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Metabolic Properties of Normal and Mutant Mannan-Binding Proteins in Mouse Plasma

Haruna Naito,* Yong Ma,* Kazuhide Uemura,* Yasushi Arano,† and Toshisuke Kawasaki*
**Department of Biological Chemistry and CREST (Core Research for Evolutional Science and Technology) Project, Japan Science and Technology Corporation (JST), and †Department of Patho-Functional Bioanalysis, Graduate School of Pharmaceutical Sciences, Kyoto University, Sakyo-ku, Kyoto 606-8501, Japan*

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Human mannan-binding protein (MBP) is a serum lectin involved in innate immunity. MBP activates the complement pathway through its interaction with mannose-rich carbohydrates on various microorganisms and a common opsonic defect has been shown to be associated with a low serum concentration of MBP. This low serum concentration is closely associated with a single base mutation in codon 52, 54 or 57 of the human MBP gene, which results in a change of Arg52 to Cys, Gly54 to Asp, or Gly57 to Gln, respectively, in the collagen-like region of the molecule and prevents the formation of higher oligomers. However, the mechanism underlying the low serum concentration in such patients is completely unknown. The levels of protein synthesis and secretion of the normal and mutant MBPs seem to be similar according to our previous *in vitro* results. In this study, we examined the plasma clearance of the normal and mutant human (Gly54Asp) MBPs in mice, and found that the half-life of the mutant MBP is about half that of the normal MBP, explaining in part the difference in the plasma levels between the two types of MBP.

Serum mannan-binding protein (MBP), a C-type serum lectin specific for mannose and N-acethylglucosamine, has been isolated from various mammalian sera and characterized [1–4]. MBP plays an important role in the first-line host defense during the early stage of a disease or infection when the complex immune system has not yet developed. MBP activates the complement pathway through its interaction with carbohydrate antigens on various microorganisms [5–7], serves as a direct opsonin [8], and also enhances FcR- and CR1-mediated phagocytosis by mononuclear phagocytes [9, 10]. MBP has a series of oligomeric structures of up to 680kDa, consising of, at the largest, 18 identical subunits of 32kDa. Each 32kDa subunit has a carbohydrate recognition domain at its COOH-terminus and a collagen-like region consisting of 19 repeats of the sequence, Gly-Xaa-Yaa, at its NH2-terminus [11]. The subunit tend to form trimers linked through disulfide bonds. These trimers, each of which is called a structural unit, assemble into higher oligomers (trimers to hexamers) through disulfide bonds. The whole structure of MBP resembles that of C1q, one of the complement components.

Recently, it was reported that a low serum MBP concentration is associated with a common opsonic defect and causes frequent unexplained infections in infants [12–14]. The patients were homo- or heterozygous for a codon 52, 54 or 57 mutation, which results in a change of Arg52 to Cys, Gly54 to Asp, or Gly57 to Gln, respectively, in the collagen-like region and prevents the assembly of MBP higher oligomers. A population study has also shown that the frequency of the opsonic defect nearly corresponds to that of mutant alleles [15–17]. Such mutant alleles lead to a low serum concentration. However, the mechanism underlying the low serum concentration in these patients remains unclear. Our previous transfection studies indicated that the levels of protein synthesis and secretion of the Gly54 Asp mutant MBP are almost the same as those of the normal MBP in COS-1 cells [18], and in hepatoma cells [19]. Accordingly, the mutant MBP may be more vulnerable to degradation or more quickly cleared from the circulation.

Here, we studied the metabolic properties of the normal and Gly54 to Asp mutant MBPs in mouse plasma. The radiolabeled normal MBP was found to have a half-life of about 6h, while that of the mutant MBP was almost half as long. These results explain in part the low serum concentration of the mutant MBP.

MATERIALS AND METHODS

Expression and purification of recombinant MBPs from a human hepatoma cell line, HLF. Recombinant MBPs were expressed and purified from a human hepatoma cell line, HLF, as described previously [19]. HLF cells (JCRB 0405) were provided by the Japanese

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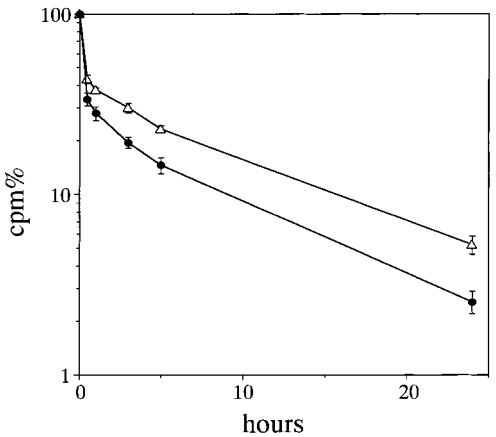

FIG. 1. Plasma clearance of the ^{125}I-normal and mutant MBPs. ^{125}I-labelled MBPs were intravenously injected into mice. Blood samples were withdrawn at 0.5, 1, 3, 5 and 24h after injection, and then the activity was measured. Three animals were used for each experiment. The graph shows the percentage of total radioactivity. Open triangles and closed circles show the normal and mutant MBPs, respectively.

Cancer Research Resources Bank (Tokyo, Japan) and were maintained in Dulbecco's modified Eagle medium (DMEM) (Nissui) supplemented with 10% fetal bovine serum (FBS) (GIBCO), from which endogenous MBP had been removed by passage through a Sepharose 4B-mannan column. HLF cells were grown at 37°C under 5% CO_2 to a concentration of 5×10^5 cells/ml in 20 ml medium. Recombinant vaccinia virus containing the human normal MBP or the human mutant MBP gene with the codon 54 mutation was added to the cells at a multiplicity of infection close to 5. Forty-eight hours postinfection the medium was harvested, from which the recombinant normal and mutant MBPs were purified on Sepharose 4B-mannan column as described previously [3].

Iodination of MBPs. Radioiodination of the MBPs was carried out with Bolton-Hunter reagent [20, 21]. ^{125}I-Labeled Bolton-Hunter reagent (135μCi; ICN Biomedical, Inc.) was added to the normal or mutant MBP (20μg) in 100μl of 0.1M phosphate buffer, pH 7.0, containing 0.5M NaCl. After incubation on ice for 4h, the reaction was terminated by the addition of 1.5M glycine to give a final concentration of 0.5M. After incubation on ice for 1h, 15mg/ml BSA was added to the reaction mixture to give a final concentration of 1mg/ml. The mixture was applied to a Sephadex G-50 column for the isolation of ^{125}I-MBP. More than 95% of the radioactivity was associated with the protein, as judged on TLC with a development solvent of methanol:H_2O = 8:2. The specific activity of ^{125}I-MBP was about 2.5×10^5 cpm/μg.

Plasma clearance in mice. Male balb/c mice weighing 20g were kept in metabolic cages with free access to water and a standardized diet. The ^{125}I-normal and mutant MBPs (3×10^5 cpm in 150μl of saline) were injected into the tail veins of these mice. When indicated, blood samples were collected from the tip of the tail using heparinized glass capillary tubes. Twenty-four hours after injection, the mice were killed by anesthesia with diethylether, and then the thymus, liver, spleen, kidneys and heart were removed and weighed, and then the radioactivity was determined. Three microliter blood samples were subjected to 3-10% gradient SDS–PAGE under nonreducing conditions, followed by BAS2000 analysis.

RESULTS

Plasma Clearance of ^{125}I-MBPs

To determine the time courses of the clearance of the ^{125}I-labeled normal and mutant MBPs, the radiolabeled proteins were injected intravenously into mice. Blood samples were collected at 0.5, 1, 3, 5 and 24hs after injection, and then the activity was determined (Fig. 1). The radioactive proteins disappeared from the circulation in two phases, i.e., a very rapid decrease in the first 30min, followed by a relatively slow decrease. The first phase corresponds to the period required for MBPs to reach equilibrium between the blood and tissue fluid. The second phase corresponds to the period of physiological turnover of MBP in the circulation. The decay curves were fitted to a two phase exponential curve, $y = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$, in which $Ae^{-\lambda_1 t}$ and $Be^{-\lambda_2 t}$ represent the first phase and second phase curves, respectively. The half lives ($t_{1/2}$) for these two phases were estimated by applying λ_1 and λ_2 to the equation $t_{1/2} = \ln 2/\lambda$. The calculated half lives are shown in Table 1. The half lives in the first phase were almost the same for the two kinds of MBPs; 1.8 ± 0.1min (mean ± S.D.) and 2.0 ± 0.3min for the wild-type and mutant MBPs, respectively. On the other hand, the half lives in the second phase were 6.4 ± 0.4h and 3.5 ± 0.1h for the normal and mutant MBPs, respectively, indicating that the turnover of the mutant MBP is twice as fast as that of the normal MBP. The intercept on the y-axis obtained by extrapolating the second slope to time zero gave 36.6% and 24.6% of the injected radioactivity for the normal and mutant MBPs, respectively. These figures suggested that 63.4% of the normal MBP, which was newly secreted into the circulation, was moved almost instantly into the extravascular spaces, 36.6% remaining in the circulation, whereas 75.4% of the mutant MBP, i.e., about 10% more than in the case of the normal MBP, moved into the extravascular space. The differential distribution of the normal and mutant MBPs was in good agreement with the difference in the molecular sizes of these two proteins; the normal MBP is more than two times larger than the mutant MBP. Consequently, the diffusion of the normal MBP into the extravascular space should be more restricted. Thus, in the equilibrium state, the mutant MBP was turned over twice as rapidly as the normal MBP,

TABLE 1

Half-Lives of the ^{125}I-Normal and Mutant MBPs

	Half-life ($t_{1/2}$)	
MBP	First phase (min)	Second phase (h)
Wild type	1.8 ± 0.1	6.4 ± 0.4
Mutant	2.0 ± 0.2	3.5 ± 0.1

Note. The half-lives were determined by fitting the data points to an exponential curve, $y = Ae^{-\lambda_1 t} + Be^{-\lambda_2 t}$, and by applying λ_1 and λ_2 to the equation, $t_{1/2} = \ln 2/\lambda$. The results are expressed as means ± S.D.

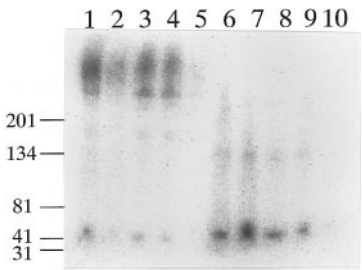

FIG. 2. SDS–PAGE of the blood samples under non-reducing conditions. Lanes 1-5 and 6-10 represent the normal and mutant MBPs, respectively. Lanes 1 and 6, 0.5h; lanes 2 and 7, 1h; lanes 3 and 8, 3h; lanes 4 and 9, 5h; and lanes 5 and 10, 24h after injection. The molecular weights, in kDa, of the standard proteins are indicated on the left.

corresponding to the low serum concentration of mutant MBP in cases who have an opsonic defect.

Chemical Stability of the Multimeric Structures of MBPs in Plasma and the Tissue Distribution

In the next experiment, to determine whether or not the injected MBPs retain their multimeric structures in the circulation, blood samples collected at the time points described above were analyzed by SDS–PAGE. As shown in Fig. 2, the apparent molecular mass corresponding to the major band of normal MBP was over 200 kDa and that of the mutant MBP was about 66 kDa throughout the experiment. Thus, the oligomeric structures of the injected MBPs did not seem to change in the plasma. Similar to the results in Fig. 1, the MBP bands were visible up to 5h, but had completely disappeared at the time point of 24h. The high chemical stability of the oligomeric structures of the MBPs in the plasma was also confirmed by *in vitro* incubation of the MBPs with mouse plasma. In this experiment, the multimer structures of both MBPs maintained for 24h (data not shown). These results indicated that the MBPs were removed the circulation into tissues such as the liver or kidneys without being degraded in the plasma.

To determine the accumulation of MBPs in tissues, the radioactivity in various tissues at 24h after injection was estimated. Every tissue, such as the thymus, liver, spleen, kidneys and heart, was found to contain less than 2% of the total radioactivity. About 40% of the radioactivity appeared in the urine and feces. These results suggested that the MBPs which disappeared from the bloodstream were not localized in any specific tissue, but were metabolized in the liver, kidneys or intestine to be excreted.

DISCUSSION

In this study we examined the plasma clearance of the normal and mutant MBPs to determine the reason for the low serum concentration of the mutant MBP which causes an opsonic defect. The results indicted that the half life of the normal MBP in plasma was about twice as long as that of the mutant MBP. It is possible that plasma proteases might degrade the mutant MBP much more easily than the normal MBP, since the mutant MBP does not form higher oligomers [22]. However, analysis of the oligomeric structures of the MBPs by SDS–PAGE indicated that both the normal and mutant MBPs were chemically stable in plasma. It was reported by Sumiya *et al.* [14] that there is a great difference in the mean serum concentration of MBP between normal homozygous (168μg/l) and mutant homozygous (1.9μg/l) cases. The results obtained in this study could not completely explain such a low serum concentration of the mutant MBP. If hepatic cells, which are responsible for the biosynthesis of serum MBP, secrete the mutant MBP at much a lower rate than the normal MBP, the difference in the serum levels of the two MBPs would be much bigger. However, this does not seem to be the case, since our previous transfection studies indicated that the levels of protein synthesis and secretion of the mutant MBP are almost the same as those of the normal MBP in COS-1 cells [18], and in hepatoma cells [19]. Therefore, there must be some other unknown mechanisms that reduces the serum level of the mutant MBP to such a low level.

The half life of human MBP in human plasma was estimated to be 5-7 days [23], which is much longer than the half life in mouse plasma found in this experiment. However, it is well accepted that, for any plasma protein, the smaller the size of the animal, the more rapid rate of turnover is. For example, the half life of a typical plasma protein, albumin, is known to be 10-18 days in man, but 0.7-1.2 days in mouse [24]. Therefore, it was reasonable to assume a similar difference in the ratio of the half lives of the normal and mutant MBPs in man.

In conclusion, we demonstrated that the normal MBP in plasma had a half life of about 6 hours but that, in contrast, the half life of the mutant MBP was almost half as long. In addition, the movement into the extravascular space was significantly higher for the mutant MBP. These metabolic properties are in part responsible for the low serum concentration of mutant MBP.

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