



## RECENT STUDIES ON HUMAN PLATELETS-GALECTIN-1 INTERACTIONS

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### Article review

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#### Abstract

Animal lectins are proteins that have the ability to bind monosaccharide or oligosaccharide carbohydrates. They were classified into five families according to their primary structure. Galectins, one of the members of this group, were previously known as S-type lectins or S-Lac. The galectin family is a phylogenetically conserved lectin family which shared amino acid sequences and a carbohydrate recognition domain. All galectins bind lactose and other  $\beta$ -galactosidic oligosaccharides. Fifteen mammalian galectins have been identified, being designated as Gal-1 to Gal-15. We have previously demonstrated that porcine spleen Gal-1 interacts with resting human platelets and after activation with thrombin (Tr) demonstrating the presence of receptors, a necessary condition in order to exert functional roles.

The discovery of endogenous Gal-1 and their potential ligands/receptors, suggest an autocrine mechanism acting in HPlt (human platelets) to produce different functional roles. The information available is very poor which demonstrates the need to deep other aspects, some of which have already been addressed (localization and ultrastructural changes, protein associations

and platelet receptors) and other are pending, as its relevance in platelet physiopathology, in particular its association with thrombotic and inflammatory processes.

**Keywords:** galectin-1, human platelets, trombin.

## Resumen

Las lectinas animales son proteínas que tienen la capacidad de unir carbohidratos, monosacáridos u oligosacáridos simples. Fueron clasificadas en cinco familias de acuerdo a su estructura primaria. Las galectinas, uno de los miembros de este grupo, fueron previamente conocidas como lectinas de tipo S o S-Lac. Las galectinas son una familia filogenéticamente conservada de lectinas que contienen secuencias de aminoácidos características compartidas y dominios de reconocimiento de carbohidratos. Todas las galectinas unen lactosa y otros oligosacáridos  $\beta$ -galactosídicos. Se han identificado 15 galectinas de mamíferos, siendo designadas como Gal-1 a Gal-15. En nuestro trabajo, demostramos que la Gal-1 esplénica porcina interacciona con plaquetas humanas (PltH) en reposo y luego de la activación con trombina (Tr) lo cual demuestra la presencia de receptores, condición necesaria para que pueda ejercer algún rol funcional.

El hallazgo de Gal-1 endógena y de posibles receptores, permite suponer que un mecanismo autócrino actúe en la PltH para ejercer diferentes roles funcionales. La información disponible es muy escasa lo que demuestra la necesidad de profundizar otros aspectos, algunos de los cuales ya han sido abordados (localización y cambios ultraestructurales, asociaciones proteicas y receptores plaquetarios) y otros están pendientes de encarar, como su relevancia en la fisiopatología plaquetaria, en particular su vinculación a procesos trombóticos y a la inflamación.

**Palabras clave:** galectina-1, plaquetas humanas, trombina.

## Introduction

### *Animal lectins*

Animal lectins are proteins that have the ability to bind monosaccharide or oligosaccharide carbohydrates [1]. They were classified into five families according to their primary structure [2] as follows:

***C-type lectins:*** They have a conserved recognition domain (CRD), the bound carbohydrate is variable (mannose, galactose, fructose and tetrasaccharide heparin among others). The binding of carbohydrates is calcium dependent.

***I-type lectins:*** They have an immunoglobulin-like CRD and also the carbohydrate binding is variable (monosialylated phosphorylated hybrid type Gal $\beta$ 1-4GlcNAc (Man6GlcNAc2), HNK-1(CD57 antigens), epitope, hyaluronic acid and  $\alpha$ 2,3/ $\alpha$ 2,6-sialyllactose, among others).

***Galectins:*** They have a conserved CRD and the link is specific for a  $\beta$ -galactosidic carbohydrates.

***P-type lectins:*** The recognition site for carbohydrates is very similar to a CRD. Proteins containing mannose-6-phosphate mediate the binding to carbohydrates.

***Pentraxins:*** The CRD is a pentameric subunit and can bind different carbohydrates (4,6-cyclic acetal of  $\beta$ -galactose, galactose, sulfated and phosphorylated monosaccharides)

We describe below the galectin family in more detail, as the Galectin-1 protein used in our research work, belongs to this family.

## **Galectins**

They were previously known as S-type lectins or S-Lac. The galectin family is a phylogenetically conserved lectin family which shared amino acid sequences and has a carbohydrate recognition domain [3]. All galectins bind lactose and other  $\beta$ -galactosidic oligosaccharides [4]. Fifteen mammalian galectins have been identified, being designated as Gal-1 to Gal-15 [5,6]. All the CRD of galectin members share affinity for the ligand N-acetyllactosamine, a disaccharide that is found in many cellular glycoproteins. However, individual galectins may also recognize modifications of this ligand, developing multiple specificities [7]. Galectins were expressed in different cell types in mammals and non-mammalian vertebrates and invertebrates and are also found in plants. They lack the secretory signal sequence, so they do not follow the classical route endoplasmic reticulum /Golgi pathway; however, some galectins are found in the extracellular space or on the cell surface. This unusual externalization could prevent the association of Gal-1 with glycoconjugates that are being processed in normal secretory vesicles [8]. Studies of galectins localization let to know that these proteins may be secreted in multiple cellular compartments depending on cell status [9,10]. They are classified according to their biochemical structure [11] as:

**Proto-type galectins:** They contain a single CRD, recognize disaccharide carbohydrate structures. Galectin 1, 2, 5, 7, 10, 11, 13, 14 and 15 are included within this group. They are found as monomers (galectin 1, 5, 7, 10) or as homodimers (Galectin 1, 2, 11, 13, 14, 15).

**Chimera-type galectin:** Galectin 3 is the only member of this group, contains a CRD and a non-lectin domain.

**Tandem repeat-type galectins:** They contain two CRD connected and structurally different, allowing the interaction with different carbohydrates. Belong to this group galectin 4, 6, 8, 9 and 12.

## **Human platelets- exogenous galectin-1 interactions**

We have previously demonstrated that porcine spleen Gal-1 interacts with resting human platelets and after activation with thrombin (Tr). Because of galectins are a family of phylogenetically conserved proteins [3,4], the identity between domains that bind carbohydrates to different galectins of a single mammalian specie is 20-40 per percent and the homology of the Gal-1 among different species is 80-90 percent [4]. Thus, as these experiments are made with porcine spleen Gal-1, a lectin very similar to human Gal-1, results are comparable to the findings with Gal-1 from other species.

Previous studies on the interaction of lectins with surface glycans, employing several plant and animal lectins, led to findings of interest. With the aim to explore the functional role of carbohydrates in the cell surface and the glycoligands, several studies of aggregation were done in vitro with lectins and blood cells. They concluded that lectins caused aggregation of these cells (red blood cells, lymphocytes, neutrophils and platelets) inducing changes that could result in the activation / expression of adhesion proteins on the cell surface, allowing in this way, the stabilization of intracellular contacts and, therefore, promoting cellular aggregation [12,13,14,15,16, 17]. With regard to Gal-1, homologous Gal-1, CG-16 (chicken galectin-16) and bovine Gal-1 from different tissues, induce the aggregation of neutrophils, thymocytes and platelets [16]. Studies previously developed in our laboratory, on human and porcine neutrophils exposed to pig spleen Gal-1, confirmed the presence of surface receptors in PMN (polymorphonuclear leukocytes) before and after activation and degranulation [18]. During IIF (Indirect Immunofluorescence) studies done with human PMN [19], we observed that platelets contaminating the PMN cellular suspension, gave positive reaction for the detection of endogenous Gal-1 (unpublished data); this finding led us to initiate human platelet studies.

In relation to our experiences with exogenous Gal-1, it was observed that pig spleen Gal-1 interacts with HPIt inducing aggregation dependent on lectin concentration.

Particularly in platelets, several authors have shown that Gal-1 produces dose-dependent aggregation in different species. Thus, Timoshenko et al. [16] showed these results using bovine Gal-1. Moreover, Pacienza et al. [20] found similar data working with human recombinant Gal-1. Platelets have different types of receptors and one of them is the receptor GPIIb / IIIa, member of the integrin receptor family; it is on the platelet surface at rest and also inside the platelet, mainly in  $\alpha$  granules, dense bodies and on the membranes that surround the open canalicular system. These receptors are able to bind to the plasmatic membrane when platelets are activated and produce the secretion reaction [21,22]. Approximately 15% of the complex GPIIb / IIIa is composed by carbohydrates [23] and the interaction of human recombinant Gal-1 with HPIt causes conformational changes in receptor GPIIb / IIIa; these changes favour the phenomenon of activation [20].

Furthermore, in Tr-activated platelets, prior to the addition of pig spleen Gal-1, the number of receptors detected increases compare to platelets incubated only with the same concentration of lectin. These results are consistent with our earlier data, and suggest that previous Tr platelet activation, synergistically promotes the interaction of Gal-1 to platelet membrane receptors.

These findings showed that Gal-1 *per se* activates washed and resting HPIt, as it was also described only for Tr. Moreover a synergism between them was observed. This agonist effect could be due to the interaction of Gal-1 with the complex GPIIb / IIIa, as both molecules interact with the family of integrins receptors. Taking into account that an enormous variety of ligands binds Gal-1 [24], it cannot be excluded that other Gal-1 receptors present on HPIt surface could be activated.

We were also interested to deep in the HPIt- Gal-1 interactions, so we assayed different Gal-1 concentrations and different degrees of platelet activation. By flow cytometry (FC) studies in resting platelets with addition of Gal-1 increasing concentrations, we demonstrated that the interaction lectin / receptor is concentration-dependent.

With regard to activated platelets, at different times of activation from 0 up to 300 min for a unique Tr concentration (0.2 U/mL), we observed that the only condition that follows a linear relation to lectin concentrations, was obtained for a period of incubation of 120 seconds. For other periods of activation, there is a minimum detection at a medium Gal-1 concentration (0.3  $\mu$ M). These results are in agreement with the diversity of behaviours that often characterize Gal-1, leading, under certain circumstances, to inhibitory or stimulatory effects such as: anti-adhesion, adhesion, stimulation or inhibition of proliferation. The response depends on cell type, their activation states, surface receptor expression and their glycosylation states, the Gal-1 monomer-dimer ratio and the intra vs. extracellular distribution. Biphasic and paradoxical behaviour of Gal-1 activity at different concentrations has been described, suggesting that its effect depends on the activation status and cell type, although some authors suggest that may be influenced by a relative distribution of monomer-dimer forms [25,26,27]. The findings of at least 13  $\beta$ -1, 3 (4)-galactosyltransferases show the delicate and dynamic regulation that occurs during glycan synthesis and the diversity of their determinants. In that sense, it has been observed that controlled glycosylation would regulate the homeostasis of T cells, modulating the TCR signaling, restricting activation and favouring the deletion of some T effector cells [28]. It has also been described that Gal-1 causes a biphasic dose-dependent effect on cell growth. At high doses (approximately 1  $\mu$ M) inhibits the proliferation activity regardless of the binding activity to carbohydrates, and at low concentrations (approximately 1 nM), it has mitogenic activity, inhibited by lactose [26,29].

### ***Endogenous galectin-1***

We also carry out studies to confirm our initial findings of the presence of endogenous Gal-1 in HPIt. Therefore, with the goal to show the expression of endogenous Gal-1 on membrane and surface receptors for Gal-1 in HPIt, we made tests by IIF and FC.

Preliminary results [30,31] showed that there was expression of endogenous Gal-1 in resting platelets. In human platelet proteomic studies done by Martens et al (32), it was found that Gal-1 is part of the platelet proteome, considering it as a minor protein. Coincident with these data, Tenedini et al [33] studied the gene expression profiles of megakaryocytic cells derived from normal and malignant CD34 cells (in patients with ET (Essential thrombocythaemia)), and found that there was a de-regulation of proapoptotic genes in malignant megakaryocytic such as BAX, BNIP3, BNIP3L and members of the mitochondrial PT complex, a channel that is involved in the permeability regulation of mitochondrial membrane during apoptosis. They also observed an increase of CFLAR expression (CASP8 and the apoptosis regulator FADD), which exert a well-known antiapoptotic activity. In the same study, a reduction of the expression of gene LGALS1 (Gal-1) was observed. The role that Gal-1 might play in normal individuals or in TE patients needs to be clarified.

Further studies for the better understanding of Gal-1 and platelets interaction, were conducted in our laboratory that extend previous works. Thus, in preliminary data recently reported [34], modifications of platelet ultrastructure, showed a biphasic behaviour of the lectin. Platelets 0.5  $\mu$ M concentration lectin added, showed fewer filopodia, decreased number of granules and moderate alterations of the open canalicular system (OCS). On the other hand, in platelets with low added concentrations of Gal-1 (less than 0.3 $\mu$ M), an increase in the number of filopodia, a greater decrease in the number of granules and OCS marked expansion were observed. Ultrastructural changes of platelets under the action of Gal-1 partially inhibited with lactose (100 mM) were mild compared to resting platelets. Besides, there were characteristic changes in dramatically activated platelets with Tr and with a single concentration of Gal-1 added (0.5  $\mu$ M). Firstly, based on these results, consistent with data mentioned above, by immunohistochemistry [30] and FC [31], we concluded that the interaction of Gal-1 with ligand / receptors, such as integrins or other receptor types, would be responsible for the ultrastructural morphological changes related to functions such as adhesion / platelet aggregation. Unfortunately, from our experiments, it was not possible to establish a dose-dependent relation.

We demonstrated the presence of endogenous platelet Gal-1 [35] for the first time and we postulated that could exert similar physiological roles to exogenous porcine spleen Gal-1, through autocrine mechanisms.

In relation to this finding, another goal we focused was to purify and characterize the endogenous Gal-1 in HPIt, so we developed a protocol recently reported for lectin purification and further biochemical characterization [36]. We demonstrated that Gal-1 purified from HPIt by HPLC-mass spectrometric analysis, two peaks were obtained; one was a 14kDa protein and the other appeared as a complex with actin. Then we performed an enzymatic digestion with trypsin and peptides analysis obtained by HPLC followed by mass spectrometry. We observed the presence of Gal-1 (3 peptides sequenced; % coverage: 30.3) and actin (15 peptides sequenced; % coverage: 36.5), in agreement with the western blot results. These are findings similar to those described for other cells, such as T lymphocytes, muscle cells and human brain [37,38,39] where Gal -1 binds to actin, probably as part of complex systems that regulate actin activity. These studies let us to extend previous data [35], and to demonstrate that Gal-1 is a protein that is in a low proportion in HPIt. They are in agreement with proteomic studies made by other authors, who determined that Gal-1 can not be found within the 50 most abundant platelet proteins [32]. Similarly, it has been described that Gal-1 interacts with polylysosamines to increase cell motility induced by a higher RhoA protein expression, which is involved in actin cytoskeleton polymerization [40].

Therefore, from our results we could postulate the hypothesis that endogenous Gal-1 forms a complex with the intracellular actin monomer, based on data from PAGE and immunoblotting in resting HPIt, and that, perhaps after the activation, the lectin interfere in the polymerization-depolymerization of actin, thus participating in the activation and subsequent platelet aggregation.

### ***Galectin-1 and cytoskeletal platelets proteins***

On the other hand, a significant number of cytoskeletal platelet proteins, including talin, gelsolina,  $\alpha$ -actinin, cofilina, and vinculin was involved in the promotion or inhibition of actin polymerization. Referred to talin, it was described as a protein of high MW (225-235 kDa) that is uniformly distributed in the cytoplasm of resting platelets [41]. It has been shown that in platelets activated with Tr, cytoplasmic localization of talin changes moving to the plasma membrane involved in platelet adhesion [42]. Beckerle et al. [41], investigated the distribution of talin in resting and Tr-activated human platelets by IIF and immunoelectron microscopy; they observed that talin changed its cellular localization according to the state of the cell (resting- activated). In resting platelets, there was a cytoplasmic distribution of talin, while in Tr-activated platelets, talin appeared on the platelet membrane. This re-distribution of talin, suggested that this protein could play an important role in platelet adhesion.

Taking into account the ability of talin to interact with proteins associated with the cytoskeleton and platelet membrane, and in order to set links between the actin cytoskeleton and the extracellular environment by means of adhesion sites to ligands, Bertagnoli et al. [42] studied the distribution and phosphorylation of talin in Tr-activated platelets. They observed that talin phosphorylation significantly increased during the first two minutes of activation, and subsequent proteolytic cleavage occurred in response to activation, with consequent re-distribution of talin to the platelet periphery. In connection with these observations, then we intended to study whether the interaction of porcine spleen Gal-1 with HPIt, triggers the redistribution of platelet talin [43]. The study was done employing IIF assays, by detection of endogenous Gal-1 and talin using polyclonal and monoclonal antibodies on Tr-activated or resting platelets or Gal-1 added as agonist. For resting platelets, there was a homogenous cytoplasmic distribution pattern for both talin and endogenous Gal-1. In Tr-activated platelets, there was a change in the homogenous patterns to peripheral distribution for both molecules. The incubation with Gal-1 provoked a partial change of talin pattern to a peripheral one. It is known that talin binding to integrin cytoplasmic domain is critical to induce the activation of GPIIb/IIIa. This is a glycoprotein that acts as fibrinogen receptor and is required for platelet aggregation. After the addition of agonists *in vivo*, a small percentage of GPIIb/IIIa appears directly associated with actin filaments mediating cellular functions of proteins [44]. Similarly, talin has an N-terminal FERM domain (F for 4.1 protein, E for ezrin, R for radixin and M for moesin), which is a widespread protein module involved in localising proteins to the plasmatic membran, that binds actin [45]. Therefore, this suggests that Gal-1 could generate an interactive complex with talin and other cytoskeletal proteins. It remains to be clarified the possible functional role of this complex interaction of Gal-1 / talin on HPIt and its relevance in adhesion / platelet-platelet aggregation or platelet-endothelial cells. Our data show time dependence for the interaction of Gal-1 and Tr-activated platelets, approximately as those for talin phosphorylation. This fact could also substantiate the hypothesis of a possible complex formation between talin and other cytoskeletal proteins.

### ***Pathological relevance***

A future study derived from our findings was to study how these patterns of response are modified in pathology. Consistent with this perspective, recently Gorudko et al. [46], using the plant lectin, *Viscum album* agglutinin (VAA) and agglutinins from wheat germ (WGA), studied isolated blood cells *in vitro* (platelets, lymphocytes and neutrophils) from healthy donors and patients with coronary acute syndrome (CAS); the aim of this study was to compare the glyco-biologic characteristics of healthy and pathological cells. They found significant differences in the aggregation produced by lectins on lymphocytes and platelets, such as aggregation of both cells induced by VAA was lower in patients with CAS. They also observed that platelet aggregates induced by WGA in CAS patients were less stable, while neutrophil aggregates were more stable

than those of control cells. These results indicate that the glyco-biologic status of blood cells undergoes a complex specific cellular remodelling in association with CAS. Therefore, they concluded that the decrease in aggregation of platelets and lymphocytes could be due to the observed decrease of ligands containing  $\beta$ -galactosidic residues on the cell surface or changes associated with the disease that cause complex spatial restrictions, damaging interactions with the lectin on the cell surface. They showed that, in CAS patients, the glyco-biologic status of blood cells was subject to specific cellular changes that could be revealed with advanced aggregating techniques, for detecting the formation of stable aggregates induced by lectins.

## Conclusions

According to our results we can conclude that, porcine spleen Gal-1 interacts with HPIt at rest and after Tr-activation, demonstrating the presence of receptors, a necessary condition in order to exert functional roles.

The discovery of endogenous Gal-1 and their potential ligands/receptors, suggest an autocrine mechanism acting in HPIt to produce different functional roles. The available information is very poor which demonstrates the need to thoroughly investigate other aspects, some of which have already been addressed (localization and ultrastructural changes, protein associations and platelet receptors) and other are pending to be considered, as its relevance in platelet physiopathology, in particular its association with thrombotic and inflammatory processes.

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