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Binding of Galectin-1 to Neutrophils Enhanced by Activation

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Human polymorphonuclear neutrophils undergo diaphoresis after a selectin-mediated rolling on the endothelial cells of the blood vessel wall. Extravasation is believed to be an integrin-mediated process. Galectin-1 is a small dimeric beta-galactoside-binding protein synthesized by the endotherial cells and present in the perivascular connective tissue. In this study we suggest the possible role of galectin-1 in extravasation of the activated neutrophils. MAL lectin binding study showed that f-MetLeuPhe-activated neutrophils decrease surface sialylation and increase galectin-1 binding via exposure of new galectin-1 binding sites. Desialylated HL-60 cells also show the same decrease in MAL binding and increase in galectin-1 binding, an increase which was not observed in the presence of lactose. Galectin-1 blotting analysis detected two possible major ligands (approximately 120 and 160 kDa) of galectin-1 from the desialylated HL-60 cell lysates.

Key words : *galectin-1, HL-60 cell, neutropil, galectin-1 ligands.*

Adhesion and migration of immune cells through the ECM towards inflammatory sites involve a multi-step process, co-ordinated by several factors such as receptors recognizing ECM glycoproteins, chemokines, and cytokines.[1] In case of neutrophils, during the inflammatory response, leukocytes adhere to the activated endothelium through sequential interactions with different receptor and counter receptors.[2,3] Adherent neutrophils are activated by local mediators, and the activated neutrophils emigrate through the endothelial barrier. The possibility of the basement membrane and extracellular matrix harboring other proteins capable of binding to the leukocyteswas explored, the candidate protein being a β-galactoside binding protein termed galectin-1.

Galectin-1 is a member of the growing family of animal lectin with affinity for β-galactoside containing glycoconjugates and show preferential binding to poly-*N*-acetyl-lactosamine.[4,5] Galectin family is highly conserved throughout the animal evolution and share remarkable sequence similarities in the carbohydrate recognition domain.[6] Although the precise function of galectin-1 *in vivo* is still under investigation, galectin-1 has been proposed to play key roles in a wide variety of biological events involving cell-cell and cell-ECM interactions,[14] metastasis,[9] immuno-modulation,[7] and cell growth regulation.[8] Galectin-1 is secreted by many cell types, including human endothelial cells[10,11] and is found in the basement membrane and extracellular matrices around capillary walls.[12,13] A variety of inter and extracellular candidate ligands such as laminin,[14] fibronectin,[15] Lamp1 and 2,[16] and CD45[17] have been reported to bind to galectin-1.

To explore the biological activity of galectin-1 toward leukocytes, we prepared a recombinant form of dimeric human galectin-1 and investigated the interactions of these galectins with HL-60 cells and also with both the resting and the activated human neutrophils. The activated human neutrophils showed two-fold increase in binding of galectins-1 to cell surface. Furthermore we also found the two possible galectin-1 ligands in the desialylated HL-60 cells and the identification of these proteins is still under investigation.

Materials and Methods

Preparation of galectin-1. The cDNA for human galectin-1 was cloned using the polymerase chain reaction (PCR) from the published sequence.[26] The cDNAs for galectin-1 was ligated into the Bam HI and Hind III sites of the plasmid PQE-50 (Qiagen). Galectin-1 was expressed at high levels in the transformed *E.coli* and was purified from the sonicated *E. coli* cell extracts on columns of lactosyl-Sepharose as described previously.[24]

Cell Lines and enzymatic desialylation. HL-60 cells were obtained from the American Type Culture Collection and maintained at 37°C and 5% CO_2 in complete RPMI 1640, which contained 10% fetal calf serum, 100 mU/ml

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Abbreviations: ECM, extracellular matrix; f-MetLeuPhe, *N*-Formyl-Met-Leu-Phe chemotactic peptide; HBSS, Hanks' balanced salt solution; HSA, human serum albumin; MAL, *Maackia amurensis* leukoagglutinin; PBS, phosphate-buffered saline.

penicillin, and 100 μg/ml streptomycin. To prepare the desialylated cells, HL-60 cells (3×10^7 cells) were treated with 100 mU *Arthrobacter ureafaciens* neuraminidase (Sigma) in 500 μl of RPMI medium containing 10% fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 1 h at 37°C. The treated cells were washed twice with RPMI before use.

Isolation and activation of neutrophils. Heparinized blood was obtained from healthy donors. Neutrophils were isolated through dextran sedimentation, hypotonic lysis, and density gradient centrifugation on Histopaque-1077 (Sigma) as described.[20] Typically, the polymorphonuclear cells were more than 90% neutrophils as assessed by Wright-Giemsa staining and over 90% $CD16^+$ by the flow cytometry. Neutrophils were activated through the treatment with 1.0 μM f-MetLeuPhe (Sigma) for 10 min at 37°C in 1 ml HBSS/HSA ($Ca^{2+}$and Mg^{2+}-free Hanks balanced salt solution with 0.2% HSA)

Iodination of galectin. The protein (100 μg) was incubated at room temperature for 15 min in 50 mM sodium phosphate, pH 7.0, with 74 MBq carrier-free $Na^{125}I$ (Amersham Pharmacia Biotech) in the presence of two Iodobeads™ (Pierce). Labeled proteins were separated from the free iodine and lactose by gel filtration on Sephadex G-25 (PD-10 column, Amersham Pharmacia Biotech). Specific radioactivity was 500 cpm/ng of galectin-1.

Myeloperoxidase assay. The activity of myeloperoxidase was determined by measuring the rate at which it oxidized 3,5,3',5'-tetramethylbenzidine (TMB) at 25°C during the first 1 min of reaction. The reaction, carried out in 100 mM acetate buffer, pH 5.4, containing 8 % dimethylformamide, was started by adding 300 μM hydrogen peroxide.

Assay for galectin-1 binding to cells. Galectin-1 binding to the activated neutrophils and HL-60 cells were examined by the following methods. (i) Galectin-1 was diluted in 0.1 M phosphate saline (PBS, pH 8.0) and immobilized on the 96-microtiter well (500 ng/well). Cells were applied to the same well, incubated for 30 min and washed with PBS three times. The amount of attached cells were determined by myeloperoxydase assay. (ii) The cells were harvested, washed three times with PBS, and resuspended in PBS containing BSA (1 mg/ml) at a concentration of 1×10^7 cells/ml. They (3.3×10^5 cells) were then incubated in the Eppendorf tubes with a constant amount of the iodinated galectin-1 and different amounts of cold galectin-1 or 20 mM lactose. After incubation at 4°C for 30 min, 0.15 ml of Apiezon: n-butyl phthalate (1 : 9) was added to the Eppendorff tube, and the mixture was centrifuged for 7.5 min. The supernatant was removed through aspiration, and the cell pellet was placed in the counting vial. The radioactivity was measured using a Beckman γ counter.

Lectin blotting. For the detection of galectin-1 ligand glycoproteins, 30 μg of the microsomal fraction was prepared as described previously[18] and subjected to electrophoresis on 7.5 % SDS-PAGE. The separated proteins were electrophoretically transferred onto nitrocellulose membranes (Hybond-ECL Western, Amersham Corp.), followed by lectin blot analysis. The ECL lectin blotting was performed as described previously,[18] except that the galectin-1 ligands were detected using 10 μg/ml of galectins-1 followed by horseradish peroxidase-conjugated anti-galectin-1 antibody.

Results

Activation of neutrophils decreases surface sialic acid and increases Gal-1 binding. To assess the effects of galectin-1 on normal human leukocytes, we used the resting and activated human neutrophils. Iodinated galectin-1 was incubated with the resting and fMLP-activated neutrophils at 4°C. Galectin-1 bound more to the activated than to the resting neutrophils (Fig. 1). Presence of excess unlabeled galectins-1 competed with the galectin-1 binding to neutrophils and abolished the binding of radiolabeled galectin-1, indicating the binding is specific. Solid phase cell adhesion assay also gave the similar results. Microplate coated with galectin-1 was incubated with the resting and activated neutrophils and adherent neutrophils were detected with myeloperoxidase assay after removing the nonspecific binding. Activation of neutrophil increased the binding of total galectin-1 and lactose inhibited the binding (Fig. 2). In all cases, binding of galectin-1 was inhibited by 20 mM lactose but not affected by the same concentration of maltose solution (Fig. 2), demonstrating that the binding of neutrophils to galectin coated well is specifically galectin-glycoconjugate mediated. These results demonstrate that galectin-1 binds more effectively to the activated human neutrophils than to the resting neutrophils.

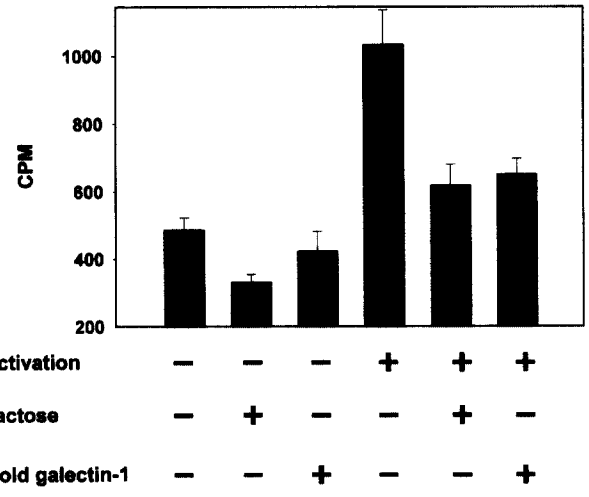

Fig. 1. Binding site for galectin-1 increase upon activation of neutrophil. Resting and fMLP activated neutrophils were incubated with iodinated galectin-1 and lactose or excess unlabeled galectin-1. The amount of galectin-1 binding was determined by measuring CPM on the cell pellet after an oil wash.

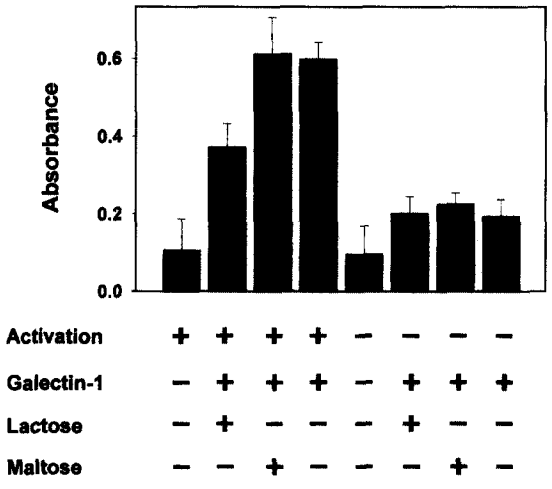

Fig. 2. Neutrophil adhesion assay on a galectin-1-coated well. Resting and activated neutrophils were incubated on a galectin-1 coated-well with lactose (20 mM) or maltose (20 mM). Adherent cells were measured though myeloperoxidase assay.

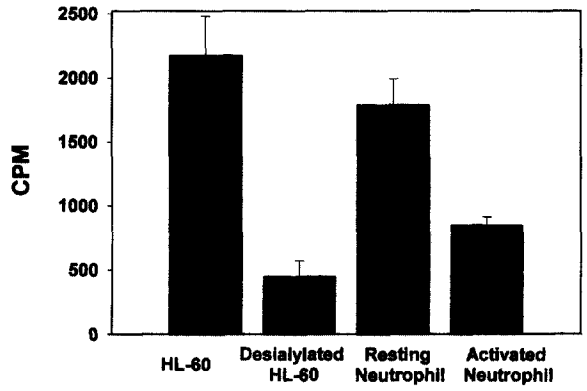

Fig. 3. Desialylation of HL-60 cells and activation of neutrophil decreases binding sites for MAL. HL-60 cells and enzymatically desialylated HL-60 cells (dsHL-60) were incubated with iodinated MAL (10 μg/ml each) for 30 min on ice and bound CPM was counted as indicated in the experimental procedure. Results are representative of three independent experiments.

Binding of galectin-1 to HL-60 cells. The promyelocyte cell-line HL-60 can be induced to differentiate into neutrophil-like cells. Such a neutrophil-like HL-60 cells are known to contain many of the neutrophil receptors and are frequently used as a model system for studying the neutrophil function. However, HL-60 cells are totally deficient in specific granules and in most of the proteins stored in these granules. It was reported that when the neutrophils were activated, various types of proteins were released from the granules, one of these being sialidase.[28] In order to simulate the changes of ligands on the activated neutrophil surface, HL-60 cells were treated with sialidase. Enzymatic desialylation of HL-60 cells (dsHL-60 cells) decreased the binding of the sialic acid-specific lectin MAL, which recognizes NeuAca2→3Galß1→4GlcNAc-R linkages (Fig. 3).[19] Conversely, we observed an increase in

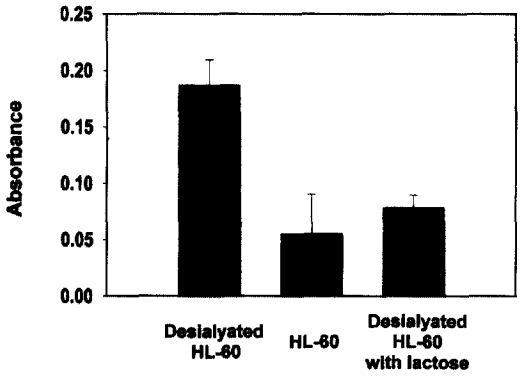

Fig. 4. Desialylation increases the binding sites for galectin-1 on HL-60 cells. Control or desialylated HL-60 cells were incubated in a galectin-1 coated-well (500 ng/well) for 30 min at 4°C in the absence or presence of 20 mM lactose. Unbound cells were then washed with PBS three times, and bound cells were measured in terms of their by myeloperoxidase activity. The results are representative of three independent experiments with triplicate samples.

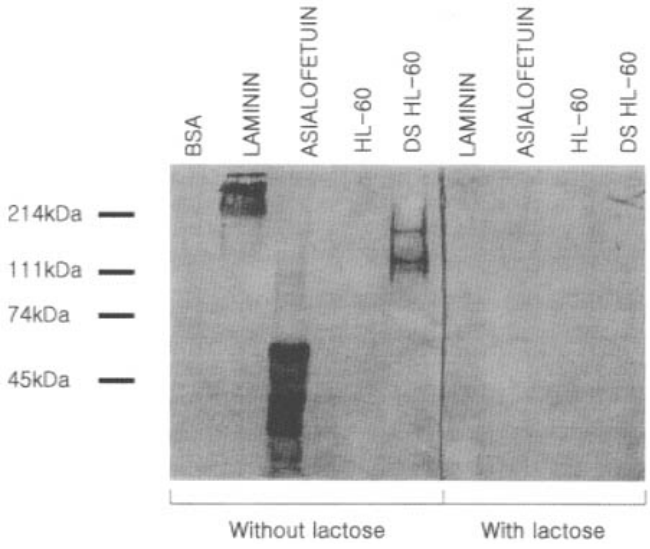

Fig. 5. Galectin-1 blotting of glycoproteins from HL-60 cells and desialyated HL-60 cells. Approximately 30 μg of microsome from each cell line was separated by 7.5% SDS-PAGE. The proteins were transferred to nitrocellulose and incubated with galectin-1, followed by 10 μg/ml horseradish peroxidase-conjugated anti galetin-1 antiboby in the absence or presence of 200 mM lactose. The blots were developed using the ECL-based Western blotting kit. Mouse laminin and asialofetuin were included as positive controls and BSA as a negative control.

the lactose-inhibited binding of enzymatically desialylated dsHL-60 cells to galectin-1 coated microtiter well (Fig. 4), indicating that both proteins bound to the specific β-galactoside-bearing glycoconjugates on the cell surface. These results demonstrated that enzymatic desialylation of the cells exposes more binding sites for galectin-1.

Galectin-1 blot analysis of glycoproteins ligands in HL-60 cells. To assess whether there are specific glycoprotein ligands for galectin-1, the microsome prepared

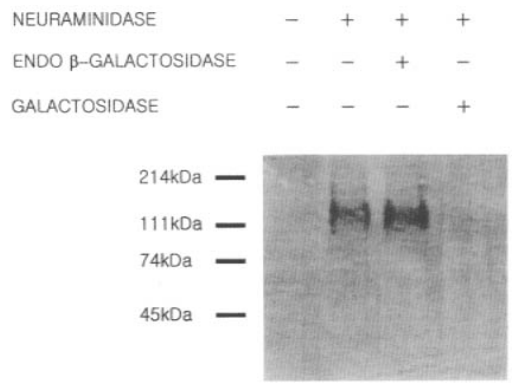

Fig. 6. Detection of carbohydrate determinants of galectin-1 ligands on HL-60. Cells were incubated with excess amount of the indicated glycosidase for 30 min at 37°C. Galectin-1 blotting was performed as in Fig. 5 after the glycosidase treatments.

from the sialidase-treated and nontreated HL-60 cells were analyzed on 7.5% SDS-PAGE, and glycoproteins were probed with galectin-1 followed by peroxidase-conjugated anti galectin-1 antibody. Unexpectedly, not many galatosylated glycoproteins bound strongly enough to galectin-1 from HL-60 cells (Fig. 5). In contrast, a significant amount of galectin-1 reactive membrane-associated glycoprotein was present in the microsomes of the desialylated HL-60 cells (Fig. 5). Two major bands, 120 and 150 kDa, were detected. In all cases the binding of galectin-1 to glycoproteins was specific, since the inclusion of hapten sugar lactose (200 mM) blocked the binding (Fig. 4). In control experiments, galectin-1 bound well, as expected, to a commercially prepared Engelbreth-Holm-Swarm laminin and asialofetuin, which is known to contain terminal β1,4-galactosyl residues.[27] These results demonstrate that desialylation of HL-60 exposed new galectin-1 ligands, which were galactosylated glycoproteins of approximately 120 and 160 kDa.

Teminal galactose residue on glycoprotein is responsible for galectin-1 binding to desialylated HL-60 cells. To address the sugar structure of glycoprotein responsible for galectin-1 binding, galectin-1 blotting was performed on HL-60 cells treated with various glycosidases. As expected, after sialidase treatment, galectin reactive band was detected. Protein of 120 kDa was predominant, while that of 160 kDa was very faint. Endo β-galactosidase treatment did not affect the galectin binding, but β1,4-galactosidase treatment abolished the galectins-1 binding to the ligands (Fig. 6). This indicated that the terminal galactose residue is more responsible for the galectin-1 binding to the ligand in desialylated HL-60 than poly-N-acetyllactosamine which is specifically cleaved by endo β-galactosidase.

Discussion

The results of this study demonstrated that binding of galectin-1 to carbohydrate determinants on leukocytes increased when the neutrophils were activated, and this increase was due to the desialylation following the exposure of a new galactosylated residue. As a model system, HL-60 cells showed similar phenomena. From the desialylated HL-60 cells, two major glycoproteins were detected on galectin-1 blotting analysis and galectin-1 and glycoprctein interactions were mainly mediated by the terminal galactose residue.

It is well-documented that galectin-1 has higher affinity to the poly-N-acetyllactosamine chain than the terminal galactose.[16,23] However, this study showed, in the activated neutrophils, the terminal galactose was somewhat more responsible for the galectin-1 binding, which can be explained by the fact that many of the suggested galectin-1 ligands in the leukocyte were mucin-type molecules which have large amount of sialylated O-linked oligosacchride containing few polylactosamine chain.[17] In the case of galectin-3, which has similar carbohydrate specificity, CD66a and b were reported as its ligand.[22] CD66a and b are LAMP1 and 2, respectively, which contain large amount of polylactosamine chains. Because the galectin binding to LAMP1 and 2 is sensitive to the endo β-galactosidase, we believe the ligands on the activated neutrophils, found in this study, are neither LAMP1 nor 2.

Galectin-1 is secreted into the extracellular environment,[24] where it recognizes ligands on ECM components,[25] and it has been postulated to act as a modulator of cell-cell and cell-ECM interaction.[14] Galectin-1, in particular, was reported to act as a T-cell modulator.[17,21] This observation is supported by the localization of galectin-1 *in vivo*. Galectin-1 was detected in the endothelial cells lining the specialized vessels, termed as the high endothelial venules.[13] These results suggest that galectin-1 expressed by endothelial cells may bind to and affect the movement of cells emigrating from blood into tissues. Another type of immune cells that migrate through vessels are activated neutrophils. The factors regulating neutrophil turnover *in vivo* are unclear. Selectins, integrins, and other adhesion molecules are required for the neutrophil adhesion, activation, and extravasation from the circulation.[20] Our study suggest that neutrophils activated by a local mediator in the inflammatory site are desialylated, which results in the exposure of new galectin-1 ligands, followed by the interaction with galectin-1 during the extravasation process. The consequences of galectin-1 binding to the activated neutrophils are still under investigation.

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