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Galectin-8 modulates neutrophil function via interaction with integrin αM

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The members of the galectin family are associated with diverse cellular events, including immune response. We investigated the effects of galectin-8 on neutrophil function. Human galectin-8 induced firm and reversible adhesion of peripheral blood neutrophils but not eosinophils to a plastic surface in a lactose-sensitive manner. Other human galectins, galectins-1, -3, and -9, showed low or negligible effects on neutrophil adhesion. Confocal microscopy revealed actin bundle formation in the presence of galectin-8. Cytochalasins inhibited both actin assembly and cell adhesion induced by galectin-8. Affinity purification of galectin-interacting proteins from solubilized neutrophil membrane revealed that N-terminal carbohydrate recognition domain (CRD) of galectin-8 bound promatrix metalloproteinase-9 (proMMP-9), and C-terminal CRD bound integrin αM/CD11b and proMMP-9. A mutant galectin-8 lacking the carbohydrate-binding activity of N-terminal CRD (galectin-8R69H) retained adhesion-inducing activity, but inactivation of C-terminal CRD (galectin-8R233H) abolished the activity. MMP-3-mediated processing of proMMP-9 was accelerated by galectin-8, and this effect was inhibited by lactose. Galectins-1 and -3 did not affect the processing. Superoxide production, an essential event in bactericidal function of neutrophils, was stimulated by galectin-8 to an extent comparable to that induced by fMLP. Galectin-8R69H but not galectin-8R233H could stimulate superoxide production. Taken together, these results suggest that galectin-8 is a novel factor that modulates the neutrophil function related to transendothelial migration and microbial killing.

Key words: galectin/inflammation/integrin/matrix metalloproteinase/neutrophil

Introduction

Recent studies have established the roles of animal lectins, especially C-type lectins, such as selectins and mannose-binding protein, in the immune system. In addition to C-type lectins, accumulating evidence implicates the members of the galectin family (formerly known as S-type lectins) as a novel class of modulators of innate and adaptive immune functions (Vasta *et al*., 1999; Lowe, 2001; Rabinovich *et al*., 2002): Galectin-1 and galectin-9 have been shown to induce apoptosis of T lymphocytes (Perillo *et al*., 1995) and thymocytes (Wada *et al*., 1997), respectively. Galectin-1 was also suggested to modulate complement receptor 3 function (Avni *et al*., 1998). We demonstrated that galectin-9 is a potent and selective chemoattractant for eosinophils (Matsumoto *et al*., 1998; Matsushita *et al*., 2000). Furthermore, galectin-3 was shown to suppress T-cell apoptosis (Yang *et al*., 1996), and to be a novel chemoattractant for monocytes and macrophages (Sano *et al*., 2000).

Currently, 10 members of the human galectin family (galectins-1–4, galectins-7–10, galectins-12 and -13) are known. These family members can be classified into three subtypes according to their structures (Hirabayashi and Kasai, 1993): the prototype (galectins-1, -2, -7, -10, and -13) and chimera-type (galectin-3) galectins have a single carbohydrate recognition domain (CRD), and they form a homodimer resulting in homobifunctional cross-linking activity. Galectins-4, -8, -9, and -12 belong to the tandem repeat type, consisting of two CRDs joined by a linker peptide. N-terminal and C-terminal CRDs of the tandem repeat type galectins generally have different sugar binding specificities (Wasano and Hirakawa, 1999; Arata *et al*., 2001; Sato *et al*., 2002; Hirabayashi *et al*., 2002), and this heterobifunctional property makes it possible to cross-link a wide variety and combination of glycoconjugates. Due to this multiple cross-linking potential, tandem repeat-type galectins are attractive targets for studying novel function of the galectin family in the immune system.

Galectin-8 was first identified in a rat liver cDNA library as the third member (mouse but not human galectin-6 is known) of the murine tandem repeat type galectin family (Hadari *et al*., 1995). Two independent studies concerning tumor-associated antigens resulted in the identification of human galectin-8 cDNAs (Su *et al*., 1996; Bassen *et al*., 1999). In contrast to other tandem repeat type galectins, galectin-8 is widely expressed in normal tissues in addition to tumor cells. The physiological role of galectin-8 is largely unknown. However, it seems that galectin-8 is involved in malignant transformation and cell–matrix interaction (Danguy *et al*., 2001; Hadari *et al*., 2000; Levy *et al*., 2001).

In our search for novel function of galectins in the immune system, especially in the innate immune system, it was found that galectin-8 induced firm and reversible adhesion of peripheral blood neutrophils *in vitro*. Neutrophils play a central role in innate immunity to bacterial infection.

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Recruitment of circulating neutrophils to an affected site proceeds through several defined steps, namely, attachment to, rolling on, and firm adhesion to endothelial cells and then transendothelial migration. Recent studies have revealed the molecular basis of the leukocyte–endothelium interaction. More than a dozen molecules, on both neutrophil and endothelial cell membranes, involved in the interaction have been identified. Here, we report the characterization of galectin-8 as a novel modulator of neutrophil function.

Results

Sugar-binding ability of recombinant galectin-8

The functionality of wild-type and mutant galectin-8 was assessed by asialofetuin affinity chromatography (Figure 1). Wild-type galectin-8 was almost completely retained by an asialofetuin-agarose column under the conditions used. The presence of small amounts of galectin-8 mutants, especially galectin-8R69H, in the unbound fraction suggest that mutant galectin-8 possessed lower affinity for asialofetuin compared to the wild-type one. On the contrary, a double mutant, galectin-8R69, 233H, was not retained by the column, showing that the R69H and R233H mutations abolished the sugar-binding activity of N-terminal and C-terminal CRD, respectively.

Induction of neutrophil adhesion by galectin-8

Human neutrophils, when incubated in the presence of serum, exhibited negligible adhesion to a tissue culture plate. The addition of recombinant galectin-8 to the culture medium resulted in rapid and almost complete adhesion of neutrophils but not eosinophils (Figure 2A), and the effect was inhibited by lactose (Table I). Galectin-8-induced neutrophil adhesion was not affected by treatment of tissue culture plates with human soluble intercellular adhesion molecule-1 (ICAM-1) (extracellular domain of ICAM-1). Confocal microscopy showed that exogenous galectin-8 was localized on the surface of adhering neutrophils (Figure 3A).

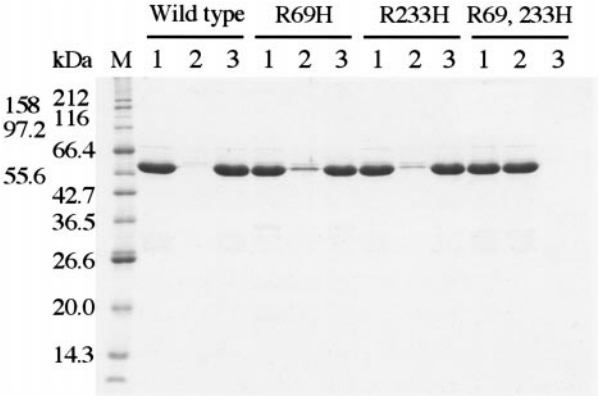

Fig. 1. Sugar-binding ability of recombinant galectin-8. Recombinant galectin-8 with or without site-directed mutation(s) was expressed in *E. coli* and then purified by affinity chromatography on a glutathione-Sepharose column. The purified GST-fusion proteins were subjected to affinity chromatography on an asialofetuin-agarose column. Proteins bound to the column were eluted with 0.2 M lactose. A control sample (lane 1), nonbound fraction (lane 2), and bound fraction (lane 3) were analyzed by SDS–PAGE. The gel was stained with CBB R-250. M, molecular weight markers.

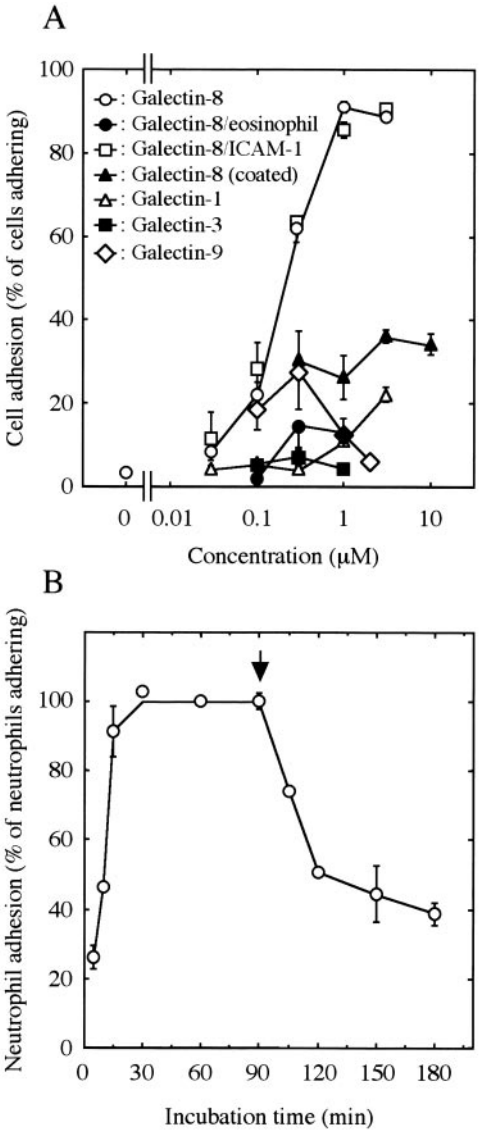

Fig. 2. Comparison of human galectins as to the promotion of neutrophil adhesion to tissue culture plates and the time course of neutrophil adhesion induced by galectin-8. Purified human neutrophils or eosinophils suspended in serum-containing medium were added to 24-well tissue culture plates. (**A**) After the addition of various concentrations of GST-fusion proteins of human galectins, the cells were allowed to adhere for 60 min at 37°C. In one experiment, neutrophil adhesion was assessed by using tissue culture plates precoated with indicated concentrations of GST-galectin-8 without addition of recombinant proteins to the medium (closed triangles). In another experiment, tissue culture plates precoated with soluble human ICAM-1 (10 μg/ml) were used to examine the effect of galectin-8 on neutrophil adhesion (open squares). In the case of galectin-8, the effect on eosinophil adhesion was also examined (closed circles). (**B**) After the addition of GST-galectin-8 (1 μM), the cells were allowed to adhere for the indicated times at 37°C. The arrow indicates the time of the change to galectin-8-free medium. Cell adhesion was determined as described in *Materials and methods.* Data represent means ± SD of triplicate measurements.

Table I. Effects of various reagents on galectin-8-induced neutrophil adhesion

Inhibitor		% Neutrophils adhering[a]
Control		2.6 ± 0.4
None (+galectin-8, 1 μM)		95.5 ± 4.5
Lactose	5 mM	35.4 ± 3.4
	10 mM	20.6 ± 0.7
	20 mM	6.4 ± 0.6
Sucrose	20 mM	90.1 ± 1.6
Anti-αL	5 μg/ml	71.7 ± 2.0
Anti-αM	5 μg/ml	20.0 ± 3.3
Cytochalasin B	5 μM	38.7 ± 0.4
Cytochalasin D	5 μM	38.5 ± 1.1

[a]Data represent means ± SD of triplicate measurements.

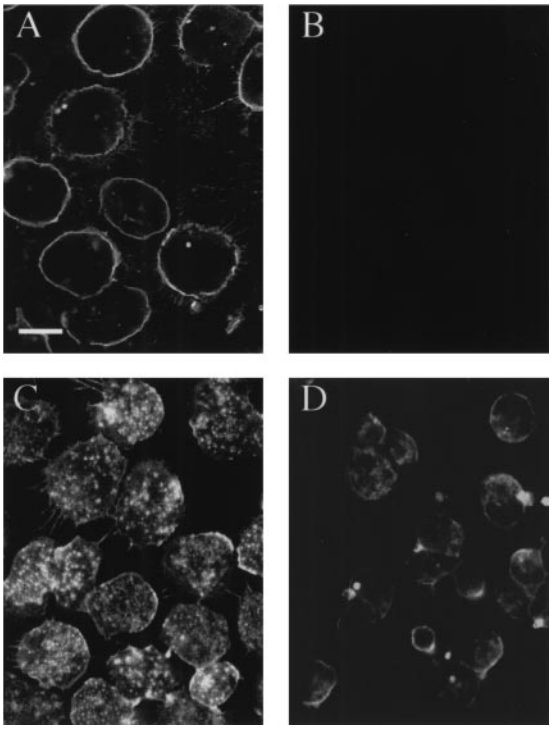

Fig. 3. Immunofluorescence localization of galectin-8 and F-actin in human neutrophils treated with galectin-8. Neutrophils were treated with GST-galectin-8 (1 μM) for 60 min at 37°C in the absence (**A**, **B**, **C**) or presence (**D**) of cytochalasin D (5 μM). Fixed and permeabilized neutrophils were treated with affinity-purified anti-galectin-8 polyclonal antibodies (**A**) or control IgG (**B**) and anti-rabbit IgG-FITC, and F-actin was stained with FITC-phalloidin (**C**, **D**), followed by confocal laser scanning microscopy. Scale bar, 10 μm.

Other members of the galectin family, galectins-1, -3, and -9, showed only a low or negligible effect. Although adhering neutrophils could not be detached by pipetting unless treated with trypsin, the adhesion was significantly reversed

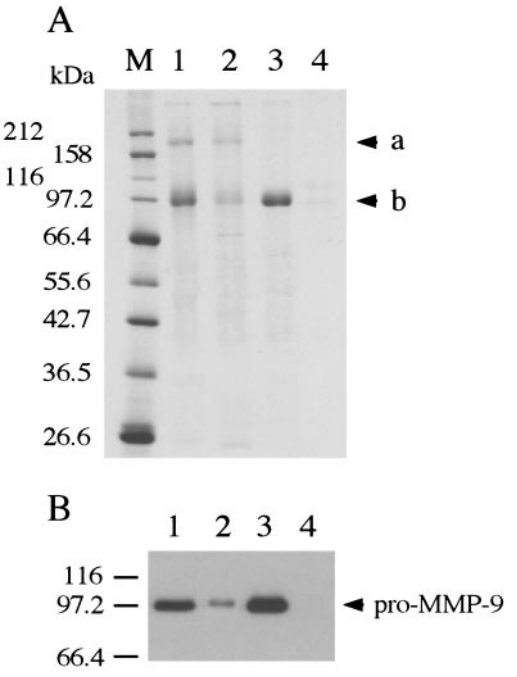

Fig. 4. Identification of galectin-8 binding proteins in neutrophils. (**A**) The solubilized membrane fraction of human neutrophils was incubated with recombinant GST-galectin-8 (lane 1), GST-galectin-8R69H (lane 2), GST-galectin-8R233H (lane 3), or GST alone (lane 4). Galectin-8-binding protein complexes were trapped with glutathione-Sepharose gel and then the binding proteins were eluted with lactose. The eluted proteins were resolved by means of SDS–PAGE and stained with CBB R-250. For N-terminal amino acid sequence analysis, the separated proteins were transferred to PVDF membranes. Amino acid sequences were determined with a gas-phase sequencer. M, molecular weight markers; a, 180-kDa band; b, 94-kDa band. (**B**) Western blot analysis of proMMP-9 in the solubilized membrane fraction of neutrophils affinity-purified with GST-galectin-8 (lane 1), GST-galectin-8R69H (lane 2), GST-galectin-8R233H (lane 3), or GST alone (lane 4).

by removal of galectin-8 (Figure 2B). Tissue culture plates pretreated with a serum-free galectin-8 solution (0.3–10 μM) supported neutrophil adhesion (Figure 2A). However, the level of adhesion was less than 40% of that induced by galectin-8 (1 μM) in the medium.

It is known that actin rearrangement occurs in neutrophils at the time of adhesion, during chemotaxis and phagocytosis. F-actin staining in neutrophils treated with galectin-8 was concentrated in distinct focal areas, showing sites of cell–substratum contact (Figure 3C). The addition of cytochalasin D, an inhibitor of actin polymerization, disrupted actin assembly and inhibited cell adhesion (Figure 3D and Table I).

Identification of galectin-8 receptors in neutrophils

To examine the molecular mechanisms by which galectin-8 induces neutrophil adhesion, proteins bound to galectin-8 in a lactose-sensitive manner were affinity-purified from the solubilized neutrophil membrane fraction. Only two major protein bands corresponding to molecular weights of about 180,000 and 94,000 were detected with sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) (Figure 4A). The N-terminal amino acid sequences of

the higher- and lower-molecular-weight band materials were determined to be FNLDTENAMTFQENAR and APRQRQSTLXLFP, respectively. These sequences completely matched with those of integrin αM/CD11b and promatrix metalloproteinase-9 (proMMP-9). A mutant galectin-8 with an inactivated N-terminal CRD (galectin-8R69H) exhibited similar affinity for integrin αM and reduced affinity for proMMP-9, compared to the wild type (Figure 4A). On the other hand, another mutant form with an inactivated C-terminal CRD (galectin-8R233H) retained affinity for proMMP-9 but did not bind integrin αM.

Integrin αM exists as a heterodimer with β subunit (integrin β2, 95 kDa) on the surface of neutrophils. Because we could not detect the β subunit by N-terminal amino acid sequence analysis, we carried out western blot analysis of the affinity-purified preparations using anti-proMMP-9 and anti-integrin β2 antibodies. proMMP-9 was detected in all the preparations in accordance with the result of amino acid sequence analysis (Figure 4B). The content of integrin β2 was below the detection limit (data not shown).

The neutrophil adhesion assay showed that galectin-8R69H possessed reduced but apparent adhesion-inducing activity and that inactivation of C-terminal CRD almost completely abolished this activity (Figure 5). In addition, anti-integrin αM antibodies but not anti-integrin αL ones strongly inhibited the neutrophil adhesion induced by galectin-8 (Table I).

Effect of galectin-8 on processing of proMMP-9 by MMP-3

As the processing of MMP precursors (proMMPs) is an important step in the control of MMP activity *in vivo*, we examined the effect of galectin-8 on the processing of proMMP-9 by MMP-3 under the conditions that permit slow processing of proMMP-9 (1 μg/ml proMMP-9; proMMP-9:MMP-3 = 1:0.1, molar ratio). In the absence of galectin-8, about 15% of proMMP-9 was processed after 1 h incubation under the conditions used (Figure 6). Galectin-8 accelerated the reaction in a dose-dependent manner: the addition of 0.25 μM and 1 μM galectin-8 resulted in about 40% and 70% processing, respectively, of proMMP-9 after 1 h incubation. The effect was inhibited by lactose almost completely. Galectin-8 mutants (R69H and

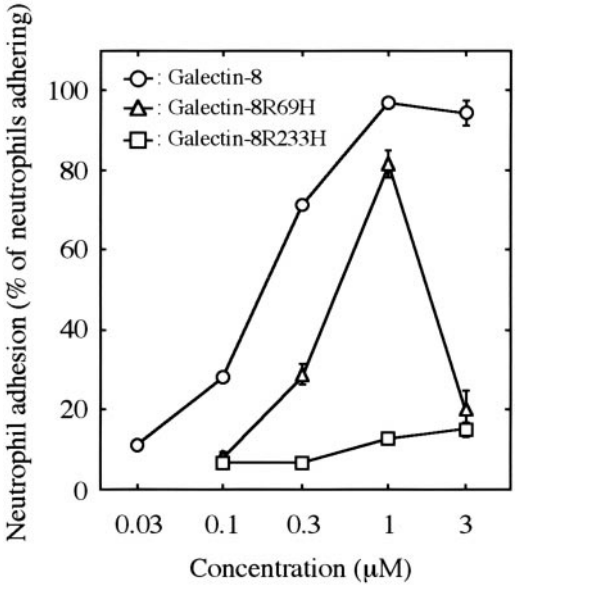

Fig. 5. Comparison of GST-fusion proteins of galectin-8 and galectin-8 mutants as to the promotion of neutrophil adhesion to tissue culture plates. The neutrophil adhesion assay was carried out as described in the legend to Figure 2. Data represent means ± SD of triplicate measurements.

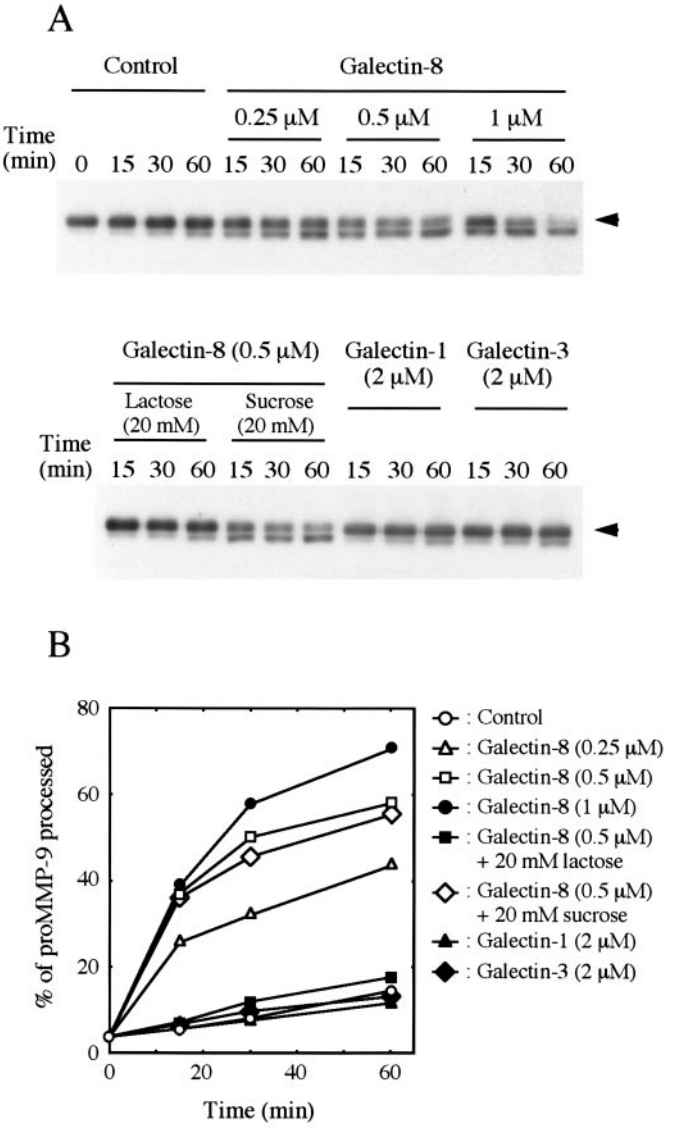

Fig. 6. Effect of galectin-8 on the processing of proMMP-9 by MMP-3. A reaction mixture comprising 100 ng proMMP-9, 5 ng activated MMP-3 and 0.25–2 μM GST-fusion protein, in a final volume 100 μl, was incubated at 37°C. Aliquots of the mixture were withdrawn as specified. Activation of proMMP-9 was assessed by western blot analysis. In the case of galectin-8 (0.5 μM), the effects of lactose and sucrose were also examined. (**A**) Representative western blot analysis of MMP-9. The arrowheads indicate the position of proMMP-9. (**B**) The integrated intensities of proMMP-9 and processed MMP-9 were obtained by densitometric scanning. Only the mean values for three independent experiments are shown.

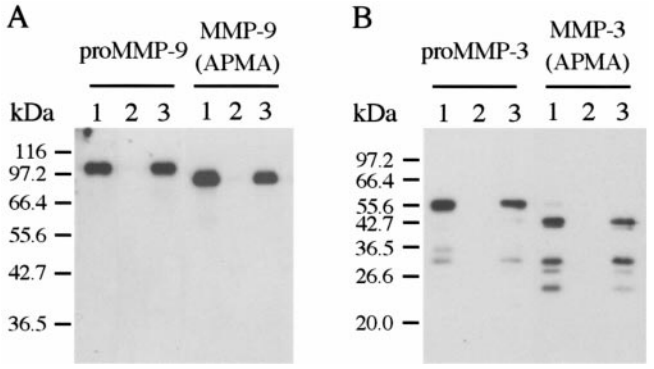

Fig. 7. Binding activity of galectin-8 with pro- and activated forms of MMP-9 and MMP-3. Purified MMPs (precursor forms and activated forms prepared by treatment with APMA) were incubated with GST-galectin-8. After incubation for 1 h at 4°C, glutathione-Sepharose gel was added to the mixture, followed by further incubation for 45 min at 4°C. The gel was washed with TBS, 0.03% CHAPS. Galectin-binding proteins were eluted with TBS, 0.2 M lactose. MMPs were detected by western blot analysis. (**A**) MMP-9; (**B**) MMP-3; lane 1, control sample; lane 2, nonbound fraction; lane 3, eluate from glutathione-Sepharose gel.

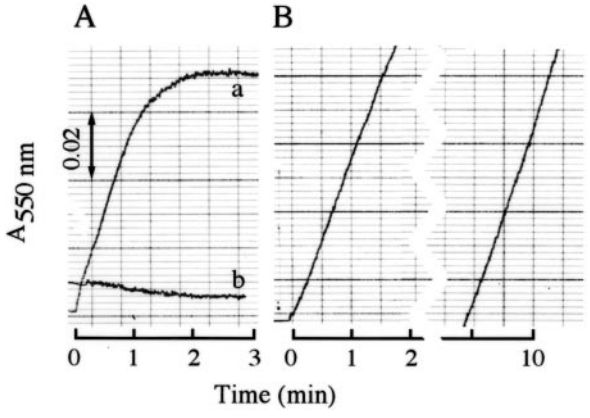

Fig. 8. Time course of superoxide production by human neutrophils induced by galectin-8 and fMLP. Purified neutrophils suspended in PBS containing 0.5 mM $MgCl_2$, 0.8 mM $CaCl_2$, 7.5 mM glucose, and 75 μM horse heart cytochrome c were placed in a cuvette. After preincubation for 5 min at 37°C, the stimulants were added, and the absorbance change at 550 nm was recorded with a double-beam spectrophotometer. (**A**) a, fMLP (0.2 μM), b, without stimulant; (**B**) GST-galectin-8 (1 μM). Representative data from three separate experiments are shown.

R233H, 1 μM) showed a negligible effect on the processing (data not shown). Galectins-1 and -3 (2 μM) were also ineffective as to acceleration of the reaction. The N-terminal amino acid sequence analysis of the processed MMP-9 revealed that the sequence, LLLQKQLSLP lies between the first and the second cleavage site of proMMP-9 by MMP-3 reported by Ogata *et al.* (1992).

To determine whether or not galectin-8 can interact with MMP-3, we carried out a binding assay using purified MMPs. Galectin-8 was capable of binding both proMMP-3 and activated MMP-3 in addition to the pro- and activated forms of MMP-9 (Figure 7A and 7B).

Table II. Effects of fMLP and GST-fusion proteins of galectin-8 and galectin-8 mutants on superoxide production by neutrophils

Additives	Superoxide production[a] (O_2^- nmoles/l × 10^6 cells/min)	
	−cytochalasin B	+cytochalasin B
None	0.00	0.00
fMLP, 0.2 μM	2.14	8.43
fMLP, 0.2 μM+lactose, 20 mM	—	8.54
fMLP, 0.2 μM+SOD	—	0.00
Galectin-8, 0.3 μM	—	2.60
Galectin-8, 1 μM	1.83	5.40
Galectin-8, 1 μM (-neutrophils)[b]	0.00	
Galectin-8, 1 μM+lactose, 20 mM	—	0.27
Galectin-8, 1 μM+sucrose, 20 mM	—	5.60
Galectin-8, 1 μM+SOD	—	0.00
Galectin-8, 3 μM		7.34
Galectin-8R69H, 1 μM	—	4.70
Galectin-8R233H, 1 μM	—	0.07

[a]Data represent means of three independent experiments.
[b]Superoxide production was measured in the absence of neutrophils.

Effect of galectin-8 on superoxide production by neutrophils

Extracellular matrix proteins are known to stimulate some main functions of neutrophils, such as secretion of lytic enzymes and liberation of superoxide, via interactions through members of the integrin family of glycoproteins. Because of the ability of galectin-8 to bind integrin αM, we examined the effect of galectin-8 on superoxide production in neutrophils. The addition of galectin-8 to a neutrophil suspension induced immediate production of superoxide. The initial rate of the reaction induced by galectin-8 (1 μM) was comparable to that induced by fMLP (0.2 μM) (Figure 8 and Table II). fMLP-induced superoxide production ceased after 2 min incubation, and that induced by galectin-8 continued for up to 10 min at a nearly constant rate. The addition of cytochalasin B enhanced the effects of both fMLP and galectin-8. Experiments involving galectin-8 mutants showed that galectin-8R69H but not galectin-8R233H retained this activity (Table II).

Discussion

It has already been shown that several types of normal and transformed cells adhere and spread on immobilized galectin-8 via interaction with integrins (Levy *et al.*, 2001). In contrast, when present in the medium, galectin-8 inhibited cell adhesion in their assay system. The authors concluded that galectin-8 functions like matrix proteins, but in a more complex manner, in promoting cell adhesion. In the present study, however, galectin-8 added to serum-containing medium induced almost complete and firm cell attachment specific to neutrophils. Immobilized galectin-8 also supported

neutrophil adhesion, but the number of neutrophils adhering was less than 40% of that with galectin-8 as a soluble ligand. In addition, the adhesion was partly reversed (about 60%) by removal of galectin-8 from the culture medium. An attempt to isolate galectin-8-interacting proteins from neutrophils revealed that integrin αM was a binding protein for C-terminal CRD and that anti-integrin αM antibodies inhibited neutrophil adhesion induced by galectin-8. Furthermore, abolition of the sugar binding activity of C-terminal CRD but not N-terminal CRD abolished the adhesion-promoting activity of galectin-8.

Integrin αM exists as a heterodimer with β subunit (integrin β2/CD18). However, we could not detect integrin β2 in the affinity-purified preparations by N-terminal amino acid sequence analysis and western blot analysis. The loss of integrin β2 indicates the possibility that the heterodimer structure of Mac-1 changes when galectin-8 interacts with the oligosaccharide moiety of α subunit. The structural change may result in lowered interaction between the two subunits, and thus, β2 subunit was lost during affinity purification. These results suggest that galectin-8 promoted cell adhesion by activating integrin αM (αMβ2/Mac-1), that is, by inducing a direct interaction between Mac-1 and the substratum, in at least about 60% of the adhering neutrophils. There is, however, the possibility that neutrophil cell surface protein(s) other than integrin αM play a role in galectin-8-induced neutrophil adhesion because galectin-8R69H did not retain full adhesion-inducing activity. Dong and Hughes (1997) reported that integrin αM is one of the predominant binding proteins for galectin-3 in macrophages. In addition, preliminary experiments showed that human galectin-7 could bind integrin α6 (unpublished data). These results and those of galectin-8 suggest that integrin α subunits are common targets of the members of the galectin family.

The expression of Mac-1 is not restricted to neutrophils; eosinophils and monocytes/macrophages also express the molecule. Currently, the reason for the inability of galectin-8 to induce eosinophil adhesion is unclear. One possible explanation for this observation is that the number of Mac-1 molecules capable of interacting with galectin-8 on eosinophils may be significantly smaller than that on neutrophils. Structural heterogeneity in the oligosaccharide moiety of Mac-1 may also result in the difference.

Kuwabara and Liu (1996) reported that galectin-3 promoted neutrophil adhesion to a laminin-coated plastic surface. In our case, galectins-1 and -3 induced apparent neutrophil adhesion at high concentrations, but the adhering cells could be detached by pipetting, showing that the binding between neutrophils and the substratum induced by galectin-8 is much stronger than that induced by galectins-1 and -3.

ICAM-1, C3bi, collagen, and fibrinogen are known as physiological ligands for Mac-1. Among them ICAM-1 plays a critical role in neutrophil adhesion to endothelial cells and subsequent migration (Diamond *et al*., 1990). Galectin-8 induced neutrophil adhesion to ICAM-1-coated plates in a manner very similar to that to untreated plates (Figure 2). Because plastic culture plates serve as good substratum in neutrophil adhesion, it is likely that the maximum effect of galectin-8 on neutrophil adhesion was achieved using untreated plates.

MMPs are considered to play a key role in normal tissue remodeling and in pathological destruction of the matrix in various diseases. MMP-9 is produced mainly by polymorphonuclear leukocytes and monocytes/macrophages. For example, transmigrating neutrophils secrete MMP-9 to degrade matrix proteins (Baggiolini and Dewald, 1984). The activities of MMPs are controlled not only through their gene expression but also through processing of their precursor forms and inhibition by endogenous inhibitors. Stromelysins, including MMPs-3 and -10, are capable of activating proMMPs (proMMPs-1, -8 and -9) and are considered to play a pivotal role in the initiation of the MMP activation cascade. In this study, we showed that galectin-8 accelerated the processing of proMMP-9 mediated by MMP-3 using purified components and the experimental conditions that permit slow processing of proMMP-9 in the absence of galectin-8. The N-terminal amino acid sequence analysis revealed that MMP-9 processed under the present conditions (1 μg/ml proMMP-9; proMMP-9:MMP-3 = 1:0.1, molar ratio) was not the fully processed form but possessed 35 extra N-terminal amino acid residues compared to the fully processed one. On the other hand, accelerated production of the fully processed MMP-9 in the presence of galectin-8 was observed in some experiments in which higher concentrations of proMMP-9 and MMP-3 (2.5 μg/ml proMMP-9; proMMP-9:MMP-3 = 1:1, molar ratio) were used (data not shown). It was difficult, however, to obtain reproducible results under the latter conditions. These results suggest that galectin-8 facilitates the processing by recruiting proMMP-9 and MMP-3 (see later discussion) when these proteins exist at low concentrations and that the production of fully processed MMP-9 is sensitive to the experimental conditions when nanomolar concentrations of the MMPs were used. The lactose-sensitive nature of the accelerating effect indicates that interaction between galectin-8 and oligosaccharide chain(s) of proMMP-9 (Rudd *et al*., 1999; Mattu *et al*., 2000) and/or MMP-3 is indispensable. Although the molecular mechanisms involved in the effect remain to be determined, the inability of monovalent galectin-8 mutants, galectin-8R69H and galectin-8R233H, to accelerate the reaction suggests that the formation of a ternary complex from proMMP-9, galectin-8, and MMP-3 is an essential step. The finding that galectin-8 can bind purified MMP-3 is consistent with this speculation.

Galectin-8 was as effective as fMLP, a potent leukocyte chemotactic factor, in inducing superoxide production by peripheral blood neutrophils. fMLP-induced superoxide production continued only for 2 min in the absence of cytochalasin B. In contrast, galectin-8 showed a long-lasting effect. The difference is probably due to rapid down-regulation of the fMLP receptor and not the galectin-8 receptor after activation. The experiments involving galectin-8 mutants suggested that Mac-1 is a major signal transducer in galectin-8-induced superoxide production. Yamaoka *et al*. (1995) reported that human galectin-3 stimulated superoxide production by neutrophils, although the maximum activity of galectin-3 was about 25% of that of fMLP. They suggested that the aggregation of NCA-160 (CD66) induced by galectin-3 might be important for neutrophil activation. However, because galectin-3 is

capable of interacting with integrin αM, it is possible that galectin-3 triggers superoxide production via interaction with Mac-1.

Neutrophil adhesion to endothelial cells and subsequent transendothelial migration proceed through defined steps: (1) circulating cells are captured and induced to roll on the stimulated endothelium; (2) surface-bound activating signal(s) cause activation of neutrophil β2 integrins, which immobilize the cells on the endothelium; and (3) cells migrate through the endothelial cell layer and interstitial space by degrading the extracellular matrix. Selectins and β2 integrins play central roles in the first and second steps, respectively. MMPs and β2 integrins are important factors in the third step. In addition to these factors, the present study suggests that galectin-8 is a new player in the process of neutrophil migration: galectin-8 may participate in the second and third steps as an activator of Mac-1 and a modulator of MMP-9 activity, respectively. This possibility must be evaluated by means of *in vivo* studies.

Materials and methods

Construction of expression vectors for galectins

Expression vectors for glutathione-S-transferase–fusion (GST-fusion) proteins of human galectins-1, -3, and -9 were prepared as described previously (Matsushita *et al*., 2000). A human galectin-8 cDNA encoding an isoform with a short linker peptide (Bidon *et al*., 2001) was amplified from first-strand cDNAs prepared from the poly(A)$^+$ RNA fraction of a human epidermoid carcinoma cell line (A431) using forward and reverse primers tagged with extra 5′ *Bgl*II (G8-F: 5′-CGTCCTAGATCTATGATGTTGTCCTTAAACAAC-3′) and *Sal*I (G8-R: 5′-CGACCGGTCGACCTACCAGCTCCTTACTTCCAG-3′) sequences, respectively. The amplified cDNA was digested with *Bgl*II and *Sal*I, and the resulting cDNA fragment was inserted into the *Bam*HI–*Xho*I site of pGEX-4T-2 (Amersham Biosciences, Piscataway, NJ).

Site-directed mutagenesis of galectin-8

The following forward and reverse primers were used to generate cDNAs for site-directed mutants of galectin-8: G8R69H-F (5′-CATTTCAATCCTCATTTCAAAAGG-3′), G8R69H-R (5′-CCTTTTGAAATGAGGATTGAAATG-3′), G8R233H-F (5′-CACTTGAACCCACACCTGAATATT-3′), and G8R233H-R (5′-AATATTCAGGTGTGGGTTCAAGTG-3′). Site-directed mutagenesis of galectin-8 residue Arg69 (and Arg233) to His was carried out as described elsewhere (Matsushita *et al*., 2000). Galectin-8 cDNA carrying a double mutation, R69H and R233H (galectin-8R69, 233H), was prepared by introducing the R233H mutation into galectin-8R69H. The resultant mutations were confirmed by DNA sequencing.

Expression, purification, and sugar-binding activity of recombinant proteins

Expression of recombinant proteins in *Escherichia coli* BL21 cells was carried out as described previously (Matsushita *et al*., 2000). Recombinant proteins were purified by affinity chromatography on a lactose-agarose column (Seikagaku, Tokyo) (galectin-8 and galectin-8R233H) or a glutathione-Sepharose column (Amersham Biosciences) (galectin-8R69H and galectin-8R69, 233H). The protein concentration was determined using BCA protein assay reagent (Pierce, Rockford, IL) and bovine serum albumin as a standard. The sugar-binding ability of recombinant galectin-8 was assessed by asialofetuin-agarose affinity chromatography. Purified GST-fusion proteins (1 mg) were applied to an asialofetuin-agarose column (bed volume, 0.5 ml; 20 mg of conjugated protein/ml) equilibrated with phosphate buffered saline (PBS). After washing the column with PBS, proteins bound to the column were eluted with PBS, 0.2 M lactose. A control sample, nonbound fraction, and bound fraction were analyzed by SDS–PAGE.

Preparation of antibodies to galectin-8

The affinity-purified GST-galectin-8 was digested with thrombin and the released GST moiety was removed by glutathione-Sepharose affinity chromatography. Anti-galectin-8 antiserum was raised in Japanese white rabbits as described (Shoji *et al*., 2002).

In vitro *cell adhesion assay*

Neutrophils and eosinophils were isolated as described previously (Matsumoto *et al*., 1998; Matsushita *et al*., 2000). Isolated cells were added to 24-well tissue culture plates (2.5×10^5 cells in 0.45 ml of medium/well) in triplicate. After the addition of 50 μl of the assay sample, the cells were allowed to adhere for 60 min at 37°C. At the end of the incubation period, loosely attached cells were removed by vigorous pipetting and washing with PBS. The attached cells were recovered by treatment with 0.25% trypsin/0.5 mM ethylenediamine tetra-acetic acid (EDTA) for 10 min at 37°C, and then sonicated for 15 s in 50 mM sodium phosphate buffer (pH 7.4) containing 2 M NaCl and 2 mM EDTA. The DNA content of the sonicate was determined by the method of Labarca and Paigen (1980) using calf thymus DNA as a standard. In some experiments, tissue-culture plates were pretreated with GST-galectin-8 or recombinant human ICAM-1 (extracellular domain; Genzyme-Techne, Minneapolis, MN) dissolved in PBS for 3 h at room temperature. The plates were washed three times with PBS before the adhesion assay.

Fluorescence microscopy of the neutrophil cytoskeleton and galectin-8

Fluorescein isothiocyanate (FITC)-phalloidin was used to stain F-actin. Neutrophils adhering to a plastic chamber slide (Nalge Nunc International, Naperville, IL) were washed with PBS and then fixed with 4% paraformaldehyde. The cells were permeabilized with 0.1% Triton X-100 in PBS prior to incubation with 1.65×10^{-7} M FITC-phalloidin (Molecular Probes, Eugene, OR) for 20 min, followed by washing with PBS. All samples were covered with 50% glycerol and a coverslip and then subjected to confocal laser scanning microscopy. Immunofluorescent localization of galectin-8 was carried out using affinity-purified anti-galectin-8 polyclonal antibodies. Paraformaldehyde-fixed permeabilized neutrophils were incubated

with anti-galectin-8 antibodies (5 μg/ml) overnight at 4°C. After washing with PBS, the cells were incubated with anti-rabbit IgG-FITC (10 μg/ml; Kirkegaard & Perry Laboratories, Gaithersburg, MD) for 1 h at room temperature. The cells were washed with PBS and then subjected to confocal microscopy as described.

Western blot analysis

Samples were electrophoretically separated in SDS/10% polyacrylamide gels and then transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA). Immunodetection was carried out with monoclonal antibodies against human MMP-3 (Daiichi Fine Chemical, Toyama, Japan) and human CD18 (Cymbus Biotechnology, Chandlers Ford, UK) and polyclonal antibodies against human MMP-9 (Chemicon, Temecula, CA) as described previously (Nishi *et al.*, 1995).

Isolation of galectin-8-interacting proteins and protein sequencing

Purified neutrophils suspended in 10 mM Tris–HCl (pH 7.5), 1 mM phenylmethylsulfonyl fluoride were subjected to a single freeze–thaw cycle and then sonicated. The total membrane fraction was isolated by centrifugation at 50,000 × *g* for 30 min and then suspended in 10 mM Tris–HCl (pH 7.5), 0.5 M NaCl, 1.5% Triton X-100 (8 ml/1 × 10^8 cells). After incubation for 1 h at 4°C, the solubilized membrane fraction was obtained by centrifugation. The resulting supernatant was incubated with recombinant proteins (1 μM) for 1 h at 4°C. After the addition of glutathione-Sepharose gel, the mixture was incubated for 45 min at 4°C with gentle mixing. The gel was washed with 20 mM Tris–HCl (pH 7.5), 0.15 M NaCl, 0.03% 3-{(3-cholamidopropyl)dimethylammonio}-1-propane sulfonate (TBS, 0.03% CHAPS). Galectin-binding proteins were eluted with TBS, 0.2 M lactose. The proteins were resolved by means of 10% SDS–PAGE and stained with Coomassie brilliant blue R-250 (CBB R-250). To determine the N-terminal amino acid sequences of the galectin-8-interacting proteins, the resolved proteins were transferred to PVDF membranes. The blotted proteins were stained with CBB R-250 and cut out, and then the N-terminal amino acid sequence was determined with a gas-phase sequencer.

Processing of ProMMP-9 with MMP-3

Activated MMP-3 was prepared by treating proMMP-3 (human synovial fibroblasts; Biogenesis, Kingston, NH) with 2 mM aminophenyl mercuric acetate (APMA; Sigma, St. Louis, MO). APMA was removed from the activated MMP-3 preparation by dialysis. The reaction mixture for the processing of proMMP-9 was mad up of 20 mM Tris–HCl (pH 7.5), 0.4 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.03% CHAPS, 100 ng proMMP-9 (human neutrophils; Calbiochem, San Diego, CA), and 0.25–2 μM recombinant protein in a final volume of 100 μl. After the addition of activated MMP-3 (5 ng/100 μl), the reaction mixture was incubated for 0–60 min at 37°C. Aliquots of the mixture were withdrawn as specified. Activation of proMMP-9 was assessed by western blot analysis.

Determination of the binding activity of galectin-8 with MMPs

Purified MMPs (1 μg) were mixed with GST-galectin-8 (25 μg) in 0.4 ml 20 mM Tris–HCl (pH 7.5), 0.4 M NaCl, 5 mM $CaCl_2$, 1 μM $ZnCl_2$, 0.1% Triton X-100. After incubation for 1 h at 4°C, glutathione-Sepharose gel was added to the mixture, followed by further incubation for 45 min at 4°C with gentle mixing. The gel was washed with TBS, 0.03% CHAPS. Galectin-binding proteins were eluted with TBS, 0.2 M lactose. A control sample, nonbound fraction, and bound fraction were subjected to western blot analysis. Activated MMP-9 was prepared by treating proMMP-9 with APMA.

Determination of superoxide production

O_2^- production was determined by the method of Yamaoka *et al.* (1995). Purified neutrophils (2.5 × 10^6/2 ml), suspended in PBS containing 0.5 mM $MgCl_2$, 0.8 mM $CaCl_2$, and 7.5 mM glucose were placed in a cuvette. The cuvette also contained horse heart cytochrome c (75 μM). The reaction was carried out in the presence or absence of cytochalasin B (5 μg/ml; Biomol Research Laboratories, Plymouth Meeting, PA). In control experiments, superoxide dismutase (SOD, 50 μg/ml) was added. After preincubation for 5 min at 37°C, the stimulants were added and the absorbance change at 550 nm was monitored. The O_2^--generating activity was calculated using a molar extinction coefficient of 20.5 × 10^3 $M^{-1}cm^{-1}$.

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Abbreviations

APMA, aminophenyl mercuric acetate; CBB, Coomassie brilliant blue; CHAPS, 3-{(3-cholamidopropyl) dimethylammonio}-1-propane sulfonate; CRD, carbohydrate recognition domain; EDTA, ethylenediamine tetraacetic acid; FITC, fluorescein isothiocyanate; GST, glutathione-S-transferase; ICAM-1, intercellular adhesion molecule-1; MMP, matrix metalloproteinase; PBS, phosphate buffered saline; PVDF, polyvinylidene difluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SOD, superoxide dismutase.

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