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Mannose-binding Protein Recognizes Glioma Cells: *In vitro* Analysis of Complement Activation on Glioma Cells via the Lectin Pathway

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The lectin pathway is a novel pathway for activation of the complement cascade, which is initiated by the binding of mannose-binding protein (MBP) to its carbohydrate ligands. We investigated whether the complement system was activated *in vitro* by glioma cells through this pathway to the C3 level. MBP was found to bind to all six glioma cell lines tested by using flow cytometric analysis. Binding of a complex of MBP-associated serine protease and MBP was observed in two of the cell lines examined, thereby resulting in C4 consumption. Activation of C3 was hemolytically evaluated in these two lines. C3 consumption was also observed in one. Based on these results, it is likely that recognition by MBP followed by complement activation occurs in certain glioma cell lines.

Key words: Glioma — Complement — Mannose-binding protein — Lectin pathway — Complement regulatory factor

The complement cascade can be initiated either by the classical or alternative pathway. Recently, another activation pathway of complement was discovered which was designated the lectin pathway (Fig. 1).[1,2] The initial event in this pathway consists of the binding of mannose-binding protein (MBP) to its ligands. MBP is an animal lectin present in blood, and has the ability to bind specifically to mannose and N-acetylglucosamine in the presence of calcium.[2,3] After binding to carbohydrate ligands, MBP activates complement via activation of a serine protease that we discovered and termed MBP-associated serine protease (MASP).[4]

Until now, investigations of the lectin pathway have been mainly carried out using bacteria, viruses and blood cells bearing mannan.[2] It has been reported that high-mannose glycopeptides are present on the surface of malignant or transformed cells.[5,6] However, it is unclear whether MBP reacts with tumor cells.

In this study, we first examined whether MBP binds to glioma cells. Next, activation of complement to the C3 stage tested *in vitro* using human glioma cell lines.

MATERIALS AND METHODS

Buffers The following buffers were prepared: phosphate-buffered saline (PBS); veronal-buffered saline (VB): veronal buffer containing 0.148 *M* NaCl; mannitol-gelatin-veronal buffer (MGVB): VB containing 0.1% gelatin, 2.3% mannitol, 2 m*M* $CaCl_2$, and 0.5 m*M* $MgCl_2$; glucose-gelatin-veronal buffer (GGVB): VB containing 0.1% gelatin, 2.5% glucose, 0.15 m*M* $CaCl_2$ and 0.5 m*M* $MgCl_2$; EDTA-GVB: VB containing 0.1% gelatin and 10 m*M* EDTA.

Cell lines Six human glioma cell lines were used in this study; T98G and A-172 were obtained from the Japanese Cancer Research Resources Bank (Tokyo), U-87MG, U251 and A-7 were from Riken Cell Bank (Tsukuba) and 1321N1 was kindly provided by Dr. N. Nakahata, Department of Pharmacology, Fukushima Medical School, Fukushima.

MBP and MASP MBP, MASP and MBP-MASP complex (MBP-MASP) were purified from normal human serum as previously reported.[4]

Monoclonal antibodies Mouse anti-MBP monoclonal antibody (mAb) 3E7 was obtained as previously described.[7] 1C6, specific for decay-accelerating factor (DAF, CD55), was established as previously reported.[8] 1F5, specific for homologous restriction factor (HRF20, CD59), was a gift from Dr. N. Okada, Fukuoka University School of Medicine, Fukuoka.[9]

Complement components, EA and EAC14 Guinea pig complement component C1[10] and human components C4,[11] oxidized C2 (^{oxy}C2),[12] C3 and C5[13] were purified as previously described. Serum was used as a source of C6, C7, C8 and C9 (C6-9 reagent) and prepared as previously described.[14] C4-deficient serum (C4D) was obtained from C4-deficient guinea pigs. EA represents sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte antibody and EAC14 represents EA bearing C1 and C4. Each preparation was suspended in GGVB and used at 10^8/ml.

Binding of MBP to tumor cells One million cells were treated with 200 μl of MGVB containing 10 μg/ml MBP for 30 min on ice. They were then incubated on ice with

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20 μl of 100 μg/ml mAb 3E7 for an additional 30 min and stained with 20 μl of 100 μg/ml FITC-conjugated rabbit anti-mouse immunoglobulins (FITC-anti-mouse Igs) (Dako Japan Co., Ltd., Kyoto) for a final 30 min on ice. The cells were washed twice with MGVB between each reaction. Reactivities were evaluated on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) and compared with two controls which were cells of each line treated with FITC-anti-mouse Igs or with mAb 3E7 and FITC-anti-mouse Igs.

Consumption of C4 Three million cells were treated with various concentrations of MBP-MASP for 30 min on ice. They were washed twice, then 50 μl of C4 (200 SFU/ml) was added to each tube and incubation was continued at 30°C for 30 min. After centrifugation, the supernatant was collected from each sample and subjected to twofold serial dilution. One hundred microliters of EA was incubated at 37°C for 1 h with an equal volume of each diluted sample and C4D. After addition of 1.2 ml of EDTA-GVB, the extent of hemolysis was determined spectrophotometrically and C4 consumption was calculated.

Consumption of C3 Three million cells were incubated with 10 μg/ml MBP-MASP for 30 min on ice. They were washed twice, and exposed to 50 μl of C4 and ^{oxy}C2 at 30°C for 30 min. After centrifugation, the supernatant was removed, 50 μl of C3 (250 SFU/ml) was added, and incubation was continued at 37°C for 30 min. Three controls were prepared as follows: A) C3 alone as a negative control, B) cells treated with only C3 to examine non-specific adhesion and C) cells treated with MBP-MASP and C3, since MASP has a little direct activity against C3, as shown in Fig. 1. After centrifugation, the supernatant was collected from each tube and subjected to twofold serial dilution. One hundred microliters of EAC14 was incubated with 50 μl of each diluted sample and ^{oxy}C2, C5 and C6-9 reagent at 37°C for 1 h. After addition of 1.2 ml of EDTA-GVB, the extent of hemolysis was determined spectrophotometrically and C3 consumption was calculated.

Expression of DAF and HRF20 One million tumor cells were treated with 100 μg/ml 1C6 or 1F5 in PBS for 30 min on ice. They were washed twice with PBS, then exposed to FITC-anti-mouse Igs for 30 min on ice, and following extensive washing, they were analyzed on a FACScan. Cells exposed to FITC-anti-mouse Igs only were used as a control.

RESULTS

Binding of MBP to tumor cells Binding of MBP to glioma cells was analyzed by flow cytometry. In all 6 cell lines, MBP binding was observed (Fig. 2). There were no differences between cells exposed to FITC-anti-mouse Igs only and those exposed to mAb 3E7 and FITC-anti-mouse Igs (data not shown). The highest mean fluorescence intensity was seen in U-87MG cells. Dose-dependency was estimated from the relationship between the

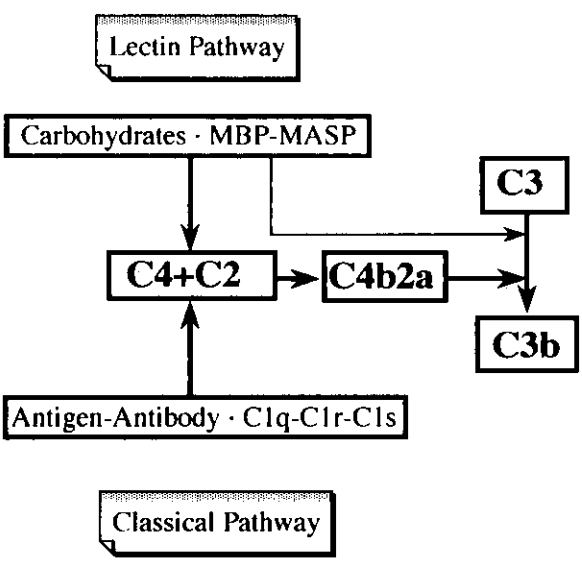

Fig. 1. Outline of the lectin pathway.

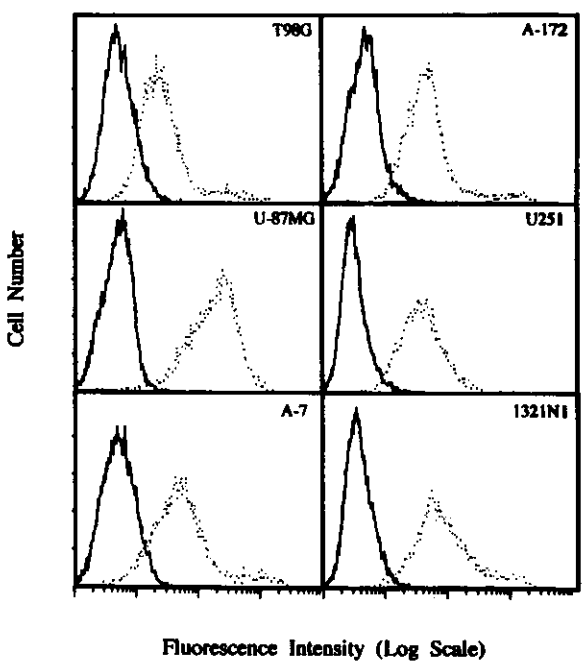

Fig. 2. Binding of MBP to glioma cells. Cells were incubated with MBP (dotted line) or buffer alone (solid line) and then exposed to mAb 3E7 followed by FITC-anti-mouse Igs. Fluorescence was recorded in arbitrary units on a logarithmic scale and plotted against the relative cell number.

concentration of MBP reacted (from 0.01 to 10 μg/ml) and mean fluorescence intensity in T98G and U251 cells. The mean intensity increased in a dose-dependent fashion in the two cell lines examined (Fig. 3). Inhibition of MBP binding by EDTA (by blocking calcium) or mannose was examined in U-87MG, U251, A-7 and 1321N1 cells. In all four cell lines, 10 m*M* EDTA was used. Forty millimolar mannose was used for A-7 and 1321N1, and 80 m*M* for U-87MG and U251. Inhibition by EDTA was observed in all four cell lines tested (Fig. 4). Although inhibition by mannose was demonstrated in 1321N1, it was very weak in other cell lines (Fig. 5). These results indicate that the binding of MBP is specific for carbohydrate at least in 1321N1.

Consumption of C4 Binding of MBP-MASP to glioma cells was evaluated by means of a C4 consumption test in U-87MG and 1321N1. The extent of C4 consumption on cells exposed to various concentrations of MBP-MASP (0, 0.33, 1.0, 3.3 and 10 μg/ml in MGVB) was determined. Consumption of C4 increased dose-dependently, reaching 100% in cells treated with 10 μg/ml MBP-MASP (Fig. 6). Therefore, it is likely that MBP-MASP complex binds to glioma cells under functionally active conditions.

Consumption of C3 C3 consumption in U-87MG and 1321N1 cells was tested as in the C4 consumption experiments. In 1321N1, C3 activity decreased by 40.1% compared with control A whereas it decreased by 24.8% in controls B and C. When controls B or C were regarded as 0%, C3 consumption was 20.4% (Table I). The reduction observed in control B was considered to be caused by non-specific binding of C3 to the cells and the decrease by 20.4% in the sample compared with control B was regarded as consumption via the lectin pathway. Since there was no difference between controls B and C, direct C3 consumption by MASP was not detectable in this experiment. However, in U-87MG cells, reduction compared with control A was observed to the same extent in the sample and in controls B and C and therefore con-

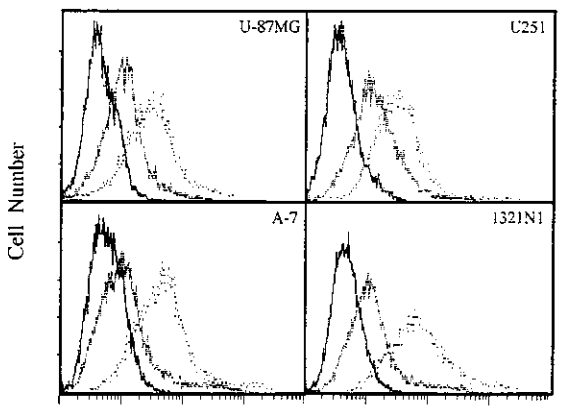

Fig. 4. Inhibition of MBP binding to glioma cells by EDTA. Cells were treated with MBP (dotted line), MBP and 10 m*M* EDTA (densely dotted line) or buffer alone (solid line) followed by mAb 3E7 and FITC-anti-mouse Igs. They were analyzed on a FACScan flow cytometer.

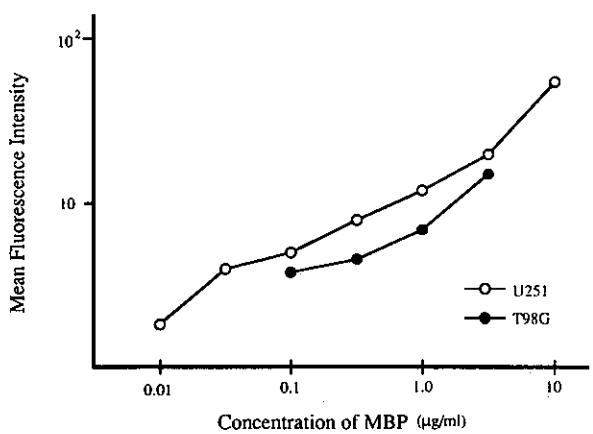

Fig. 3. Dose-dependency of MBP binding to glioma cells. Cells were incubated with various concentrations of MBP then exposed to mAb 3E7 followed by FITC-anti-mouse Igs. Fluorescence was recorded in each sample and the mean intensity is plotted against the concentration of MBP reacted in U251 (open circles) and T98G (closed circles).

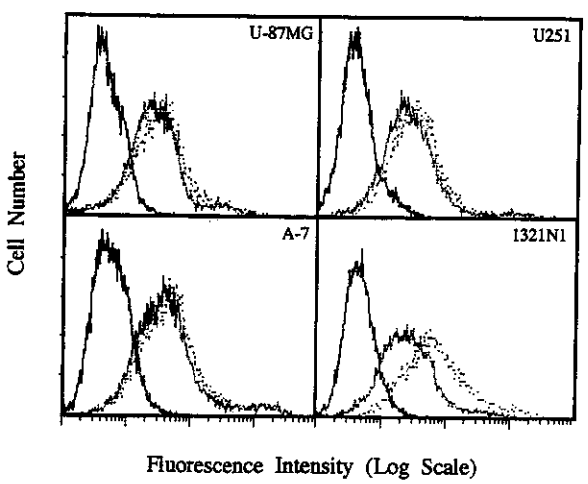

Fig. 5. Inhibition of MBP binding to glioma cells by mannose. Cells were treated with MBP (dotted line), MBP and mannose (densely dotted line) or buffer alone (solid line) followed by mAb 3E7 and FITC-anti-mouse Igs. Forty millimolar mannose was used for A-7 and 1321N1 and 80 m*M* for U-87MG and U251. The cells were analyzed on a FACScan.

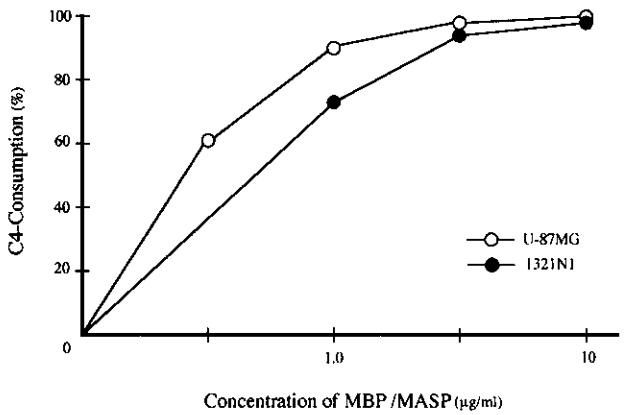

Fig. 6. C4 consumption on glioma cells via the lectin pathway. Equal amounts of C4 were added to cells treated with various concentrations of MBP-MASP. After incubation at 30°C for 30 min, residual C4 activity was measured in each sample by hemolytic assay and the amount of C4 consumed was calculated. C4 consumption is plotted against the concentration of MBP-MASP reacted with the cells in U-87MG (open circles) and 1321N1 (closed circles).

Table I. C3 Consumption in 1321N1

		Tumor cells	MBP-MASP	C4, C2	C3	C3 consumption (%)
Control	A	−	−	−	+	0
	B	+	−	−	+	24.8
	C	+	+	−	+	24.8 (0)[a]
Sample		+	+	+	+	40.1 (20.4)[a]

a) C3 consumption calculated by regarding the consumption in control B as zero.

sumption via the lectin pathway was not detected (data not shown).

Expression of DAF and HRF20 There are some complement regulatory factors on nucleated cells which prevent lysis by homologous complement, such as DAF and HRF20. DAF is a molecule which prevents the assembly of C3 convertases.[8] HRF20 prevents C5-8 complex-mediated C9 insertion into the cell membrane.[9] These inhibitors may be responsible for the difference in C3 consumption between U-87MG and 1321N1 as shown above. Therefore, expression of DAF and HRF20 on glioma cells was analyzed by flow cytometry. As shown in Fig. 7, all six cell lines were found to express HRF20 on their surfaces and four (T98G, A-172, A-7 and 1321N1) also carried DAF. However, DAF expression was not related to C3 consumption in 1321N1 or U-87MG.

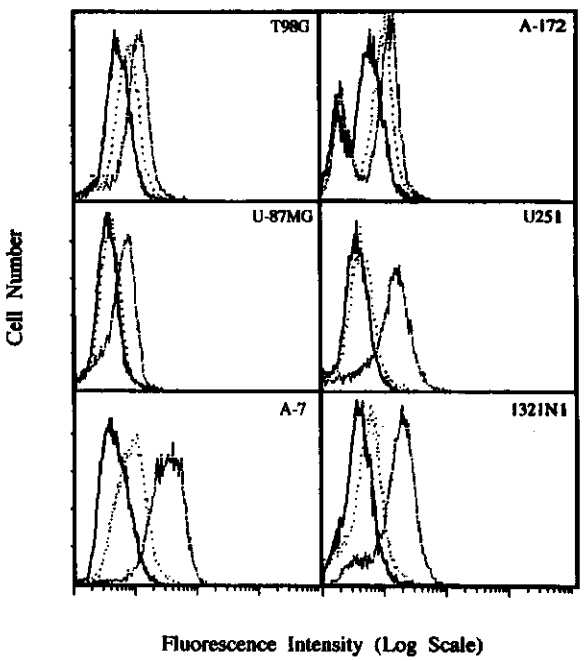

Fig. 7. Expression of DAF and HRF20 on glioma cells. Cells were incubated with mAb 1C6 specific for DAF (dotted line), 1F5 specific for HRF20 (densely dotted line) or buffer alone (solid line) followed by FITC-anti-mouse Igs. They were analyzed on a FACScan flow cytometer.

Lastly, we examined whether 1321N1 cells were lyzed by complement. These cells were treated with excess amounts of MBP-MASP, C4, C2 and C3 and subsequently with C5-C9. Viability was measured in terms of dye exclusion with Trypan blue. However, no lysis was observed (data not shown), indicating that HRF20 may protect the cells from lysis by autologous complement.

DISCUSSION

MBP is an animal lectin which was first reported by Kawasaki *et al.* in 1978.[15] Purified MBP is composed of a mixture of trimers, tetramers, pentamers and hexamers in an approximately 90 kDa structural unit, which is composed of three polypeptide chains. The hexamer was found to be very similar in structure to complement component C1q by electron microscopic analysis.[16] It is known that MBP plays a role in host defence, i.e., 1) it displays opsonizing activity by binding to microorganisms and mediating phagocytosis via the C1q-receptor (C1q-R),[17,18] 2) it protects cells from HIV infection by binding to gp120, a high-mannose type glycoprotein on HIV,[19] and 3) it activates complement after binding specifically to mannose and N-acetylglucosamine.[20–22]

It was formerly thought that MBP acts in a manner similar to C1q and activates the complement cascade by the classical pathway. Matsushita and Fujita, however, identified a new serine protease termed MBP-associated serine protease (MASP) which participates in the activation of the complement cascade after the binding of MBP to its ligands.[4] This pathway of complement activation was termed the lectin pathway.[23]

If MBP binds to glioma cells, it may initiate activation of complement and/or eliminate them from the host via C1q-R. It is known that high-mannose glycopeptides are present on malignant or transformed cells. Glioma cells in the brain are thought to be free of MBP since this protein cannot pass through the blood-brain barrier and is present only at extremely low levels in cerebrospinal fluid.[24] Therefore, we surmised that MBP might be useful in the treatment of malignant glioma by means of local administration and a preliminary study was carried out.

In the present study, we show that MBP binds to glioma cells in a dose-dependent manner. Inhibition of binding by EDTA and mannose was observed in the 1321N1 cell line, showing that the binding is specific for a carbohydrate moiety. U-87MG had the highest mean intensity on flow cytometric analysis, as shown in Fig. 2, but the inhibition by mannose was not observed. This may be explained by a difference in the quantity of terminal mannose expressed and/or by the difficulty of binding to mannose in the fluid phase. Another possibility is that some MBP might bind to tumor cells through C1q-R since MBP is similar in structure to C1q, though these receptors were not detected by flow cytometry (data not shown). In any case, this is the first report concerning the binding of MBP to tumor cells.

The reactivities of C4 and C3 were determined by studying consumption in two cell lines, U-87MG and 1321N1. C4 consumption was observed in both cell lines and C3 consumption in one. It was, however, confirmed that C3 was activated by the lectin pathway in 1321N1 *in vitro*. Thus, it is thought that glioma cells are eliminated by phagocytosis by means of not only C1q-R but also C3 receptors.

In conclusion, we demonstrated complement activation via the lectin pathway to the C3 stage on glioma cells *in vitro* as well as expression on these cells of complement regulatory factors. It was reported that when DAF function is blocked by anti-DAF mAb, C3 uptake and complement-mediated lysis of resistant melanoma lines are markedly enhanced.[25] Since our future investigations will focus on lysis and phagocytosis against glioma cells mediated by MBP and complement, a strategy for blocking inhibitors will also be required.

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