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Galectin-9 in physiological and pathological conditions

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We first cloned galectin-9 (Gal-9)/ecalectin as a T cell-derived eosinophil chemoattractant. Gal-9 plays a role in not only accumulation but also activation of eosinophils in experimental allergic models and human allergic patients, because Gal-9 induces eosinophil chemoattraction *in vitro* and *in vivo* and activates eosinophils in many aspects. Gal-9 requires divalent galactoside-binding activity but not the linker peptide of Gal-9 to exhibit its biological functions, and an unidentified matrix metalloproteinase is involved in the release of Gal-9. Our recent studies also showed that Gal-9 has other functions, such as cell differentiation, aggregation, adhesion, and death. Now, we and other groups are on the way of investigating the regulation and function of Gal-9 in a variety of physiological and pathological conditions. In this article, we will show the possible role of Gal-9 in physiological and pathological conditions by using our recent findings.
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Introduction

In early work, we found that an eosinophil-specific chemoattractant (ECA) is produced and released by concanavalin A- and antigen-activated CD4+ T lymphocytes from patients infected with *Schistosoma mansoni* [1] or spontaneously produced and released from T lymphocytes of Kimura's disease [2,3]. Moreover, we have established a T cell line STO-2 that produced the ECA by viral transformation [4]. Although most ECA reported by other investigators are positively charged proteins that are quite small in size [5,6], gel filtration and electrophoretic analysis showed that one of the ECA produced by the above T cell populations is a 40-kDa protein possessing a relatively neutral isoelectric point [4]. We described the purification, molecular cloning, and functional expression of the "ecalectin", a novel lectin-like ECA [7]. We noted previously that ecalectin represents a variant form of human Gal-9, since there are as many as five amino acids that are different between the two proteins [8]. However, our further studies revealed that Gal-9 is ecalectin and ECA activity is a function of Gal-9 [9].

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Galectins exhibit a variety of biological functions, such as cell aggregation and adhesion, proliferation, cell death, and modulation of inflammation. Gal-9 was first cloned from tumor tissues of Hodgkin's disease that is frequently characterized by blood and tissue eosinophilia [8]. It is thus reasonable that Gal-9 has been discovered as an ECA. Moreover, Gal-9 causes thymocyte apoptosis in mouse, suggesting a possible role in the process of negative selection occurring during T cell development [10,11]. Recently, Lipkowitz *et al.* reported that Gal-9 might act as a urate transporter (refer to his chapter).

Gal-9 belongs to tandem-repeat type galectins that consist of 2 homologous carbohydrate recognition domains (CRD) connected by a linker peptide [12]. Compared to galectin-1 and -3, we don't have enough information on Gal-9 yet, since there are only 24 published manuscripts so far. In this chapter, we will raise the possible role of Gal-9 in physiological and pathological conditions by using our recent data including unpublished data.

Distribution

Several galectins such as Gal-1, -3, and -8 have a broad tissue distribution [13], whereas Gal-7 is localized in stratified and

pseudostratified epithelia that are detected in skin, esophagus, stomach, anus, tongue, trachea and ovaries [14].

In the case of Gal-9, it seems widely distributed in liver, small intestine, thymus, kidney, spleen, lung, cardiac and skeletal muscle [10]. It is barely detectable in reticulocyte and brain [10]. In our experiments, we found that Gal-9 is not detectable in endothelial cells, fibroblast, and astrocytes in physiological conditions [15–17]. However, Gal-9 expression is up regulated by some cytokines, such as IFN-γ or IL-1β, suggesting that the functions of Gal-9 in physiological conditions differ from those in pathological conditions as described below.

Structure

As mentioned above, human Gal-9 consists of 2 homologous CRDs (N-terminal, 148 amino acids; C-terminal, 149 amino acids) connected by a linker peptide similar to Gal-4 and -8. According to the size of the linker peptide , Gal-9 is classified into 3 types, namely long-sized (58 amino acids), medium-sized (26 amino acids), and short-sized (14 amino acids) Gal-9 [12]. Although such Gal-9 isoform is probably the result of alternative splicing (unpublished data), there is little information at present as to whether the Gal-9 isotypes exhibit different biological functions. However, we have found that long-sized Gal-9 is preferentially localized on the cell surface of Jurkat cells than the cytoplasm in our preliminary experiments (unpublished data), suggesting that respective Gal-9 isoform exhibits different functions.

Binding characteristics

Gal-9 is a β-galactoside binding lectin. Both of two CRDs (N- and C-terminal CRD), of course, bound to β-galactoside [9,12]. We characterized Gal-9 binding molecules in various immune cell lines such as Jurkat (T cell), MOLT-4 (T cell), BALL-1 (B cell), Daudi (B cell), RPMI-8866 (B cell), THP-1 (macrophage), HMC-1 (mast cell), and HL-60 (promyelocyte). Among of them, Jurkat, BALL-1, HMC-1, and THP-1 exhibited evident endogenous surface Gal-9, whereas MOLT-4 and HL-60 did not (Figure 1A). When the cell line cells were

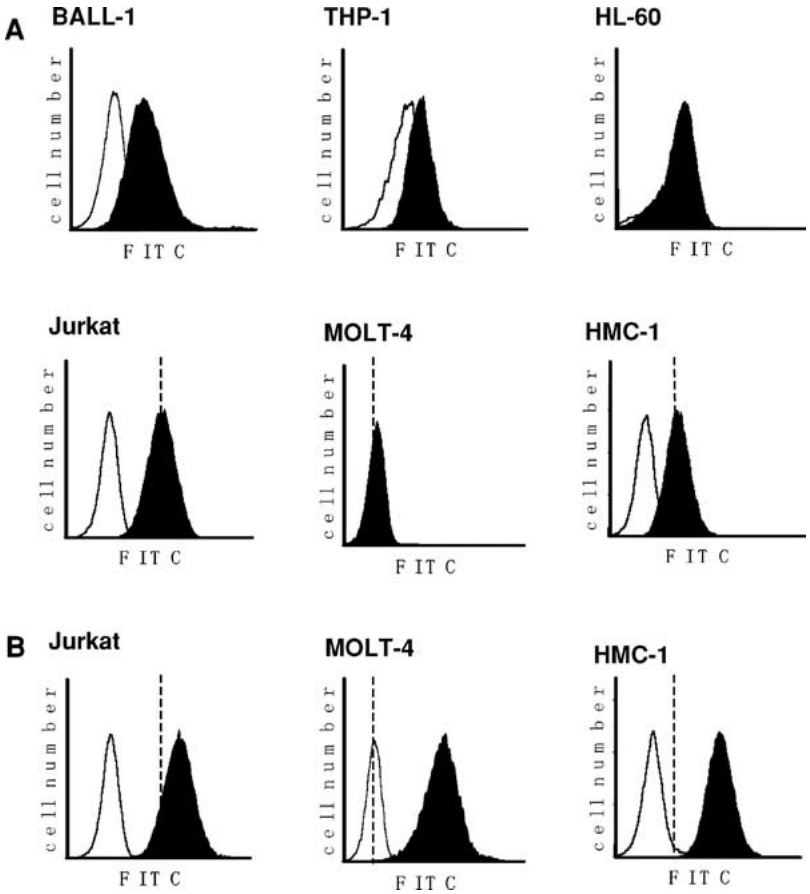

Figure 1. Gal-9 surface expression and binding by immune cells lines. (A) Surface Gal-9 expression on immune cell lines. Gal-9 staining was analyzed by flow cytometry after labeling with 50 μg/ml rabbit-anti-human Gal-9 antibody for 30 min on ice. After several washings, cells were incubated with FITC-conjugated goat-anti-rabbit antibody for 30 min on ice. (B) Binding of Gal-9 by immune cell lines. Cells were incubated with 0.1 μM recombinant Gal-9 for 90 min on ice before staining with antibody. Unshaded area is normal IgG control and filled is anti-Gal-9 antibody. Vertical bars indicate surface Gal-9 level on each cell. Data are representative of three separate experiments.

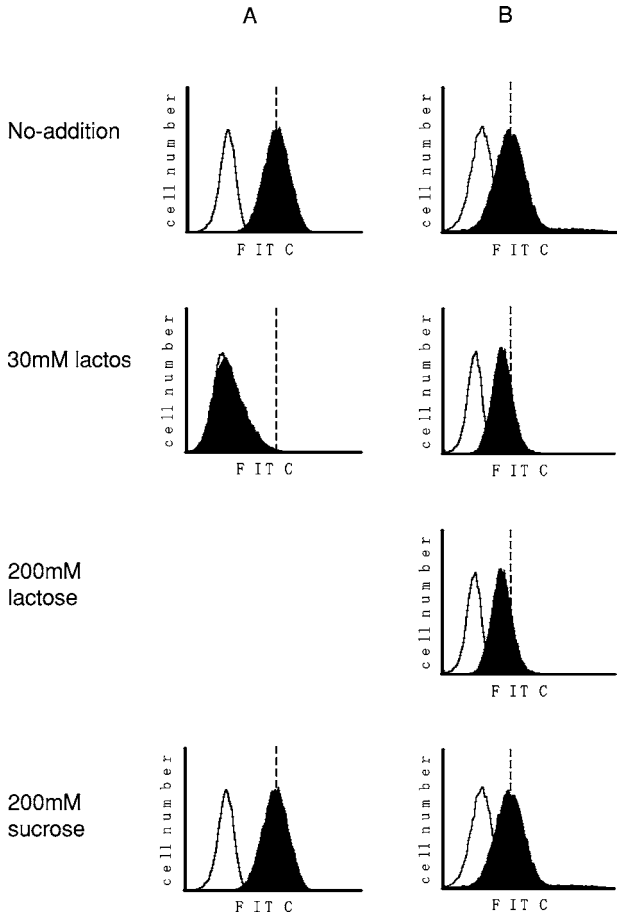

Figure 2. Release of endogenous cell surface Gal-9 by lactose. Both Jurkat (A) and HMC-1 (B) were incubated with 30 or 200 mM lactose for 30 min on ice before incubation with anti-Gal-9 antibody for 30 min on ice. As a control, we used 200 mM sucrose. Vertical bars indicate surface Gal-9 levels on each cell line. Data representative of three experiments are shown.

incubated with Gal-9, additional Gal-9 on the surface was observed, and this binding was most evident in Jurkat, MOLT-4 and HMC-1 (Figure 1B) The levels of Gal-9 mRNA and protein in Jurkat and HMC-1 were higher than those in MOLT-4. Both the surface and bound Gal-9 on Jurkat and MOLT-4 were almost completely shed by 30 mM lactose (Figures 2 and 3). In contrast, lactose even at 200 mM did not shed all the Gal-9 from the surface of HMC-1 (Figures 2 and 3). Gal-9 was almost completely released from a Jurkat cell surface by 30 mM lactose, whereas its release (not all) from a HMC-1 cell surface required 200 mM lactose, suggesting the presence of binding molecules with different affinity in the two cell lines.

The fact that only eosinophils are preferentially attracted by Gal-9 suggests the presence of several types of Gal-9 binding molecules, such as ECA-associated binding molecules, apoptosis-associated molecules and others.

Both N-terminal and C-terminal CRDs of Gal-9 are required to exhibit ECA activity [9]. We did frontal affinity chromatography analysis of the two separate domains of Gal-9 and whole Gal-9 to clarify oligosaccharide specificity [17]. Thus, it was found that Gal-9 has striking affinity to both branched N-glycans and repeated oligolactosamines. In fact, affinities to biantennary, triantennary and tetraantennary N-glycans were more than 100 times higher than the average of functionally monovalent N-glycans, and those to oligolactosamines drastically increased as the repeating number increased. Furthermore, Gal-9 exhibited particular preference for two glycolipid-type glycans, such as Forsmann pentasaccharide and A-hexasaccharide. Such special affinity is apparently attributed to N-terminal CRD. N-terminal CRD also exhibited significant affinity to other glycolipid-type glycans. In contrast, enhanced affinity to branched N-glycans and oligolactosamines are shared by both CRDs. The fact that Gal-9 exhibits particular preference for Forsmann saccharide and A-hexasaccharide as well as repeated oligolactosamines may be associated with the selective activity of Gal-9.

Regulation of Gal-9 production and release

Galectin members are localized on the cell membrane, in the cytoplasm and in the nucleus of cells. So far, the secretory pathway of galectins has not been fully understood. One reason is that all known galectins including Gal-9 lack signal peptides for their insertion into the endoplasmic reticulum membrane and its subsequent secretion via the classical pathway. Recently, a sequence in the N-terminal domain of Gal-3 was proposed as a determinant for its secretion [18]. However, the presence of such secretion signal sequence in other galectins and the precise mechanism of Gal-3 secretion remain unknown. We have previously described that antigen-stimulated T cells produce and release Gal-9 in guinea pig and human systems [19], and recently we assessed the regulation of Gal-9 production and release in Jurkat cells [12]. Phorbol 12-myriastate 13-acetate (PMA) up-regulated the level of Gal-9 mRNA in Jurkat cells. Western blot and FACS analyses revealed that PMA induce up-regulation of both intracellular and surface Gal-9 protein. The stimulated Jurkat cells simultaneously released evident ECA that was adsorbed by both lactose and anti-Gal-9 antibody affinity columns, indicating that the ECA was mainly Gal-9. When Jurkat cells were stimulated with PMA in the presence of a matrix metalloproteinase (MMP) inhibitor, BB94, the release of Gal-9 was suppressed in a dose-dependent manner. Calphostin c, a protein kinase c inhibitor (PKC), also suppressed the release of Gal-9 weakly but significantly, suggesting partial involvement of MMP and PKC in the release of Gal-9 from Jurkat cells.

Not only T cells but also other types of cells expressed Gal-9, and its production was up-regulated with some stimuli. For example, Gal-9 expression in normal human astrocytes is enhanced by IL-1β [15]. This up-regulation by IL-1β is suppressed by treatment with glucocorticoid. We also found that IFN-γ up-regulated the Gal-9 expression in endothelial cells [20]. In contrast, IL-4 failed to induce Gal-9 production.

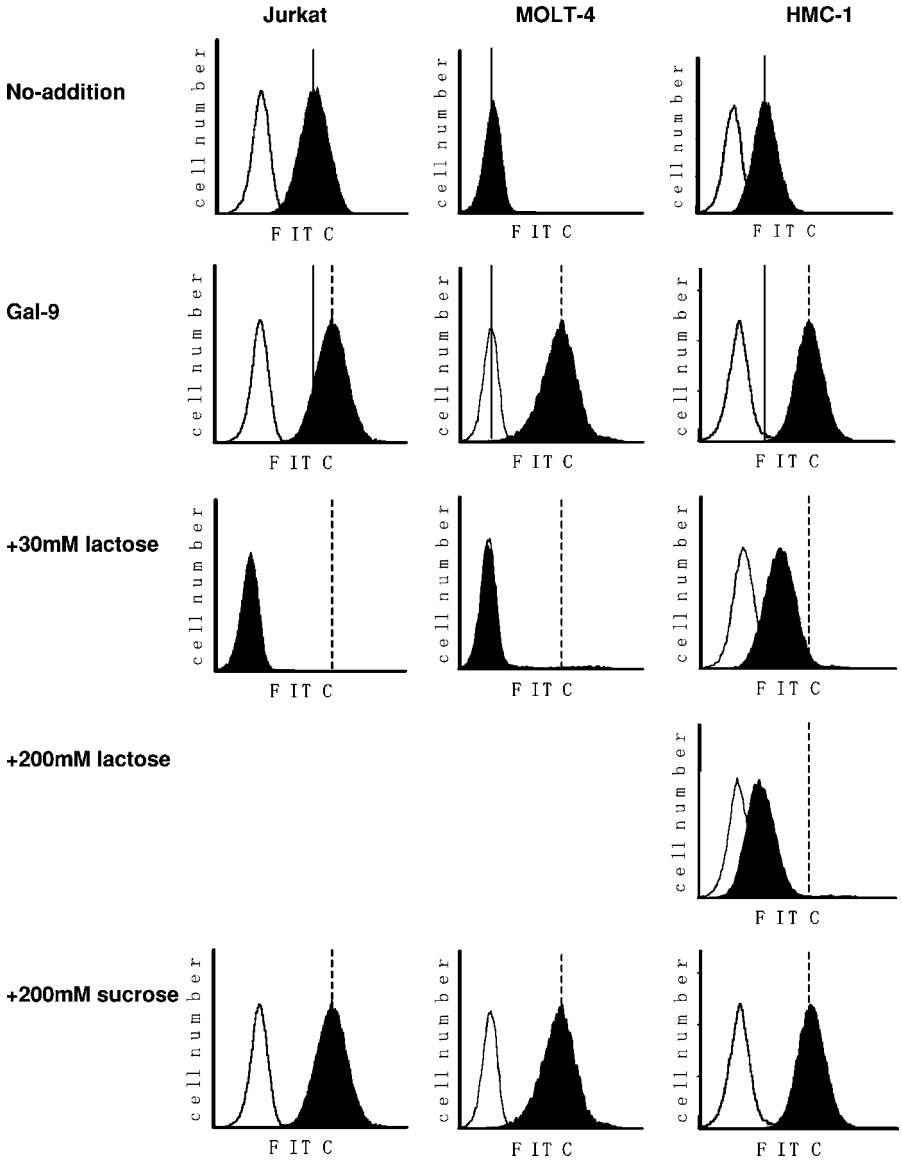

Figure 3. Removal of bound Gal-9 by lactose. Jurkat, MOLT-4, and HMC-1 were incubated with recombinant Gal-9 for 90 min on ice and then incubated with 30–200 mM lactose for 30 min on ice before incubation with anti-Gal-9 antibody for 30 min on ice. As a control, 200 mM sucrose was added to cells. Solid lines indicate surface Gal-9 level and dotted indicate amount of bound Gal-9. Data are representative of three separate experiments.

Furthermore, we recently found that IFN-γ enhanced Gal-9 production and surface expression in fibroblasts [21]. However, we did not find any ECA activity and Gal-9 in the supernatant from IFN-γ-treated cells though the supernatant from PMA- or antigen-stimulated T cells exhibit evident ECA activity, suggesting that endothelial cells and fibroblast lack the ability (maybe some MMP) to release Gal-9.

Cell differentiation

HL-60 cells could differentiate into eosinophil-, monocyte- and neutrophil-like cells when they are cultured with sodium butyrate under mild alkaline condition, PMA, and dimethyl sulfooxide, respectively [22–24]. During differentiation into 3 lineages, the changes in galectin levels were assessed in HL-60 cells. Before differentiation, RT-PCR analysis revealed that HL-60 cells expressed Gal-1, -3, -8, -9, and -10 (identical to Charcot Leyden crystal) mRNAs [25]. The levels of Gal-1 and -8 do not change evidently during differentiation. However, Gal-9 mRNA gradually decreased, and Gal-10 mRNA increased during eosinophilic differentiation. Since Gal-10 is probably identical with Charcot Leyden crystal (CLC) protein [26], it is conceivable that Gal-10 up-regulation is observed during eosinophilic differentiation. Furthermore, Gal-9 mRNA decreased and Gal-3 mRNA increased during differentiation

into monocytes [25]. The close relation between Gal-3 expression and monocytic lineage cells is very expectable because of the fact that Gal-3 is closely associated with monocytes and macrophages [27]. During differentiation into neutrophils, Gal-10 mRNA unexpectedly increased [25]. One report showed that mononuclear cells and neutrophils also contain CLC protein though the amounts in those types of cells were less than that in eosinophils [28]. Further, we found by FACS analysis that neutrophils exhibit Gal-9 protein on the surface and in the cytoplasm (unpublished data).

Eosinophil activation

Besides having ECA activity, Gal-9 acts as an eosinophil activator [7]. Other eosinophil chemoattractant such as eotaxin could modulate the expressions of surface antigens on eosinophils [29]. Therefore, we performed experiments to clarify the effects of Gal-9 on eosinophil surface antigens. First, CD11b expression on eosinophils stimulated with or without Gal-9 was analyzed by flow cytometry with FITC-conjugated CD11b antibody. CD11b expressions (% and MFI) of untreated eosinophils were 64.3% and 2.8 MFI, respectively. The CD11b expression was up-regulated in eosinophils after addition of Gal-9 in a dose dependent manner; the maximal effects were observed at 300 nM. The up-regulation by Gal-9 (Gal-9; 96.5% and 8.7 MFI) was lower than that by C5a (97.8%, 12.0 MFI) and eotaxin 97.4%, 10.1 MFI).

Gal-9 also up-regulates CD69 and VLA-4 expressions of eosinophils. CD69 (29.4% and 7.9 MFI) and VLA-4 (34.7% and 8.2 MFI) expressions of untreated eosinophils from 7 healthy donors were significantly enhanced by treatment with Gal-9 (CD69, 39.9% and 10.8 MFI; VLA-4, 48.6% and 11.5 MFI).

Superoxide production by eosinophils was measured by superoxide dismutase-inhibitable reduction of cytochrome *c* [16]. Gal-9 induced a concentration-dependent superoxide production from eosinophils though Gal-9 did not induce eosinophil degranulation. Superoxide production and degranulation are usually induced by IL-5 and other eosinophil affecting factors. Taken together, these results suggest the existence of separate pathways for superoxide production and degranulation. In addition, Gal-9 did not induce a Ca^{2+} influx in eosinophils [16], but we recently found that Gal-9 induce it in Jurkat cells resulting in apoptosis [30]. From these results, it can be raised a hypothesis that binding molecules involved in eosinophil activation differ from those involved in apoptosis of T cells.

FACS analysis using dihydrorhodamine123 was performed to confirm the enhancing effects of Gal-9 on oxygen radical production from eosinophils. Unexpectedly, PMA-induced oxygen radical production was suppressed by pretreatment with Gal-9 in a dose dependent manner. In contrast, C5a and eotaxin enhanced the PMA-induced oxygen radical production (unpublished data). Exact mechanism of such discrepant findings on superoxide production remains unclear.

Cell aggregation

One of the characteristics of galectins is their hemagglutination activity, which is attributable to their bivalent carbohydrate-binding property. In the case of galectins with two CRDs, the bivalency is likely due to carbohydrate binding manifested by each domain. Therefore, hemagglutination activity of recombinant Gal-9 and domain fragments was assessed. The minimum concentration of whole Gal-9 required for the hemagglutination was 0.0125 μM, which was comparable to that for Gal-1 (0.025 μM). The separate CRDs (Gal-9-NT and- CT) also exhibited hemagglutination activity, although it was lower (0.2 microM) than that of whole Gal-9 [9].

Gal-9 also induced aggregations of eosinophil and malignant melanoma cells [31] and the addition of lactose inhibited this aggregation. Next, because eosinophil aggregation in guinea pigs is induced by Ca^{2+}-dependent interaction between Mac-1 (CD11/CD18) and ICAM-1, which are expressed on eosinophils after stimulation [32,33], we examined the effects of EDTA and anti-CD18 mAb on Gal-9-induced eosinophil aggregation. Gal-9-induced aggregation was inhibited by EDTA, but not by anti-CD18 mAb suggesting Gal-9-induced eosinophil aggregation, which needs divalent cations, is not related to interaction between Mac-1 (CD11/CD18) and ICAM-1. Analyses of purified peripheral blood eosinophils, neutrophils and mononuclear cells showed that Gal-9 induces aggregation of only eosinophils.

In melanoma cells, we found that a melanoma line (MM-RU), that proliferate without evident colony formation, do not express detectable Gal-9 mRNA and surface Gal-9 protein whereas a colony-forming melanoma (MM-BP) has Gal-9 mRNA and surface Gal-9 [33]. When MM-RU cells were cultured in the presence of Gal-9, cell aggregation was induced in a dose- and time-dependent manner. We next examined the function of Gal-9 in a breast cancer cell line, MCF-7, that had been over expressed Gal-9 by transfection, and observed growing styles of transplanted tumors in nude mice. It was thus found that MCF-7 cells with higher expression of Gal-9 exhibit stronger aggregation *in vitro* and *in vivo* [33].

Cell adhesion

We have recently found that Gal-9 also plays as an adhesive factor. In endothelial cells, Gal-9 expression was up regulated when endothelial cells were stimulated with IFN-γ. IFN-γ enhanced the adhesion of human eosinophilic cells (EOL-1) to Gal-9-stimulated endothelial cells [20].

Eosinophils selectively adhere to IFN-γ-treated fibroblasts though neutrophils fail [21]. Such adhesion was suppressed by lactose and by anti-Gal-9 antibody. Further experiments revealed that eosinophils adhered to Gal-9-coated dish but not to Gal-1-coated dish, indicating the selective relation between Gal-9 and eosinophils. At present, it is still unclear whether there is biological significance of Gal-9-mediated

adhesion between eosinophils and endothelial cells or fibroblasts.

Cell death

As is shown by Wada *et al*., Gal-9 induces mouse thymocyte apoptosis, as do other galectins, such as Gal-1, Gal-7, and Gal-8. This Gal-9-mediated apoptosis is not limited to thymocytes [10].

Experiments were done to clarify whether Gal-9 is associated with eosinophil survival. It was thus found that Gal-9 prolonged eosinophil survival concentration-dependently [16]. Since fibronectin [34] and other molecules [35,36] prolong eosinophil survival indirectly by triggering eosinophils to secrete cytokines that prolong eosinophil survival, we examined whether this effect of Gal-9 was direct. Gal-9-induced prolonged eosinophil survival was inhibited by lactose but not by antibodies to IL-5, IL-3, or GM-CSF, suggesting that Gal-9 directly prolongs eosinophil survival *in vitro*.

We assessed the Gal-9 expression in peripheral blood eosinophils of eosinophilic patients (E-Eos) in comparison with those of normal volunteers (N-Eos) [37]. Surface and intracellular immunoreactive Gal-9 was more evident in E-Eos than N-Eos, respectively. Incubation of eosinophils with IL-5 *in vitro* results in down-regulation of Gal-9 expression of E-Eos and in no significant change in N-Eos. Treatment of eosinophils with dexamethasone and anti-Fas antibodies results in the significant up-regulated Gal-9 expression of E-Eos. However, dexamethasone down-regulated Gal-9 expression of N-Eos. We further found that Gal-9 suppresses apoptosis of E-Eos, though it enhanced apoptosis of N-Eos. Furthermore, we found that Gal-9 suppresses DEX-induced apoptosis of N-Eos whereas it does not affect dexamethasone-induced apoptosis of N-Eos. On the other hand, apoptosis induced by anti-Fas antibodies in both N-Eos and E-Eos was enhanced by Gal-9, indicating possible association of Gal-9 with eosinophil apoptosis, and that E-Eos and N-Eos differ in the response to Gal-9 during apoptosis.

Gal-9 is involved in cell death not only of eosinophils but also of other immune cells. For instance, we found that Gal-9 induces apoptosis of immune cell lines, such as Jurkat (T cell), MOLT-4 (T cell), BALL-1 (B cell), THP-1 (macrophage), and HL-60 (promyelocyte) [30]. Our further studies revealed that Gal-9 induces the apoptosis via the calcium-calpain- caspase-1 pathway that is similar to that of the glucocorticoid. We also found that Gal-9 induces apoptosis of CD3-activated peripheral CD4-positive T cells than CD8-positive T cells. Furthermore, Gal-9 fails to induce evident apoptosis of resting peripheral T cells [30].

Gal-9 as a prognostic factor in malignant tumors

Some glycoproteins appear to be deeply involved in metastasis of cancer cells [38]. Some galectins, *e.g*. Gal-1 and Gal-3, also enhance cancer invasion and/or protect cancer cells from apoptosis [39]. In contrast, Gal-9 expression in tumor cells is associated with better clinical features in tumor-bearing patients as follows.

As mentioned above, MM-BP, a melanoma cell line, proliferated with colony formation, but MM-RU failed. RT-PCR analyses revealed evident expression of Gal-9 mRNA in MM-BP, but not in MM-RU. MM-BP expressed Gal-9 protein both on the surface and in the cytoplasm, whereas MM-RU expressed it only in the cytoplasm. Exogenous Gal-9 *in vitro* induced both cell aggregation and apoptosis of MM-RU proliferating without colony formation [31]. Therefore, association of Gal-9 expression with prognosis of 75 patients bearing melanocytic tumors was further examined. Gal-9 protein was strongly and homogeneously expressed in melanocytic nevi, but down-regulated in melanoma cells especially in metastatic lesions, implying the down-regulation of Gal-9 expression with malignant transformation in the melanocytic cell lineage. High Gal-9 expression was inversely correlated with the progression of this disease, indicating that high Gal-9 expression in primary melanoma lesions links to a better prognosis, especially in lymphnode metastasis, recurrence and mortality, and is associated with significantly higher 5-year disease-free survival and overall 5-year survival [31].

We also examined the expression of Gal-9 protein in 87 cases of breast cancer patients by immunohistochemistry using anti-Gal-9 polyclonal antibody, and analyzed the correlation between Gal-9 expression and distant metastasis of breast cancer in comparison with node status, a conventional predictive factor for metastasis [33]. The percentage of Gal-9-positive cases was about 50%. Gal-9 expression inversely correlates with distant metastasis, but neither to lymph node metastasis nor to hormone receptor status. Eighteen cases out of 21 metastatic cases were Gal-9 negative ($p = 0.0003$). Disease-free survival ratios (DFS) of each of Gal-9 positive and negative cases were 93% and 47%, respectively ($p = 0.0008$). Furthermore, DFS of Gal-9 positive and negative cases even within the node-positive group was 89% and 19%, respectively ($p = 0.0017$). Multivariate analysis using Cox's proportional-hazards regression model revealed that hazard ratio of Gal-9 expression (13.159) is higher than that of node status (5.927), and that Gal-9 is an independent prognostic factor from other factors. From the above data, it is suggested that Gal-9 expression correlates positively with homotypic tumor cell aggregation, but negatively with distant metastasis.

The above findings suggest that both adhesion and apoptosis are probably required for the Gal-9-induced suppression of melanoma and breast cancer. Therefore, Gal-9 could be an important molecule to implement a novel therapeutic strategy through up-regulation of cell aggregation and apoptosis in tumor cells. Furthermore, we are preparing an immunostaining kit for determining the prognosis of malignant neoplasm to determine whether melanoma and breast cancer will metastasize

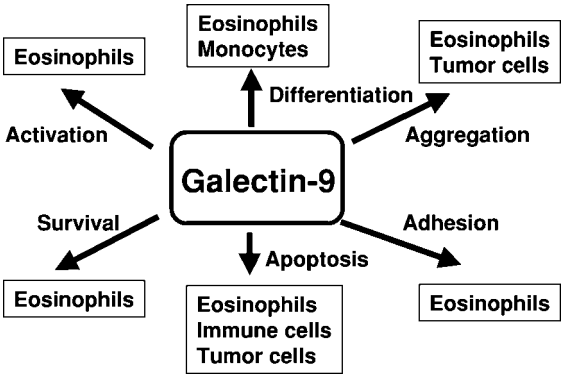

Figure 4. Multifunctions of Gal-9 on various cells.

in future. We also believe that Gal-9 will give us a master key to open the door toward the establishment of new cancer therapies.

Conclusion

In this chapter, we have presented our recent information of Gal-9. As Gal-9 has been first found as an ECA, there is no doubt that Gal-9 is involved in eosinophil functions. However, it is clear that Gal-9 also has a variety of other biological functions as described in this paper (summarized in Figure 4). Therefore, further clarification of Gal-9 regulation and functions can lead us to understand the immunomodulatory activities and significance of Gal-9 in physiological and various pathological conditions.

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