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Dual Function of Macrophage Galactose/N-Acetylgalactosamine-specific Lectins: Glycoprotein Uptake and Tumoricidal Cellular Recognition

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We investigated whether the interaction of peritoneal macrophages with extracellular ligands is mediated by C-type lectins specific for galactose and N-acetylgalactosamine. The carbohydrate-binding domain of mouse galactose/N-acetylgalactosamine-specific lectin was prepared in a recombinant form. The purified recombinant lectins were tested for competitive inhibition against glycoprotein uptake and against tumoricidal effect. Thioglycolate-elicited macrophages internalized galactosylated bovine serum albumin *in vitro*. The internalization was blocked by recombinant macrophage lectins. Activated macrophages obtained after intraperitoneal injection of a nonspecific immune potentiator, OK432, did not internalize galactosylated bovine serum albumin. These cells elicited a cytotoxic effect against P815 murine mastocytoma cells, and the effect was blocked by recombinant macrophage lectins. These results indicated that galactose/N-acetylgalactosamine-specific C-type lectins expressed on the surface of inflammatory macrophages and on activated tumoricidal macrophages mediate two distinct functions, i.e. glycoprotein uptake and tumoricidal effector mechanisms.

Key words: Macrophage — Lectin — Cellular recognition — Tumoricidal function

Macrophages (Mϕs) perform a variety of immunological functions, such as antigen presentation, tumoricidal effect, cytokine production, and phagocytosis of foreign substances. We previously showed that activated tumoricidal Mϕs from the mouse peritoneal cavity expressed galactose/N-acetylgalactosamine (Gal/GalNAc)-specific lectins (MMGL) on their surfaces.[1)] MMGL is likely to be involved in the recognition of tumor cells.[2)] Purified MMGL consisted of glycoproteins with M_r ranging from 45k to 60k, depending on the source of Mϕs. We have recently cloned the cDNA for MMGL and have found that the primary structure of MMGL is highly homologous to rat hepatic lectins and to rat macrophage Gal/GalNAc-specific macrophage asialoglycoprotein-binding proteins.[3–6)] These latter C-type lectins (hepatic lectins and the asialoglycoprotein-binding proteins) from rats were thought to function as receptors for glycoprotein uptake. Thus, it seemed plausible that MMGL also functioned as asialoglycoprotein receptors, which could initiate endocytosis. If this is the case, then how these same molecules can mediate the tumoricidal activity of Mϕs is a mystery. We hypothesized that these molecules, expressed on different Mϕ populations, possessed two distinct functions. MMGL has already been shown to present itself on the surface of both inflammatory Mϕs and tumoricidal Mϕs.[3)] Furthermore, it has been shown that both inflammatory Mϕs and tumoricidal Mϕs adhere to malignant cells, yet tumoricidal activity was exhibited only by the activated Mϕs. We designed several experiments using recombinant soluble MMGL (rML) to test our hypothesis. It has not previously been examined whether tumoricidal Mϕs execute asialoglycoprotein uptake. Thus, we investigated whether MMGL could function as a receptor for glycoprotein uptake by mouse Mϕs at different stages of activation, and whether these molecules mediated Mϕ adhesion to mastocytoma cells, which we thought was a prerequisite to cytotoxicity. The results suggested that cell surface MMGL has dual functions and that tumoricidal Mϕs but not inflammatory Mϕs adhere to tumor cells through these molecules.

MATERIALS AND METHODS

Materials OK-432, a penicillin-treated and lyophilized preparation of the Su-strain of *Streptococcus pyrogenes*, was kindly donated by Chugai Pharmaceutical Co. (Tokyo). One KE (Klinische Einheit) unit corresponds to 0.1 mg of dried streptococci, Bacto-thioglycolate medium was purchased from Difco Laboratories (Detroit, Mich., USA). Neoglycoproteins (Gal-BSA and GlcNAc-BSA) were prepared as described previously.[1)]

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Orosomucoid (Sigma Chemical Co., St. Louis, Mo., USA) was desialized by acid hydrolysis with 0.05 *M* H_2SO_4 at 80°C for 1 h to obtain asialoorosomucoid (ASOM). Glycoproteins were labeled with ^{125}I-NaI by the chloramine-T method.[7]

Cells Peritoneal exudate cells were harvested from 6- to 10-week-old C3H/He female mice (Charles River Japan, Kanagawa), which had been treated for 4 days with OK-432 (2 KE/mouse) or with 3% Bacto-thioglycolate medium (1 ml/mouse). Adherent cells in peritoneal exudate preparations were collected after adhering to plastic tissue culture plates. Adhesive cells were collected by EDTA treatment. OK-432-elicited adhesive cells and thioglycolate-elicited adhesive cells were designated as OK-macrophages (OK-Mϕs) and TG-macrophages (TG-Mϕs), and used as tumoricidal (activated) Mϕs and inflammatory Mϕs, respectively. The murine mastocytoma cell line, P815, was maintained in RPMI1640 medium supplemented with 10% fetal calf serum (10% FCS-RPMI). A soluble form of the mouse macrophage lectin (rML) was expressed and purified from *E. coli* transformed by a cDNA encoding the extracellular regions including a carbohydrate recognition domain (CRD), as described previously.[4]

Endocytosis of ^{125}I-labeled glycoproteins by Mϕs Peritoneal exudate adherent cells (1×10^7) were suspended in 500 μl of Eagle's minimum essential medium with 10 m*M* HEPES, pH 7.4 containing 300 ng of ^{125}I-labeled glycoproteins (Gal-BSA, GlcNAc-BSA or ASOM). After incubation at 37°C for 30 min with shaking, the cells were washed three times with PBS, pH 7.2 containing 5 m*M* EDTA and 0.1% BSA. Radioactivity associated with the cells was counted on a gamma counter. For MMGL competition experiments, competitive inhibitors (400 μg/ml rML or 0.5% rat anti-MMGL serum[4]) were added throughout the incubation period.

Binding of mastocytoma cells to Mϕs P815 mastocytoma cells were labeled with 1 μCi/ml of [^{3}H]thymidine at 37°C for 4 h in 10% FCS-RPMI. The ^{3}H-labeled P815 cells were preincubated with or without recombinant MMGL at 4°C for 30 min. OK-Mϕs or TG-Mϕs were suspended in 10% FCS-RPMI and cultured for 2 h in 96-well culture plates (1.5×10^5 cells/well). P815 cells at a concentration of 2×10^4 cells/well were added to monolayers of Mϕs in PBS, pH 6.2 containing 1 m*M* $CaCl_2$, 0.02% NaN_3 and 0.1% BSA. The cells were incubated at 4°C for 30 min, then the plate was centrifuged for 1 min at 200*g*. Thirty minutes later, each well was washed four times. The P815 cells attached to Mϕs in each well were lysed by 2% sodium laurylsulfate, and the radioactivity was determined on a scintillation counter.

Tumoricidal activity TG-Mϕs or OK-Mϕs (1.5×10^5 cells/100 μl/well) were allowed to adhere to 96-well flat plates. The [^{3}H]thymidine-labeled P815 cells were preincubated at 37°C for 30 min in 10% FCS-RPMI containing recombinant MMGL. Then, the preincubated P815 cells (1×10^4 cells/100 μl/well) were added to the Mϕ monolayers. After a 48 h culture, the radioactivity of an aliquot (100 μl) of the culture supernatant was determined in a scintillation counter. Cytolytic activity was determined as follows.

$$\%\ \text{cytolysis} = \frac{\text{released radioactivity} - \text{spontaneous release}}{\text{total radioactivity} - \text{spontaneous release}} \times 100$$

The spontaneous release was radioactivity released without macrophages, and the total radioactivity was deter-

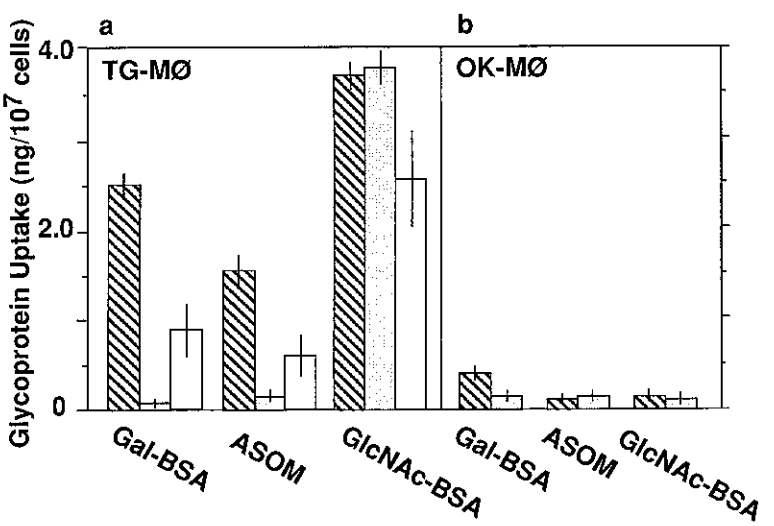

Fig. 1. Inhibition of the uptake of glycoproteins by recombinant MMGL. Mϕs (1×10^7 cells) were incubated without (hatched columns) or with recombinant MMGL (shaded columns), or with anti-MMGL antisera (open columns) in Eagle's minimum essential media with the ^{125}I-labeled glycoproteins. The values are shown as mean ± SD from triplicate experiments. (a) TG-Mϕs; (b) OK-Mϕs.

mined as the radioactivity of target cells lysed with 2% sodium laurylsulfate.

RESULTS

Effect of rML or anti-MMGL on galactosylated glycoprotein uptake by Mφs The uptake of ^{125}I-labeled glycoproteins having carbohydrate chains with terminal galactose residues was tested in the absence or presence of rML. To assess whether the interaction of Gal/GalNAc-binding lectins on Mφ surfaces with ^{125}I-labeled ASOM, Gal-BSA or GlcNAc-BSA induces the incorporation of these substances, they were incubated with 1×10^7 Mφs at 37°C for 30 min (Fig. 1). Inflammatory Mφs elicited by thioglycolate (TG-Mφs) incorporated Gal-BSA, GlcNAc-BSA, and ASOM. The amount of these glycoproteins taken up by tumoricidal Mφs (OK-Mφs) was significantly lower than that by TG-Mφs. The uptakes of Gal-BSA and ASOM by TG-Mφs were inhibited by rML added to the incubation medium. Polyclonal rat antisera against MMGL also inhibited the uptake of galactosylated glycoproteins (Fig. 1). The uptake of GlcNAc-BSA was not inhibited by rML or anti-MMGL antisera, indicating that the inhibition was competitive and specific. These results demonstrated that MMGL was involved in the uptake of galactosylated glycoproteins by TG-Mφ. MMGL is also expressed on the surface of OK-Mφ. However, these cells did not significantly internalize galactosylated glycoproteins.

Effect of rML on the binding of Mφs to tumor cells rML was used to assess whether MMGL competitively inhibited the adhesion of Mφs to P815 mastocytoma cells (Fig. 2). P815 mouse mastocytoma cells were preincubated in the absence or presence of rML and tested for their ability to adhere to Mφ monolayers at different stages of activation. P815 cells bound TG-Mφs or OK-Mφs regardless of preincubation with lipopolysaccharide (LPS). rML did not significantly reduce the number of P815 cells bound to TG-Mφs in a dose-dependent manner when they were not activated with LPS. The degree of inhibition by rML was significant for OK-Mφs. rML at a concentration of 600 ng/ml inhibited the adhesion of P815 cells to LPS-treated TG-Mφs approximately 70%. The degree of inhibition of adhesion to OK-Mφs (LPS-treated or untreated) was 90%. These results indicated that P815 cell adhesion to TG-Mφs was not mediated by MMGL, whereas P815 cell adhesion to OK-Mφs was highly dependent on MMGL. The molecules that mediate P815 cell adhesion to TG-Mφs are unknown.

Tumoricidal activity of Mφs at different stages of activation against P815 mastocytoma cells We examined the tumoricidal capacity of TG-Mφs and OK-Mφs, untreated or treated with LPS, against P815 cells. As shown

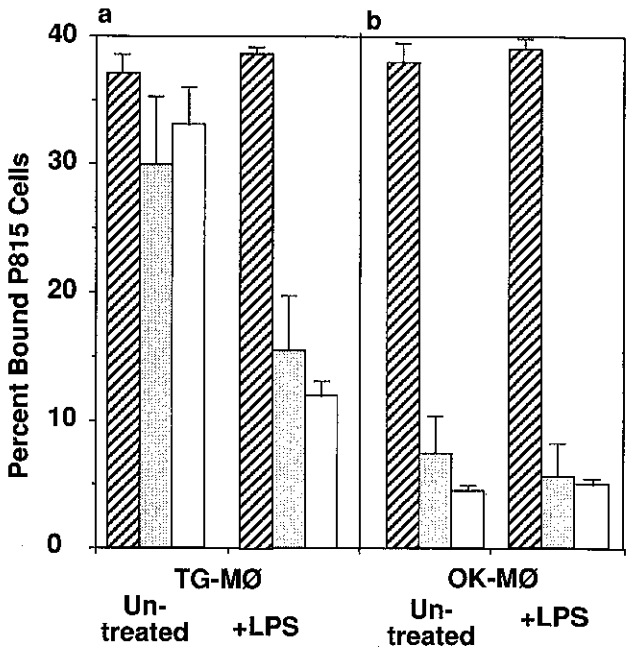

Fig. 2. Effect of recombinant MMGL on the binding of TG-Mφs or OK-Mφs to P815 mastocytoma cells. Mφ monolayers (1.5×10^5 cells/well) were incubated in the absence or presence of 100 ng/ml LPS for 18 h. P815 cells (2×10^4 cells/well) were added and further incubated without recombinant MMGL (hatched columns), with 300 ng/ml recombinant MMGL (shaded columns), or with 600 ng/ml recombinant MMGL (open columns) at 4°C for 60 min in PBS, pH 6.2, containing 1 m*M* $CaCl_2$ and 0.1% BSA. The values are shown as mean±SD from triplicate experiments. (a) TG-Mφs; (b) OK-Mφs.

in Fig. 3, TG-Mφs did not lyse P815 cells unless these cells were treated with LPS. When TG-Mφs were treated with LPS, they acquired the ability to lyse P815 cells. Untreated OK-Mφs were tumoricidal against P815 cells. Their tumoricidal capacity was enhanced by LPS treatment. Thus, Mφ populations obtained by exposure to different stimulators, thioglycolate and OK-432, showed different levels of tumoricidal activity to P815 cells, although their capacity to adhere to P815 cells was similar. Before stimulation with LPS *in vitro*, the tumoricidal activity appeared to correlate to MMGL-dependent adhesion. However, the involvement of MMGL in the adhesion of P815 cells to LPS-treated TG-Mφs appeared to be less than that to OK-Mφs (Fig. 2), yet LPS-treated TG-Mφs were more cytotoxic to P815 cells than untreated OK-Mφs (Fig. 3).

Effects of rML on Mφ cytotoxicity The effects of rML on the cytotoxicity of TG-Mφs and OK-Mφs are shown in Fig. 4. P815 cells labeled with [^{3}H]thymidine were incubated with rML at 37°C for 30 min, then added to

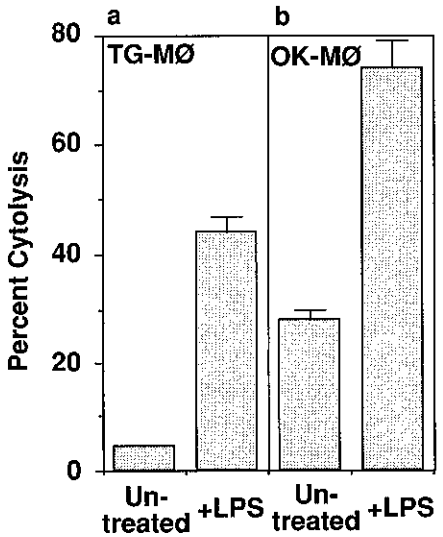

Fig. 3. Comparison of the tumoricidal activity of TG-Mφs (a) and OK-Mφs (b). Mφ monolayers (1.5×10^5 cells/well) were incubated without or with 100 ng/ml LPS for 18 h in 10% FCS-RPMI. The cytolysis of [^{3}H]thymidine-labeled P815 cells (1×10^4 cells/well) was determined after incubation with Mφs at 37°C for 48 h. The values are shown as mean±SD from triplicate experiments.

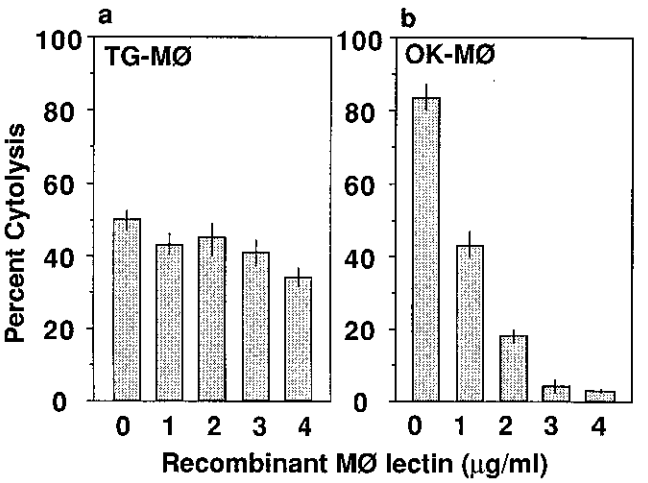

Fig. 4. Effect of recombinant MMGL on the tumoricidal activity of TG-Mφs and OK-Mφs. TG-Mφs (a) or OK-Mφs (b) were treated with LPS, and incubated with [^{3}H]thymidine-labeled P815 cells. The effect of soluble recombinant MMGL was assessed after incubation at 37°C for 48 h. The values are shown as mean±SD from triplicate experiments.

Mφ monolayers. The release of [^{3}H]thymidine from P815 cells induced by LPS-stimulated TG-Mφs was inhibited by rML in a dose-dependent manner. The reduction in the presence of 1 μg/ml lectin was approximately 50%. The cytotoxicity of LPS-treated OK-Mφs was effectively inhibited by the preincubation of target cells with rML. Thus, it is strongly suggested that OK-Mφs, effective tumoricidal Mφs, recognize and bind P815 cells through C-type lectins, and that this interaction is required for their cytotoxic effect.

DISCUSSION

A variety of mammalian cells are known to produce membrane-bound lectins. Many of them are implicated in cellular recognition. Mφs of various tissue origins express a wide variety of lectins. For example, Gal/GalNAc-specific lectin,[6,8,9] Man/GlcNAc/Fuc-specific lectin,[10-12] and Gal(GalNAc)/Fuc-specific lectin,[13] have been described as C-type lectins. Most of them were postulated to be mediators of binding and phagocytosis of glycoproteins and microorganisms. The S-type lectins known as Mac-2 antigens are among the major Mφ surface molecules.[14,15] Sialoadhesin is a specific splenic Mφ lectin without known gene family association.[16] We have previously purified and characterized an MMGL from mouse peritoneal tumoricidal Mφs induced by OK432.[1] From the sequence homology between MMGL and hepatic asialoglycoprotein receptors such as rat hepatic lectins and rat macrophage asialoglycoprotein-binding proteins, it is hypothesized that these molecules function as receptors for glycoproteins with terminal galactose at the oligosaccharide residues. These lectins might also be involved in an unknown recognition that distinguishes tumor cells from normal cells.[2,4] The present study demonstrated that MMGL on different Mφ subpopulations performs distinct functions. On OK-Mφs, MMGL appeared to mediate adhesion to malignant cells. Furthermore, this adhesive mechanism is likely to mediate the Mφ-induced lysis of tumor target cells.

OK-Mφs internalized galactosylated proteins to a much lesser extent than TG-Mφs. The reason for the difference is not clear. When labeled Gal-BSA was incubated with OK-Mφs and TG-Mφs at 4°C, the amounts of Gal-BSA bound on the surface were very similar. After raising the temperature to 37°C and incubating for 30 min, a significant portion of radioactivity associated with OK-Mφs was still removable by EDTA-treatment. Therefore, OK-Mφs may be incapable of glycoprotein uptake. Cell surface MMGL of activated Mφs may not be linked to intracellular structures that initiate endocytosis. As an alternative function, MMGL on OK-Mφs is likely to recognize malignant cells. The results presented in this paper indicate that MMGL-dependent adhesion was necessary for Mφs to lyse P815 mastocytoma cells. TG-Mφs adhered to P815 cells to essentially the same extent as OK-Mφs, but did not induce the lysis of P815

cells. It remains to be elucidated whether the MMGL-dependent adhesion induces a specific metabolic change in activated Mφs, as seen in platelet-Mφ interactions mediated by P-selectin.[17]

There is an apparent discrepancy between the inhibition of Mφ adhesion to P815 cells and the inhibition of Mφ cytotoxicity against these cells when LPS-activated TG-Mφs were examined. rML significantly inhibited the adhesion (Fig. 2) but not the cytotoxic effect (Fig. 4). These puzzling results suggested that the recognition mechanism necessary for the tumoricidal functions of LPS-activated TG-Mφs was at least in part distinct from that of OK-Mφs. It seemed that the cytotoxic effects of OK-Mφs were more dependent on MMGL than those of LPS-activated TG-Mφ.

The involvement of carbohydrate-protein interactions in Mφ-tumor cell adhesion and subsequent tumor cell destruction has been suggested in a number of experiments. For example, various monosaccharides were shown to inhibit Mφ-tumor cell adhesion and cytotoxicity.[18-20] Treatment of P815 mastocytoma and other tumor cells with inhibitors of glycoprotein carbohydrate-chain processing was shown to decrease macrophage-mediated lysis.[21] The carbohydrate-dependent recognition of tumor cells by Mφs might be mediated by C- or S-type lectins, or through multifunctional adhesion molecules such as laminin.[22] Alternatively, galactosyltransferases[23] or carbohydrate chains[24] may function as the counterparts of carbohydrate chains, although Mφ-tumor cell interactions through these molecules have not been reported. The present results strongly suggest that C-type Gal/GalNAc-specific lectins on activated Mφs mediate tumor cell adhesion and recognition.

We have recently investigated the binding specificity of this lectin expressed in *E. coli* by means of immobilized lectin affinity chromatography with various glycopeptides having defined carbohydrate structures (to be published elsewhere). Glycopeptides carrying three constitutive GalNAc-Ser/Thr (Tn-antigen) had a very strong affinity, whereas those with fully sialylated carbohydrate chains showed a weak binding. We also compared the elution profiles of high mannose-type and complex-type Asn-linked carbohydrate chains. A strong retardation was observed with tetraantennary complex-type glycopeptides terminated with galactose obtained from human α1-acid glycoprotein. These results indicated that the carbohydrate specificity of Mφ lectin was promiscuous, yet it shows the strong tendency to interact with carbohydrate chains that is characteristic of malignant cells. Tn-antigen is known to be expressed on a variety of carcinoma cells.[25] Also, highly branched complex-type oligosaccharides are thought to be specific for transformed and tumorigenic cells.[26,27] It remains to be elucidated whether tumor cells with these carbohydrate chains show distinct survival and malignant behavior *in vivo*.

The present study clearly demonstrated that MMGL mediated two distinct Mφ functions, i.e. glycoprotein uptake and tumor cell recognition, depending on the type of Mφ populations. There are few examples of cell surface molecules that potentially play dual functions in cellular recognition. For example, insulin-like growth factor II (IGF-II)/mannose-6-phosphate receptor recognizes mannose-6-phosphate in lysosomal enzymes and mediates endocytosis of IGF-II.[28,29] MMGL may serve as another example of dual recognition functions, when it is expressed on different Mφ populations.

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