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Regulation of T lymphocyte function by galectin-1 and its dependence on the carbohydrate binding activity of lectin

Summary of Ph.D. thesis

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Introduction

The glycosylation of proteins and lipids is a common posttranslational modification that regulates many physiological processes, like cell to extracellular matrix, cell to cell adhesion, assembly and activation of receptor complexes and protein trafficking. The role of glycosylation in cell physiology becomes better understandable with the characterization of the lectins, proteins that have binding sites for the specific mono- di- or oligosaccharides linked to proteins or lipids. The galectins are the beta-galactoside binding lectins with a carbohydrate recognition domain that has many conserved sequence elements. Galectin-1 was the first member of the galectin family to be described. It specifically recognizes beta-galactosides and has conserved sequence elements characteristic of galectins. The chain of 134 amino acids folds into the typical galectin CRD globule (Fig. 1). Two identical galectin-1 globules can form a dimer. Dimerization involves self-association of monomer subunits via hydrophobic surfaces opposite to the sugar-binding pocket.

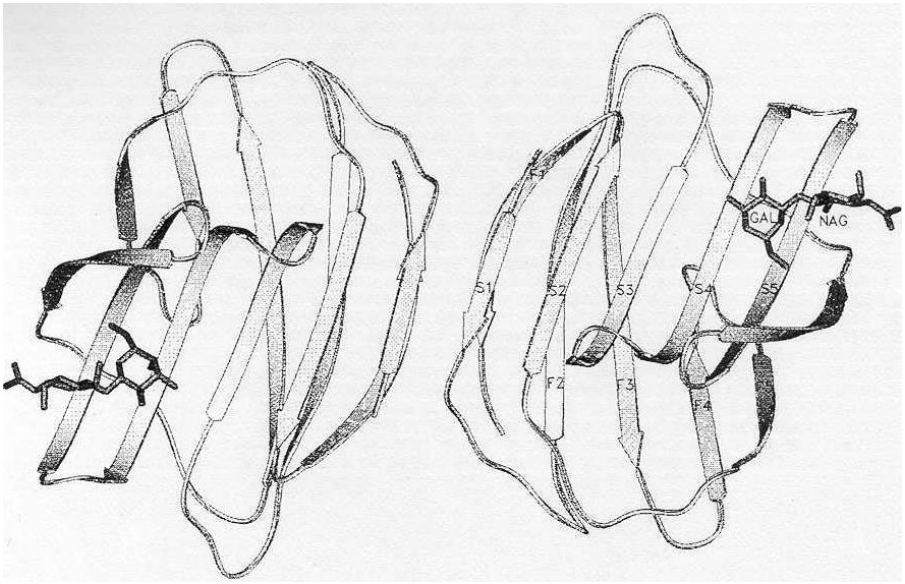

Fig. 1. Crystal structure of galectin-1 dimer complexed with lactose(Bianchet et al., 2000)

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The activity of the galectin-1 gene is strongly modulated under a variety of physiological or pathological conditions. A small genomic region of no more

than 100 base pairs surrounding the transcription start site (-50/+50), accounts for most of the transcriptional activity of the galectin-1 gene.

The expression pattern of galectin-1 varies among different adult tissues, changes during development and is altered in many neoplastic transformations and other pathological conditions. In human embryogenesis, it is found prevalently in connective tissues and some epithelia, such as in the basal layers of the skin and in the epithelial cells of the gonads. In adult tissues, galectin–1 is abundantly expressed in many cell types, such as skeletal, smooth and cardiac muscle and other cells of mesenchymal origin. High galectin-1 expression has been detected in sensory, motor and olfactory neurons and it has been demonstrated to participate in the regulation of olfactory axon fasciculation and targeting. In hematopoietic and lymphoid organs galectin-1 is expressed by stromal cells and activated T cells. In murine thymus, the lectin is expressed by thymic epithelial cells and is detected throughout the stromal framework of the thymus.

Galectin-1 expression is strongly modulated in human tumors. Lectin mRNA levels are very low in normal thyroid non-neoplastic goiters, adenomas and follicular carcinomas, while they are increased (5-20-fold) in a large proportion of papillary carcinomas and in almost all the aggressive anaplastic carcinomas.

Targeted disruption of the galectin-1 gene alone, or galectin-1 and galectin-3 genes together, was performed in mice. Galectin-1-/-, or galectin-1-/- together with galectin-3-/-, homozygous null mutants appear normal and fertile. The potential role of galectin-1 in the developing olfactory system was investigated in newborn mice. This analysis revealed that the absence of lectin results in major defects in primary olfactory neuron outgrowth. Because galectin-1 has been postulated to participate in regulating immune function, including T-lymphocyte selection in the thymus, complete blood counts were compared for control and galectin-1 “null” mutant mice. No significant

differences were found in white cell numbers or differential distribution. However, potential differences in the prevalence of lymphocyte subtypes have not been investigated.

Galectin-1 exhibits dual localization, both in the intracellular and extracellular compartments. Immunohistochemical studies demonstrated the presence of lectin in cytoplasm of smooth muscle and thymic epithelial cells, as well as in the nucleus of the same smooth muscle cells and osteoblasts. Indirect immunofluorescence studies showed the presence of galectin-1 on the surface of K562 cells. The protein is also present in the extracellular environment in thymus and bone marrow. In accordance with the dual localization, galectin-1 functions can be divided intracellularly and extracellularly.

Extracellularly, galectin-1 participates in the regulation of cell activation, proliferation, adhesion and apoptosis.

Galectin-1 induces apoptosis of T cells during their development in the thymus and after immune stimulation of the peripheral activated, but not resting, T cells. The triggering of cell death does not occur via the T cell antigen receptor (TCR), Fas or tumor necrosis factor receptor. As an early cell response, the lectin initiates tyrosine phosphorylation in T and B cell lines.

An important role of galectin-1 is that it participates in the generation of central and peripheral tolerance utilizing its growth inhibitory and apoptotic properties. Human thymic epithelial cells express galectin-1, which binds to core 2-O-glycans on immature cortical thymocytes and modulates their survival. Galectin-1 kills non-selected and negatively selected immature thymocytes. Induced apoptosis is independent of that induced by steroids, but is synergistic with TCR engagement. In this sense the epithelial lectin participates in positive and negative selection growth and cytotoxic signals in the thymus.

The peripheral T cells are also exposed to the cytotoxic effect of galectin-1. It shows specific growth inhibitory properties towards different cell types, such as phytohemagglutinin (PHA)-activated human T cells, concanavalin

(ConA)-stimulated rat T cells, activated chicken lymphocytes, human leukemia T cells. Following an antigenic challenge, galectin-1 is secreted from activated T cells and reduces clonal expansion of antigen-stimulated CD8+ T cells and IL-2 production in an autocrine manner. It has been demonstrated that under certain inflammatory conditions, activated macrophages, antigen-stimulated T cells, activated B cells and alloreactive T cells secrete high level of galectin-1, and downregulate the immune response.

The immunosuppressive and anti-inflammatory effects of galectin-1 have been demonstrated in several experimental models of autoimmunity and chronic inflammation. Galectin-1 ameliorates phospholipase A2-induced edema in a selective and dose-dependent manner, when pre-injected or co-injected together with the enzyme. The lectin inhibits the release of arachidonic acid and prostaglandin production from lipopolysaccharide-stimulated macrophages and blocks neutrophil extravasation, mast cell degranulation and nitric oxide synthesis. It prevents clinical and histopathological manifestations of autoimmune encephalomyelitis, ameliorates the inflammatory and autoimmune response in collagen-induced arthritis by increasing T cell susceptibility to activation induced cell death.

Galectin-1 is expressed in immune privileged tissues, such as retina, placenta, testis and ovary. Immune privileged tissues do not encounter the differentiating immune cells, therefore they are potential targets for the immune response. They have to protect their integrity, prohibiting the survival and proliferation of specific lymphocytes and thereby the inflammation reaction in these tissues. Galectin-1 might contribute to immune privileged mechanisms, ensuring the rapid elimination of the autoreactive T-cells by an apoptotic pathway.

Questions remain open about the mechanisms of the galectin-1 function. One of the most important is the involvement of its sugar binding activity in extracellular function, particularly in the induction of apoptosis. As has been

mentioned above, T cells lacking C2GnT are resistant to galectin-1 induced cell death. This enzyme is responsible for creating the branched structure on O-glycans of T cell surface glycoproteins. Blocking of the CRD by lactose gives controversial results, inhibiting or not inhibiting the lectin induced apoptosis. Experiments where the lectin CRD specificity is abolished would give additional valuable data on importance of sugar binding in the induction of apoptosis.

In spite of the more or less well characterized functions of secreted galectin-1, its intracellular function remains poorly defined. The lectin has been implicated in the regulation of the membrane localization of oncogenic H-Ras(12V). Overexpression of galectin-1 results in cell transformation, while antisense RNA inhibits such transformation. Farnesylthiosalicylic acid, which disrupts Ras membrane anchorage, disrupts H-Ras(12V)-galectin-1 interactions as well. Further studies demonstrated that the lectin diverts the Ras signals to Raf-1 at the expense of PI3 kinase.

Galectin-1 is also involved in pre-mRNA processing. Nuclear extracts (NE) from HeLa cells used for cell free splicing assays contain galectin-1. Depletion of galectin-1 and galectin-3 from NE inhibits splicing, and reconstitution of the proteins reconstitutes splicing activity. A component of the spliseosome complex, Gemin4 has been shown to interact with galectin-1 in this process. This function is inhibited by lactose. Intracellular galectin-1 has also been shown to promote proliferation of human neuroblastoma cells by an unknown mechanism.

Aims

Our aim was to study two principal aspects of galectin-1 function:

1. It has not been clearly defined whether the carbohydrate binding of galectin-1 is essential for the apoptotic function of the protein. The main reason for this is that the purification of the non-binding mutants with classical sugar-matrix affinity chromatography encountered difficulties. Therefore our purpose was:

a) To establish a system that would provide a way to isolate this kind of mutant proteins.

b) To analyse whether or not mutants characterized previously as sugar "non-binding" are able to trigger apoptosis of T lymphocytes.

c) To investigate how particular mutations of amino acids, that directly bind carbohydrate, affect galectin-1 induced apoptosis.

2. To obtain a deeper insight into the intracellular function of galectin-1 we have also asked the questions:

a) How *de novo* expressed galectin-1 modulates T cell function?

b) What is the role of the lectin property of galectin-1 in intracellular function?

Materials and Methods

3.1. Recombinant protein production

Human wild type and mutant galectin-1 cDNAs (GAL1) were a generous gift from J. Hirabayashi and K. ichi Kasai Teikyo University, Japan. Figure 3.1. shows the sequence of the protein and the positions of the mutations. Wild type

and R48H (Arg48 changed to His), E71Q (Glu71 changed to Gln), R73H (Arg48 changed to His) mutant GAL1s were excised from pUC540 vector with NcoI, and inserted into a bacterial expression vector pETHis at the NcoI site, in the sense direction. The resulting protein has a His_6 tag at the N-terminus and three linker amino acids. The His tag allows for affinity purification of the proteins on a Ni-resin. Wild type GAL1 cDNA, after excision from pUC540, was also inserted into bacterial expression vector pQE60 (QUIAGEN, Hilden, Germany) at the NcoI site. The proper orientation of the cDNA inserts was confirmed by restriction enzyme analysis. Quality of the cloning and "in frame" position of the His_6 tag in pETHis vector was confirmed by sequencing.

ACGLVASNLN LKPGECLRVR GEVAPDAKSF VLNLGKDSNN LCLHFNP(R48H)FN AHGDANTIVC NSKDGGAWGT (E71Q)Q(R73H)EAVFPFQ PGSVAEVCIT FDQANLTVKL PDGYEFKFPN RLNLEAINYM AADGDFKIKC VAFD

Fig.3.1. Protein sequence of galectin-1 with indicated mutations. Each mutated version of galectin-1 carries one of the substitutions.

GAL1 subcloned to pQE60 or to pETHis was transformed into BL-21 (BF^-*dcm ompT hsdS*($r_B^-m_B$) gal λ(DE3)) (Stratagene), inoculated from a single colony into 2ml of LB medium containing 200 μg/ml ampicillin, and grown overnight. The obtained culture in 1:1000 dilution was inoculated in LB medium containing 200 μg/ml ampicillin and grown for 8 hours at 37^0C.

3.2. Purification of recombinant galectin-1 (rGal1)

200ml of bacterial culture producing rGal1 was centrifuged at 2500g for 30 min at 4^0C in a Sorvall RC3B centrifuge, washed twice with 80ml of lysis buffer (2500g 15 min, 4^0C, Hettich Universal 30RF, rotor 1424A) containing 4 mM beta-mercaptoethanol, then disrupted at 4^0C by "French" press (AMINCO,

Maryland, USA) in 50ml of lysis buffer supplemented with 1 mM PMSF. All following steps were done at 4^0C. The lysate was spun at 10 000g for 30 min, the supernatant was filtered through GF/C membrane, and loaded on an alpha-lactose gel (EY Laboratories) column pre equilibrated with 20ml of lysis buffer supplemented with 1mM PMSF and 4mM beta-mercaptoethanol. The lysate was recirculated three times and the bound protein was washed with 20 ml of washing buffer supplemented with 0,25 mM PMSF and 4mM beta-mercaptoethanol. Protein was eluted with 20ml of elution buffer supplemented with 0,1M iodoacetamide, and 1ml fractions were collected. Eluted fractions were analyzed on sodium dodecyl sulfate 12% polyacrylamide gel electrophoresis (SDS/12%PAGE) and dialyzed against phosphate buffered saline (PBS) containing 100 μM beta-mercaptoethanol. Protein concentration was estimated with Bradford assay reagent (Biorad) and by the absorbance at 280nm.

3.3. Purification of recombinant His-tagged galectin-1 (rHisGal1)

200ml of bacterial culture producing rHisGal1 or its mutants was processed as described in section 3.2. All following steps were done at 4^0C. Lysate was spun at 10 000g for 30 min, supernatant was filtered through GF/C membrane (Whatman), and supplemented with iodoacetamide to final concentration of 0,1M. Then the lysate was loaded onto a Ni-NTA-agarose (Invitrogen) column pre equilibrated with 20ml of lysis buffer supplemented with 1mM PMSF. It was recirculated three times and washed with 20ml of washing buffer containing 0,1 M iodoacetamide. Bound protein was eluted with 20ml of elution buffer . Collected 1ml fractions were tested on SDS/12%PAGE and dialyzed against PBS containing 100μM beta-mercaptoethanol. Protein concentration was estimated with Bradford assay reagent (Biorad) and by the absorbance at 280nm.

3.4. Alpha-lactose binding assay

Purified recombinant wild type rHisGal1 and its mutants in PBS containing 100μM beta-mercaptoethanol were incubated with alpha-lactose gel (EY Laboratories) at 4^0C for 1 hour on a rotating platform, washed with washing buffer three times. Resin, together with bound protein, was subjected to SDS/12%PAGE and Western blotting (see below) with rabbit anti-galectin-1 antibody produced in our laboratory.

3.5. Cell Culture and transfection

The human Jurkat T cell line was cultured in RPMI 1640 (GIBCO) tissue culture medium supplemented with 5% heat inactivated FCS and glutamine . Human embryonic kidney epithelium based cell lines ProPak-A (PPA) and Phoenix gp (generous gift of G. Nolan) were cultured in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% heat inactivated FCS and glutamine . All cell lines were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO_2. GAL1 and mutant (R48H) GAL1 cDNA were excised from pETHis with BamHI/NcoI, and ligated into EcoRI/BamHI treated mammalian expression vector pLXSN together with preannealed oligonucleotides (G-1.Kozak"+": 5’ - AAT TCT CTT CGC TTC AGC TTC AAT - 3'; G-1.Kozak"-"5' - CAT GAT TGA AGC TGA AGC GAA GAG - 3'). The proper orientation and position of the cDNA inserts was confirmed by restriction enzyme analysis and sequencing. Phoenix gp cell line was transfected by conventional Ca/phosphate method with obtained constructs together with pCiGLwt vector containing viral envelope cDNA (kindly provided by G. Veres). Virus containing supernatant was collected 48 hours after transfection, filtered through 0.45 μm filter (Corning, SFCA membrane) and used for infection of PPA cells by centrifugation in the presence of 4μg/ml polybrene. Infected PPA cells were selected on 400μg/ml of G418 (Invitrogen) for two weeks. Selected cells were grown to confluence, and supplied with a fresh medium. Cells were

fed with fresh medium and supernatant was collected after 16 hours culture. The Jurkat cells were transduced by centrifugation at 1000g for 90 minutes with virus containing supernatant in presence of 4μg/ml polybrene and selected for two weeks on 1mg/ml of G418. Protein expression was analyzed by Western blotting (section 3.6).

3.6. Western blotting

Triton X-100 soluble extracts prepared from 2×10^5 Jurkat cells per sample or proteins after binding to alpha-lactose gel (EY Laboratories) were separated on a SDS/12%PAGE and transferred to nitrocellulose membrane (Schleicher & Schuell) in transfer buffer . The membranes were blocked using Tris buffered saline (TBS) containing Tween20 (TBST) , 3% Teleostean gelatin and subsequently probed with 20μg/ml of rabbit anti-galectin-1 antibody in TBST for 1 hour at 4^0C and with 275μg/ml biotinylated goat anti-rabbit IgG (DAKO) in TBST for 1 hour at 4^0C, and then with 18,75 μg/ml streptavidin-HRPO conjugate (DAKO) in TBST for 1 hour at 4^0C. Immunoreactive proteins were visualized by an enhanced chemiluminescence (ECL plus) detection system (Amersham Pharmacia Biotech). Prestained molecular weight marker was purchased from GIBCO-BRL.

3.7. Indirect immunofluorescence

Jurkat cells were washed once in PBS, then incubated for 1 hour at 4^0C in PBS containing 20μg/ml of the indicated monoclonal antibody, washed three times with cold PBS containing 0,1% NaN_3, and then incubated for 30 min at 4^0C with goat anti-mouse FITC conjugate. Finally, the cells were washed three times with cold PBS containing 0,1% NaN_3 and subjected to cytofluorimetric analysis with FACScalibur (Becton Dickinson). The dead cells were discriminated by propidium iodide (PI) uptake (1μg/ml) prior measurement.

3.8. Assays for cell growth

To assess the growth and proliferation of Jurkat transfectants, cells were plated at a density of 10^5 cells/ml in RPMI 1640 medium containing 5% FCS in triplicates. Viable cell numbers were determined on day 4 after plating by the 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay (145) and by trypan blue exclusion counting. MTT assay is based on a reduction of tetrazolium salt to formazan by mitochondrial dehydrogenase. It was performed as follows. MTT solution was added to each sample to a final concentration of 1mg/ml. Samples were incubated for 4 hours at 37^0C then the medium was removed and a solution of 0,05M HCl in isopropanol was added for 10 min to solubilize formazan crystals. Absorbance of the samples was measured at 570nm. Trypan blue exclusion counting was performed as follows. Cell suspension was diluted 1:10 in trypan blue solution for cell counting and the living cells were discriminated by trypan blue exclusion. Average, standard deviation, and Student test parameters were calculated.

3.9. Cell cloning

The Limiting dilution method was used for cloning. Transfected cells were diluted to 10, 100, 1000 cells/ml in RPMI 1640 tissue culture medium supplemented with 5% FCS and antioxidants, then plated in 96-well flat bottom plates in 100 μl/well. After three weeks growing colonies were expanded and analyzed.

3.10. Measurement of intracellular Ca^{2+} concentration

Jurkat cells were suspended in 10^7 cells/ml concentration in RPMI-1640 supplemented with 5% FCS and incubated with 7.5 μM of Fluo-3AM and 7.5 μM of FuraRed-AM (Molecular Probes, Eugene, OR, USA) for 30 min at 37°C. Cells were then adjusted to a final concentration of $5x10^5$ cells/ml with the addition of cell culture medium and incubated for a further 30 min at 37°C. The

cells were washed twice with RPMI and resuspended in fresh cell culture medium at a concentration of 10^6 cell/ml. Intracellular Ca^{2+} was measured using FACScalibur (Becton Dickinson) and data were presented as the ratio of Fluo-3 and FuraRed fluorescence intensity at 530 and 650 nm, respectively, versus time.

3.11. Apoptosis assay

For the apoptosis assay we used cytofluorimetric analysis of the DNA content and sub-G1 cell population was considered as apoptotic cells. The cells (1-3 10^6 cells/ml) were treated with various apoptosis inducing agents for 16 hours in RPMI 1640 medium containing 2% FCS in a CO_2 thermostat. After washing with PBS, the cells were resuspended at a concentration of 1×10^6 cells/ml with staining solution . The samples were then incubated at room temperature for 20 min in the dark and analyzed by flow cytometry (FACScalibur; Becton Dickinson). CellQuest (Becton Dickinson) and ModFit LT (Verity Software House, Topsham, ME) were used for the analysis.

3.12. Binding assay to cell surface glycoconjugates

Jurkat cells were incubated for 1 hour at 4^0C in PBS containing 25μg/ml of rHisGal1 or its mutants, then washed with cold PBS or PBS containing 100mM lactose and incubated further at 4^0C in PBS containing 20μg/ml of rabbit anti-galectin-1 antibody for 30 minutes. After washing with cold PBS containing 0,01% of NaN_3, the cells were stained with 20μg/ml of goat anti-rabbit biotin conjugate (DAKO) in PBS at 4^0C for 30 min, then it was washed out and they were stained with the 2μg/ml of streptavidin-FITC in PBS for 30 min. Finally, the cells were washed three times with cold PBS containing 0,01% NaN_3 and subjected to cytofluorimetric analysis with FACScalibur (Becton Dickinson). Propidium iodide was added to cells to the final concentration of 1μg/ml to discriminate the dead cells.

3.13. Mitochondrial dehydrogenase activity assay

The cells were plated in triplicates at a concentration 10^6 cells/well in flat bottom 96-well plates in RPMI supplemented with 5% FCS and glutamine. MTT assay was performed as described in the section 3.8.

Results

- Recombinant Gal1 induces apoptosis is not diminished by lactose. Jurkat cell line was treated with rGal1 for 24 hours in the presence or absence of 100mM lactose and apoptosis was estimated by measuring the DNA content. Apoptosis induced by rGal1 was not significantly inhibited by lactose.
- Recombinant HisGal1 is purified on Ni-NTA agarose resin. We subcloned wild type cDNA and cDNA of the R48H, E71Q, R73H mutants into pETHis vectors in order to be able to purify the recombinant proteins via His_6-tag independently of their carbohydrate-binding activity. Wild type and mutant proteins were named rHis-Gal1 for wild type, rHisGal1.71 for E71Q, rHisGal1.73 for R73H, rHisGal1.48 for R48H.
- Rabbit anti-galectin-1 antibody recognizes both His-tagged and non-His-tagged galectin-1 and its mutants. To test whether the extra nine aminoacids affected the antigenic properties of rHisGal1, we performed immunoblotting analysis using rabbit anti-galectin-1 antibody. The antibody equally recognized both proteins. This suggested that the antigenic properties of rHisGal1 were not changed compared to rGal1 and the antibody can be used to detect rHisGal1. Antigenic properties of the recombinant His-tagged mutants were tested in comparison to wild type

rHisGal1 with Western blotting. The antibody to rGal-1 recognized the mutant rHis-Gal1 proteins similarly to the wild type protein.

- Recombinant HisGal1 binds to lactose and cell surface glycoproteins, and induces apoptosis of Jurkat cells. To test whether addition of the his-tag affected the biological activity of rHisGal1, we compared the sugar binding ability of rGal1 and rHisGal1. Both proteins, rGal1 and rHisGal1, bound to the lactose and surface glycoconjugates of Jurkat cells as was shown by indirect immunofluorescence staining. Apoptosis inducing ability of rHisGal1 was not affected by the his-tag and was similar to that of rGal1. rHisGal1 mutants, R48H, E71Q, R73H, have reduced or abrogated ability to bind alpha-lactose, no binding activity towards cell surface glycoconjugates, and have reduced or abrogated ability to induce apoptosis. Recombinant HisGal1.71 and HisGal1.73 had a slightly reduced and rHisGal1.48 an abrogated lactose-binding activity. The "non-binding" mutants, rHisGal1.73, rHisGal1.71, and rHisGal1.48 did not bind to the cells. Recombinant HisGal1 and HisGal1.73 induced apoptosis, although rHisGal1.73 triggered a lower level of apoptosis. Recombinant HisGal1.48 did not induce a significant degree of apoptosis.
- Galectin-1 expression in stably transfected Jurkat cells. For the investigation of intracellular function of galectin-1 and its dependence on carbohydrate binding activity, we transfected Jurkat cells with wild type and R48H mutant galectin-1 using retroviral transfection. In the control, Jurkat cells were transfected with an empty vector. The transfectants were named: Jmock – for Jurkat transfected with pLXSN vector, Jgal – for Jurkat transfected with galectin-1 cDNA cloned into pLXSN vector, and Jgal48 – for Jurkat transfected with R48H mutant galectin-1 cDNA cloned into pLXSN vector. After selection of the transfected cells expression of galectin-1 was detected by Western blotting using rabbit anti-galectin-1 antibody. Later on, the selected cells were cloned. 60 clones of the Jmock

cells, 59 clones of the Jgal cells, and 56 clones of the Jgal48 cells were obtained. The clones were analysed by Western blotting for galectin-1 expression.

- Galectin-1 is not present on the surface of transfected cells. In order to determine whether galectin-1 is present on the surface of expressing cells, we performed indirect immunofluorescence staining of the surface proteins with the rabbit anti-galectin-1 antibody. The exogenously added rGal1 was bound to the surface of Jurkat cells. However galectin-1 produced by the transfectants did not appear on the surface of Jgal cells. Jmock cells were used as negative control.
- Presence of intracellular galectin-1 influences the proliferation of Jurkat cells. Both Jgal and Jgal48 showed significantly lower proliferation compared to the Jmock cells in a four day proliferation assay. The result was obtained with two different methods. The MTT assay represents the metabolic activity of the living cells, and the trypan blue exclusion assay shows the number of the viable cells. Expression of the wild type galectin-1 in the Jurkat cells resulted in a more dramatic effect on proliferation then did the mutant protein, since galectin-1 caused a 38% decrease while the mutant only a 20% decrease in the cell division compared to the proliferation of mock transfected cells measured with MTT.
- Galectin-1 expression upregulates cell surface level of TCR. Expression of the surface molecules determines T cell function. We examined the expression of the most important surface markers. The expression levels of CD45, CD95, CD2, LFA-1, and MHC1 were not changed on Jgal compared to Jmock, as was shown by the indirect immunofluorescence labeling with respective antibodies. In contrast, TCR expression level was upregulated by galectin-1 expression. Expression of the mutant galectin-1

in the Jurkat cells resulted in an intermediate elevation of TCR expression between the TCR level of mock and wild type galectin-1 transfected cells.

- Ca^{2+} influx is affected by galectin-1 expression. To analyse whether galectin-1 expression affected the TCR function, we performed the measurement of the intracellular Ca^{2+} concentration in response to TCR stimulation. Elevation of the intracellular Ca^{2+} concentration is one of the responses to stimulation of T cells through the TCR. The calcium acts as a second messenger molecule directly and indirectly regulating the function of various proteins, such as calmodulin, calcineurin, transcription factors, and others. The Jmock, Jgal, and Jgal48 cells were activated via the TCR with anti-TCR antibody, OKT3. Clone 3g6 (Jgal) cells had a higher elevation of intracellular Ca^{2+} concentration in response to TCR stimulation then clones 5c11 (Jmock) and 8g12 (Jgal48). The Jgal and Jgal48 transfected cells expressed higher level of TCR that the Jmock transfected cells. To show whether the elevation of Ca^{2+} level is a result of this, we used a calcium ionophore, which acts independently of TCR in triggering Ca^{2+} influx. The calcium ionophore treatment resulted in Ca^{2+} elevation with a similar pattern to TCR stimulation, indicating that Jgal cells give a higher response to the stimulations triggering Ca^{2+} influx. The increase of intracellular calcium concentration may come from two sources: the release of Ca^{2+} from intracellular stores or from the extracellular space. We added EGTA to the tissue culture medium to deplete the extracellular Ca^{2+} and to inhibit the latter source. Release from intracellular stores observed in absence of extracellular Ca^{2+} depleted by 5mM EGTA was also higher in 3g6 Jgal cells in either induced via the TCR or with calcium ionophore suggesting that Ca^{2+} comes from both sources. Analysis of the second set of clones 5h12, 3f6, and 8e10 gave a similar result.

- Susceptibility to apoptosis is increased in Jurkat cells expressing galectin-1. Galectin-1 induces apoptosis of activated T cells when applied extracellularly. It is not known, however, how endogenous expression of galectin-1 influences the susceptibility of T cells to apoptosis. We examined the uncloned transfected cells for their ability to respond to extracellular rGal1 stimulation. Treatment with recombinant galectin-1 induced a higher degree of apoptosis in cells expressing galectin-1.
- Mitochondrial activity is higher in cells expressing galectin-1. Mitochondria are a target for galectin-1 induced apoptosis, as was shown in our laboratory. To examine whether endogenously expressed galectin-1 affected mitichondrial activity, a possible reason for the increased sensitivity to galectin-1 induced apoptosis of the Jgal cells, we analysed mitochondrial activity by labeling the cells with MTT. Expression of galectin-1 resulted in an increase in the activity of the mitochondrial dehydrogenase.

Summary

1. We fused galectin-1 to His_6-tag, expressed in E. coli, and purified with Ni^{2+} affinity chromatography. The His-tagged wild type recombinant protein retained the affinity to the disaccharide, lactose, and complex glycoconjugate ligands and induced apoptosis on Jurkat cells. The wild type and the mutant His-galectin-1 forms, rHisGal1.48, rHisGal1.71, rHisGal1.73 were recognized by the rabbit anti-galectin-1 antibody similarly to the recombinant galectin-1 without His-tag.

2. Two mutants, rHisGal1.71 and rHisGal1.73, but not rHisGal1.48 coupled to solid phase lactose but not to complex glycoconjugates on the surface of the Jurkat cells. The mutants maintaining the lactose binding capacity, induced

apoptosis of the Jurkat cells, in contrast to the “null-binding” mutant, rHisGal1.48.

3. For analysis of the function of the intracellular galectin-1, the cDNA was stably transfected and overexpressed in Jurkat cells. The *de novo* expressed galectin-1 caused the upregulation of TCR expression but did not effect the expression of CD45, CD2, LFA1, MHCI, and CD95.

4. The galectin-1 expressing Jurkat cells had lower proliferation rate, higher amplitude of the Ca^{2+} response upon TCR stimulation or using Ca ionophore, and higher susceptibility to galectin-1 induced apoptosis in comparison to galectin-1 non-expressing cells.

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List of publications.

1. **Demydenko D.** Galectin-1 expression in tumors and its role in cancer cell adhesion. Review. Exp Oncol 2002 Sept; 24: 163-166.

2. Fajka-Boja R, Hidvegi M, Shoenfeld Y, Ion G, **Demydenko D**, Tomoskozi-Farkas R, Vizler C, Telekes A, Resetar A, Monostori E. Fermented wheat germ extract induces apoptosis and downregulation of major histocompatibility complex class I proteins in tumor T and B cell lines. Int J Oncol 2002 Mar; 20(3):563-70.

3. Violeta Chitu, **Dmytro Demydenko**, Zoltán Hegedüs and Éva Monostori. Conditions for permeabilization of cells used for intracellular tyrosine phosphorylation studies.BioTechniques 1999, 27:435-437

4. Telegeev G. D., Dybkov M.V., Bogko M.V., **Demydenko D.V.**, Maluita S.S., Tretiak N.M., Bondar M.V. Diagnostic of Chronic Myelogenous Leukemia by molecular biological methods (methodical recommendations). Kyiv, 1997