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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March 2000

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ABSTRACT

Lymphocytes from patients with B-chronic lymphocytic leukemia (B-CLL) express large numbers of $P2X_7$ receptors for extracellular adenosine triphosphate (ATP). Activation of $P2X_7$ 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 EC_{50} of ATP (35 μM) and BzATP (10 μM) was identical for shedding of both molecules. Inactivation of the $P2X_7$ receptor by pre-incubation with OxATP, an irreversible inhibitor of $P2X_7$ purinoceptor, abolished ATP-induced shedding of both molecules. Moreover, KN-62, the most potent inhibitor for the $P2X_7$ receptor inhibited ATP-induced shedding of both CD23 and L-selectin with the same IC_{50} (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 IC_{50} 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 ($r^2=0.6$).

This study shows that the adhesion molecules L-selectin and CD23 can be lost from lymphocytes by two different physiological pathways. One is by $P2X_7$ 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.

ACKNOWLEDGMENTS

This work was supported by grants from the National Health and Medical Research Council (NHMRC) of Australia and the New South Wales Cancer Council, Australia.

I wish to sincerely thank my supervisor Prof. James S. Wiley, Professor of Haematology, Department of Medicine, Nepean Hospital, University of Sydney, for his support and guidance both before and during my master candidature. His never-failing sense of enthusiasm has been a continuing source of inspiration.

I wish to thank my co-supervisor Dr. Linda J. Bendall, Research Officer, Leukemia Research Unit, Institute of Clinical Pathology and Medical Research, University of Sydney, for her intellectual and technical support.

I also thank my former colleague Dr. Joan R. Chen, Department of Haematology, Austin Hospital, Victoria, for her intellectual and technical contributions, and many useful suggestions on transmigration study.

I would like to thank my colleague Mrs. Phuong Dao-Ung for her help on leukemia immunophenotyping and transmigration study.

Much gratitude also goes to the many other members of Haematology Research Group, Austin Hospital, Nepean Hospital, and Westmead Hospital, who provided their technical expertise and knowledge, in particular: Dr. Gary Jamieson, Mrs. Mary Snook and Ms. Monique Spencer for the correlation of L-selectin MESF with molecules per cell; Dr. Christopher J. Bradley for the electron-microscope photos of transmigration; Dr. Stephen P. Mulligan for organising the source of subjects for study; Mrs. Mary Sartor for technical support on flow cytometry and kind contribution of monoclonal antibodies.

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.

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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 (ß-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)

Gu, B., Bendall, L.J. & Wiley, J.S. ATP-induced shedding of CD23 and L-selectin (CD62L) from lymphocytes is mediated by the same receptor but different metalloproteases. *Blood,* 92, 946-951. 1998

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