REGULATION OF T CELL SIGNALLING BY GALECTIN-1 AND LYSO-PHOSPHATIDYLCHOLINE

Ph.D. thesis

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Articles related to the thesis

I. *Legradi A*, Chitu V, Szukacsov V, Fajka-Boja R, Szekely Szucs K, Monostori E.: ***Lysophosphatidylcholine is a regulator of tyrosine kinase activity and intracellular Ca(2+) level in Jurkat T cell line***, Immunol Lett. 2004 Jan 30; 91(1):17-21, 2004, IF:2,136
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II. Fajka-Boja R, Szemes M, Ion G, *Legradi A*, Caron M, Monostori E.: ***Receptor tyrosine phosphatase, CD45 binds galectin-1 but does not mediate its apoptotic signal in T cell lines***, Immunol Lett. 2002 Jun 3; 82(1-2):149-54, 2002, IF:1,847
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III. Kiss J, Kunstar A, Fajka-Boja R, Dudics V, Tovari J, *Legradi A*, Monostori E, Uher F: ***A novel anti-inflammatory function of human galectin-1: inhibition of hematopoietic progenitor cell mobilization***, Exp Hematol. 2007 Feb; 35(2):305-13, 2007, IF:3,408

Citable abstracts and posters

I. J. Kiss, A. Kunstár, R. Fajka-Boja, V. Dudics, J. Tóvári, *Á. Légrádi*, Ė. Monostori and F. Uher: ***Galectin-1 inhibits hematopoietic stem and progenitor cell mobilization***, Blood Reviews 2007 (21) S88 IF: 5,756

II. E. Monostori, G. Ion, R. Fajka-Boja, *A. Legradi* : ***Human galectin-1 induces T cell apoptosis via ceramide mediated mitochondrial pathway*****,** Tissue Antigens 64 (4): 423-424 Oct. 2004, IF: 1,990

III. *Légrádi Ádám***,** Demydenko Dmytro, Ion Gabriela, Frankó András, Monostori Éva: ***A galektin-1 fehérje – immunmoduláló humán lektin –1 struktúra-funkció vizsgálata,*** Magyar Immunológiai Társaság 33. Vándorgyűlése, Győr, 2003. október 15-17.

IV. Ion Gabriela, Fajka-Boja Roberta, *Légrádi Ádám*, Monostori Éva: ***A galektin-1 mitochondriális úton indukál apoptózist a Jurkat-T-sejtekben,*** A Magyar Immunológiai Társaság 33. Vándorgyűlése, Győr, 2003. október 15-17.

V. Gabriela Ion, *Ádám Légrádi*, Roberta Fajka-Boja, Michel Caron, Dmytro Demydenko, Éva Monostori: ***Biological effect of galectin-1 on different cell lines of bone marrow origin,*** A Magyar Immunológiai Társaság XXXII. Kongresszusa, Kaposvár, 2002 szeptember 30.-október 2.

VI. Monostori Éva, Fajka-Boja Roberta, Gabriela Ion, *Légrádi Ádám*, Michel Cáron, Dmytro Demydenko: ***Galektin-1, egy gyulladáscsökkentő endogén lektin apoptótikus hatásának molekuláris mechanizmusa,*** Kaposvár, Magyar Immunológiai Társaság 32. Kongresszusa 2002. szeptember. 30-október 2.

VII. R. Fajka-Boja, M. Szemes, *Á. Légrádi*, G. Ion, M. Caron, É. Monostori: ***Galectin-1 binds to and internalizes in T leukemia cells,*** 11th Symposium on Signals and Signal Processing in the Immune System, Pécs, 2-6 September, 2001.

TABLE OF CONTENTS

1.INTRODUCTION 2

1.1. SIGNAL TRANSDUCTION EVENTS IN T CELLS 2
1.2. THE STRUCTURE AND FUNCTION OF LYSOPHOSPHATIDYLCHOLINE (LYSO-PC) 3
1.2.1. Origin of lyso-PC 3
1.2.2. Biological functions of lyso-PC 5
1.3. GALECTIN-1 8
1.3.1. Correlation between structure and function of Gal-1 8
1.3.2. Biological role of Gal-1 11

2. AIMS OF THE STUDY 16

3. MATERIALS AND METHODS 17

4. RESULTS 21

4.1. ROLES OF $P56^{LCK}$ AND ZAP70 TYROSINE KINASES IN LYSO-PC-INDUCED SIGNALLING EVENTS 21
4.2. $P56^{LCK}$ AND ZAP70 KINASES, BUT NOT CD45 PHOSPHATASE, HAVE A CRUCIAL ROLE IN GAL-1-INDUCED APOPTOSIS IN JURKAT CELLS 24
4.3. STRUCTURE-FUNCTION STUDY OF GAL-1 26
4.3.1. Purification of human recombinant Gal-1 26
4.3.2. Effect of substitution of cysteine with serine on carbohydrate binding 27
4.3.3. Effect of cysteine to serine substitution on apoptosis-induction capacity of Gal-1 29
4.3.4. Effects of Gal-1-derived synthetic oligopeptides on Gal-1-induced apoptosis 30

5. DISCUSSION 32

5.1 NEW ASPECTS OF $P56^{LCK}$ AND ZAP70 ON T CELL SIGNALLING 32
5.1.1. Lyso-PC, a regulator of T cells 32
5.1.2 Gal-1, a regulator of T cell apoptosis 33
5.2. STRUCTURE-FUNCTION CORRELATION OF GAL-1 34

6. ACKNOWLEDGEMENTS 37

8. NOVEL RESULTS PUBLISHED IN THIS THESIS 38

7. ABBREVIATIONS 39

9. REFERENCES 40

1. INTRODUCTION

1.1. Signal transduction events in T cells

T lymphocytes play a central role in the immune response, as effector cells or regulatory cells modulating the functions of numerous other cell types, primarily those participating in the body's immune defence mechanisms. Thus, the proper functioning of T cells is essential for the normal operation of the immune system (1).

The T cell receptor (TCR) consists of two highly polymorphic heterodimer αβ or γδ subunits, responsible for specificity, three invariant CD3 (λ, δ and ε) and two additional polypeptides, ζ and/or η. The invariant chains form homo- or heterodimers within the TCR complex and transmit antigen-stimulated signals (2).

Protein tyrosine kinases (PTKs) and protein tyrosine phosphatases (PTPases) have central roles in the T cell signal transduction. The key initiating event in T cell signal transduction is the increased phosphorylation of immunoreceptor-tyrosine-based activation motif (ITAM) tyrosines in TCR subunits by the Src family kinase $p56^{lck}$(leukocyte-specific protein tyrosine kinase) (3). The phosphorylated ITAMs serve as docking sites for the tandem SH2 (Src homology 2 domain) domains of the ZAP70 (zeta-chain associated protein kinase 70) kinase (4). Double-phosphorylated ITAMs bind ZAP70 with high affinity, and hence other SH2 domain-containing signalling molecules are excluded (5). Once recruited, ZAP70 is activated via $p56^{lck}$ -mediated phosphorylation at Tyr-493 in the activation loop of ZAP70 (6). The activated ZAP70 molecules then autophosphorylate in the trans position to create docking sites for SH2 domain-containing signalling proteins (7). One of the well-known substrates of ZAP70 is LAT (linker for activation of T cells) (8). LAT then ligates phospholipase Cγ1 (PLCγ1), the Grb/Sos/Ras complex, and the Gads/SLP-76/VAV complex and activates the respective downstream signalling pathway of IP3, Ras/Raf/Erk and RhoA, leading to gene regulation, proliferation and actin-reorganization responses (Fig. 1) (9).

CD45 transmembrane protein tyrosine phosphatase (10) maintains $p56^{lck}$ tyrosine kinase in dephosphorylated form at the position Tyr-505, which is needed for the "answer ready" form if the activation signal comes (11).

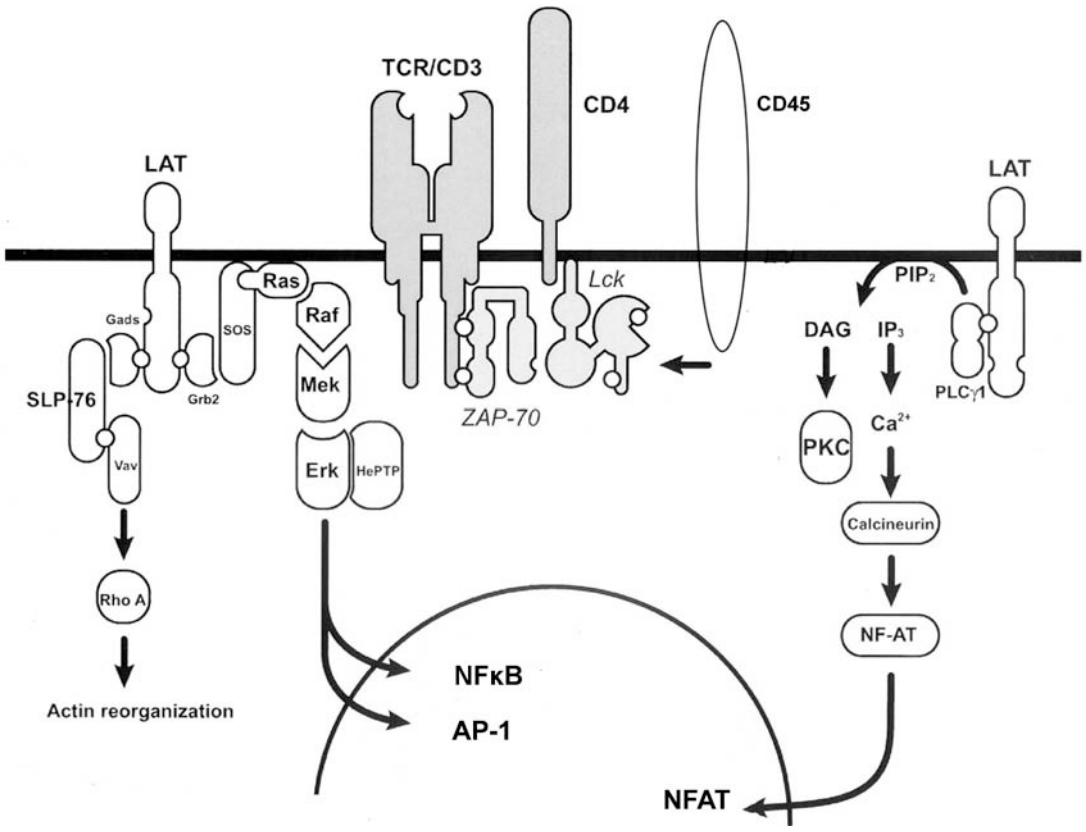

Fig. 1. **Simplified outline of the intracellular signalling pathways initiated by the T cell receptor complex and its co-receptor.** The TCR complex and co-receptor (in this example the CD4 molecule) are associated with Src family protein kinase $p56^{lck}$. It is thought that the binding of a peptide:MHC (major histocompatibiliy complex) ligand to the TCR and co-receptor brings together CD4, the TCR complex and CD45. This allows the CD45 tyrosine phosphatase to remove inhibitory phosphate groups and thereby allow the activation of $p56^{lck}$. Phosphorylation of the ζ chains enables them to bind the cytosolic tyrosine kinase ZAP70. The subsequent activation of bound ZAP70 by phosphorylation leads to three important signalling pathways. ZAP70 phosphorylates the adaptor proteins LAT and SLP-76, which in turn leads to the activation of PLC-γ by kinases and the activation of Ras by guanine-nucleotide exchange factors. As illustrated, activated PLC-γ and Ras initiate three important signalling pathways that culminate in the activation of transcription factors in the nucleus. Together, NFκB, NFAT (nuclear factor of activated T cells) and AP-1 (activator protein-1) act on the T-cell chromosomes, initiating new gene transcription that results in the differentiation, proliferation and effector actions of T cells. The picture is taken from Ref. 1 with modification.

1.2. The structure and function of lysophosphatidylcholine (lyso-PC)

1.2.1. Origin of lyso-PC

The main origins of oxidized lipids are lipoproteins, components of the lipid transport system. Lipoproteins consist of a spherical or oblate ellipsoidal outer envelope of

phospholipids and cholesterol, encasing apolipoproteins, cholesterol esters and triglyceride in the interior. They can be classified by density, configuration and electrophoretic mobility.

Chylomicrons contain only traces of protein and cholesterol; very low-density lipoprotein (VLDL) contains slightly more protein and some cholesterol. Human low-density lipoprotein (LDL) and high-density lipoprotein (HDL) contain considerable protein and a great deal of cholesterol, but little triglyceride, and mainly protein and a small amount of cholesterol, respectively (12). LDL (Fig. 2) is the main source of blood cholesterol in the human.

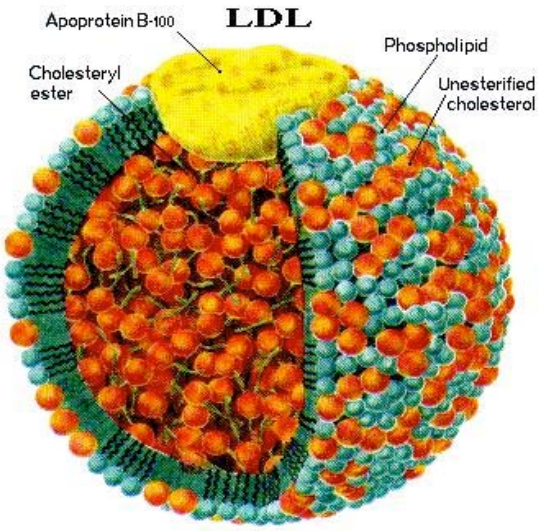

Fig 2. **LDL contains four main components**: phospholipids (i.e. phosphatdylcholine), cholesterol esters, apoprotein B-100 and unesterified cholesterol. The picture is taken from www.puntofape.com.

Lyso-PC normally accounts for 1-5% of the total phosphatidylcholine (PC) content of LDL. The oxidative modification of LDL (oxLDL) is associated with a dramatically increased formation of lyso-PC, which can reach 40-50% of the total PC content (13). In the presence of reactive oxygen species, the ester bond at position 2 of glycerol is hydrolysed, resulting in a free fatty acid and lyso-PC (14) (Fig. 3).

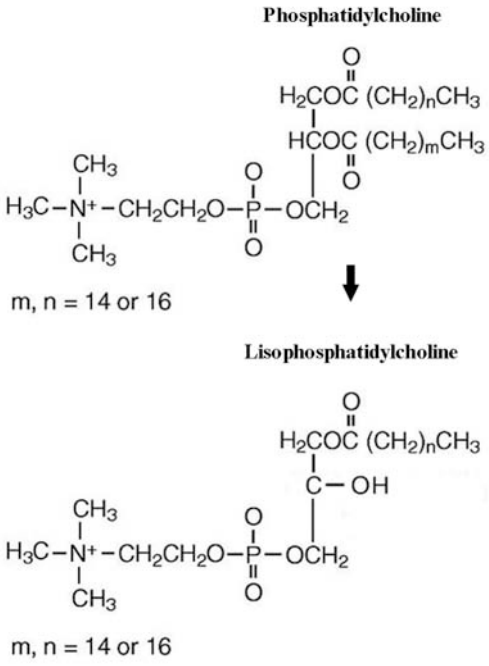

Fig. 3. **Origin of lyso-PC**. PC carries two long fatty acyl chains esterified to positions 1 and 2 of glycerol, whereas the position 3 contains a hydrophilic phosphate diester anion, choline. Lyso-PC is formed from PC in an oxidative reaction with the contribution of phospholipase A_2 or a reactive oxygen species.

There are three different pathways for lyso-PC production during LDL oxidation:

- lyso-PC production occurs after hydrolysis of phospholipids by phospholipase A_2 (PLA_2) (15);

-lecitin-cholesterol acyl-transferase (LCAT) can hydrolyse and transfer the fatty acid of PC to cholesterol and produce lyso-PC (16);

-lyso-PC can also be formed when LDL is oxidatively altered *in vitro* by a number of different mechanisms: prolonged storage (17) LDL exposure to different agents such as dissolved oxygen (18), free radical initiators (19), transition metal ions (20), specific iron or copper-containing proteins (21), UV irradiation (22), lipooxygenase (23) and myeloperoxidase (24).

Lyso-PC exists in several physiological forms, including free, micellar, LDL, bound to hydrophobic serum proteins such as albumin, consumed within immune complexes, and incorporated into plasma membranes. Indeed, physiological concentrations of lyso-PC in body fluids are high (up to 100 μM) and it therefore probably exists predominantly in an inactive form. It is likely, therefore, that the action of lyso-PC is temporally and spatially constrained, possibly occurring in an autocrine or paracrine fashion. For example, the activation of receptors by lyso-PC produced from plasma membranes of neighbouring cells may reflect spatially restricted paracrine actions (25).

1.2.2. Biological functions of lyso-PC

The functions of lyso-PC are examined intensively in the context of atherosclerosis. Atherosclerosis, an underlying cause of myocardial infarction, stroke and other cardiovascular diseases, consists of focal lesions of the arterial intima which are characterized by cholesterol deposition, fibrosis and inflammation. These lesions begin as local infiltrates of monocyte-derived macrophages, T lymphocytes, and lipoproteins. The experimental data suggest, that the local endothelial expression of the vascular endothelial adhesion molecule-1 (VCAM-1) and chemotactic stimulation by oxLDL may be important for the formation of this early lesion. During the subsequent progression of the lesion, macrophages are transformed into lipid-laden foam cells, presumably by the uptake of oxLDL, and smooth muscle cells migrate into the lesion to form a fibrous cap around the lipid rich core. By means of their cytokine secretion, activated macrophages and T lymphocytes may regulate foam cell transformation, smooth muscle proliferation and the generation of free oxygen radicals (26).

Lyso-PC acts at least 5 points of the atherosclerotic process:

(a) Lyso-PC modifies the functions of endothelial cells (ECs): Initial observations on the effect of oxLDL with a higher lyso-PC content revealed a greater impairment of the endothelium-dependent relaxation than that of LDL with a lower lyso-PC content, in rat aortic rings (27). Others confirmed these results in a rat mesenteric artery model (28). It is known, that oxLDL, but not native LDL causes a progressive time-dependent decrease in steady-state endothelial NO synthase (eNOS) mRNA levels. The results were reproducible in human saphenous vein and aortic EC models (29). The dysfunctioning ECs express adhesion molecules (i.e. VCAM-1) that favour the migration and adherence of leukocytes into the arterial intima, a sequence of events comprising a key step in early atherogenesis (30). Lyso-PC is the lipid primarily responsible for the oxLDL-mediated inhibition of EC movement (31). The mechanisms by which Lyso-PC inhibits EC motility are not yet known.

(b) Lyso-PC induces migration and proliferation in vascular smooth muscle cells (SMCs): Lyso-PC induces vascular SMC proliferation in growth-arrested rabbit vascular SMCs. The effects of lyso-PC were significantly inhibited by the phospholipase C inhibitor U73122, the intracellular antioxidant NAC, and the NADPH (nicotinamide adenine dinucleotide phosphate) oxidase inhibitor diphenylene iodonium (32, 33). Redox-sensitive protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) pathways have been implicated as potential underlying mechanisms (34). Lyso-PC activates PLCγ and through this activates PKC, this signal transduction pathway leading to an increased intracellular Ca^{2+} level in renal arterial SMCs. A really intriguing feature of lyso-PC is that, in addition to its mitogenic effect, it also promotes apoptosis and exhibits cytotoxic properties towards SMCs, at least partly, through its detergent action, causing membrane leakiness and a resultant intracellular Ca^{2+} level overload (35). Lyso-PC not only influences the proliferation, but additionally induces the migration of rat vascular SMCs (36).

(c) Lyso-PC initiates the chemotaxis of macrophages: Lyso-PC is a potent chemoattractant for human monocytes and T lymphocytes. (37,38). Monocytes trapped in the intima, initiate a strong inflammatory response and release active oxygen intermediates in the vessel wall, which might amplify lipid peroxidation, thereby resulting in further intimal damage and the influx of inflammatory cells (39). Lyso-PC induces the expression of monocyte-chemoattractant protein-1 (MCP-1), which is a proatherogenic factor responsible

for approximately 60% of plaque macrophages in mouse models of atherosclerosis (40). Cytokines produced by macrophages in atherosclerotic lesions are important in the initiation and amplification of inflammation. IL-1β (interleukin-1β) has been demonstrated in human atherosclerotic lesions (41) and its production is stimulated by lyso-PC (42). IL-1β promotes its own production in macrophages and promotes further lyso-PC production by activating PLA_2 (43). Following their recruitment and migration into the subendotheleial space, monocytes differentiate into macrophages and take up oxLDL via scavenger receptors, leading to the formation of cholesterol ester-loaded foam cells. One of the important features pertinent to foam cell formation is the regulation of macrophage lipoprotein lipase (LPL) secretion and mRNA expression by atherogenic lipoproteins. LPL is present in arterial lesions and constitutes a bridging ligand between lipoproteins, proteoglycans and cell receptors, thus favouring macrophage lipoprotein uptake and lipid accumulation. Lyso-PC is responsible for downregulating LPL activity and mRNA abundance in human monocyte-derived macrophages, indicating that they may modulate LPL-mediated pathways of lipoprotein uptake during the conversion of macrophages to foam cells (44).

(d) Lyso-PC modifies the platelet activation and coagulation pathway: Lyso-PC promotes the expression of the tissue factor (TF), the principal initiator of coagulation (45), and increases the production of the plasminogen activator inhibitor-1 (PAI-1), an important regulator of fibrinolysis, by SMCs in atherosclerotic arteries (46). Lyso-PC has been shown to upregulate the mRNA expression of the urokinase type plasminogen activator (uPA) and its cell surface receptor (uPAR), which have both been shown to be present in macrophages in atherosclerotic plaques (47).

(e) Lyso-PC modifies the functions of T lymphocytes: Lyso-PC has a central role in the immune response caused by oxLDL. T lymphocytes isolated from human atherosclerotic plaques recognize oxLDL, and respond with proliferation or interferon-γ (IFN-γ) secretion (48). $CD4^+$ T lymphocytes participate in the immunologic processes of atherosclerosis. $CD4^+$ T lymphocyte depletion by an anti-CD4 monoclonal antibody reduces post-angioplastic atherosclerosis in rats by inhibiting the accumulation of mononuclear and SMCs in the intima of the arterial wall (49), and also reduces the incidence of spontaneous atherosclerosis in hyperlipidaemic mice (50).

Lyso-PC increases IFN-γ production and CD40L expression in $CD4^+$ T cells stimulated with anti-CD3 antibody and recombinant CD80 molecules, whereas it has no effect on IL-2 and IL-4 production. These results suggest that lyso-PC in combination with other stimuli may regulate $CD4^+$ T cell functions to propagate local inflammatory reactions, and also imply a novel role played by a modified lipid in the selection of Th1/Th2 immune response and in the T cell-mediated pathogenesis in atherosclerosis (51).

Recent data have suggested that the effect of lyso-PC is mediated via G2A, the G protein-coupled immunoregulatory receptor (52). Lyso-PC interacting with its receptor G2A induces an elevation of the intracellular Ca^{2+} concentration and also the transcriptional activation of the serum response via the MAPK pathway (53).

In spite of the accumulating data, the exact mechanism by which lyso-PC exerts the signalling function in T lymphocytes has not been well established yet.

1.3. Galectin-1

Galectin-1 (Gal-1) a prototype homodimer lectin, is able to bind β- galactosides, like the other type of proto-type galectins, it contains two slightly conservative carbohydrate binding domain (CRD) (54).

1.3.1. Correlation between structure and function of Gal-1

The Gal-1 protein, is a beta-sandwich of about 134 amino acids (Fig. 4). The two sheets are slightly bent with 6 strands (S1-S6) forming the concave sides and 5 strands (F1-F5) forming the convex sides. The concave side forms a groove in

(A)

ACGLVASNLNLKPGECLRVRGEVAPDAKSFVLNLGKDSNN
LCLHFNPRFNAHGDANTIVCNSKDGGAWGTEQREAVFPFQ
PGSVAEVCITFDQANLTVKLPDGYEFKFPNRLNLEAINYMA
ADGDFKIKCVAFD-COOH

(B)

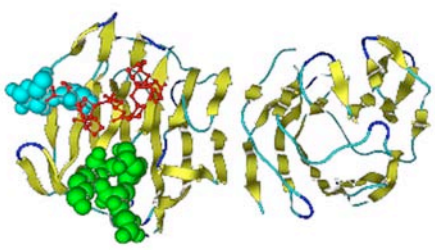

Fig. 4. **The structure of Gal-1. (A)** The amino acids labelled with green caps (DAKSF) are totally conserved in the mammalian Gal-1-s. The amino acids labelled with blue caps are responsible for carbohydrate binding. **(B)** The worms model of Gal-1 where the DAKSF sequence is labelled with green, the amino acid responsible for carbohydrate binding is labelled with blue, and a bound beta -galactoside is shown in red.

which carbohydrate is bound, and which is long enough to hold a linear tetrasaccharide (55,56).

The roles of selected amino acid residues of human Gal-1 have been studied by site-directed mutagenesis (Table 1). All the mutant lectins in which one of the cysteine residues has been substituted by serine (C2S, C16S, C42S, C60S, C88S, and C130S) prove to have a sugar-binding ability comparable with that of wild-type lectin. The C2S mutant retains asialofetuin-binding capacity for over a week in the absence of β-mercapthoethanol, whereas the wild-type lectin loses it within a day. Substitution of the highly conservative tryptophan by tyrosine in position 68 (W68Y) slightly reduces the lactose-binding ability, but the mutant is still strongly adsorbed to asialofetuin agarose. Other mutant lectins in which conservative hydrophilic amino acids are substituted (N46D, E71Q and R73H) fail to bind to asialofetuin agarose, with no sign of retardation (57).

Table 1. **Functionally characterized amino acid residues in Gal-1 sequence**

Amino acid residues	Functions related to the amino acid residues
Asn^{46}, Trp^{68}, Arg^{48}, Glu^{71}, Arg^{73}	Responsible for carbohydrate binding
Asp^{26},Ala^{27},Lys^{28}	Responsible for cell growth-inhibitory activity
Cys^{2}, Leu^{4}, Val^{5}, Ala^{6}, Ile^{128}, Phe^{133},	Responsible for dimerization

Oxidation of the Gal-1 molecule causes the formation of unfavourable intra- and intersubunit disulfide bonds, resulting in destruction of the native conformation of the lectin.

Mass spectrometric analysis of peptide fragments from Gal-1-transfected COS-1 cells that secrete Gal-1 shows that the secreted protein exists in an oxidized form containing three intramolecular disulfide bonds (Cys-2-Cys-130, Cys-16-Cys88 and Cys42-Cys60). Oxidized recombinant Gal-1 enhances axonal regeneration from the transected nerve sites of adult rat dorsal root ganglion explants with associated nerve stumps, but it lacks lectin activity. In contrast, mutant Gal-1, where all cysteines have been replaced by serine, induces the haemagglutination of rabbit erythrocytes, but lacks axonal regeneration-promoting activity. These results indicate that the oxidation state of Gal-1 regulates its functions (58,59).

Gal-1 is able to form a homodimer of 14 kDa subunits, each subunit having a single carbohydrate-binding site. The functional lectin exists in a monomer-dimer equilibrium with

$K_d \approx 7$ μM, the attainment of equilibrium being rather slow ($t_{1/2} \approx 10$ h). The hydrophobic amino acids at the N- and C-termini of the monomers are responsible for this association. To explore the mechanism of dimerization and the functional differences between the monomeric and dimeric forms of Gal-1, specific mutations were made in the extreme N-terminus involved in subunit interactions. Two mutants, termed N-Gal-1 (which contains C2S, L4Q, V5D and A6S mutations in one protein) and V5D Gal-1 are functional monomers at low concentrations (≤ 60 μM), but they dimerize at high concentrations. The dimeric forms of native and mutated Gal-1 can be covalently cross-linked to generate non-dissociable forms of Gal-1 that are extremely potent agglutinins. In contrast, the monomeric forms lack agglutining activity, but can compete with the dimeric forms of lectin and block binding of the dimers (60,61). This C2SV5D monomer mutant form is unable to cause phosphatidylserine (PS) exposure (which is one of the most important marks of early apoptotic events) on Molt-4, HL-60 cell lines and activated neutrophils (62).

It has been reported that mouse β-galactoside binding protein (m-GBP) has a strong growth-inhibitory effect. A surface loop, comprising amino acid residues 25-30, and joining two internal β-strands, forms part of the growth-inhibitory site. The amino acids forming this loop are totally conserved in the mammalian Gal-1-s. Point mutation analysis has shown that the growth inhibitory effect is independent of the β-galactoside-binding property, because replacing the amino acids from 26 to 28 one by one (D26N, A27R, and K28T) results in reduced cell growth-inhibitiory activity, but full carbohydrate-binding activity. There are also mutants with full growth-inhibitory activity and reduced lectin activity (R47G and D46N) (63).

From the detailed studies, it is clear there are 3 main parts of the Gal-1 structure, which are sterically separate from each other:

(a) N and C-terminal hydrophobic component of Gal-1 are responsible for the dimerization;

(b) hydrophobic amino acids are responsible for the carbohydrate binding;

(c) highly conservative amino acid residues in positions 25-30 are responsible for cell growth inhibition.

1.3.2. Biological role of Gal-1

The functions of Gal-1 in the body are pleiotropic; it acts both intra-(mRNA splicing (142,143)) and extracellularly (cell growth regulation, apoptosis induction, immunomodulation, cell transformation and cancer):

(1) *Regulation of cell growth by Gal-1*. Gal-1 has been shown either to promote or to inhibit cell growth in different cell types, when added exogenously. For example, it has a mitogenic effect on vascular endothelial cells (64) and 3T3 fibroblasts cells (65). Inhibition of cell growth has been documented for neuroblastoma cells, this phenomenon depending on the carbohydrate-binding property of Gal-1 (66). Gal-1 also inhibits the growth of other cell types, independently of its lectin properties, including mouse embryonic fibroblasts (67), Concavalin-A (Con A)-stimulated rat spleen mononuclear cells (68) phytohaemagglutinin (PHA)-activated T lymphocytes (69) and stimulated T lymphoma cells (70). As these examples show, the cell-growth inhibitory function of Gal-1 can be carbohydrate dependent or independent, depending on the target cell type and the concentration. The carbohydrate-dependent activities are typically demonstrated at a high concentration of Gal-1. In contrast, the carbohydrate-independent activities require a relatively low amount of gal-1 (71, 72).

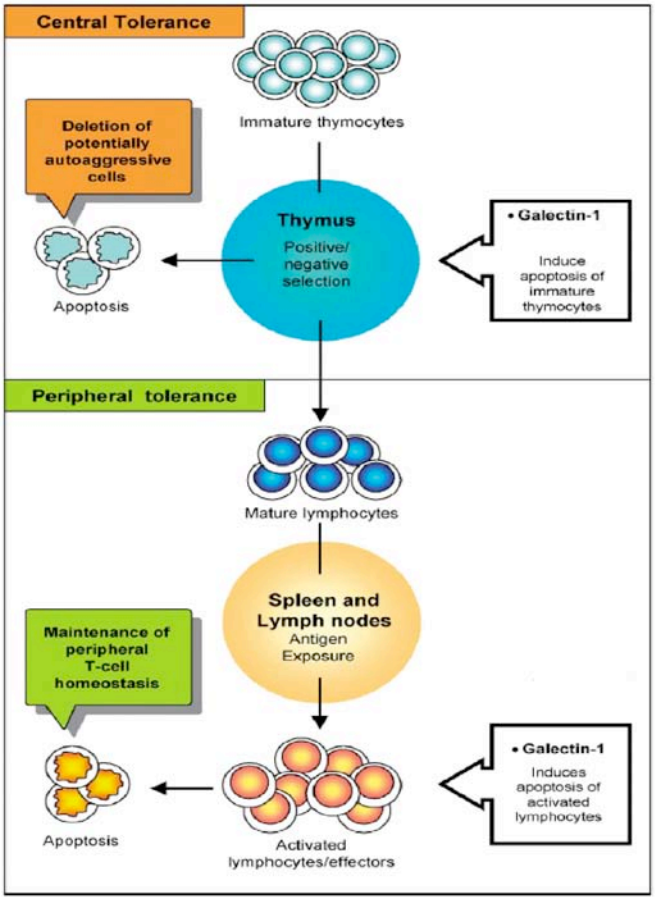

Fig. 5. **The role of Gal-1-induced apoptosis in the immune homeostasis.** Gal-1 has important roles in both the central (killing of the autoaggressive T cell clones) and the peripheral tolerance (killing of the activated T cells after the immune response) through its apoptosis- induction capacity. The picture is taken from Ref. 144 with modification.

Exogenously added Gal-1 has been shown to induce cell cycle arrest during the S to G2 transition of mammary cell lines, and also to arrest T lymphocytes in the S and G2/M

phases of the cell cycle. The mechanism underlying this effect remains to be determined (73). In mouse GBP (galactoside-binding protein)-treated human mammary carcinoma cell lines, the cell cycle arrest is followed by apoptosis, though only after a delay of 2-4 days, indicating a possibility of cross-talk between the apoptosis-induction capacity and the cell growth arrest (74).

(2) Role of Gal-1 in apoptosis induction. Apoptosis (programmed cell death) has a central role in the immunoregulation. This process is involves in the maturation of T lymphocytes (75) and also in the regulation of the immune response through the elimination of activated T cells after the immune response (76). Gal-1 added extracellularly has been shown to induce apoptosis in activated human T cells (77), T leukaemia cell lines (78), and subsets of CD4(lo) CD8(lo) immature thymocytes (79). Gal-1 produced in the thymus induces apoptosis of the immature and autoreactive lymphocytes (80). Gal-1 induces the apoptosis of activated T lymphocytes at the periphery. Through this apoptosis-induction capacity Gal-1 is able to regulate the immune homeostasis and the immune response (Fig. 5) (77).

In mammalian cells, the apoptotic response is mediated through either the intrinsic pathway or the extrinsic pathway, depending on the origin of the death stimuli. The intrinsic pathway is triggered in response to a wide range of death stimuli that are generated from within the cells, such as oncogene activation or DNA damage. This pathway is mediated by mitochondria and the proteins released from the intermembrane space of mitochondria into the cytoplasm (81). Perhaps the most intriguing of these released proteins is cytochrome c, which binds and activates the protein APAF1 (apoptotic peptidase-activating factor) in the cytoplasm (82). The binding of cytochrome c to APAF1 induces a conformational change that allows APAF1 to bind to ATP/dATP and to form the apoptosome, which mediates the activation of caspase-9, the mediator caspase of the intrinsic pathway. The caspases are a conserved family of enzymes that irreversibly commit a cell to die. The initiator caspases (caspase-8 and caspase-9) cleave and activate the effector caspases (caspase-3 and caspase-7), which destroy the substrates needed for the cells to remain alive (83-85). The extrinsic pathway is initiated by the binding of an extracellular death ligand to its cell surface death receptor, such as FasL to Fas (86). The ligand binding leads to the formation of a minimally homotrimeric ligand–receptor complex that recruits further cytosolic factors, such as FADD (Fas-associated death domain) and caspase-8, forming an oligomeric death-inducing

signalling complex (DISC) (87). Formation of the DISC leads to the activation of the initiator caspase, caspase-8, which then cleaves and activates the effector caspase, caspase-3 (88).

During the past several years, scores of data have been published on the mechanism of Gal-1 induced apoptosis; however, these data remain largely controversial. It is still not clear which receptor transmits the apoptotic signal into the T cells. It appears to be distinct from the Fas/FasL pathway, as it has been demonstrated in a Fas-resistant T cell line and Fas-deficient *lpr* mice (89,90). Interestingly, it seems that Gal-1 cooperates with Fas-induced apoptosis in resting and activated peripheral T cells (91). The Gal-1-binding cell surface glycoproteins (CD2, CD7, CD43 and CD45) have been presumed as signal transmitters (92). Gal-1-induced apoptosis is regulated by the expression of specific glycosyltransferases such as core 2 β-1,6-N-acetylglucosaminyltransferase (C2GNT), which creates a core 2 branch on O-glycans, allowing the addition of lactosamine sequences (93,94). O-Glycans transported with C2GNT increase the susceptibility of C2GNT-transfected $CD45^+$ cells to Gal-1 (50). N-Glycans on CD45 also have a very important role in Gal-1-induced apoptosis: sialization of the N-linked glycans on CD45 by SA6-Gal-1 sialyltransferase inhibits Gal-1-induced apoptosis (95).

Caspases have been implicated as death effectors in the Gal-1-induced death pathway, though, in contrast with others, one group has recently reported that the caspase cascade is not involved in the Gal-1-induced apoptotic process (96). Bcl-2 downregulation, activation of the AP-1 transcription factor and the translocation of endonuclease G from the mitochondria to the nucleus have also been indicated to be involved in Gal-1-induced apoptosis (97).

It is known from our previous study that Gal-1 initiates the acid sphingomyelinase-mediated release of ceramide, an event critical for the further apoptotic steps. Elevation of the ceramide level coincides with exposure to phosphatidylserine on the outer cell membrane. The downstream events, decrease of the amount of Bcl-2 protein, depolarization of the mitochondria and the activation of caspase-9 and caspase-3, depend on the production of ceramide. All downstream steps, including the production of ceramide, require the generation of membrane rafts, and as presented in this thesis, the presence of two tyrosine kinases, $p56^{lck}$ and ZAP70 (98,99).

(3) *Imunomodulatory function of Gal-1*. There are some examples where Gal-1 has a direct role in the modification of the immune response. For example, Gal-1 ameliorates phospholipase A2-induced odema in a selective and dose-dependent manner, when pre-

injected or co-injected with the enzyme. This lectin inhibits arachidonic acid release and prostaglandin production from lipopolysaccharide-stimulated macrophages and blocks neutrophil extravasation, mast cell degranulation and nitric oxide synthesis (100). Gal-1 also acts through the modification of cytokine secretion: it inhibits the IL-2 (101) IFN-γ and tumor necrosis factor-α (TNF-α) (79), but induces IL-10 production (102). There are several autoimmune diseases where extracellularly added Gal-1 is able to inhibit the manifestation of the disease in experimental animal models. For example, Gal-1 prevents the clinical and histopathological manifestations of autoimmune encephalomyelitis (EAE), an experimental model of multiple sclerosis in Lewis rats (103). Gal-1 also ameliorates the inflammatory and autoimmune responses in collagen-induced arthritis, an experimental model of rheumatoid arthritis (104). Investigations of the mechanisms involved in the anti-inflammatory effects of Gal-1 revealed that this β-galactoside-binding protein increases T cell susceptibility *in vivo* to AICD (antigen–induced cell death) (105). It has been clearly demonstrated that, under certain inflammatory conditions, activated macrophages (106), antigen-stimulated T cells (90), activated B cells (107) and alloreactive T cells (108) secrete high levels of Gal-1 to kill effector T cells after the completion of an immune response. Gal-1 is also overexpressed in immunoprivileged tissues such as the retina (109), testis (110) and ovary (111). Since immunoprivileged tissues have been identified by their ability to inhibit the spread of inflammation to protect their integrity and their function (112), Gal-1 might contribute to immunoprivileged mechanisms, ensuring the rapid elimination of inflammatory T cells by a novel apoptotic pathway.

The first step during inflammation is mobilization of the haematopoetic progenitor cells (HPCs) in the bone marrow. Gal-1 dramatically inhibits cyclophosphamide and granulocyte-colony stimulating factor-induced HPC migration to the periphery, and decreases peripheral neutrophilia and monocytosis in a dose- and time-dependent manner. In contrast, Gal-1 stimulates HPC expansion and accumulation within the bone marrow. The possible mechanism of HPC migration inhibition is not dependent on the apoptosis-induction capacity of Gal-1, because Gal-1 inhibits HPC mobilization by obstructing the transendothelial migration of bone-marrow-derived cells, as shown in *in vitro* experiments, where galectin-1 inhibited the transendothelial migration of bone marrow-derived HPC migration in response to SDF-1 (stromal cell-derived factor-1) (113).

(4) *Gal-1 and cancer*. Gal-1 influences the life of tumour cells at three points:

a) Gal-1 facilitates the tumour transformation: There is direct evidence, that Gal-1 expression is necessary for the initiation of the transformed phenotype of tumours. Inhibition of the Gal-1 expression suppresses the transformed phenotype of human glioma cells (114). The correct mechanism whereby Gal-1 is involved in cell transformation is not fully understood. One underlying mechanism could be that overexpression of Gal-1 in tumour cells results in an increase in the membrane association of oncogenic Ras, enhancing Ras signalling (115).

(b) Gal-1 facilitates tumour metastasis: It has been extensively documented that the loss of intercellular adhesion is correlated with tumour invasion and metastasis. This loss of adhesion occurs during the initiation of metastasis, allowing tumour cells to leave the initial tumour site. Gal-1 secreted by a tumour cell may favour tumour cell aggregation over adhesion to ECM (extracellular matrix) and promote tumour embolization in the case of ovarian carcinoma cells (116). Gal-1 is able to form bridges between cells and the N-acetyl lactosamine component of the extracellular matrix, like laminin, as reported in the case of melanoma cells (117). During the process of metastasis, Gal-1 suppresses the detachment of the tumour cells from the primary tumour site and also promotes the attachment of the tumour cells to the endothelium at distal sites through these two antagonistic cell adhesion mechanisms (118).

(c) Gal-1 suppresses the immune response against tumour cells: Cancer cells protect themselves against the immune response of the body. The glycoconjugates have a central role in this protection (119). The amount of expressed Gal-1 correlates with the invasiveness of the tumours. For example, glioma xenografts which express more Gal-1 are more invasive and have a higher metastatic potential than those gliomas which express less Gal-1 (120).

As mentioned in this thesis, Gal-1 inhibits T cell activation (121), induces the growth arrest and apoptosis of activated T cells (77) and suppresses the proinflammatory cytokines (79). All of these activities are demonstrated by exogenously added Gal-1. The correlation between Gal-1 and the aggressiveness of different tumour types, in conjunction with its immunoregulatory effects, indicates that tumour cells might impair T cell effector functions by secreting Gal-1, thereby tilting the immunosuppressive environment at the tumour site.

2. AIMS OF THE STUDY

(1) We found that **lyso-PC**, a component of atherogenic oxLDL, induces early tyrosine phosphorylation in T cells. One of our aims, therefore, was to identify the particular kinases responsible for this process and further signalling events.

(2) Human **Gal-1**, an endogenous lectin is cytotoxic for activated and leukaemic T cells.

- Accordingly, we set out to identify early signalling events during Gal-1-induced apoptosis and to determine the effector tyrosine kinases participating in Gal-1-triggered tyrosine phosphorylation.

3. A further aim was to find a correlation between the structure and function of Gal-1, and particularly to establish how mutations at conserved cysteines and the inhibition of functions coupled to the N terminus modify the apoptotic effect of Gal-1.

3. MATERIALS AND METHODS

Cells and reagents

The leukaemic T cell line Jurkat was cultured in RPMI 1640 (GIBCO-BRL) medium supplemented with 5% heat-inactivated fotal calf serum (FCS) (Protein GMK) at 37 °C in an incubator with 5% CO_2. The $p56^{lck}$-deficient Jurkat variant JCaM1.6 and the ZAP70-deficient T cell line P116 were cultured in RPMI 1640 with 10% FCS under the same conditions. Rabbit polyclonal antibodies to $p56^{lck}$ or ZAP70 and mouse monoclonal anti-$p56^{lck}$ and anti-galectin-1 antibodies were produced in our laboratory. The AKNL and ACGE peptides were synthetized in the laboratory of Prof. Gábor Tóth (Department of Medical Chemistry, Szent-Györgyi Medical University, Szeged). Anti-mouse IgG conjugated with fluorescein isothiocyanate (FITC) or horseradish peroxidase (HRPO) and streptavidin-FITC were purchased from DAKO. Mouse monoclonal anti-phosphotyrosine (4G10) was from Upstate Biotechnology Inc. Other reagents were purchased from Sigma.

Stimulation with lyso-PC

Jurkat and JCaM1.6 cells were stimulated with lyso-PC as described in a publication attached to the thesis (Legrádi *et al*., Immunol. Lett. 91:17-21). Briefly, cells were stimulated with the indicated amounts of lyso-PC for 1 min at 37 ˚C in a buffer containing 10 mM $MnCl_2$, 10 mM $Mg(OAc)_2$, 296 μM $CaCl_2$, 2 mM EGTA and 40 mM HEPES (pH 7.4). The activation was stopped with 2 times concentrated ice-cold lysis buffer (50 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 20 mM NaF, 200 μM Na_3VO_4, 10 mM Na-pyrophosphate, 2 mM EGTA, 1 mM PMSF and 1 μg/ml aprotinin or leupeptin). Cell lysates were incubated on ice for 30 min and spun at 12000 g for 15 min. The difficulty in the use of lyso-PC as an immunomodulatory reagent *in vitro* is the narrow border between the concentrations of lyso-PC acting as a physiological modulator and as a cytolytic detergent. The latter is efficiently used to permeabilize cells for functional studies (122).

Immunoprecipitation and Western blotting

For immunoprecipitation of the kinases, the cell lysates of 1 x 10^7 lyso-PC stimulated or non-stimulated cells were incubated for 1 hour at 4 ˚C with preformed complexes of protein A-Sepharose beads (Pharmacia) and the appropriate antibody, rabbit anti-$p56^{lck}$ or rabbit anti-ZAP70. The cell lysates of $2x10^5$ cells or the immunoprecipitates were separated on a 10% SDS polyacrylamide gel, and Western blot analysis was performed to visualize the tyrosine phosphorylated proteins and $p56^{lck}$ and ZAP70 kinases, as described in Legrádi *et al*., Immunol. Lett. 91:17-21.

Intracellular Ca^{2+} concentration measurement

The changes in intracellular Ca^{2+} concentration in Jurkat cells in response to lyso-PC were measured by using Ca^{2+}-sensitive fluorochromes and flow cytometry, as described in Legrádi *et al*., Immunol. Lett. 91:17-21.

Sub-G1 cell population analysis

For the determination of late apoptotic cells, we carried out cell cycle analysis by flow cytometry. The sub-G1 (hypodiploid) population was considered apoptotic. The detailed protocol is described in Fajka-Boja *et al*., Immunol Lett. 82:149-54, attached to the thesis.

Production of human recombinant Gal-1

cDNA containing the wild type and the cysteine-serine mutation of Gal-1 were cloned into pQE-60 bacterial protein expression vector. The vector was transformed into the Bl-21 *E. coli* strain. The bacterial cultures were grown till OD_{600}=1.5 in LB medium containing 200 μg/ml ampicillin, centrifuged at 2500 g for 15 min and washed 2 times with TE buffer (1 M Tris pH 7.4, 200 mM EDTA). The pellet of 100 ml bacterial culture was lysed with a French press in 25 ml lysis buffer (50 mM Tris pH 7.5, 200 mM EDTA, 1 mM PMSF, 14 mM β-mercaptoethanol) and spun at 10000 g for 30 min at 4 ˚C. The supernatant was filtered through Whatman 3 MM filter paper. The lysate was loaded onto a lactosyl-agarose column, circulated 2 times, and the column was then washed twice with 10 ml of washing buffer to remove the non-bound components of the bacterial lysate. The β-mercaptoethanol concentration of the washing buffer (50 mM Tris pH 7.5, 1 mM PMSF) was decreased from

14 mM to 4 mM to minimallize the reaction with iodacetamide in the elution buffer. The bound Gal-1 was eluted with 10 ml elution buffer (50 mM Tris pH 7.5, 100 mM lactose, 100 mM iodoacetamide). The eluate was collected in 1 ml fractions, and the Gal-1 content and purity of the fractions were analysed with 12% SDS-PAGE. Finally, the fractions were collected and dialysed against PBS supplemented with 250 μM β-mercaptoethanol for 24 h, sterilized by filtering through Millex GV low protein-binding filters (0.22 μm, Millipore) and then lyophilized. Before use, the lyophilized protein was dissolved in distilled water containing 250 μM β-mercaptoethanol. The concentration of the purified Gal-1 was determined by measuring the optical density at 280 nm.

The protein concentration was calculated via the following formula (123):

abs= (nW x 5500 + nY x 1490 + nC x 125)/ MW, .

where W= tryptophan, Y=tyrosine, and C=cysteine .

According to the formula, the absorption of 1 mg/ml Gal-1 solution at 280 nm is 0.566. The protein concentrations of purified mutant Gal-1-containing samples were confirmed with a BCA protein measurement kit (Pierce).

Western blotting on bacterial lysate

The bacterial culture was centrifuged at 13000 rpm and the pellet was lysed in SDS-PAGE sample buffer (125 mM Tris/HCl pH 6.8, 20% glycerine, 4% SDS, 2% β-mercaptoethanol, bromophenol blue). 100 ng purified Gal-1 or 2 μl bacterial lysate was loaded onto 12% SDS-PAGE. The proteins were then transferred to nitrocellulose membrane and detected with anti-Gal-1 monoclonal antibody (clone number 3C1/A1, 1 μg/ml) and anti-mouse IgG-HRPO. The signals were detected with the ECL plus detection system (Amersham). To visualize the proteins in the gel, they were stained with Coomassie-Brilliant Blue for 15 minutes, then differentiated with 10% acetic acid for 4-16 h.

Carbohydrate-binding test

The binding of Gal-1 and its mutants to glycoproteins (asialofetuin) was determined with an ELISA test. The ELISA plate wells were coated with 5 μg/ml asialofetuin in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, 3 mM NaN_3) overnight at 4 °C. The blocking solution was PBS containing 0.05% Tween-20 and 3% Teleostean gelatin. The wild-type and mutant Gal-1

(5 μg/ml) were added in the absence or presence of lactose (concentration range between 0 and 1 mM), and incubated for 1 h at 37°C. The bound Gal-1 was detected with monoclonal anti-Gal-1 antibody (clone: 2C1/6, 5 μg/ml), and anti-mouse Ig-HRPO. Finally, the peroxidase was detected in ELISA substrate buffer (63 mM Na_2HPO_4, 26.6 mM citric acid pH 6.0, 0.25 mg/ml OPD, 0.16 μl/ml H_2O_2) and stopped with 50 μl of 4 N H_2SO_4. The intensity of the colour reaction was measured with an ELISA reader at 492 nm.

Analysis of Gal-1 binding to cell surface

Jurkat cells were incubated with wild-type or mutant Gal-1 (1.8 μM) for 1 h at 4 °C in 100 μl of FACS buffer (PBS, 1% FCS, 0.1% NaN_3). In lactose competition assays, the cells were resuspended in FACS buffer containing 100 mM lactose after Gal-1 binding. The cell surface-bound Gal-1 was detected with monoclonal anti-Gal-1 antibody (clone number 2C1/6, 10 μg/ml). Anti-mouse Ig-FITC was added in the final step. For dead cell exclusion, propidium iodide was added before data acquisition.

The fluorescence intensity was measured with a flow cytometer (FACSCalibur, Becton, Dickinson & Co.). The median of the fluorescence intensity was analysed with CELLQuest software (Becton, Dickinson & Co.). The signal/noise ratio was calculated via the formula

$$\text{fluorescent signal} = 10^{\{(\text{Ch\# sample} - \text{Ch\# control})/256\}}$$

where Ch# sample is the average fluorescence intensity of the sample, and Ch# is the average fluorescence intensity of the antibody control in channel number.

Annexin-V (AnnV) labelling

To detect PS exposure on the outer cell membrane, Jurkat cells were treated as indicated, then washed twice with PBS and resuspended in binding buffer (0.01 M HEPES, 0.14 M NaCl and 2.5 mM $CaCl_2$). Annexin V-FITC (Pharmingen) and propidium iodide (10 μg/ml) were added to the cells for 15 min in the dark, at room temperature. The cells were analysed on a FACSCalibur cytofluorimeter, and the percentage of apoptotic Ann V^+ cells was determined with CellQuest software (Becton, Dickinson & Co.)

4. RESULTS

4.1. Roles of p56^{lck} and ZAP70 tyrosine kinases in lyso-PC-induced signalling events

Treatment of the leukaemic T cell line Jurkat with lyso-PC induced the tyrosine phosphorylation of a set of intracellular proteins in a concentration-dependent fashion. Maximum phosphorylation was achieved by the addition of lyso-PC to the cells in concentrations of 25 and 50 μg/ml (Fig. 6.A). Induction of tyrosine phosphorylation with 25 μg/ml lyso-PC occurred within 15 sec, and declined after 5 min of stimulation (Fig. 6.B).

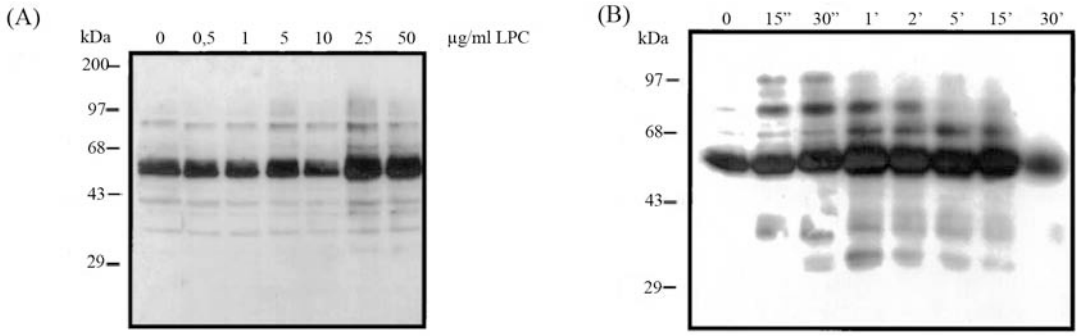

Fig. 6. **Lyso-PC induces tyrosine phosphorylation in Jurkat cells in a concentration- and time-dependent fashion.** Jurkat cells were treated with different concentrations of lyso-PC for 1 min (**A**) or with 25 μg/ml lyso-PC for different times **(B)** at 37 ˚C, or incubated without lyso-PC. The samples were then analysed by Western blotting using a monoclonal antibody to phosphotyrosine. The anti-phosphotyrosine reactive bands were visualized with anti-mouse Ig-HRPO and ECL reagent.

To define the contribution of p56^{lck} in the lyso-PC-triggered tyrosine phosphorylation events we analysed the consequence of the deficiency of p56^{lck} in Jurkat cells. Jurkat and its p56^{lck}-deficient variant JCaM1.6 were stimulated for 1 min and the induction of protein tyrosine phosphorylation was investigated. In the absence of p56^{lck} tyrosine kinase, the lyso-PC-induced phosphorylation was greatly reduced (Fig. 7.A) as compared with the wild-type Jurkat cells (Fig. 7.B), suggesting that p56^{lck} is largely required for the lyso-PC-triggered tyrosine phosphorylation in Jurkat T cells.

To identify prominent tyrosine phosphorylated bands with 56 and 70 kDa apparent molecular mass, immunoprecipitates of the p56^{lck} and ZAP70 kinases from non-activated and lyso-PC-activated Jurkat cell lysates were analysed by anti-phosphotyrosine blotting. Lyso-PC induced increases in the phosphorylation of p56^{lck} and ZAP70 as compared with the normal phosphorylation pattern of these proteins (Fig. 7.C).

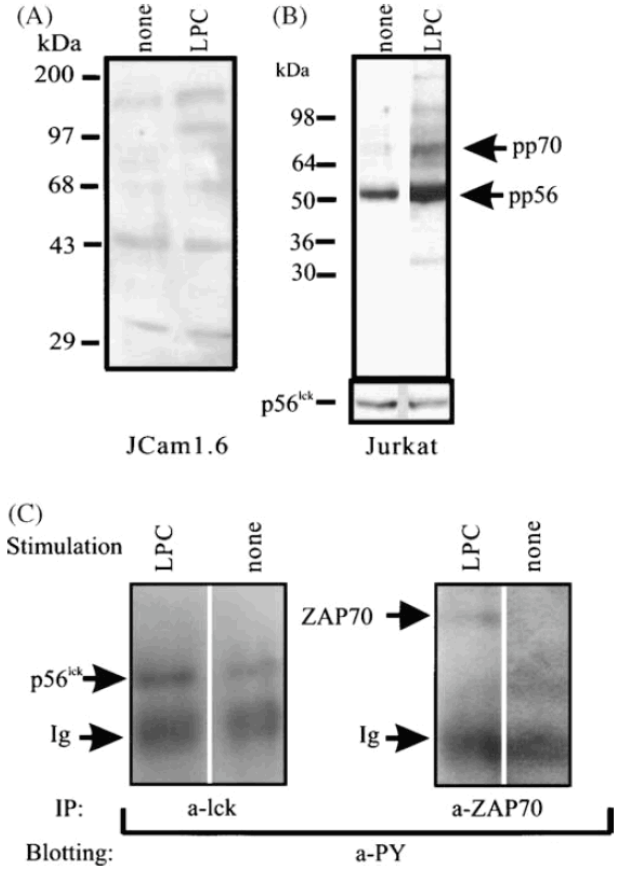

Fig. 7. **The protein kinases p56^{lck}and ZAP70 are involved in the lyso-PC-triggered signalling events.** Lysates were prepared from cells of the p56^{lck}-deficient Jurkat variant, JCaM1.6 **(A)** or Jurkat cells **(B)**, which were stimulated with 25 μg/ml lyso-PC for 1 min at 37 ˚C or left unstimulated. Bottom panel: the quantity of p56^{lck} in stimulated and unstimulated samples was analysed by immunoblotting. For panel **(C)**, the lysates obtained from stimulated or unstimulated Jurkat cells were immunoprecipitated with anti-p56^{lck} or anti-ZAP70 antibodies. The samples were then analysed by anti-phosphotyrosine Western blotting.

One of the most important signalling pathways, triggered upon ZAP70 activation is the activation of PLCγ. The activated PLCγ cleaves phosphatidylinositol bisphosphate (PIP_2) into two second messengers: inositol trisphosphate (IP_3) and diacylglycerol (DAG). IP_3 is water-soluble, and is able to diffuse into the cytoplasm. There it binds to ryanodine receptors on the endoplasmatic reticulum, and releases Ca^{2+} from the internal stores leading to an increased cytoplasmatic Ca^{2+} concentration (124).

Lyso-PC induces a rise in the intracellular Ca^{2+} concentration in different cell types (52,125,126), including Jurkat T cells as shown in Fig. 8.A. To establish whether phosphorylation events are involved in the Lyso-PC induced Ca^{2+} signal, the broad-spectrum, cell-permeable inhibitors of Ser/Thr and tyrosine kinases, staurosporin and genistein, respectively, were added to the cells during the Ca^{2+} assay. Suppressing Ser/Thr or tyrosine kinase activity with the broad-spectrum inhibitors, straurosporin or genistein, respectively, diminished the increase in the intracellular Ca^{2+} concentration in Jurkat cells (Fig. 8.A).

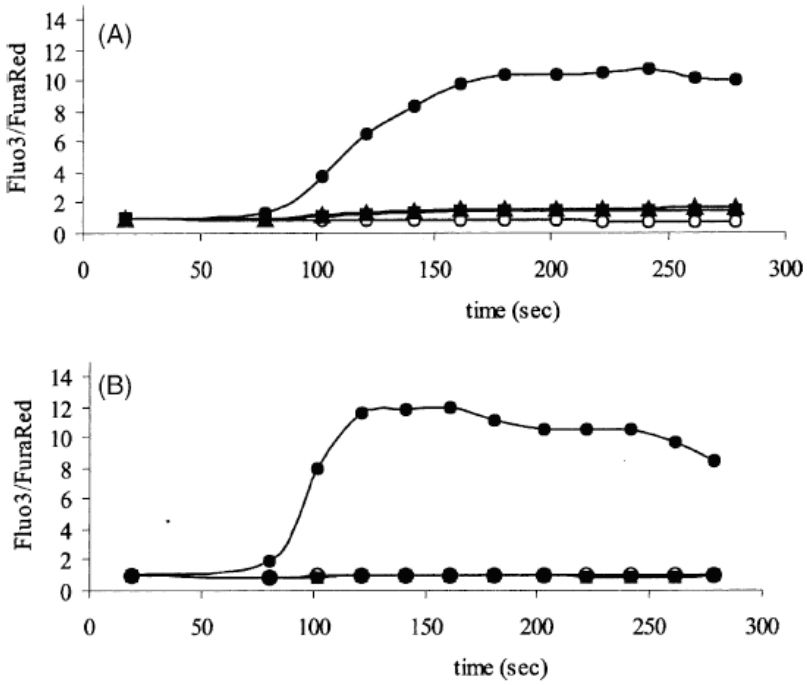

Fig. 8. **Treatment of Jurkat cells with lyso-PC results in an elevation of the intracellular Ca^{2+} concentration in a protein kinase and G-protein-coupled receptor-dependent fashion.** (**A**) Jurkat cells were loaded with Ca^{2+} sensitive dyes, and Fluo-3AM and FuraRed-AM, and then stimulated with DMSO (open circles) or 45 μg/ml lyso-PC (closed circles) in the presence or absence of protein kinase inhibitors, genistein (50 μM) (closed triangles) or straurosporine (1 μM) (closed squares). (**B**) The cells were preincubated with (closed squares) or without (closed circles) 100 ng/ml pertussis toxin, then loaded with fluorescence dyes and finally activated with 20 μg/ml lyso-PC. The control sample was treated with DMSO (open circles). The change in the intracellular Ca^{2+} concentration was measured by FACS as the ratio of the Fluo-3 and FuraRed fluorescence intensities versus time.

Table 2. **Absence of $p56^{lck}$ in Jurkat cells results in a delay of the Ca^{2+} response upon lyso-PC stimulation**

	Time of maximum Ca^{2+} signal upon lyso-PC stimulation (sec)	Time of maximum Ca^{2+} signal upon Ca^{2+} ionophore stimulation (sec)	Maximum level of Ca^{2+} signal upon lyso-PC stimulation	Maximum level of Ca^{2+} signal upon Ca^{2+} ionophore stimulation
Jurkat	161±20.66	278	8.42+0,91	9.98
JCaM1.6	261+42.03	278	11+2,9	12.3

Jurkat or JCAM1.6 cells were loaded with fluorescence Ca^{2+} dyes (see in Materials and Methods) and stimulated with 45 μg/ml lyso-PC or with 2 μg/ml Ca-ionophore A23187.

To examine the role of $p56^{lck}$ induced phosphorylation events of the lyso-PC-induced intracellular Ca^{2+} mobilization, the $p56^{lck}$ deficient Jurkat cell line (JCam1.6) was used in a

Ca^{2+} elevation test. The $p56^{lck}$-deficient Jurkat cell line reacted with a similar magnitude of Ca^{2+} response to lyso-PC or Ca^{2+} ionophore stimulation, as did its wild-type counterpart, but we observed that the maximum in the Ca^{2+} response in JCaM1.6 to lyso-PC, but not ionophore treatment, was always delayed as compared with that of Jurkat cells (Table 2).

It has been shown that G2A, a G protein-coupled seven transmembrane receptor, is the receptor of lyso-PC on T cells. Accordingly, the presence of the best-known G protein inhibitor, pertussis toxin, completely abrogated the lyso-PC-induced Ca^{2+} elevation (Fig. 8.B).

4.2. $p56^{lck}$ and ZAP70 kinases, but not CD45 phosphatase, have a crucial role in Gal-1-induced apoptosis in Jurkat cells

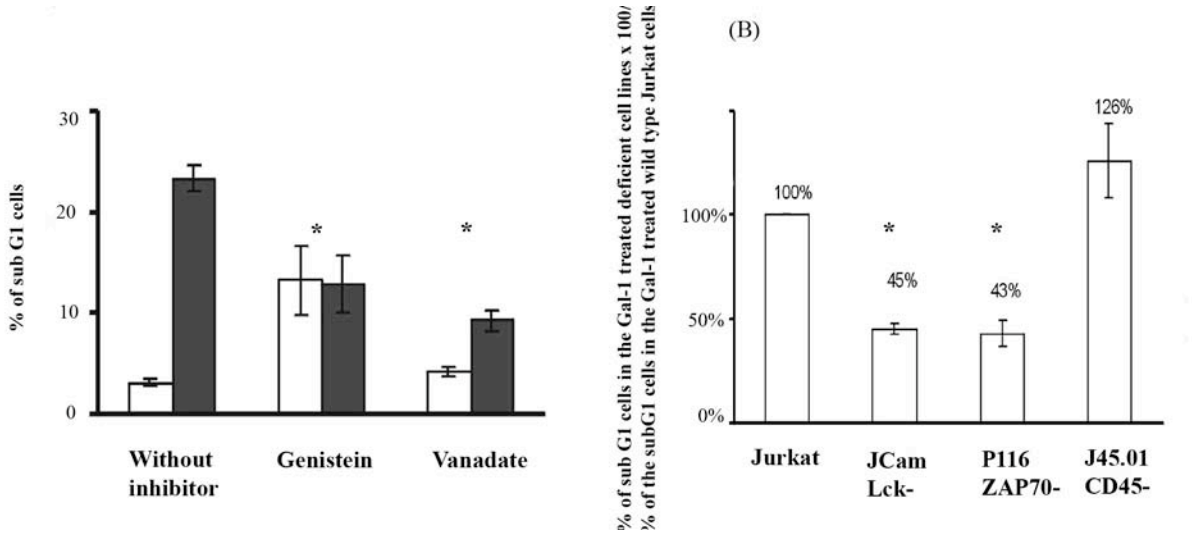

Fig. 9. **Protein tyrosine kinases and phosphatases play roles in Gal-1-induced apoptosis. (A)** Jurkat cells were treated with 1.8 μM Gal-1 for 24 h in the presence of tyrosine kinase (genistein 250 μM) and tyrosine phosphatase (vanadate 250 μM) inhibitors. The empty columns show the percentage of sub-G1 cells without Gal-1 treatment, and the black columns the percentage of apoptotic cells after Gal-1 treatment. (**B**) Wild-type and mutant Jurkat derivatives were treated with 1.8 μM Gal-1 for 24 h. The columns depict the percentage of the sub-G1 cell population relative to the sub-G1 cell population caused by Gal-1 on wild-type Jurkat (% of sub-G1 cells in the deficient cells x100/% of sub-G1 cells in Jurkat).

It has been shown previously in our laboratory that Gal-1 treatment induces tyrosine phosphorylation in Jurkat T cells (Fajka-Boja *et al.*, Immunol Lett. 82:149-54). To determine whether this event is important in Gal-1-induced apoptosis, Jurkat cells were treated with Gal-1 in the presence of tyrosine kinase (genistein) and protein phosphatase (vanadate) inhibitors for 24 h, and the percentage of the sub-G1 cell population was then determined (Fig. 9.A). The Gal-1-induced apoptosis was significantly reduced in the presence of the inhibitors,

suggesting the role of tyrosine kinases and phosphatases in Gal-1-induced apoptotic events (Fig. 9.A).

In our experiments, we focused on the three best-characterized signalling molecules in T cells: $p56^{lck}$ and ZAP70 tyrosine kinases and CD45 tyrosine phosphatase. To demonstrate the role of these signalling molecules in Gal-1-induced apoptosis,

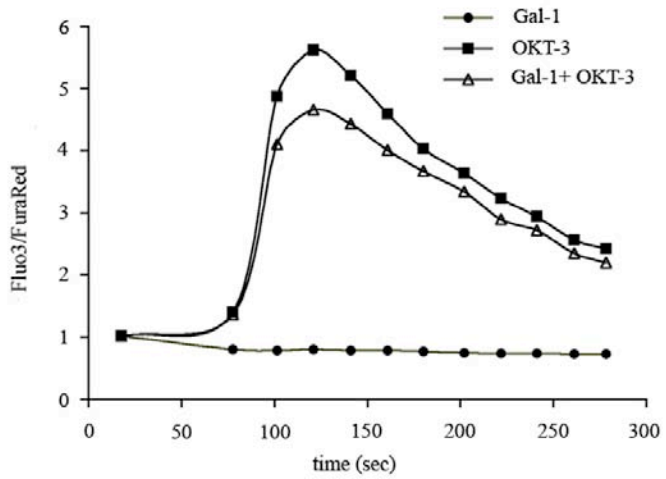

Fig. 10. **Gal-1 does not induce an intracellular Ca^{2+} level elevation.** Jurkat cells were loaded with Ca^{2+}-sensitive dyes (Fluo3-AM and FuraRed-AM), then stimulated with 1.8 μM Gal-1 (circle) or with anti-CD3 (squares, OKT-3) or both (open triangles). The change in intracellular Ca^{2+} concentration was measured by FACS and demonstrated as the ratio of the Fluo-3 and FuraRed fluorescence intensities versus time.

mutant Jurkat T cells, JCaM1.6, P116 and J45.01 cell lines deficient in $p56^{lck}$, ZAP70 and CD45, respectively, were used in apoptosis tests. (Fig. 9.B). The kinase mutant cell lines P116 and JCaM1.6 showed lower apoptotic responses than did the wild-type Jurkat, indicating that $p56^{lck}$ and ZAP70 kinases play roles in the Gal-1-induced apoptotic pathway. The CD45 receptor tyrosine phosphatase has been indicated to be a major Gal-1 receptor mediating the Gal-1 cytotoxic effect (77,94). In conflicting with these studies, we proved that, although Gal-1 is bound to CD45, its presence is not required to trigger apoptosis since CD45-deficient J45.01 cells responded similarly to wild-type Jurkat cells on Gal-1 treatment (Fig. 9.B).

As shown in the previous section, activation of $p56^{lck}$ and ZAP70 by lyso-PC resulted in an elevation of intracellular Ca^{2+}, a process similar to that of triggering the TCR (Fig. 10). To determine whether modulation of the activity of these kinases was followed by Ca^{2+} signalling during Gal-1-induced apoptosis, the intracellular Ca^{2+} level was measured upon stimulation with Gal-1 (Fig. 10). Stimulation of Jurkat cells with Gal-1 did not result in an elevation of the intracellular Ca^{2+}, but rather reduced the Ca^{2+} signal generated via TCR.

4.3. Structure-function study of Gal-1

Monomeric Gal-1 contains 6 conserved cysteine residues. Oxidation of these amino acids may influence functions such as the haemagglutination activity of Gal-1. However, the oxidized form of Gal-1 also has biological activity, since it causes axonal nerve growth on transected nerve sites of adult rat dorsal root ganglion explants (58). In this chapter, I describe how the substitution of individual cysteine residues by serine affects the carbohydrate-binding and apoptosis-induction capacities of Gal-1.

4.3.1. Purification of human recombinant Gal-1

To ascertain the functions of particular cysteine amino acids in the apoptosis-induction capacity of Gal-1, 5 cysteine residues were substituted one by one with serine residues at positions 2; 16; 42; 60; and 130 . The cysteine-serine mutants (C2S, C16S, C42S, C60S and C130S) and wild-type Gal-1 proteins were expressed and purified as described in the Materials and Methods section. Although purification occurred under the same conditions (an identical amount of bacterial lysate, the same size of the affinity column and so on), the yield of the C60S mutant was reproducibly lower than those of the other constructs (Fig. 11.A, Table 3). Moreover, the C60S mutant migrated anomalously on SDS PAGE with an apparent MW of 13.5, which indicates a conformational change caused by this mutation (Fig.11.B). This finding is supported by Western blotting analysis, since anti-Gal-1 mAb produced in our laboratory, recognizes the wild-type and all mutants but C60S with similar affinity (Fig. 13.C). Although the C60S mutant was interesting because of its anomalous migration in SDS PAGE (Fig.11.B) and its modified antigenic structure indicated by the low signal obtained with Gal-1 mAb (monoclonal

Table 3. **The amount of the purified Gal-1**

Gal-1	Amount of the purified protein (mg)
Wild type	4.67
C2S mutant	3.82
C16S mutant	4.05
C42S mutant	4.20
C60S mutant	1.73
C130S mutant	3.92

The concentration of the purified Gal-1 was determined by spectrophotometer, described in Materials and Methods.

antibody) (Fig.11.C), we could not carry out further studies with it because of the very low expression (Fig. 11.A, and Table 3).

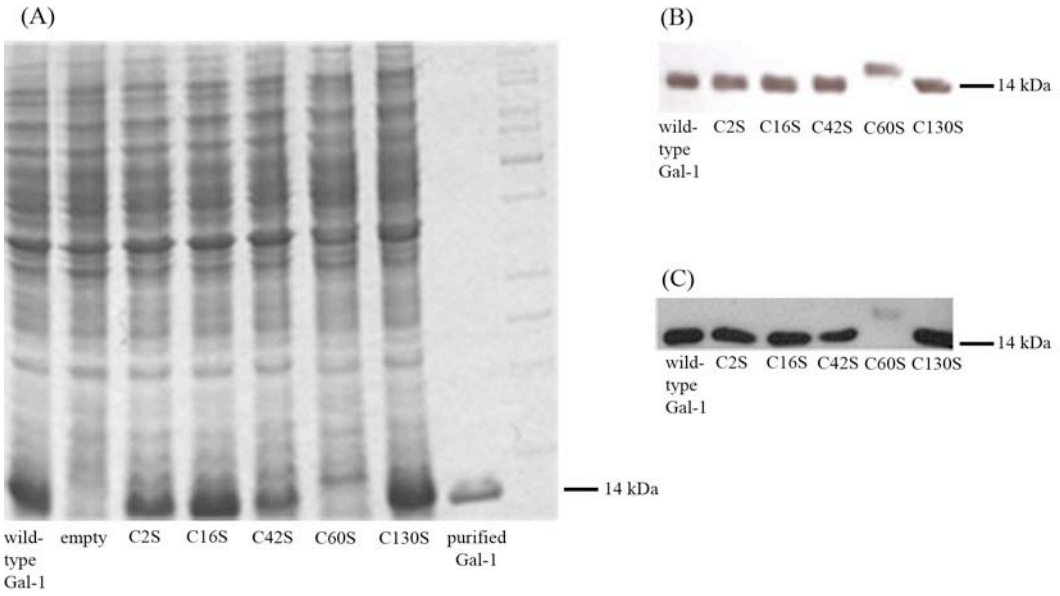

Fig. 11. **Cysteine-serine replacement at the position 60 causes a lower protein expression level and slower migration capacity on 12% SDS-PAGE.** (**A**) The bacterial culture was grown till OD_{600}=1.5, 100 µl was then centrifuged and the pellet was lysed with SDS-PAGE sample buffer and loaded onto 12 % SDS-PAGE. The protein bands were visualized with Coomassie Brilliant Blue staining. (**B**) 2.5 µg of the purified wild-type and cysteine-serine mutant Gal-1 were loaded onto 12% SDS-PAGE. The protein bands were visualized with Coomassie Brilliant Blue staining. (**C**) 100 ng of the purified wild-type and cysteine-serine mutant Gal-1 were loaded onto 12% SDS-PAGE and the protein was visualized by Western blotting experiment with the help of monoclonal anti-Gal-1 antibody.

4.3.2. Effect of substitution of cysteine with serine on carbohydrate binding

The sugar-binding ability of each mutant Gal-1 was analysed by lectin-asialofetuin binding assay. Asialofetuin is a Gal-1-binding glycosylated protein in fotal calf serum. In our experiment, we measured the asialofetuin-binding capacities of the mutants in an ELISA system. The strength of Gal-1 binding to asialofetuin exhibited the following order: C42S > C16S > C130S > wild type > C2S mutant (Fig. 12.A). These results suggested that cysteine-serine mutations had a changed the binding ability to bind Gal-1 to asialofetuin. Relative to the wild-type Gal-1, the asialofetuin-binding capacity of the C2S mutant was decreased.

There was no change for the C130S mutant, and the binding capacities of the C16S, and C42S mutants were higher than that of the wild-type.

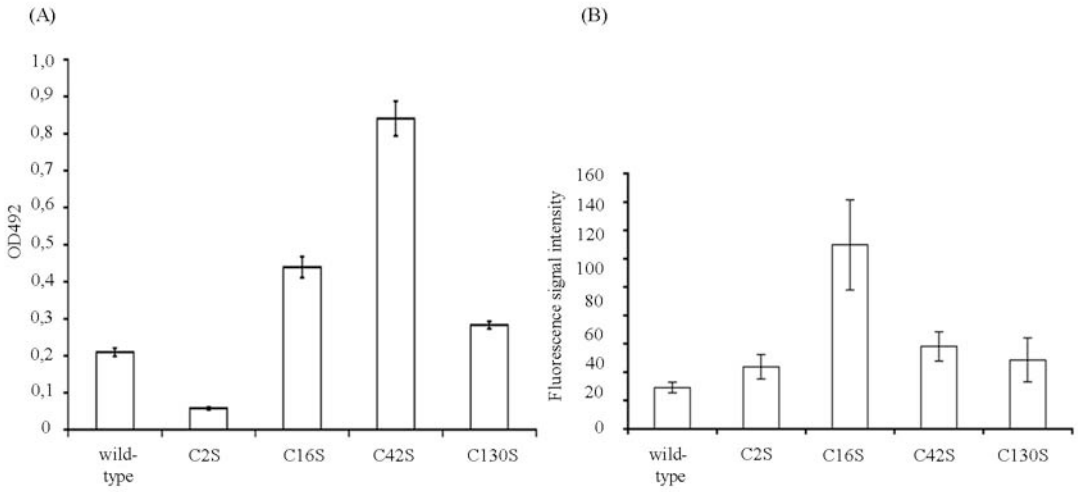

Fig. 12. **Cysteine-serine replacement modifies the asialofetuin and cell surface-binding capacity of Gal-1 (A)** 5 μg/ml asialofetuin was bound to an ELISA plate, 0.36 μM of the wild-type or mutant Gal-1 was added and the bound Gal-1 was detected with monoclonal anti-Gal-1 antibody (2C1/6) and peroxidase-conjugated anti-mouse Ig. The intensity of the colour reaction was measured with an ELISA reader at 492 nm. The experiments were performed with 2 parallels and the background was subtracted from the samples. **(B)** 2.5 x 10^5 cells were plated to a 96-well plate, and 1.8 μM wild-type or cysteine-serine mutant Gal-1 was incubated with the cells for 1 h at 4 °C. The bound Gal-1 was detected with monoclonal anti-Gal-1 antibody (2C1/6) and the specifically bound Gal-1 antibody was detected with FITC-conjugated anti-mouse Ig in a flow cytometer. The intensity of the fluorescence signal caused by the bound Gal-1 was calculated as described in the Materials and Methods section. The experiment was performed with 3 parallels.

Gal-1 interacts with cell surface glycoconjugates, which was analysed by flow cytometry. Jurkat cells were incubated with the wild-type and cysteine-serine mutants and the amount of bound Gal-1 was detected by indirect immunocytometry. The rank of binding, calculated from the medians of fluorescence intensities, was as follows: C16S > C42S > C130S > C2S > wild-type (Fig. 12.B). Unexpectedly, the binding to the cell surface glycoconjugates did not correspond to the results obtained with asialofetuin. C16S coupled to asialofetuin similarly to the wild-type. In contrast, the affinity of the C16S mutant to cell surface glycoconjugates was higher relative to the wild-type Gal-1.

Lactose, the minimal ligand of Gal-1, inhibits the binding of Gal-1 to cells or asialofetuin by competing for β-galactoside-containing glycoproteins (127). A high molar excess (1.8 μM Gal-1 versus 100 mM lactose) reduced the Gal-1 binding to the cells by >95% for all constructs (Fig. 13. A). In the asialofetuin-binding assay, the signal, caused by the wild-type,

and the C2S, C16S, C42S and C130S mutant Gal-1 decreased greatly in the presence of an increasing concentration of lactose in the solution (Fig. 13.B).

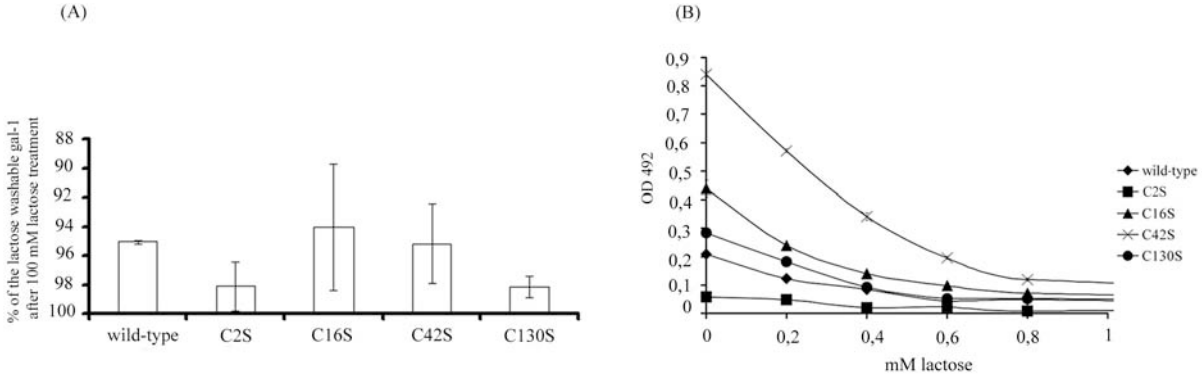

Fig. 13. **Lactose inhibits the carbohydrate-binding ability of the mutants. (A)** Jurkat cells were incubated with 1.8 μM of the wild-type or cysteine-serine mutant Gal-1 for 1 h at 4 °C, and the cells were then treated with 100 mM lactose containing FACS buffer for 1 h at 4 °C. The remaining Gal-1 was labelled with monoclonal anti-Gal-1 antibody (2C1/6), and the signal caused by the cell surface-bound Gal-1 was detected with FITC-conjugated anti-mouse Ig flow cytometrically. The values in the Table were calculated via the following formula: fluorescence intensity from the 100 mM lactose treated sample / fluorescence intensity from the non-lactose treated sample x 100. The intensity of the fluorescence signal caused by the bound Gal-1 was calculated as described in the Materials and Methods section. The columns depict the average of three independent experiments. **(B)** 5 μg/ml asialofetuin was bound to the ELISA plate in 50 μl final volume, 0.36 μM wild-type or cysteine-serine mutant Gal-1 was then added to the asialofetuin without or in the presence of different concentrations of lactose, and the bound Gal-1 was detected with monoclonal anti-Gal-1 (2C1/6) antibody and peroxidase-conjugated anti-mouse Ig. The intensity of the colour reaction was measured with an ELISA reader at 492 nm. The experiment was performed with 2 parallels and the background was subtracted from the samples.

4.3.3. Effect of cysteine to serine substitution on apoptosis-induction capacity of Gal-1

Apoptosis was measured either by detecting PS on the outer surface of the cell membrane or by DNA content analysis of low DNA content cells. The loss of membrane asymmetry and hence the exposure of PS on the outer surface of the cell membrane is one of the major consequences of the apoptotic trigger (128). The PS exposure caused by Gal-1 treatment was detected by FITC-labelled AnnV on Jurkat cells. The final step of the apoptosis, the degradation of the nuclear DNA, was analysed by the formation of the sub-G1, hypodiploid cell population. The results showed that the degrees of early (AnnV-positive cells, Fig. 14.A.) and late (sub-G1 cell population, Fig. 14.B) apoptotic population were

similar in the wild-type and the cysteine mutants, C2S, C16S and C42S, but not C130S, which displayed a slightly reduced apoptosis.

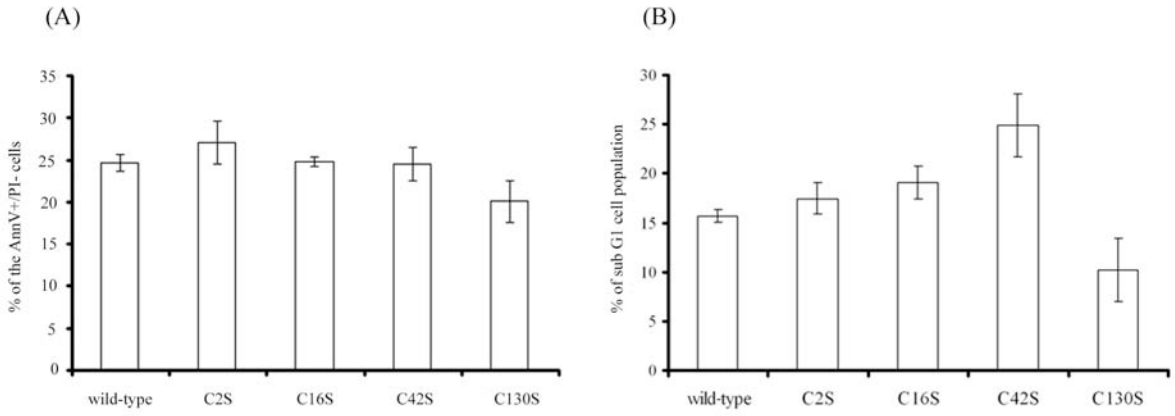

Fig. 14. **A cysteine-serine amino acid mutation does not have a strong effect on the apoptotic induction of Gal-1.** (**A**) Jurkat cells were incubated with 1.8 μM wild-type or mutant Gal-1 for 6 h. The cells were then labelled with FITC-conjugated AnnV and propidium iodide (see Materials and Methods section), and the fluorescence intensity was measured by flow cytometry. The columns show the percentage of the early apoptotic population (AnnV+, PI-) The experiment was performed with 3 parallels, and the result on the non-treated sample was subtracted from those for the Gal-1-treated samples. (**B**) Jurkat cells were incubated with 1.8 μM wild-type and mutant Gal-1 for 12 h. The cells were then permeabilized and the DNA content of the cells was labelled with propidium iodide. The fluorescence signal caused by the DNA bound propidium iodide was measured on a flow citometer. The colums show the percentage of sub-G1 cell population. The experiment was performed with 3 parallels, and the percentage for the non-treated sample was subtracted from those for the Gal-1 treated samples.

4.3.4. Effects of Gal-1-derived synthetic oligopeptides on Gal-1-induced apoptosis

Two oligopeptides (ACGE and AKNL; see sequences in Fig. 15) representing different parts of the Gal-1 sequence were chemically synthetized and used in further studies.

ACGE reflects the N terminal hydrophobic part of the Gal-1 sequence implicated in the dimerization of Gal-1. The short oligopeptide AKNL contains some of the amino acids required for cell growth inhibition (29).

ACGLVASNLNLKPGECLRVRGEVAP
DAKSFVLNLGKDSNNLCLHFNPRFNA
HGDANTIVCNSKDGGAWGTEQREAV
FPFQPGSVAEVCITFDQANLTVKLPD
GYEFKFPNRLNLEAINYMAADGDFKI
KCVAFD-COOH

Fig. 15. **The amino acid sequence of Gal-1**. Green caps show the peptide which contains the N-terminal hydrophobic amino acids required for the dimerization (ACGE), while red caps show the peptide (AKNL) which contains a part of the sequence required for cell growth inhibition, and common in all Gal-1 from different species (DAKSF, underlined caps).

Apoptosis was tested by sub-G1 cell population measurement. The peptides did not induce apoptosis on Jurkat T cells alone (Fig. 16). However, when the peptides were added together with Gal-1, used in an apoptotic concentration (1.8 μM), the Gal-1-induced apoptosis slightly but reproducibly decreased or increased in the presence of ACGE or AKNL (Fig. 16), respectively.

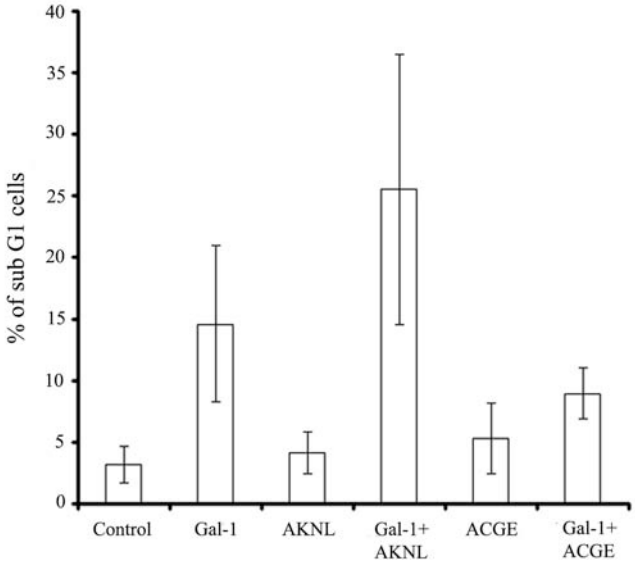

Fig. 16. **The presence of ACGE peptide partially inhibits, whereas the presence of AKNL peptide stimulates the apoptosis-induction capacity of Gal-1.** Jurkat cells were incubated with 1.8 μM Gal-1 and 90 μM ACGE **(A)** or AKNL **(B)** peptide for 24 h. The cells were then permeabilized and the DNA content of the cells was labelled with propidium iodide. The percentages of sub-G1 cells were measured on a flow cytometer. The data are the results of three independent experiments.

5. DISCUSSION

5.1 New aspects of $p56^{lck}$ and ZAP70 on T cell signalling

5.1.1. Lyso-PC, a regulator of T cells

Lyso-PC, the major phospholipid component of oxLDL, plays a significant role in the pathological process of atherosclerosis. It functions as a chemoattractant for monocytes (37) and T lymphocytes (38). In addition to attracting the T cells to the site of atherosclerotic lesion and sites of inflammation, lyso-PC enhances the production of IFN-γ by the activated T cells and in this manner promotes the inflammatory reaction (51). The possible involvement of tyrosine kinases has been described in the regulation of ICAM-1 (intercellular adhesion molecule 1) expression following lyso-PC treatment of cultured human umbilical vein endothelial cells (127). Also, lyso-PC stimulated the tyrosine phosphorylation of the proline-rich tyrosine kinase, PYK2, a mediator of the phospholipids-induced Ras signalling pathway in vascular endothelial cells (128). Recent characterization of G2A and GPR4 as receptors for lyso-PC has provided important steps promoting an understanding of lyso-PC signalling (53). However, the early effects of lyso-PC on T cells has still not been adequately described.

In the present work, we show that marked tyrosine phosphorylation is induced as early as 15 s after addition of lyso-PC to the Jurkat T cell line. Among others, two proteins are tyrosine phosphorylated in this response, $p56^{lck}$ and ZAP70, the intracellular tyrosine kinases that play central roles in T cell signalling. Implication of $p56^{lck}$ in the lyso-PC-triggered signalling pathway is demonstrated by the dramatic reduction of tyrosine phosphorylation in the $p56^{lck}$-deficient Jurkat variant JCaM1.6 upon lyso-PC stimulation. During the TCR signalling, the activation of $p56^{lck}$, and subsequently ZAP70, results in the formation of an active signalosome (129). This signalling complex contains activated phospholipase Cγ-1 (PLCγ-1), which controls the Ca^{2+} mobilization and the activation of PKC (124). Lyso-PC treatment of the Jurkat cells also increases the intracellular Ca^{2+} concentration. However, this change in the intracellular Ca^{2+} level does not seem to occur via the TCR signalling pathway, since the absence of $p56^{lck}$ does not abrogate it, although it modulates the kinetics of the Ca^{2+} signal. The tyrosine kinase inhibitor, genistein diminishes the Ca^{2+} response, indicating that other tyrosine kinase(s) regulate the process.

The role of PKC activation in the lyso-PC-induced cell response has been confirmed in several studies (130-132). Staurosporine, an inhibitor of Ser/Thr kinases impedes the lyso-PC-induced Ca^{2+} elevation, suggesting that both Ser/Thr and tyrosine kinase pathways are involved in the regulation of the intracellular Ca^{2+} concentration.

Multiple factors contribute to the pathogenesis of atherosclerosis, including activated T lymphocytes and T cell-derived cytokines (133). Chemotactic and antigenic activation of T cells by oxidized LDL (oxLDL) and, as a result, production of the pro-inflammatory cytokine, IFN-γ occurs at the site of the atherosclerotic lesion (134,135). OxLDL serves as a source of lyso-PC, the main lipid component of ox-LDL, which contributes to the immunomodulatory effect of oxLDL. In this work, we show that lyso-PC triggers early signalling events in T cells, such as the activation of $p56^{lck}$ and probably other protein tyrosine kinases, and then the elevation of the intracellular Ca^{2+} concentration. The latter is regulated by multiple signalling components, such as the presence of $p56^{lck}$ and other, uncharacterized tyrosine and Ser/Thr kinases, and also by the activity of a G-protein coupled lyso-PC receptor.

5.1.2 Gal-1, a regulator of T cell apoptosis

It has been shown in recent studies that the binding of Gal-1 to cells results in a series of signal transduction events in T and B cells as regards CD45 signalling. The most pronounced change following the binding is a decrease of the tyrosine phosphatase activity of CD45 (136,137) and as a consequence the elevated tyrosine phosphorylation and decreased kinase activity of B cell tyrosine kinase, lyn (136). However, it has not been well documented whether the regulation of the CD45 activity and the following signalling events are a direct outcome of the ligation of CD45 by Gal-1 or a concomitant result of the trigger of other receptors. Although Gal-1 is secreted from the producing cells (138) and couples to CD45 in T and B lymphocytes, it is not known whether the biological effect of the extracellular Gal-1 is initiated via coupling to CD45.

Gal-1 treatment results in a definite tyrosine phosphorylation in both wild type and CD45 deficient Jurkat cells, although the constitutive tyrosine phosphorylation is much higher in the phosphatase-deficient J45.01 cells (data not shown; Fajka-Boja *et al.*, Immunol Lett. 82:149-54, attached to the thesis). The Gal-1-induced tyrosine phosphorylation is essential for the subsequent apoptosis, since the tyrosine kinase inhibitor genistein blocks the apoptosis when added to the cells together with Gal-1 (98,99). The data published so far have supported

the idea that CD45 may be the major receptor mediating the Gal-1 induced apoptosis in lymphocytes (77). Our results do not confirm this, since the Jurkat variant, J45.01, readily responds to a Gal-1 stimulus with apoptosis in the absence of functional CD45.

On the other hand, the induction of tyrosine kinase activity is essential for the further events of apoptosis. The tyrosine phosphorylation is attributed to $p56^{lck}$ and ZAP70 since a deficiency of these enzymes abolishes the Gal-1-induced cell death, and restoration of $p56^{lck}$ and ZAP70 expression restores apoptosis (98,99). Although the contribution of $p56^{lck}$ to the ceramide and mitochondrion-mediated apoptotic processes has recently been confirmed (139), the immediate receptor(s) mediating $p56^{lck}$ activation and the direct target(s) of $p56^{lck}$ activity have not yet been identified. The involvement of ZAP70 suggests that it may be at least one of its targets. The function of the $p56^{lck}$/ZAP70 kinases has been supported by the recent finding that, upon Gal-1 treatment, the TCRζ chain is partially phosphorylated (140), a biochemical step that occurs during T-cell apoptosis via this pathway. This observation underlines a potential mechanism for ZAP70 activation and the subsequent tyrosine phosphorylation: the participation of the $p56^{lck}$/ZAP70 pathway in the Gal-1-induced apoptosis suggests the feasible involvement of TCR as a mediator of the Gal-1 effect. However, our results do not confirm this, since TCR-negative CEM cells (TCRneg) or T-cell lines expressing different amounts of TCR, such as MOLT-4 (TCRlow), Jurkat (TCRmedium) or HPB-ALL (TCRhigh), respond equally well to the Gal-1 cytotoxic effect (data not shown). Moreover, the pattern and kinetics of tyrosine phosphorylation are well distinguishable from those of TCR stimulation (data not shown). The failure of triggering of the Ca^{2+} signal by Gal-1 also indicates that TCR is not the mediator of Gal-1-induced apoptosis.

5.2. Structure-function correlation of Gal-1

One of the well-known characteristics of vertebrate β-galactoside-binding lectins is their requirement for a reducing reagent such as β-mercaptoethanol for maintenance of the carbohydrate-binding ability (141). Although this and other observations suggest the presence of an essential free thiol group in the binding site, some apparently conflicting results have also been reported. For example, rat and human 14-kDa lectins are not inactivated by chemical modification with monoiodoacetamide (59), but the stability of the modified lectin

is significantly increased. This suggests that chemical modification of some reactive thiol groups by a small modifying group such as a carboxamidomethyl group protects these thiol groups against oxidation. Oxidation may cause the formation of unfavourable intra- or intersubunit disulfide bonds and result in destruction of the native conformation of the lectin. Thus, the true role of a thiol-reducing reagent added for stabilization of the lectin seems to be not to maintain the reduced state of a putative key thiol group, but to protect against destruction of the native conformation caused by the formation of disulfide bonds (57,59). The stabilities of the mutant lectins, and especially that of C2S, under non-reducing conditions are greatly increased. This finding is also consistent with the observation of Clerch *et al.* (56) that Cys2 of rat 14-kDa lectin is the most reactive of the 6 cysteine residues, suggesting that Cys2 is exposed on the surface of the lectin molecule and is most readily oxidized. This has been verified by determination of the X-ray structure of Gal-1 (55,59). Our results are mostly consistent with the literature data, since all the investigated mutants, C2S, C16S, C42S and C130S bind to the carbohydrate present on the non-physiological ligand asialofetuin, although the binding efficiencies differ slightly. Using physiological ligands expressed on the cell surface, similar results have been obtained with the exception of the C16S mutant, which showed higher degree of binding to cells. Interactions between glycoconjugates and Gal-1 clearly depends on the sugarbinding of the mutant lectins since the minimal ligand, lactose, inhibited this binding. It is noteworthy that mutation of the cysteine at position 60 results in an obvious conformational change, reflected by an anomalous migration in SDS PAGE and modified antigenic properties. Unfortunately, we could not study this mutant because of the very low bacterial expression.

The effects of specific mutations in the Gal-1 sequence have been studied only with respect to the carbohydrate-binding ability of the lectin. In this work, we analysed whether a particular cysteine mutation modulates the biological activity of Gal-1. The results show, that the C2S, C16S and C42S mutants are as cytotoxic to T cells as wild-type Gal-1 is. Mutation at position 130 causes a slightly reduced apoptotic activity as compared with the wild-type and other Cys mutants.

The above data indicate that cysteines at positions 2, 16 and 42 are not determining factors in the biological activity of Gal-1. C130S is worthy of further study and construction

of a mutant in Cys60, the most conserved cysteine among the vertebrate Gal-1 sequences, must be investigated.

In the study of the structure-function correlation, Gal-1-related synthetic oligopeptides have also been used. The peptides ACGE and AKNL are N-terminal fragments involved in dimerization and growth regulation, respectively. The presence of ACGE decreases Gal-1-induced apoptosis, indicating that the dimerization of Gal-1 is important in its biological function. A possible explanation of the inhibitory property of ACGE is that the peptide competes for the dimerization site of Gal-1, resulting in less functional monomers. The AKNL peptide synergizes with Gal-1-induced apoptosis, an effect that could occur by stimulation of the Gal-1- binding site. However, these results are very preliminary and an understanding of the mechanism via which the peptides modulate-Gal-1 induced apoptosis requires extensive further research.

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8. NOVEL RESULTS PUBLISHED IN THIS THESIS

(a) First in the literature we have identified the roles of $p56^{lck}$ and ZAP70 kinases in the atherogenic lyso-PC signalling in T cells. Stimulation of T cells with lyso-PC results in an elevation of the intracellular Ca^{2+} level, a process which depends on the activity of Ser/Thr and tyrosine kinases, but is independent of the presence of $p56^{lck}$. Pertussis toxin, an inhibitor of G-protein coupled receptors blocks the Ca^{2+} signal, indicating the role of the G-protein-coupled seven transmembrane receptor, G2A, a receptor for lyso-PC on T cells.

(b) We have provided novel data showing that two tyrosine kinases, $p56^{lck}$ and ZAP70, but not tyrosine phosphatase, CD45, are essential components of the apoptotic signalling pathway triggered by β-galactoside-binding human Gal-1. It is also presented that Gal-1 does not induce the increase of the intracellular Ca^{2+} level in T cells, but reduces the signal stimulated via TCR.

(c) The preliminary results obtained from structure–function studies show that all the investigated mutants, C2S, C16S, C42S and C130S, bind to carbohydrate present on the non-physiological ligand asialofetuin, though their binding efficiencies differ slightly. Using physiological ligands expressed on the cell surface, similar results have been obtained with the exception of the C16S mutant, which exhibited a higher degree of binding to cells. Mutations of the investigated cysteines did not affect the degree of T cell apoptosis, with the exception of position 130, which caused a slightly reduced apoptotic activity as compared with the wild type and other Cys mutants.
N-terminal synthetic oligopeptides modified Gal-1 induced apoptosis, indicating a role of these sequences in the biological function of Gal-1.

7. ABBREVIATIONS

APAF-1- apoptotic peptidase activating factor
C2GNT-2β-1,6-N-acetylglucosaminyltransferase
DISC- death inducing signalling complex
DMSO- dimethil-sulfoxide
EC- endothelial cells
ECL- enhanced chemiluminescence substrate
EDTA- ethylenediamine- tetraacetic acid
EGTA- ethylene glycol tetraacetic acid
ELISA- enzime linked immonoadsorbent
FACS- flourescent activated cell sorter
FITC- fluorescein izothiocianate
Gal -1- galectin- 1
HEPES- 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HPC- haematopoetic stem cell
HRPO- horseradish peroxidase
IFN-γ- interferon-γ
Ig- immunglobulin
IL- interleukin
IP3- inositol triphosphate
ITAM- immunoreceptor tyrosine based motif
LAT- linker for activation of T cells
LB media- lysogeny broth media
Lck- leukocyte-specific protein tyrosine kinase
LDL- lowdensity lipoprotein
Lyso-PC- lysophosphatidyl choline
LPL- lipoprotein lipase
mAb- monoclonal antibody
MAPK- mitogen activated protein kinase
OPD- orto phenyl diamine
oxLDL- oxidized LDL
PLA_2- phospholipase A_2
PLC-γ- phospholipase C γ
PKC- protein kinase C
PMSF- phenyl metal sulfoxid
PTPase- protein tyrosine phosphatase
PS- phosphatidylserine
SDS-PAGE- sodium dodecyl sulfate polyacrylamide gel electrophoresis,
assay
SH2 domain- src homology 2 domain
SMC- smooth muscle cells
TCR- T cell receptor
ZAP70- Zeta-chain-associated protein kinase 70
VCAM-1-vascular cell adhesion molecule

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Lysophosphatidylcholine is a regulator of tyrosine kinase activity and intracellular Ca^{2+} level in Jurkat T cell line

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Abstract

Lysophospholipids, particularly lysophosphatidylcholine (lyso-PC), have been implicated in modulating T cell functions at the sites of inflammation and atherosclerosis. Although the chemotactic and immunomodulatory effects are well documented, the exact signaling pathway of lyso-PC action is poorly defined. In this work, we studied the earliest biochemical events in T cells triggered by lyso-PC. A marked and immediate tyrosine phosphorylation was induced in the leukemic T cell line, Jurkat. Phosphorylation of cellular substrates included *src* family kinase, $p56^{lck}$ and *syk* family kinase, ZAP70. The lyso-PC induced tyrosine phosphorylation was largely dependent on the presence of functional $p56^{lck}$. Tyrosine phosphorylation was followed by the elevation of intracellular Ca^{2+} concentration. The magnitude of the mobilization of the intracellular Ca^{2+} was similar in the absence of the $p56^{lck}$ activity in JCaM1.6 cells as in Jurkat cells, however, it was slightly but reproducibly delayed compared to that in the wild type cells. Inhibition of the Ser/Thr kinases and tyrosine kinases with staurosporine and genistein, respectively, decreased the rise in the intracellular Ca^{2+} content. Moreover, pertussis toxin completely blocked the Ca^{2+} signal supporting the role of the G-protein coupled LPC receptor in this event.
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Keywords: Lysophosphatidylcholine; Tyrosine phosphorylation; $p56^{lck}$; Intracellular Ca^{2+}

1. Introduction

Lysophosphatidylcholine (lyso-PC) is an intrinsic, intracellular messenger generated by the hydrolysis of membrane phosphatidylcholine by intracellular phospholipase A2 (PLA2). Lyso-PC has been described as an enhancer of cellular responses including proliferation and differentiation, when additional second messengers, diacyl glycerol (DAG) and Ca^{2+} are concomitantly present [1].

In addition to the intracellular lyso-PC, it is locally generated and accumulates at the site of wounds, inflammation and atherosclerosis. Moreover, it is a major phospholipid component of the chemically modified low density lipoproteins (LDL), particularly oxidized LDL (oxLDL), that is implicated in atherosclerotic and inflammatory processes [2,3]. Recent data suggest that lyso-PC is directly involved in inflammatory and atherosclerotic response because lyso-PC induces a wide range of cellular responses in macrophages [4,5], dentritic cells [6] and T lymphocytes whose infiltration occur in these tissues [7,8].

Exogenously added lyso-PC enhances interferon-γ (IFN-γ) production in $CD4^+$ T cells when stimulated via the T cell receptor (TCR) [9] and it has chemotactic effect on T lymphocytes [10,11] and monocytes [12] and mitogenic effect on macrophages [5]. Treatment of fibroblast and endothelial cells with lyso-PC activates the activator protein-1 (AP-1) and the c-jun N terminal kinase [13]. The adhesion molecule, ICAM-1 expression is elevated on endothelial cells upon lyso-PC treatment on a PKC-independent and tyrosine kinase(s)-dependent fashion [14]. Other lyso-PC-mediated cellular responses involve

Abbreviations: ECL, enhanced chemiluminescence; FCS, fetal calf serum; Ig, immunoglobulin; HRPO, horse radish peroxidase; lyso-PC, lysophosphatidylcholine; PAGE, polyacrylamide gel electrophoresis; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinine; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCR, T cell receptor

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protein kinase C (PKC) as a signaling effector molecule [1,15]. For example, phorbol ester-induced differentiation of HL-60 cells into macrophages is efficiently potentiated by the presence of lyso-PC [16]. Moreover, lyso-PC induces T cell proliferation in the presence of membrane permeable diacyl glycerol and Ca^{2+} ionophore [1].

Recent data have shown that the effect of lyso-PC is mediated via G2A, the G-protein coupled immunoregulatory receptor [30]. Lyso-PC, interacting with its receptor, G2A, induces the elevation of the intracellular Ca^{2+} concentration and also the transcriptional activation of the serum response factor via the MAPK pathway (reviewed in [31]). In spite of the accumulating data, the exact mechanism by that lyso-PC exerts the signaling function in T lymphocytes has not yet been well established.

In this work, we studied the earliest biochemical events triggered by lyso-PC in T cells. A marked and immediate tyrosine phosphorylation was induced in the leukemic T cell line, Jurkat. Phosphorylation of cellular substrates included *src* family kinase, $p56^{lck}$ and *syk* family kinase, ZAP70. The lyso-PC induced tyrosine phosphorylation was largely dependent on the presence of functional $p56^{lck}$. Tyrosine phosphorylation was followed by the elevation of intracellular Ca^{2+} concentration that was regulated by tyrosine and Ser/Thr kinases and G-protein coupled receptor.

2. Materials and methods

2.1. Cells

Human leukemic T cell line, Jurkat was cultured in RPMI 1640 (GIBCO-BRL) medium supplemented with 5% fetal calf serum (FCS) (Protein GMK, Hungary) in an incubator with 5% CO_2 at 37 °C. The $p56^{lck}$ deficient Jurkat variant, JCaM1.6 was cultured in RPMI 1640 with 10% FCS under the same conditions.

Polyclonal antibodies to $p56^{lck}$ or ZAP70 were produced in rabbit as previously described [29]. Monoclonal anti-$p56^{lck}$ was produced in our laboratory. Reagents were purchased from Sigma if not stated otherwise.

2.2. Cell stimulation and lysis

Cells were harvested from growth medium by centrifugation, washed once in RPMI and were resuspended at 4×10^7 cells/ml in a buffer containing 10 mM $MnCl_2$, 10 mM $Mg(OAc)_2$, 296 μM $CaCl_2$, 2 mM EGTA, 40 mM HEPES (pH 7.4) [17]. The cell viability was not affected in this hyposmotic buffer for the short period of activation. However, this helped to keep the background phosphorylation at low level. Stimulation was initiated by adding the indicated amounts of lyso-PC. Cells were further incubated for 1 min at 37 °C and activation was stopped with 2× concentrated ice-cold lysis buffer (50 mM HEPES (pH 7.4), 1% Triton X-100, 150 mM NaCl, 20 mM NaF, 200 μM Na_3VO_4, 10 mM Na pyrophosphate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 μg/ml aprotinin and leupeptin). Lysates were incubated on ice for 30 min and the soluble fraction was recovered following removal of nuclear and cytoskeletal components by centrifugation at $12{,}000 \times g$ for 15 min. It must be noted that various batches of lyso-PC with different lot numbers purchased from Sigma induced signaling events in much different concentrations in a range from 20 to 45 μg/ml. The difficulty using lyso-PC as an immunomodulatory reagent in vitro is the narrow border between the concentration of lyso-PC acting as a physiological modulator and as a cytolytic detergent. The latter is efficiently used to permeabilize cells for functional studies [18]. Therefore, each new purchase of the lyso-PC was always tested for optimal concentration and toxicity.

2.3. Immunoprecipitation

Triton X-100 soluble extracts prepared from 1×10^7 stimulated or non-stimulated cells were incubated for 1 h at 4 °C with preformed complexes of protein A-Sepharose beads (Pharmacia) and the appropriate antibody, rabbit-anti-$p56^{lck}$ or rabbit-anti-ZAP70. The beads were then washed three times with 1 ml of ice-cold lysis buffer and the proteins eluted with non-reducing SDS-PAGE sample buffer.

2.4. Western blotting

Triton X-100 soluble extracts prepared from 2×10^5 cells per sample or immunoprecipitates were separated on a 10% SDS polyacrylamide gel and transferred to nitrocellulose membrane (Schleicher & Schuell) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). The membranes were blocked using Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% Teleostean gelatin or 3% milk powder and subsequently probed with anti-phosphotyrosine mAb, 4G10 (Upstate Biotechnology Inc.) or mouse monoclonal antibody to $p56^{lck}$ (produced in our laboratory) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (DAKO). Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL plus) detection system (Amersham Pharmacia Biotech). Prestained molecular weight marker was purchased from GIBCO-BRL.

2.5. Measurement of changes in intracellular Ca^{2+} concentration, $[Ca^{2+}]_i$

Elevation of intracellular Ca^{2+} concentration was measured accordingly to Novak and Rabinovitch [21] with slight modification. Briefly, Jurkat cells were suspended in 10^7 cells/ml concentration in culture medium (RPMI 1640 supplemented with 5% FCS) and incubated with 7.5 μM of Fluo-3AM (fluorescence increased upon Ca^{2+} binding) and 7.5 μM of FuraRed-AM (fluorescence decreased upon Ca^{2+} binding, Molecular Probes) for 30 min at 37 °C. Cells were then adjusted to a final concentration of 5×10^5 cells/ml

with cell culture medium and incubated for 30 min at 37 °C. The cells were washed twice with RPMI and resuspended in fresh cell culture medium at 10^5 cells/ml. After stimulation with lyso-PC, the intracellular $[Ca^{2+}]_i$ was measured by cytofluorimetry using FACS Calibur (Beckton Dickinson). The data were presented as the ratio of Fluo-3 and FuraRed fluorescence intensity at 530 and 650 nm, respectively, versus time.

3. Results

3.1. Lysophosphatidylcholine induces rapid tyrosine phosphorylation of intracellular proteins in T lymphocytes

Lyso-PC induced tyrosine phosphorylation of a set of intracellular proteins in a concentration-dependent fashion in leukemic T cell line, Jurkat (Fig. 1A). The maximal phosphorylation was achieved with the addition of lyso-PC to the cells in concentrations of 25 and 50 μg/ml. Induction of tyrosine phosphorylation with 25 μg/ml lyso-PC occurred within 15 s and after 5 min of stimulation the level of phosphorylation declined (Fig. 1B). In the further phosphorylation experiments, we stimulated the cells for 1 min.

The tyrosine kinase, $p56^{lck}$ plays an indispensable role in T lymphocyte activation. To define the contribution of *lck* in the lyso-PC-triggered tyrosine phosphorylation events we analyzed the consequence of the deficiency of *lck* in Jurkat cells. Jurkat and its *lck* deficient variant, JCaM1.6 were stimulated for 1 min and induction of protein tyrosine phosphorylation was investigated. In the absence of $p56^{lck}$ tyrosine kinase, the lyso-PC induced phosphorylation was greatly reduced (Fig. 2A) compared to the wild type Jurkat cells (Fig. 2B), suggesting that $p56^{lck}$ is largely required for the lyso-PC-triggered tyrosine phosphorylation in Jurkat T cells. The phosphorylation signal in JCaM was not enhanced with long (24 h) exposure time of the X-ray film. In the wild type Jurkat cells, among others, two specific proteins were phosphorylated, a 56 and a 70 kDa proteins, likely representing the $p56^{lck}$ and the ZAP70 tyrosine kinases. Blotting $p56^{lck}$ with specific antibody showed the equal amounts of protein loaded to the gel. To identify the bands with 56 and 70 kDa apparent molecular mass, immunoprecipitates of the $p56^{lck}$ and ZAP70 kinases from non-activated and lyso-PC activated Jurkat cell lysates were analyzed by anti-phosphotyrosine blotting. As Fig. 2C shows, lyso-PC induced an increase in the phosphorylation of $p56^{lck}$ and ZAP70 compared to the phosphorylation of these proteins

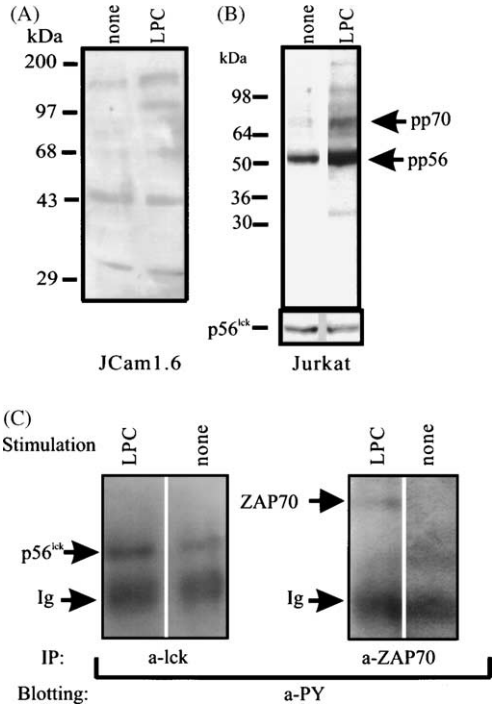

Fig. 2. The protein kinase $p56^{lck}$ is involved in the lyso-PC-triggered signaling events. Lysates were prepared from cells of the $p56^{lck}$ deficient Jurkat variant, JCaM1.6 (A) or Jurkat cells (B) which were stimulated with 25 μg/ml lyso-PC for 1 min at 37 °C or left unstimulated. Bottom panel (B): the quantity of *lck* in stimulated and unstimulated samples was analyzed by immunoblotting. For panel (C), the lysates obtained from stimulated or unstimulated Jurkat cells, were immunoprecipitated with anti-lck or anti-ZAP70 antibodies. The samples were then analyzed by SDS-PAGE and anti-phosphotyrosine Western blotting. The anti-phosphotyrosine reactive bands were visualized with anti-mouse Ig-HRPO and ECL reagent.

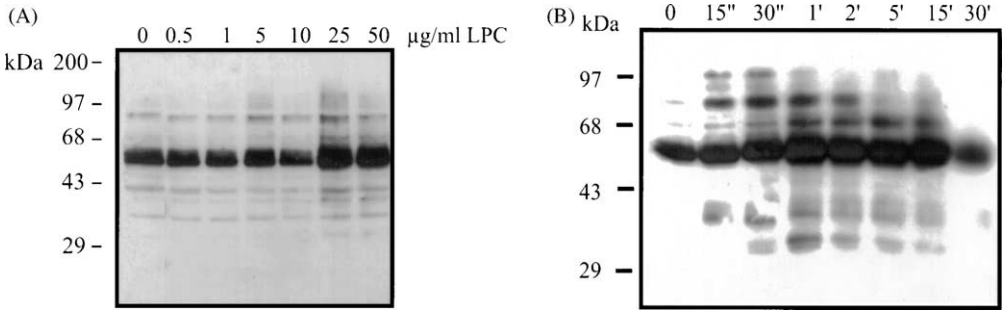

Fig. 1. Lyso-PC induces tyrosine phosphorylation in Jurkat cells in a concentration- and time-dependent fashion. Jurkat cells were treated with different concentrations of lyso-PC for 1 min (A) or with 25 μg/ml lyso-PC for different times (B) at 37 °C or incubated without lyso-PC. The samples were then analyzed by SDS-PAGE and Western blotting with a monoclonal antibody to phosphotyrosine. The anti-phosphotyrosine reactive bands were visualized with anti-mouse Ig-HRPO and ECL reagent.

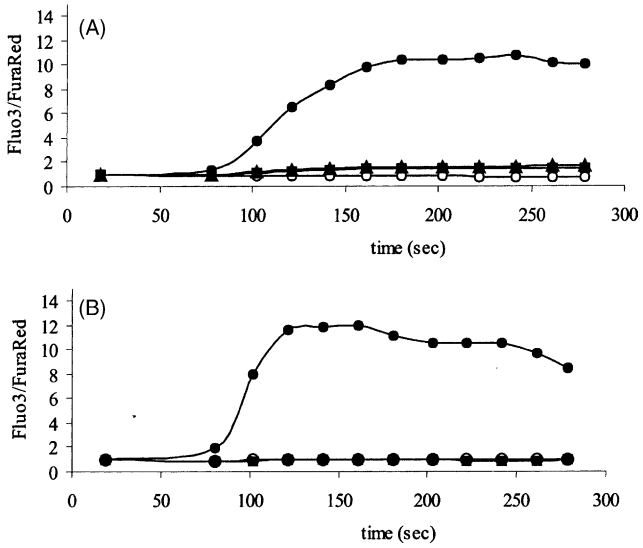

Fig. 3. Treatment of Jurkat cells with lyso-PC results in an elevation of the intracellular Ca^{2+} concentration on a protein kinase and G-protein coupled receptor-dependent fashion. (A) Jurkat cells were loaded with calcium sensitive dyes, Fluo-3AM and FuraRed-AM, then stimulated with lyso-PC solvent, DMSO (open circles) or 45 μg/ml lyso-PC (closed circles) in the presence or absence of protein kinase inhibitors, genistein (50 μM) (closed triangles) or straurosporine (1 μM) (closed squares). (B) The cells were pre-incubated with (closed squares) or without (closed circles) 100 ng/ml pertussis toxin, then loaded with fluorescence dyes and finally activated with 20 μg/ml lyso-PC. Control sample was treated with DMSO (open circles). The change in the intracellular Ca^{2+} concentration was measured by FACS as the ratio of Fluo-3 and FuraRed fluorescence intensity versus time.

in the unstimulated controls suggesting that both PTKs become activated as a result of lyso-PC stimulation.

3.1.1. Lyso-PC induces an increase of the intracellular Ca^{2+} concentration in Jurkat cells in a PTK- and Ser/Thr kinase-dependent manner

Lyso-PC induces a rise in the intracellular Ca^{2+} concentration in different cell types [15,23–25] including Jurkat T cells as it is shown in Fig. 3A. Whether phosphorylation events are involved in the LPC induced Ca^{2+} signal, the broad spectrum, cell permeable inhibitor of Ser/Thr and tyrosine kinases, staurosporin and genistein, respectively, were added to the cells during the Ca^{2+} assay. Suppressing Ser/Thr kinase activity diminished the increase of the intracellular Ca^{2+} concentration in Jurkat cells (Fig. 3A). Activation of *lck* by stimulation of the T cell receptor initiates the signal transduction cascade in which the activation of phospholipase Cγ and as consequence, the elevation of the intracellular Ca^{2+} concentration is triggered. Since $p56^{lck}$ is involved in the lyso-PC induced tyrosine phosphorylation (Fig. 2A) we investigated whether the lyso-PC induced Ca^{2+} signal is a result of the activation of this tyrosine kinase. The $p56^{lck}$ deficient Jurkat cell line reacted with a similar magnitude of Ca^{2+} response to lyso-PC or Ca^{2+} ionophore stimulation compared to its wild type counterpart, but we observed that the maximum of the Ca^{2+} response in JCaM to lyso-PC but not ionophore treatment was always delayed compared to that of Jurkat (Table 1).

Recently it has been shown that G-protein coupled seven transmembrane receptor, G2A is a receptor for lyso-PC on T cells and stimulation of this receptor results in the elevation of the intracellular Ca^{2+} level [31]. According to this, pertussis toxin completely abrogated the LPC induced Ca^{2+} response in Jurkat cells (Fig. 3B).

4. Discussion

Lyso-PC, the major phospholipid component of oxidized LDL, plays significant role in the pathological process, atherosclerosis. It functions as a chemoattractant for monocytes [10] and T lymphocytes [11,12]. In addition to attracting the T cells to the site of atherosclerotic lesion and sites of inflammation, lyso-PC enhances the production of interferon-γ by the activated T cells and in this manner promotes the inflammatory reaction [7,9]. Possible involvement of tyrosine kinases has been described in regulation of ICAM-1 expression following lyso-PC treatment of cultured human umbilical vein endothelial cells [14]. Also, lyso-PC stimulated the tyrosine phosphorylation of the proline-rich tyrosine kinase, PYK2, a mediator of the phospholipid induced Ras signaling pathway in vascular endothelial cells [19]. Recent characterization of G2A and GPR4 as receptors for lyso-PC has provided important steps to understand the lyso-PC signaling [30–32]. However, the early effects of lyso-PC on T cells has still not been well-described.

In the present work we show that a remarkable tyrosine phosphorylation is induced as early as 15 s after addition of lyso-PC to the Jurkat T cell line. Among others, two proteins are tyrosine phosphorylated in this response,

Table 1
Absence of $p56^{lck}$ in Jurkat cells results in the delay of the Ca^{2+} response upon lyso-PC stimulation

	Time of maximum Ca^{2+} signal upon lyso-PC stimulation (sec)	Time of maximum Ca^{2+} signal upon Ca^{2+} ionophore stimulation (s)	Maximum level of Ca^{2+} signal upon lyso-PC stimulation	Maximum level of Ca^{2+} signal upon Ca^{2+} ionophore stimulation
Jurkat	161 ± 20.66	278	8.42 ± 0.91	9.98
JCaM1.6	261 ± 42.03	278	11 ± 2.9	12.3

Jurkat or JCaM1.6 cells were loaded with fluorescence Ca^{2+} dyes (see Section 2) and stimulated with 45 μg/ml lyso-PC or with 2 μg/ml Ca-ionophore A23187.

p56^{lck} and ZAP70, the intracellular tyrosine kinases that play central roles in T cell signaling. Implication of p56^{lck} in the lyso-PC-triggered signaling pathway is demonstrated by the dramatic reduction of tyrosine phosphorylation in *lck*-deficient Jurkat variant, JCaM1.6, upon lyso-PC stimulation. During the TCR signaling, the activation of p56^{lck} and subsequently ZAP70, results in the formation of an active signalosome [20]. This signaling complex contains activated phospholipase Cγ-1 (PLCγ-1) that controls the Ca^{2+} mobilization and the activation of PKC (reviewed in [22]). Lyso-PC treatment of the Jurkat cells also increases the intracellular Ca^{2+} concentration. However, this change in the intracellular Ca^{2+} level does not seem to occur via the TCR signaling pathway, since the absence of p56^{lck} does not abrogate although it modulates the kinetics of the Ca^{2+} signal. The tyrosine kinase inhibitor, genistein diminishes the Ca^{2+} response indicating that other tyrosine kinase(s) regulates the process.

The role of the PKC activation in lyso-PC induced cell response has been confirmed in several studies [1,15,16]. Staurosporine, the inhibitor of Ser/Thr kinases impedes the lyso-PC induced Ca^{2+} elevation suggesting that both Ser/Thr and tyrosine kinase pathways are involved in the regulation of the intracellular Ca^{2+} concentration.

Multiple factors are involved in the pathogenesis of atherosclerosis including activated T lymphocytes and T cell derived cytokines (reviewed in [26]). Chemotactic and antigenic activation of T cells by oxidized LDL (oxLDL) and as a result, production of pro-inflammatory cytokine, interferon-γ occurs at the site of the atherosclerotic lesion [27,28]. OxLDL serves as a source of lyso-PC, the main lipid component of ox-LDL, that contributes to the immunomodulatory effect of oxLDL. In this work we show that lyso-PC triggers early signaling events in T cells, such as activation of p56^{lck} and likely of other protein tyrosine kinases and then the elevation of the intracellular Ca^{2+} concentration. The latter is regulated by multiple signaling components, such as the presence of p56^{lck} and other, uncharacterized tyrosine and Ser/Thr kinases and also by the activity of a G-protein coupled lyso-PC receptor.

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II.

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Receptor tyrosine phosphatase, CD45 binds galectin-1 but does not mediate its apoptotic signal in T cell lines

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Abstract

Galectin-1 (Gal-1) is an endogenous mammalian S-type lectin with highly pleiotropic effect on different tissues. The viability of the lymphoid cells is reduced by gal-1 by triggering apoptosis, however, the mechanism of the gal-1 induced apoptosis is still under investigation. The receptor tyrosine phosphatase, CD45, a heavily glycosylated cell surface molecule binds to gal-1 with high affinity, however, its contribution to the gal-1 induced apoptosis is still controversial. In this study we show that galectin-1 binds to cells deficient for CD45, although CD45 is one of the galectin-1-binding cell surface proteins on T cells. Moreover, the CD45 deficient Jurkat variant, J45.01 responds readily with tyrosine phosphorylation and subsequent apoptosis to galectin-1 treatment in a similar degree as its wild type counterpart, Jurkat does. These results strongly indicate that CD45 is not the receptor via gal-1 mediates the apoptotic signal into the cells as it was suggested in previous studies. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Galectin-1; CD45 tyrosine phosphatase; Apoptosis; Internalization; Tyrosine phosphorylation

1. Introduction

Galectin-1 (gal-1) is a prominent member of the S-type mammalian lectins, the galectin family. These proteins are characterized by high affinity for β-galactoside on complex glycoconjugates and by a highly conserved carbohydrate recognition domain (CRD) [1]. Gal-1 is expressed in different tissues [2] and secreted by an unconventional way [3]. Galectin-1 is present at the sites of apoptosis during normal T-cell development and maturation secreted by thymic epithelium cells [4]. According to this report, gal-1 induces apoptosis of immature thymocytes, activated T-cells, and several T leukemia cell lines in vitro [5,6]. Recently, different T cell surface receptors have been identified as gal-1 binding proteins, including CD45, CD43, CD2, CD3, CD4 and CD7 [7,8]. From these receptors CD45 has been implicated as a putative mediator of the biological effect of gal-1 in T lymphocytes [9,10].

CD45 is a transmembrane tyrosine phosphatase [11,12] with isoforms that are generated by alternative splicing of the N terminal extracellular part [13]. All isoforms are heavily glycosylated on the extracellular part [14]. Function of the intracellular phosphatase activity has been well established, since it primarily regulates T cell activation via the T-cell receptor [15]. Although the large N terminus of the molecule displays several features of a receptor, the role of the extracellular part in the CD45 function and the ligand for CD45 still remains unidentified. Monoclonal antibodies (MoAbs) to the CD45 modulate the T-cell receptor (TCR) induced proliferation [16]. The tyrosine phosphatase activity of a chimera molecule composed of the

Abbreviations: FCS, fetal calf serum; Gal-1, galectin-1; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate; TCR, T-cell receptor complex.

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extracellular part of the epidermal growth factor receptor (EGFR) and the intracellular part of the CD45 is negatively regulated by epidermal growth factor [17]. Lectins such as B cell surface receptor, CD22β [18,19] or plant lectins, phytohemagglutinin (PHA) and concanavalin-A (Con A) bind to CD45 [20–22]. Recent studies showed that galectin-1 also couples to CD45 and decreases its phosphatase activity [9,10], therefore, gal-1 was suggested to be a natural ligand for this phosphatase. It has also been indicated that gal-1 triggered apoptosis in T cells is mediated via CD45. The role of CD45 in apoptotic processes have been suggested in other studies, as well [23,24]. However, a recent report has not strongly supported the importance of the CD45 in galectin-1 induced apoptosis [8].

To determine the role of the CD45 in gal-1 induced apoptosis we analyzed galectin-1 binding to Jurkat T cell line and its CD45 deficient counterpart, J45.01. Both cell lines bound and internalized gal-1. Moreover, similar sets of proteins were isolated from Jurkat and J45.01 by the lectin, except that the CD45 bands were absent from the gal-1 binding proteins isolated from CD45 deficient cells. Stimulation of both cell lines with gal-1 resulted in a rapid tyrosine phosphorylation of intracellular proteins. In spite of the absence of CD45 in J45.01, gal-1 readily induced a pronounced and similar degree of apoptosis in both wild type Jurkat and J45.01. Our results prove that gal-1 triggered apoptosis is not mediated by the CD45, however, it cannot be excluded that other gal-1 signals are transmitted via the phosphatase.

2. Materials and methods

2.1. Cells and reagents

Leukemic T cell lines, Jurkat and MOLT-4 were cultured in RPMI 1640 (GIBCO-BRL) medium supplemented with 5% heat inactivated fetal calf serum (FCS) (Protein GMK) at 37° C in an incubator with 5% CO_2. The CD45 deficient Jurkat variant, J45.01, was cultured in RPMI 1640 with 10% FCS under the same conditions. Reagents were purchased from Sigma if not stated otherwise. Human recombinant galectin-1 was cloned and purified as previously described [25].

2.2. Analysis of galectin-1 binding to cell surface by flow cytometry

The cells were resuspended in cold phosphate buffered salt (PBS) supplemented with 1% FCS and 0.1% NaN_3 (FACS buffer) at 5×10^6 cells/ml and incubated with 50 μg/ml (3.6 μM) FITC conjugated galectin-1 for 1 h at 4° C. The cells were then washed in FACS buffer and analyzed on FACS Calibur cytofluorimeter (Becton Dickinson).

2.3. Precipitation of biotinylated cell surface proteins by galectin-1 Sepharose beads

Jurkat cells (8×10^7) were cell surface biotinylated with 500 μg/ml sulfo-NHS biotin (Pierce) in ice cold PBS supplemented with 1 mM $MgCl_2$ and 0.1mM $CaCl_2$ for 20 min at 4° C, as previously reported [26]. The cells (2×10^7 cells/sample) were then lysed for 30 min in ice cold lysis buffer (50mM HEPES pH 7.4, 1% Triton X-100, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF) and 10 μg/ml leupeptin). Postnuclear supernatants were mixed with 10 μl Sepharose 4B beads (Pharmacia) covalently coupled to galectin-1 or an indifferent protein (control) and incubated for 1 h at 4° C. The immunoprecipitates were washed two times in lysis buffer and then mixed with equal volumes of 2× Laemmli buffer containing 2-mercaptoethanol and loaded onto a 7.5–15% gradient SDS polyacrylamide gel. Proteins were then transferred to nitrocellulose membrane (Schleicher and Schuell) in transfer buffer (25 mM Tris, 192 mM glycine, 20% methanol). Membranes were blocked using Tris-buffered saline (TBS) containing 0.05% Tween 20 and 3% cold fish gelatin and subsequently probed with streptavidin-horseradish peroxidase (DAKO) to detect the biotin labeled membrane proteins in galectin precipitation. Alternatively, the membranes were probed with CD45 monoclonal antibodies, KD3, GB3 [27] and rabbit anti-mouse IgG-HRPO (DAKO). Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL plus) detection system (Amersham). Prestained molecular weight marker was purchased from GIBCO-BRL.

2.4. Tyrosine phosphorylation analysis in Western blot

The cells were stimulated at 5×10^7 cells/ml in RPMI without FCS by adding the indicated amounts of galectin-1. Activation was stopped by addition of equal volume of 2× concentrated, ice cold lysis buffer, supplemented with the following phosphatase inhibitors: 20 mM NaF and 200 μM Na_3VO_4. The cells were lysed on ice for 30 min and cleared off nuclear/cytoskeletal components by centrifugation at 12 000 × *g* for 15 min. Postnuclear supernatants were separated on a 7.5–15% gradient SDS polyacrylamide gel and then transferred to nitrocellulose membrane. Membranes were blocked using TBS containing 0.05% Tween 20 and 3% cold fish gelatin and subsequently probed with anti-phosphotyrosine mAb 4G10 (Upstate Biotechnology Inc.) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (DAKO).

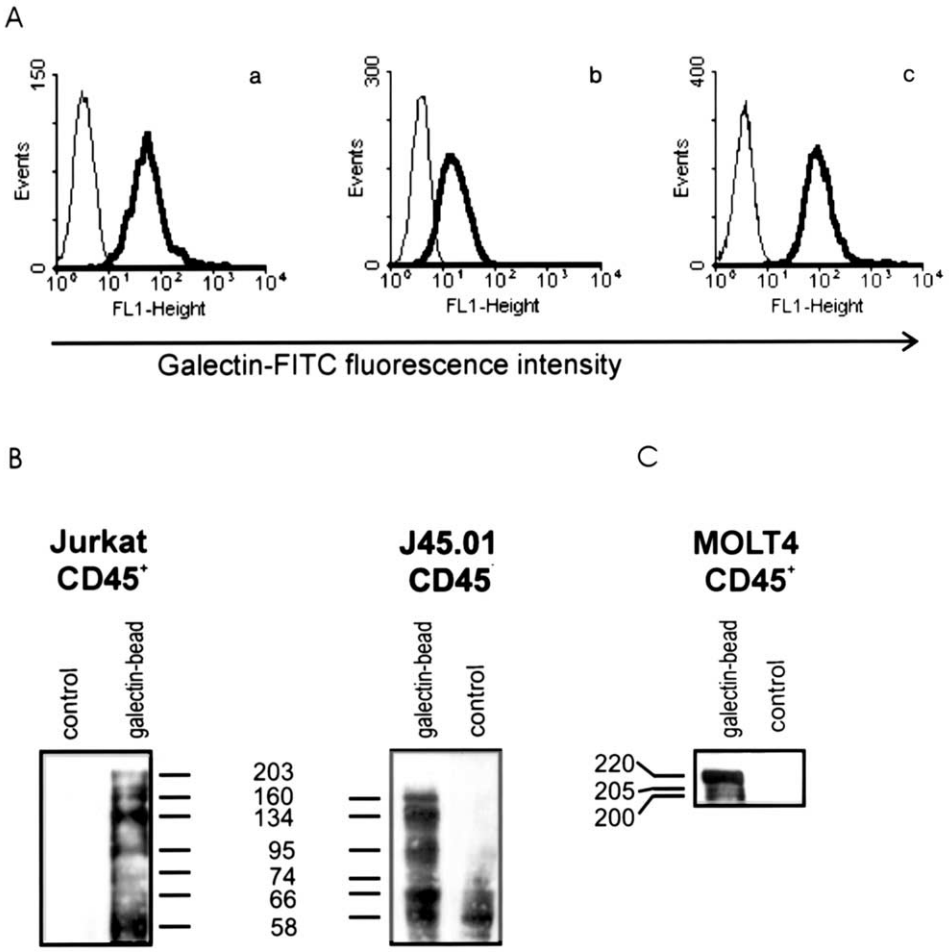

Fig. 1. Galectin-1 binds to T cells and interacts with a number of cell surface proteins including CD45. (A) The T cell lines, Jurkat (a), CD45 deficient Jurkat, J45.01 (b) and MOLT-4 (c) were incubated with 50 μg/ml (3.6 μM) galectin-1-FITC for 1 h at 4° C. The cells which bound galectin-1 were analyzed by flow cytometry. (B) The cells were cell surface biotinylated then extracted in lysis buffer. The cell lysates were precipitated with galectin-1-Sepharose 4B beads, and the proteins were analyzed in Western blotting, using streptavidin-HRPO. Molecular masses of the precipitated proteins are indicated. (C) Galectin-1 precipitates prepared from MOLT-4 cells as described for Jurkat cells were probed with a cocktail of CD45 monoclonal antibodies (KD3, GB3) followed by anti-mouse IgG-HRPO. Molecular masses of CD45 isoforms are indicated on the left side.

2.5. Measurement of apoptosis

Cells were treated as indicated and subjected to DNA content analysis. Briefly, the cells were harvested and washed two times with PBS containing 0.1% glucose, then permeabilized and stained in the following solution: PBS supplemented with 0.1% Triton X-100, 0.1% Na_3 citrate, 10 μg/ml RNase and 10 μg/ml propidium iodide. After incubation for 30 min at room temperature in dark, the cells were analyzed on FACS Calibur cytofluorimeter using MODFIT software program (Becton Dickinson). The sub-G1 population in the cell cycle analysis was considered as apoptotic. The percentage of apoptotic cells was counted as follows: apoptosis% = apoptosis (galectin)-apoptosis (control).

2.6. Galectin-1 internalization

Cells were resuspended in 5×10^5 cells/ml concentration in cell culture medium and incubated with 50 μg/ml (3.6 μM) biotinylated galectin-1 at 4° C for 1 h. The cells were then washed twice in RPMI and incubated for subsequent 3 h either at 4° C to inhibit or 37° C to promote the internalization process. The cells were then washed in ice cold FACS buffer, and were incubated with the MoAbs anti-CD45RA (KD3) or anti-CD45 (GB3) [27] for 30 min at 4° C followed by goat anti-mouse IgG-FITC and streptavidin-Quantum Red for 30 min at 4° C. The cells were then washed in FACS buffer and analyzed on FACS Calibur cytofluorimeter.

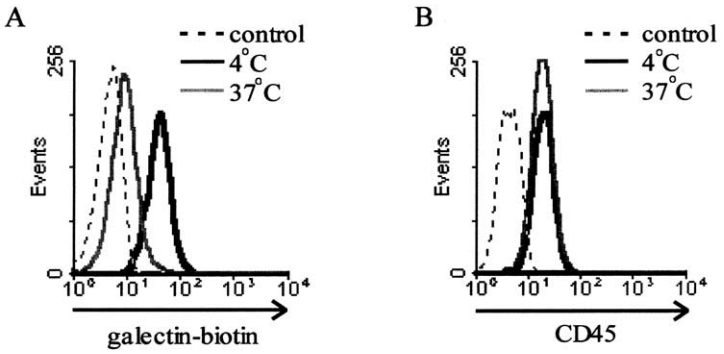

Fig. 2. Galectin-1 does not co-internalize with CD45. Jurkat cells were incubated with 50 μg/ml (3.6 μM) of biotinylated galectin-1 for 1 h at 4° C, then washed in cell culture medium. The cells were then incubated at either 4 or 37° C. The cells were then double stained: cell surface biotinylated galectin was detected with streptavidin-Quantum Red (left panel) and level of CD45 was estimated using monoclonal antibodies against CD45 followed by goat anti-mouse Ig-FITC (right panel).

3. Results and discussion

The extracellular part of the CD45 has been implicated as a major functional receptor for galectin-1, mediating the gal-1 induced apoptosis in T cell lines [5,9,10]. To analyze the consequence of galectin-1-CD45 interaction first we identified the cell surface proteins that bound to the gal-1 affinity sorbent. As Fig. 1A shows, two different T cell lines, Jurkat and MOLT-4, bound similar amount of FITC-labeled gal-1, however, the CD45 deficient Jurkat variant bound much less gal-1, indicating a role of CD45 in gal-1 binding. Affinity precipitation form Jurkat cells using Sepharose coupled gal-1 beads resulted in the detection of a series of cell surface proteins including proteins with molecular weight about 200 kDa (Fig. 1B). Patterns of gal-1 binding proteins isolated from CD45 deficient Jurkat variant, J45.01 and wild type Jurkat cells were similar, except that the proteins with 200 kDa were missing from J45.01. The latter proteins turned out to be CD45 glycoproteins, when the gal-1 binding proteins were hybridized with CD45 antibodies (Fig. 1C). These results were in accordance with the previous reports which showed that CD45 binds gal-1 with high affinity [5,9,10]. However, it is noteworthy that in our experiment CD45 was not the major gal-1-binding protein since several proteins with molecular mass of 160, 134, 95 and 58 kDa were bound with similar strength (Fig. 1B).

It has been shown in recent studies that binding of gal-1 to cells resulted in a series of signal transduction events in T and B cells which regarded CD45 signaling. The most pronounced change following the binding was a decrease of the tyrosine phosphatase activity of CD45 [9,10] and as a consequence the elevated tyrosine phosphorylation and decreased kinase activity of B cell tyrosine kinase, *lyn* [9]. However, it has not been well documented whether the regulation of the CD45 activity and the following signaling events were the direct outcome of the ligation of CD45 by galectin-1 or a concomitant result of the trigger of other receptors. Although gal-1 is secreted from the producing cells [3] and couples to the above mentioned receptors in T and B lymphocytes, it is not known whether the biological effect of the extracellular gal-1 is initiated via coupling to cell surface receptors or intracellularly after internalization. As Fig. 2A shows the large majority of the cell surface-bound gal-1 is internalized at 37° C. This internalization does not occur at 4° C indicating that internalization is mediated via cell surface receptor(s). The receptor, transmitting gal-1 into the cell, is not identical with CD45, because the reduction of the cell surface amount of CD45 does not accompany the decrease of that of gal-1 (Fig. 2B).

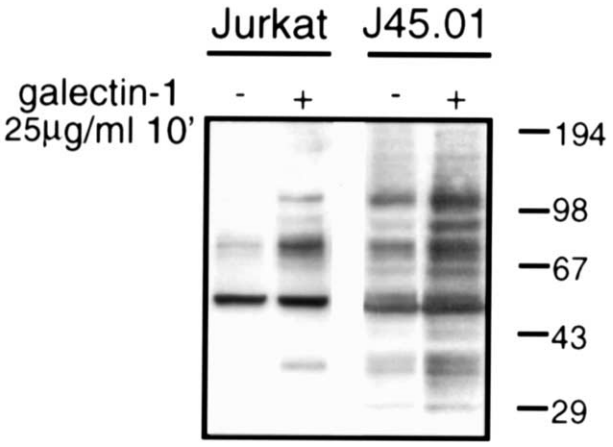

Fig. 3. Galectin-1 induces tyrosine phosphorylation in wild type and CD45 deficient Jurkat cells. Jurkat cells and the CD45 deficient Jurkat variant, J45.01, were stimulated with 25 μg/ml (1.8 μM) galectin-1 for 10 min at 37° C or left unstimulated. The samples were then analyzed in Western blotting with monoclonal antibody to phosphotyrosine and anti-mouse Ig-HRPO. Molecular weight standards are indicated on the right side of the blotting.

To understand the role of the CD45 in the gal-1 effect regarding apoptosis, we compared the gal-1 induced cell response in Jurkat T cells and their CD45 deficient counterpart, J45.01. Gal-1 treatment resulted in a

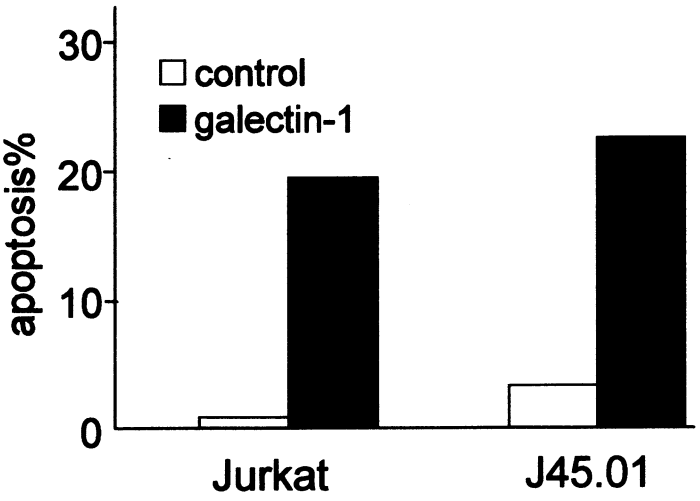

Fig. 4. Galectin-1 induces apoptosis in Jurkat cells and in CD45 deficient Jurkat cells. Jurkat and J45.01 cells were co-cultured with galectin-1 (1.8 μM) at 37° C. The cells were harvested after 24 h and analyzed for DNA content by flow cytometry. The percentage of sub-G1 population was considered as apoptotic cells based on the calculation with MODFIT program. The experiment presented is a representative of three separate experiments.

definite tyrosine phosphorylation in both wild type and CD45 deficient Jurkat cells although the constitutive tyrosine phosphorylation was much higher in the phosphatase deficient J45.01 cells (Fig. 3). The gal-1 induced tyrosine phosphorylation was essential for the subsequent apoptosis, since tyrosine kinase inhibitor, genistein blocked the apoptosis when added together with gal-1 to the cells (R. Fajka-Boja, submitted for publication). Jurkat and CD45 deficient J45.01 responded with a similar degree of apoptosis when co-cultured for 24 h with gal-1 under identical conditions (Fig. 4). The data published so far have supported the idea that CD45 can be the major receptor mediating the gal-1 induced apoptosis in lymphocytes [5,10]. Our result has not confirmed this since the Jurkat variant, J45.01 readily responded to gal-1 stimulus with apoptosis in the absence of functional CD45. The disagreement between our results and results of others could be explained by the difference between experimental approaches. Most of the studies were based on the inhibition of gal-1 induced apoptosis by specific antibodies to CD45 [5,7]. However, this approach may not be the adequate one because immununoglobulins are glycosylated and may compete for the galectin-1 as it was observed in our experiments (M. Szemes, unpublished observation).

It is an attractive theory that galectin-1 is the physiological ligand for CD45 because in spite of the intensive search, the natural ligand of CD45 isoforms has not been identified, yet. The glycosylation likely fulfills important role in the regulation of CD45 via the extracellular part, since lectins bind to CD45 and some of them affect its phosphatase activity [18–20]. Moreover, it has been shown that CD45 cross-linking may be a target for apoptosis induction in immature thymocytes and T and B lymphocytes [23,24]. Our results prove that gal-1 does not induce apoptosis via the CD45, however, it cannot be excluded that other signals are transmitted via CD45. It remains elusive whether gal-1 induces apoptosis after internalization or what is the receptor that mediates such an effect of galectin-1.

Acknowledgements

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III.

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A novel anti-inflammatory function of human galectin-1: inhibition of hematopoietic progenitor cell mobilization
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Experimental Hematology 35 (2007) 305–313

A Novel Anti-Inflammatory Function of Human Galectin-1: Inhibition of Hematopoietic Progenitor Cell Mobilization

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Objective. **The immunosuppressive and anti-inflammatory activity of mammalian galectin-1 (Gal-1) has been well established in experimental in vivo animal models and in vitro studies. Since the proliferation and migration of leukocytes represent a necessary and important step in response to the inflammatory insult, we have investigated whether Gal-1 affects the mobilization of hematopoietic progenitor cells (HPC) induced by cyclophosphamide (CY) and granulocyte colony-stimulating factor (G-CSF).**

Methods. **Bone marrow HPCs were mobilized with CY/G-CSF or CY/G-CSF plus human recombinant Gal-1 in BDF1 mice. Bone marrow (BM) and blood cells were taken at different time points and analyzed for their in vivo repopulating ability in lethally irradiated syngeneic animals. The number of myeloid progenitor cells in BM and blood samples was determined by colony-forming cell assay. Expression of surface markers (Sca-1, CD3ϵ, CD45R/B220, Ter-119, GR-1, and CD11b) on nucleated marrow cells was measured by flow cytometry. The lymphocytes, granulocytes, and monocytes in blood samples were counted after Giemsa staining.**

Results. **Gal-1 dramatically inhibited CY/G-CSF-induced HPC migration to the periphery as well as decreased peripheral neutrophilia and monocytosis in a dose- and time-dependent manner. In contrast, Gal-1 itself stimulated HPC expansion and accumulation within the BM. The presence of the lectin for inhibition of HPC mobilization was essential during the second half of the treatment. Moreover, Gal-1 inhbited transendothelial migration of BM-derived HPCs in response to SDF-1 in vitro.**

Conclusion. **Gal-1 blocked BM progenitor cell migration induced by CY/G-CSF treatment, indicating a novel anti-inflammatory function of the lectin. We suggest that the inhibition of HPC mobilization occurs mainly via obstructing the transendothelial migration of BM-derived cells including primitive hematopoietic and committed myeloid progenitor cells and mature granulocytes and monocytes.** **© 2007 International Society for Experimental Hematology. Published by Elsevier Inc.**

Galectins are a growing family of animal lectins defined by their affinity for β-galactosides and by conserved sequence elements in their carbohydrate recognition domains [1]. One of the best-studied family members is galectin-1 (Gal-1), a homodimeric protein composed of noncovalently bound subunits with one carbohydrate recognition domain of 134 amino acids. Expression of Gal-1 has been identified in lymphoid organs and in immune-privileged sites such as placenta and cornea, suggesting an important role in generating and maintaining immune tolerance [2,3]. In vitro, Gal-1 induces cell-cycle arrest and apoptosis of activated T cells or T-cell lines [4–8]. However, the in vivo functions of Gal-1 are currently unclear because targeted disruption of the *Gal-1* gene in null mutant mice results in the absence of major phenotypic abnormalities, perhaps because of compensation by other family members [9]. On the other hand, exogenous administration of recombinant Gal-1 prevents the development of experimental autoimmune encephalomyelitis in rats [10]. The injection of fibroblasts

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genetically engineered to secrete Gal-1 abrogates the manifestation of collagen-induced arthritis in DBA/1 mice [11]. This anti-inflammatory/immunosuppressive outcome has also been observed in concanavalin-A-induced hepatitis [12], in experimental colitis [13], and in graft-vs-host disease [14]. Moreover, it has been also shown that Gal-1 inhibits chemotaxis and trans-endothelial migration of polymorphonuclear leukocytes in vitro [15]. Expression of Gal-1 in bone marrow–derived mesenchymal (stromal) cells has been recently detected and implicated in bone marrow (BM) cell differentiation [16,17]. Accordingly, we described a biphasic growth-promoting and apoptosis-inducing capacity of the lectin on BM cells of different differentiation stage [18]. Since hematopoietic progenitor cells (HPC) are massively mobilized during severe inflammatory response we analyzed whether the anti-inflammatory Gal-1 affects the immune reaction at the level of BM cell mobilization in vivo. We triggered BM cell release using cyclophosphamide (CY) and granulocyte colony-stimulating factor (G-CSF) in a murine model. Here we demonstrate that Gal-1 is able to efficiently inhibit the CY/G-CSF-induced HPC, granulocyte, and monocyte migration from BM into the blood flow.

Materials and methods

Mice

Male and female (C57Bl/6 × DBA/2)F1 (BDF1) mice were obtained from the animal facility of the National Institute of Oncology (Budapest, Hungary) and were used at 8 to 12 weeks of age. The animals were fed with commercial rodent chow and acidified water ad libitum. All experimental protocols were approved by the institutional ethical committee on animal experiments.

Mobilization protocol

On days 0 and 2, mice were injected intraperitoneally with ~200 mg/kg (generally 3–4 mg/mouse) CY (Cytoxan, Bristol-Myers Squibb, Baar, Switzerland). Then, on each successive day, animals were injected subcutaneously with ~250 μg/kg (generally 5 μg/mouse) human recombinant G-CSF (Neupogen, F Hoffman-La Roche, Basel, Switzerland). On day 5, mice were sacrificed, and bone marrow and blood samples were collected (Fig. 1).

Galectin-1 treatment

Human recombinant galectin-1 was cloned and purified as previously described [5]. For in vivo experiments, galectin-1 was diluted to the desired concentration in phosphate-buffered saline (PBS) containing 250 μM β-mercaptoethanol (Sigma, St. Louis, MO, USA) and administered as indicated (Fig. 1).

Cell preparation and staining

Marrow was flushed from the femurs and tibias of experimental animals. Single-cell suspensions were prepared by drawing the bone marrow cells through a 25-gauge needle, then expelling them back through the needle and through a nylon mesh screen. After standard erythrocyte lysis, nucleated cells were washed twice before further use.

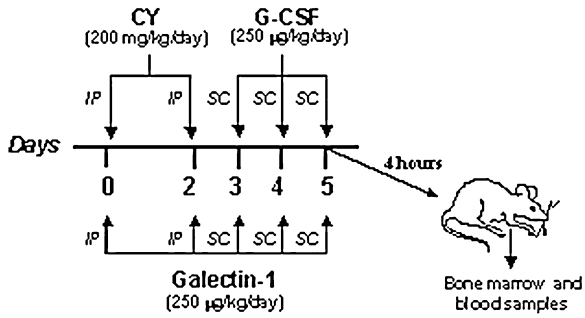

Figure 1. Experimental protocol. BDF1 mice were injected with CY (4 mg/mouse/day) intraperitoneally (IP) and with human G-CSF (5 μg/mouse/day) subcutaneously (SC) on successive days. Recombinant Gal-1 (5 μg/mouse/day) was injected together with CY and/or G-CSF, IP and SC, respectively. Mice were sacrificed 4 hours after the last G-CSF injection on day 5.

For phenotypic analysis, marrow cells were stained with PE-conjugated monoclonal anti-Sca-1 (clone D7) and a panel of biotinylated lineage-specific antibodies against CD3ϵ (clone 145-2C11), CD45R/B220 (clone RA3-6B2), CD11b (clone M1/70), Ly-6G (clone RB6-8C5), and TER-119 (clone TER-119) (BD PharMingen, San Diego, CA, USA) for 30 minutes at 4°C. Cell-bound biotinylated antibodies were detected with streptavidin-PE (Sigma). Stained cells were washed with PBS and analyzed immediately on a FACScan flow cytometer using Cell Quest software (Becton-Dickinson, Mountain View, CA, USA).

Blood samples were collected by cardiac incision in the presence or absence of heparin (Sigma) (for functional assays or for counting blood smears, respectively). Blood smears were stained with May-Grunwald-Giemsa solution.

Stem cell transplantation

Recipient BDF1 mice were lethally irradiated with 9 Gy from a ^{60}Co gamma source and injected intravenously with 50 or 100 μL of blood obtained from BM-mobilized animals. Survival was followed for at least 90 days.

Progenitor (colony-forming cell) assay

Quantification of the number of colony-forming units granulocyte-macrophage (CFU-GM) was performed using a semisolid CFC assay. Mouse bone marrow cells or blood samples were plated in IMDM supplemented with 1% methylcellulose, 30% horse serum (Gibco, Grand Island, NY, USA), 10% WEHI-3B conditioned medium as source of growth factors, 4×10^{-3} M/L L-glutamine, 2.5×10^{-4} M/L a-thioglycerol (Gibco), 1% deionized bovine serum albumin (Sigma), and antibiotics (Gibco). Cells were cultured in 35-mm petri dishes (BD Falcon, Bedford, MA, USA) at 37°C in 5% CO_2 in air. Colony formation in methylcellulose was scored using an inverted microscope after 7 days of culture.

Annexin V labeling

To detect cell-surface exposure of phosphatidyl serine, nucleated BM cells were washed twice with PBS and resuspended in binding buffer (0.01 M HEPES, 0.14 M NaCl, and 2.5 mM $CaCl_2$). Annexin V–FITC (Sigma) and propidium iodide (10 μg/mL) (Sigma) or annexin V–FITC and PE-labeled anti-Sca-1 antibody were added to the cells for 15 minutes in the dark at room temperature.

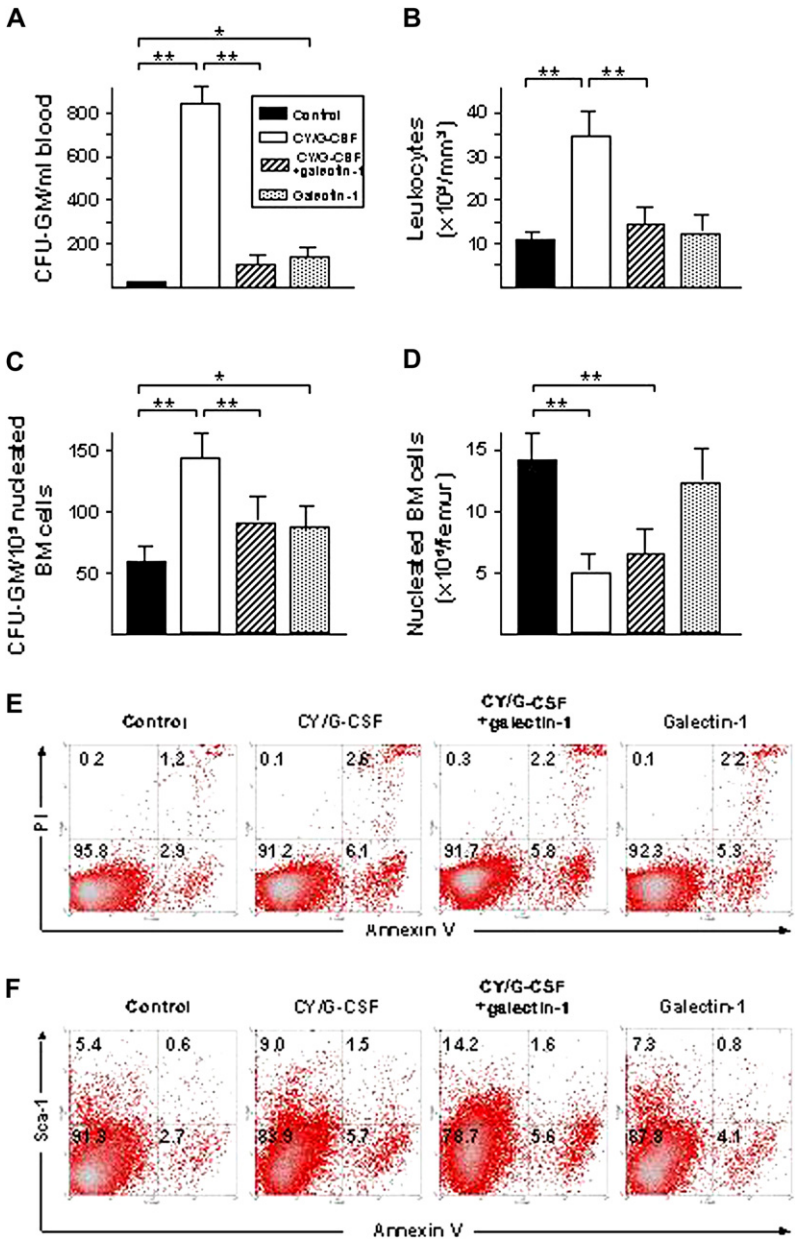

Figure 2. Effect of Gal-1 on the mobilization of granulocyte-macrophage colony-forming cells. Total leukocyte counts and CFU-GM frequencies were determined in blood **(A,B)** and BM **(C,D)** samples isolated from mice treated with either saline, CY/G-CSF, CY/G-CSF+Gal-1, or Gal-1 alone. The results are expressed as mean ± SD of 7 independent experiments. Statistically significant differences were indicated by asterisks (*$p < 0.05$, **$p < 0.001$). The percentage of apoptotic BM cells **(E)** and Sca-1$^+$ marrow cells **(F)** was measured by annexin V labeling. Representative data from one typical experiment is shown.

After washing, the cells were analyzed on a FACScan cytofluorimeter.

Chemotaxis and transendothelial migration assays

Chemotaxis and transendothelial migration assays were performed in 24-well tissue culture plates (BD Falcon) using Millicell culture plate inserts (Millicell-PCF, Millipore Co., Cork, Ireland) of 12-mm diameter, with 3-μm pore filters as described by La et al. [15], with some modifications. Briefly, in transendothelial migration experiments, KS-Imm cells (a Kaposi sarcoma–derived CD31, CD34, vWF, and c-kit$^+$ immortalized endothelial cell line) [19,20] were plated at 100,000 cells/Millicell inserts and placed in 24-well tissue culture plates. Nonadherent cells were removed after 18 hours and the adherent cells were cultured for an additional 2 to 3 days to obtain confluent endothelial monolayers. On the day of the experiment, 10^6 freshly prepared nucleated BM cells were added to the upper compartment in 400 μL medium (DMEM with 10% FCS) with or without Gal-1, and 600 μL medium containing100 ng/mL recombinant mouse SDF-1 (R&D Systems, Minneapolis, MN, USA) was added to the lower compartment.

After 4 or 6 hours incubation at 37°C in 5% CO_2, cells migrating through the empty filters (chemotaxis) or the endothelial monolayers (transmigration) were retrieved from the lower chamber, counted by light microscopy, washed, and further tested in CFU-GM assay (see above).

Statistics

Data were analyzed for statistical significance using the Student's *t*-test (Microsoft Excel, Microsoft Corporation, Seattle, WA, USA). Differences were considered significant at $p < 0.05$ or $p < 0.001$.

Results

Galectin-1 inhibits mobilization of committed hematopoietic progenitor cells

To elucidate the effect of exogenously injected Gal-1 in HPC mobilization, we determined the total leukocyte number and CFU-GM frequency in BM and blood samples isolated from BM cell–mobilized mice. Treatment of animals with CY/G-CSF caused an approximately 40-fold increase of CFU-GM frequency in the blood (Fig. 2A). This elevation resulted in threefold increase in the total number of blood leukocytes (Fig. 2B). Co-administration of Gal-1 with CY/G-CSF markedly ameliorated the migration of progenitor cells from BM to the periphery (Fig. 2A,B). Inhibition of Gal-1-induced BM cell mobilization was not complete (~85%), since concomitantly with the inhibitory effect, Gal-1 itself induced a low but significant mobilization. In the marrow, CFU-GM frequency increased about twofold after CY/G-CSF treatment, which was inhibited with Gal-1 co-administration (Fig. 2C). In contrast, the total number of nucleated cells decreased upon CY/G-CSF treatment and Gal-1 had no significant effect on these changes (Fig. 2D). Although the frequency of CFU-GM in femur decreased when Gal-1 was co-administered with mobilizing agents, the total number of the progenitor cells did not change significantly. Comparing the total number of CFU-GM in the femur of CY+G-CSF vs CY+G-CSF+Gal-1 groups, the frequency of these cells was 142.0 ± 15.6 vs 93.9 ± 8.6 / 100,000 BM cells, the total nucleated BM cell number was 5.2 million ± 1.1 million vs 6.8 million ± 1.9 million; hence 7384 and 6385 CFU-GM cells were present in the femur from CY+G-CSF vs CY+G-CSF+Gal-1 groups, respectively. Inhibition of BM cell mobilization did not occur due to Gal-1 cytotoxicity since Gal-1 treatment did not cause apoptosis of BM cells or the Sca-1^+ fraction of marrow cells under these experimental conditions (Fig. 2E,F).

Dose and time dependence of Gal-1-mediated inhibition of BM cell mobilization

Next we analyzed the dose and time dependence of Gal-1-mediated inhibition of clonogenic myeloid progenitor cell mobilization in response to CY and G-CSF. As seen in Figure 3A, as little as 2.5 μg/mouse (corresponding to 125 μg/kg body weight) of Gal-1 resulted in maximal inhibition of CFU-GM number in the blood, verifying that Gal-1 was a highly effective inhibitor of HPC mobilization in vivo.

Increase of the number of CFU-GM progenitor cells in blood was detected on the fourth day of induction of mobilization, reached its maximum on the fifth day, then rapidly decreased. The inhibitory capacity of Gal-1 was sustained at all three time points (Fig. 3B) without changing the time kinetics of CY/G-CSF-induced progenitor cell mobilization.

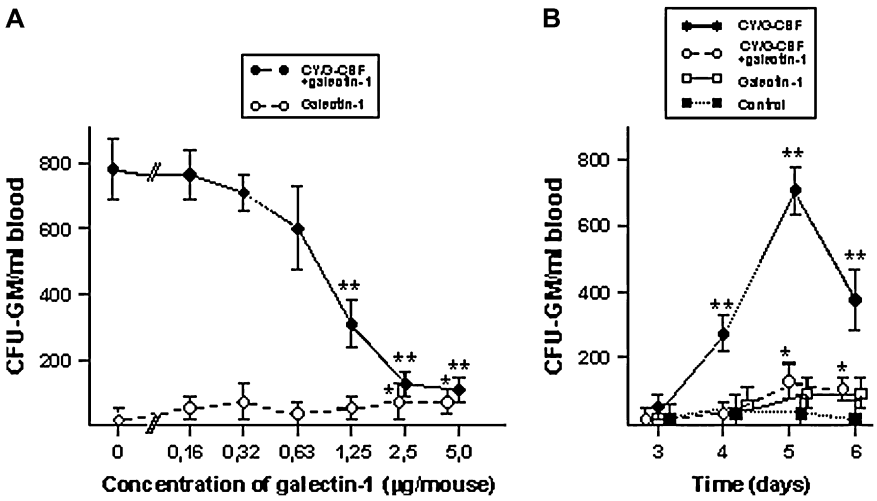

Figure 3. Gal-1-mediated inhibition of myeloid progenitor cell mobilization is dose and time dependent. **(A)** CFU-GM frequencies were determined in blood samples isolated from mice treated with either CY/G-CSF or CY/G-CSF and different amount of Gal-1. **(B)** Mice were treated with either CY/G-CSF or with CY/G-CSF+Gal-1 (5 μg/mouse/day) and blood CFU-GM frequencies were determined at the indicated time points of mobilization. The results are expressed as mean ± SD of 3 independent experiments. Statistically significant differences were indicated by asterisks (*$p < 0.05$, **$p < 0.001$).

Effect of Gal-1 treatment on the distribution of leukocyte subpopulations in blood and BM during HPC mobilization

The total number of leukocytes significantly elevated in circulation of mobilized animals compared to that of nontreated animals (Fig. 2), and concomitantly the ratio of cell types, markedly changed. As shown in Table 1, the ratio of granulocytes and monocytes increased (from 22% to 33% and 1.2% to 19%, respectively) at the expense of lymphocytes, which in turn decreased from 78% to 48% on day 5 of mobilization. This finding indicated that BM cell mobilization predominantly affected the granulocyte/monocyte cell population, resulting in peripheral granulophilia and monocytosis. Administration of Gal-1 with mobilizing agents quantitatively inhibited granulophilia and partially blocked monocytosis.

Analyzing the BM cell populations, we found that primitive (Sca-1^+) and granulocyte-macrophage-committed (GR-1^+ and CD11b$^+$) progenitor cells expanded on the fifth day of mobilization, suggesting an intensive marrow regeneration. Co-injection of Gal-1 and mobilizing agents resulted in an even more profound HPC cell growth compared to the effect of CY/G-CSF alone (11.7% vs 30.8% for Sca-1^+, 54.8% vs 73.9% for GR-1^+, and 55.1% vs 70.1% for CD11b$^+$ cells). The percentage of progenitor and/or recirculating B cells (B220$^+$) decreased in mobilized marrow and Gal-1 did not modify this effect, supporting that the major targets of Gal-1 inhibition of BM cell mobilization are HPCs, granulocytes, and monocytes. Interstingly, Gal-1 alone significantly reduced the frequency of B cell progenitors and/or recirculating B cells compared to the untreated control (27.6% ± 1.8% in control vs 19.9% ± 2.1% in the marrow of Gal-1-treated mice). It might reasonable to hypothesize that this observation was partially associated with the proposed functional role of Gal-1 in the marrow stroma as a pre-B cell receptor (surrogate light chain) ligand [21,22].

Presence of galectin-1 is critical for inhibition during the second half of mobilization period

We determined whether the presence of Gal-1 was required during the entire period of BM cell mobilization. As shown in Figure 4, inhibition was not affected by the absence of Gal-1 on the first two days. When Gal-1 was administered from the third day, BM cell mobilization was still highly blocked. Then Gal-1 presence was obligatory as lack of Gal-1 injection resulted in the failure of inhibition. If the animal obtained Gal-1 on all days but the last, inhibition was reduced to 50%, indicating the necessity of Gal-1 presence on this day as well. These results showed that the presence of Gal-1 for inhibition was indispensable during the most intensive HPC mobilization occurring between days 3 and 5 (see Fig. 3B).

Requirement of Gal-1 during only the later mobilization events was not due to the limitation of the lectin effect on

Table 1. Analysis of blood and bone marrow leukocyte populations[a]

Experimental groups	Blood leukocytes (%)[b]			Bone marrow nucleated cells (% positive)[c]					
	Ly	Gr	Mo	Sca-1	CD3	B220	Ter-119	GR-1	CD11b
Control (saline)	76.7 ± 4.5	22.1 ± 1.3	1.2 ± 0.2	6.9 ± 0.5	1.8 ± 0.2	27.6 ± 1.8	10.7 ± 0.8	51.1 ± 2.9	49.9 ± 4.4
CY/G-CSF	47.6 ± 5.3*	33.1 ± 4.3*	19.3 ± 1.5*	11.7 ± 1.1*	1.9 ± 0.2	5.8 ± 0.6*	16.2 ± 0.9*	54.8 ± 3.7*	55.1 ± 4.5*
CY/G-CSF+Gal-1	65.4 ± 5.9*	23.2 ± 1.3	11.4 ± 1.4*	30.8 ± 2.9*	1.6 ± 0.5	5.9 ± 0.4*	15.1 ± 1.1*	73.9 ± 5.4*	70.1 ± 3.9*
Gal-1	79.8 ± 5.7	19.1 ± 1.8	1.1 ± 0.1	5.5 ± 0.7	1.9 ± 0.3	19.9 ± 2.1*	9.6 ± 1.1	52.7 ± 3.2	51.6 ± 2.8

[a]Data are the mean ± SD of three independent experiments. Figures marked with an asterisk (*) are significantly different from the values of control ($p < 0.05$).
[b]Serial manual counts of blood smears were performed after May-Grunwald-Giemsa staining.
[c]Expression of lineage markers was measured by flow cytometry.

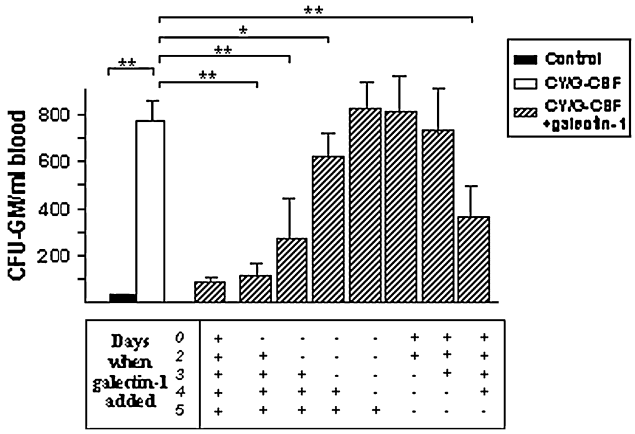

Figure 4. Presence of Gal-1 is required at the second half of mobilization period. Myeloid progenitor cell frequencies were analyzed in blood samples isolated from mice treated with either CY/G-CSF or with CY/G-CSF+Gal-1. In these experiments Gal-1 (5 μg/mouse/day) was added only at the indicated time points of mobilization. The results are expressed as mean ± SD of 3 independent experiments. Statistically significant differences were indicated by asterisks (*$p < 0.05$, **$p < 0.001$).

the G-CSF-induced myeloid progenitor cell mobilization, as mobilization of granulocyte-macrophage colony-forming cells with CY alone was also markedly inhibited by repeatedly added Gal-1 (Fig. 5).

Gal-1 inhibits SDF-1-induced trans-endothelial migration of marrow-derived HPCs in vitro

Then we tested the effect of Gal-1 on SDF-1-induced chemotaxis and trans-endothelial migration of nucleated BM cell and granulocyte-macrophage colony-forming cell. As shown in Figure 6, neither BM cell (A) nor CFU-GM (B) chemotaxis was inhibited by Gal-1 in a transwell assay in which cells migrated in response to a gradient of SDF-1 for 4 hours. In contrast, trans-endothelial migration of BM cells (Fig. 6C), as well as CFU-GMs (Fig. 6D), was blocked by Gal-1 in a dose-dependent manner within a 6-hour period.

Rescue of lethally irradiated mice fails when transplanted with blood derived from CY/G-CSF-mobilized and Gal-1 treated animals

Lethally irradiated mice were transplanted with 100 μL or 50 μL of blood obtained from CY/G-CSF-treated donors (Fig. 7). Recipients survived (5 of 7 or 4 of 7, respectively) longer than 90 days. In contrast, when 100 μL blood isolated from CY/G-CSF+Gal-1-treated donors was injected into irradiated recipients, all mice died within 5 to 22 days after transplantation. None of the irradiated mice injected with blood from untreated control animals survived more than 14 days. These data also suggested that Gal-1 was able to inhibit the CY/G-CSF-induced progenitor cell mobilization and long-term repopulating ability of hematopoietic stem cells as well.

Discussion

In adult mammals, hematopoietic stem and progenitor cells migrate from bone marrow to the periphery in response to several stimuli, including cytoreductive drugs, such as CY, and cytokines, such as G-CSF. G-CSF is widely used clinically to mobilize BM cells for collection and transplantation, often in combination with CY, which augments its effect [23,24]. In rodents and humans CY/G-CSF induces mobilization in a time- and dose-dependent fashion characterized by rapid neutrophilia followed by a delayed increase of HPC number in blood, peaking at levels 10-fold to 100-fold above baseline. This process mimics enhancement of the physiological release of stem and progenitor cells from the BM reservoir in response to stress signals during injury and inflammation [25,26].

Despite the common use, the molecular mechanisms of CY/G-CSF-induced mobilization of primitive hematopoietic cells are poorly understood. Recent experiments suggest that mobilization is initiated by stress-induced activation of neutrophils and osteoclasts [27,28]. This results in shedding and release of membrane-bound stem cell factor (SCF), proliferation of progenitor cells, and activation and/or degradation of adhesion molecules such as VLA-4 and P/E selectins [29]. The dynamic secretion and

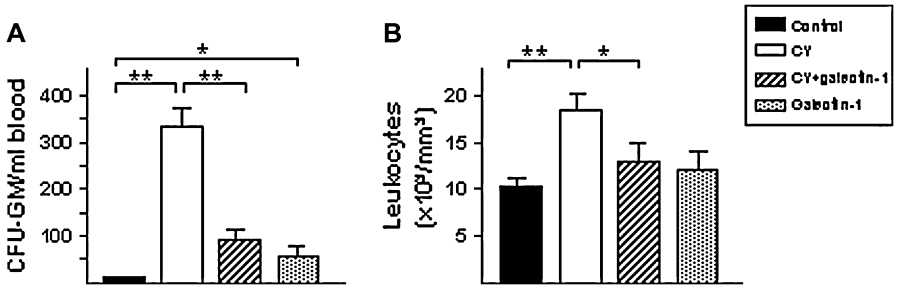

Figure 5. Effect of Gal-1 on the CY-induced mobilization of granulocyte-macrophage colony-forming cells. CFU-GM frequencies **(A)** and total leukocyte counts **(B)** were determined in blood samples isolated from mice treated with either saline, CY, CY+Gal-1, or Gal-1 alone. The results are expressed as mean ± SD of 3 independent experiments. Statistically significant differences were indicated by asterisks (*$p < 0.05$, **$p < 0.001$).

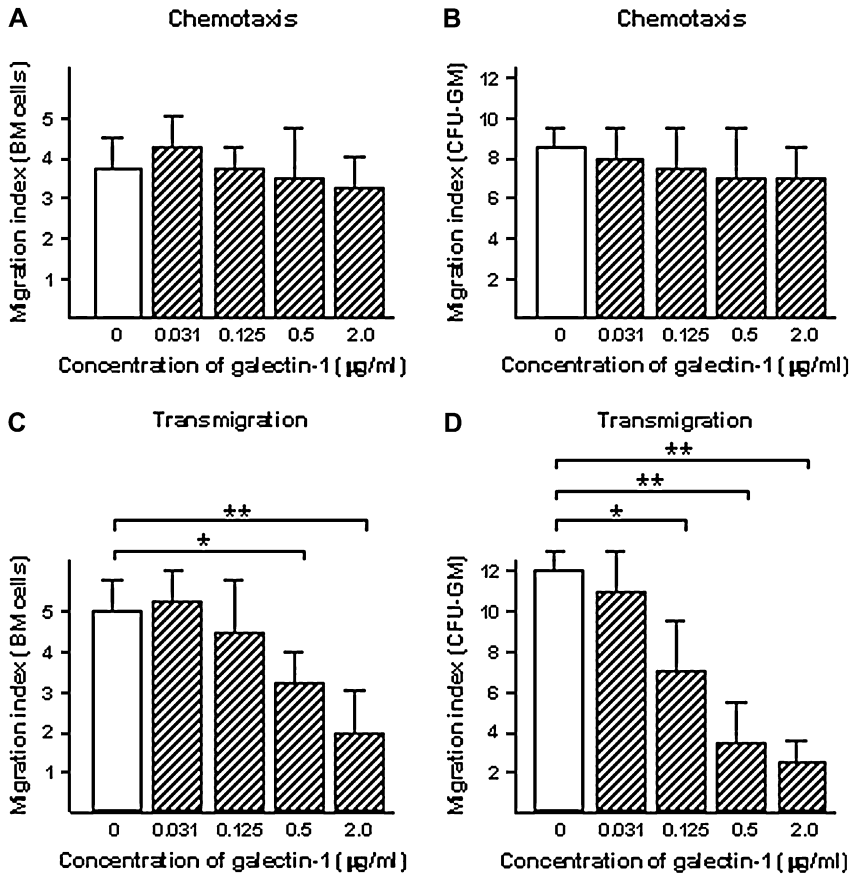

Figure 6. Effects of Gal-1 on HPC chemotaxis and transendothelial migration. BM cells were added to empty **(A,B)** or to endothelial monolayer covered **(C,D)** Millicell culture plate inserts in the absence or presence of increasing concentrations of Gal-1. Mouse recombinant SDF-1 (100 ng/mL) was added to the lower chambers **(A–D)**, and the number of BM cells **(A,C)** and granulocyte-macrophage colony-forming cells **(B,D)** migrating to the bottom well were assessed after 4 or 6 hours, respectively. Migration index was calculated by dividing the number of cells migrating to SDF-1 by the number of cells migrating spontaneously. The results were expressed as mean ± SD of 3 independent experiments. Statistically significant differences were indicated by asterisks (*$p < 0.05$, **$p < 0.001$).

inactivation of chemokines, such as stromal cell–derived factor-1 (SDF-1)/CXCL12 and interleukin-8 (IL-8)/CXCL8, and multiple cycles of inactivation and degradation of BM components by proteolytic enzymes such as elastase, cathepsin G, proteinase 3 [27,28,30–32], CD26 (dipeptidyl peptidase IV) [33], and various matrix metalloproteinases (MMPs), especially MMP-9 [28,34], are implicated as major regulatory components in the mobilization process.

In this study, the number and quality of mobilized leukocytes in BM and blood derived from BDF1 mice that underwent a mobilizing regimen by CY/G-CSF in the presence or absence of human recombinant Gal-1 have been determined by an array of phenotypic and functional assays. We have found that Gal-1 is able to inhibit CY/G-CSF-induced HPC mobilization as well as peripheral neutrophilia in a dose- and time-dependent fashion (Figs. 2–4). Despite the reduced HPC mobilization, the frequency and absolute number of Sca-1$^+$, GR-1$^+$, and CD11b$^+$ cells are markedly higher in the BM from animals treated by CY/G-CSF+Gal-1 than in the marrow from mice treated by CY/G-CSF alone (Table 1). Moreover, although the number of nucleated BM cells is not affected by Gal-1 alone, CFU-GM frequency slightly increases in marrow treated with Gal-1 alone or along with the mobilization protocol (Fig. 2). Thus Gal-1 does not inhibit, and rather stimulates, HPC expansion and accumulation in the BM during CY/G-CSF treatment in accordance with our previous finding showing that a low amount of Gal-1 has an enhancing activity on the growth of committed hematopoietic progenitor cells in tissue culture [18]. Accordingly, this low concentration of Gal-1 does not exert cytotoxic effect on BM cells (Fig. 2).

Previous studies have shown that accumulation of diverse cell-bound and soluble neutrophil proteases, especially MMPs in the BM, is a critical step of stem and progenitor cell mobilization [27,32]. Without appropriate proteolytic activity in the marrow, HPCs are unable to detach from the surrounding stromal elements, which is necessary [35,36] for their observed expansion in the BM

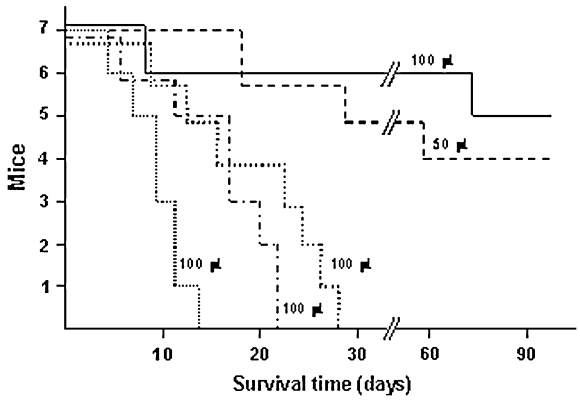

Figure 7. Effect of Gal-1 on the in vivo repopulating ability of mobilized primitive hematopoietic cells. Lethally irradiated (900 cGy) BDF1 mice were injected with the indicated volume of blood from syngeneic animals treated with either saline (·····), CY/G-CSF 100 µL (——) or 50 µL (– –), CY/G-CSF+Gal-1 (– · –), or Gal-1 alone (• • • •). Data are representative of 2 independent experiments.

(Fig. 2 and Table 1). Gal-1-mediated HPC mobilization unlikely occurs by this mechanism since we have not observed differences in gelatinase activity in BM samples from mobilized animals vs marrow samples from CY/G-CSF+Gal-1-treated mice (data not shown).

The other regulatory milestone in BM cell release to the periphery is their migration step through endothelium. La et al. [15] have reported that Gal-1 inhibits interleukin-1β-induced recruitment of polymorphonuclear leukocytes into the mouse peritoneal cavity as well as their chemotaxis and transendothelial migration [15]. In addition, He and Baum found reduced transendothelial migration of T cells when Gal-1 expression on endothelial cells was increased [37]. Therefore, we suggest that Gal-1 inhibits the trafficking of HPCs, mature granulocytes, and monocytes from the BM into the circulation during CY/G-CSF-induced mobilization by blocking transendothelial migration of leukocytes. This view is supported by our present findings: 1) in vitro, Gal-1 inhibits transendothelial migration of BM-derived HPCs in response to SDF-1 (Fig. 6); 2) Gal-1 has opposite effects on the marrow and blood cellularity during CY/G-CSF treatment in vivo (Fig. 2B and D and Table 1) as co-administration of Gal-1 results in increase of nucleated cell number in BM and decrease of that in blood; 3) Gal-1 has to be present in mice only during the later stage of mobilization for inhibition, when the migration of BM cells occurs (Fig. 4).

In this report we demonstrate a novel anti-inflammatory function of human galectin-1. It inhibits HPC, granulocyte, and monocyte trafficking to the periphery and so it may contribute to attenuation of acute and chronic inflammation. Since expression of Gal-1 in the marrow stromal cells is remarkably high [16,17], it is reasonable to speculate that the dynamic equilibrium between the slow efflux of early hematopoietic cells from the BM into the blood and the rapid recapture (homing) of these cells into the BM under steady-state conditions may also be regulated, at least in part, by Gal-1. However, further experiments will be required to validate this possibility.

Acknowledgments

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