inhibitor p21 (el-Deiry et al., 1994; Harper et al., 1993).

In relation to its apoptotic function, an important finding was the discovery of a p53-dependent negative response element in the bcl-2 promoter through which, p53 can down-regulate bcl-2 gene expression (Miyashita et al., 1994a; 1994b). It is therefore possible that p53 inactivation, and thus the loss of p53-mediated repression of the bcl-2 gene, can account in part for the high levels of Bcl-2 found in some malignancies that do not exhibit gross chromosomal alterations of the bcl-2 gene (Miyashita et al., 1994b). In addition, p53 can simultaneously up-regulate the pro-apoptotic bax gene (Miyashita et al., 1994b; Selvakumaran et al., 1994; Zhan et al., 1994), through p53-responsive elements in the bax promoter (Miyashita and Reed, 1995). This pro-apoptotic regulator seems to be a component of the p53-mediated apoptotic pathway in some cell types, as Bax-deficient mice exhibit accelerated tumour growth and decreased p53-mediated apoptosis (Yin et al., 1997). However, other unidentified p53 regulated genes must play important roles in a cell-specific manner as bax-deficient thymocytes are not defective in p53-dependent apoptosis (Knudson et al., 1995). It is probable that other pro-apoptotic members of the Bcl-2 family such as Bad or Bik will be components of the p53-mediated apoptotic pathway.

1.6.8d c-Myc

Substantial evidence implicates the c-myc proto-oncogene in the control of cell proliferation; it is one of the immediate early response genes rapidly induced upon mitogenic stimulation, and the c-Myc protein is continuously expressed in proliferating but not in quiescent cells. Deregulated expression of this gene, resulting in constitutive expression, is frequent in cancer (Facchini and Penn, 1998). In addition to its proliferative effects, c-myc has also been found to promote apoptosis (reviewed by Thompson, 1998). This apparently contradictory activity of c-Myc has been
demonstrated with the use of growth factor (GF)-dependent cell lines. When over-expressed in the absence of GF, c-Myc induces apoptosis, but when excess GF is present cell death does not occur (Askew et al., 1991; Evan et al., 1992). c-Myc-induced apoptosis can however be prevented by the simultaneous over-expression of Bcl-2 (Bissonnette et al., 1992; Fanidi et al., 1992). This confirmed earlier studies that demonstrated the cooperation of these two oncogenes in the transformation of lymphoid cells (Vaux et al., 1988; Strasser et al., 1990). The simultaneous appearance of both bcl-2 and c-myc translocations is common in B cell neoplasms (Facchini and Penn, 1998). A 'dual signal' hypothesis has emerged to explain c-Myc-dependent apoptosis (Harrington et al., 1994). This suggests that c-Myc elicits two signals, one for proliferation, and one for apoptosis. In the presence of survival factors (such as constitutive Bcl-2 expression), the apoptotic signal is blocked, resulting in potent transformation due to the presence of both survival and growth advantages. Cooperation between c-myc and bcl-2 is further illustrated by the ability of these oncogenes to antagonise p53. Co-expression of these oncogenes has been shown to block both growth arrest and apoptotic functions of p53, whereas bcl-2 alone is only able to block the latter (Ryan et al., 1994b).

In tumour development, c-Myc has also been found to cooperate with mutant p53. Co-expression of mutant p53 in myeloid leukaemic cells expressing deregulated c-myc, exhibited increased leukaemogenicity in vivo, compared to cells only expressing deregulated c-myc (Lotem and Sachs, 1995). These results demonstrate another cooperative mechanism causing malignant transformation.

In conclusion, tissue homeostasis requires the coordinated regulation of the antagonistic processes of proliferation and apoptosis. Genes involved in the intracellular signalling and the final elucidation of these processes are prone to genetic alterations, and as such contribute to the multi-causal process of malignancy. In addition, mechanisms of malignant cell survival may involve genes involved in cell surface interactions, such as those encoding cytokines and their receptors, and cell adhesion molecules. This may lead to the deregulated production of cytokines, or changes in the ability to receive incoming signals, i.e., an increased number and/or affinity of cytokine receptors, and the expression of receptors or adhesion molecules.
not normally present on the cell, thus changing the repertoire of signals to which the cell is able to respond.

1.7 SMS-SB CELLS: A CHILDHOOD PRE-B ACUTE LYMPHOBLASTIC LEUKAEMIA CELL LINE

1.7.1 Acute lymphoblastic leukaemia

Acute lymphoblastic leukaemia (ALL), is a malignant disorder mainly regarded as a childhood disease. It is characterised by unrestricted cell growth and maturational arrest of haematopoietic precursor cells in bone marrow, peripheral blood and other tissues (reviewed by Cortes and Kantarjian 1995; Lilleyman, 1997). The disease is currently classified on the basis of immunophenotyping, allowing the cell lineage and the step in differentiation in which transformation occurred to be determined. This process partly involves the identification of various clusters of differentiation (CD) antigens. For example, CD10, also known as the common ALL antigen (CALLA), was the first antigen to be used in the classification of ALL (Greaves et al., 1975). This antigen is a lineage-independent marker and is not universally expressed in ALL, with lack of expression being often associated with poor prognosis (Cortes and Kantarjian, 1995). In addition to CD antigens, other characteristics assessed for classification purposes include cytoplasmic and surface immunoglobulins, TdT, and HLA-DR which represents the expression of class II histocompatibility antigens.

Based on immunophenotyping, ALL can be broadly classified as having a T or B-lineage origin. The latter includes a number of subtypes: early-pre-B (cytoplasmic (c) Ig^−, surface (s) Ig^−), pre-B (c Ig^+, s Ig^−), transitional B (c Ig^+, s Ig^−, surface μ heavy chains but no light chains), and mature B (s Ig^+) (Cortes and Kantarjian 1995). In addition, some ALL’s have been found to also express myeloid markers, and may thus represent the transformation of a pluripotent cell or a rare progenitor cell expressing markers and features from several lineages (Smith et al., 1983; Greaves et al., 1986).
1.7.2 The SMS-SB cell line

The SMS-SB cell line was initially characterised by Smith and colleagues in 1981. The cells were derived from a 16 year old African-American girl (SB) in the leukaemic phase of a lymphoblastic lymphoma. The original lymphoma was unusual as it originated in and around skeletal bone and bone marrow rather than in lymphatic tissue, and did not express any T-lymphocyte markers which are usually typical of lymphoblastic lymphomas (Smith et al., 1981; Smith, 1984). This distribution of disease is typical of lymphomas induced in mice by the Abelson Leukaemia Virus (Abelson and Rabstein, 1970). The cultured leukaemic SMS-SB cells are designated as pre-B lymphocytes by virtue of the expression of B-cell markers, the presence of cytoplasmic \( \mu \) heavy chains, and the absence of both surface Ig and any T-cell specific markers. SMS-SB cells seem to most closely resemble the major population of pre-B cells found in normal bone marrow, and differ from other cultured pre-B ALL cell lines in that they do not express high levels of CD10 (CALLA), or the enzyme TdT (Smith, 1984).

SMS-SB cells do not contain the Epstein Barr Virus nuclear antigen, demonstrating that this virus did not cause transformation of these cells (Smith et al., 1981). Karyotypic analysis has shown that SMS-SB cells do not exhibit any gross chromosomal abnormalities, such as the \( t(1;19) \) translocation which is commonly seen in pre-B ALL. This translocation results in the fusion of E2A (an immunoglobulin enhancer-binding protein), with PBX (a homeobox protein that acts as a transcription factor), to create a constitutively expressed PBX gene not normally found in pre-B cells (Nourse et al., 1990). The \( t(9;22) \) translocation (the Philadelphia chromosome), is also implicated in acute leukaemia. This transposes the \( c-abl \) gene (the cellular homologue of the Abelson viral oncogene), to the \( bcr \) (breakpoint cluster region) gene. The resulting chimeric protein has increased tyrosine kinase activity compared to the normal \( c-ABL \) protein (Konopka et al., 1994). Upon analysis of \( c-abl \) in SMS-SB cells, Ozanne and colleagues discovered no gross rearrangements but found 2 additional abl-related transcripts not found in normal cells. The researchers suggested that this aberrant expression may have assisted in the initiation of malignancy in these cells, and may have synergised with other activated oncogenes (Ozanne et al., 1982).
The development of new molecular diagnostic techniques have improved the characterisation of genetic abnormalities in ALL, and have identified abnormalities not identified by routine karyotyping (Rubnitz and Pui, 1997). In the light of these recent improvements, it is possible that SMS-SB cells do contain as yet undetected abnormalities.

In a further attempt to characterise SMS-SB, Tsai and colleagues examined a number of nuclear proto-oncogenes, the expression of which are often altered in leukaemias/lymphomas. The researchers discovered that compared with other cell types, SMS-SB cells exhibit normal expression of c-myc, c-myb and c-jun, but over-express the c-fos proto-oncogene and the corresponding protein p55<sup>c-fos</sup>. The c-fos gene did not appear to harbour any mutations, and the elevated levels of c-fos transcripts were found to be due to up-regulated transcription, which could be further induced by serum. Consistent with the over-expression of c-fos mRNA, the p55<sup>c-fos</sup> protein is also elevated in SMS-SB compared to another ALL cell line, Nalm-6. This protein is known to heterodimerise with members of the Jun family of proteins to form the transcription factor AP-1 (Activator protein 1) (reviewed by Karin <i>et al</i>, 1997).

SMS-SB cells do not however exhibit elevated amounts of AP-1 DNA binding activity compared to Nalm-6 cells (Tsai <i>et al</i>, 1991).

1.7.2a SMS-SB cells produce growth promoting factors

After removal from patient SB, the leukaemic cells adapted easily to tissue culture without going through a crisis phase. The cells were able to be grown in both serum-free and protein-free media without the addition of exogenous mitogens (Smith <i>et al</i>, 1981). The immediate establishment of human leukaemic cells is unusual, and thus prompted Zack and colleagues to investigate the growth-regulatory factors produced by SMS-SB cells. Two growth activities were identified from culture supernatants, one which acts as an autocrine factor to enhance the growth of SMS-SB cells placed at low cell density in serum-free medium, and another, termed leukaemia-derived-transforming growth factor (LD-TGF), promotes the growth of fibroblasts, but does not act on SMS-SB cells or other haematopoietic cells (Zack <i>et al</i>, 1987).
More recent investigations by White (1995), further studied SMS-SB cell growth characteristics, and the production of the SMS-SB autocrine growth factor (SB-AF). SB-AF present in the supernatant from normal cultures, was found not to be directly mitogenic for SMS-SB cells, but was able to promote the growth of cells placed at low cell density in protein-free medium, conditions at which the cells would normally cease growth and undergo apoptosis. In an attempt to identify the autocrine factor, numerous cytokines were tested for the ability to substitute for SB-AF activity. Two potential candidates, platelet-derived growth factor (PDGF), and the soluble form of CD23 (sCD23), were identified. Although SMS-SB cells had previously been found to secrete PDGF and express PDGF receptors (Tsai et al., 1994), White demonstrated by the use of anti-PDGF neutralising antibodies, that PDGF cannot account for the autocrine activity of SB-AF. In addition, the cells were found not to express CD23, therefore also negating this cytokine as the SMS-SB autocrine factor (White, 1995).

1.7.2b SMS-SB cells express a novel receptor for CD23

As previously mentioned, sCD23 was found to promote the growth of low cell density SMS-SB cells but did not constitute the autocrine factor as the cells do not express CD23 (White, 1995). In addition, SMS-SB cells were found not to express any of the known receptors for CD23 namely, CD21, CD11b or CD11c (Aubry et al., 1992, Lecoanet-Henchoz et al., 1995), but could specifically bind to full-length (45kDa) CD23a-containing fluorescent liposomes. This demonstrated that SMS-SB cells express a novel receptor for CD23 (CD23NR), stimulation of which can prevent apoptosis of these cells. Preliminary results from CD23 affinity columns suggest that the novel receptor has a molecular weight of approximately 80kDa, but further characterisation is required. (White, 1995).

1.8 RESEARCH AIMS

The original aim of this thesis was to further study the SMS-SB autocrine growth factor. This included, an investigation into the mechanism by which the factor influenced the cells, the characterisation and isolation of the factor, and the determination of its role in SMS-SB and normal B cell growth and development.
A second aim was to study the effect of 45kDa CD23a on SMS-SB, and address whether, like sCD23, this membrane-associated form of CD23 could also transmit anti-apoptotic signals. These studies were also hoped to generate a suitable screening procedure for the production of monoclonal antibodies towards the novel CD23 receptor component(s), since the absence of any readily available 45kDa CD23 to be used in liposomes prevented antibodies from being screened for the ability to inhibit CD23 liposome binding to SMS-SB.

It was also hoped to investigate the existence of the novel receptor for CD23 on other B cell types, with the hope of investigating the role of this receptor in leukaemic and normal B cell growth and development.
CHAPTER 2

MATERIALS AND METHODS
2.1 MATERIALS

2.1.1 General chemicals and materials

Unless otherwise stated below, all chemicals/reagents were of ‘AnalaR’ grade and purchased from Sigma-Aldrich or BDH Chemicals, both Poole, Dorset, England.

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<th>Chemical/Reagent</th>
<th>Supplier</th>
</tr>
</thead>
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</tr>
<tr>
<td>α-[³²P]-dCTP (3000 Ci/mmol)</td>
<td>Amersham Life Science Ltd, Bucks, UK</td>
</tr>
<tr>
<td>Methyl-[³H] Thymidine (20-30 Ci/mmol)</td>
<td></td>
</tr>
<tr>
<td>Rainbow protein molecular markers</td>
<td></td>
</tr>
<tr>
<td>Hybond™ ECL nitrocellulose membrane</td>
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</tr>
<tr>
<td>Hybond™-N</td>
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</tr>
<tr>
<td>BIACore Sensor Chip CM5</td>
<td>BIACORE AB, Herts, UK</td>
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<td>RNAzol B</td>
<td>Biogenesis Ltd, Poole, UK</td>
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<tr>
<td>dNTP’s</td>
<td>Boehringer Mannheim Ltd, Sussex, UK</td>
</tr>
<tr>
<td>Restriction enzymes and Buffers</td>
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</tr>
<tr>
<td>Express Hyb hybridization solution</td>
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</tr>
<tr>
<td>Saponin</td>
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</tr>
<tr>
<td>1kb DNA ladder</td>
<td>Life Technologies Ltd, Paisley, UK</td>
</tr>
<tr>
<td>0.24-9.5kb RNA ladder</td>
<td></td>
</tr>
<tr>
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<td>Bacto-tryptone</td>
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<td>Yeast extract</td>
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<td>MicroSpin ™ S-200 HR Columns</td>
<td>Pharmacia Biotech, Herts, UK</td>
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<td>T4 DNA Ligase and buffers</td>
<td>promega UK Ltd, Southampton, UK</td>
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<td>Restriction enzymes and buffers</td>
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<td>JM109 Bacteria</td>
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<td>Cloned Pfu DNA Polymerase</td>
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<tr>
<td>Vectashield Mounting Medium</td>
<td>Vector Laboratories Inc, Peterborough, UK</td>
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2.1.2 Kits

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<th>Kit</th>
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<tr>
<td>Oligo-labelling kit</td>
<td>Pharmacia Biotech, Herts, UK</td>
</tr>
<tr>
<td>pCR-Script™ SK (+), cloning vector kit</td>
<td>Stratagene Ltd, Cambridge, UK</td>
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<tr>
<td>BigDye™ DNA Sequencing kit</td>
<td>PE Applied Biosystems, Warrington, UK</td>
</tr>
<tr>
<td>QIAGEN Plasmid Maxi Purification Kit</td>
<td>QIAGEN Ltd, West Sussex, UK</td>
</tr>
<tr>
<td>QIAGEN, QIAquick Gel Extraction Kit</td>
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2.1.3 Cell lines

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<th>Human B cell lines</th>
<th>Description/Supplier</th>
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<tr>
<td>Blin-1</td>
<td>Pre-B acute lymphoblastic leukaemia (ALL)</td>
</tr>
<tr>
<td>Nalm-1</td>
<td>Pre-B ALL</td>
</tr>
<tr>
<td>Nalm-6</td>
<td>Pre-B ALL</td>
</tr>
<tr>
<td>697</td>
<td>Pre-B ALL</td>
</tr>
<tr>
<td>207</td>
<td>Pre-B ALL</td>
</tr>
<tr>
<td>1E8</td>
<td>Mature B ALL</td>
</tr>
<tr>
<td></td>
<td>Gift from Professor R.E. Callard, Institute of Child Health, London, UK</td>
</tr>
<tr>
<td>SMS-SB</td>
<td>Pre-B ALL, Gift from Professor B Ozanne, CRC Beatson, Glasgow, UK</td>
</tr>
<tr>
<td>EDR</td>
<td>EBV transformed Human Mature B cell</td>
</tr>
<tr>
<td></td>
<td>Gift from Professor C Watts, Dundee University, UK</td>
</tr>
<tr>
<td>Non Human lines</td>
<td></td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------------------------------------------------------</td>
</tr>
<tr>
<td>COS7</td>
<td>SV40 transformed, African green monkey kidney cells. Gift from Professor G Milligan, Glasgow University, UK</td>
</tr>
<tr>
<td>NRK-49F</td>
<td>Normal Rat Kidney fibroblasts, Gift from M Lacy, CRC Beatson, Glasgow, UK</td>
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### 2.1.4 Cell culture materials and reagents

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<td>Disposable plastic pipettes</td>
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<td>BM-Cyclin (mycoplasma removal agent)</td>
<td>Boehringer Mannheim Ltd, Sussex, UK</td>
</tr>
<tr>
<td>G418</td>
<td>Calbiochem-Novabiochem, Nottingham, UK</td>
</tr>
<tr>
<td>Tissue culture flasks</td>
<td>Corning Costar, Buckinghamshire, UK</td>
</tr>
<tr>
<td>Disposable cell scrapers</td>
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</tr>
<tr>
<td>96 and 6 well plates</td>
<td></td>
</tr>
<tr>
<td>RPMI-1640 medium</td>
<td>Life Technologies Ltd, Paisley, UK</td>
</tr>
<tr>
<td>Protein-Free Hybridoma Medium II (PFHMI)</td>
<td></td>
</tr>
<tr>
<td>Optimum</td>
<td></td>
</tr>
<tr>
<td>Glutamine</td>
<td></td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
</tr>
<tr>
<td>Trypsin</td>
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</tr>
<tr>
<td>Foetal calf serum</td>
<td></td>
</tr>
<tr>
<td>Cryovials</td>
<td></td>
</tr>
<tr>
<td>Haemocytometer</td>
<td>Phillip Harris Scientific, Lanarkshire, UK</td>
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<tr>
<td>Cell Dissociation Media</td>
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</tr>
<tr>
<td>Trypan blue</td>
<td>Sigma-Aldrich, Dorset, UK</td>
</tr>
<tr>
<td>Dulbecco’s Modified Eagles Medium (DMEM)</td>
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</tr>
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### 2.1.5 Transfection reagents

<table>
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<th>Reagent</th>
<th>Supplier</th>
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<tr>
<td>DOTAP</td>
<td>Boehringer Mannheim Ltd, Sussex, UK</td>
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<tr>
<td>Gene Pulser Electroporation Cuvette</td>
<td>Bio-Rad Laboratories Ltd, Herts, UK</td>
</tr>
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### 2.1.6 Antibodies

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Supplier</th>
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<tbody>
<tr>
<td>Protein A horse-radish peroxidase-linked</td>
<td>Amersham Life Science Ltd, Buckinghamshire, UK</td>
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<tr>
<td>Mouse anti-human B cell, CD19-FITC</td>
<td>DAKO, Denmark</td>
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<tr>
<td>Mouse anti-human Bcl-2 monoclonal</td>
<td>Pharmingen, San Diego, USA</td>
</tr>
<tr>
<td>Rabbit anti-human : Bcl-2 (N-19)</td>
<td>Santa Cruz Biotechnology Inc, USA</td>
</tr>
<tr>
<td>: Bax (I-19)</td>
<td></td>
</tr>
<tr>
<td>: Bcl-xL (L-19)</td>
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<tr>
<td>Sheep anti-mouse IgG-FITC</td>
<td>SAPU (Scottish Antibody Production Unit)</td>
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<td>Lanarkshire, UK</td>
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<tr>
<td>Mouse anti-human CD21-FITC</td>
<td>Serotec, Oxford, UK</td>
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<tr>
<td>Mouse anti-human CD11a-FITC</td>
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<tr>
<td>Mouse anti-human CD11b/c -FITC</td>
<td>Sigma-Aldrich, Poole, Dorset, UK</td>
</tr>
<tr>
<td>Rabbit anti-mouse IgG-FITC</td>
<td></td>
</tr>
<tr>
<td>Mouse anti-human CD23-FITC</td>
<td>The Binding Site Ltd, Birmingham, UK</td>
</tr>
<tr>
<td>Rabbit-anti-human CD23</td>
<td>Gift from Dr J-Y Bonnefoy, Geneva Biomedical Research Institute, Switzerland</td>
</tr>
<tr>
<td>Mouse anti-human CD23</td>
<td>Gift from Professor J Gordon, Birmingham University, UK</td>
</tr>
</tbody>
</table>
2.1.7 CD23 cytokine

Initial stocks of affinity-purified 25kDa sCD23 were a gift from Dr J-Y Bonnefoy, Geneva Biomedical Research Institute, Switzerland. Subsequent stocks were produced by Dr. J. Matheson, University of Glasgow, UK. The cytokine was produced in baculovirus-infected Sf9 cells as previously described (Jansen et al., 1991; Flores-Romo et al., 1993).

2.1.8 Plasmids

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description/Supplier</th>
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<tr>
<td>pcDL SRα296CD23a</td>
<td>Full length CD23a cDNA in expression vector pcDL SRα. A gift from Dr. J-Y Bonnefoy,</td>
</tr>
<tr>
<td></td>
<td>Geneva Biomedical Research Institute, Switzerland</td>
</tr>
<tr>
<td>pcDNA3.1(+)</td>
<td>Expression vector from Invitrogen, Netherlands</td>
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2.1.9 cDNA probes for Northern blotting

<table>
<thead>
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<th>Probe</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>c-fos</td>
<td>Full-length cDNA probes. Gifts from Dr. J Winnie, CRC Beatson, Glasgow, UK</td>
</tr>
<tr>
<td>c-myc</td>
<td></td>
</tr>
<tr>
<td>c-jun</td>
<td></td>
</tr>
<tr>
<td>7S</td>
<td></td>
</tr>
</tbody>
</table>
2.2 METHODS

All solutions and buffers referred to in the following sections are detailed in the appendix.

2.2.1 Cell culture

2.2.1a Culture of Human B Lymphocytes

SMS-SB cells were maintained routinely in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated foetal calf serum (heated at 57°C for 50 minutes), 2mM L-glutamine and 0.05mg/ml gentamicin. For some experiments the cells were also cultured in Protein-Free Hybridoma Medium II (PFHMII) or in the serum-free medium Optimem. All other B cell lines were cultured in supplemented RPMI-1640 medium only. All cells were routinely sub-cultured every 2-3 days depending on experimental requirements. Cells were cultured in 25 or 75cm² flasks at 37°C in a humidified 6% CO₂ incubator and manipulated aseptically in Laminar-flow-hoods.

2.2.1b Culture of non-B cell lines

COS7 and NRK-49F cells were maintained in Dulbecco’s Modified Eagles Medium (DMEM), supplemented with 10% (v/v) foetal calf serum, 2mM glutamine and 0.05mg/ml gentamicin. Cells were sub-cultured every 3-4 days using trypsin (0.25% w/v in PBS). Stably transfected CD23aCOS7 cells were maintained in supplemented DMEM medium and 800µg/ml G418, and sub-cultured using non-enzymatic Cell Dissociation Solution, to prevent cleavage of the CD23 membrane protein. All cells were cultured in 75cm² flasks or 9cm tissue culture treated petri dishes and maintained under conditions as for B cell lines.

2.2.1c Frozen cell stocks

Frozen stocks of each cell line routinely used in culture were maintained in liquid nitrogen. 10⁷ logarithmically growing cells were centrifuged and resuspended in 1ml of chilled freezing media (90% (v/v) heat-inactivated FCS and 10% (v/v) DMSO (dimethyl sulfoxide)), and quickly transferred to 1ml cryovials. Vials were then kept at
-70°C for 3-4 days before being transferred to liquid nitrogen. Cells removed from nitrogen storage were rapidly thawed and immediately washed in 10ml of appropriate medium to remove residual DMSO. The cells were then resuspended in medium and allowed to recover overnight before further manipulation.

### 2.2.1d Detection of mycoplasma

Cell lines were tested for the presence of mycoplasma by staining with the fluorescent dye Hoechst 33258 (2- [2- (4-hydroxy-phenyl) -6- benzimidazolyl] -6- (1-methyl-4-pierpazyl) -benzimidazol -tri-hydrochloride) as previously described (Chen, 1977). Cell culture supernatants where tested for their ability to infect NRK-49F, a cell line consistently negative for mycoplasma infection. Briefly, NRK-49F cells were incubated for 2-3 days in 50% (v/v) ‘test’ supernatant, which had been in contact with cells for 4 days before removal. NRK-49F cells were then fixed with 3:1 methanol:glacial acetic acid, and stained with Hoechst 33258 (0.05μg/ml in PBS) for 10 minutes in the dark. Cells were then rinsed with dH₂O and visualised under a Leitz Laborluxs fluorescent microscope using filter position 2. Mycoplasma contamination was identified by the presence of green/yellow fluorescent speckles in and around the cells, additional to the main fluorescent nuclear components of normal healthy cells.

Mycoplasma was detected in all SMS-SB cell stocks and so these were treated with BM-Cyclin in accordance with the manufacturers instructions. The cells were then frequently re-tested for the return of infection and re-treated when necessary. All other cell lines used were consistently negative for mycoplasma.

### 2.2.1e Determination of cell number and viability of B cell lines

Before every experiment cell number and viability were determined using trypan blue staining. Dead cells have lost membrane integrity and thus allow the uptake of trypan blue and can be easily discriminated from healthy cells. Twenty microliters of cell suspension was mixed with an equal volume of trypan blue, loaded onto a haemocytometer and counted. All experimental set-ups were based on the number of live cells per ml in the original culture. This was estimated by multiplying the average live count of 8x16 square area grids by 2 (which accounts for trypan blue dilution), and then by 1x10⁶. The number of dead (blue) cells were also calculated in this way.
and the cells only used if viability was above 90% of the total number of cells in culture.

2.2.1f Preparation of Conditioned Medium (CM)

SMS-SB cells routinely grown in Protein Free Hybridoma Medium II (PFHMII) were seeded at 5x10^5/ml in fresh medium and cultured for 3 days. The cells were then removed by centrifugation at 250 xg and returned to culture for further uses. The supernatant (i.e., conditioned medium) was re-centrifuged at 1710 xg to remove any residual cells and then sterile filtered through a 0.22μm filter, aliquotted and stored at -20°C.

2.2.2 Transfection of mammalian cells

2.2.2a Transfection of human B cell suspension cultures

SMS-SB cells were transiently transfected with a CD23a expression vector using DOTAP (N-[1-(2,3-Dioleoyloxy) propyl]-N, N, N-trimethylammonium methylsulphate), a cationic liposome-mediated transfection reagent. The reagent was used in accordance with the manufacturers recommended cell densities and DNA:DOTAP concentrations (5-10μl DOTAP per μg of DNA). Transfections were performed using DNA prepared from a QIAGEN Maxi Plasmid Kit (section 2.2.7b). SMS-SB cells were prepared the day prior to transfection in 10ml of fresh serum-supplemented RPMI-1640 at a density of 5x10^5/ml.

For the transfection procedure, 10μg of plasmid DNA was diluted in 90μl of Heps buffer (20mM, pH 7.4) in a sterile glass bijou bottle. In a separate bottle 70μl of DOTAP was added to 130μl of Heps buffer. The nucleic acid solution was then transferred to the DOTAP and carefully mixed by gentle pipetting, and incubated for 15 minutes at room temperature. After incubation, the DOTAP/nucleic acid mixture was added dropwise into the cells and gently mixed. The cells were then incubated for 4 hours after which the DOTAP containing medium was replaced with fresh culture medium. After 24 or 48 hours the cells were assessed for the expression of CD23 by flow cytometric analysis (see section 2.2.3b), and for the formation of homotypic adhesions. Comparisons were made with untransfected and mock-transfected cells.
2.2.2b Transfection of non-human cell lines

COS7 cells were transfected for the generation of stable CD23a-expressing transfectants using electroporation. A confluent plate of cells were harvested using trypsin, washed in serum-free DMEM and resuspended in 0.8ml of serum-free medium. The CD23 expression vector (30µg) was added and the cells transferred to an electroporation cuvette. After a 5 minute incubation on ice, the cells were electroporated in a Bio-rad Gene Pulsar System at 0.3kV (960µFD). The cells were then returned to ice for a further 5 minutes and plated back into a 9cm culture dish in serum-supplemented DMEM. After 24 hours the cells were sub-cultured by a 1:10 dilution. At 48 hours, the culture medium was replaced with selection medium containing 800µg/ml of G418. The cells were then left for 3-4 weeks with occasional replacement of selection medium. The CD23aCOS7 cells were then analysed for CD23 expression by flow cytometry and Western blotting compared to untransfected COS7 cells (section 2.2.3b and 2.2.15).

2.2.3 Flow cytometry

All analytical flow cytometric analysis was performed on a Becton-Dickinson FACScan Flow Cytometer fitted with an argon laser. All samples were prepared in 6ml Falcon tubes (Becton Dickinson Labware, Plymouth, UK), suitable for use on the cytometer.

2.2.3a Analysis of cell cycle and intracellular proteins

To analyse the expression of intracellular Bcl-2 levels as a function of cell cycle and apoptosis, cells were subjected to a saponin treatment to permeabilise the cells thus allowing entry of the antibodies.

Each centrifugation in the following procedure was performed at 250 xg for 10 minutes. For each stain, cells were harvested from culture (see individual experiments), washed once in PBS and then twice with SBP buffer (0.1% (w/v) saponin / 0.5% (w/v) BSA / PBS), and incubated for 20 minutes on ice with 1µg of Bcl-2 primary antibody. After two further SBP washes, 10µl of a 1:100 dilution of
sheep anti-mouse IgG-FITC was added to the cells and incubated as before. The cells were washed twice in SBP and stained with 0.5ml propidium iodide solution (100μg/ml PI in PBS). All samples were immediately analysed on the Flow Cytometer, in two fluorescence channels FL-1/530nm (FITC), and FL-2/585nm (Propidium iodide, PI).

Both unstained cells, and cells incubated with secondary antibody alone were also analysed to test for autofluorescence and non-specific secondary antibody binding, respectively.

2.2.3b Cell phenotyping

Flow cytometry was used to analyse the surface expression of CD (cluster of differentiation) antigens on a variety of different cell lines. For each stain, 10^6 cells were removed from culture and washed twice with ice-cold PBS. Three microliters of FITC-conjugated antibody (CD19, CD23, CD11a, CD11b, CD11c, CD21), was added to the cells and incubated for 20 minutes on ice protected from light. After incubation, cells were washed twice in PBS and resuspended in 0.4ml of PBS ready for analysis. All samples were analysed in cytometer channel FL-1/530nm to detect FITC fluorescence against cell number. Unstained cells were also analysed to test for autofluorescence.

2.2.4 Flow cytometric cell sorting

Flow cytometric cell sorting was performed on a Becton-Dickinson FACstar Flow Cytometer that was fitted with an argon laser supplied by Coherent Lasers Ltd, Cambridge, UK.

CD23a-expressing COS7 cells (5x10^6) were stained with 15μl of FITC-conjugated anti-CD23 antibody using the procedure outlined in section 2.2.3b. Finally, the cells were resuspended in 5ml of PBS in 6ml Falcon tubes (Becton Dickinson Labware, Plymouth, UK). The cells were then analysed in the FL-1/530nm cytometer channel to detect FITC fluorescence against cell number. Cells exhibiting a fluorescence above
1.2×10^1 were separated from the rest of the population and subsequently returned to cell culture.

2.2.5 Conditioned medium manipulation

2.2.5a CM activity assay (recovery of LCD SMS-SB cells from apoptosis)

The activity of SMS-SB autocrine factor (SB-AF), contained within the cell culture supernatant, was determined by a basic assay which assessed the ability of the conditioned medium (CM) to rescue low cell density (LCD) SMS-SB cultures from death. This basic LCD assay was then used to assess activity after all CM manipulations.

SMS-SB cells grown in Protein-Free Hybridoma Medium II (PFHMII) were washed twice in fresh medium by gentle centrifugation (250 g for 10 minutes), due to the delicate nature of the cells. The cells were counted and set up at 2×10^5/ml representative of LCD by diluting the cells in appropriate volumes of PFHMII ± CM as presented in the table below. In addition to LCD, cells were also set up at intermediate density (1×10^5/ml), to determine the effect, if any, of CM on cells at a higher density. The cultures were set up in 75cm^2 flasks in a final volume of 20ml and incubated for 18 hours. The cells were then harvested and stained for intracellular Bcl-2 expression and cell cycle status and analysed by flow cytometry (section 2.2.3a).

<table>
<thead>
<tr>
<th>Cell density</th>
<th>Final % CM</th>
<th>CM</th>
<th>Cells (2×10^5/ml)</th>
<th>PFHMII</th>
</tr>
</thead>
<tbody>
<tr>
<td>ICD (1×10^5/ml)</td>
<td>0</td>
<td>0</td>
<td>5ml</td>
<td>15ml</td>
</tr>
<tr>
<td>ICD</td>
<td>20</td>
<td>4ml</td>
<td>5ml</td>
<td>11ml</td>
</tr>
<tr>
<td>LCD (2×10^4/ml)</td>
<td>0</td>
<td>0</td>
<td>1ml</td>
<td>19ml</td>
</tr>
<tr>
<td>LCD</td>
<td>5</td>
<td>1ml</td>
<td>1ml</td>
<td>18ml</td>
</tr>
<tr>
<td>LCD</td>
<td>10</td>
<td>2ml</td>
<td>1ml</td>
<td>17ml</td>
</tr>
<tr>
<td>LCD</td>
<td>20</td>
<td>4ml</td>
<td>1ml</td>
<td>15ml</td>
</tr>
<tr>
<td>LCD</td>
<td>50</td>
<td>10ml</td>
<td>1ml</td>
<td>9ml</td>
</tr>
</tbody>
</table>

67
2.2.5b Enzymatic and heat treatment of conditioned medium

Conditioned medium was prepared as in section 2.2.1f. Aliquots, each of 5ml, were then independently treated with RNase, DNase and trypsin all at a final concentration of 1μg/ml and incubated at 37°C for 4 hours. Nucleic acid digestion was stopped by cooling on ice, and trypsin inhibitor (final concentration of 2μg/ml) added to the trypsin treated sample with a further 1 hour incubation at room temperature. Aliquots of CM were also heated to 65°C and 90°C for 1 hour and then cooled. All treated conditioned media were sterile filtered through a 0.2μm filter and assayed at 20% of the final culture volume to rescue LCD cells from apoptosis (section 2.2.5a).

2.2.5c Lentil Lectin Chromatography

Lentil lectin-agarose equivalent to 0.6ml of packed beads was washed 5 times in 10ml of Protein-Free Hybridoma Medium supplemented with 0.1M CaCl₂/MgCl₂ (PFHMII-Ca²⁺/Mg²⁺). The packed beads (0.5ml) were then transferred to a V-bottomed centrifuge tube and 10ml of SMS-SB conditioned medium added and incubated with rotation for 3 hours at room temperature. The beads were then pelleted by centrifugation at 420 xg for 10 minutes, washed 3 times in 15ml PFHMII-Ca²⁺/Mg²⁺ medium and resuspended in 10ml of PFHMII supplemented with 0.2M α-methyl-D-mannopyranoside and rotated for 1 hour at 37°C. The beads were then pelleted and the supernatant retained as the eluate sample. Meanwhile, the supernatant obtained from the first bead incubation was transferred to a new tube containing 0.1ml of packed washed beads and incubated for 2 hours at room temperature. After the incubation the beads were removed by centrifugation and the supernatant retained as the flow-through sample.

Both the eluate and flow-through media were filter sterilised through a 0.2μm filter and assayed for the ability to rescue LCD SMS-SB cells from apoptosis compared to unmanipulated conditioned medium. Cells were also incubated with media containing 0.2M α-methyl-D-mannopyranoside ± conditioned medium to control for the possible effects of the added saccharide. All media were assayed at 20% of the final culture volume.
2.2.5d G25 Chromatography

A G25 column was equilibrated with 30ml of PFHMII. Conditioned medium (3ml) was added to the top of the column and allowed to pass into the matrix. PFHMII media was then used to elute 8 x 3ml fractions from the column, which were sterilised through a 0.2μm filter and assayed at 20% final volume for ability to rescue LCD SMS-SB cells from apoptosis.

2.2.6 Proliferation assay

Tritiated-thymidine uptake assays were performed to assay the response of SMS-SB cells to membrane CD23 expressed on COS7 ‘feeder’ cells. Untransfected COS7 cells were used to assay the effect of the COS7 cells alone. To prepare the ‘feeder’ layers, cells were seeded into the wells of a 96-well flat bottomed plate to achieve 80-90% confluency after 24 hours. To allow SMS-SB cell proliferation to be studied independently of the feeder layers, the mono-layers were fixed in 1% (w/v) paraformaldehyde/PBS for 2 hours. After the incubation, the cells were extensively washed in PBS supplemented with 100mM glycine to quench the paraformaldehyde, and then further washed a number of times with RPMI-1640 medium supplemented with 2% (v/v) FCS.

SMS-SB cells were washed, counted and resuspended in fresh medium (2% (v/v) FCS RPMI-1640) and dispensed onto the feeder layers at varying densities (5x10⁵, 1x10⁶, 5x10⁴, 1x10⁴, 5x10³, 1x10³ cells/ml) in a final volume of 100μl. In addition, SMS-SB cells were dispensed into wells without feeders layers (to assess normal proliferation), and feeder layers were left without SMS-SB cells (to assess background proliferation after fixation). The cells were then placed in the incubator for 24 or 48 hours. For the final 4-5 hours of the incubation, each well was pulsed with 0.33μCi of tritiated methyl thymidine (³H-TdR) and then harvested onto glass fibre mats using a LKB Wallac 1295-001 Cell Harvester. The mats were then air dried and counted in a LKB 1205 Betaplate Liquid Scintillation Counter. All determinations were made in triplicate.
2.2.7 Isolation and preparation of DNA

Composition of buffers used are detailed in the appendix.

2.2.7a Small scale preparation of DNA (mini-preps)

A 1ml overnight *Escherichia coli* bacterial culture was spun in a microfuge tube for 3 minutes at 9500 xg and the subsequent pellet resuspended in 100μl of solution I and left at room temperature for 5 minutes (see appendix for solution details). Solution II (200μl) was then added and incubation for 10 minutes on ice. Pre-chilled solution III (150μl), was added with a further 5 minute incubation on ice. To remove insoluble debris, the tubes were microfuged for 10 minutes at 16060 xg and the supernatant transferred to fresh tubes containing an equal volume of phenol:chloroform (1:1). The sample was then mixed, centrifuged at 16060 xg, and the resulting aqueous phase precipitated with 2 volumes of ethanol at -20°C for 30 minutes. The sample was then centrifuged as before at 4°C and the pellet washed in 70% ethanol, air-dried and resuspended in 20μl of dH2O and stored at -20°C. DNA from cloning procedures was subjected to restriction enzyme digestion and electrophoresis to analyse transformants (sections 2.2.8a/b).

2.2.7b Large scale preparation of DNA (maxi-preps)

After analysis of mini-prep samples, large quantities of ‘clean’ DNA for use in salt sensitive applications such as PCR, sequencing and transfection, were prepared using a QIAGEN Maxi Plasmid Kit in accordance with the manufactures instructions using the buffers supplied.

Briefly, 200ml cultures (supplemented with 100μg/ml of ampicillin), were grown overnight and harvested at 6000 xg. The bacterial pellet was resuspended in 10ml of solution PI with a further addition of 10ml of PII and incubated at room temperature for 5 minutes. Chilled PIII solution (10ml) was then added and lysate mixed by inversion and incubated for 20 minutes on ice. Samples were then centrifuged at 20,000 xg for 30 minutes at 4°C. The supernatant was then passed through a muslin gauze and loaded on to a pre-equilibrated QIAGEN maxi column. The sample was
allowed to pass into the column under gravity, and the column washed three times with 30ml of wash buffer (QC). Plasmid DNA was eluted from the column with the addition of 10ml of elution buffer (QE). Isopropanol (10.5ml = 0.7 volumes) was then added to the eluate and the sample centrifuged at 15,000 xg for 30 minutes at 4°C. The subsequent pellet was washed twice with 70% ethanol and air dried. The DNA was then resuspended in 300ul of TE buffer and the DNA concentration determined by spectrophotometry. The DNA was stored aliquotted at -20°C.

2.2.8 Basic manipulation of DNA

2.2.8a Restriction endonuclease DNA digestion
Digestion conditions varied depending on the concentration and volume of DNA used. A general rule was followed that states that under appropriate conditions, 1 unit of enzyme digests 1µg of DNA in 1 hour. Digests were set up in 1X restriction buffer with the volume of enzymes not exceeding 1:5 of the total reaction volume. Samples were incubated for 90 minutes at 37°C, and then mixed with gel loading buffer ready for analysis.

2.2.8b Agarose gel electrophoresis
1% agarose gels were prepared using 1X TBE with the addition of 0.5µg/ml ethidium bromide and submerged in 1X TBE electrophoresis buffer. DNA samples were mixed with an appropriate volume of 5X DNA loading dye to give a 1X final concentration and ran at 100V/60mA in a BRL (Cambridge, UK) horizontal electrophoresis tank. All gels were run with 1kb DNA ladder standards. The DNA was visualised on a UV transilluminator and photographed.

2.2.8c DNA fragment purification
DNA fragments obtained by PCR were purified for further cloning manipulations by agarose gel extraction. The appropriate DNA band was excised from a gel and purified with a QIAGEN-QIAquick gel extraction kit in accordance with the manufacturers instructions using the solutions supplied. Briefly, 300µl of QIAGEN solubilisation (QXI) buffer was added to each 100mg of gel (400mg maximum), and incubated at
50°C for 10 minutes. The dissolved gel mixture was then applied to a QIAquick column/collection tube and centrifuged for 1 minute at 10,000 xg. The flow-through was discarded, and the column washed with 0.75ml of PE buffer. The flow-through was again discarded and the column re-centrifuged to remove residual buffer. Fifty microliters of dH2O was then directly added to the centre of the column and centrifuged as before to elute the DNA into a fresh tube. The DNA was stored at -20°C.

2.2.8d Ligation of plasmid vector and insert DNA

The vector and insert DNA were prepared with appropriate restriction digestion and purification. Liguations were then carried out in 1X ligation buffer with 1 unit of T4 DNA Ligase in a final volume of 10µl. A maximum of 250ng of vector DNA was used, the amount of insert to be added was calculated as a function of the molar concentration of free ends in the reaction. Ligurations were set up equivalent to 1:1 and 1:3 molar ratios of vector:insert.

\[
\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{molar ratio of insert} = \text{ng of insert}
\]

Ligation reactions were then carried out at 14°C overnight and then 2µl of each reaction used for the transformation of E. coli.

2.2.9 Transformation of DNA into E. coli

2.2.9a Preparation of competent cells (rubidium chloride method)

An overnight culture of JM109 E. coli bacteria was diluted 1:100 in 250ml of LB containing 20mM MgSO4, and incubated with shaking until the A600 reached 0.4-0.6. The cells were then centrifuged at 4,500 xg for 5 minutes at 4°C. The cell pellet was resuspended in 100ml of ice-cold TFB1 (see appendix for solution details), incubated on ice for 5 minutes, and centrifuged as before. For all subsequent steps, the cells were kept on ice and all solutions and plasticware were chilled before use. The cells were
resuspended in 10ml of TFB2 and incubated for 30-60 minutes before being aliquotted and quick frozen in a dry ice/isopropanol bath. Cells were stored at -70°C and were stable for up to 3 months.

2.2.9b Transformation of competent cells
Ten nanograms of plasmid DNA (in a maximum of 10μl) or 2μl of a ligation reaction, was added to 100μl of competent cells in a microfuge tube and incubated for 30-60 minutes on ice. The cells were heat shocked at 42°C for 1 minute and cooled on ice for 2 minutes. SOC medium (1ml) was then added and the cells incubated with shaking for 1 hour at 37°C to allow expression of antibiotic resistance. Aliquots (50-100μl) of the transformation mix were plated onto LB-agar selection plates (50μg/ml ampicillin), and incubated overnight at 37°C.

2.2.9c Analysis of transformants
To identify colonies of E. coli containing plasmid or recombinant vector, individual colonies were picked and grown up overnight in a 5ml culture of selective LB (50μg/ml ampicillin). Mini-preps and restriction digest analysis were then performed (see sections 2.2.7a, 2.2.8a/b), to allow the plasmid to be mapped and the presence of cloned insert confirmed.

2.2.10 Polymerase Chain Reaction (PCR)

2.2.10a Oligonucleotide design and preparation
Using the published cDNA sequence of the CD23 gene (Entrez accession number: M14766), a series of synthetic oligonucleotides were designed to allow the PCR of the different forms of CD23 (a, b, 37kDa and 25kDa). Oligonucleotides were designed with either BamH[AGATCT] or NcoI[GCGGCCGC] restriction sites within them to assist with subsequent cloning steps.

CD23a (sense) 5'- GTAGAGATCCACCGCCATGGAGGAAGGTC -3'
CD23b (sense) 5'- GTAGAGATCCAGCATAATGAAATCCTCCAAGCCAGGAGATC GAGGAGCTTCCCAGG -3'
Oligos were synthesised on an Applied Biosystems 392 RNA/DNA synthesiser. The lyophilised DNA produced was resuspended in 50μl of MilliQ water, its concentration determined by spectrophotometry, then aliquotted and stored at -20°C.

### 2.2.10b PCR reaction

All PCR amplification reactions were performed using *Pfu* DNA Polymerase using the plasmid pcDL Srø296CD23a as a template. For each gene amplification, optimal components and conditions were determined for both the reaction mixture and the cycle parameters as detailed below. All reactions were carried out in 0.5ml microcentrifuge tubes and were carried out in a Techne Genius PCR machine. After the PCR, samples were analysed on a 1% (w/v) agarose gel, purified, digested and cloned into an appropriate vector and then sequenced (sections 2.2.8a/b/c and 2.2.11). A negative control (no DNA template), was also performed with each PCR reaction.

**Reaction mixture:**

<table>
<thead>
<tr>
<th>Component</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>template DNA</td>
<td>50ng</td>
</tr>
<tr>
<td>upstream primer</td>
<td>1μM</td>
</tr>
<tr>
<td>downstream primer</td>
<td>1μM</td>
</tr>
<tr>
<td>DNTP mix</td>
<td>0.2mM</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>1X</td>
</tr>
<tr>
<td>DMSO</td>
<td>10% (v/v)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>5% (v/v)</td>
</tr>
<tr>
<td><em>Pfu</em></td>
<td>0.025U/μl</td>
</tr>
<tr>
<td>dH2O</td>
<td>(to final volume 100μl)</td>
</tr>
</tbody>
</table>
### Cycle parameters:

- **94°C 5 minutes** 1 cycle
- **94°C 30 seconds** (denaturation)
- **62°C 30 seconds** 20 cycles (annealing)
- **72°C 1 minute** (extension)
- **72°C 10 minutes** 1 cycle
- **4°C** HOLD

#### 2.2.10c Blunt ended PCR cloning of 37kDa and 25kDa CD23 forms

The PCR products for these transcripts were blunt-end cloned using the pCR-Script™SK(+) cloning kit which utilises simultaneous action of T4 DNA ligase, and SrfI restriction digestion of non-recombinant, re-ligated vector. The kit was used in accordance with the manufacturers instructions using all the reagents/vectors supplied. Briefly, the ligation reaction was set up as advised and incubated for 1 hour at room temperature. The reaction was then heated to 65°C for 10 minutes and transformed into XL-1 Blue MRF’ Kan supercompetent cells. The transformation mixture was then plated out onto antibiotic selection plates (50μg/ml ampicillin, 40μg/ml X-gal (5-Bromo-4-Chloro-3-Indoly]-β-D-galactoside)) and incubated for 16 hours at 37°C. Plates were then placed at 4°C to allow blue/white colour selection to develop. White colonies were then analysed further for the presence of an insert as described in section 2.2.9c, and the DNA sequenced.

#### 2.2.11 Nucleotide sequence analysis

The cloned CD23 constructs were sequenced using Biosystems ABI 373A automated DNA sequencer. Fifty nanograms of plasmid was mixed with 3.2pmoles of sequencing primer and 8μl of Bigdye™ termination reaction mix, in a total volume of 20μl. DNA was subjected to ‘cycle sequencing’ in a DNA thermal cycler for 25 cycles (each cycle comprises 10 seconds at 96°C, 5 seconds at 50°C, and 4 minutes at 60°C). The products were then ethanol precipitated, washed in 70% ethanol, and dried in a Speedivac for 5 minutes prior to being resuspended in loading buffer (95%
formamide, 25mM EDTA pH8, 1.5mg/ml dextran blue). The samples were then denatured by heating to 94°C, chilled on ice, and subject to electrophoresis on the ABI 373A sequencer, operated as a core service by staff at the CRC Beatson Institute, Glasgow. For nucleotide sequencing in pcDNA3.1(+) and pCR-Script™ SK(+), the T7 and RP, and the T7 and T3 primers were used respectively:

T7 5’- GTAATACGACTCACTATAGGGC -3’  
T3 5’- AATTAACCTCACTAAAGGG -3’  
RP (reverse primer) 5’- TAGAAGGCACAGTCGAGGC -3’

2.2.12 RNA manipulation

2.2.12a SMS-SB cell stimulation

To assay the response of SMS-SB cells to membrane CD23a expressed on COS7 cells at the RNA level, SMS-SB cells were exposed to the ‘feeder layer’ for different periods of time (0, 15, 30, and 60 minutes) and RNA prepared. SMS-SB cells were also placed on untransfected COS7 cells to assay the effect of COS7 cells alone. The SMS-SB cells used in this assay were grown for a week prior to the experiment in Optimem, a serum-free medium, to rule out serum-stimulated effects.

24 hours prior to use, both types of COS7 cells were sub-cultured into 75cm² flasks to allow 3 flasks of both type for each time point, each achieving 80-90% confluency at the time of use. On the day of the assay, CD23aCOS7 and COS7 feeder layers were fixed as described in section 2.2.6, and SMS-SB cells counted and diluted in Optimem to give 8x10⁵/ml. Diluted SMS-SB culture (10ml) was then placed in each feeder flask (i.e., a total of 30ml of SMS-SB cells for each time point on each COS7 cell type), and incubated appropriately for each time point. Cells were then removed from the feeder layers, and each time point from each and feeder cell type pooled together. The resulting 30ml, a total of 2.4x10⁷ cells, was centrifuged at 270 x g for 10 minutes.

2.2.12b Isolation of RNA from SMS-SB cells

Total RNA was extracted from cells using RNAzol B according to the manufacturers instructions using RNase free filter tips and plasticware. Briefly, the pelleted cells
(2.4x10^7), were washed once with PBS and lysed with 4.8ml of RNAzol B (0.2ml per 10^6 cells) by passing the cells through a pipette. Chloroform was then added (0.1ml per 1ml homogenate) and the samples shaken vigorously for 15 seconds and put on ice for 5 minutes before being centrifuged at 12,000 xg for 15 minutes at 4°C. After centrifugation the homogenate forms two phases; a lower blue phenol-chloroform phase and a colourless upper aqueous phase where the RNA resides. The upper phase was carefully transferred to a fresh tube and an equal volume of isopropanol added and the sample stored on ice for 15 minutes to allow RNA precipitation. The sample was then centrifuged as before, and the resultant pellet washed with 75% ethanol, air dried and resuspended in 30µl of RNase-free water. The samples were then stored at -70°C until analysis. Before use, samples concentrations were determined by spectrophotometer readings at A_{260} and A_{280}. Samples had A_{260}/A_{280} ratios greater than 1.9 indicative of DNA and protein-free preparations.

2.2.12c Northern blotting
All gel equipment used was treated with RNaseOUT before use by liberally spraying the reagent over surfaces, leaving it for 10 seconds and washing with RNase-free water. RNase-free filter tips were also used to prevent contamination. Twenty micrograms (approximately 2µl), of total RNA was added into sample buffer (0.75µl 10X MOPS, 2.25µl formaldehyde, 7.5µl formamide), and made up to 15µl with RNase-free water. The sample was then heated for 15 minutes at 65°C and placed on ice for 5 minutes. Ethidium bromide (0.5µl of a 10mg/ml stock) and 0.5µl of RNA loading dye (see appendix for details) were added and the sample loaded onto a 50ml 1% (w/v) agarose gel containing 5ml of 10X MOPS and 9ml of formaldehyde, and electrophoresed slowly (50V) in 1X MOPS for approximately 3 hours. The gel was then washed for 2 x 15 minutes in dH2O and visualised/photographed on a transilluminator to assess RNA quality and to align RNA markers. RNA was then transferred to Hybond N+ nitrocellulose membrane by overnight capillary blotting (Southern, 1975) using 20X SSC. The RNA was UV crosslinked using a UV Stratalinker 1800, and stored wrapped in clingfilm at 4°C until probe hybridisation.

2.2.12d Radiolabelling of cDNA probes
Complementary DNA (cDNA) probes were labelled using a random priming oligo-
labelling kit and radiolabelled dCTP. For each probe, 50ng of DNA, in a volume of 34μl of dHzO, was denatured by heating to 100°C for 5 minutes and then cooled on ice for 2 minutes. Ten microliters of reagent mix (containing dATP, dGTP, dTTP and random hexadeoxyribonucleotides), 5μl (3000Ci/mmole) of α-[32P]-dCTP and 1μl of Klenow fragment were all added. The reaction was mixed gently and incubated for 1 hour at 37°C. After incubation, unincorporated nucleotides were removed by centrifugation at 735 x g for 2 minutes through a MicroSpin Sephacryl S-200 Column as these are retained in its bead matrix. The probe was boiled for 5 minutes, placed on ice for 10 minutes, and then added to the pre-hybridized membrane as detailed below.

2.2.12e Hybridisation with [32P]-labelled cDNA

Nylon membranes were placed in 20ml plastic tubes and pre-hybridised in 5ml of Express Hyb hybridization solution, and incubated with rotation for 1 hour at 65°C in a Hybaid oven. The [32P]-labelled probe was then added directly into the buffer and the membrane incubated as before. The filter was then washed for 15 minutes at 65°C with shaking in 1X SSC/0.1% (w/v) SDS, and then in 0.1X SSC/ 0.1% (w/v) SDS. The filter was then sealed in saran wrap and exposed to Kodak XAR-5 film for 1-5 days in autoradiography cassettes with intensifying screens at -70°C. Films were then processed in a Kodak X-omat developer.

Membranes to be reprobed were stripped by placing them in boiling 0.1% (w/v) SDS solution and leaving them to cool to room temperature with shaking. All membranes were reprobed with 7S cDNA as an RNA loading control.

2.2.13 BIAcore analysis of the novel receptor for CD23

BIAcore surface plasmon resonance technology was employed to study the interaction of the novel CD23 binding receptor on SMS-SB cells to sCD23, and to investigate the presence of the receptor on other cell lines. For this technique, cell membrane extracts were passed over sCD23 immobilised on a biosensor chip and the binding of any cell material analysed. The binding of cellular material, i.e., the novel receptor for CD23, creates a mass change at the chip surface, which in turn changes the resonance angle.
of light emitted from chip, thereby allowing the receptor-ligand interaction to be analysed.

2.2.13a Preparation of cell membrane extracts

1x10⁶ cells were removed from logarithmically growing cultures, washed twice in 50ml of PBS and resuspended in 1.6ml of ice-cold membrane extraction buffer (see appendix for buffer details). The cells were then homogenised (approximately 30 strokes) in a glass hand held homogeniser, transferred to microfuge tubes and kept on ice for 1 hour. The extract was centrifuged at 380 x g to remove nuclei and any unbroken cells. The supernatant was then centrifuged at 45,000 x g at 4°C for 40 minutes, and the resulting supernatant stored at 4°C overnight until analysis. Immediately prior to analysis, extracts were diluted 1:10 in HBS buffer.

2.2.13b Surface plasmon resonance measurements

Real-time analysis of the interaction between sCD23 and CD23NR was performed with a BIAcore 2000 instrument. sCD23 was covalently immobilised onto a flow cell of a CM5 (carboxyl methyl dextran-coated) sensor chip using carbodi-imide coupling. This method involves the use of carbodi-imide cross-linking reagents to form a covalent bond with carboxylate groups on the sensor chip surface, leaving a bond sensitive to attack by free amino groups on the ligand. This procedure was performed in association with Dr. J Matheson, University of Glasgow.

Diluted extracts were run individually through the BIAcore machine in a continuous flow of HBS buffer using the automated injection procedures and fluidics. Each sample was simultaneously monitored in real time for the binding to the sCD23-immobilised sensor chip flow cell, and also to a blank flow cell to assess non-specific binding to the chip surface. After analysis, the sensor chip surface was regenerated to remove non-covalently bound material from the surface by injecting 0.1M HCl, and then washed with a continuous flow of HBS buffer. Other extract samples were then analysed.

Association and dissociation kinetics were determined using the BIAevaluation 2.1 analysis package supplied with the BIAcore 2000. From these kinetics, the overall equilibrium constant of the interaction was determined.
2.2.14 Immunocytochemistry

To assess the expression of CD23a on adherent transfected CD23aCOS7 cells, immunocytochemistry was performed. CD23aCOS7 and mock-transfected COS7 cells were plated onto coverslips in the wells of 12 well plates to achieve 70-80% confluency after 24 hours. The monolayers were washed once in sterile PBS, fixed for 15 minutes at 37°C in 4% (w/v) paraformaldehyde/PBS and washed 5 times in 3ml of PBS. To prevent non-specific antibody binding, the cells were incubated for 40 minutes at room temperature in blocking buffer (0.5% BSA/10% FCS/PBS), and then incubated in 1ml of mouse anti-human CD23 antibody (4μg/ml) in blocking buffer for 1 hour at room temperature. The cells were washed in PBS as before and then incubated with a 1:200 dilution of rabbit anti-mouse IgG-FITC antibody for 20 minutes in the dark. Cells were washed as before in PBS, mounted onto slides and stored in the dark at 4°C until examined. Slides were examined on a Biorad MRC-600 laser scanning confocal microscope.

Both CD23aCOS7 and control COS7 cells were also stained with the FITC-conjugated antibody alone to assess non-specific secondary antibody binding.

2.2.15 Western blotting

2.2.15a Preparation of cell extracts

Lysates were prepared from logarithmically growing cultures. 1x10^7 cells were washed once in PBS, resuspended in 0.5ml of RIPA buffer and incubated for 30 minutes on ice. Lysates were then centrifuged at 16,000 xg to remove cellular debris. The supernatant was carefully removed, aliquotted and stored at -70°C until use.

Protein concentration was determined by the Lowry method (Lowry et al., 1951). Known amounts of protein were mixed with an appropriate volume of 4X protein loading dye supplemented with β-mercaptoethanol to give a 1X final dilution. Samples were then boiled for 5 minutes and loaded onto an SDS-PAGE gel.
2.2.15b SDS-PAGE electrophoresis and Western blotting

A 10 or 12% (w/v) acrylamide separating gel was prepared using a Bio-rad mini-gel rig and left to set with an isopropanol overlay. A 5% (w/v) acrylamide stacking gel was then poured on top. Both gels were made to the specifications listed in the table below. Protein samples were loaded into the stacking gel and run at 15mA in electrophoresis buffer. When samples had reached the separating gel, the current was increased to 25mA. Rainbow protein molecular weight markers were also run alongside samples to allow molecular weights to be determined.

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Final concentration of reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>separating gels</td>
</tr>
<tr>
<td>Acrylamide (40% w/v) /bis acrylamide (3.3% w/v)</td>
<td>10% or 12% (w/v)</td>
</tr>
<tr>
<td>Tris pH8.8</td>
<td>0.375M</td>
</tr>
<tr>
<td>Tris pH6.8</td>
<td>-</td>
</tr>
<tr>
<td>SDS</td>
<td>0.1% (w/v)</td>
</tr>
<tr>
<td>Ammonium persulphate</td>
<td>0.05% (w/v)</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.0003% (v/v)</td>
</tr>
</tbody>
</table>

Following gel separation, the protein was transferred to nitrocellulose in transfer buffer using Bio-rad mini-wet blotting apparatus at 100mA/70V for 2 hours. After transfer, the nitrocellulose was blocked for either 3 hours at room temperature or overnight at 4°C in 10% (w/v) marvel/PBS. Following blocking, membranes were briefly rinsed with PBS and incubated at room temperature on a shaker with the appropriate primary antibody for 3-4 hours. Bcl-2, Bax, Bcl-xL antibodies were used at 0.5μg/ml, and the rabbit anti-human CD23 antibody at 0.8μg/ml, and were diluted in 15ml of 1% (w/v) marvel/ 0.1% (w/v) thiomersol/ PBS. After incubation, membranes were washed for 3 x 10 minutes with 0.1% (v/v) Tween 20/ 1% (w/v) marvel /PBS and incubated with a 1:1000 dilution of Protein A-HRP antibody for 1 hour with shaking at room temperature (in 1% (w/v) marvel/ 0.1% (w/v) thiomersol/ PBS). The membrane was then washed as before.
2.2.15c ECL detection system

All blots were developed using the ECL (Enhanced Chemiluminescence) detection kit in the darkroom and in accordance with the manufacturers instructions. Equal volumes of the two ECL reagents were mixed and poured onto a washed membrane and incubated for exactly one minute. Excess reagent was then removed with tissue and the membrane wrapped in cling film. The blot was exposed to Fuji RX film for between 20 seconds and 30 minutes depending on the intensity of the signal. Films were processed in a Kodak X-omat developer.
CHAPTER 3

RESULTS

STUDIES OF AN ANTI-APOPTOTIC AUTOCRINE FACTOR PRODUCED BY SMS-SB CELLS
3.1 INTRODUCTION

Throughout B cell development, the majority of cells initially produced undergo apoptosis/programmed cell death. This may be due to the absence of essential cytokines or growth factors, or may be as a result of developmental problems at a specific differentiation stage. For example, the extensive cell death evident at the pre-B cell stage of development is due to the non-productive rearrangement of heavy chain genes (Osmond et al., 1994). Several genes, and their protein products, have been implicated in the regulation of apoptosis. The Bcl-2 family is now considered to be one of the most important, consisting of both pro and anti-apoptotic members. The founding member, Bcl-2, was initially identified as a result of a translocation in a human cancer resulting in the over-expression of the gene, and the prevention of apoptosis (Tsujimoto et al., 1984; Cleary et al., 1986; Tsujimoto and Croce, 1986). Over-expression of Bcl-2 has now been found to prevent apoptosis induced in a wide variety of conditions (Reed, 1994).

The regulation of apoptosis, and indeed proliferation and differentiation of B cells is governed by the action of various growth factors and cytokines. These factors can regulate the expression and activity of proteins in the Bcl-2 family and thereby direct the cell either into or away from apoptosis (Franke and Cantley, 1997). Many of these factors act in a paracrine fashion, and in the case of precursor B cell regulation, are produced by the stromal cells of the bone marrow. In addition, cells can also produce factors that can regulate their own fate, so called autocrine factors. The production of these autocrine factors has been implicated in the development of cancers, allowing the cells to avoid external growth signals and partake in autonomous growth, but alone are not sufficient for transformation (Young et al., 1991). Although not solely responsible, the identification of B cell autocrine factors and the mechanism by which they act to influence a cell is of obvious importance in determining their role in leukaemogenesis, and therefore for the development of potential targets for cancer treatment.

The data in this chapter demonstrate a potential mechanism for the action of an autocrine factor produced by the pre-B acute lymphoblastic leukaemia cell line SMS-
SB, and an attempt to characterise and isolate the factor from culture supernatant. When removed from the patient, these cells grew spontaneously in culture without going through a crisis phase. Previous studies into the growth characteristics of these cells performed by White (1995), demonstrated that the cells are: i) able to grow in medium devoid of all protein at the same rate as cells in serum-containing media, ii) cells are density-dependent for growth, with low density cultures undergoing apoptosis and iii) supernatant from normal density cultures (referred to hereafter as conditioned medium (CM)), apparently promotes the growth of the low density cultures. All these characteristics demonstrated the production of an autocrine growth factor by SMS-SB cells, which is required at a minimum concentration to maintain cell growth. In addition, White (1995), showed that the SMS-SB autocrine factor (SB-AF), could be concentrated over a 100kDa filter, but could not identify the cytokine responsible. Based on these results it was decided that a more directed isolation procedure using various chromatography techniques was required for the study of this factor.

All manipulations of the SMS-SB cells in this chapter were performed in Protein-Free Hybridoma Medium II (PFHMII), to eliminate any possible interactions between the autocrine factor and serum proteins and to prevent the interference of these proteins in the isolation procedures. In addition, all assays were based on the ability of CM to rescue low cell density (LCD) SMS-SB cultures from apoptosis, as analysed by flow cytometric studies of Bcl-2 and cell cycle status.

3.2 RESULTS

3.2.1 SMS-SB cells produce an autocrine factor

White (1995), demonstrated that SMS-SB cells produce an autocrine factor by taking CM from normal density SMS-SB cultures and adding it to low cell density cultures, at which cells would usually cease proliferation and die by apoptosis.

Figure 3.1 (taken and modified from White, 1995), represents the incorporation of $[^3]H$-thymidine by various SMS-SB cell densities in the presence and absence of 20%
The final volume CM over 3 days of culture. The data demonstrate that CM can promote the proliferation of SMS-SB cells at 2.5 and 5x10^4 cells/ml, although at these densities the cells can grow alone after 2 or 3 days in culture. At 1x10^4 cells/ml however, CM promotes cell growth for 2 days but cannot sustain the effect beyond this time. An explanation of these growth differences is the presence of a lag phase in proliferation after initial seeding in fresh unconditioned medium. At the two higher densities the CM could maintain proliferation during this phase, thus allowing the cells to produce enough of their own autocrine factor to continue growth, thus explaining the enhanced growth of these cell density cultures in the presence of CM. The short-lived effect on proliferation for 1x10^4 cells/ml, suggests that the exogenous CM is used initially during what would be the lag period, but by 3 days it has been depleted and the cells have not produced enough of their own to maintain subsequent growth.

Therefore these data suggest that the exogenous CM is 'saving' low cell density cell cultures until they can produce a sufficient concentration of their own CM. If the cells do not reach this critical concentration they no longer proliferate and undergo apoptosis. Thus, this experiment demonstrates that SMS-SB cells produce an autocrine growth factor, which if present at high enough concentrations allows the cells to grow autonomously under protein-free conditions. The factor does not act as a mitogenic signal per se, but rather seems to rescue the cells from death which then allows their subsequent proliferation.

3.2.2 SMS-SB cells express Bcl-2

The autocrine factor produced by SMS-SB cells rescues LCD cells from death. A potential mechanism for its action could be the regulation/influence of Bcl-2 family members. Therefore, to investigate how the autocrine factor may influence apoptosis, the expression of Bcl-2 in LCD SMS-SB cells in the presence and absence of the autocrine factor (present in CM) was investigated.

Flow cytometry was employed to allow the simultaneous analysis of Bcl-2 expression and cell cycle status in SMS-SB cells. For this technique the cells had to be permeabilised with saponin to allow staining of the intracellular markers. Cells were
stained with anti-human Bcl-2 antibody (and with an appropriate FITC-conjugated secondary antibody), and then with propidium iodide to assess cell cycle status.

Figure 3.2 represents the basic stain for Bcl-2 and cycle analysis. As the Bcl-2 antibody is not fluorescent, a fluorescently-conjugated secondary antibody has to be used which binds to the primary antibody, thus allowing visualisation of Bcl-2 expression. Dotplot A illustrates the profile achieved when SMS-SB cells are stained with both antibodies, while B represents staining with secondary antibody alone. The difference in y-axis fluorescence demonstrates that the secondary antibody is specifically binding to the primary and not non-specifically to cellular components, and, is therefore directly representative of Bcl-2 expression. Propidium iodide (PI) is a non-specific fluorescent dye that intercalates between the bases of double stranded DNA. The fluorescence of PI (linear x-axis), is therefore proportional to the DNA content of the cell and thus indicative of cell cycle status.

In normal cell density (NCD) cultures (figure 3.3), PI staining demonstrates that the majority of the cells are cycling (panel A), and can be sub-divided into the distinct phases of the cell cycle (panel B). G1 therefore has PI fluorescence of 2n and G2 a fluorescence of 4n, due to doubling of DNA content during replication in S phase. Cells to the left of the dotplot represent debris and cells undergoing apoptosis in pre-G1. The cell phase marked >G2 represents aneuploid cells and are not counted as normal cycling cells. With regard to Bcl-2 expression, NCD cells show strong staining in all cycle phases, with a small increase in cells of the G2 phase. Culture debris and cells undergoing apoptosis, have low y-axis fluorescence indicative of being negative for the anti-apoptotic protein. Panel C represents histogram analysis of the cell cycle and shows a characteristic profile indicative of asynchronously cycling cells, as would be expected for normal density healthy cultures.

3.2.3 SMS-SB autocrine factor sustains Bcl-2 expression and promotes cell cycle progression

To test the hypothesis that the SMS-SB autocrine factor regulates the survival of low cell density cultures via the regulation of Bcl-2, CM was added to LCD cultures (at
which apoptosis normally occurs), and Bcl-2 expression and cell cycle status analysed.

The dot plots in figure 3.4 represent intermediate cell density (ICD) cultures where only just above half the cells are actively cycling. The addition of 20% (v/v) of conditioned medium to these cultures caused no increase in the expression levels of Bcl-2, but promoted cells to cycle rather than undergo apoptosis. Statistical analysis (panel C) based on the number of cells in the distinct cycle phases (as detailed in figure 3.3 panels A and B), confirms this effect. There are 13.06% more cells in cycle in the presence of CM and is seen throughout all the distinct cycle breakdown phases. The most obvious difference is the 18.39% reduction in culture cell debris which represents cells that have already undergone apoptosis. There are however more cells undergoing apoptosis in pre-G1 in the presence of CM. An probable explanation of this result is that upon seeding in fresh medium, the cells become stressed due to CM removal and go through a lag phase in proliferation and growth. Some of the cells residing in G1 proceed into apoptosis within the first few hours and so present themselves 24 hours later at the time of analysis as cell debris (on the far left side of the dotplot). However, in the presence of exogenous CM the onset of apoptosis is initially delayed so more cells are kept within the cycle and can begin to produce their own autocrine factor. Later in the assay, some cells still die as the CM is quickly depleted and therefore present themselves in pre-G1 at the time of analysis. Bcl-2 levels are reduced in these cells as they are undergoing apoptosis.

In low cell density cultures (figure 3.5 panel A and table 2), only very few of the cell are still cycling after 24 hours (11.14%), with the majority having either undergone apoptosis (76.24% debris), and showing no staining for Bcl-2, or are undergoing apoptosis in pre-G1 (10.74%). In the presence of increasing amounts of exogenous CM, the expression of Bcl-2 is not changed in cycling cells, but there is however a profound effect on the number of cells in cycle. Figure 3.5 clearly shows that cells are restored into the cycle in a dose dependent manner, from 15.86% with 5% final volume CM, to 32.28% with 50%, restoring the cycle profile to that seen in healthier ICD cultures (figure 3.5 panel f). With this increase of cycling cells, there is a corresponding decrease in cell debris within the culture, a difference of 29.78% from LCD cells alone to cells with 50% CM. There is also a slight increase in the number of
apoptotic pre-G1 cells as CM concentration increases. This phenomenon may again be explained if cells undergo apoptosis later on in the assay due to initial rescue by the CM, whereas in LCD cultures devoid of CM, the cells have already committed to or undergone apoptosis.

Figure 3.6 shows more closely the expression of Bcl-2 in SMS-SB cultures. Histogram analysis of Bcl-2 expression in each cell analysed by flow cytometry shows, that in LCD cultures, only 13.07% of the cells are positive for Bcl-2 expression (panel A), compared to 87.73% for normal density healthy cultures (panel C). In the presence of exogenous CM the percentage of LCD cells positive for the anti-apoptotic protein was increased to 41.48%. These results confirmed that CM was rescuing cells from apoptosis by maintaining the expression of Bcl-2 to the level seen in healthy cultures.

3.2.4 SMS-SB cells express a number of Bcl-2 family members

Concerns arose that the monoclonal antibody used for flow cytometric analysis was directed against the Bcl-2 family as a whole and was therefore not specific for the Bcl-2 protein itself. The effect of CM seen by flow cytometry may therefore represent the total expression of a number of family members and not give details of individual proteins. For instance, the CM may in fact be inducing Bcl-2 expression and simultaneously reducing the expression of the pro-apoptotic protein Bax which when assayed using this antibody may look like no overall change in expression. In the absence at this time of commercially available monoclonal antibodies to each member of the Bcl-2 family, Western blot analysis was performed to try and address this problem by using antibodies that did not cross react with other members of the Bcl-2 family.

Figure 3.7 shows the Western blot analysis of SMS-SB cells, grown at normal density in PFHMI, using a peroxidase-linked secondary antibody and ECL detection system to detect expression of Bcl-2, Bcl-xL and Bax proteins. The results show that at normal density, SMS-SB cells express similar amounts of the anti-apoptotic proteins Bcl-2 and Bcl-xL (25-26kDa), and also of the pro-apoptotic protein Bax (21kDa). The
identity of the additional bands on the Bcl-xL blot are unknown but the size of the major band makes the anti-apoptotic protein Mcl-1 (37kDa), a candidate, although the antibody is reported not to cross-react with other Bcl-2 family members.

Now that the status of Bcl-2, Bcl-xL and Bax had been determined in the cells by Western blot analysis, it was hoped that the effect of CM on their expression in LCD cultures could be determined, giving a more accurate account of the state of the apoptotic proteins in the cell and on how the autocrine factor may act to save the cells from apoptosis. Unfortunately, extracts could not be successfully made from low density cultures. As seen in figure 3.5 panel A and in table 2, nearly 90% of LCD culture is debris or cells undergoing apoptosis and are therefore very difficult, if not impossible to make extracts from that are suitable for use in Western blotting.

**3.2.5 SMS-SB autocrine factor has an essential protein component that is heat stable to 90°C**

In an attempt to identify the nature of the autocrine factor, CM was exposed to enzyme and heat treatment in an attempt to remove the activity, therefore indicating a component involved. The graph of figure 3.8, compares the ability of treated CM to recover LCD cells from apoptosis and maintain them in the cycle to the recovery seen with untreated CM. The data show that the only treatment to have an effect on activity was treatment with trypsin, reducing to activity of the CM to that seen when no CM is added. The fact that all the activity is removed by this treatment means that the autocrine factor must have a protein component that is essential for its activity. In addition, the protein component is heat stable to 90°C as after such treatment the medium retained activity akin to the untreated CM.

**3.2.6 SMS-SB autocrine factor is a multi-component factor**

In attempts to further characterise and crudely isolate the autocrine factor, various chromatographic procedures were performed, using the basic Bcl-2/PI staining to test for the activity of the CM.
To assess if the autocrine factor had a glycoprotein component which could potentially be used for further isolation procedures lentil lectin affinity chromatography was performed. The method was based on the theory that any glycoproteins in the CM would bind to lentil lectin-agarose and these could then be eluted from the beads with a mannose-containing medium. The eluate and the flow-through media were then tested for autocrine factor activity.

Figure 3.9 demonstrates that neither the eluate or flow-through samples had any capacity to recover LCD SMS-SB cells from apoptosis, as both had Bcl-2/PI staining profiles akin to that of LCD cells alone. The conditioned medium used for the chromatography was however active, as cells could be rescued in the presence of unmanipulated CM (panel B). In addition, the use of mannose did not have any undesirable effects on the procedure, as alone it did not induce cell recovery (panel E), or adversely effect the activity of unmanipulated CM (panel F). There are two plausible explanations for the loss of autocrine activity during this procedure. Firstly, the factor may be a lectin and is therefore binding to the beads with high affinity and can not be easily eluted by the addition of mannose. Secondly, the autocrine factor may be a multi-component factor, part of which is a glycoprotein. If this is the case, the glycoprotein component would bind to the beads and ultimately appear in the eluate, while the rest of the factor would remain in the flow-through. Essential components of the factor would therefore be separated preventing the effect of combined component activity, and the rescue of LCD cells from apoptosis. To test this theory, the eluate and flow-through samples could have been pooled together and tested for the restoration of activity. Unfortunately, this idea was not tested at the time of the investigation. However, this experiment may not have allowed any further interpretations to be made, as the absence of pooled sample activity, thereby negating the activity of a multi-component factor, would possibly be due to irreversible changes caused by binding of components to the lectin beads, thereby preventing any subsequent activity.

Previous studies found that SB-AF could be concentrated over a 100kDa size filter (White, 1995). Therefore, in an attempt to define the native molecular size of the autocrine factor, conditioned medium was subjected to G25 gel filtration.
chromatography. Eight 3ml fractions were collected from the column and tested for the ability to rescue LCD cells from apoptosis compared to unmanipulated CM. Figure 3.10 shows that none of the fractions were able to rescue SMS-SB cells from death, each having similar percentages of cells in cycle as LCD cultures alone. The conditioned medium put through the G25 column used was active however, as unmanipulated CM could rescue cells from death. These data suggest that the autocrine activity could be attributed to a multi-component factor, thus supporting the results and interpretations of the lentil lectin chromatography experiment. The loss of activity from all the G25 column fractions suggests that perhaps at least one of the essential components is of a different size to the others, and is therefore residing in a different fraction, or is smaller than 25kDa and is being retained within the column. The procedure has thus separated the essential components which combine to give the overall activity.

3.3 DISCUSSION

The pre-B cell line SMS-SB has previously been found to produce an autocrine factor which is able to prevent the cell death of low cell density cultures (Zack et al., 1987; White, 1995). The results presented in this chapter demonstrate that this autocrine factor rescues the cells from apoptosis and promotes cell cycle progression in a titratable manner. In addition, the results suggest that a potential mechanism of action for this factor is the maintenance of the anti-apoptotic protein Bcl-2, which seems to be over-expressed in SMS-SB cells. In addition, SB-AF seems to be a multi-component factor that has an essential protein component that is heat stable to 90°C.

Previous studies by White (1995), found that sCD23 and PDGF were the only tested cytokines with the ability to promote LCD SMS-SB cell growth but did not represent the autocrine factor. The results of the chromatographic procedures presented here suggest that SB-AF is a multi-component factor which requires the presence of all its individual components to exhibit anti-apoptotic activity. This may therefore explain why the other individual cytokines tested by White failed to show growth promoting activity. Synergistic effects between autocrine factors has previously been described
for immortalised human B cells, which were found to require IL-1, IL-6, TNF-α, and TNF-β for continued growth (Abken et al., 1992). To investigate which cytokines possibly contribute to SB-AF, numerous cytokines, in all possible combinations, would have to be tested. This method of investigation however may not identify the factors responsible as one, or a number of components, may be an as yet unidentified cytokine/growth factor.

The ability of SB-AF to prevent the apoptosis of LCD cultures of cells may be due to its effects on the anti-apoptotic protein Bcl-2. Flow cytometric analysis demonstrated that conditioned medium maintained the expression level of Bcl-2 to that seen in healthy normal cell density cultures. The autocrine factor may therefore provide a survival signal allowing the cells to subsequently proliferate after the initial lag phase in growth. Other researchers have reported that autocrine factors may be responsible for anti-apoptotic signals (Francia di Celle et al., 1996; Kulmburg et al., 1998), an effect that can be mediated by the upregulation of Bcl-2 (Francia di Celle et al., 1996). As Bcl-2 levels were only maintained and not up-regulated by SB-AF, it is possible that other anti-apoptotic members of the Bcl-2 family may be more significant targets for regulation.

Western blot analysis demonstrated that, in addition to Bcl-2, SMS-SB cells also express Bcl-xL and the pro-apoptotic protein Bax. Unfortunately, this method could not be used to assess the status of these proteins in LCD cultures preventing the specific effect of SB-AF on these proteins from being determined. However, the analysis revealed that normal cell density SMS-SB cells have an abnormal expression pattern of Bcl-2 family members. During normal development, B cells exhibit a reciprocal pattern of Bcl-2 and Bcl-xL expression, which is believed to be essential for the survival of cells at each developmental stage. At the pre-B cell stage, Bcl-2 levels are low, whereas Bcl-xL levels are high (Merino et al., 1994; Grillot et al., 1996). SMS-SB cells express similar levels of both these anti-apoptotic proteins thus demonstrating that either Bcl-2 levels are high, or Bcl-xL levels are low. In support of the former, SMS-SB cells have similar levels of Bcl-2 expression as the EDR cell line (data not shown), which represent the mature B cell developmental stage known to express high levels of Bcl-2 (Merino et al., 1994). In addition, other B cell acute
lymphoblastic leukaemias have been found to over-express Bcl-2 (Campana et al., 1993; Pontvert-Delucq et al., 1996; Coustan-Smith et al., 1996). To determine conclusively if SMS-SB cells over-express Bcl-2, the cells would have to be compared with normal bone marrow pre-B cells from healthy individuals.

All the flow cytometric data presented in this chapter rely on the ability of the autocrine factor to rescue LCD SMS-SB cells from apoptosis as an indication of its activity. Although all the data are representative of numerous repeats for each experiment, the assay was very sensitive to the number of cells used. Although exactly the same procedure was used each time, the set up of low cell density was based upon a cell count using a haemocytometer. Even though a number of averages were used in calculating cell number, an error of even ± 1 in the original count results in a big difference in cell number when the cells are diluted down to LCD. If too many cells were present in the assay, LCD cells alone would have cells in the cycle preventing the activity of exogenous CM from being clearly seen. If there are too few cells in the assay, no amount of CM could rescue them from death. A very small window therefore exists to test for the activity of the CM by flow cytometry.

As the effects of SB-AF can only be seen when the cells are at low cell density, alternative methods for assessing activity, such as Western blot analysis, could not be successfully performed. Investigations into the effects of growth factor effects at low cell densities are therefore very difficult to perform. A further hindrance to the study of SB-AF was having to grow the cells in protein-free medium to prevent any synergistic reactions between SB-AF and serum proteins. Although able to be cultured in this medium, SMS-SB cells are extremely fragile, making even basic cell culture procedures such as centrifugation difficult.

Based on these problems, and the discovery that SB-AF activity seems to be mediated by a number of individual components/factors, which as such would be difficult to isolate, it was decided to terminate the investigation into the SMS-SB cell autocrine factor. However, as previously discussed it would be interesting in the future to further investigate whether SMS-SB cells over-express Bcl-2, a potentially significant finding from this study.
Figure 3.1  [$^3$H]-Thymidine incorporation by SMS-SB cells cultured at various densities in the presence and absence of conditioned medium.

(Modified from White, 1995)

SMS-SB cells grown in Protein-Free Hybridoma Medium II (PFHMII) were seeded in the wells of a 96-well plate at 1x10^4, 2.5x10^4 and 5x10^4 cells/ml in PFHMII, in a volume of 100μl. Each density was cultured in the absence and presence of conditioned medium (CM) at 20% of the final culture volume. The cultures were incubated for 1, 2 or 3 days and pulsed with 0.33μCi/well [$^3$H]-thymidine for 6 hours prior to harvest. All cultures were in triplicate and the experiment is representative of four independent repeats. The error bars represent the standard deviation of triplicate data.
(Thymidine incorporation (cpm))
Figure 3.2 Flow cytometric analysis of Bcl-2 expression in SMS-SB cells by specific antibody staining.

$1 \times 10^6$ SMS-SB cells grown in PFHMII were washed in PBS, permeabilised in saponin containing buffer, and stained with mouse anti-human Bcl-2 antibody for 20 minutes. To visualise primary antibody staining, the cells were washed and incubated with an FITC-conjugated anti-mouse-IgG secondary antibody. After further washing, cells were stained with 100μg/ml propidium iodide for DNA analysis and analysed by flow cytometry. Cells were also stained with secondary antibody alone to test for non-specific antibody binding to the SMS-SB cells leading to false readings in Bcl-2 expression.

The dotplots illustrate propidium iodide (PI) staining on the $x$-axis with a linear scale, while Bcl-2 expression is represented logarithmically on the $y$-axis. Panel A represents staining with both the Bcl-2 and FITC-conjugated secondary antibody, while panel B is secondary antibody staining alone.
Figure 3.3 Flow cytometric analysis of Bcl-2 expression and the cell cycle status in SMS-SB cells.

Normal cell density (NCD=5x10^5/ml), SMS-SB cells grown in PFHMI were stained for intracellular Bcl-2 expression and cell cycle status (propidium iodide staining), as described in figure 3.2. The three panels represent the same cell sample and illustrate the features that can be determined by flow cytometric analysis. Panel A- all cells in cycle, Panel B- breakdown analysis of the cell cycle into distinct phases, Panel C- histogram representation of cell cycle status (PI staining) as a function of each cell in the sample.
A: Cells in cycle

B: Debris, pre-G1, G1, S, G2

C: Number of cells
Figure 3.4 Effect of conditioned medium on Bcl-2 expression and cell cycle status of intermediate density SMS-SB cultures.

SMS-SB cells grown in PFHMII at intermediate cell density (ICD=1x10^5/ml), were cultured overnight in the presence and absence of 20\% final volume conditioned medium (prepared as detailed in Materials and Methods section 2.2.1f). The cells were then stained with Bcl-2 and PI as described for figure 3.2. Panel A- ICD cells alone, Panel B- ICD cells with 20\% (v/v) CM, Panel C- statistical analysis of cell cycle status, based on the distinct phases illustrated in figure 3.3.

The data is representative of six independent repeats, each using different preparations of CM.
A. ICD - CM

B. ICD + CM

<table>
<thead>
<tr>
<th></th>
<th>% debris</th>
<th>% pre-G1</th>
<th>% cells in cycle</th>
<th>% G1</th>
<th>% S</th>
<th>% G2</th>
<th>% &gt;G2</th>
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<td>54.07</td>
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<td>ICD+CM</td>
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<td>33.28</td>
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Figure 3.5  Effect of conditioned medium on Bcl-2 expression and cell cycle status in low cell density SMS-SB cultures.

SMS-SB cells grown in PFHMII were cultured overnight at low cell density (2x10^5/ml) supplemented with varying amounts of CM. Panel A - cells alone, B - cells + 5% final volume CM, C - cells + 10% CM, D - cells + 20% CM, E - cells + 50% (v/v) CM and F - intermediate density culture (1x10^5/ml) with no CM addition. The cells were removed from culture using a cell scraper as many apoptotic cells had adhered to the flask, and stained for Bcl-2 expression and cell cycle status as described for figure 3.2.

The data shown is representative of at least six independent repeats, each using different preparations of CM.
A. LCD

B. LCD + 5% CM

C. LCD + 10% CM

D. LCD + 20% CM

E. LCD + 50% CM

F. LCD
Table 2. Statistical analysis of the effect of conditioned medium on cell cycle status in low cell density SMS-SB cultures.

The table shows the percentage analysis of the cell cycle status for the dotplot samples in figure 3.5. The analysis was performed on the basis of the total number of cells in the cycle (depicted in figure 3.3 panel A), and of the distinct cell cycle phases that can be identified by flow cytometric analysis (figure 3.3 panel B).
<table>
<thead>
<tr>
<th>% debris</th>
<th>% cells in cycle</th>
<th>% cells</th>
<th>% G1</th>
<th>% G2</th>
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<tr>
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<td>32.28</td>
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</tr>
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</table>
Figure 3.6 Flow cytometric histogram analysis of Bcl-2 expression in low density SMS-SB cultures in the presence and absence of conditioned medium.

The histograms represent an alternative presentation and analysis of the levels of Bcl-2 expression in SMS-SB cells at varying densities. Cells were removed from culture and stained for Bcl-2 and cell cycle status as previously described and then analysed in the FITC fluorescence channel only. The histograms illustrate Bcl-2 expression in logarithmic fluorescence on the x-axis, against a linear scale of cell number on the y axis. Panel A represents low cell density, Panel B- low cell density + 20% final volume of conditioned media, Panel C- normal cell density cultures. Each histogram shows the percentage of cells that are positive for Bcl-2 expression as denoted by the horizontal bar.
A. LCD

% cells positive for Bcl-2 = 13.07%

B. LCD + 20% CM

% cells positive for Bcl-2 = 41.48%

C. NCD

% cells positive for Bcl-2 = 87.73%

Bcl-2 (FITC)
Figure 3.7 Western blot analysis of specific Bcl-2 family members in SMS-SB cells.

SMS-SB extracts were prepared from cells grown in PPHMII as detailed in Materials and Methods section 2.2.15a. Protein samples were loaded onto a 12% (w/v) acrylamide separating gel: Lane 1= 30μg, Lane 2= 50μg of protein. After electrophoresis and transfer to nitrocellulose, blots were incubated with 0.5μg/ml of either Bcl-2, Bcl-xL, or Bax antibody. To detect antibody binding, the membranes were incubated with 1:1000 dilution of protein-A-HRP and developed with an ECL detection system. Rainbow protein molecular weight markers (15μl) were also run to determine molecular size, as illustrated. The experiment is representative of over eight independent repeats with different cell preparations.
Figure 3.8 The effects of enzymatic and heat treatment on conditioned medium activity.

Aliquots of conditioned medium were independently treated with 1μg/ml of DNase, RNase or trypsin, or heat-treated at 65 and 90°C as described in Materials and Methods section 2.2.5b. The treated media was then assayed at 20% final volume for ability to rescue LCD SMS-SB cultures from apoptosis, as analysed by Bcl-2 expression and cell cycle analysis compared to LCD cells alone and cells with untreated CM. A percentage activity value was calculated for each sample based upon the percentage of cells in cycle (as illustrated in figure 3.3 panel A), with untreated CM having 100% activity.

\[
\text{% activity of a sample} = \frac{\text{% cells in cycle for the sample}}{\text{% cells in cycle for untreated CM}} \times 100
\]

The experiment is representative of four independent repeats, with different preparations of CM.
Culture conditions and treatment

- LCD
- LCD+20% CM
- LCD+20% trypsin CM
- LCD+20% RNase CM
- LCD+20% DNase CM
- LCD+20% 65°C CM
- LCD+20% 90°C CM

% activity

- 0%
- 58.9%
- 59%
- 105.5%
- 100.5%
- 99.9%
- 103.8%
Using SMS-SB cell CM, eluate and flow-through samples were prepared from lentil lectin chromatography as described in Materials and Methods section 2.2.5c. Using the basic Bcl-2/PI stain (as detailed in figure 3.2), the samples were assayed for ability to rescue LCD SMS-SB cells from apoptosis compared to LCD cells alone, and cells cultured with unmanipulated CM. Cells were also incubated with α-methyl-D-mannopyranoside in the presence and absence of CM to control for the effects of the elution saccharide. All media were assayed at 20% final culture volume. Panel A- cells alone, Panel B- cells + unmanipulated CM, Panel C- cells + eluate, Panel D- cells + flow-through, Panel E- cells + mannoside medium, Panel F- cells + mannoside + CM.

The experiment is representative of three independent repeats with different CM preparations.
Figure 3.10 G25 Gel Filtration Chromatography.

SMS-SB cell CM was applied to a G25 column and 8 x 3ml fractions collected. The fractions were then tested at 20% final volume for the ability to rescue LCD cultures from apoptosis as analysed by Bcl-2/PI staining compared to LCD cells alone, and cells in the presence of unmanipulated CM. A percentage activity value was derived for each sample based on the percentage of cells in cycle (as illustrated in figure 3.3 panel A), with unmanipulated CM having 100% activity (see legend of figure 3.7 for equation).

These data are representative of three independent repeats, each performed with different preparations of CM.
CHAPTER 4

RESULTS

ANALYSIS OF THE EFFECTS OF 45kDa MEMBRANE-ASSOCIATED CD23 ON SMS-SB CELLS
4.1 INTRODUCTION

The 45kDa membrane form of CD23 has been shown to be involved in a number of B cell functions including: antigen presentation, IgE regulation, T-B cell conjugate formation, and B cell homotypic adhesion (reviewed by Bonnefoy et al., 1997). The influence of the CD23 molecule is broadened by the release of various soluble factors, all derived from cleavage of the 45kDa form. When used in conjunction with IL-1α, the 25kDa form of human sCD23 has been ascribed various cytokine functions including the rescue of germinal centre B cells from apoptosis (Liu et al., 1991a), and the growth and maturation of early human myeloid and thymocyte precursors (Mossalayi et al., 1990a; 1990b). In addition, sCD23 has also been implicated as a BCGF due to its ability to promote the proliferation of EBV-transformed and normal receptor-stimulated B lymphocytes (Swendeman and Thorley-Lawson, 1987; Gordon et al., 1989). However, a number of researchers have disputed this function especially with regard to the 25kDa form (Uchibayashi et al., 1989). Cairns and Gordon have found that CD23 may mediate its BCGF effects as a membrane-associated cytokine, as intact 45kDa CD23 was found to be consistently mitogenic for pre-activated B cells (Cairns and Gordon, 1991).

Previous work by White (1995), demonstrated that the 37, 33, 29 and 25kDa forms of soluble CD23 (sCD23), can prevent apoptosis and allow the subsequent growth of low cell density SMS-SB cells. This response was found to be paracrine, not autocrine, since SMS-SB cells do not express CD23. These results were of great interest as there had not been any previous reports of sCD23 acting as a growth factor for pre-B cells, and the effect did not require synergy with IL-1α. In addition, White demonstrated that SMS-SB cells do not express any of the known receptors for CD23 namely, CD21, CD11b or CD11c (Aubry et al., 1992; Lecoanet-Henchoz et al., 1995), but could specifically bind to full-length (45kDa) CD23a-containing fluorescent liposomes. This demonstrated that SMS-SB cells express a novel receptor for CD23 (referred to hereafter as CD23NR), which may be involved in the regulation of apoptosis and growth of these cells.
Although biochemical and molecular isolation studies for CD23NR were being performed outwith this study, it was also decided to produce monoclonal antibodies against the putative receptor components(s). These would be valuable tools to aid in the isolation studies, and also for studying the distribution of CD23NR on other human haematopoietic cells. Obvious screening procedures for the generation of these antibodies were to assess the inhibition of either CD23 liposome binding to SMS-SB cells, or the growth promoting effects of sCD23. However, in the absence of any readily available source of purified 45kDa CD23a protein to use in liposomes, or sCD23 to use in growth assays, alternative screening procedures were sought.

This chapter of results describes experiments performed to investigate the effect of membrane form (45kDa) CD23a on SMS-SB cells, in the hope of generating an alternative screening procedure for the generation of monoclonal antibodies towards the component(s) of novel receptor for CD23. Investigations were performed to assess the effect of CD23a when it was expressed in SMS-SB cells themselves, or when CD23a was delivered as a paracrine signal from feeder monolayers. Also described are the construction of various CD23 constructs, and the generation of a CD23a-expressing COS7 monolayer used in the investigation.

4.2 RESULTS

4.2.1 The effect of 45kDa CD23a transiently expressed in SMS-SB cells

The initial investigation into the effect of 45kDa CD23 was based upon the expression of CD23a in SMS-SB cells. Based on the previous findings by White (1995), it was hypothesised that expression of CD23a in SMS-SB cells would render the cells density-independent for growth, as the survival signal utilised at the low cell density would be constitutively present. In addition, it was envisaged that the transfected CD23a would permit adhesions between itself and the novel receptors on adjacent cells, leading to the formation of homotypic adhesions. It was hoped that monoclonals
against the novel CD23 receptor could be screened by assessing their ability (like an anti-CD23 antibody), to disrupt the formation of SMS-SB cell aggregations generated by the novel receptor and the transfected CD23.

To test these hypotheses, SMS-SB cells were transfected with a CD23a isoform expression vector (pcDL SRα296CD23a), using the liposome-mediated transfection reagent DOTAP. Figure 4.1 illustrates SMS-SB cell culture phenotype at 24 hours after CD23a transfection. Cells transfected with CD23a (panel B), exhibited the formation of large cell clumps within the culture compared to the single cell phenotype of untransfected SMS-SB cells (panel A). Hopes that this effect was solely a function of CD23a were dismissed upon analysis of the mock-transfected cells (i.e., cell transfected with an empty expression vector) (panel C). These cells also exhibited clump formation, suggesting that the phenomenon was mainly being caused by the transfection reagent or procedure. Although CD23a may have been causing some adhesions, these could not be distinguished from the effects of the transfection procedure.

Although a CAT assay of a transfected reporter construct confirmed that SMS-SB cells could be transiently-transfected (data not shown), flow cytometric analysis of the cells (figure 4.2), demonstrates that the frequency of transfection is very low. The results show that both untransfected (panel B) and mock-transfected control (panel C) SMS-SB cells exhibit a profile very similar to unstained cells (panel A), demonstrating that, as expected, they do not express CD23a. Cells transfected with CD23a (panel D), exhibit a slight shift in the histogram profile to the right on the x-axis compared to untransfected and mock-transfected cells, demonstrating that some of the cells are weakly positive for CD23a. Meaningful statistical analysis could not be performed on these data as the exact point where a cell is regarded as CD23a positive was difficult to determine. The data therefore illustrate that the transfection frequency of SMS-SB cells is very low, with only a few SMS-SB cells exhibiting weak CD23a expression. These findings thus support the data from figure 4.1, which indicate that CD23a is not responsible for the extensive adhesion formation within the cultures. Based on these results, it was decided not to try and assess whether CD23a could render SMS-SB cells density-independent, as transfection at such a low frequency in
normal density cultures would not affect the cells when they were subsequently seeded to low cell density.

In order to try and enhance transfection efficiency, and prevent reagent-associated changes to SMS-SB phenotype, transfections were performed using different DNA:DOTAP ratios, other transfection reagents (DOSPER, Tfx50, Tfx20, Transfectamine, FUGENE-6 and SuperFect), and also electroporation (data not shown). Unfortunately, these changes yielded no significant improvements to SMS-SB cell transfection efficiency. In addition, no significant differences were seen in the effects of any of the reagents, or in the efficiency of transfection, when the cells were assayed at 48 hours post transfection (data not shown).

As the effects of the transfection reagent were still a problem within the time of a transient transfection assay, it was decided to try and make cells stably expressing CD23a. To do this, CD23a was cloned into an expression vector with a drug resistance gene for the selection of resistant stable cells.

### 4.2.2 Generation and cloning of CD23 constructs

CD23a cDNA was generated by the polymerase chain reaction (PCR), using synthetic oligonucleotides and the vector pcDL SRαz96CD23a as a template. It was also decided at this time to produce other forms of CD23, namely CD23b, and the 37kDa and 25kDa soluble forms to allow the subsequent bulk production of CD23 proteins in baculovirus, for use in future experiments.

#### 4.2.2a PCR amplification of CD23 constructs

Figure 4.3 shows a diagrammatic representation of the different CD23 forms, and the synthetic oligonucleotides used for the generation of their cDNAs by PCR. The oligonucleotides were designed to amplify specific forms, and included BamHI or NotI restriction enzyme sites to assist with subsequent cloning steps. After 20 cycles of PCR amplification, products of each reaction were electrophoresed and visualised on a UV transilluminator (figure 4.4). The expected sizes for CD23a, b, 37kDa and 25kDa, based on their published sizes (Entrez accession number M14766), plus the
\( \textit{NotI} \) and \( \textit{BamHI} \) restriction sites, were 992, 989, 755 and 543bp, respectively. For each PCR reaction only a single band was visualised on the gel that was consistent with the theoretical size of that specific CD23 cDNA. There were no fragments generated in the negative (no DNA template) control, showing that there was no contaminating DNA present, and that the amplifications had been specific for each form of CD23.

4.2.2b Cloning the CD23 constructs

The CD23 fragments generated by PCR were purified from the agarose gel and cloned into plasmid vectors. Using the intrinsic \( \textit{BamHI} \) and \( \textit{NotI} \) restriction sites, CD23a and b were inserted into the pcDNA3.1(+) mammalian expression vector containing a gene for neomycin resistance. As the soluble forms of CD23 were not going to be expressed in mammalian cells they were blunt end cloned into the pCR-Script™ SK(+) cloning vector, to allow nucleotide sequencing to be carried out.

The CD23 fragments were ligated into their appropriate vectors, and the plasmids transformed into \textit{E. coli}. To identify transformants containing the CD23 inserts, restriction digests were performed on DNA preparations derived from individual bacterial colonies. Restriction digests using \( \textit{BamHI} \) and \( \textit{NotI} \) should directly liberate the cloned insert from its vector, as restriction sites for these enzymes were included at the ends of each CD23 construct. Figure 4.5 demonstrates that this restriction digest produced two DNA bands after electrophoresis, one corresponding to the CD23 construct, and the other to the vector, 5.4kb or 2.96kb for pcDNA3.1(+) or pCR-Script™ SK(+), respectively.

4.2.2c Sequence analysis of the CD23 constructs

Figure 4.6 shows the nucleotide sequence, and the corresponding conceptually-translated amino acids, for the cloned CD23 constructs. The identity of these cloned fragments and the fidelity of the PCR reaction was confirmed by nucleotide sequence analysis and comparison to the published CD23 sequence (Kikutani et al., 1986a; Entrez accession number M14766), by GCG pairwise alignment. The sequences of all the cloned CD23 forms were shown to be identical to the published sequence except for two nucleotide changes at positions 690 (A→G) and 717 (T→C). Analysis of the
corresponding amino acid sequence showed that these nucleotides were third base
nucleotides in the codons encoding the amino acids glycine (GGA→GGG), and
histidine (CAT→CAC), respectively. However, these changes were degenerate, and so
did not alter the amino acid sequence of the peptide.

Therefore, each form of CD23 was successfully generated and cloned. Sequence
analysis of the genes confirmed their identity and revealed only degenerate base
changes. Outwith this study, 45kDa CD23a, and the 37kDa and 25kDa soluble forms,
were subcloned into the baculovirus expression system for the generation of CD23
proteins. The 25kDa sCD23 was subsequently used for BLAcore analysis as detailed in
Chapter 5.

4.2.3 Stable transfection of 45kDa CD23a into SMS-SB cells

The CD23a expression vector (pcCB-CD23a), was transfected into SMS-SB cells
using the transfection reagent DOTAP and, as before, caused the cells to form
adhesions (data not shown). At 48 hours post transfection, the cell culture medium
was replaced with medium containing 200µg/ml of G418. After three weeks of
selection, the majority of the culture had died leaving very few viable drug resistant
cells, consistent with the low transfection frequency of this cell line. In an attempt to
enhance the growth and survival of the transfectants, which were essentially at low
cell density with regard to other live cells in the culture, the transfection was repeated
with cells in selection medium containing 50% (v/v) conditioned medium. This
procedure did not have any obvious beneficial effects.

The main problem with this method was how to retrieve the small number of
transfected cells from the dead ones in a suspension culture. It was feared that the live
cells would be lost by centrifugation through a Ficoll gradient, and due to local safety
regulations the cells could not be sorted by flow cytometry. A method put forward by
Andersen and Junker (1994), exploits the specificity of the lectin concanavalin A for
α-glucosyl and α-mannosyl residues present on mammalian cells. The researchers
found that only living cells would adhere to surfaces covalently coated with the lectin,
allowing dead cells to be discarded, and the live cells removed by forced pipetting and
returned to culture. This method was attempted for SMS-SB cells (data not shown), but unfortunately did not successfully separate any live cells from the culture, suggesting that the number of viable drug resistant cells was too low for successful separation.

During these attempts to transfect SMS-SB cells, the cells were found to be contaminated with mycoplasma, which is known to have adverse effects on transfection. Although the cells were treated and cleared of contamination as assessed by staining with the fluorescent dye Hoechst 33258, SMS-SB cell transfection was unfortunately not improved. Therefore, after many attempts of trying to transfect SMS-SB cells, transiently or stably, it could not be determined whether the transfection of CD23a into SMS-SB cells caused adhesions to form via their novel receptor and transfected CD23. The knock-on effect of this was that this system could not be used as a screening technique for the generation of a monoclonal antibody towards the novel receptor. This line of work was therefore terminated.

4.2.4 The effect of 45kDa CD23a when presented to SMS-SB cells from monolayer cell cultures

As the addition of soluble CD23 to low cell density cultures of SMS-SB cells increases the $[^3]H$-TdR incorporation by cells in proliferation assays (White, 1995), it was hypothesised that 45kDa membrane-associated CD23 would have a similar effect on SMS-SB cells. Indeed, previous studies have shown that purified 45kDa CD23 is stimulatory for normal and transformed mature B cells, and is more reliable in its activity than the soluble CD23 forms (Cairns and Gordon, 1990). As purified 45kDa CD23 protein was not available at the time of this study, CD23 was presented to low cell density SMS-SB cells using a monolayer cell line expressing CD23a-CD23a 'feeder' cells. It was hoped that if CD23a expressed on these cells could promote SMS-SB cell $[^3]H$-TdR incorporation, monoclonal antibodies against the SMS-SB cell novel receptor for CD23 could be tested for their ability to inhibit the growth-promoting effect, thus providing an alternative screening technique. It was also envisaged that SMS-SB cells would bind to these monolayers, as the cells are able to bind 45kDa CD23a-containing liposomes.
4.2.4a Generation of the CD23a expressing ‘feeder’ cells

COS7 cells were chosen for the generation of a monolayer expressing membrane form CD23a as these cells are easy to maintain and transfect, and give high levels of transgene expression. The cells were transfected with pcCB-CD23a or an empty pcDNA3.1(+) expression vector (mock-transfected), using electroporation and subjected to G418 selection for 3-4 weeks. The cells were then assayed for CD23 expression by flow cytometric analysis (Figure 4.7 panel A). Mock-transfected cells exhibited a histogram profile essentially identical to unstained cells (panel Ai), demonstrating that they do not express CD23a. Although some pcCB-CD23a transfected cells expressed CD23 at their membrane (panel Aii), others seemed to be negative for protein expression. This was an unexpected result as after drug selection, the surviving cells must be resistant to G418 due to transformation with the vector, and so by default should also express CD23a. This result suggested that some of the cells were drug resistant but were not expressing membrane CD23. An explanation of this may be that as COS7 cells are transformed with the SV40 virus and pcDNA3.1(+) uses an SV40 origin of replication, the transfected vector would exist episomally, and as such may have variable plasmid propagation leading to low CD23a expression levels in some cells. Alternatively, part of the vector containing the drug resistant cassette, but not the CD23a coding sequence, may have integrated into the genome resulting in CD23a negative drug resistant cells.

As the monolayer was not being used to study directly CD23a, and was only serving as a stimulatory layer, it was decided that rather than transfecting another cell line, the cells could be sorted on the basis of their CD23a expression using a FACstar Fluoresently Activated Cell Sorter. This would produce a population of COS7 cells expressing CD23a at a high level. The cells were stained with anti-CD23-FITC antibody as for basic analysis, and then those exhibiting a CD23 FITC-fluorescence above 1.2x10^1 were separated from the rest of the population and retained for subsequent cell culture. Figure 4.7 Aiii, shows the flow cytometric analysis of these cells, demonstrating that the majority now have very high expression levels of CD23 at the cell membrane. Western blot analysis further confirms the CD23 expression of the sorted CD23aCOS7 cells (figure 4.7 panel B).
As the cells were to be used as adherent monolayers, the cells were assayed for CD23 expression by immunocytochemistry. Cells fixed onto coverslips were stained with mouse anti-human CD23 antibody, and then with rabbit anti-mouse IgG-FITC. Figure 4.8 shows the confocal microscopic images obtained. Sorted CD23aCOS7 cells (panel A), are highly positive for CD23a all over their surface, clearly showing the outline of the cells. Mock-transfected COS7 cells (panel B), only demonstrate background fluorescence. Both sets of cells did not exhibit any fluorescence when stained with the FITC-conjugated antibody alone (data not shown), demonstrating the specificity of the secondary antibody used.

Thus, overall, a population of monolayer cells expressing 45kDa CD23a had been generated for use as a stimulatory layer for SMS-SB suspension cells. In a biosynthetic pulse-chase experiment, no radiolabelled CD23, either as 45kDa or the soluble forms, could be immunoprecipitated from the culture supernatant at the end of a 24 hour chase period (data not shown). Only 45kDa membrane-associated CD23 was thus available to the SMS-SB cells.

4.2.4b SMS-SB cells bind to CD23aCOS7 cells
To determine whether SMS-SB cells would bind CD23a expressing monolayers, 10ml of SMS-SB cells, at a density of 5x10^6/ml, were placed onto confluent cultures of CD23aCOS7 and untransfected COS7 cells in 75cm^2 tissue culture flasks. After 60 minutes incubation without disturbance, the cultures were examined by phase contrast microscopy. Upon gentle movement of the flasks, the SMS-SB cells cultured with the control (untransfected) COS7 cells moved freely, as in cultures of SMS-SB cells alone. In contrast, SMS-SB cells cultured with the CD23a expressing COS7 cells did not move within the culture media, suggesting that the cells could be binding to the monolayer via their novel receptor for CD23 (results not shown).

4.2.4c 45kDa CD23a on COS7 cells does not promote SMS-SB cell proliferation at low cell density
To investigate whether 45kDa CD23a, like sCD23, could promote an increase in [^3H]-TdT incorporation by SMS-SB cells, the cells were seeded at various low cell densities onto the 'feeder' cells. It was decided to use SMS-SB cells grown in serum-
containing medium (2% (v/v) FCS/RPMI-1640), as cells grown in protein-free medium, as used for the sCD23 studies previously, are very fragile and sensitive to manipulation. However, the addition of protein is able to enhance the proliferation of SMS-SB cells, although not to the extent of sCD23 or conditioned medium (White, 1995). Based on this, it was hypothesised that in the presence of this additional stimulus, SMS-SB cell growth would be enhanced at densities lower than those for cells grown in protein-free media.

The graphs of figure 4.9 show the \(^3\)H-TdR incorporation of various densities of SMS-SB cells cultured alone or in the presence of either CD23a-expressing or untransfected (control) monolayers. So that SMS-SB cell proliferation could be assessed independently, the \(^3\)H-TdR incorporation value of the fixed monolayers (on average 300cpm), was subtracted from the values of SMS-SB proliferation in the appropriate culture wells. The data demonstrate that 45kDa CD23a had no effect, either positively or negatively, on the LCD growth of SMS-SB cells over 24 and 48 hours, as the mean \(^3\)H-TdR incorporation was the same as SMS-SB cells cultured alone. In addition, the results show that the monolayer cells themselves were not stimulatory for growth, as untransfected COS7 control cells did not initiate a change in \(^3\)H-TdR incorporation. These results therefore suggest that 45kDa CD23a expressed on COS7 monolayers, in contrast to sCD23, does not promote the growth of LCD SMS-SB cells.

An explanation of this result might be that although the COS7 cells seem to have no direct effect on cell growth, it is possible that their presence is affecting the SMS-SB cells by preventing them from responding normally to CD23. Without any purified 45kDa protein to address this possibility, it cannot be concluded that 45kDa CD23a is not stimulatory for SMS-SB cell growth. In addition, it is also possible that the presence of serum proteins in the SMS-SB culture medium may have affected the sensitivity of the assay. Future experiments would therefore have to be repeated using cells grown in protein-free medium.

Although the SMS-SB cells being used for this assay had been treated and cleared for mycoplasma, it is possible that a low level of contamination, undetectable by Hoechst
33258 staining, was still present in the culture. This low level contamination may have affected the response of the SMS-SB cells to CD23a.

Overall, these results are inconclusive as to whether 45kDa CD23a is stimulatory for SMS-SB cell growth. However, the absence of any noticeable response prevents this assay from being used a screening procedure for the generation of a monoclonal antibody against the novel receptor.

4.2.4d 45kDa CD23 on COS7 monolayers does not specifically induce changes in the expression of the immediate early genes c-fos, c-jun or c-myc in SMS-SB cells

As 45kDa CD23a expressed on COS7 'feeder' cells did not seem to be stimulatory for the growth of LCD SMS-SB cells, it was decided to investigate whether 45kDa CD23a could mediate any changes in SMS-SB cells, at the level of expression of the immediate early genes (IEG's) c-fos, c-jun, and c-myc, which encode important cellular transcription factors. c-Fos and c-Jun are members of the Fos and Jun families of transcription factors, which dimerise with each other to form the AP-1 (Activator Protein 1) transcription factor (reviewed by Karin et al., 1997). c-Myc mediates its transcriptional activity as a heterodimer complex with a related protein Max (Blackwood and Eisenman, 1991). These IEG-encoded transcription factors are known to mediate multiple cellular signals in haematopoietic cells including the induction of cellular proliferation, differentiation, and apoptosis (reviewed by Liebermann et al., 1998; Facchini and Penn, 1998). The effects of these transcription factors on cell behaviour seems to be dependent on cell type, differentiation state, and surrounding environment.

Due to the difficulties in making lysates from low cell density cultures of SMS-SB cells (discussed in chapter 3), it was decided to assess changes in the immediate early genes in SMS-SB cells at normal cell densities (8x10^5/ml), when cultured with either CD23αCOS7 or control untransfected COS7 cells. In addition, the SMS-SB cells were cultured in the serum-free medium Optimem, to rule out any serum-stimulated effects. Previous studies have demonstrated that the addition of serum to cell cultures mediates an increase in the expression of c-fos RNA (Tsai et al., 1991; Lallemand et al., 1997), and various Fos and Jun-related proteins (Lallemand et al., 1997). As IEG
expression has been shown to usually change within 30 minutes after stimulation (Tsai et al., 1991; Lallemand et al., 1997), SMS-SB cells were placed onto confluent 'feeder' layers for 0, 15, 30 and 60 minutes, and RNA then prepared.

Figure 4.10 shows the results of Northern blot analysis. The transilluminator images (panel A), show that the RNA used for blotting was not degraded. Each membrane probed with a specific cDNA probe was stripped of radioactivity and reprobed with a 7S probe (panel C) to demonstrate RNA loading. The results show that stimulation of SMS-SB cells with either control COS7 or CD23aCOS7 cells resulted in the down-regulation of c-fos over 60 minutes (c-fos probe, panel B). As RNA loading was essentially equal, this demonstrated that the COS7 cells themselves were affecting the SMS-SB cells. The expression levels of c-jun transcripts (c-jun probe, panel B), were similar for all time points and for SMS-SB cells on both types of feeder layers. Although after 30 minutes of CD23aCOS7 cell stimulation, the SMS-SB cells had a slightly lower level of expression of c-jun, this was not a concern as the 7S probe (c-jun probe, panel C), demonstrated that a lower amount of RNA was loaded for that sample. With respect to c-myc (c-myc probe, panel B), there was again no clear difference between any of the samples, any slight differences being attributable to RNA loading.

Overall, the results of Northern blot analysis demonstrated that using this method of stimulation, a CD23a-specific signal was not being transduced by c-fos, c-jun or c-myc in SMS-SB cells. However this does not necessarily mean that the cells were not responding to CD23a. In addition to c-Fos and c-Jun, other Fos related (FosB, Fra1, Fra2), and Jun related (JunB, JunD) proteins dimerise to form AP-1. It is possible that complexes of these proteins are the predominant regulators of transcription in SMS-SB cells, and as such, transcripts for these proteins would also have to be tested.

The results also demonstrate that the monolayer cells alone were affecting the SMS-SB cells, as the control untransfected COS7 cells caused a decrease in c-fos expression. This possibly explains why SMS-SB cells did not respond to 45kDa CD23 in the proliferation assay. The presence of the COS7 cells, may in someway be affecting the response of the low cell density cultures to the 45kDa CD23 expressed
on the monolayers. To further address this, an alternative cell line such as CHO, could be generated as a feeder line, in conjunction with using purified 45kDa CD23a as a control.

4.3 DISCUSSION

The results presented in this section demonstrate the successful cloning of a number of CD23 constructs, and the generation of a 45kDa CD23a-expressing monolayer. Although SMS-SB cells bind to this monolayer, it does not however seem to promote the growth of SMS-SB cells at low cell density as analysed by \(^{3}H\)-thymidine incorporation. As CD23a could not be efficiently transfected into SMS-SB cells, it was impossible to determine whether CD23a could generate homotypic adhesions by binding to the novel CD23 receptor on adjacent cells. The absence of any effect of 45kDa CD23a on SMS-SB cells prevents these functional assays from being used as screening techniques for the generation of monoclonal antibodies against the novel CD23 receptor.

Despite numerous attempts using different transfection reagents, SMS-SB cells were not able to be efficiently transiently-transfected with CD23a. The cells are very sensitive to this type of manipulation, highlighted by the fact that a number of commonly used reagents, and electroporation, caused excessive cell death of SMS-SB cell cultures. Although able to tolerate DOTAP, this transfection reagent caused SMS-SB cells to form into large clumps, which was unfortunately the predicted effect of the transfected CD23. Stable transfection of the cells was also unsuccessful. Although some viable cells seemed to be present in the culture after selection, these could not be successfully separated from the dead cells in the suspension culture. The SMS-SB cell line is therefore not particularly amenable to either transient or stable transfection.

Although 45kDa CD23a expressed on COS7 monolayers failed to increase the incorporation of \(^{3}H\)-thymidine by LCD SMS-SB cells, it is not possible to state unequivocally that this form of CD23 is not stimulatory for SMS-SB cell growth. The results of the Northern analysis suggest that the COS7 cells are affecting the SMS-SB
cells. Therefore, the signal mediated by the binding of 45kDa CD23 to the SMS-SB novel CD23 receptor may be masked by the COS7 cells in this assay. The mechanism of sCD23 growth stimulation is believed to be an anti-apoptotic signal, rather than a direct mitogenic signal, which subsequently allows the SMS-SB cells to proliferate (White, 1995). If the same signal is generated by 45kDa CD23a, it is possible that in the monolayer system used here, CD23a is mediating an anti-apoptotic signal but the COS7 cells are preventing the subsequent growth of the SMS-SB cells, so that no effects are seen in the proliferation assay. To investigate this possibility, 45kDa CD23a ‘stimulated’ SMS-SB cells could be analysed by two-colour flow cytometry using the DNA-binding fluorochromes propidium iodide and Hoechst 33342. This technique (detailed by Dive et al., 1992), discriminates and quantifies viable and apoptotic cells, and was previously used to directly show that sCD23 prevents the apoptosis of low cell density SMS-SB cultures (White, 1995).
Figure 4.1 Photomicrographs of SMS-SB cells transiently-transfected with CD23a.

SMS-SB cells were transfected with the CD23 expression vector pcDL SRα 296CD23a, using the liposome-mediated transfection reagent DOTAP as detailed in section 2.2.2a. At 24 hours post transfection, the cells were examined by phase-contrast microscopy using a 40X magnification lens. Panel A- untransfected cells, Panel B- CD23a-transfected cells, Panel C- cells transfected with an empty expression vector (mock-transfected).
Figure 4.2 Flow cytometric analysis of CD23a-transfected SMS-SB cells.

SMS-SB cells were transfected with CD23a as described for Figure 4.1. At 24 hours post-transfection 1x10⁶ cells were removed from culture, washed twice in PBS and stained with a FITC-conjugated anti-CD23 antibody for 20 minutes protected from the light. After further washing, the cells were analysed by flow cytometry in the FITC fluorescence channel only. The histograms illustrate CD23 expression in logarithmic fluorescence on the x-axis, against a linear scale of cell number on the y-axis. Panel A- unstained untransfected cells, Panel B- antibody stained untransfected cells, Panel C- stained mock-transfected, Panel D- stained CD23-transfected cells.

The data shown is representative of 3 independent repeats.
CD23 (FITC)
Figure 4.3 Diagramatic representation of the different CD23 forms and the oligonucleotide primers designed for their PCR generation.

An illustration of the two membrane (45kDa), and the two soluble (37 and 25kDa), forms of CD23 showing the major structural features, namely the leucine zipper and lentil lectin homology domains.

\[ \text{transmembrane spanning domain} \]

Oligonucleotide primers, corresponding to the amino acid sequences indicated by the arrows A→E, were designed using the published cDNA nucleotide sequence (Entrez accession number M14766). These were used for the PCR generation of each CD23 form as indicated. Details of each oligonucleotide sequence, including the intrinsic restriction sites are given.

ATG : transcriptional start codon

\[ \text{GGATCC} \] : BamHI restriction site

\[ \text{GCGGCCC} \] : NotI restriction site
A: 5'-GTAGGATCCACCGCC**ATG**GAGGAAGGTC -3
B: 5'-GTAGCGGCCGCTCAAGAGTGGAGAGGGGCAG -3
C: 5'-GTAGGATCCAGCATA**ATG**AATCCTCCAAGCCAGGAGATCGAGGAGCTTCCCAGG -3
D: 5'-CACGGGATCCAG**ATG**GCACGAAATC -3
E: 5'-GACAGGATCCAGG**ATG**GAGTTGCACGT -3
Figure 4.4 Gel electrophoresis showing the PCR amplification of the CD23 forms.

The different forms of CD23 were generated by PCR amplification using the pcDL SRα296CD23a plasmid as template DNA, Pfu DNA polymerase, and the primers detailed in figure 4.3. For each set of reactions, a negative control (no DNA template) was also performed to control for contaminating DNA. Details of reaction mixtures and PCR cycle parameters are given in section 2.2.10b. After the reactions were complete, the amplification products were separated by gel electrophoresis and visualised on a UV transilluminator. 1kb DNA ladder standards were also run for the verification of PCR product identity (shown here in panel B as bp fragments).

Panel A- PCR amplification of CD23a, Panel B- CD23b, Panel C- CD23 37 and 25kDa. Lane 1 (a/b)- positive PCR amplification, Lane 2 (a/b)- negative control. Each of the amplification products and their molecular sizes are indicated.
Figure 4.5  Gel electrophoresis showing the CD23 forms excised from their cloning vectors.

The CD23 constructs generated by PCR amplification were cloned into plasmid vectors. The ‘a’ and ‘b’ isoforms were cloned into pcDNA3.1(+) using the intrinsically designed BamHI and NotI restriction sites, whereas CD23 37kDa and 25kDa were blunt-end cloned into pCR-Script SK(+). Maxi-prep preparations of each of the plasmid constructs were subjected to BamHI/NotI digestion. The digest was then separated by electrophoresis and visualised on a UV transilluminator. 1kb DNA ladder standards were also run (shown as bp fragments in panel A).

Panel A- restriction digest of the membrane CD23 isoforms, Lane 1- CD23a, Lane 2- CD23b. Panel B- digest profile of the soluble CD23 forms, Lane 1- CD23 25kDa, Lane 2- CD23 37kDa.
Each of the cloned forms of CD23 were sequenced using the T7 and T3, or the T7 and RP primer binding sites present in pCR-Script SK(+) and pcDNA3.1(+) vectors, respectively. The sequence for each one was then compared using GCG pair-wise alignment analysis to the published CD23 sequence (Entrez accession number M14766).

The nucleotide sequence and the corresponding amino acids are represented in full for 45kDa CD23a, and for the soluble 37kDa and 25kDa forms. Only the N terminal region of CD23b is detailed, as the remaining sequence is identical to the ‘a’ isoform.

\[\text{transcriptional start codon}\]

\[\text{transcriptional stop codon}\]

\[\text{regions of the ‘a’ and ‘b’ isoforms that differ}\]

\[\text{alterations in the cloned nucleotide sequence compared to the published sequence}\]

\[\text{nucleotide 690: } A \rightarrow G = \text{GGA (glycine)} \rightarrow \text{GGG (glycine)}\]

\[\text{nucleotide 717: } T \rightarrow C = \text{CAT (histidine)} \rightarrow \text{CAC (histidine)}\]
Figure 4.7 Analysis of 45kDa CD23a-transfected COS7 cells.

Panel A represents the flow cytometric analysis of the cells. CD23a transfected COS7 and mock-transfected COS7 cells were removed from culture using non-enzymatic cell dissociation solution and washed twice in PBS. 1x10^6 cells were then stained with a FITC-conjugated anti-human CD23 antibody and analysed. The solid line represents unstained cell autofluorescence, the dotted line is anti-CD23 antibody stained cells. Aii- mock-transfected COS7 cells, Aii- CD23a-transfected COS7 cells (CD23aCOS7), Aiii- fluorescently sorted CD23aCOS7 cells. The histograms illustrate CD23 expression in logarithmic units of fluorescence on the x-axis, against a linear scale of cell number on the y-axis. The results are representative of 5 independent repeats.

Panel B represents the Western blot analysis of the fluorescently sorted CD23aCOS7 cells. CD23aCOS7 and mock-transfected COS7 (mCOS7) cells were removed from culture as for panel A, and extracts prepared. 30 and 60μg of protein were subjected to electrophoresis through a 10% (w/v) acrylamide separating gel. After transfer to nitrocellulose, blots were incubated with 0.8μg/ml of rabbit anti-human CD23 antibody, and then with a 1:1000 dilution of protein-A-HRP. The blot was then developed with an ECL detection system. The results are representative of 3 independent repeats with different cell preparations.
A i)  

A ii)  

A iii)  

CD23(FITC)  

B  

<table>
<thead>
<tr>
<th></th>
<th>mCOS7</th>
<th>CD23aCOS7</th>
</tr>
</thead>
<tbody>
<tr>
<td>30µg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>60µg</td>
<td></td>
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</tbody>
</table>

CD23 (45kDa)
Figure 4.8 Analysis of 45kDa CD23a expression in transfected COS7 cells by immunocytochemistry.

Mock-transfected COS7 and sorted CD23aCOS7 cells adhered to coverslips were fixed with 4% (w/v) paraformaldehyde/PBS and then washed. After blocking in 0.5%BSA/10% (v/v) FCS/PBS buffer, the cells were incubated with mouse anti-human CD23 antibody. In order to visualise primary antibody staining the cells were washed and incubated with rabbit anti-mouse IgG FITC-conjugated antibody. The coverslips were then mounted onto slides, and the cells examined on a Biorad MRC-600 confocal microscope.

Panel A- CD23aCOS7 cells, panel B- mock-transfected COS7 cells. The data shown are representative of 3 independent repeats.
Figure 4.9 Effect of 45kDa CD23a on $[^{3}\text{H}]$-Thymidine incorporation by SMS-SB cells at various densities.

SMS-SB cells grown in serum-supplemented RPMI-1640 medium were seeded at $5 \times 10^5$, $1 \times 10^5$, $5 \times 10^4$, $1 \times 10^4$, $5 \times 10^3$ and $1 \times 10^3$ cells per ml onto paraformaldehyde-fixed 'feeder' cells. The graphs represent the incorporation of $[^{3}\text{H}]$-TdR for various seeding densities of SMS-SB cells alone, or in the presence of either untransfected COS7 or CD23a-expressing COS7 cells. To correct for the background proliferation of the monolayer cells, the $[^{3}\text{H}]$-TdR incorporation value for fixed COS7 or CD23aCOS7 cells alone was subtracted from the appropriate incorporation values of the SMS-SB cells. The cultures were pulsed with 0.3μCi/well $[^{3}\text{H}]$-thymidine for 4-5 hours prior to harvest, after 24 hours or 48 hours of incubation. All cultures were in triplicate and the error bars represent the standard deviation of corrected triplicate data.

The experiment is representative of 4 independent repeats.
[3H]-Thymidine incorporation (cpm)
Figure 4.10 Northern blot analysis of the expression of c-fos, c-jun and c-myc in SMS-SB cells, upon stimulation with CD23a expressed on COS7 cells.

SMS-SB cells were grown in the serum-free medium, Optimen, were placed on CD23aCOS7 and COS7 cells at a density of 8x10^5/ml for 0, 15, 30, or 60 minutes. Total RNA was then isolated from the cells and 20μg subjected to electrophoresis. The RNA was then blotted onto nylon membrane, hybridised with a [32P]-labelled probe for either c-fos, c-jun or c-myc, and visualised by autoradiography. The membranes were stripped and re-hybridised with a 7S probe and visualised. The positions of the 1.35, 2.37, 4.4, and 7.46 kb RNA markers are indicated to the right of each image in panel B.

Panel A - transilluminator image of the separated RNA; panel B - hybridisation of membranes with specific probe (c-fos, c-jun or c-myc); panel C - hybridisation of membranes with 7S probe. The experiments shown are representative of 3 independent repeats using different preparations of RNA made from stimulated SMS-SB cells.
CHAPTER 5

RESULTS

STUDIES OF THE NOVEL RECEPTOR FOR CD23
ON VARIOUS B CELL LINES
5.1 INTRODUCTION

In addition to IgE, other known ligands/receptors for CD23 are CD21 (Aubry et al., 1992), CD11b and CD11c (Lecoanet-Henchoz et al., 1995). The former is also the receptor for EBV (Tanner et al., 1987), interferon-α (Delcayre et al., 1991), and C3 complement proteins (Weis et al., 1984). CD23 and CD21 function as adhesion molecules which are important in the B cell/T cell interaction necessary for IgE production (Aubry et al., 1992, 1993), and for B cell antigen presentation to T cells (Grosjean et al., 1994). The CD23/CD21 interaction is also proposed to be important for the development of B cells undergoing selection within the germinal centre. Anti-CD21 antibodies have been found, like follicular dendritic cell-derived sCD23 (Liu et al., 1991a), to rescue germinal centre B cells from apoptosis (Bonnefoy et al., 1993), suggesting that the survival signal is mediated by CD21. In this regard, CD23 derived from bone marrow stromal cells (Fourcade et al., 1992), may deliver anti-apoptotic signals to developing precursor B cells. However, B cells do not express CD21 until the late pre-B cell stage, raising the possibility that other receptors for CD23 may mediate early B cell development.

CD11b and CD11c represent the α chains of the β2 integrin adhesion molecules, CD11b-CD18 and CD11c-CD18, that act as CD23 receptors on monocytic cells (Lecoanet-Henchoz et al., 1995). Upon binding to monocytes via CD11b/c, CD23 causes a marked increase in nitric oxide production and the release of proinflammatory cytokines. Within the B cell compartment, these adhesion molecules are only found on activated cells, whereas the CD11a-CD18 member of this integrin family is often expressed on unactivated cells. CD11a however is not a receptor for CD23 (Lecoanet-Henchoz et al., 1995).

Studies on the SMS-SB cell line by White, 1995, demonstrated that although these cells do not express any of the known receptors for CD23, they can specifically bind to full-length (45kDa) CD23a-containing fluorescent liposomes, an effect that can be partially inhibited by sCD23. This suggested that the rescue of LCD SMS-SB cells from death by sCD23 was mediated via a novel receptor for CD23 (CD23NR), which is able to bind both soluble and membrane forms of CD23. Although preliminary
results from CD23-affinity-columns suggested that the novel receptor has a molecular weight of approximately 80-85kDa, antibodies to CD molecules of approximately this size could not inhibit liposome binding, thus failing to identify the novel CD23 receptor (White, 1995).

More recent studies on SMS-SB cells have begun to further characterise CD23NR using BIAcore surface plasmon resonance technology. This method allows the interactions of macromolecules to be studied and, as binding is measured over time, kinetic constants for the interaction can be determined. Initial work has demonstrated that the binding of the SMS-SB novel receptor to sCD23 can be studied using this technique, and that the interaction seems to be calcium-independent, and of a protein-protein nature as it is not inhibited by fucose-1-phosphate. The interactions of CD23 with CD23NR are therefore very different from those of CD21 or CD11b/c, which involve the C-type lectin activity of CD23 (Aubry et al., 1994; Lecouanaet-Henchoz et al., 1995). In addition, preliminary experiments have recovered biotinylated SMS-SB membrane proteins, i.e., CD23NR, from the binding interaction and has been visualised by Western blotting as a 80-90kDa protein (Dr. J Matheson -personal communication), thus supporting the previous findings by White (1995). This method also potentially provides a means of isolating protein(s) for microsequencing analysis, to determine the identity of the novel receptor. Using BIAcore technology for isolation purposes has a significant advantage over standard biochemical procedures, in that CD23NR binding can be monitored throughout the isolation.

As SMS-SB cells express a novel receptor for CD23, it was decided to investigate whether this receptor was unique to this pre-B cell line. The results presented in this chapter thus describe an investigation into the presence of CD23NR on other human B cell lines. The cell lines used for this study were primarily other pre-B ALL lines (Blin-1, Nalm-1, Nalm-6, 697 and 207), but also a mature-B ALL (1E8), and an EBV-transformed mature B cell line (EDR). The cells were analysed for the expression of CD23 and the known receptors, the ability to bind to CD23a-expressing monolayers, and for the interaction with sCD23 as analysed by BIAcore technology.
5.2 RESULTS

5.2.1 Phenotypic analysis of B cell lines

In order to investigate the presence of the novel receptor(s) for CD23 on other B cell lines, the cells firstly had to be analysed for the expression of CD23 and the known receptors for CD23. In addition to these CD antigens, the cells were also analysed for the other β2-integrin, CD11a, and also for the B cell marker CD19. Each cell line was stained with a FITC-conjugated antibody specific for each antigen and analysed by flow cytometry. The cells were not tested for CD18 expression as this subunit of the integrin receptors is not involved in the binding to CD23 (Lecoanet-Henchoz et al, 1995).

Figure 5.1 shows the analysis of the cell line Blin-1. The cells are positive for CD19 expression, but are negative for all the other surface antigens tested as they exhibited histogram profiles essentially identical to unstained cells. In comparison, 207 cells (figure 5.2), are positive for CD23 and CD21, and weakly positive for CD11a and CD19, demonstrated by the increases of FITC fluorescence on the x-axis. Each of the other B cell lines were analysed in this manner, the results of which are summarised in Table 3, along with the culture phenotype of each line. The results demonstrate that like SMS-SB, a number of the other cell lines are negative by flow cytometry for surface expression of CD23 and the known CD23 receptors, namely, Blin-1, Nalm-6 and 697 (pre-B cell lines), and 1E8 (mature-B cells). Of these lines, only 697 exhibited a clumping phenotype in culture, which must be mediated by other adhesion interactions.

The other cell lines, Nalm-1, 207 (pre-B), and EDR (mature-B), express all, or a combination of CD23, CD21 or CD11a, potentially explaining the clumping phenotype of these cell lines in culture. Although not a receptor for CD23, the β2-integrin CD11a is involved in cell adhesion and as such may contribute to the clumping displayed by these cell lines.
The absence of CD11b and CD11c from all the cell lines analysed was not surprising as these antigens are not normally found on precursor or resting B cells, but act as CD23 receptors on monocytic cells (Lecoanet-Henchoz et al., 1995). The antibodies against these antigens had previously been tested on known antigen-positive cell lines, eliminating concerns that the negative result was due to inefficient antibody binding or fluorescence.

5.2.2 Other B cell lines bind to CD23aCOS7 cells

As SMS-SB cells were found to bind to CD23a-expressing monolayer cultures (section 4.2.4b), it was decided to assess whether the other B cell lines under investigation could also bind to the monolayers. Each cell line, at a density of 5x10^5/ml, were placed for 60 minutes onto confluent cultures of CD23aCOS7 and control COS7 cells. The B cells were then compared to SMS-SB cells for their ability to bind to the monolayers.

The results (summarised in Table 3), show some inconsistency between the expected results based on cell surface phenotype, and what was observed in the assay. The CD21 positive cells, 207 and EDR, bound to both of the monolayers, suggesting that binding was not necessarily specific for CD23, whereas the weakly CD21-positive Nalm-1 cells did not seem to bind to either monolayer. Of the cells lines that do not express any of the know receptors for CD23, 697 cells bound to both monolayers, while 1E8 cells did not bind to either. However, the Blin-1 and Nalm-6 cell lines bound to the CD23a-expressing monolayer, with no or only weak binding, respectively, to the control COS7 cells. This suggests that these cell lines, like SMS-SB cells, are binding to CD23 on the monolayer cells via a novel receptor for CD23.

Although inconclusive for some of the known CD23 receptor-negative cell lines, this basic assay indicated that other cell lines may indeed express the novel receptor for CD23 found on SMS-SB cells.

As all subsequent investigations were based on the ability of the novel receptor to bind CD23, none of the other CD23 receptors could be present on the cell lines being
studied. Based on this, and the encouraging results of CD23aCOS7 cell binding assay, Blin-1, Nalm-6 and 1E8 cells were chosen for further investigation.

5.2.3 BIAcore analysis of SMS-SB, Blin-1, Nalm-6 and 1E8 cell lines

In order to directly demonstrate the existence of a novel CD23 binding receptor on other B cell lines, cell extracts were analysed by BIAcore technology for interactions with sCD23. The basis of BIAcore analysis, surface plasmon resonance (SPR), is an optical phenomenon which measures changes in the refractive index close to the surface of a sensor chip. The refractive index is changed by the binding of material to the surface immobilised ligands on the chip, thus allowing binding interactions to be monitored by changes in SPR signal.

Extracts were made of each cell line to be analysed using a buffer based on octyl-β-D-glucopyranoside (OGP), a detergent which solubilises membrane-bound proteins in their native state and preserves protein-protein interactions. Diluted membrane extracts were individually injected in continuous flow over a sCD23 immobilised flow cell, and then over a blank flow cell on a sensor chip. SPR measurements, expressed in resonance units (RU), were monitored over time to produce sensorgram profiles of the interactions between the analytes (the cell extracts) and the sCD23 ligand (details of procedure are outlined in section 2.2.13). Previous analysis using monoclonal antibodies specific for CD23 had demonstrated that sCD23 was successfully immobilised on the chip and so could be used for cell extract analysis (Dr. J Matheson -personal communication).

Figure 5.3 shows the BIAcore analysis of SMS-SB cells. The sensorgram illustrates the real time binding of cell membrane extracts to sCD23-immobilised and blank flow cells of a sensor chip. Four characteristic phases of SPR change can be identified: i- represents the initial increase in RU seen immediately after extract injection which results from a change in the buffer, ii- is the association of the analyte material to the sensor chip surface, iii- decrease in response due to re-introduction of normal flow buffer, and iv- the natural dissociation of the analyte from the chip after injection is terminated.
The amount of analyte bound to each flow cell is represented by the difference in resonance (RU) from the pre-injection baseline to that seen after dissociation has occurred, and an exact value for this can be determined (presented in table 5). The results show that analyte has bound to the sCD23 flow cell, giving a change in response of 202 RU (indicated in figure 5.3 as $B_{T1}$). A response change (78.8 RU), is also seen for the blank flow cell ($B_{T2}$), demonstrating that non-specific binding to the sensor chip was occurring. The specific binding ($B_{S}$), of SMS-SB cell extract to sCD23 is therefore 123.2 RU ($B_{S} = B_{T1} - B_{T2}$), which according to current consensus is a significant result. Analysis of the binding kinetics shows that the CD23NR expressed on SMS-SB cells and sCD23 has an equilibrium constant ($K_D$) of $8.1 \times 10^{-10}$ M.

Figure 5.4 shows the results of the BIAcore analysis of Blin-1 (panel A), Nalm-6 (panel B), and 1E8 (panel C). Each set of sensorgram profiles is similar to those obtained for SMS-SB cells in figure 5.3. All the extracts showed binding to both the sCD23 flow cell and to the blank flow cell. Specific binding of Blin-1, Nalm-6 and 1E8 extracts to sCD23 was 62.3, 72.9, and 75.3 RU, respectively (table 5). These data demonstrate that like SMS-SB cells, Blin-1, Nalm-6 and 1E8 cells are able to bind to sCD23 in the absence of any of the known receptors, suggesting that these cell lines express a novel binding receptor for CD23. Kinetic analysis of the novel CD23 receptors on these cells and sCD23 (table 5), demonstrates that the equilibrium constants are within a similar range to that of SMS-SB cells. This suggests that the interaction with sCD23 may be mediated by a similar receptor species.

### 5.3 DISCUSSION

The data presented in this chapter demonstrate that in addition to SMS-SB cells, the pre-B cell lines Blin-1 and Nalm-6, and, the mature B cell line 1E8, do not express any of the known receptors for CD23, namely CD21, CD11b or CD11c, but are able to bind to 45kDa CD23a-expressing monolayers, and significantly bind to sCD23 as analysed by BIAcore analysis. These results suggest that these cell lines also express a novel binding receptor for CD23. CD23NR may therefore not be exclusive to the pre-B cell line SMS-SB.
The CD23NR on SMS-SB cells is able to bind to both full-length 45kDa CD23 and sCD23 (White, 1995). Binding to the latter has now been further confirmed using BIAcore technology. In addition to binding sCD23 on the BIAcore, Blin-1 and Nalm-6, but not 1E8 cells, seem to bind to 45kDa CD23a expressing monolayers. However, to formally demonstrate that the CD23NR on these cells can bind the full-length form of CD23, all these cell lines would have to be analysed for binding to 45kDa CD23a-containing liposomes. In addition, antibodies to the known CD23 receptors could be tested for their inability to inhibit liposome binding, thereby confirming that these receptors are not responsible for CD23 binding by these cells. Although Blin-1, Nalm-6 and 1E8 cells do not express CD21, CD11b or CD11c as analysed by flow cytometry, the possibility exists that the cells may express very low levels of these receptors undetectable by this method of analysis. Unfortunately, liposome binding analysis was not performed at the time of this study due to the unavailability of purified 45kDa CD23a to use in liposomes.

Although the BIAcore kinetic data suggest that the interaction between sCD23 and the novel binding receptor is similar for SMS-SB, Blin-1, Nalm-6 and 1E8 cells, it is not possible to state unequivocally at this stage whether the same molecular species is present on all the cell lines. It is possible that CD23 binding maybe mediated by a number of different novel receptors. To investigate this possibility, experiments could be performed to characterise the nature of the sCD23/CD23NR interactions for each of these cells, as analysis of SMS-SB cells has demonstrated that receptor-ligand binding seems to be mediated by protein-protein interactions. In addition, SMS-SB sCD23 binding material has been successfully recovered from the BIAcore and identified a 80-90kDa protein by Western blotting (Dr. J Matheson- personal communication). Although 2-D electrophoresis still needs to be performed to ensure that the 80-90kDa species corresponds to a single protein, the potential exists for protein microsequencing to be performed. If successful, this procedure could be performed with the other B cell lines, allowing their novel receptors for CD23 to be directly compared.

As all the B cell lines analysed on the BIAcore were found to bind sCD23, it is possible that the result is an artefact of use of the BIAcore instrument itself. This is
however unlikely, as analyte binding to the sCD23 flow cell was significantly greater than that on the blank flow cell. To rule out this possibility, a cell line known not to bind to CD23 should also be analysed. Unfortunately the cell line Raji, previously found not to bind CD23a-liposomes (White, 1995), was not available at the time of this study. Future analysis of this cell line as a negative control is paramount to confirm the validity of the BIAcore approach.

In the SMS-SB cell line, sCD23 acts via the novel receptor to inhibit apoptosis (White, 1995). It will be of great interest to determine the consequences of stimulating CD23NR on Blin-1, Nalm-6 and 1E8 cells in order to determine the biological importance of the novel receptor on these cells. As these, like SMS-SB cells, are B cell acute lymphoblastic leukaemia cell lines, it is possible that CD23NR may play a role in the transformation or subsequent maintenance of the cells by delivering survival signals. It will therefore be important to analyse normal precursor B cells for the expression of CD23NR, to determine if it potentially has a role in normal early B cell development. The discovery of a novel CD23 binding receptor, although potentially not the same species, on the mature B cell line 1E8, suggests that the novel receptor may not be restricted to the early stages of B cell development. Normal B cells from all developmental stages will therefore have to be screened for the expression of CD23NR.

Although cell lines expressing CD23 and the known receptors for CD23 may also express the novel receptor, it is not possible to test these cells using the currently available assays. Investigations of these cell lines can only be performed when a specific monoclonal towards the novel receptor is available.

At present the identity of the novel CD23 receptor on SMS-SB cells remains to be fully determined. However, the demonstration that novel binding receptors exist on other B cell lines allows these to be studied as alternative models. The function of the novel receptor on these cells is also unknown making the study of this receptor(s) very interesting.
Figure 5.1 Flow cytometric analysis of Blin-1 cells.

1x10^6 Blin-1 cells were removed from culture, washed twice in PBS and stained with a FITC-conjugated antibody (CD19, CD21, CD23, CD11a, CD11b, or CD11c). After a 20 minute incubation, the cells were washed, then analysed for surface expression of each CD antigen. The histograms illustrate CD antigen expression in logarithmic units of fluorescence on the x axis, against a linear scale of cell number on the y-axis.

The results are representative of 3 independent repeats.
Figure 5.2 Flow cytometric analysis of 207 cells.

$1 \times 10^6$ 207 cells were removed from culture, and analysed for the expression of various CD antigens as detailed for figure 5.1. The results are representative of 3 independent repeats.
Table 3. Flow cytometric analysis of various B cell lines.

Each of the cell lines under investigation were analysed for the expression of various CD antigens as described and illustrated in figures 5.1 and 5.2. Each cell line was then classified as either: positive (+ +), weakly positive (+), or negative (−) for cell surface expression of each antigen. The culture phenotype of each cell line is also indicated as analysed using phase contrast microscopy.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>Cell type</th>
<th>Phenotype</th>
<th>CD19</th>
<th>CD23</th>
<th>CD24</th>
<th>CD41a</th>
<th>CD11b</th>
<th>CD11c</th>
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<tbody>
<tr>
<td>SMS-SB</td>
<td>pre-B</td>
<td>single cell</td>
<td>++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>small clumps</td>
<td>++</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Blm-1</td>
<td>pre-B</td>
<td>single cell</td>
<td>++</td>
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<td>Nalm-6</td>
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<td>697</td>
<td>pre-B</td>
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<td>-</td>
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<td>1E6</td>
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Table 4. Binding of various B cell lines to 45kDa CD23a expressed on COS7 cells.

B cells at $5 \times 10^5$/ml were placed onto confluent cultures of CD23aCOS7 and untransfected (control) COS7 cells. The cultures were then left without disturbance for 60 minutes and then examined by phase contrast microscopy. The B cell lines were assessed for binding to the COS7 feeder layers by agitating the flasks, and designated as either: binding to the monolayers (+ +), weak binding (+), or no binding (−).
<table>
<thead>
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<th>Cell line</th>
<th>Feeder layer</th>
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<td></td>
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<td>++</td>
</tr>
<tr>
<td>Nalm-1</td>
<td>-</td>
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<td>Bln-1</td>
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</tr>
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<td>207</td>
<td>++</td>
</tr>
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<td>1B8</td>
<td>-</td>
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<tr>
<td>EDR</td>
<td>++</td>
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</table>
Membrane extracts of SMS-SB cells were prepared as described in section 2.2.13a. Diluted extract (the analyte), was injected over the surface of a BIAcore sensor chip in a continuous buffer flow. The overlaid sensorgrams illustrate the real time binding of SMS-SB membrane extract, expressed as resonance units (RU), to sCD23-immobilised on a CM5 sensor chip flow cell (sCD23-FC), and to a blank flow cell (blank-FC).

The four characteristic stages of the analysis are indicated on the sCD23-FC sensorgram: i- bulk contribution; ii- association of analyte to chip; iii- bulk contribution; iv- dissociation of analyte from chip. ▲ and ▼ represent the points at which analyte injection was started and stopped, respectively. \( B_{T1} \) represents the total binding of SMS-SB extract to the sCD23-FC; \( B_{T2} \) total binding to the B-FC; \( B_S \) specific binding to sCD23.

The sensorgrams are representative of 3 independent repeats of BIAcore analysis using different SMS-SB extract preparations and different sensor chips.
Figure 5.4 BIAcore analysis of Blin-1, Nalm-6 and 1E8 cell lines.

Other B cell lines were analysed by BIAcore technology as described in figure 5.3. Panel A- Blin-1 cells; panel B- Nalm-6 cells; panel C- 1E8 cells. $B_s$ represents specific binding to sCD23. The sensorgrams are representative of 3 independent repeats using different cell extract preparations and different sensor chips.
A

B

C

sCD23-FC

BS

blank-FC

response (RU)

time (seconds)
Table 5. Binding responses and kinetics of BIAcore interactions.

The total binding (in RU), of each cell extract, to the sCD23-immobilised (B_{T1}) and the blank flow cell (B_{T2}) was determined by BIAcore analysis. The specific binding to the sCD23 was then calculated (B_S = B_{T1} - B_{T2}). The association (k_a (M^{-1}s^{-1})) and dissociation (k_d (s^{-1})) kinetics were determined using the BIAevaluation 2.1 analysis package. The values presented are those derived from the sensorgrams illustrated in figures 5.3 and 5.4.

Equilibrium constant; K_D (M) = k_d / k_a
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<th>B&lt;sub&gt;2&lt;/sub&gt;: total blank-TC binding (RU)</th>
<th>B&lt;sub&gt;s&lt;/sub&gt;: specific CD23 binding (RU)</th>
<th>K&lt;sub&gt;S&lt;/sub&gt; (M&lt;sup&gt;-1&lt;/sup&gt;)</th>
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<td>123.2</td>
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<td>62.3</td>
<td>3.5x10&lt;sup&gt;-10&lt;/sup&gt;</td>
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<tr>
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<tr>
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<td>43.1</td>
<td>75.3</td>
<td>1.2x10&lt;sup&gt;-9&lt;/sup&gt;</td>
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</table>
CHAPTER 6

GENERAL DISCUSSION
6.1 Main conclusions

The main findings of this thesis are that the autocrine factor previously identified in culture supernatants of the pre-B cell line SMS-SB (Zack et al., 1987; White, 1995), possibly rescues low cell density cultures from apoptosis by maintaining the expression of the anti-apoptotic protein Bcl-2 and promoting cell cycle progression. During attempts to identify the specific Bcl-2 family members influenced by SB-AF, SMS-SB cells were found to express high levels of Bcl-2, which is unusual for cells at the pre-B developmental stage. Experiments performed to characterise and isolate the autocrine factor suggest that SB-AF maybe a multi-component factor, whose anti-apoptotic activity results from the synergistic action of a number of factors. At least one, if not all, of the essential components of SB-AF is a protein which is heat stable to 90°C.

Although the soluble forms of CD23 have been found to act as paracrine survival signals for SMS-SB cells (White, 1995), the results of the current investigation suggest that, in comparison, full-length 45kDa CD23a does not seem to elicit the same response in SMS-SB cells. However, as discussed in chapter 4, it is possible that the COS7 cell monolayer used to present 45kDa CD23a to SMS-SB cells may be influencing the response of the SMS-SB cells to CD23.

The pre-B cell lines Blin-1 and Nalm-6, and the mature B cell line 1E8, do not express the known receptors for CD23, namely CD21, CD11b or CD11c, but are able to bind, like SMS-SB cells, to sCD23 as analysed by BIAcore technology. Therefore, in addition to SMS-SB cells, other B cells express a novel binding receptor for CD23. At present it is not however known whether the same molecular species is present on all the cell lines, or what the functional significance of CD23NR is on the Blin-1, Nalm-6 or 1E8 cell lines.

6.2 SMS-SB cell autocrine factor

The constitutive production of an autocrine growth factor by SMS-SB cells, allows the cells to grow in vitro in serum-free and protein-free media without the addition of
exogenous mitogens or stromal cell support. In the patient, the autocrine factor could have allowed the leukaemic pre-B cells to survive and actively proliferate in the peripheral blood resulting in a very aggressive leukaemia. Although autocrine factors are regarded not to be the sole criterion for transformation (Young et al., 1991), and can be produced by normal cells (Gordon and Cairns, 1991), the ability to independently regulate growth and survival may still significantly contribute to the development of malignancies. The constitutive production on an autocrine factor may possibly extend normal survival or growth thus allowing other cooperating genetic mutations to occur (Levy and Bost, 1996).

There are a number of mechanisms which could result in the constitutive expression of the SMS-SB autocrine factor. Firstly, if SB-AF is a factor normally produced by the stromal cells to control B cell growth in a paracrine fashion, the aberrant expression of SB-AF in SMS-SB cells would render the cells independent of stromal cell regulation, and thus achieve autonomous growth. Secondly, SB-AF may represent a normal B cell autocrine factor, the effects of which have become deregulated by: i) increased production of the factor, ii) inappropriate expression of the receptors for the factor, or iii) the loss of action or response to an inhibitory autocrine or paracrine factor.

At present, the identity of the SMS-SB cells autocrine factor remains unknown. None of the cytokines tested by White were able to substitute for SB-AF activity (White, 1995), although the growth promoting effects of IL-10 and II-8 have not been tested. The results of this current study suggest that SB-AF may be the result of the synergistic action of a number of factors, all of which are required for anti-apoptotic activity. The discovery that SB-AF potentially mediates its survival effect by maintaining the levels of the anti-apoptotic protein Bcl-2, are in line with a previous report by Francia di Celle et al., 1996, suggesting that Bcl-2 is a potential target for the regulation of cell survival mediated by autocrine growth factors.

In addition to its survival function, Bcl-2 is also able to restrain cell cycle entry (O'Reilly et al., 1996; Huang et al., 1997). In this respect, the ability of the autocrine factor to promote both survival and cell cycle progression is unusual. It is therefore possible that in addition to Bcl-2, the autocrine factor may be influencing the
expression of other proteins, namely those involved in the regulation of cell proliferation. A potential candidate is c-Myc, which, has been found to cooperate with deregulated Bcl-2 in the transformation of lymphoid cells (Vaux et al., 1998; Strasser et al., 1990). It would therefore be of interest to determine whether SB-AF can influence the expression levels of the c-Myc protein.

In addition to the production of autocrine factors, transformed lymphocytes may also produce factors which stimulate the production of cytokines by normal cells that may further enhance malignant cell growth. In this respect, SMS-SB cells produce leukaemia-derived transforming growth factor (LD-TGF), which acts in a paracrine manner to activate the growth of fibroblasts (Zack et al., 1987). LD-TGF has also been found to stimulate the production of growth inhibitors by macrophage-like cells in LTMC (Prof. B.W Ozanne - personal communication). Thus, a hypothetical role for LD-TGF in the bone marrow of patient SB is the activation of stromal cell fibroblasts to produce B cell growth factors, and the simultaneous activation of macrophages to produce growth inhibitors. This apparent contradictory action of LD-TGF may be explained if SMS-SB cells have lost the ability to respond to inhibitory cytokines. Thus by producing LD-TGF, the leukaemic cells may have indirectly stimulated their own growth, whilst inhibiting the growth of normal B cells, giving themselves a further selective advantage.

6.3 Bcl-2 over-expression in SMS-SB cells

The pre-B cell stage of normal progenitor B cell development is characterised by low levels of expression of Bcl-2 (Merino et al., 1994), which is consistent with many cells at this stage undergoing apoptosis due to non-functional rearrangement (Lu and Osmond, 1997). Therefore, the finding that SMS-SB cells, as pre-B ALL cells, have high levels of Bcl-2, suggests that this may have contributed to the transformation and/or subsequent survival of these cells in the patient.

Studies of other precursor B cell ALL malignancies have found that Bcl-2 expression was not only markedly higher than that of normal progenitor B cells, but generally surpassed the levels of expression found in mature resting B lymphocytes (Constan-
Smith et al., 1996). The relative levels of the Bcl-2 protein in leukaemic cells have also been found to correlate with the duration of their survival when placed into in vitro culture, suggesting that leukaemic cells with higher levels of Bcl-2 enjoy a selective survival advantage (Hanada et al., 1993; Pontvert-Delucq et al., 1996). The high levels of Bcl-2 in SMS-SB cells may therefore explain why, once removed from the patient, the cells adapted spontaneously to in vitro culture (Smith et al., 1981). Recent studies have demonstrated that over-expression of Bcl-2 correlates with a decrease in the apoptosis of precursor B cells, namely pre-B, leading to an significant increase (from 25 to 60%) in cell number and production rate of these cells in the bone marrow of Bcl-2 transgenic mice (Janani et al., 1998). These findings support the theory that the over-expression of Bcl-2 at the pre-B cell stage is important to the development of precursor B cell leukaemia.

SMS-SB cells have been found to have non-productively re-arranged Ig light chain genes (Prof. B.W Ozanne -personal communication). In normal pre-B cells, this occurrence prevents any further differentiation of the cell and results in apoptosis. However in the presence of high levels of Bcl-2, as in SMS-SB cells, a cell could be protected from cell death, thus allowing the continued survival of a malignant cell blocked at the pre-B cell stage of development.

Bcl-2 over-expression has been found in a number of B-ALL cases (Campana et al., 1993; Pontvert-Delucq et al., 1996; Coustan-Smith et al., 1996). Of the cases analysed by Pontvert-Delucq and colleagues, the only ALL to over-express Bcl-2 was a pre-B ALL. These cells had no exogenous growth factor requirement, were able to support their own survival/growth by the production of an autocrine factor, and did not harbour any chromosome 18 translocations (Pontvert-Delucq et al., 1996). These ALL cells thus exhibit the same characteristics as SMS-SB cells, demonstrating a potentially common mechanism involved in malignancy.

As SMS-SB cells do not harbour any gross chromosomal abnormalities (Smith et al., 1981), the over-expression of Bcl-2 cannot be the result of the t(14:18) translocation commonly associated with this proto-oncogene (Tsujimoto et al., 1984). There are however suggestions that high levels of Bcl-2 in malignancy may be due to p53
inactivation, which in its normal wild-type form down-regulates bcl-2 expression (Miyashita et al., 1994a, 1994b). In addition, wt-p53 has also been found to regulate the expression of growth factor and receptor genes, suggesting a role for mutant p53 in the gain of autocrine and paracrine growth advantages (reviewed by Asschert et al., 1998). By acting as a proliferation-suppressor, wt-p53 is important for the differentiation of haematopoietic cells during development (Shaulsky et al., 1991a, 1991b; Soddu et al., 1994). Conversely, transduction of haematopoietic progenitor cells with mutant-p53 prevents differentiation but promotes survival and proliferation (Shounan et al., 1996). Based on these reports, it would be of great interest to assess the status of p53 in SMS-SB cells, as the absence of p53, or the presence of a mutant protein, may partly explain the malignant phenotype of SMS-SB cells, with respect to the high expression levels of Bcl-2, the constitutive production of an autocrine factor, and the enhanced survival and proliferation of this aggressive leukaemia.

6.4 SMS-SB cell CD23NR

In addition to the autocrine factor, SMS-SB cells are rescued from apoptosis by sCD23 which, in the absence of any of the known receptors for CD23, mediates its effects via a novel CD23 receptor (CD23NR) (White, 1995). Previous in vitro studies have reported that when used in conjunction with IL-1α, sCD23 is able to rescue germinal centre B cells from cell death (Liu et al., 1991a). In addition, the sCD23/IL-1α combination is able to promote the proliferation and maturation of early myeloid precursors (Mossalayi et al., 1990a), thus suggesting a role for CD23 in haematopoiesis. In line with this theory, CD23 is expressed on the stromal cells of the bone marrow (Fourcade et al., 1992). However, CD21, the CD23 receptor believed to mediate the anti-apoptotic signals on germinal centre B cells (Bonnefoy et al., 1993), is not expressed until the late pre-B stage of development. In this respect, the presence of a novel receptor for CD23 on SMS-SB presents the possibility that stromal cell-derived CD23 could have influenced the survival, and therefore the transformation, of these malignant cells. If normal precursor B cells express CD23NR, CD23, in either soluble or membrane associated form, may have a role in normal precursor B cell survival and development.
In comparison to sCD23, full-length 45kDa CD23a, although able to bind to SMS-SB cells, does not seem to mediate any anti-apoptotic signals to the cells at low cell density. However, as discussed in Chapter 4 the lack of effect may be attributed to the COS7 cells used to generate the CD23a-expressing monolayer, making it possible that full length CD23 does mediate the same effects as the soluble forms. It is unfortunate that 45kDa CD23a could not be transfected into SMS-SB cells as this would have demonstrated whether the presence of this form of CD23 would render the cells density-independent for growth by providing a survival signal. However, if 45kDa CD23 does not mediate the anti-apoptotic effects of the soluble forms, it is possible that the potential effects of CD23 on precursor B cell development are mediated only by stromal cell-derived sCD23. However, it is not known whether the anti-apoptotic effect of sCD23 represents a true in vivo function of this cytokine. The role of CD23 in precursor B cell development is thus purely hypothetical at the present time.

In addition to potentially being important for the initial establishment of leukaemic SMS-SB cells in the bone marrow, it is possible that CD23NR may have had a role in the subsequent survival of the cells within the peripheral blood of the patient. However, it is not known whether patient SB had elevated levels of sCD23 in her serum to support this theory. Soluble CD23 levels are, however, elevated in B cell chronic lymphocytic leukaemia (Sarfaty et al., 1988), and as such would be an interesting model in which to investigate the possible expression and function of the novel CD23 receptor.

The anti-apoptotic and differentiation effects mediated by sCD23 are only seen when the responder cells are treated with IL-1α (Mossalayi et al., 1990a; Liu et al., 1991a). In this respect SMS-SB cells are unusual, as sCD23 alone was sufficient to mediate the rescue of cells from apoptosis (White, 1995). A potential explanation for this is that, as a leukaemic cell line, SMS-SB cells are in a constitutively activated state, potentially due the production of the autocrine factor, thus allowing the cells to aberrantly respond to sCD23. In addition, CD23NR expression may be a direct function of the cells being leukaemic, thus allowing them to respond to survival signals which cannot be utilised by normal B cells giving the malignant cells a further growth advantage. However, it is presently not known whether CD23NR is expressed.
on normal cells. If this is the case, these cells would possibly have to be activated, which would prevent CD23 from having non-directed and inappropriate survival effects.

### 6.5 CD23NR on other B cells

In addition to SMS-SB, the pre-B cell lines Blin-1 and Nalm-6, and the mature B cell line 1E8, also seem to express a novel CD23 binding receptor. As all these B cells were acute lymphoblastic leukaemia lines, CD23NR may be a common feature of this malignancy allowing the cells to respond aberrantly to sCD23 signals. This hypothesis will be tested by investigating the existence of the novel receptor on normal B cells.

The expression of CD23NR on the mature, albeit leukaemic, 1E8 cell line, suggests that the novel receptor may possibly mediate sCD23 signals outwith the bone marrow environment. Normal mature B cells express CD21 which, in a complex with CD19 and TAPA-1, is involved in the survival of centrocytes within the germinal centre. In this context, B cell CD21 is bound to the complement component C3d, which is associated with antigen held on the follicular dendritic cells (FDC). This interaction reduces the threshold for B cell activation by the B cell antigen receptor and provides a survival signal (reviewed by Fearon and Carter, 1995; Carroll, 1998). Soluble CD23 has been shown in vitro to provide an anti-apoptotic signal to activated germinal centre B cells (Liu et al., 1991a), a function believed to be mediated by B cell CD21 (Bonnefoy et al., 1993). However, in vivo, CD21 maybe occupied by C3b and is thus unavailable to bind sCD23. Therefore, the expression of a distinct receptor for CD23, in addition to CD21, would allow the cells to respond to FDC-derived CD23, and thus receive further survival signals within the germinal centre. Alternatively, if CD23NR expression is purely a function of the cells being leukaemic, it may allow the cells to receive survival signals. In the absence of CD21 expression on 1E8 cells, an unusual feature for a mature B cell line, the CD23NR pathway of anti-apoptotic signalling may be very significant.
6.6 Identity of the novel receptor for CD23

Preliminary BIAcore studies have found that the interaction between SMS-SB CD23NR and sCD23 is calcium-independent and is not inhibited by fructose-1-phosphate (Dr. J Matheson - personal communication). These results suggest that the interaction between the novel receptor and CD23 is of a protein-protein nature, and therefore different from the interactions of CD23 with CD21 or CD11b/c (Aubry et al., 1994; Lecoanet-Henchoz et al., 1995). Previous attempts to identify CD23NR on SMS-SB cells by antibody inhibition of CD23-liposome binding were not successful (White, 1995). It is hoped that the use of the BIAcore recovery procedure will allow the amino acid sequence of CD23NR to be determined. Until this information is available, there are a number of potential candidate molecules for CD23NR.

One possible candidate is the CD11d α chain of the β2 integrin adhesion molecule CD11d-CD18. Relatively little information is available at present regarding this adhesion molecule, although it is known to be more closely related to CD11b/c than to CD11a (Van der Vieren et al., 1995). As the former but, not the latter, are able to bind to CD23 (Lecoanet-Henchoz et al., 1995), this raises the possibility that CD11d will also be able to bind CD23 and thus act as a CD23 receptor. It will therefore be interesting to determine whether the B cell lines analysed in this investigation express CD11d, and whether an anti-CD11d antibody could inhibit liposome binding.

A recent study by Hermann and colleagues has identified the vitronectin receptor (VnR), αVβ3 integrin, and its associated CD47 molecule, as a novel receptor for sCD23 on monocytic cells (Hermann et al., 1999). Anti-CD47 and anti-CD61 (β3) monoclonal antibodies, and vitronectin were found to significantly suppress sCD23-induced proinflammatory cytokines production by monocytes. These antibodies did not however inhibit the binding of sCD23 to the cells, in comparison to an anti-CD51 (αV) antibody. To explain this result, the researchers postulate that sCD23 directly interacts with the αV component of the vitronectin receptor, but the β3 and CD47 components are involved in mediating receptor signalling (Hermann et al., 1999).
If CD23NR on SMS-SB cells is VnR/CD47, this may provide an alternative explanation for the apparent failure of 45kDa CD23a expressed on COS7 cells to stimulate SMS-SB cells at low cell density. This experiment was performed using SMS-SB cells grown in serum-containing media, and as serum is a source of vitronectin, this ECM component may have bound to the receptor thus preventing CD23 from binding and mediating its anti-apoptotic effects. However, the αv sCD23 binding component of VnR/CD47 is approximately 135kDa (Hermann et al., 1999), and so does not correspond to the 80-90kDa size band obtained after Western blot analysis of recovered SMS-SB BIAcore material. Although this suggests that VnR/CD47 does not represent CD23NR, SMS-SB, and the other B cell lines examined, will have to be tested for the expression of this receptor complex, and if positive, for the ability of antibodies to inhibit liposome binding.

6.7 Concluding remarks

Although the identity of the SMS-SB cell autocrine factor could not be determined, the study revealed that SMS-SB cells express high levels of the anti-apoptotic protein Bcl-2. It would be interesting in the future to investigate this potentially significant phenomenon further, especially with respect to determining the p53 status of the cells.

In addition to SMS-SB cells, other B-ALL cell lines, including a mature B cell line, also seem to express a novel binding receptor for CD23. This suggests that, in addition to possibly having a role in haematopoiesis, CD23 may influence the survival of cells within germinal centres, and therefore, perhaps, throughout their differentiation pathway. It will be important to determine whether, i) the novel binding receptor on all the B cells examined is the same molecular species, ii) the expression of CD23NR in other B cell differentiation stages, and iii) whether CD23NR is expressed on normal B cells. Ultimately, the importance of CD23NR outwith the B cell lineage will also need to be determined.

The presence of novel CD23 binding receptors on Blin-1, Nalm-6 and 1E8 cells allows these lines to be used as additional/alternative models for studying the
CD23NR phenomenon. In the light of the numerous problems experienced with the SMS-SB cell line during this investigation, this is of great benefit and will perhaps allow the investigations into the CD23NR to progress more successfully, whilst potentially uncovering as yet unknown features of the novel CD23 receptor.
REFERENCES


Bettler, B., Maier, R., Ruegg, D. & Hofstetter, H. (1989b). Binding site for IgE of the human lymphocyte low-affinity Fc epsilon receptor (Fc epsilon RII/CD23) is confined to the domain homologous with animal lectins. Proc Natl Acad Sci USA, 86, 7118-22.


characterization of a newly established Philadelphia-positive acute lymphoblastic leukemia cell line (Z-33) with an autocrine response to GM-CSF. *Leukemia*, **10**, 1534-43.


SOLUTIONS AND BUFFERS

All solutions were made up with sterile distilled water unless otherwise stated.

10X PBS STOCK
(pH 7.2)

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<td>SDS</td>
</tr>
<tr>
<td></td>
<td>Bromophenol blue</td>
</tr>
</tbody>
</table>

193
<table>
<thead>
<tr>
<th>Component</th>
<th>DNA MINI-PREPARATION SOLUTIONS</th>
<th>DNA MAXI-PREPARATION SOLUTIONS (QIAGEN SUPPLIED)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Glucose</td>
<td>Tris-HCl (pH 8)</td>
</tr>
<tr>
<td>Glycerol</td>
<td>40% (v/v)</td>
<td>50mM</td>
</tr>
<tr>
<td>β-mercaptoethanol</td>
<td>20% (v/v)</td>
<td>EDTA</td>
</tr>
<tr>
<td>Solution 1</td>
<td></td>
<td>RNase A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10mM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>200µg/ml</td>
</tr>
<tr>
<td>NaOH</td>
<td>0.2M</td>
<td>SDN</td>
</tr>
<tr>
<td>Solution 2</td>
<td></td>
<td>1% (w/v)</td>
</tr>
<tr>
<td>K acetate</td>
<td>3M</td>
<td></td>
</tr>
<tr>
<td>Solution 3</td>
<td>(pH to 4.8 with acetic acid)</td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (pH 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris-HCl (pH 8)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RNase A</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaOH</td>
<td>200mM</td>
<td></td>
</tr>
<tr>
<td>SDS</td>
<td>1% (w/v)</td>
<td></td>
</tr>
<tr>
<td>K acetate</td>
<td>3M</td>
<td></td>
</tr>
<tr>
<td>(pH to 4.8 with acetic acid)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaCl</td>
<td>750mM</td>
<td></td>
</tr>
<tr>
<td>MOPS (pH 7)</td>
<td>50mM</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>15% (v/v)</td>
<td></td>
</tr>
<tr>
<td>Triton X-100</td>
<td>0.15% (v/v)</td>
<td></td>
</tr>
<tr>
<td>QC (wash buffer)</td>
<td>NaCl</td>
<td>1M</td>
</tr>
<tr>
<td>MOPS (pH 7)</td>
<td>50mM</td>
<td></td>
</tr>
<tr>
<td>Isopropanol</td>
<td>15% (v/v)</td>
<td></td>
</tr>
</tbody>
</table>
**QF (elution buffer)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.25 mM</td>
</tr>
<tr>
<td>Tris-HCl (pH 8.5)</td>
<td>50 mM</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>15% (v/v)</td>
</tr>
</tbody>
</table>

**Bacterial Media**

**L-B (1L)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>10.0 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>5.0 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>10.0 g</td>
</tr>
</tbody>
</table>

**L-agar (1L)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-agar</td>
<td>15.0 g/L</td>
</tr>
</tbody>
</table>

**SOC (100ml)**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacto-tryptone</td>
<td>2.0 g</td>
</tr>
<tr>
<td>Bacto yeast</td>
<td>0.5 g</td>
</tr>
<tr>
<td>NaCl (autoclave)</td>
<td>1 ml of 1M</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1 ml of 1M</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>1 ml of 1M</td>
</tr>
<tr>
<td>Glucose</td>
<td>1 ml of 2M</td>
</tr>
</tbody>
</table>

*Note: (sterile filtered through 0.2um filter)*

**Competent Cell Preparation**

**TFB1**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>K acetate</td>
<td>30 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>10 mM</td>
</tr>
<tr>
<td>MnCl₂</td>
<td>50 mM</td>
</tr>
<tr>
<td>Rubidium chloride</td>
<td>100 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15% (v/v)</td>
</tr>
</tbody>
</table>

**TFB2**

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPS</td>
<td>100 mM</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>75 mM</td>
</tr>
<tr>
<td>Rubidium chloride</td>
<td>10 mM</td>
</tr>
<tr>
<td>Glycerol</td>
<td>15% (v/v)</td>
</tr>
</tbody>
</table>