TWO PATHWAYS OF SHEDDING OF L-SELECTIN
AND CD23 FROM HUMAN B-LYMPHOCYTES

A thesis submitted to fulfil the requirements for the degree of

Master of Science in Medicine

by

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Lymphocytes from patients with B-chronic lymphocytic leukemia (B-CLL) express large numbers of P2X7 receptors for extracellular adenosine triphosphate (ATP). Activation of P2X7 receptors induces multiple downstream effects, of which the best documented is the opening of an ionic channel that is selective for divalent cations. Another effect of ATP is to induce the shedding of L-selectin (CD62L), a molecule which is involved in the adhesive interactions of lymphocytes on endothelial cells. High levels of soluble L-selectin and CD23 are found in the serum of patients with B-CLL, although the mechanisms involved in their production are poorly characterized. Because extracellular ATP causes shedding of L-selectin, we studied the effect of ATP on shedding of CD23, an adhesion molecule expressed on the surface of B-CLL lymphocytes. ATP induced the shedding of CD23 at an initial rate of 12% of that for L-selectin, while the EC50 of ATP (35 µM) and BzATP (10 µM) was identical for shedding of both molecules. Inactivation of the P2X7 receptor by pre-incubation with OxATP, an irreversible inhibitor of P2X7 purinoceptor, abolished ATP-induced shedding of both molecules. Moreover, KN-62, the most potent inhibitor for the P2X7 receptor inhibited ATP-induced shedding of both CD23 and L-selectin with the same IC50 (12 nM). Ro 31-9790, a membrane permeant zinc chelator which inhibits the phorbol-ester stimulated shedding of L-selectin also inhibited shedding of CD23 from B-CLL lymphocytes, but the IC50 was different for the two shed molecules (25 versus 1 µg/ml respectively). Although L-selectin was completely shed by incubation of cells with phorbol-ester no
CD23 was lost under these conditions. Also, Ca\(^{2+}\) inhibits ATP-induced CD23 shedding but not L-selectin shedding.

Since soluble CD23 and L-selectin are found in the serum of normal subjects and B-CLL patients, the expression of these two adhesion molecules on lymphocytes before and after transendothelial migration was studied in an *in vitro* model of this process. In normal and B-CLL subjects, 71±5\% of L-selectin from both T and B cells and 90\% of CD23 from B cells was lost following transmigration, while the expression of a range of other adhesion molecules such as VLA-4, ICAM-1, LFA-1 and CD44 was unchanged. Lymphocytes incubated with OxATP retained their capacity for transendothelial migration and showed the same loss of L-selectin as control leukaemic lymphocytes. Ro 31-9790, which can protect ATP-induced both L-selectin and CD23 shedding, had no effect on inhibiting L-selectin and CD23 lost during transmigration. These data show the presence of a second pathway for the downregulation of L-selectin and CD23 from the lymphocyte surface.

Data *in vivo* from 'knock-out' mice show that L-selectin is essential for the emigration of lymphocytes through high endothelial venules into lymph nodes. The migration of normal and B-CLL lymphocytes across confluent human umbilical vein endothelial monolayers was studied in an *in vitro* model of this process. Lymphocytes treated with ATP or BzATP showed 56±25\% or 67±16\% loss of L-selectin on the surface and 36±24\% or 64±19\% decrease of transmigration, respectively, while OxATP, which does not alter the L-selectin level, had no effect on lymphocyte transmigration. Further experiments examined this correlation between L-selectin expression and lymphocyte
transendothelial migration in this model system. A quantitative assay for cell surface L-selectin showed that expression of L-selectin was lower on B-CLL lymphocytes (8,880±5,700 molecules/cell) than on normal lymphocytes (29,500±7,500 molecules/cell, p<0.001). Also the rate of transmigration of B-CLL lymphocytes (1.5±0.9 migrated cells/HUVEC) was lower than normal peripheral lymphocytes (2.4±0.9 migrated cells/HUVEC, p=0.04). Incubation of lymphocytes in complete medium for 24 hrs increased the expression of L-selectin on B-CLL lymphocytes by 1.5 to 2 fold while the normal lymphocyte L-selectin remained at the initial level. This upregulation of B-CLL L-selectin correlated with a 2 fold increased rate of transendothelial migration. A correlation was found between L-selectin expression on lymphocytes and their ability for transendothelial migration (r²=0.6).

This study shows that the adhesion molecules L-selectin and CD23 can be lost from lymphocytes by two different physiological pathways. One is by P2X7 receptor activation by extracellular ATP while the second is activated by transendothelial migration of these cells. A second finding is that B-CLL lymphocytes have lower level of L-selectin expression and an impaired ability for transendothelial migration compared with normal peripheral blood lymphocytes. Do these results explain the high serum levels of soluble L-selectin and CD23 observed in B-CLL? Although B-CLL lymphocytes do not recirculate as rapidly as normal peripheral blood lymphocytes, the greatly increased number of leukaemic cells in B-CLL ensures that much more soluble L-selectin and CD23 is generated during the recirculation of these cells through the body.
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DECLARATION

The work described in this thesis was carried out on a full-time basis by the candidate under the supervision of Prof. James S. Wiley and Dr. Linda J. Bendall in the University of Sydney, Department of Medicine, Nepean Hospital, New South Wales. The studies described in this thesis have not previously been submitted for a degree at this or any other university. The experiments undertaken are my own original work except where due acknowledgement has been made.

Baijun Gu
ABBREVIATIONS

The abbreviations listed below are frequently used in the thesis.

µM $10^{-6}$ M
ATP adenosine 5'-triphosphate
BAPTA-AM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)
BSA bovine serum albumin
BzATP 3'-O-(4-benzoyl)benzoyl-adenosine 5'-triphosphate
CLL chronic lymphocytic leukemia
dATP 3'-deoxy adenosine 5'-triphosphate
EC50 concentration of a drug that produce 50% of the maximum response
ECGS endothelial-cell growth supplement
ECL enhanced chemiluminescence
EDTA ethylenediaminetetraacetic acid
EGTA ethylene glycol-bis (β-aminoethyl ether) N,N,N',N'-tetraacetic acid
ELISA enzyme-linked immunosorbent assay
Fig figure
FITC fluorescein isothiocyanate
Fura-2 AM Fura-2 acetoxymethyl ester
HBSS Hanks balanced salt solution
HEPES N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HEV high endothelial cells present in postcapillary venules
HMA 5-(N,N-hexamethylene)-amiloride
HRP horseradish peroxidase
HUVEC human umbilical vein endothelial cell
IFN-γ Interferon-γ
KN-62  1-[N,O-bis(5-isoquinoline sulfonyl)N-methyl-L-tyrosyl]-4-phenylpiperazine
2-MeSATP  2-methylthio- adenosine 5'-triphosphate
α,β-meATP  α,β-methylene adenosine 5'-triphosphate
MESF  molecules of equivalent soluble fluorescein
mM  10^{-3} M
MoAb  monoclonal antibody
nM  10^{-9} M
OxATP  adenosine 5'-triphosphate-2',3'-dialdehyde
PAGE  polyacrylamide gel electrophoresis
PBS  phosphate buffered saline
PLD  phospholipase D
PMA  phorbol 12-myristate 13-acetate
PTX  pertussis toxin
Ro 31-9790  N-2-((2s)-[(hydroxycarbamoyl)4-methylvaleryl]-N-1,3-dimethyl-L-valinamide
R.T.  room temperature
SDS  sodium dodecyl sulfate
TMA  trimethylammonium chloride
TNF-α  Tumour Necrosis Factor-α
**PUBLICATIONS**

( arising from work in this thesis )

