Chapter 1

GENERAL INTRODUCTION

1.1 ATP

The role of ATP within the cell has been central to the metabolic pathways of all organisms, but it is only in the last two decades that receptors for extracellular ATP have been described. These receptors have gained increasing importance in our knowledge of the responses of both neuronal, smooth muscle cells and cells of hematopoietic origin. The structure of ATP is shown in Fig.1-1. At physiological pH, ATP is chiefly negatively charged as ATP$^4$ (~60%) or HATP$^3$ (~30%). The negative charge on ATP is crucial for its binding to metal ions and to positively charged moieties on proteins. This high negative charge also prevents ATP permeation through the plasma membrane.

![Fig. 1-1 The Structure of ATP](image-url)
There are four major sources of extracellular ATP\(^1\). Firstly, it has been demonstrated that ATP is co-packaged in granules in both adrenergic and cholinergic nerve terminals and is released during neurotransmission into synaptic spaces. Secondly, cytosolic ATP stores can be released by sudden lysis of intact cells, which occurs during rupture of blood vessels and other tissue injury. Thirdly, ATP is co-packaged with serotonin in platelet dense granules which has been shown to be released in significant amounts during platelet activation. Finally, ATP has been reported to be released by intact vascular endothelial cells in tissue culture; the mechanism underlying this release has not been defined. From these sources, pericellular ATP concentrations can attain biologically active levels (0.1~0.5 µM concentrations). However, the local ATP concentration may be much higher at points of cell-cell contact and will depend on the amount released, the effect of dilution and the efficiency of adjacent ectonucleotidases.

Exposure of many intact tissues/organs to extracellular ATP produces complex changes in the biological response, and these actions have been correlated with the diversity of ATP receptor subtypes on the different cells. Receptors for extracellular ATP include ligand-gated ion channels\(^2\)~\(^3\) and G-protein-coupled receptors where their activation serves in cell-cell communication, often by depolarizing effector cells\(^4\)~\(^6\).

### 1.2 Historical Aspects of ATP Receptors

Since ATP was shown to be released during antidromic stimulation of sensory nerves supplying the rabbit ear artery in 1959 by Holton,\(^7\) there have been more and more studies on extracellular ATP and its role as a signal transmitter. Implicit in the concept of purinergic transmission was the existence of
postjuncional receptors for ATP. Two major purinoceptors have been recognized and classified as adenosine or P1 purinoceptors which couple to adenylate cyclase and are competitively antagonized by low concentrations of methylxanthines, and ATP or P2 purinoceptors which are activated preferentially by ATP or other nucleotides.

In 1985, Burnstock & Kennedy proposed the first subdivision of P2 purinoceptors\(^8\) into P2X purinoceptors (which mediate vasoconstriction and contraction of visceral smooth muscle) and P2Y purinoceptors (which mediate extracellular ATP.

![Diagram](from P2 PURINOCEPTORS: LOCALIZATION, FUNCTION AND TRANSDUCTION MECHANISMS, Ciba Foundation Symposium 198, Page 7, 1996)

**Fig. 1-2** Mechanisms underlying the increase in cytosolic [Ca\(^{2+}\)] activated by different P2 purinoceptors for extracellular ATP. (from P2 PURINOCEPTORS: LOCALIZATION, FUNCTION AND TRANSDUCTION MECHANISMS, Ciba Foundation Symposium 198, Page 7, 1996)
vasodilatation as well as relaxation of the smooth muscle of the gut). This subdivision into P2X and P2Y receptors was based on pharmacological characteristics in that α,β-meATP was the most potent agonist for P2X, and 2-meSATP for P2Y. Soon after, other subclasses of P2 purinoceptors were proposed. They were the P2T purinoceptor, which was ADP-selective and involved in platelet aggregation; the P2Z purinoceptor, which was activated by ATP$^{4-}$ and permeabilized cells such as macrophages, lymphocytes and mast cell;¹ and the P2U receptor where ATP and UTP are equipotent as agonists.⁹ Yet another subclass, the P2D receptor has been proposed to account for the biological effects of diadenosine polyphosphates.¹⁰(Fig.1-2)

While this initial classification depended on pharmacological studies, a major step came from advances in molecular biology.¹¹-¹⁴ Thus mainly based on homology between the structure of cloned purinoceptors, seven subclasses of the P2X receptor family were reported. (Table 1-1). These seven subclasses have extensive homology (30-40%) in their primary structure but differ considerably in the length of their carboxyl termini ranging from as short as 30 amino acids for P2X₆ and 48 amino acids for P2X₁ to as long as 240 amino acids for P2X₇.¹⁵

This molecular approach has also applied to the classification of P2Y receptors of which there are now 11 members, all of which are 7-transmembrane, G-protein coupled receptors. Based on this molecular classification, the P2T receptor became the P2Y₁ receptor and the P2U receptor became the P2Y₂ receptor.
Table 1-1 Classification of P2X subtypes.

<table>
<thead>
<tr>
<th>P2X Subtypes</th>
<th>Tissue Cloned from</th>
<th>Most Potent Agonist</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2X₁</td>
<td>Bladder</td>
<td>2-MeSATP &gt; ATP &gt; α, β-meATP</td>
</tr>
<tr>
<td>P2X₂</td>
<td>PC12 Cells</td>
<td>2-MeSATP &gt; ATP</td>
</tr>
<tr>
<td>P2X₃</td>
<td>Spinal cord</td>
<td>2-MeSATP &gt; ATP</td>
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<tr>
<td>P2X₄</td>
<td>Brain</td>
<td>ATP &gt; 2-MeSATP</td>
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<tr>
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<td>Brain</td>
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<tr>
<td>P2X₆</td>
<td>Brain</td>
<td>ATP</td>
</tr>
<tr>
<td>P2X₇</td>
<td>hematopoietic</td>
<td>BzATP &gt; ATP</td>
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Recently the genes for both the rat and human macrophage P2Z/P2X₇ purinoceptors were cloned and expressed in HEK-293 cell.\textsuperscript{16; 17} On the basis of extensive homology with the six described members of the P2X family of receptors, it was proposed that P2Z receptor of macrophage be termed P2X₇.\textsuperscript{16; 18} The P2Z receptor of lymphocytes shares many features with this cloned P2X₇ receptor, and the evidence below suggests that the two receptors are identical. First the receptors are specific for the fully ionized ATP\textsuperscript{4⁻} species and show a rank order of agonist potency of benzoylbenzoyl ATP (BzATP) $\gg$ ATP $>$ 2-meSATP $>$ ATP-$\gamma$-$\text{S}$.\textsuperscript{19} Second both receptor operated channels are highly Ca\textsuperscript{2⁺} selective although Ba\textsuperscript{2⁺} as well as fluorescent dyes such as ethidium\textsuperscript{+} or Yo-Pro are also permeants.\textsuperscript{18; 20} Third both receptors are inhibited partially by extracellular Na\textsuperscript{+} and totally by the isoquinoline sulphonamide derivative, KN-62 as well as by the irreversible inhibitor 2', 3' dialdehyde ATP (OxATP).\textsuperscript{19; 21} Finally molecular RT-PCR analysis shows identity between the lymphocyte and monocyte P2X₇ genes.\textsuperscript{22} All members of P2X family have protein structures with two putative transmembrane segments linked by a long
extracellular loop rich in cysteines and intracellular NH$_2$- and COOH-termini. (Fig. 1-3)

**Fig. 1-3** P2X$_7$ Structure

### 1.3 Properties of P2X$_7$ Receptor and Its Channel

The P2X$_7$ purinoceptor and its associated channel is widely expressed in cells of hematopoietic origin and shows strong selectivity for the divalent cations Ca$^{2+}$ and Ba$^{2+}$ over monovalent cations. An unusual feature of this receptor is the slow kinetics of channel dilatation: after immediate (< 1s) channel opening, a second permeability state develops which allows larger organic cations to pass, a process termed “pore” formation. This larger permeability state allows permeation by ethidium$^+$ cation (314 Da) or YoPro$^{2+}$ (375 Da) but excludes passage of propidium$^{2+}$ (414 Da).
The P2X7 purinoceptor appears to be the most sensitive of all P2X receptors to steric aspects of ATP structure. Active agonists require the adenine base, D-ribose sugar and the 5'-triphosphate side chain. 3'-O-(4-benzoyl) benzoyl ATP (BzATP), a photoactivatable ATP analogue (Fig. 1-4), is 10-100 times more potent than ATP in activating the P2X7 purinoceptor expressed in number of cell types. The rank order of agonist potency of ATP and its analogues as P2X7 receptor agonists is BzATP > ATP > 2-MeSATP>ATPγS which differs from other P2X receptors. In lymphocytes from patients with chronic lymphocytic leukemia (CLL), the natural ligand ATP, is a partial agonist, producing only 70% of the maximal response in ethidium cation influx observed for the full agonist BzATP.

![BzATP Structure](image)

**Fig. 1-4** BzATP Structure

Activation of P2X7 receptors induces multiple downstream effects in addition to the opening of an ionic channel. One effect of ATP is to stimulate a phospholipase D which on attachment to the plasma membrane catalyses hydrolysis of phosphatidylcholine to phosphatidic acid and choline. Yet
another effect of ATP is to induce the shedding of L-selectin (CD62L), a C-type lectin which is involved in the adhesive interactions and rolling behaviour of lymphocytes on endothelial cells. Recently, the P2X7 purinoceptor has been shown to play an important role on ATP induced lymphocyte apoptosis.

The most widely used inhibitors of P2 purinoceptors are suramin and reactive blue 2. However, their inhibitory potency towards lymphocyte P2X7 purinoceptors is weak, with IC50 values of 60 μM for suramin and 70 μM for reactive blue 2. The 2',3'-dialdehyde derivative of ATP, also called oxidized ATP (OxATP), reacts with lysine residues at the ATP binding site to form a covalent Schiff base. Reaction with OxATP gives total and irreversible inhibition of P2X7 purinoceptors on mouse macrophages without effect on either P2Y or P2U purinoceptors present on these cells. Recently, isoquinolinesulphonamide, KN-62, a selective and potent inhibitor of the

Fig. 1-5 Structure of KN-62
calcium-calmodulin kinase II (CaM-kinase II)\(^{36, 37}\) (Fig. 1-5), was found as a potent antagonist of the P2X\(_7\) receptor.\(^{21}\) It inhibits all known responses mediated by the P2X\(_7\) receptor of human lymphocytes at IC\(_{50}\) 10~20 nM. In contrast, KN-62 had no effect on responses mediated by the P2Y\(_2\) receptor of neutrophils. A structural analogue of KN-62, KN-04, which has no effect on CaM-kinase II shows an almost equipotent effect on inhibition of P2X\(_7\) receptor of human lymphocytes. To date both KN-62 and KN-04 are the most potent known inhibitors of the P2X\(_7\) receptor.

1.4 Advantages of Using Cells from Chronic Lymphocytic Leukemia (CLL) to Study B-lymphocytes

Chronic lymphocytic leukemia (CLL), a malignant disorder of the lymphocyte, is characterised by an abnormal accumulation of monoclonal well-differentiated lymphocytes in the bone marrow and peripheral blood with eventual infiltration of organs such as spleen and liver. This abnormally prolonged lifespan of CLL-lymphocyte may be due to altered lymphocyte recirculation, however the molecular mechanisms are unknown. Large numbers can be obtained from patients with CLL, in whom lymphocyte counts can be up to 100x10\(^9\)/L in peripheral blood (25 ~ 100 fold of normal), due to clonal proliferation and accumulation of mature cells of B phenotype (CD5\(^+\), CD19\(^+\), CD20\(^+\)).

Cells often co-express several P2 receptor subtypes, so that multiple pathways may be available for the biological effects of ATP. However, competitive experiments with BzATP and ATP in lymphocytes from patients with CLL show that the permeability responses are dominated by the P2X\(_7\) receptor.\(^{28}\)
Specifically, lymphocytes show no ATP-induced release of internal Ca\(^{2+}\) (a P2Y effect) and no response to UTP or ADP, agonists which are specific for the P2U/P2Y\(_2\), and P2T/P2Y\(_1\) purinoceptor, respectively. These results confirm that lymphocytes possess only P2X\(_7\) purinoceptors and are an ideal cell type to study the properties and physiological function of this receptor and its associated ion channel. Lymphocytes from patients with CLL over-express this P2X\(_7\) receptor\(^{29}\) and with their well defined profile of adhesion molecules\(^{38;\ 39}\) are an ideal cell to study the role of this receptor in human B-lymphocytes. Lymphocytes and monocytes from normal peripheral blood also express P2X\(_7\) purinoceptors.

1.5 In Vitro Model For Lymphocyte Transendothelial Migration

Lymphocytes in the body undergo continuous recirculation between the blood and tissues\(^{40}\). A rapid turnover of lymphocytes in the blood was found half a century ago, and there is good evidence they enter spleen, lymph nodes and Peyer’s patches as well as non-lymphoid organs (lungs, liver and intestinal wall) in large numbers. Under normal conditions lymphocytes do not accumulate within vessel walls \textit{in vivo} which suggests that once migration is initiated it goes to completion. The first step of lymphocyte emigration to the lymph node is its adhesion to vascular endothelial cells (step 1, Fig.1-6). Then they migrate over the surface of endothelial cells towards inter-endothelial junctions (step 2). Cells move through these junctions (step 3) and then across the basement membrane and interwoven pericytes (step 4) to complete migration into the tissues.
The movement of leukocytes across vessel walls into tissues has been difficult to study \textit{in vivo} and a number of in vitro models have been developed in which lymphocyte and neutrophil transmigration across confluent monolayers of cultured endothelial cells are quantitated.\textsuperscript{41} Human umbilical vein endothelial cells (HUVECs) are the most commonly used source of endothelial cells for study of lymphocytes transmigration due to their ready availability. Endothelial cells from various type of rat tissue (brain capillary, retinal capillary and lymph node) have also been used to study lymphocyte transmigration.\textsuperscript{42-44} Endothelial cells can be propagated by serial subculture after primary isolation from tissues.
For the transmigration assay, an endothelial monolayer is required and used within 24-72 hr of confluence.

Several markers for human vascular endothelial cells can be used to study the cultured endothelial cells or in their separation from contaminating cells, they are pan-endothelial markers such as CD31 (PECAM-1), EN-4, PAL-E, Ulex europaeus lectin; uptake of acetylated low-density lipoprotein; expression of von Willebrand factor; E-selectin (CD62E), inducible by cytokines (IL-1, TNF, LPS) and P-selectin (CD62P) which is also expressed after activation.

Pre-treatment of the endothelial monolayer with cytokines such as TNF-α or IFN-γ increases the lymphocyte migration. Treatment of lymphocytes with PMA, PHA, antigen or IL-2 activated lymphocytes show higher levels of adhesion or migration than unactivated lymphocytes. It is not clear whether increased migration of activated lymphocytes reflects higher levels of binding to the surface of endothelial cells or more efficient migration of lymphocytes across the monolayer once they have bound to the surface. Meanwhile, Greenwood & Calder showed that transendothelial migration of activated lymphocytes is not increased by IFN-γ-pre-treatment of the endothelial monolayer whereas the migration of unactivated lymphocytes is increased by the same treatment. These results suggest that transendothelial migration of activated and unactivated lymphocytes may be independently regulated or that activated endothelial cells activate lymphocytes.

Transmigration assay has been performed in at least 2 ways: in the first, endothelial cells are grown to confluence on glass slides, cell culture dishes or flat bottom 24- or 96-well plates (Fig. 1-7, Assay 1). After the plastic adhesion
to remove monocytes, lymphocytes are then plated onto endothelial monolayers and allowed to interact for 2 to 8 hours with endothelial cells under static conditions. Non-adherent lymphocytes are washed away and the adherent or migrated lymphocytes are fixed with endothelial cells together. The surface-bound and migrated lymphocytes can be easily distinguished under a phase-contrast inverted microscope (phase II with 40X objective). Lymphocytes bound to the surface of endothelial cells (type I) are phase-bright in small size and can be stained with toluidine blue whereas migrated lymphocytes (type II) are phase dark, large in size and difficult to stain. The phenotypic and functional changes in migrated lymphocytes can be studied immediately after transmigration by pre-staining lymphocytes with probes like CFSE, a fluorescent dye and harvesting them with EDTA detachment. The second type of transmigration assay is using transwell system (Fig. 1-7, Assay 2). Endothelial cells are grown to confluence on a fibronectin-coated polycarbonate membrane with 3~5 µm
size pores. Lymphocytes are plated onto the top of the endothelial monolayer and migrated lymphocytes can be collected in the low chamber for analysis. Lymphocytes that are bound to the surface of endothelial cells or have migrated across the monolayer but not into the low chamber can be harvested using EDTA detachment. This assay model is particularly useful for chemotactic or chemokinetic factors.

1.6 L-selectin

The continuous recirculation of lymphocytes throughout the body and leukocyte transmigration into sites of inflammation is an essential component of the body's primary defence against infection and tissue injury. The first step of this cascade is leukocyte adhesion to endothelial cells. This involves the interactions of distinct surface expressed leukocyte adhesion molecules on the endothelial cells and their respective counter-receptors present on the emigrating leukocytes. At least three families of adhesion molecules and their receptors are involved in this process: 1) the three selectins: L-selectin (CD62L, LAM-1, LECAM-1, MEL-14), E-selectin (CD62E, ELAM-1), P-selectin (CD62P, PAGDEM, GMP-140). 2) multiple leukocyte integrins: LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), VLA-4 (CD49d/CD29) and VLA-5 (CD49e/CD29). 3) members of the immunoglobulin superfamily, usually the ligands for some integrins: Vascular Cell Adhesion Molecule-1 (VCAM-1, INCAM-110) which binds VLA-4; Intercellular Cell Adhesion Molecule-1 (ICAM-1, CD54), ICAM-2 which bind LFA-1 and Mac-1; ICAM-3 which binds LFA-1 and platelet endothelial cell adhesion molecule 1 (PECAM-1, CD31) which binds αvβ3 integrin in a homotypic or heterotypic manner.
L-selectin is one of the key adhesion molecules in the initial steps of leukocyte transmigration into peripheral lymph nodes\textsuperscript{60} and it is implicated as one of the key adhesion molecules involved in the cascade of events leading to leukocyte recruitment into inflammatory sites.\textsuperscript{61} The central importance of L-selectin in regulating leukocyte emigration has been shown in L-selectin “knockout mice” which show impaired lymphocyte recirculation and impaired neutrophil emigration to sites of inflammation.\textsuperscript{62-64} In L-selectin deficient mice, the impaired migration of lymphocytes into peripheral lymph nodes results in the accumulation of lymphocytes within the circulation and other tissues, specifically, 30 to 55% increase in splenic cellularity, and circulating lymphocyte numbers are increased nearly threefold.\textsuperscript{64}

L-selectin has a mosaic structure (Fig.1-8). This includes an amino-terminal C-type lectin domain, an epidermal growth factor-like domain, two short consensus repeat units (SCR) and a transmembrane domain and cytoplasmic tail. L-selectin from human, mouse, rat and cow have similar structure and are 75\% identical in amino acid sequence.\textsuperscript{50; 53; 65-67} The L-selectin protein isolated from lymphocytes has an apparent molecular weight of 68 kD after reduction and 74 kD under non-reducing condition,\textsuperscript{68} whereas that of neutrophils is higher, 90~100 kD after

![Fig. 1-8 Structure of L-Selectin (CD62L)](image-url)
reduction. It is likely that the difference in MW between various leukocytes is solely a function of the carbohydrate side-chains since the sequencing of cDNA isolated from B, T lymphocytes and neutrophils reveals no differences. This indicates that L-selectin protein expressed in different cell types is identical and surface L-selectin is heavily glycosylated or may undergo additional post-translational processing.

L-selectin expression is limited to hematopoietic cells and depends on the stage of differentiation, activation status and tissue location of the cells. Blood B cells are uniformly L-selectin positive, as well as the thymocytes and the majority of T cells. L-selectin is also expressed in a subpopulation of NK cells. However, CD4⁺CD8⁺ T cells express little or no L-selectin while in the germinal centre B cells are L-selectin negative. Monocytes and neutrophils express similar levels of cell-surface L-selectin as lymphocytes. In bone marrow, early erythroid progenitor cells are also L-selectin positive, but negative on the later stages, whereas myeloid progenitor cells at all levels of maturation are L-selectin positive. The earliest hematopoietic progenitor cells express not only L-selectin but also its ligand. This suggests that L-selectin may also play a broader role in stem cell traffic.

Significant amounts of soluble L-selectin (sL-selectin) are present in the serum of normal healthy subjects. Using Western blot analysis and a quantitative ELISA, a mean sL-selectin level of 1.6±0.8 µg/ml was found in serum from normal human blood donors. Increased level of serum sL-selectin is found in diseases such as CLL, acute leukemia, common variable immunodeficiency (CVID), and HIV infection. This high level of serum sL-
selectin level may be due to excess shedding of L-selectin from these lymphocytes which may in turn affect lymphocyte trafficking.  

Like many other membrane molecules, soluble forms of L-selectin have been described although the mechanisms involved in their production are poorly characterised. The soluble fragment of L-selectin contains the known functional lectin and EGF domains and inhibits L-selectin specific attachment of lymphocytes to cytokine-activated endothelial cells at a concentration of 8–15 µg/ml. This indicates a potential role for sL-selectin in the regulation of leukocyte attachment, as it is possible that sL-selectin serves as a natural buffering system to prevent the attachment of leukocytes to minimally activated endothelial cells.

The source of sL-selectin is still unclear. One possibility is due to the rapid loss of L-selectin from the surface of leukocytes following cellular activation. Non-physiological mediators such as phorbol esters are known to cause shedding of L-selectin by activating a zinc containing membrane metalloprotease termed L-selectin sheddase. Our group also found extracellular ATP induces shedding of L-selectin from the surface of both normal and leukaemic lymphocytes via activation of the P2X7 purinoceptor of these cells. Other cells such as neutrophils and monocytes also shed L-selectin following their activation. Thus, granulocyte-macrophage colony-stimulating factor (GM-CSF), lipopolysaccharides (LPS), phorbol ester (PMA), various chemoattractants and calcium ionophore all induce L-selectin shedding in neutrophils, and this decrease is accompanied by a several fold increase in CD11b surface expression. Moreover, GM-CSF causes dramatic changes in leukocyte migration and L-selectin expression in vivo. Monocytes also lose L-selectin following
activation, and even their adherence to plastic at 37°C causes a complete loss of L-selectin from the cell surface.

Since the L-selectin level is significantly higher on blood lymphocytes than lymphocytes isolated from thymus, bone marrow and spleen,\textsuperscript{71} it is possible that \textit{in vivo} lymphocyte entry into lymphoid tissues cause L-selectin loss from the cell surface. Therefore, this downregulation process could become a major source of sL-selectin. One of the aims of this thesis is to examine if there is significant loss of L-selectin from migrated lymphocytes. Such a process would require lymphocytes to resynthesize L-selectin on entry back into the circulation.

It has been shown that overnight incubation with IFN-γ increases L-selectin expression on human eosinophils and this can be inhibited by a protein synthesis inhibitor, cycloheximide.\textsuperscript{84} Moreover, cycloheximide treatment of rats and rabbits produced a lymphocytosis due to decreased lymphocyte homing to mesenteric nodes, Peyer's patches, and spleen, but not lung. This effect was not specific for distinct lymphocyte subsets, and included both T cells, B cells, or lymphocytes expressing L-selectin.\textsuperscript{85} Thus, it is likely that L-selectin is continuously shed at a slow rate with its expression kept constant by continuous synthesis.

The mechanism for shedding of L-selectin may involve in part, the specific activation of a membrane-bound protease followed by enzymatic cleavage of this cell surface adhesion molecule. Alternatively, activation-induced conformational changes may expose nascent sites on L-selectin susceptible to cleavage by proteinases. A hydroxamic acid-based inhibitor of zinc-dependent metalloproteases, Ro 31-9790 (Fig. 1-10) has been shown to inhibit phorbol dibutyrate-stimulated L-selectin shedding from mouse lymphocytes with an IC\textsubscript{50}
of 2–4 μM.\textsuperscript{81} Another hydroxamic acid-based metalloprotease inhibitor, KD-IX-73-4, has a similar effect on PMA-stimulated L-selectin shedding from human lymphocytes with an IC\textsubscript{50} of 3 μM.\textsuperscript{86}

L-selectin shedding may result from cross-linking with its ligands on endothelial cells during adhesion and transmigration of lymphocytes. L-selectin has been shown to be an endothelial cell receptor. Anti-L-selectin monoclonal antibodies block the binding of L-selectin positive lymphocytes to HEV\textsuperscript{60} and inhibit the binding of lymphocytes, monocytes and neutrophils to cytokine-treated HUVEC under conditions of flow. Further evidence that L-selectin serves as a leukocyte adhesion receptor for endothelial cells is provided by the finding that a recombinant L-selectin / IgG heavy chain chimeric protein (LEC-IgG) binds specifically to HUVEC activated for 6 or 24 hours with TNF-α, but not to resting HUVEC.\textsuperscript{87} Various possible ligands are also inducible on cultured HUVEC following treatment with specific inflammatory stimuli.\textsuperscript{88, 89} Several sulfated carbohydrate L-selectin ligands on endothelial cells are revealed soon after. They are glycosylation-dependent cell adhesion molecule 1 (GlyCAM-1), a ~50 kD glycoprotein previously known as Sgp50;\textsuperscript{90, 91} sialomucin CD34, a ~90 kD glycoprotein previously known as Sgp90, also a marker for hematopoietic stem cells;\textsuperscript{92} a ~200 kD glycoprotein, Sgp200;\textsuperscript{93} Podocalyxin-like protein (PCLP), a transmembrane sialomucin that is similar in structure to CD34;\textsuperscript{94} a ~105 kD peripheral lymph node addressin (PNAd).\textsuperscript{95} All the above ligands bind to an anti-PNAd monoclonal antibody named MECA-79, which blocks L-selectin-dependent adhesion and selectively stains lymph node HEV.\textsuperscript{93, 94} The Sialyl-LewisX epitope is found as a common ligand for all three selectins (E-, L-, P-selectin) on both endothelial cells and neutrophils.\textsuperscript{96-98} Another L-selectin
ligand on neutrophils is P-selectin glycoprotein ligand-1 (PSGL-1), a mucinlike protein that binds P- and E-selectin\textsuperscript{99-101} and also acts as an L-selectin ligand on monocytes and CD34\textsuperscript{+} hematopoietic progenitor cells.\textsuperscript{102} Another L-selectin ligand was also found on hematopoietic progenitor cells exhibiting sulfate-independent function which is different to all previously described L-selectin ligands.\textsuperscript{103} These various L-selectin ligands on different types of cell suggest that structural determinants conferring L-selectin binding may vary in a cell- and tissue-specific manner. Moreover, the affinity of L-selectin for ligand can be measured using polyphosphomannan ester (PPME), a soluble carbohydrate which binds to the lectin domain of L-selectin, and blocks lymphocyte attachment to both HEV and cytokine treated HUVEC\textsuperscript{88} but does not inhibit leukocyte rolling \textit{in vivo}.\textsuperscript{104, 105}

1.9 CD23

CD23, also known as Fc\v RII, B6, Leu-20, BLAST-2, is a low affinity receptor for IgE, and belongs to a superfamily of type-II integral membrane proteins (Fig. 1-10a) with a short N-terminal intracytoplasmic tail (23 amino acid), a single transmembrane domain (20 amino acid), and a large C-terminal extracellular region (277 amino acid) which recognises four different ligands: IgE, CD21, CD11b and CD11c and is separated from the transmembrane domain by a coiled-coil stalk which facilitates dimer or trimerization (Fig. 1-10b).\textsuperscript{106, 107} CD23 is a 45 kD glycoprotein and can be cleaved into soluble 37-, 33-, 25~27 and 16 (or 12) kD fragments.\textsuperscript{108, 107, 109} The 25~27 kD fragment is derived from the cleavage of soluble 33~37 kD precursors. The 33 and 37 kD fragments are
capable of binding to IgE\textsuperscript{110}, and the 37 kD fragment also regulates the synthesis of human IgE\textsuperscript{111}.

In humans, CD23 antigen is highly expressed on B-lymphocytes and monocytes but small amounts may be found on a large variety of other cells such as T cells, eosinophils, platelets, Langerhans cells and some epithelial cells\textsuperscript{112}. On B-cells, CD23 is a differentiation marker that is selectively expressed on surface-bound sIgM/sIgD double-bearing cells\textsuperscript{113} and is lost upon differentiation into Ig-secreting cells. CD23 may also be considered as a B-cell activation marker because its expression is strikingly increased following T cell-B cell interactions\textsuperscript{114; 115}, direct contact with CD40 ligand\textsuperscript{116} or immobilised anti-CD40 monoclonal antibody\textsuperscript{117}. Some human T- and B-cell lines and an eosinophil cell line contain multiple forms of CD23 transcripts\textsuperscript{118}. There are type A and B CD23 isoforms, which have 6 amino acid differences in their cytoplasmic Fig. 1-10 Structure of CD23 (FceRII)
domain. The expression of CD23 type A is restricted to resting B cells, while that of type B is strongly induced in response to IL-4, IL-13, or B-cell activation. Malignant B cells mainly express CD23 isoform type A.

CD23 expression is increased by several B cell mitogens, including PMA, Epstein-Barr virus, anti-immunoglobulin (Ig), and IL-4. PGE2 and IFN-\(\gamma\) inhibit the IL-4-mediated induction of CD23 on B lymphocytes. Tyrosine protein kinase (TPK) inhibitors, such as herbimycin A and genistein, decreased both the IL-4- and 12-O-tetradecanoylphorbol 13-acetate (TPA)-induced CD23 expression by 50-80%. This indicates that signals through TPK pathways might play the major role in CD23 expression.

CD23 is involved in various functions: 1) The pairing of CD23-CD21 mediated by T-B cell contact may participate in the control of IgE production; 2) Cross-linking of CD23 antigen by its natural ligand (IgE) or by anti-CD23 antibody prevents B lymphocyte proliferation and differentiation; 3) CD23 interacts specifically with CD11b and CD11c, which are the alpha chains of the beta 2 integrins, LFA-1,Mac-1/Mo1 and gp150/95. Such a ligation on monocytes causes a marked increase in production of nitrite oxide and pro-inflammatory cytokines (IL-1 beta, IL-6, and TNF alpha). 4) Ligation of CD23 triggers cyclic AMP generation in human B lymphocytes. 5) Cross-linking of CD23 delivers a negative growth signal to the leukaemic B cells.

Soluble CD23 (sCD23) enhances IgE synthesis and also induces cell adhesion. However, intact CD23 has no demonstrable role in cell adhesion; instead, the portion of CD23 remaining on the cell surface following cleavage appears to mediate cell adhesion. The transendothelial migration of a human
eosinophilic leukemia cell line (EoL-3.12) toward a chemokinetic gradient of soluble CD23 (sCD23; 29 kD fragment) closely paralleled the density of membrane CD23 expressed on EoL-3.12 cells. Soluble CD23 also inhibits the spontaneous migration of U937 monocytic cells. This suggests a possible role for soluble CD23 at sites of inflammation where B cells and monocytes accumulate and are activated.

The CD23 gene is abnormally regulated in B-CLL and other lymphoproliferative diseases. B lymphocytes from patients with B-CLL, strongly express CD23. As expected, sCD23 levels in the serum from B-CLL patients are 3~500 times higher than from normal individuals (Table 1-2). Mean sCD23 levels are normal in some types of non-Hodgkin's lymphoma (but elevated in follicular lymphocytic types; see table 1-2), Hodgkin's disease, hairy cell leukemia, acute lymphoblastic leukemia (ALL), acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), multiple myeloma, and solid tumours. sCD23 level in CLL may reflect disease activity and correlates with the tumor mass and clinical stage, and could be helpful in monitoring these patients.

Therefore, with other cell markers such as CD5 and CD38, soluble CD23 has become an important diagnostic and prognostic indicator for B-CLL.

**Table 1-2  Soluble CD23 Level**

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<tr>
<th>Condition</th>
<th>sCD23 (U/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B-CLL (typical)</td>
<td>3,650 ± 4,654</td>
</tr>
<tr>
<td>B-CLL (atypical)</td>
<td>3,440 ± 4,671</td>
</tr>
<tr>
<td>Follicular Cell Lymphoma</td>
<td>3,200 ± 1,511</td>
</tr>
<tr>
<td>Splenic Lymphoma with Villous Lymphocytes</td>
<td>8,236 ± 7,294</td>
</tr>
<tr>
<td>Normal Control</td>
<td>137 ± 128</td>
</tr>
</tbody>
</table>
Chapter 2

MATERIALS AND METHODS

2.1 Materials

Ficoll-Paque (density 1.077) was obtained from Pharmacia (Uppsala, Sweden). ATP, UTP, BzATP, OxATP, PMA, BSA, HEPES, ECGS, ethidium bromide, collagenase, TNF-α, IFN-γ, gelatin, glutaraldehyde (25% aqueous solution), glutamine, bovine calf serum (iron-supplemented), RPMI 1640 medium and Medium 199 were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Adenosine was from C.F.Boehringer & Soehne GmbH Mannheim (Germany). KN-62, and KN-04 was from Research Biochemicals, Inc. (Natick, MA, U.S.A.). Ro 31-9790 was a gift from Roche Products (Dee Why, NSW, Australia). 0.25% Trypsin plus 1 mM EDTA, heparin, penicillin, streptomycin and Fungizone were from Gibco-BRL (Gaithersburg, MD, U.S.A.). BAPTA-AM and Fura-2 AM were from Molecular Probes (Eugene, OR, U.S.A.)

Monoclonal antibodies to human L-selectin (CD62L) were the DREG 56 clone from Immunotech (Marseilles, France) or the FMC46 clone (DAKO, Carpinteria, CA, U.S.A.). Fluorescein (FITC) labelled antibodies to L-selectin (CD62L) were the DREG.55 clone (Bender MedSystems, Vienna, Austria). FITC or phycoerythrin (PE) conjugated and purified mouse anti-human monoclonal antibodies to CD23 were the Tü-1 clone from Bender MedSystems (Vienna, Austria), the Leu-20 clone and flow cytometry calibration beads were from Becton Dickinson Co. (San Jose, CA, USA) and the MHM6 clone from DAKO (Carpinteria, CA, USA). Horseradish peroxidase (HRP) conjugated goat anti-
mouse IgG was obtained from DAKO. Enhanced chemiluminescence (ECL) kit was a product of Amersham (Buckinghamshire, UK). Flow cytometry standard beads (Quantum 26) were from Flow Cytometry Standards Co. (San Juan, U.S.A.). PerCP-conjugated monoclonal antibody to CD20 and FITC- or PE-conjugated monoclonal to CD3 were from Becton Dickinson Co.

2.2 Solutions

**Na Buffer**

145 mM NaCl  
5 mM KCl  
10 mM HEPES  
adjust to pH 7.5 at 20°C with TMA

**Na Medium**

Na Buffer as above with 0.1% BSA and 5 mM D-glucose added on day of experiment, and filtered with 0.2 μM filter for transmigration assay.

**K Buffer**

150 mM KCl  
10 mM HEPES  
adjust to pH 7.5 at R.T. with TMA

**K Medium**

K Buffer as above with 0.1% BSA and 5 mM D-glucose added on day of experiment, and filtered with 0.2 μM filter for transmigration assay.

**Isotonic Mg^{2+} 'stopping' buffer** (for stopping ATP or BzATP effect)

20 mM MgCl₂ in Na buffer, pH 7.5

**Complete M199 medium**

To 500 ml of basic M199 media add:

1. 20% bovine calf serum
2. 2 mM glutamine
3. 100 U/ml penicillin
4. 100 µg/ml streptomycin sulphate
5. 30 µg/ml ECGS
6. 5 U/ml heparin
7. 2.5 µg/ml Fungizone

**Complete RPMI1640 medium**

To 500 ml of basic RPMI1640 media add:
1. 10% fetal calf serum
2. 2 mM glutamine
3. 5 µg/ml gentamycin

**Transport Medium**

To 500 ml of basic M199 media add:
1. 20% bovine calf serum
2. 4 mM glutamine
3. 100 U/ml penicillin
4. 2.5 µg/ml Fungizone
5. 10 U/ml heparin

**HBSS**

To 500 ml of basic Hank's buffer add:
1. 10 mM HEPES
2. 10 µg/ml gentamycin
3. 100 U/ml penicillin
4. 100 µg/ml streptomycin sulphate

**Phosphate Buffered Saline (PBS) (0.01 M, pH 7.2)**

<table>
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<tr>
<th>Component</th>
<th>Concentration</th>
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</thead>
<tbody>
<tr>
<td>NaCl</td>
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<tr>
<td>Na₂HPO₄·2H₂O</td>
<td>7.5 mM</td>
</tr>
<tr>
<td>NaH₂PO₄·H₂O</td>
<td>2.5 mM</td>
</tr>
<tr>
<td>KCl</td>
<td>5 mM</td>
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**10~20% Gradient Gel (unit: ml)**

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<thead>
<tr>
<th></th>
<th>10%</th>
<th>20%</th>
<th>Stacking Gel</th>
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<tbody>
<tr>
<td>H₂O</td>
<td>8.1</td>
<td>3.28</td>
<td>8.0 ml</td>
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<tr>
<td>50% Glycerol</td>
<td>0.18</td>
<td>0.98</td>
<td></td>
</tr>
<tr>
<td>1.5 M Tris</td>
<td>4.5</td>
<td>4.5</td>
<td>0.833 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>0.18</td>
<td>0.18</td>
<td>0.1 ml</td>
</tr>
<tr>
<td>40% Acrylamide</td>
<td>4.5</td>
<td>9.0</td>
<td>0.975 ml</td>
</tr>
<tr>
<td>TEMED</td>
<td>7.5</td>
<td>7.5</td>
<td>15 µl</td>
</tr>
<tr>
<td>10% Ammonium Persulfate</td>
<td>52.5</td>
<td>52.5</td>
<td>200 µl</td>
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mix 10% and 20% Gel with Gradient Gel Maker.

**Non-Reduced Sample Buffer for Western Blot**

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<td>Glycerol</td>
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</tr>
<tr>
<td>0.5 M Tris</td>
<td>2.5</td>
<td>ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>4.0</td>
<td>ml</td>
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<tr>
<td>H₂O</td>
<td>1.1</td>
<td>ml</td>
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<tr>
<td>0.1% Bromophenol Blue</td>
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<td>ml</td>
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Mix 1:1 with sample

**SDS-PAGE Tray Buffer (2 Litre)**

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<tr>
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<tbody>
<tr>
<td>Tris</td>
<td>12 g</td>
</tr>
<tr>
<td>Glycine</td>
<td>57.6 g</td>
</tr>
<tr>
<td>SDS</td>
<td>2 g</td>
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**Electrophoresis Transfer Buffer (3 Litre)**

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<td>25 mM</td>
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<tr>
<td>Glycine</td>
<td>192 mM</td>
</tr>
<tr>
<td>Methanol</td>
<td>20% (v/v)</td>
</tr>
</tbody>
</table>

**Tris Buffered Saline containing Tween (TTBS) (2 Litre, pH 7.5)**

<p>| | |</p>
<table>
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<tbody>
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<td>Tris</td>
<td>20 mM</td>
</tr>
<tr>
<td>NaCl</td>
<td>500 mM</td>
</tr>
<tr>
<td>Tween-20</td>
<td>2 ml</td>
</tr>
</tbody>
</table>

**Blocking Buffer**

5% skim milk powder in TTBS
2.3 Preparation Of HUVEC Culture Flasks And Plates

Gelatin solution (1%) was autoclaved and allowed to completely liquify at 37°C. The culture surface of flasks and 24-well plates was coated with the gelatin solution. Containers were incubated at 37°C for 30 min, and solution removed by aspiration then allowed to dry at least 2 hours before introducing cells and medium. The coated flasks and plates could be stored at 4°C for up to 6 months.

2.4 Isolation of Human Umbilical Vein Endothelial Cells (HUVEC)

A human umbilical cord was severed from the placenta soon after birth, placed in a sterile container filled with transport medium and stored at 4°C until processing (within 24 hours). The umbilical cord is comprised of three non-branching vessels: two arteries and one vein. Any damaged part was removed to avoid other cell types such as fibroblasts being isolated.

The cord was placed in a sterile petri dish and the vein was perfused with 20 ml of HBSS to remove residual blood and allow to drain. 10 ml of pre-warmed medium (at 37°C) containing 2 mg/ml collagenase (type 1) was then infused into the vein and the other end of the cord was closed using a haemostat. The cord was then incubated for 15 min at room temperature. After this incubation, the collagenase solution containing the endothelial cells was flushed from the cord into a sterile 50 ml tube by perfusion with 30 ml of HBSS. The cells were centrifuged at 200 g for 10 min and resuspended in 10 ml M199 complete medium within a 25 cm² culture flask pre-coated with 1% gelatin. The flasks were incubated at 37°C under 5% CO₂. The medium was changed twice a week commencing the next day until cells reached confluence.
2.5 Subculture HUVEC

The confluent HUVEC monolayer was harvested with 0.25% trypsin plus 1 mM EGTA for 30~90 seconds at 37°C, and transferred to a tube containing an equal volume of complete M199. Cells were then centrifuged for 3 min at 200 g and resuspended into M199 complete medium. Cell suspensions were divided approximately at a 1:4 ratio into culture flasks or to 24-well plates. After 3~7 days of culture, cells had grown to confluence with medium changes every 3 days. All experiments involving HUVECs monolayers were carried out using passage number between 3 to 6. In some experiments HUVEC monolayers were activated with the cytokine TNF-α (5 ng/ml) or IFN-γ (10 ng/ml) for 4~6 hr at 37°C and then washed twice to remove cytokine prior to assay of lymphocyte transendothelial migration.

2.6 Cell Preparation

Heparinized venous blood from patients with B-cell chronic lymphocytic leukemia (CLL) or from normal blood donors was diluted with 2 vol. of RPMI 1640 basic medium, and mononuclear cells were separated by density-gradient centrifugation over Ficoll-Paque. Monocytes were depleted by plastic adhesion for 60 min at 37°C. Cells in the supernatant were washed once and resuspended in NaCl medium plus 1 mM CaCl₂. Cell morphology showed that >99% of the mononuclear cells from B-CLL patients were small mature lymphocytes and immunophenotype showed 93.3±8.8% of these lymphocytes typed as CD5+, CD19+ while 5.7±2.5% typed as CD3+ except an early stage B-CLL patient with 34% T cells and 66% B cells (patient 4).
2.7 Lymphocyte Transmigration Assay By Phase Contrast Microscopy

HUVECs were grown to confluent monolayers in 24 well tissue culture plates. Lymphocytes were resuspended to 1.0-2.0x10^7 cells/ml in M199 medium, 1.0 ml of the suspension added to each well and incubated for 2 to 6 hours at 37°C under 10% CO₂. Non adherent lymphocytes were removed by aspiration, the HUVEC layer washed five times in NaCl medium plus 1mM CaCl₂. HUVEC layers were fixed using 2% glutaraldehyde in PBS for 20 min at room temperature and examined by high power (x400) phase contrast microscopy. For each well, the numbers of adherent (phase light) and migrated (phase dark) cells were counted in a 4 mm² area while HUVEC numbers in the same area were counted after counterstaining with 0.1% toluidine blue. The index of migration was calculated as the numerical ratio of migrated cells per HUVEC.

Measurements of L-selectin and CD23 were made on lymphocytes which had not been fixed and recovered from parallel wells on the same plate. The non-adherent lymphocytes were removed by gentle repetitive washing and retained for analysis while the tightly adherent and transmigrated lymphocytes were recovered by 5 mM EDTA in PBS dispersion of the monolayer at 37°C for 5 min.

2.8 Lymphocyte Transmigration Assay by Transmission Electron Microscopy

Millipore 0.22 µm cellulose acetate filters (Millipore, Bedford, MA, USA) were sterilised by immersion in 70% ethanol for 60 min, washed in sterile distilled water for 5 min x 3, placed in a petri dish and coated with 1% gelatin as described for endothelial cell culture above. Endothelial cells were cultured on the filter until confluent and the lymphocyte transmigration assay was then
carried out. After completion of the lymphocyte transmigration assay the filters were washed twice with washing buffer and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffered saline (PBS) for 30 min at room temperature. Filters were kept in PBS at 4°C prior to processing. Filters were cut into 2 mm squares, post-fixed in 2% osmium tetroxide and processed through graded acetone to Araldite-Epon resin (Ciba Geigy, Lane Cove, NSW, Australia). Thin sections were cut and stained with saturated aqueous uranyl nitrate and lead citrate. Sections were viewed on a JEOL JEM 1200EX electron microscope (JEOL, Tokyo, Japan) at an acceleration voltage of 80 kV.

2.9 Monitoring L-selectin Expression
Lymphocytes separated by Ficoll-Paque were resuspended at 1x10^6 cells/ml in Na medium. Following preliminary antibody titration, an optimal titre of 5 µl of FITC conjugated anti-L-selectin MoAb (from Bender MedSystems) was added in 100 µl cell suspension and cells were incubated for 15 min at room temperature followed by one wash. The lymphocyte population was gated using forward and side scatter and the mean channel fluorescence of 5,000 cells was collected using linear amplification on an ELITE flow cytometer (Coulter Electronics, Hialeah, FL, USA) with 488 nm excitation. To allow for day-to-day variation in the performance and settings of the flow cytometer, fluorescent standard beads containing known numbers of fluorescein molecules were analyzed under the same conditions as the lymphocytes. Calibration curves of molecules of equivalent soluble fluorescein (MESF) against mean fluorescence channel number were constructed following each assay to enable specific binding of the fluorescein anti-L-selectin to be converted to MESF per cell.
2.10 L-selectin and CD23 Shedding Induced by ATP, BzATP or PMA

To study L-selectin shedding induced by ATP, BzATP or PMA, aliquots of lymphocytes (1.0x10^6/ml) were incubated for up to 30 min in NaCl or KCl medium at 37°C with either ATP, BzATP or PMA. In some experiments, lymphocytes were pretreated with 300 µM OxATP for 1 hr. The inhibitors Ro 31-9790 or KN-62 were added 15 min prior to the addition of ATP or BzATP. The incubation was stopped by adding an equal volume of cold isotonic Mg^{2+} buffer (20 mM MgCl, 145 mM NaCl, 5 mM KCl, 10 mM Hepes, pH 7.5). Cells were washed once and stained with FITC or PE conjugated anti-L-selectin or CD23 monoclonal antibodies. The mean channel fluorescence was collected on a Becton Dickinson FACScan flow cytometer in linear mode. The binding of Leu-20 monoclonal antibody to CD23 was independent of Ca^{2+} concentration in the medium.

2.11 Measurement of soluble L-selectin using ELISA

In the sL-selectin ELISA kit (version 2) from Bender MedSystems (Vienna, Austria), an anti-sL-selectin MoAb is coated onto microwells and captures sL-selectin which is then measured by a HRP-conjugated anti-sL-selectin MoAb. Following incubation with this MoAb, unbound antibody is removed by a wash step and then substrate solution reactive with HRP is added to the wells. A coloured product is formed in proportion to the amount of soluble L-selectin present in the sample. The colour reaction is terminated by addition of acid and absorbance is measured at 450 nm. A standard curve is prepared from seven sL-selectin standard dilutions and sL-selectin sample concentration determined. Lymphocytes from each individual studied (10^8 cells/ml) were incubated with
and without 50 nM PMA for 15 min at 37°C to induce near complete shedding (>99%) of L-selectin. The supernatant was collected and stored at -70°C. By following the protocol provided with the kit, total sL-selectin in each supernatant was determined from the standard ELISA curve which was run at the same time.

2.12 Correlation between L-selectin MESF and Molecules/Lymphocyte

To quantitate the cell surface L-selectin in molecules per cell, a correlation was established between L-selectin which could be shed by PMA (molecules per lymphocyte) and L-selectin surface reactivity measured by an FITC-conjugated anti-L-selectin antibody. Soluble L-selectin shed with PMA was measured in

![Graph](image)

**Fig. 2-1** Correlation between L-selectin Molecules/Cell and MESF. Linear mean channel of fluorescence intensity on lymphocytes surface was converted to MESF by using standard beads on each test at the same parameters on flow cytometry to enable the comparison between samples tested on different days. ELISA was used to measure the total L-selectin (ng) on the cell surface and values were transferred to molecules/cell.
the supernatants by ELISA in microtitre plates while cell-associated L-selectin of
cells in the pellet was measured by flow cytometry. For each individual, the
PMA-induced reduction in the cell-associated L-selectin was compared to the
PMA-induced shedding of soluble L-selectin in supernatants from the same
cells. The quantity of soluble L-selectin released by PMA was converted from
ng/ml to molecules per lymphocyte assuming a molecular weight of 68 kD.140 A
good correlation was found between these two parameters measured on the
same cells from 5 separate B- CLL patients and normal subjects
(Y=0.335X+1113, r²=0.97, where X is the PMA-induced reduction in the
fluorescein-antibody binding expressed as MESF per cell, and Y is the PMA-
induced shedding of L-selectin expressed as molecules per cell).

2.13 Measurement of L-selectin and CD23 Downregulation during
Transmigration
Washed lymphocytes were resuspended for analysis at 5 x 10⁶ cells/ml in 100
µl of buffered saline medium. Cells were incubated with the appropriate
combination of monoclonal antibodies for 10 min at room temperature. Cells
were then washed and fixed in 1% fresh paraformaldehyde at 4°C and 5,000
lymphocytes analyzed on a Becton Dickinson FACScan flow cytometer. Non-
specific staining was obtained by analysing cells incubated for 10 min at 37°C
with an equivalent amount of PE- or FITC-labelled isotype control
immunoglobulin. L-selectin or CD23 expression levels on control lymphocytes
were similar before and after incubation with EDTA (5 mM) in PBS at 37°C for
up to 2 hr. In all experiments L-selectin and CD23 were analyzed by gating first
on lymphocytes using forward and side-scattering properties. The value for L-
selectin or CD23 expression was measured by three colour analysis in which
CD3 and CD20 antibodies were added to each tube in addition to the PE- or FITC-labelled antibody of interest.

2.14 Ethidium$^+$ Influx Measurement by Time Resolved Flow Cytometry. Mononuclear cells (2x10⁶) pre-labelled with FITC-conjugated cell markers were washed once and resuspended in medium at 37°C. Cells were gated by forward and side scatter and by cell type specific antibodies. Ethidium (25 µM) was added, followed 40 s later by addition of 1.0 mM ATP. Mononuclear cells were analyzed at about 1000 events per second by a Becton Dickinson FACSCalibur flow cytometer and the linear mean channel of fluorescence intensity for each gated subpopulation over successive 5 s intervals was analyzed by WinMDI software (Joseph Trotter, version 2.7) and plotted against time.

2.15 Preparation of Cell Lysates
Lymphocytes (1.0x10⁸/ml) were treated with either 0.2 mM BzATP or 100 nM PMA at 37°C in NaCl medium without BSA plus proteinase inhibitors (5 mM EDTA, 2 µg/ml aprotinin, 0.5 mM phenylmethylsulfonylfluoride (PMSF), 0.2 mg/ml leupeptin, 10 µM trans-Epoxysuccinyl-L-leucylamido(4-guanidino)-butane (E-64), 1 µg/ml pepstatin A (from Sigma). Following centrifugation and collection of supernatant, the cells were washed once and lysed in 1% Triton X-100 containing 50 mM Hepes, pH 7.0, plus proteinase inhibitors as above for 10 min in ice. Cell lysates were clarified by centrifugation at 4000g x 15 min at 4°C.
2.16 Western Blots for L-Selectin and CD23

Samples and protein standard markers were mixed 1:1 with non-reduced sample buffer and separated on a 10-20% gradient SDS-PAGE gel in tray buffer overnight and transferred onto nitrocellulose membranes in transfer buffer for 4 hours. Membranes were blocked overnight in TTBS containing 5% skim milk powder. Membranes were washed 3 times with TTBS and probed with 1:200 unlabelled mouse anti-human L-selectin MoAb (FMC46 clone, DAKO) or anti-human CD23 MoAb (MHM6 clone, DAKO) as indicated. Membranes were washed 5 times with TTBS and bound antibodies were detected by 1:1000 HRP conjugated goat anti-mouse IgG (DAKO). After another 5 times washing with TTBS, an ECL kit (Amersham) was used to detect enhanced chemiluminescence according to manufacturer's instructions.

2.17 Statistics

Mean values ± S.D. are shown while significance of difference between mean values was calculated using a two tailed Students t-test.
Chapter 3

ATP-INDUCED SHEDDING OF CD23 AND L-SELECTIN (CD62L) FROM LYMPHOCYTES IS MEDIATED BY THE SAME RECEPTOR BUT DIFFERENT METALLOPROTEASES

3.1 INTRODUCTION

Recently extracellular ATP has been shown to mediate shedding of L-selectin from the surface of both normal and leukaemic lymphocytes via activation of the P2X7 purinoceptor of these cells.\textsuperscript{33} High levels of soluble CD23 are also found in the serum of patients with B-CLL suggesting that shedding of CD23 from malignant lymphocytes may occur by a continuous physiological process similar to that for L-selectin. This study reports that extracellular ATP induces shedding of CD23 from B-CLL lymphocytes and compares the characteristics of CD23 shedding with L-selectin shedding.
3.2 RESULTS

3.2.1 Extracellular ATP Causes Loss of CD23

Fig.3-1a and Fig.3-1b shows that both ATP and BzATP, a more potent agonist for P2X7 receptor, caused rapid shedding of L-selectin from the surface of B-CLL lymphocytes, as previously described. Both agonists for P2X7 receptors also caused disappearance of CD23 from the lymphocytes surface (Fig.3-1a, b), but at a far slower rate than for L-selectin (7% versus 60% per min respectively). Similar results were observed for B-CLL cells from 5 separate patients. Lymphocytes incubated in either isotonic NaCl or KCl showed a small but significant rate of spontaneous CD23 shedding with values of 1-3% over 5 min. In contrast to the above results, extracellular ATP did not alter the expression of CD44, CD45, CD47, CD49d or HLA-DR on B-CLL lymphocytes (data not shown).
Fig. 3-1. Time course of ATP and BzATP induced loss of CD23 and L-selectin. B-CLL lymphocytes were incubated with (a) 500 μM ATP or (b) 100 μM BzATP for the indicated times prior to labeling with anti-CD23 (○) or anti-L-selectin (●). Results are expressed as the percentage of initial expression.
3.2.2 ATP releases soluble CD23 and L-selectin

Western blotting showed the presence of soluble CD23 and L-selectin in the supernatants of B-CLL cells treated with BzATP. Soluble L-selectin was detected as a single band in the supernatants of PMA treated neutrophils (lane I, Fig. 3-2a) and lymphocytes (lane II, Fig. 3-2a) and BzATP treated lymphocytes (lane III, Fig. 3-2a) with approximate molecular weights of 90, 67 and 67 kD, respectively. However, soluble CD23 was not detected in the supernatants of cells treated with PMA (Fig. 3-2b). The appearance of soluble CD23 (lane 3, Fig. 3-2b) was associated with a loss of cell associated CD23 in the triton X-100 lysates (lane 4, Fig.3-2b). Analysis of soluble CD23 in previous

![Figure 3-2](image-url)  
**Fig.3-2** Appearance of (a) soluble L-selectin (b) soluble CD23 in the supernatant following neutrophils (Lane I, fig.a) or lymphocytes (Lane II, III in fig.a and all in fig.b) exposure to BzATP or PMA. Lymphocytes were treated with 100 nM PMA (lanes I &II in fig.a, lanes 1 & 2 in fig.b) or 0.2 mM BzATP (lane III in fig.a, lanes 3 & 4 in fig.b) for 30 min. L-selectin was detected in supernatants (lanes I, II, III), but not cell lysates (data not shown). CD23 was detected in supernatants (lanes 1 & 3) or cell lysates (lanes 2 & 4) by Western blotting. Lane 5 is a cell lysate of untreated lymphocytes. No bands were detected using an irrelevant control antibody.
studies has identified multiple fragments of molecular sizes 37, 33, 25 and 12 kD.\textsuperscript{109} The principal band identified in Fig.4-2a was 33 kD with a lesser band at 27 kD. The bands at 33 kD and 27 kD appeared as doublets. The significance of this is uncertain.

3.2.3 Agonist dose-response for CD23 shedding

Our previous results show that ATP induces shedding of L-selectin by agonist occupancy of P2X\textsubscript{7} receptors.\textsuperscript{33} ATP induced the loss of CD23 from B-CLL lymphocytes with an EC\textsubscript{50} of 50 \(\mu\text{M}\), a value identical to the EC\textsubscript{50} for ATP-induced loss of L-selectin (Fig.3-3a). A similar result was obtained with BzATP which caused loss of CD23 with an EC\textsubscript{50} of 10 \(\mu\text{M}\) and loss of L-selectin with an EC\textsubscript{50} of 15 \(\mu\text{M}\) (Fig.3-3b).
Fig. 3-3 Dose response of ATP and BzATP induced shedding of CD23 and L-selectin. B-CLL lymphocytes were incubated with the indicated concentration of ATP (a) or BzATP (b) for 7 min prior to labeling with anti-CD23 (○) or for 1.5 min prior to labeling with anti-L-selectin (●). Results are expressed as percentage of initial expression.
3.2.4 CD23 shedding is mediated via P2X7 receptors

The isoquinolinesulfonamide derivative, KN-62 is the most potent inhibitor of the P2X7 receptor described to date.\textsuperscript{21} The ATP-induced shedding of both L-selectin and CD23 was inhibited by KN-62 with an identical IC\textsubscript{50} of 12 nM (Fig. 3-4). Similar IC\textsubscript{50} for KN-62 have been described for ATP-induced Ba\textsuperscript{2+} and ethidium\textsuperscript{+} influxes\textsuperscript{21}. Pre-incubation of lymphocytes with OxATP gives irreversible inhibition of the P2X7 receptor of both murine macrophages and human lymphocytes.\textsuperscript{19; 35} Lymphocytes were pre-incubated with or without OxATP (300 \textmu M for 60 min at 37\textdegree C) prior to incubation with BzATP (100 \textmu M) in NaCl medium. Fig.3-5 shows that the shedding of both CD23 and L-selectin was completely abolished by OxATP pre-exposure. The downstream effects of

![Graph showing KN-62 inhibition of ATP induced shedding of CD23 and L-selectin. B-CLL lymphocytes were treated with the indicated concentrations of KN-62 prior to exposure to 500 \textmu M ATP (7 min for CD23 and 1.5 min for L-selectin measurements). Cells were then labeled with anti-CD23 (O) or anti-L-selectin (●) and the results expressed as the percentage of inhibition of shedding.](image)

ATP on lymphocytes are attenuated in high NaCl media which slows but does not abolish the shedding of L-selectin from B-CLL lymphocytes. Similarly ATP-induced shedding of CD23 was slower in NaCl medium than in KCl medium (data not shown).

Fig. 3-5  Inhibition of CD23 and L-selectin shedding by OxATP. B-CLL lymphocytes were treated with (▲) or without (△) 300 µM OxATP prior to exposure to 100 µM BzATP. Expression of CD23 (a) and L-selectin (b) were measured and results expressed as the percentage of initial expression.
3.2.5 Phorbol esters induce shedding of L-selectin but not CD23

Phorbol esters such as PMA are known to induce shedding of L-selectin from B-CLL lymphocytes. Fig. 3-6 shows that PMA induced shedding of L-selectin with an EC<sub>50</sub> of 2 nM. However PMA did not affect the expression of CD23 and even at doses as high as 100 nM, no significant shedding of CD23 was observed.

![Graph showing PMA-induced shedding of L-selectin and CD23](image)

**Fig. 3-6** PMA induces shedding of L-selectin but not CD23. B-CLL lymphocytes were exposed to the indicated concentrations of PMA for 15 min before measurement of CD23 (O) or for 1.5 min before measurement of L-selectin (●). Results are given as the percentage of initial expression.

3.2.6 The shedding of CD23 and L-selectin is inhibited by Ro 31-9790

The hydroxamic acid derivative Ro 31-9790 is a lipophilic inhibitor of L-selectin sheddase, effective in the 2-10 μM range in blocking phorbol-ester induced shedding of L-selectin in human lymphocytes. Ro 31-9790 inhibited ATP-
induced shedding of L-selectin with an IC₅₀ of 20 µg/ml. This inhibitor was even more potent in inhibiting ATP-induced shedding of CD23 with an IC₅₀ of 1.0 µg/ml (Fig.3-7).

3.2.7 CD23 shedding is inhibited by extracellular Ca²⁺
Extracellular ATP-induced shedding of L-selectin is independent of the presence or absence of Ca²⁺ in the medium, a finding confirmed in the present study (Fig.3-8a). However ATP-induced loss of CD23 was inhibited by more than 30–40% in the presence of 1 mM Ca²⁺ (Fig.3-8b). The inhibitory effect of other divalent cations on the shedding of CD23 by ATP was studied. Mn²⁺ was the most potent inhibitor of CD23 loss and the order of inhibitory potency (at 1
mM divalent concentration) was Mn$^{2+}$ > Ca$^{2+}$ = Mg$^{2+}$ > Ba$^{2+}$ (data not shown). Thus the presence of Ca$^{2+}$ and especially Mn$^{2+}$ increased the resistance of CD23 to proteolytic shedding by 3.4 fold.

![Graph](image-url)

**Fig. 3-8** Effect of Ca$^{2+}$ on the shedding of CD23 and L-selectin. B-CLL lymphocytes were incubated with 500 µM ATP in the presence (■) or absence (▲) of 1 mM Ca$^{2+}$. Cells were then labeled with anti-L-selectin (a) or anti-CD23 (b) and the results expressed as the percentage of initial expression.
3.3 DISCUSSION

Lymphocytes from patients with CLL express large numbers of receptors for extracellular ATP (P2 purinoceptors), and agonist competitive studies have shown one of those purinoceptors, the P2X\textsubscript{7} receptor is expressed on the surface of these cells.\textsuperscript{28} Activation of P2X\textsubscript{7} receptors induces multiple downstream effects of which the best documented is the opening of an ionic channel which is selective for divalent cations.\textsuperscript{29, 30} A second effect of ATP is to stimulate a phospholipase D, which on attachment to the plasma membrane, catalyses hydrolysis of phosphatidylcholine to phosphatidic acid and choline.\textsuperscript{31, 32} A third effect of ATP is to induce the shedding of L-selectin (CD62L),\textsuperscript{33} a C-type lectin which is involved in the adhesive interactions and rolling behaviour of lymphocytes on endothelial cells.\textsuperscript{144} This study has identified another effect of ATP on B-CLL lymphocytes: the shedding of CD23 and appearance of soluble CD23 in the supernatant. In contrast to the rapid shedding of L-selectin, the loss of CD23 induced by ATP was about an order of magnitude slower. Despite this difference in kinetics, several lines of evidence demonstrate that the ATP-induced loss of CD23, like L-selectin, is mediated by agonist activation of the P2X\textsubscript{7} receptor. Thus CD23 and L-selectin showed identical dose response curves for shedding by either ATP or the more potent agonist BzATP (Fig. 3-2). Moreover the potent P2X\textsubscript{7} receptor inhibitor, KN62 blocked the shedding of L-selectin and CD23 with identical IC\textsubscript{50} (Fig. 3-3) while pre-treatment of lymphocytes with OxATP irreversibly inhibited the ability of P2X\textsubscript{7} agonists to induce shedding of either surface molecule.
There has been much recent interest in the nature of the protease responsible for shedding of L-selectin. This enzyme does not cleave soluble substrates and is insensitive to a wide range of commonly employed protease inhibitors, including DFP, TIMP-1 and phosphoramidon.\textsuperscript{81, 86} The only known inhibitors are the hydrophobic hydroxamic acid derivatives such as Ro 31-9790 or KD-IX-73-4 which chelate zinc whereas the water soluble zinc-chelator o-phenanthroline is ineffective (unpublished observation). This requirement for lipid solubility of the inhibitor suggests that the enzyme which sheds CD23 contains zinc in a hydrophobic location, probably within the plasma membrane. How this enzyme is activated when agonist occupies the P2X\textsubscript{7} receptor is unknown. Members of the P2X receptor family have protein structures with two putative transmembrane segments linked by a long extracellular loop and intracellular N- and C-termini. The P2X\textsubscript{7} receptor is unusual in having a long C-terminal tail which not only confers unique permeability properties to large fluorescent cations\textsuperscript{16, 18} but also has the potential for interactions with other membrane molecules such as proteases.

The initial rate of decrease of L-selectin immunoreactivity was 60% per min but only 7% per min for CD23 with either agonist (Fig.3-1) indicating an 8.5-fold greater rate of shedding for L-selectin over CD23. It has been demonstrated that phorbol ester-induced shedding of L-selectin from human leukocytes by “L-selectin sheddase” involves proteolytic cleavage at a K-S peptide bond.\textsuperscript{81, 140} The structure of CD23 also includes three K-S bonds in its extracellular domain, although the most proximal cleavage site is 35 residues distal from the transmembrane domain compared with 10 residues for L-selectin. Assuming the catalytic site for proteolysis is membrane-associated,\textsuperscript{81} and L-selectin
sheddase cleaves both membrane proteins, then purely steric factors may account for the slower rate of CD23 shedding compared to L-selectin. However, recent data using site directed mutagenesis has cast some doubt about the specificity of L-selectin sheddase for the K-S bond. Our data supports a different hypothesis, namely that two different proteases are involved in the shedding of L-selectin and CD23. This latter possibility is supported by the failure of phorbol ester to shed CD23 (Fig.3-6) and the different sensitivity of shedding of L-selectin and CD23 to inhibition by Ro 31-9790 (Fig.3-7). Considering the wide range of membrane molecules which are known to be shed from the surface of leukocytes it is likely that many different proteases are involved.

A number of membrane molecules require extracellular Ca\(^{2+}\) to maintain their tertiary or quaternary structure. Our data show that extracellular divalent ions inhibit the ATP-induced shedding of CD23. This result may reflect the stabilisation of CD23 structure by Ca\(^{2+}\) such as shown for the resistance of the plasma cell membrane glycoprotein, PC-1 to proteolysis and thermal denaturation. However, the structure of the stalk region of CD23 (where enzymatic cleavage occurs) is an \(\alpha\)-helical coiled-coil which does not contain a recognized Ca\(^{2+}\)-binding domain and is unlikely to be stabilised by Ca\(^{2+}\)-ions. Thus the mechanism by which Ca\(^{2+}\) inhibits ATP-induced CD23 shedding remains uncertain.

ATP-induced loss of CD23 immunoreactivity from the surface of B-CLL lymphocytes is associated with the appearance of soluble CD23 in the supernatant (Fig.3-2). Western analysis of the supernatant revealed two bands
of molecular weights 33 and 27 kD, similar to those previously described in human plasma.\textsuperscript{111} High levels of both soluble CD23 and soluble L-selectin are found in the sera of patients with B-CLL, while levels of soluble CD23 have been related both to the tumour load and the clinical stage of this disease.\textsuperscript{139; 148-153} Moreover soluble CD23 has been proposed as a sensitive and specific marker for disease activity\textsuperscript{149-151} while in a complementary study high levels of cellular CD23 have been correlated with a favourable prognosis in B-CLL.\textsuperscript{153} Recent evidence suggests that L-selectin is cleaved from the surface of leukocytes within seconds during the process of rolling under hydrodynamic flow\textsuperscript{86} and it has been shown that hydroxamic acid-based inhibitors of zinc-dependent metalloproteases not only slow the rate of leukocyte rolling\textsuperscript{86} but also inhibit ATP-induced shedding of both L-selectin and CD23 (Fig.3-7). Since hydrodynamic shear causes release of ATP from endothelial cells,\textsuperscript{154} it is possible that the transient tethering which characterizes the rolling behaviour of leukocytes may also provide the rapid signal for endothelial ATP release. This in turn could activate the P2X\textsubscript{7} receptor in the microenvironment of the tethered lymphocyte, stimulate the proteinases which are coupled to this receptor and generate both soluble L-selectin and soluble CD23.
Chapter 4

DOWNREGULATION OF L-SELECTIN AND CD23 ON LYMPHOCYTE TRANSENDOTHELIAL MIGRATION

4.1 INTRODUCTION

Flow cytometric analysis of L-selectin shows this molecule is lost from the surface of neutrophils following their migration across human umbilical vein endothelial cell (HUVEC) monolayers \(^ {155}\) and there is one report that L-selectin is decreased on mouse lymphocytes after their interaction with high endothelial cells (HEV).\(^ {156}\) Downregulation of L-selectin from neutrophils is thought to be mediated by chemoattractants such as IL-8, which is synthesized by HUVECs and which can induce rapid shedding of L-selectin.\(^ {69; 82}\) Recently our group has shown that extracellular ATP can induce shedding of L-selectin within several minutes from the surface of both normal and leukaemic human lymphocytes.\(^ {33}\) Moreover, Chapter 3 shows that agonist occupancy of this P2X\(_7\) receptor not only opens an ionic channel but also activates a second membrane metalloproteinase which causes shedding of the adhesion molecule, CD23. Since ATP can be secreted by endothelial cells,\(^ {154}\) the expression of L-selectin and CD23 on lymphocytes before and after transmigration across monolayers of HUVECs during short \textit{in vitro} incubations was measured.
4.2 RESULTS

4.2.1 Time and Concentration Dependence of Transmigration

Lymphocytes from normal subjects and patients with B-CLL (1.0 x 10⁷/ml) were plated over confluent endothelial monolayers at 37°C and examined by phase-contrast microscopy at various time intervals. Fig. 4-1 shows a typical appearance after 2 hr incubation in which two populations of lymphocytes were visible; phase light lymphocytes which were adherent to the upper surface of the HUVEC monolayer and phase dark lymphocytes which had migrated beneath the HUVEC monolayer. Electron microscopy confirmed the sub-endothelial location of the migrated lymphocytes (Fig. 4-2). The kinetics of the migration process was studied for B-CLL cells. After 2 hours incubation the number of transmigrated lymphocytes per HUVEC (index of transmigration) reached a steady state value which did not change over the subsequent 2 hours (Fig. 4-3). Increasing the density of lymphocytes from 0.5 to 1.5 x 10⁷ per well resulted in a 2-3 fold increase in transmigration index after 2 hr (Fig. 4-4). However the migration index did not further increase when higher densities of lymphocytes were plated. All subsequent transmigration assays were performed using 1.0 x 10⁷ lymphocytes per well and at this cell concentration a mean value of 3.30 ± 0.81 % (mean ± S.D. n = 8) of the plated cells had transmigrated after 2 hr. The index of transendothelial migration averaged 2.15 ± 0.36 lymphocytes per HUVEC after 2 hr incubation (mean ± S.D., range 0.5-6.1, n=12). In contrast, the index of transendothelial migration for peripheral blood lymphocytes from normal subjects ranged from 3 to > 10 cells per HUVEC after 2 hr incubation although exact quantification was difficult because of cell crowding beneath the monolayer. (Fig. 4-1b).
Fig. 4-1 (a) Phase-contrast micrograph of B-CLL lymphocytes after 2 hr \textit{in vitro} transendothelial migration. Two populations of lymphocytes were seen: adherent phase light cells above the monolayer and phase-dark cells which have migrated beneath the monolayer. (b) Phase-contrast micrograph of normal peripheral blood lymphocytes after 2 hr \textit{in vitro} transendothelial migration. Large groups of phase dark cells are seen beneath the monolayer. (Original Mag.x400)
Fig. 4-2 (a) Electron Micrograph of a lymphocyte adherent to an endothelial Cell. A cytoplasmic tongue from the lymphocyte is protruding beneath the endothelial monolayer (L: adherent lymphocyte, E: endothelial cell); (b) Electron microscope of a migrated lymphocyte beneath the endothelial monolayer (E: endothelial cell, L: migrated lymphocyte)
Fig. 4-3 Time dependence of lymphocyte transendothelial transmigration is expressed as number of migrated lymphocytes per HUVEC. Values (±S.D.) are the mean of quadruplicate wells and are representative of 3 different experiments.

Fig. 4-4 Concentration dependence of lymphocyte transendothelial migration. Lymphocytes (0.5 - 4.0 x10⁷/well) were plated over monolayers of HUVECs (1.0 ml) and incubated at 37°C for 2 hr. Values (±S.D.) are the mean of quadruplicate wells and are representative of 3 different experiments.
4.2.2 Index of Transmigration Increased on Activated HUVECs

Previous studies have shown that transendothelial migration is enhanced after activation of HUVEC with TNF-α. The present results confirm this observation. The transmigration index for lymphocytes (0.5-2.0 $10^7$/ml) was by 2-3 fold greater following TNF-α or IFN-γ activation of HUVECs (Fig. 4-5).

![Graph showing index of transmigration for lymphocytes on HUVECs](image)

**Fig. 4-5** 4 hr lymphocyte transmigration on HUVECs which were non-activated or activated by 5 ng/ml TNF-α or 10 ng/ml IFN-γ for 6 hr. Values (±S.D.) are the mean of quadruplicate wells and are representative of 3 different experiments.
4.2.3 Comparison of L-selectin expression on normal and leukaemic lymphocytes

To quantitate L-selectin expression on lymphocytes, these cells were first incubated with FITC-conjugated anti-L-selectin MoAb. Linear mean channel of fluorescence intensity of bound MoAb was measured on gated lymphocyte populations and the value was converted to molecules of equivalent soluble fluorescein (MESF) by comparison with standard fluorescein-containing beads which were run with samples to correct for day-to-day variations in machine performance. This MESF value of bound FITC-anti-L-selectin antibody was then converted to molecules per cell according to the correlation between L-
selectin antibody MESF and molecules per cell described in the Methods. Lymphocytes from patients with B-CLL expressed 8,878 ± 5,700 molecules L-selectin per cell (Mean ± S.D., n=11). This was significantly lower than for lymphocytes from normal subject which was 29,506 ± 7,526 (Mean ± S.D., n=9, p< 0.001) (Fig. 4-6).

4.2.4 Impaired transendothelial migration on leukaemic lymphocytes

Lymphocytes from normal subjects and patients with CLL (1.0x10^7/ml) were plated over confluent endothelial monolayers at 37°C and examined for migration beneath the monolayer after a 4-hour incubation. The index of transendothelial migration of lymphocytes from patients with B-CLL (1.48±0.94, n=28) was lower than that for predominantly T-lymphocytes of normal subjects (2.34±0.90, n=9, p=0.04). However, lymphocytes from one B-CLL patient had a dramatically high index (>8). The T-cell percentage of the transmigrated population was also measured. Lymphocytes prepared from B-CLL patients always contain a minor population of normal T-cells (6-34%) and this population of T-cells was enriched by 2.5 fold (range 1.6-5.4 fold) in the lymphocytes recovered from beneath the monolayer. In contrast, normal lymphocytes maintain the same T:B cell ratio before and after transmigration (Table 4-1).

This result suggests that the B-CLL lymphocytes have a significant reduction (p<0.05) in their ability to undergo transendothelial migration compared with the normal T-cells present in the same mononuclear preparation. Even after endothelial activation by cytokine, the enrichment of T-cells in the migrating B-CLL population was still observed, indicating an intrinsic impairment of leukaemic B-cell migration (Table 4-1).

Table 4-1 Enrichment of T cells after 2 hours migration
<table>
<thead>
<tr>
<th>Subjects</th>
<th>T cells (% of population)</th>
<th>Enrichment of T cells after migration (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>In starting population</td>
<td>Beneath monolayer</td>
</tr>
<tr>
<td>Unactivated HUVEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 1</td>
<td>82</td>
<td>52</td>
</tr>
<tr>
<td>Normal 2</td>
<td>86</td>
<td>87</td>
</tr>
<tr>
<td>CLL 1</td>
<td>6.1</td>
<td>11.1</td>
</tr>
<tr>
<td>CLL 2</td>
<td>8.2</td>
<td>13.5</td>
</tr>
<tr>
<td>CLL 3</td>
<td>9.4</td>
<td>26.4</td>
</tr>
<tr>
<td>CLL 4</td>
<td>34</td>
<td>63</td>
</tr>
<tr>
<td>TNF-α-activated HUVEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 3</td>
<td>94</td>
<td>80</td>
</tr>
<tr>
<td>CLL 5</td>
<td>6.4</td>
<td>7.6</td>
</tr>
<tr>
<td>CLL 6</td>
<td>10.0</td>
<td>54</td>
</tr>
</tbody>
</table>

Lymphocytes (1.0x10⁷/ml) were plated to a monolayer of endothelial cells and allowed to transmigrate for 2 and 4 hours in M199 complete medium at 37°C, followed by 5 washes to remove any non-migrated cells and 5 mM EDTA-PBS was then used to harvest migrated lymphocytes with endothelial cells. B cell (gated on CD20-positive lymphocytes) and T cell (gated on CD3 positive lymphocytes) counts represent the mean of quadruplicate wells containing at least 5000 lymphocytes in the starting cell population at 0 hr or in cells beneath the endothelial monolayer at 2 hr. T-cell enrichment was calculated as the percentage of T cells beneath the monolayer divided by the percentage of T cells in the starting population.

### 4.2.5 Correlation Between L-selectin Expression and Transmigration Rate

A weak correlation was found between L-selectin expression on B-CLL lymphocytes and their index of transendothelial migration (Fig.4-7, \( r^2=0.615, \) \( n=15, p<0.001 \) ). A noteworthy observation was made with three CLL patients with very low expression of L-selectin on their lymphocytes which showed very poor transendothelial migration. All three patients had massive splenomegaly, hepatomegaly, and enlargement of lymph nodes (Rai Stage 4). In contrast, two
CLL patients with high L-selectin expression level on lymphocytes had a higher index of transendothelial migration and a quiescent disease course.

4.2.6 Regulation of L-selectin Expression Affects Lymphocyte Transendothelial Migration

Extracellular ATP and its analogue BzATP have been shown to stimulate the shedding of L-selectin from both normal and CLL lymphocytes.\(^{33}\) The effect of ATP-mediated shedding of L-selectin on the subsequent transmigration of lymphocytes was studied. Cells were pre-incubated for 30 min at 37°C with ATP (0.5 mM) or BzATP (100 µM) after which the cells were washed and assayed for L-selectin expression and index of transmigration. All three

![Graph showing correlation between L-selectin expression and transmigration index. L-selectin value was calibrated to enable the comparison between different subjects on different days. All transmigrations were done in M199 complete medium at 37°C for 4 hr with lymphocyte concentration at 1.0 x 10⁷/ml. B-CLL patients or normal donors with an extremely high index of transmigration (>8) were excluded.](image-url)
agonists downregulated L-selectin and this effect was greater for BzATP than for ATP (Fig.3-1). Both agonists decrease the index of transmigration, and this effect was proportional to the reduction in L-selectin expression (Fig.4-8). As a control, OxATP, which does not alter the expression of L-selectin, also had no

**Fig.4-8** Effect of ATP, BzATP, PMA, OxATP and Ro 31-9790 on both lymphocyte transmigration and surface L-selectin expression. Lymphocytes (1.0 x10⁷/ml) from B-CLL and normal donors were incubated with or without:
1) 0.5 mM ATP in Na medium for 15 min, wash,
2) 0.1 mM BzATP in Na medium for 15 min, wash,
3) 100 nM PMA in Na medium for 15 min, wash,
4) 0.3 mM OxATP in Na medium plus 1 mM Ca²⁺ for 60 min, wash,
5) 50 µg/ml Ro 31-9790 in M199 complete medium, no wash,
Cells were then resuspended in M199 complete medium and surface L-selectin level was measured. Results were presented as % of transmigration index (migrated lymphocytes / HUVEC) and % of L-selectin level compared with control in each experiment.
effect on lymphocyte transmigration (Fig.4-8). Ro 31-9790, which has been shown to inhibit L-selectin sheddase, was added to CLL lymphocytes for 20 min prior to their addition to HUVEC monolayers and produced a significant increase in transmigration (160±40% of control, p<0.001) (Fig.4-8). Presumably Ro 31-9790 protects L-selectin on lymphocytes from spontaneous shedding or by other unknown mechanisms and therefore increases transmigration. As shown previously, the phorbol ester, PMA produced almost complete downregulation of L-selectin, but the effect of this agent on transmigration was difficult to measure because of the large numbers of lymphocytes adherent to the endothelial monolayer.

**Fig.4-9** Effect of KN-62 on lymphocyte transmigration. Lymphocytes (1.0x10^7/ml) were incubated with or without 1 µM KN-62 in Na medium for 15 min followed by addition of 0.1 mM BzATP for 30 min at 37°C. Cells were then washed once and resuspended in M199 complete medium and KN-62 was added back. Cells were plated on to a monolayer of HUVECs for 4 hr at 37°C. Values (±S.D.) are the mean of quadruplicate wells and are representative of 3 different experiments.
Neither KN-62 nor KN-04, which inhibit ATP-induced L-selectin shedding,\textsuperscript{21} had any effect on lymphocyte transmigration. However, KN-62 or KN-04 protected lymphocytes against the effects of BzATP treatment on transmigration (Fig.4-9).

### 4.2.7 L-selectin Resynthesis Increases Transmigration

Little is known of the factors which regulate the expression of L-selectin on the lymphocyte surface although evidence suggests that shedding of L-selectin is a continuous physiological process\textsuperscript{77} which must be balanced by resynthesis of this molecule. The expression of L-selectin was studied before and after overnight incubation of lymphocytes in complete RPMI medium. L-selectin level on normal lymphocytes was not significantly different after 24 hours incubation in complete medium. Lower values of L-selectin expression were found on CLL lymphocytes but these low initial values for L-selectin increased after 24 hours incubation in complete RPMI1640 medium. This increase in L-selectin represented new protein synthesis since it was inhibited by cycloheximide (50 µg/ml) (Table 4-2). A higher index of transmigration was observed for lymphocytes with upregulated L-selectin compared with the same cells on the day of preparation (Fig.4-10).
**Table 4-2.** Effect of Cycloheximide on L-selectin resynthesis of lymphocytes

<table>
<thead>
<tr>
<th>Subject</th>
<th>L-selectin (molecules/cell)</th>
<th>Initial</th>
<th>No Cycloheximide</th>
<th>With Cycloheximide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>24 hr</td>
<td>48 hr</td>
<td>24 hr</td>
</tr>
<tr>
<td>B-CLL</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6250</td>
<td>16000</td>
<td>17626</td>
<td>6380</td>
<td>4656</td>
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<tr>
<td>1420</td>
<td>2800</td>
<td>4071</td>
<td>940</td>
<td>2298</td>
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<tr>
<td>2450</td>
<td>6910</td>
<td>21180</td>
<td>1050</td>
<td>2565</td>
</tr>
<tr>
<td>Normal</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>16148</td>
<td>17206</td>
<td>21606</td>
<td>9990</td>
<td>14553</td>
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</tr>
<tr>
<td>32426</td>
<td>32207</td>
<td>28909</td>
<td>17788</td>
<td>24496</td>
</tr>
</tbody>
</table>

Lymphocytes from normal subjects and CLL patients (5x10^6/ml) were incubated in RPMI 1640 complete medium with or without cycloheximide (50 µg/ml) for 24 and 48 hours.

**Fig.4-10** Concentration dependence of lymphocyte transmigration. Fresh isolated lymphocytes and lymphocytes incubated in RPMI complete medium for 24 hr (0.5 - 3.0 x10^7/well) were washed and resuspended into M199 complete medium and plated over monolayers of HUVECs and incubated at 37°C for 4hr. Values (±S.D.) are the mean of quadruplicate wells.
**4.2.8 Intracellular Ionized Ca\(^{2+}\) Is Essential for Transmigration**

Transendothelial migration of B-CLL lymphocytes was measured after loading the cells with BAPTA-AM (from Molecular Probes), a cell-permeant highly selective Ca\(^{2+}\) chelator, in order to buffer any changes in intracellular ionized Ca\(^{2+}\) levels. Pre-treatment of lymphocytes with 50 µM BAPTA-AM for 30 min completely abolished transmigration (Fig.4-11) while L-selectin level in those cells still remained unchanged (data not shown). A short contact of endothelial cells with BAPTA-AM caused those cells to detach from the plate.

![Figure 4-11](image)

**Fig.4-11** Effect of BAPTA on lymphocyte transmigration. Lymphocytes (1.0x10⁷/ml) were incubated with or without 10~50 mM BAPTA-AM in RPMI1640 basic medium for 30 min, washed 3 times, and resuspended in M199 complete medium and plated on to monolayer of HUVECs for 4 hr at 37°C. Values (±S.D.) are the mean of quadruplicate wells and are representative of 6 different experiments.
4.2.9 Pertussis Toxin Inhibits Transmigration

Pertussis Toxin (PTX), which catalyses the ADP-ribosylation of specific G-protein alpha subunits of the Gi family and prevents the occurrence of the receptor-G-protein interaction,\textsuperscript{157} was also used to study the lymphocyte transmigration. Both lymphocytes from B-CLL patients or endothelial cells which were pre-treated with 100 ng/ml PTX for 60 min showed significant decrease of transmigration (p<0.001).

Fig. 4-12 Effect of PTX on lymphocytes transmigration. Lymphocytes (1.0x10\textsuperscript{7}/ml) (PTX-CLL and PTX-TM) or endothelial cells (PTX-Endo) were incubated with or without 100 ng/ml PTX in Na medium for 60 min, washed twice and resuspended in M199 complete medium. Lymphocytes were plated on to a monolayer of HUVECs for 4 hr at 37°C. (Control: no PTX; PTX-TM: PTX was added back after wash and kept during transmigration). Values (±S.D.) are the mean of quadruplicate wells and are representative of 4 different experiments.
4.2.10. Loss of L-selectin Expression from all Lymphocyte Subsets after Transendothelial Migration

The expression of L-selectin on the surface of freshly isolated lymphocytes from normal subjects and CLL patients was measured before and after 2 hr of incubation on HUVEC monolayers. After repetitive washing to remove non-adherent lymphocytes, phase contrast examination established that the majority (90 ± 3%) of these remaining lymphocytes had undergone transendothelial migration. For B-CLL the adherent and transmigrated lymphocytes showed a major loss of 71 ± 5% of L-selectin expression while non-adherent cells recovered from above the HUVECs showed no change of their L-selectin expression (0.9 ± 6.9% loss) (Table 4-3, Fig.4-13, Fig.4-15). Moreover T- and B- cells from normal subjects also showed a major loss of L-selectin after transendothelial migration (Table 4-3). Non-adherent lymphocytes recovered from above the endothelial monolayer, showed no change in L-selectin expression. When the HUVEC monolayer was activated by TNFα, the B-CLL lymphocytes which had transmigrated through the monolayer showed the same loss of L-selectin (73 ± 4%) as transmigrated lymphocytes on unactivated HUVECs (71 ± 5%).
Table 4-3  Loss of L-selectin expression after transmigration

<table>
<thead>
<tr>
<th></th>
<th>B-cell selectin</th>
<th>T-cell selectin</th>
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<tbody>
<tr>
<td></td>
<td>0h</td>
<td>2h</td>
</tr>
<tr>
<td>Unactivated HUVEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 1</td>
<td>143</td>
<td>84</td>
</tr>
<tr>
<td>Normal 2</td>
<td>86</td>
<td>55</td>
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<tr>
<td>CLL 1</td>
<td>69</td>
<td>23</td>
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<tr>
<td>CLL 2</td>
<td>76</td>
<td>30</td>
</tr>
<tr>
<td>CLL 3</td>
<td>62</td>
<td>33</td>
</tr>
<tr>
<td>CLL 4</td>
<td>65</td>
<td>26</td>
</tr>
<tr>
<td>CLL 5</td>
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</tr>
<tr>
<td>TNF-α-activated HUVEC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal 3</td>
<td>165</td>
<td>93</td>
</tr>
<tr>
<td>CLL 5</td>
<td>122</td>
<td>35</td>
</tr>
<tr>
<td>CLL 6</td>
<td>82</td>
<td>42</td>
</tr>
</tbody>
</table>

Lymphocytes (1.0x10^7/ml) after plastic adhesion to remove monocytes contamination were plated to a monolayer of endothelial cells and allowed to transmigrate for 2 and 4 hours in M199 complete medium at 37°C, followed by 5 washes to remove any non-migrated cells. 5mM EDTA-PBS was used to harvest migrated lymphocytes with endothelial cells. L-selectin expression was expressed in linear mean channels (in log scale) of fluorescence for the B-cells (gated on CD20 positive lymphocytes) and T-cells (gated on CD3 positive lymphocytes) in the starting cell population at 0 hr or in cells beneath the endothelial monolayer at 2 and 4 hr.

4.2.11 Effect of Migration on CD23 and other Cell Adhesion Molecules

B-CLL lymphocytes also express CD23, the low affinity receptor for IgE and which also functions as an adhesion molecule binding to CD21, CD11b and CD11c.\textsuperscript{124; 127; 158} Table 4-4 shows that expression of this adhesion molecule was also lost on B-CLL cells after their transendothelial migration. Moreover the constitutive expression of CD23 on B-cells in the mononuclear preparation from normal subjects was also downregulated after transmigration (Table 4-4). In contrast, the expressions of VLA-4 (CD49d/CD29), ICAM-1 (CD54), LFA-1
(CD11a/CD18) and CD44 were unchanged on the migrated cells (data not shown). Neither T-cells in the B-CLL populations nor T-cells in the mononuclear preparations from normal subjects showed significant expression of CD23.

**Table 4-4 Loss of CD23 expression after transmigration**

<table>
<thead>
<tr>
<th>Unactivated HUVEC</th>
<th>B-cell CD23</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0h</td>
</tr>
<tr>
<td>Normal 1</td>
<td>37</td>
</tr>
<tr>
<td>Normal 2</td>
<td>41</td>
</tr>
<tr>
<td>CLL 1</td>
<td>110</td>
</tr>
<tr>
<td>CLL 2</td>
<td>164</td>
</tr>
<tr>
<td>CLL 3</td>
<td>392</td>
</tr>
<tr>
<td>CLL 3*</td>
<td>356</td>
</tr>
<tr>
<td>CLL 4</td>
<td>128</td>
</tr>
<tr>
<td>TNF-α-activated HUVEC</td>
<td></td>
</tr>
<tr>
<td>Normal 3</td>
<td>87</td>
</tr>
<tr>
<td>CLL 6</td>
<td>207</td>
</tr>
</tbody>
</table>

Lymphocytes (1.0x10^7/ml) after plastic adhesion to remove monocyte contamination were plated to a monolayer of endothelial cells and allowed to transmigrate for 2 and 4 hours in M199 complete medium at 37°C, followed by 5 washes to remove any non-migrated cells. 5 mM EDTA-PBS was then used to harvest migrated lymphocytes with endothelial cells. CD23 expression was expressed in linear mean channels (in log scale) of fluorescence for the B-cells (gated on CD20 positive lymphocytes) in the starting cell population at 0 hr or in cells beneath the endothelial monolayer at 2 and 4 hr. (*: cells were pre-incubated with 300 µM OxATP for 60 min at 37°C, washed once and resuspended in M199 complete medium)

4.2.12 **Ro 31-9790 cannot Inhibit L-selectin Loss in Transmigration**

Since Ro 31-9790 can inhibit L-selectin shedding induced by ATP or BzATP (see Chapter 3), the effect of Ro 31-9790 on transmigration was studied. Lymphocytes were incubated with 50 µg/ml Ro 31-9790 for 15 min prior to the
transmigration assay. Fig. 4-13 shows that Ro 31-9790 did not protect lymphocyte L-selectin from downregulation during transendothelial migration.

**Fig. 4-13** L-selectin expression on lymphocytes during the transendothelial migration assay. B-CLL lymphocytes (○,□) were plated (at 1.0x10^7/well) onto HUVEC monolayers. Other B-CLL lymphocytes (●,■) were preincubated with 50 μg/ml Ro 31-9790 for 15 min at 37°C, and plated onto HUVEC monolayers as above. After 0, 2 and 4 hr incubation at 37°C, the non-adherent cells were removed by washing and their L-selectin measured. The transmigrated and tightly adherent cells were recovered by 5 mM EDTA in PBS dispersion of the monolayer and analyzed for L-selectin. The non-adherent cells were also treated with EDTA saline before L-selectin measurement. Data presented as % of initial L-selectin level ± S.D. (n=18 for untreated, n = 6 for Ro 31-9790 treated).

### 4.2.13 P2X7 Purinoceptor Is Not Involved in Transmigration

Our group has shown that ATP induces shedding of L-selectin via occupancy of P2X7 receptors.33 Thus, the role of P2X7 receptors in lymphocyte transmigration was studied. Lymphocytes pretreated with either OxATP, an irreversible inhibitor of P2X7 purinoceptor or KN-62, a more potent inhibitor for
P2X₇ receptor, retained the same ability for transmigration (Fig. 4-8, 4-9). Recently, a mouse anti-P2X₇ monoclonal antibody which inhibits P2X₇ receptor function was developed by Dr. Gary Buell and his colleagues¹⁵⁹. B-CLL Lymphocytes pre-incubated with the antibody showed greatly reduced P2X₇ function measured by ATP induced ethidium uptake but the monoclonal antibody had no effect on lymphocyte transendothelial migration (Fig.4-14a, b)
Fig.4-14  (a) Lymphocyte transmigration. Lymphocytes (1x10^7/ml) were incubated at 37°C with or without 0.3 mM OxATP for 60 min, or anti-P2X7 MoAb (100 µg/ml) for 15 min, or an irrelevant MoAb for 15 min, or 1 µM KN-62 for 15 min and plated onto a monolayer of endothelial cells. Values (±S.D.) were the mean of quadruplicate wells. (b) Ethidium uptake by flow cytometry. Pretreated lymphocytes (2x10^6) were washed and resuspended in 1.0 ml KCl medium. 25 µM ethidium was added 40 s prior to addition of 1 mM ATP. Mean channel of cell-associated fluorescence intensity was measured for lymphocyte populations at 5 s intervals.
Moreover, inhibition of P2X\(_7\) receptor by OxATP did not protect against L-selectin loss during transmigration. Prior to the assay, lymphocytes were pre-incubated for 60 min at 37°C with OxATP. Fig. 4-15 shows that the much reduced level of L-selectin on the lymphocytes after transmigration was the same for cells pretreated with or without OxATP. The loss of L-selectin from normal lymphocytes after transendothelial migration and the lack of effect of pre-treatment with OxATP was also observed for peripheral blood lymphocytes from normal subjects (Fig. 4-15). So far, we have not found any direct evidence showing P2X\(_7\) receptor involved in transmigration.

**Fig. 4-15** L-selectin expression on lymphocytes during the transendothelial migration assay. B-CLL lymphocytes (O,□) at 1.0x10⁷/well were plated onto HUVEC monolayers. Other B-CLL lymphocytes (●, ■) were preincubated with 300 µM OxATP for 60 min in Na medium with Ca\(^{2+}\) at 37° and washed, resuspended into M199 complete medium and plated over HUVEC monolayers as above. After 0, 2 and 4 hr incubation at 37°C, the non-adherent cells were removed by washing and their L-selectin measured. The transmigrated and tightly adherent cells were recovered by 5 mM EDTA in PBS dispersion of the monolayer and analyzed for L-selectin. The non-adherent cells were also treated with EDTA saline before L-selectin measurement. Data presented as % of initial L-selectin level ± S.D. (n=18 for untreated, n = 4 for OxATP treated).
4.3 DISCUSSION

In this study human lymphocytes from patients with B-CLL were able to undergo transendothelial migration across unactivated HUVECs and this capacity was enhanced 2-3 fold by prior activation of the HUVEC’s with TNFα. Nevertheless B-CLL lymphocytes have a lower index of transmigration than normal T-lymphocytes which formed a sub-population (6-10% with 34% in one patient) in the lymphocyte preparation from each patient. Table 4-1 shows that these normal T-lymphocytes were selectively enriched in the transmigrated cells since the percentage of T-cells found beneath the endothelial monolayer was significantly increased by 2.5 fold (range 1.6-5.4) when compared to the initial population. These slower kinetics of transmigration of B-CLL cells agrees with in vivo evidence that B-CLL lymphocytes have an impaired capacity to undergo recirculation. Thus, B-CLL lymphocytes labelled by H3-thymidine infusion in one patient survived in the circulation for many weeks without evidence of dilution in the far larger extravascular lymphoid tissue. Results of studies with B-CLL lymphocytes labelled with 51chromium also show that these cells leave the circulation less rapidly than lymphocytes from normal subjects. Several factors may account for the impaired capacity of B-CLL cells to migrate across endothelial cell monolayers. Biochemical studies show that B-CLL has lower metabolic rate and ATP concentration and reduced activity of many membrane enzymes. Moreover Fig. 4-6 and Table 4-3 show that B-CLL cells have lower expression of L-selectin on their surface, since L-selectin expression on lymphocytes has been shown to correlate with the number of cells attached to the high endothelial venules (HEV) and with the ability of
lymphocyte transendothelial migration (Fig. 4-7), this may explain the impaired transmigration of B-CLL lymphocyte.

The main finding is that lymphocytes both from normal subjects and patients with B-CLL which had migrated across the HUVEC monolayer, showed low expression of cell surface L-selectin. This downregulation of L-selectin was observed with both the B-cell and T-cell components of both the normal and leukaemic population, and averaged $71 \pm 5\%$ loss compared with the initial expression of this adhesion molecule. A similar loss of CD23 expression on the B-CLL cells was also observed after their transendothelial migration in contrast to the lack of change in expression of other adhesion molecules VLA-4, ICAM-1, LFA-1 and CD44. Changes in CD23 expression on normal peripheral blood T-lymphocytes could not be tested as we found no expression of this molecule. However, normal B-cells expressed CD23 at a low level and the level of expression was also downregulated after transendothelial migration (Table 2).

It has been proposed that a sub population of lymphocytes in the starting population with little expression of L-selectin is more efficient at transendothelial migration compared with lymphocytes with high L-selectin levels.\textsuperscript{166} This possibility is unlikely to account for the downregulation of CD23 expression on B-CLL cells after migration and our findings in Table 4-1 and Table 4-3 which suggest the opposite.

Other factors affecting lymphocyte transendothelial migration were also studied. Pre-treatment of lymphocytes with BAPTA-AM, which chelates the intracellular Ca$^{2+}$, showed a dose-dependent inhibition of lymphocyte transmigration (Fig. 4-
suggesting that mobilization of intracellular Ca\textsuperscript{2+} is required for transmigra

The stimulus for this downregulation of L-selectin and for B-lymphocyte transmigration across cultured HUVECs has not been identified. A possible candidate is a “chemoattractant/chemokine” synthesised by endothelial cells. Although IL-8 stimulates rapid downregulation of L-selectin from neutrophils it has no effect on lymphocyte L-selectin or the transendothelial migration of this cell\textsuperscript{155} (Preece G and Ager A, unpublished). However, it is selectively involved in the enhanced migration of the cutaneous lymphocyte-associated antigen (CLA) positive T cells across TNF-\(\alpha\) activated endothelial cells in a pertussis toxin sensitive manner.\textsuperscript{167} Another chemokine, the secondary lymphoid-tissue chemokine (SLC) (also known as 6Ckine, Exodus-2, and thymus-derived chemotactic agent 4), which is expressed in Peyer's patches and lymph nodes, rapidly activates \(\beta_2\) integrin-mediated lymphocyte adhesion and is inhibited by pertussis toxin.\textsuperscript{168; 169} Moreover, chemokines are shown to trigger integrin leukocyte function-associated antigen-1 (LFA-1) through cytoskeletal rearrangement induced by G-protein-dependent activation and resulting in strong adhesion of T-cells to the endothelial cells and spontaneous transendothelial migration.\textsuperscript{170} Fig. 4-12 favours this “chemoattractant/chemokine” hypothesis. Either lymphocytes or endothelial cells pre-treated with pertussis toxin (PTX), which prevents the occurrence of the receptor-G-protein interaction, showed greatly reduced transmigration. Thus, lymphocyte transendothelial migration may involve regulating G-protein-dependent chemokine activation as well as the mobilization of intracellular Ca\textsuperscript{2+},\textsuperscript{171} or inhibition of lymphocyte chemotaxis.\textsuperscript{172}
Extracellular ATP can stimulate the shedding of L-selectin as well as CD23 from lymphocytes (see chapter 3) and since ATP can be secreted from endothelial cells, the role of purinergic mechanisms in transendothelial migration was studied. Thus lymphocytes were pretreated with OxATP to irreversibly inactivate the P2X\textsubscript{7} receptor prior to the migration assay. The results in Fig. 4-8,9,14 and 15 show that blocking the P2X\textsubscript{7} receptor had no effect either on the downregulation of L-selectin associated with transmigration or on the number of lymphocytes which transmigrated during the assay. Thus ATP derived from endothelial cells is neither a chemoattractant which stimulates transmigration of lymphocytes nor an agonist which mediates the global L-selectin loss during transendothelial migration. Alternatively, another possible explanation is the interaction between lymphocytes and endothelial cells. Various sulfated carbohydrate L-selectin ligands such as GlyCAM-1, CD34, Podocalyxin-like protein (PCLP) and peripheral lymph node addressin (PNAd) are found on endothelial cells. Cross-linking with these ligands may result in L-selectin shedding as well. However, different proteinases are involved in the loss of L-selectin during transendothelial migration since Ro 31-9790, which inhibits ATP induced L-selectin and CD23 shedding (see chapter 3), failed to show inhibition of downregulation of L-selectin during transmigration.

High levels of soluble L-selectin and soluble CD23 are found in the serum of patients with B-CLL and the level of soluble CD23 has been related to the tumour load. Moreover soluble CD23 has been proposed as a marker of disease activity, while in a complementary study high levels of cellular CD23 have been correlated with a favourable prognosis in B-CLL. Our data suggests that soluble CD23 and L-selectin are derived from the circulating pool
of B-CLL lymphocytes, perhaps during their recirculation and emigration across
the vascular endothelial layer. Although the kinetics of recirculation of B-CLL
lymphocytes are slower than for normal lymphocytes, the expanded lymphocyte
numbers in this disease ensures that high values of soluble L-selectin are found
in the serum.
Chapter 5

GENERAL DISCUSSION

High levels of both soluble CD23 and L-selectin are found in the serum of patients with B-CLL, while only tiny amounts of these soluble molecules can be detected in the serum of normal subjects. Lymphocyte counts in B-CLL are 2-100 fold higher than normal. These data suggest that both soluble CD23 and L-selectin in B-CLL are derived from the circulating pool of lymphocytes and shedding of these two molecules from malignant and normal lymphocytes is a continuous physiological process. Is there a common mechanism in vivo involving the shedding of these two molecules? It has been well documented that phorbol esters cause shedding of L-selectin from lymphocytes, but this stimulus is non-physiological and does not lead to shedding of CD23. In this study, two physiological pathways leading to shedding of both L-selectin and CD23 have been described. One pathway is activated by extracellular ATP, via occupancy of P2X7 receptors which are highly expressed in both lymphocytes and monocytes. A second pathway operates during lymphocyte transendothelial migration, which is a regular and frequent event during the recirculation of lymphocytes from blood into lymph nodes or other lymphoid tissues of the body. Thus 70% of L-selectin and 90% of CD23 is lost from lymphocytes which have migrated beneath a monolayer. These two pathways seem independent from each other since the P2X7 receptor is not involved in lymphocyte transendothelial migration, the downregulation of lymphocyte L-selectin and CD23 observed after transmigration. Thus, this study indicates that extracellular ATP is not involved in shedding of L-selectin and CD23 in transendothelial migration and favours the hypothesis that various endothelial
ligands for L-selectin and CD23 (perhaps clustering these molecules) or various chemoattractant/chemokine synthesised by endothelial cells somehow transduce signals to the lymphocyte to cause shedding of L-selectin and CD23 from lymphocyte surface.
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