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Galectin-8 interacts with podoplanin and modulates lymphatic endothelial cell functions

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Abstract

Podoplanin is a small, mucin-like membrane glycoprotein highly expressed by lymphatic but not by blood vascular endothelial cells. Although it was shown to be indispensable for the correct formation and function of the lymphatic vasculature, its precise molecular function has remained unknown. In the present study, we identified the mammalian lectin galectin-8 as a novel, glycosylation-dependent interaction partner of podoplanin. Galectin-8 is a tandem-repeat type galectin, which interacts with cell surface glycoproteins, including certain integrins, as well as with extracellular matrix molecules such as fibronectin. Here we show that, similar to podoplanin, galectin-8 is more highly expressed by lymphatic than by blood vascular endothelial cells, and that it promotes lymphatic endothelial cell adhesion as well as haptotactic migration when immobilized onto a surface, while inhibiting the formation of tube-like structures by lymphatic endothelial cells in a collagen matrix when incorporated into the matrix. Importantly, functions of blood vascular endothelial cells, which lack podoplanin expression, are not affected by galectin-8. These data suggest a role for galectin-8 and podoplanin in supporting the connection of the lymphatic endothelium to the surrounding extracellular matrix, most likely in cooperation with other glycoproteins on the surface of lymphatic endothelial cells.

Keywords

podoplanin; galectin-8; lymphatic endothelial cell; lymphangiogenesis

Introduction

The lymphatic vascular system has become the subject of intense research during the last two decades, since diverse molecular markers for lymphatic vessels were discovered and a number of *in vitro* and *in vivo* models became available to study various aspects of lymphatic biology. Lymphatic vessels are found in nearly all organs and play important physiological roles, including the drainage of tissue fluid, the transport of immune cells to lymph nodes and the uptake of dietary fat in the intestine. Moreover, the lymphatic system is crucially involved in a number of pathological conditions, such as cancer metastasis and certain inflammatory diseases. Despite the growing interest in lymphatic biology, however,

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the molecular events underlying the development and the function of the lymphatic system are still much less well understood than those taking place in the blood vascular system.

A long-standing open question in the field of lymphatic biology concerns the presumable molecular function of podoplanin, a sialomucin-like membrane glycoprotein, which is highly expressed on the surface of lymphatic endothelial cells (LECs) but not blood vascular endothelial cells (BECs) *in vitro* as well as *in vivo* [1,2]. Human podoplanin is a 162 amino acid type-I transmembrane protein, comprising a short cytoplasmic tail, a single membrane-spanning domain and an extensively O-glycosylated, sialylated extracellular portion [3]. Although studies in podoplanin knockout mice demonstrated that this protein is essential for the correct formation and function of the lymphatic vascular system [2], and it is one of the most commonly exploited markers for lymphatic vessels [4], the exact molecular function of podoplanin on the lymphatic endothelium remains unclear. Several reports suggested a role for podoplanin in endothelial cell cytoskeletal organization and migration. Podoplanin overexpression induces the formation of filopodia-like plasma membrane extensions in a variety of cell types [2,3,5,6], and a conserved cluster of three basic amino acids in its cytoplasmic domain mediates its interaction with the membrane cytoskeleton linkers ezrin and moesin [7]. Additionaly, two binding partners of the extracellular domain of podoplanin have been identified so far. The podoplanin ectodomain and the lymphatic-specific chemokine CCL21 form a complex, which presumably contributes to the recruitment of CCR7-positive immune cells towards the lymphatic vessels after being shed from the lymphatic endothelium into the perivascular stroma [8]. The interaction of podoplanin with the C-type lectin like receptor 2 (CLEC-2) on platelets is responsible for the platelet-aggregating capacity of podoplanin [9], which is of relevance mainly for podoplanin-expressing cancer cells, but might also be of importance in the lymphatic vasculature, to prevent mixing of blood and lymphatic vessels. The interactions with CCL21 and with CLEC-2 both require the glycosylation of the podoplanin ectodomain [8,9].

Galectins are a subgroup of animal lectins – i.e. non-enzymatic, sugar-binding proteins – which are defined by their binding specificity for β-galactosides and the presence of at least one structurally conserved carbohydrate-recognition domain (CRD) [10]. Based on their domain organization, galectins can be classified into prototypes, chimera types and tandem-repeat types [11]. They are found throughout all animal kingdoms and exert extraordinarily diverse functions both inside and outside the cell, through protein-carbohydrate as well as protein-protein interactions (reviewed in [12]). Fifteen mammalian galectins have been identified up to date, eleven of which are also expressed in humans. One of those is galectin-8, which is found in many tissues including lung, liver, kidney, spleen and others. Galectin-8 is a soluble, 35 kDa protein belonging to the subclass of tandem-repeat type galectins, since it has two homologous CRDs, connected by a short, unconserved linker peptide. Alternative mRNA splicing gives rise to at least six different potential galectin-8 protein isoforms, including proteins with only one CRD which have, however, never been isolated so far [13]. Despite having features of a cytosolic protein, galectin-8 – similar to other galectins – may be externalized under certain conditions through a non-classical secretion pathway, without the requirement for a signal peptide [14,15]. Upon secretion, it is retained at the cell surface due to its interaction with certain integrins, including $\alpha_3\beta_1$ and $\alpha_6\beta_1$ and to a very limited extent also α_4 and β_3 [15]. The binding of galectin-8 to integrins is sugar-dependent (in contrast to the protein-protein interactions of integrins with their ligands) [15] and triggers integrin-mediated signaling cascades and cytoskeletal re-organization [16]. Additionally, galectin-8 also associates with extracellular matrix (ECM) glycoproteins, such as fibronectin [17]. When immobilized onto a surface, galectin-8 promotes cell adhesion and spreading as potently as fibronectin [17]. Soluble galectin-8, however, was shown to inhibit cell adhesion to ECM molecules such as fibronectin and laminin, possibly by masking the ligand binding sites of integrin receptors and thereby

preventing cell-matrix interactions. Its ability to both positively and negatively regulate cell adhesion qualifies galectin-8 as a matricellular protein [17], i.e. an adhesion-modulating protein similar to SPARC, thrombospondin, tenascin and others [18].

In the present study, we identified galectin-8 as a novel, glycosylation-dependent interaction partner of podoplanin. Moreover, we found that galectin-8 is more highly expressed by LECs than by BECs and that it promotes LEC adhesion and haptotactic migration while inhibiting the formation of tube-like structures by LECs. Our results suggest a role for galectin-8 and podoplanin in supporting the connection of the lymphatic endothelium to the surrounding ECM, most likely in cooperation with other glycoproteins on the surface of LECs.

Materials and methods

Cell culture

Primary human lymphatic (LECs) and blood vascular endothelial cells (BECs), isolated from neonatal foreskins as previously described [1], as well as human umbilical vein endothelial cells (HUVECs; ScienCell, Carlsbad, CA) were kept in type I collagen-coated (50 μg/ml in PBS; Inamed, Fremont, CA) culture dishes in endothelial cell basal medium (EBM; Lonza, Walkersville, MD) supplemented with 20% fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine (all from Gibco, Grand Island, NY), 10 μg/ml hydrocortisone and 25 μg/ml N-6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (Sigma, St. Louis, MO). Endothelial cells were subcultured at a split ratio of 1:2 to 1:3 shortly before they reached confluence. Chinese hamster ovary (CHO) wildtype cells were cultured in DMEM (Gibco) supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Before collection of conditioned medium FBS was omitted for three days. CHO ldlD cell (ATCC, Manassas, VA) suspension cultures were kept in ProCHO protein-free medium (Lonza) supplemented with 8 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Human embryonic kidney (HEK) 293 cell suspension cultures were kept in EXCELL 293 serum-free medium (Sigma) supplemented with 6 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. All cells were grown at 37°C in a 5% CO_2 humidified atmosphere.

Expression and purification of podoplanin-Fc

Podoplanin-Fc cDNA was constructed as previously described [19], subcloned into the pcDNA3.1(+) vector (Invitrogen, Carlsbad, CA) and transfected into CHO wildtype, CHO ldlD and HEK 293 cells using the SuperFect transfection reagent (Qiagen, Hilden, Germany). Polyclonal cells, selected with 600 μg/ml (CHO ldlD) or 800 μg/ml (CHO wildtype, HEK 293) G418 (Sigma), were cultured for three days in serum-free medium before conditioned medium was collected. Podoplanin-Fc was affinity purified using protein A-columns (Bio Rad, Hercules, CA) and dialyzed against PBS. The quality of the purified proteins was assessed by SDS PAGE and silver staining and by gel filtration chromatography. For glycan analysis, proteins were digested with neuraminidase (0.5 mU/μg of protein; Roche Diagnostics, Mannheim, Germany) for 2 hrs at 37°C, followed by O-glycosidase (0.025 mU/μg of protein; Roche Diagnostics) for 21 hrs at 37° in 20 mM phosphate buffer pH 6.

Protein microarray

ProtoArray Human Protein Microarrays nc v3.0 (Invitrogen) were probed with either 50 μg/ml podoplanin-Fc expressed in CHO wildtype cells or 50 μg/ml recombinant human IgG_1 Fc (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Arrays were scanned on a GenePix 4200A scanner (Molecular Devices, Sunnyvale, CA) and results

were analyzed using the ProtoArray Prospector v3.2 software (Invitrogen). Duplicates were performed.

Binding assay and co-immunoprecipitation

30 pmoles of recombinant human galectin-8 (R&D Systems) were incubated in a total volume of 40 μl with either PBS alone, 50 pmoles podoplanin-Fc from HEK 293 cells in PBS, 50 pmoles podoplanin-Fc from CHO ldlD cells in PBS or 50 pmoles human IgG_1 (Sigma) in PBS, for 3 hours at room temperature. Protein A-sepharose (GE, Little Chalfont, UK) was prepared according to the manufacturer's instructions and blocked in 1% bovine serum albumin (BSA) in PBS for 2 hours at room temperature before use. 20 μl protein A-sepharose in PBS containing 0.1% BSA was added to each protein sample and incubated for 2 hours at room temperature with occasional mixing. Then, supernatants were collected and the sepharose beads were washed three times with PBS. Beads and supernatants were boiled in Laemmli buffer for subsequent gel electrophoresis. For co-immunoprecipitation experiments, 80 pmoles recombinant human galectin-8 were added to 80 μl LEC lysate (approximately 1 μg/ml total protein, prepared in 1% CHAPS, 25 mM HEPES, 150 mM NaCl, 5mM $MgCl_2$, 1 mM phenylmethanesulfonyl fluoride and protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics)). After overnight incubation at 4°C, goat anti-human galectin-8 antibody (4 μg; R&D Systems) was added for 3 hours at 4°C, followed by 20 μl immobilized protein G (Pierce, Rockford, IL) and subsequent procedures as described above.

Real-time reverse transcription PCR

RNA was isolated from subconfluent cultures of human dermal LECs and BECs using TRIzol reagent (Invitrogen) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay for human galectin-8 (Hs00374634_m1; Applied Biosystems). 20 ng cDNA were used as template. Cycling parameters were as follows: 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 15 sec denaturation at 95°C and 1 min annealing and extension at 60°C. All reactions were performed in triplicates using the 7900HT Fast Real-Time PCR system (Applied Biosystems). β-actin served as internal control for normalization of signals.

Immunoblot

Proteins were extracted from primary human endothelial cells for 30 min on ice, using cell lysis buffer containing 20 mM Tris pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, 25 mM sodium fluoride, 1 mM phenylmethanesulfonyl fluoride, 1 mM sodium metavanadate, 10% glycerol and protease inhibitor cocktail (Complete Mini EDTA-free; Roche Diagnostics). Protein samples were boiled in Laemmli buffer, resolved in 10% polyacrylamide gels (Invitrogen) and transferred to nitrocellulose membranes (Bio Rad) which were blocked in 10% nonfat dry milk in PBS for 90 min at room temperature. Antibody incubation was done for 3 hours at room temperature or overnight at 4°C using antibodies against galectin-8 (10 μg/ml; kind gift of Dr. Y. Zick, Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel. Or 2.5 μg/ml; R&D Systems), podoplanin (D2-40, 1:1000; Covance, Emeryville, CA), integrin β_1 (1:500; Chemicon, Temecula, CA) or β-actin (clone AC-15, 1:5000; Sigma), diluted in 5% nonfat dry milk in PBS. Horseradish peroxidase-coupled anti-mouse or anti-rabbit antibodies (GE) were applied at 1:2000 or 1:10 000 dilutions, respectively, in 5% nonfat dry milk in PBS for 1 hour at room temperature. For detection, the ECL Plus Detection System and ECL chemiluminescence films (GE) were used.

Immunofluorescence

LECs were grown on collagen-coated glass chamber slides (BD Falcon, Bedford, MA), fixed with 4% PFA in PBS at 4°C, and stained using antibodies against galectin-8 (goat IgG, 3 μg/ml; R&D Systems) and podoplanin (D2-40, 1:50; Covance), or corresponding control IgGs (Sigma), followed by Alexa Fluor 488- or 594-conjugated secondary antibodies (1:200) and Hoechst nuclear dye (10 μg/ml in PBS; Invitrogen Molecular Probes, Eugene, OR). All antibodies were diluted in 10% BSA, 5% donkey serum and 0.1% Triton X-100 in PBS. Images were taken with a SP1 TCS confocal microscope (Leica Microsystems, Wetzlar, Germany) equipped with a 63× 1.32NA Oil Ph3 HCX PlanApo objective. Adobe Photoshop was used for image overlay.

Adhesion assay

24 well cell culture plates were coated with 25 μg/ml recombinant human galectin-8 or 10 μg/ml fibronectin (BD, Franklin Lakes, NJ) in PBS for 2 hours at room temperature. Remaining protein binding sites were blocked with 100 μg/ml BSA in PBS for 2 hours at room temperature. Subconfluent endothelial cells were harvested with accutase (Sigma). 5×10^4 cells in 0.5 ml EBM containing 0.2% BSA were seeded into each well and left to attach for 20 min at 37°C. Unattached cells were washed away by three gentle washes with PBS and the remaining cells were fixed in 4% PFA in PBS at 4°C. For evaluation, attached cells were stained with Hoechst nuclear dye (10 μg/ml in PBS; Invitrogen Molecular Probes), three images per well were taken at 5× magnification using an Axiovert 200M inverted microscope equipped with a AxioCam MRm camera (Carl Zeiss, Göttingen, Germany), and cells in each well were counted using IPLab image analysis software (BD Biosciences Bioimaging, Rockville, MD). Triplicates were performed for each sample and the unpaired Student's t-test was used for statistical analysis of the results. For staining of F-actin in attached cells, adhesion assays were performed as described, but using tissue culture treated glass slides (8 chambers, BD Falcon) and 1.5×10^4 cells per chamber. Fixed cells were stained with Alexa Fluor 488-conjugated phalloidin (Invitrogen Molecular Probes) according to the manufacturer's instructions. Nuclei were counterstained with Hoechst dye (10 μg/ml in PBS).

Migration assay

Transwell migration assays were performed using HTS FluoroBlok Insert System 96 well plates (pore size 8 μm; BD). The underside of the membranes was coated with 25 μg/ml recombinant human galectin-8 in PBS or with PBS alone for 1.5 hours at room temperature, followed by blocking of remaining protein binding sites with 100 μg/ml BSA in PBS for 1.5 hours at room temperature. Subconfluent LECs were harvested with accutase after 3 hours starvation in EBM containing 0.2% BSA, and 2.5×10^4 cells/well in 50 μl EBM containing 0.2% BSA were seeded in the upper chambers of the FluoroBlok plate. The lower chambers contained either EBM with 0.2% BSA, or complete endothelial cell growth medium (with 20% FBS) as a positive control for migration. Plates were incubated for 5 hours at 37°C, before cells on the underside of the membrane were stained with 3.85 μg/ml calcein AM (Fluka, Buchs, Switzerland) in PBS for 10 min at 37°C and fluorescence was measured at λ_{ex} 485 nm/λ_{em} 538 nm using a SpectraMax Gemini EM plate reader (Molecular Devices). Six replicates were performed for each sample and the unpaired Student's t-test was used for statistical analysis of the results.

Tube formation assay

Endothelial cells were grown in fibronectin-coated (10 μg/ml in PBS) 24 well culture plates in normal medium. When confluence was reached, cells were starved overnight in EBM supplemented with 5% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-

glutamine and 10 μg/ml hydrocortisone. The next day, the starvation medium was replaced by 0.5 ml/well of a mixture of two parts 2.5 mg/ml type I collagen in PBS pH 7.4 and three parts EBM with 100 U/ml penicillin and 100 μg/ml streptomycin, containing or not 250 nM recombinant human galectin-8. The plate was incubated for 12 to 24 hours at 37°C before three images per well were taken at 5× magnification using an Axiovert 200M inverted microscope equipped with a AxioCam MRm camera (Carl Zeiss). The total tube length per well was determined using IPLab image analysis software. Triplicates were performed for each sample and the unpaired Student's t-test was used for statistical analysis of the results.

Results

Galectin-8 interacts with podoplanin in a glycosylation-dependent manner

In order to clarify the yet unknown molecular function of podoplanin on the lymphatic endothelium, we sought to identify novel interaction partners of this membrane glycoprotein. To this aim, we probed a human protein microarray with a recombinant soluble fusion protein consisting of the extracellular domain of podoplanin linked to the Fc domain of human IgG_1 (podoplanin-Fc), or the human IgG_1 Fc domain alone as a control. Podoplanin-Fc used for probing the array was produced in Chinese hamster ovary (CHO) wildtype cells and thus glycosylated. Since we were looking for ligands of the podoplanin ectodomain, we focused on proteins known to be located at the cell surface or in the extracellular space, which was a minority among the approximately 5000 human proteins printed on the array. The only interaction for which the detected signal exceeded the threshold Z-score value of 1 in both of the duplicate spots on each array was with galectin-8, a sugar-binding protein. Although also several other galectins were present on the array, none of them gave a positive signal (Table 1).

To assess whether the interaction of podoplanin with galectin-8 depends on carbohydrates, we expressed podoplanin-Fc in several mammalian cell lines differing in their glycosylation capacity, thus producing different glycoforms of podoplanin-Fc (Figure 1A). While human embryonic kidney (HEK) 293 cells assemble extended, branched O-linked oligosaccharides, which are resistant to sequential digestion with neuraminidase and O-glycosidase, CHO wildtype cells are able to make only short O-glycans of the core 1 structure with up to four sugars [20, 21], which can be completely removed with neuraminidase and O-glycosidase (Figure 1A). CHO ldlD cells – when cultured in a medium with glucose as the sole sugar source – due to their deficiency in UDP-galactose and UDP-N-acetylgalactosamine 4-epimerase activity are not able to produce any O-linked carbohydrate chains at all [22]. Using recombinant human galectin-8 and different podoplanin-Fc glycoforms in a pull-down experiment with protein A-sepharose, we were able to confirm the binding of galectin-8 to glycosylated podoplanin-Fc derived from HEK 293 cells, but not to unglycosylated podoplanin-Fc derived from CHO ldlD cells or human IgG_1 (Figure 1B), demonstrating that the interaction of galectin-8 with podoplanin depends on carbohydrate structures present in the extracellular domain of the latter. Moreover, the fact that no galectin other than galectin-8 interacted with podoplanin on the protein array confirms that galectin-8 has a unique sugar-binding specificity, as was previously reported [15, 23, 24].

To determine whether galectin-8 also interacts with endogenous podoplanin, we performed co-immunoprecipitation experiments using recombinant human galectin-8 and lysates of primary human lymphatic endothelial cells (LECs), which strongly express podoplanin. Indeed, LEC-derived podoplanin co-immunoprecipitated with galectin-8, revealing that the podoplanin glycoform expressed by LECs is recognized by galectin-8 (Figure 1C). Furthermore, we could show the previously reported interaction of galectin-8 with the integrin β_1 subunit [15], for integrin β_1 expressed by LECs (Figure 1C).

Galectin-8 is more highly expressed by lymphatic than by blood vascular endothelial cells

Since podoplanin is within the vasculature specifically expressed by lymphatic endothelial (LECs) but not blood vascular endothelial cells (BECs) and appears to have a crucial function in the lymphatic system, we assessed galectin-8 expression levels in LECs as compared to BECs. We quantified galectin-8 mRNA in three matched pairs of human dermal LECs and BECs by real-time reverse transcription PCR. 1.5 to 3.5 fold higher galectin-8 mRNA levels were detected in LECs compared to the matched BECs in all three pairs (Figure 2A). Accordingly, more galectin-8 protein was present in LECs than in BECs, as revealed by immunoblotting of LEC and BEC lysates using an anti-galectin-8 antibody (Figure 2B). In addition to the band representing the full-length galectin-8 protein at 35 kDa, a second band at approximately 30 kDa with a similar intensity in LECs and BECs was detected in the immunoblot. Most likely, this band arises from an unrelated protein being recognized by the antibody.

Although galectin-8 may be secreted under certain conditions, its concentration in the cell supernatant of either type of endothelial cell was below the detection limit of the immunoblot (not shown). This indicates that in cultured endothelial cells the protein is either not externalized or remains bound to the cell surface, as was described before [15], and hence is detected in the cell lysates in addition to the intracellular pool of galectin-8.

To rule out the possibility that contaminating cells represent the source of the galectin-8 mRNA and protein detected in LECs and to determine its subcellular localization, we also performed immunofluorescence analyses of galectin-8 expression in primary human LECs, which revealed the presence of galectin-8 mainly in the cytoplasmic compartment of LECs (Figure 2C).

Galectin-8 promotes adhesion and haptotactic migration of lymphatic endothelial cells, but inhibits formation of tube-like structures

We assessed adhesion of LECs and BECs to culture plates coated with recombinant human galectin-8 or human fibronectin as a reference. While all LECs rapidly and firmly adhered and spread on galectin-8, only few BECs were able to adhere to galectin-8 within 20 minutes after seeding (Figure 3A), and among the attached BECs many were not spread yet, but still having round shapes (Figure 3B). In contrast to this, both endothelial cell types adhered equally well to fibronectin (Figure 3A). When added to the cell culture medium in soluble form, galectin-8 nearly completely prevented adhesion of both LECs and BECs to any substrate, due to aggregation of the cells (data not shown).

To test whether galectin-8 might also promote haptotactic migration of LECs, we performed a transwell migration assay in which the underside of the membrane separating the two chambers was coated with galectin-8 or bovine serum albumin (BSA) and LECs were seeded in the upper chamber. FBS-supplemented medium in the lower chamber served as a positive control. Galectin-8 on the underside of the membrane significantly enhanced haptotactic migration of LECs to the lower chamber, as compared to BSA (Figure 4).

Finally we determined the effect of galectin-8 on the formation of tube-like structures by LECs. In this assay, a LEC monolayer is overlaid with a type I collagen gel in which endothelial cells align to form elongated structures which appear as tubes – a process involving adhesive and migratory events as well as changes in cell shape. Interestingly, despite its beneficial effect on LEC adhesion and migration, the addition of galectin-8 efficiently inhibited the formation of tube-like structures by LECs in collagen gel (Figure 5A), while it did not affect the formation of tube-like structures by human umbilical vein endothelial cells (HUVECs; Figure 5B). Possibly, the presence of galectin-8 in their immediate environment stabilizes LECs in their position thereby preventing the

rearrangement of cells required for tube formation. Given the effects of galectin-8 on LECs and its interaction with podoplanin, we also assessed the expression level and subcellular localization of podoplanin in LECs after galectin-8 treatment (250–500 nM for 24 hours). No major changes in podoplanin mRNA or total protein levels were detected by real-time RT PCR or immunoblot analysis, respectively, and also the abundance and distribution of podoplanin on the cell surface was unaltered upon treatment with galectin-8, as determined by flow cytometry and immunofluorescence (data not shown).

Discussion

In the present study we identified galectin-8 as a novel interaction partner of podoplanin, an extensively O-glycosylated membrane protein which is within the vasculature specifically expressed on the lymphatic endothelium. As expected for a lectin, the interaction of galectin-8 with podoplanin required sugar-moieties, which as such are naturally present in the ectodomain of podoplanin, as shown by the interaction of endogenous podoplanin from primary human lymphatic endothelial cells (LECs) with galectin-8 which we detected. Interestingly, only galectin-8 but not galectin-1, -2, -3 or -7 interacted with podoplanin, indicating that galectin-8 binding occurs through carbohydrate structures that fail to efficiently interact with other galectins having different ligand specificities. While the β-galactose-binding subsite of galectin carbohydrate recognition domains (CRDs) is conserved, galectins differ in their ability to accommodate various saccharides attached to galactose in other subsites of their CRDs. The two CRDs of galectin-8 display different fine specificities for ligands, the N-terminal CRD having a particularly strong affinity to β-galactosides with sialic acid or sulfate linked to position 3 of the galactose [23,24]. Indeed, (di-)sialylated core 1 structure glycans were reported to be present in the ectodomain of human podoplanin [3,21,25], which might serve as ligands for galectin-8. Alternatively, the selective binding of galectin-8 but not other galectins to podoplanin might be explained by the fact that galectin-8 features two CRDs, while all the non-interacting galectins tested belong to the groups of prototype or chimera type galectins with only one CRD (Table 1). Possibly, efficient binding to podoplanin requires the combined interaction of both galectin-8 CRDs due to moderate affinity or structural constraints. Although on the protein microarray we did not detect any significant interactions of the podoplanin ectodomain other than the one with galectin-8, this result does of course by no means exclude the existence of other ligands to the extracellular part of podoplanin, since actually only relatively few of the proteins spotted on the array were membrane-bound or extracellular proteins and thus likely binding partners of a protein ectodomain.

We found that primary human dermal LECs express galectin-8 and that the level of expression is higher than in matched blood vascular endothelial cells (BECs), as similar to podoplanin. In agreement with previous reports defining galectin-8 as a matricellular modulator of cell adhesion [17], it potently promotes the adhesion of LECs when immobilized to a surface, while inducing cell aggregation and thereby preventing cell adhesion to any substrate when present in solution. Notably, LECs much more readily attach to and spread on immobilized galectin-8 than BECs. Moreover, LECs haptotactically migrate towards galectin-8, but form less tube-like structures in a collagen gel if galectin-8 is incorporated in the gel, indicating that LECs firmly adhere to matrix-bound galectin-8 in their immediate vicinity which prevents major cell re-localizations such as required for the formation of tubes. Interestingly, incorporation of galectin-8 into the collagen gel does not prevent human umbilical vein endothelial cells (HUVECs), i.e. blood vasculature-derived cells, from forming tube-like structures.

Although these experiments were performed using recombinant human galectin-8 and it remains to be determined whether the demonstrated functions represent those of endogenous

galectin-8, our results suggest that in the tissue galectin-8 – since it also associates with glycoconjugates in the extracellular matrix (ECM) [17] – might play a role in anchoring the lymphatic endothelium to the surrounding ECM through interaction with glycoproteins such as podoplanin on the surface of LECs. This is of particular interest because lymphatic capillaries, in contrast to blood capillaries, lack a complete basement membrane and may require additional molecular “hooks” in their environment for stability. Galectin-8 is produced and – in the tissue – might be secreted by LECs themselves but is widely expressed and thus might also be provided by other cells in the vicinity of lymphatic vessels, causing LECs to haptotactically migrate towards the source of galectin-8. Once the galectin-8-mediated contact of LECs with the ECM is established, they appear to be retained in their position as suggested by the apparent reduction in the motility of LECs in a galectin-8 containing collagen matrix. Thus, the presence or absence of secreted galectin-8 might define spaces available for either LEC adhesion or migration, respectively. It appears likely, that LECs can adjust their galectin-8 expression levels or the cellular location of galectin-8 (and other galectins) according to their activation status or their specific microenvironment, in analogy to what has been described for the galectin profiles of the blood vascular endothelium [26,27].

Given the fact that galectin-8 also interacts with abundant integrins such as $\alpha_3\beta_1$ and $\alpha_6\beta_1$ [15], which as we could show is true also for integrins on the surface of LECs, the contribution of the interaction of galectin-8 with podoplanin to the above mentioned effects on LECs is most likely minor, but might nevertheless be of relevance. Podoplanin knockout mice display abnormally patterned, dilated lymphatic vessels [2], which one might speculate could at least partially be due to the loss of the stabilizing galectin-8-podoplanin interaction. Moreover, it is conceivable that galectin-8 triggers podoplanin-mediated transmembrane signalling events, possibly by cross-linking podoplanin with other cell-surface glycoconjugates, similar to the clustering and activation of integrins that has been observed in response to the binding of galectin-8 to integrin sugar-moieties [17]. In line with this idea is the observation that in a number of cell lines, adhesion to galectin-8 induces a particular pattern of cytoskeletal organization, which involves the formation of F-actin-containing microspikes and reduction of stress fibres, and differs from what is seen upon adhesion to classical integrin ligands such as fibronectin [16,17]. Thus, integrins possibly cooperate with other proteins such as podoplanin to transmit the specific signals elicited by galectin-8 into the cell. This appears likely especially because formation of F-actin-containing cell protrusions and the reduction of stress fibres was also observed as a consequence of podoplanin overexpression in various cell types [2,3,5,6], and our preliminary results suggest co-localization of podoplanin with integrins $\alpha_3\beta_1$ and $\alpha_6\beta_1$ (unpublished data). There are certainly also other molecules than podoplanin which might cooperate with integrins to process galectin-8 signals in different cell types. BECs, however, appear to lack certain molecular components required to efficiently respond to galectin-8 signals, as suggested by their poor adhesion to galectin-8 and the failure of galectin-8 to inhibit tube formation of HUVECs.

Collectively, our findings shed light on the potential function of podoplanin, for which despite intense research in several laboratories no precise molecular mechanism or general physiological role has yet emerged. As an interaction partner of galectin-8 on the surface of LECs, it might be involved in connecting LECs to the surrounding ECM, possibly in cooperation with members of the integrin family. Moreover, galectin-8 is the first galectin revealed to specifically play a role in the lymphatic endothelium, an area of research that definitely deserves attention given the importance of galectins for the blood vascular endothelium, e.g. in the context of tumor angiogenesis.

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Abbreviations

LEC	lymphatic endothelial cell
BEC	blood vascular endothelial cell
CHO	Chinese hamster ovary
HEK	human embryonic kidney
ECM	extracellular matrix

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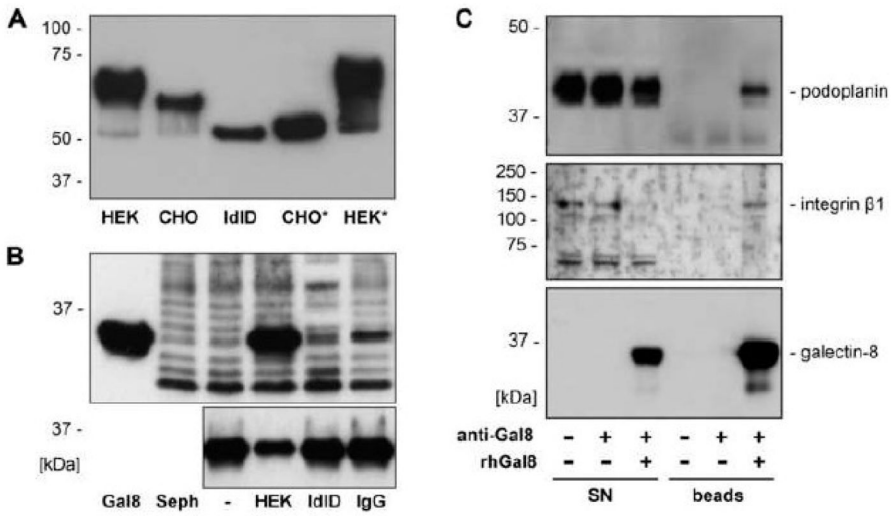

Figure 1. Galectin-8 interacts with podoplanin in a glycosylation-dependent manner

(A) Different glycoforms of recombinant soluble podoplanin-Fc fusion protein were expressed in mammalian cell lines. Proteins were purified from serum-free cell supernatants by protein A affinity chromatography, resolved by 10% SDS PAGE, transferred to nitrocellulose membranes and detected with anti-human podoplanin antibody (D2-40). *HEK* human embryonic kidney 293 cells; *CHO* Chinese hamster ovary wildtype cells; *ldlD* Chinese hamster ovary ldlD cells. * Proteins were submitted to sequential digestion with neuraminidase and O-glycosidase. (B) Recombinant human galectin (30 pmoles) was incubated with PBS alone (−), podoplanin-Fc from HEK 293 cells (*HEK*), podoplanin-Fc from CHO ldlD cells (*ldlD*) or human IgG_1 (50 pmoles each) for 3 hrs at room temperature, followed by pull-down of podoplanin-Fc with protein A-sepharose. Proteins associated with sepharose beads after washing (upper panel) or remaining in supernatants after pull-down (lower panel) were submitted to 10% SDS PAGE, transferred to nitrocellulose membrane, and detected with anti-galectin-8 antibody (R&D). *Gal8* recombinant, human galectin-8 (250 ng); *Seph* unused protein A-sepharose boiled in Laemmli buffer. (C) Recombinant human galectin-8 was added to primary human LEC lysate, and after overnight incubation pulled-down using anti-human galectin-8 antibody and immobilized protein G. Podoplanin, integrin β_1 and galectin-8 remaining in supernatants after pull-down (*SN*) or associated with protein G-beads after washing (*beads*) were detected by immunoblot.

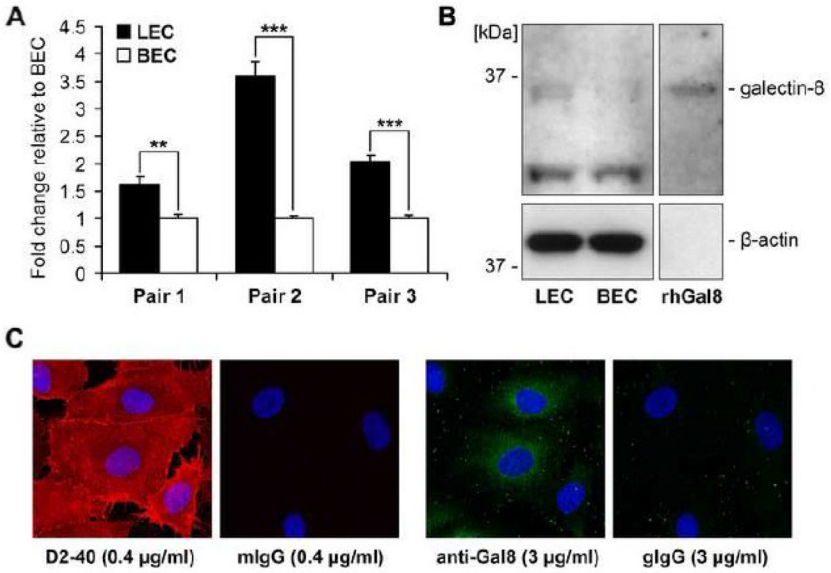

Figure 2. Galectin-8 is more highly expressed in LECs than in BECs

(A) Galectin-8 mRNA levels were determined by real-time RT PCR in three matched pairs of primary human dermal LECs and BECs as described in Materials and Methods. Data represent means +/− standard deviations (n=3), *** $p<0.001$, ** $p<0.01$. (B) Detection of galectin-8 protein in LEC and BEC lysates by immunoblot. Proteins were extracted from primary human endothelial cells (pair 3) at passage number 6 to 7. 32 μg (upper panel) or 4 μg (lower panel) protein were resolved by 10% SDS PAGE, transferred to nitrocellulose membrane, and detected with anti-galectin-8 (kind gift of Dr. Y. Zick; upper panel) or anti-β actin antibodies (lower panel). Recombinant, human galectin-8 (10 ng) was used as positive control. (C) Podoplanin (red, D2-40 antibody) and galectin-8 (green, goat anti-human galectin-8 antibody) were detected by immunofluorescence in primary human LECs. No staining was detected for corresponding control IgGs. Magnification 120×.

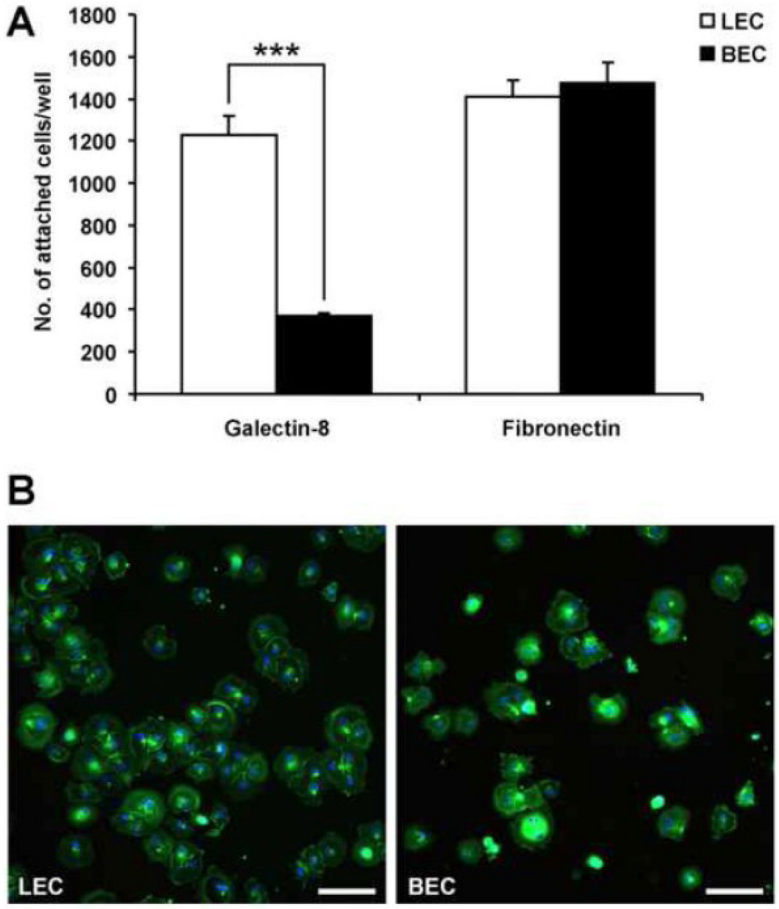

Figure 3. Immobilized galectin-8 promotes adhesion and spreading of LECs but not BECs

(A) 5×10^4 cells were seeded in serum-free medium into 24 well cell culture plates, previously coated with either recombinant human galectin-8 (25 μg/ml) or human fibronectin (10 μg/ml) and blocked with BSA (100 μg/ml). After 20 min at 37°C, unattached cells were washed away and remaining cells were fixed, stained with Hoechst nuclear dye and counted in three random images per well, taken at 5× magnification. Data represent means +/− SEM (n=3), *** $p<0.001$. (B) LECs and BECs attached to galectin-8 coated glass slides 20 min after seeding were stained for F-actin using phalloidin-Alexa Fluor 488 conjugate (green). Nuclei were counterstained with Hoechst dye (blue). Scale bar 100 μm.

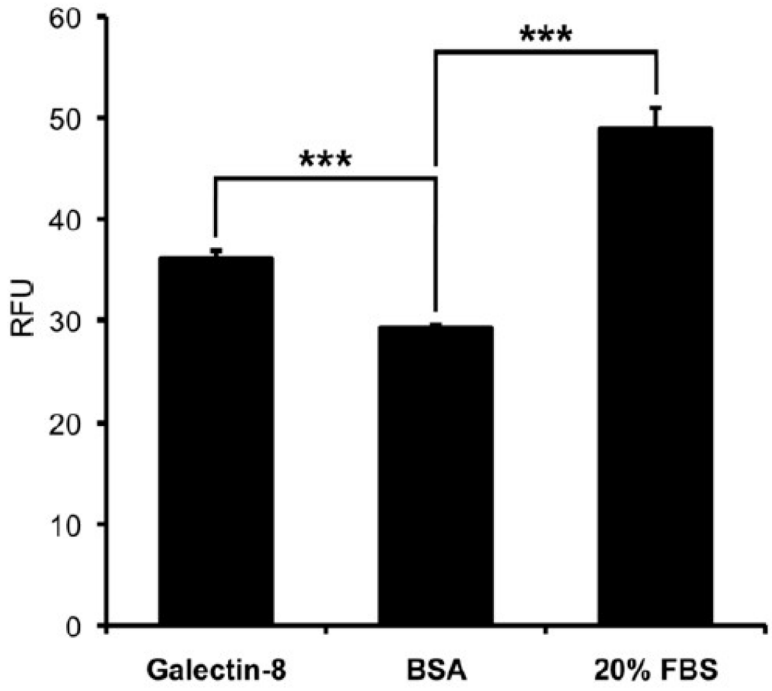

Figure 4. Galectin-8 enhances haptotactic migration of LECs

2.5×10^4 LECs were seeded in serum-free medium into the upper chambers of a HTS FluoroBlok Insert System 96 well plate, in which the underside of the insert membrane had previously been coated with galectin-8 (25 μg/ml) followed by blocking with BSA (100 μg/ml), or only blocked with BSA. Plates were incubated at 37°C for 5 hours, before cells on the underside of the membrane were stained with calcein and fluorescence intensity (RFU) at λ_{ex} 485 nm/λ_{em} 539 nm was measured. Data represent means +/− SEM (n=6), *** $p<0.001$.

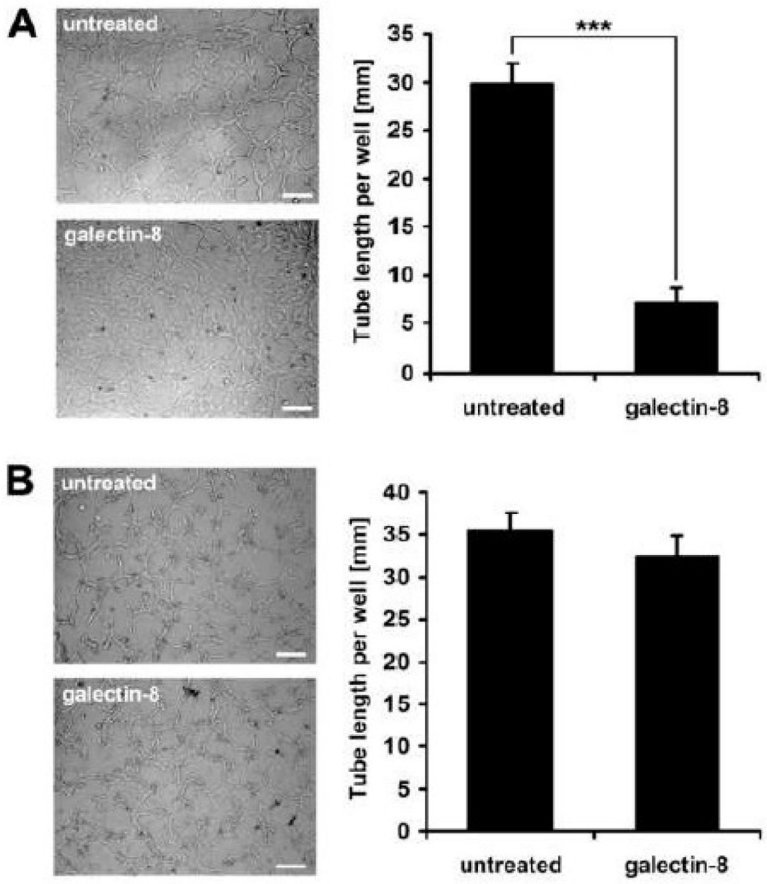

Figure 5. Galectin-8 inhibits tube formation of LECs but not HUVECs

Confluent monolayers of (A) LECs or (B) HUVECs, grown on fibronectin (10 μg/ml) coated 24 well cell culture plates, were overlaid with a collagen gel (prepared as described in Materials and Methods) containing or not recombinant human galectin-8 (250 nM). After incubation at 37°C for 12 (HUVECs) or 24 hours (LECs), the length of tube-like structures was determined in three pictures per well (5× magnification) using IPLab image analysis software. Data represent means +/− SEM (n=3), *** $p<0.001$. Scale bar 100 μm.

Table 1

Podoplanin binds to galectin-8 but not other galectins on a protein array.

Spotted Protein			Array 1		Array 2	
Name	**Subgroup**[a]	**Accession no.**	**Z-score**[b]	**CV**[c]	**Z-score**[b]	**CV**[c]
galectin 1	P	NM_002305	−0.28	0.024	−0.16	0.057
galectin 2	P	NM_006498	−0.23	0.038	−0.18	0.681
galectin 3	C	BC053667	−0.17	0.124	−0.13	0.461
galectin 3	C	BC001120	0.17	0.031	−0.05	0.020
galectin 7	P	NM_002307	−0.22	0.087	−0.16	0.872
galectin 8	TR	BC015818	1.08	0.030	1.36	0.057

[a] *P* prototype; *C* chimera; *TR* tandem-repeat

[b] distance of data point value from the mean of the population in units of standard deviations

[c] coefficient of variation between duplicate spots