Biochemical and Biophysical Research Communications 458 (2015) 77–81

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Limited effect of recombinant human mannose-binding lectin on the infection of novel influenza A (H7N9) virus *in vitro*

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ARTICLE INFO

Article history:
Received 23 December 2014
Available online 26 January 2015

Keywords:
Mannose-binding lectin
Influenza A virus
Innate immunity
H7N9

ABSTRACT

Mannose-binding lectin (MBL), a pattern-recognition molecule in serum, recognizes specific hexose sugars rich in mannose and N-acetylglucosamine on bacterium, yeasts, viruses as well as apoptotic cells. It has been well-identified that MBL has antiviral effects via binding to seasonal influenza H1 and H3 subtype viruses. Influenza A (H7N9) virus, a novel reassortant virus to human population, possesses the surface hemagglutinin (HA) and neuraminidase (NA) genes from duck and wild-bird influenza viruses and internal genes from poultry H9N2 viruses. As of Dec 7th, 2014, a total of 467 human infections and 183 fatal cases have been identified. Here, recombinant human (rh) MBL was tested for its binding and effects on hemagglutination inhibition (HI) and NA activity inhibition (NAI) of avian H7N9, H9N2 and human H3N2 viruses. We discovered that rhMBL exhibited a strong binding to H7N9 virus as human H3N2 did at high virus titers. However, it performed a significantly weaker HI activity effect on H7N9 comparing to those of H3N2 and H9N2, even at a much higher concentration (3.67 ± 0.33 vs. 0.026 ± 0.001 and 0.083 ± 0.02 μg/mL, respectively). Similarly, minor NAI effect of rhMBL, even at up to 10 μg/mL, was found on H7N9 virus while it displayed significant effects on both H3N2 and H9N2 at a lowest concentration of 0.0807 ± 0.009 and 0.0625 μg/mL, respectively. The HI and NAI effects of rhMBL were calcium-dependent and mediated by lectin domain. Our findings suggest that MBL, the host innate molecule, has differential interference effects with human and avian influenza virus and limited antiviral effect against H7N9 virus.

1. Introduction

Host innate immunity plays a critical role in the early phase of infection. This first-line defense against pathogens is mediated by a variety of pattern-recognition molecules including collectins, toll-like receptors and ficolins as well as inflammatory cytokines and type I interferon or macrophages and natural killer cells. Mannose-binding lectin (MBL) is one of collectins circulating in the serum and synthesized by liver. It consists of collagenous domains and carbohydrate recognition domains (CRD). The CRDs recognize sugars including D-mannose, N-acetylmannosamine, N-acetylglucosamine and L-fucose on the surface of many pathogens in a calcium-dependent manner [1]. Previous studies showed that MBL can bind to a range of clinically relevant microorganisms such as *Staphylococcus aureus*, *Candida Albicans* [2], HIV, SARS-CoV, Ebola virus, HSV, influenza virus [3–6]. The binding of MBL to microorganisms is presumed to induce MBL conformational changes that allow the molecule to initiate viral neutralization or kill virus via opsonization or complement activation [7].

Influenza A virus, a segmented single-stranded negative-sense RNA virus, belongs to *orthomyxoviridae* and is subtyped according to the antigenic properties of their envelope glycoproteins, HA and NA. Currently, 16 HA subtypes and 9 NA subtypes circulate in birds. Among them, only seasonal H1N1 and H3N2 viruses circulate in human population [8]. Occasionally, some subtypes of avian

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http://dx.doi.org/10.1016/j.bbrc.2015.01.070

influenza A virus can jump into human and cause diseases with a range of clinical symptoms and outcomes, such as conjunctivitis, mild upper respiratory tract disease, as well as severe pneumonia and death [9–12]. Viral HA and NA assist virus binding, entry and releasing during infection cycle. Their potential N-linked glycosylation sites (NGS) can be glycosylated, which might allow their binding to host MBL. It has been found that the glycan at residue 165 in H3N2 HA was of high-mannose and MBL neutralized viral infectivity via it. Many lines of evidences have shown that the MBL plays an important role in fighting against seasonal flu [13–15]. However, little is known about the interactions between avian influenza virus and the innate molecules. Avian influenza H7N9 virus is novel to human population [16,17], which contains the surface HA and NA genes from duck and wild-bird influenza viruses and internal genes from poultry H9N2 viruses. Unlike other H7 viruses that generally cause mild symptoms such as conjunctivitis or influenza-like illness (except one fatal case infected with H7N7 in Netherlands in 2003), H7N9 virus usually results in severe pneumonia or respiratory failure in human. Here, we examined the interactions of MBL with avian influenza virus H7N9, H9N2 and human virus H3N2. Furthermore, we studied the molecule mechanisms for them by structure modeling.

2. Materials and methods

2.1. Virus

The vaccine strain A/Anhui/1/2013(H7N9) (NIBRG-268) was obtained from National Institute for Biological Standards and Control (UK), namely H7N9Vac. The virus bears the HA and NA of A/Anhui/1/2013(H7N9) and internal genes of A/Puerto Rico/8/1934 (PR8, H1N1); A/Brisbane/10/2007(H3N2) was named as H3N2WT in the study; H9N2 virus, a reassortant bearing the HA, NA from A/Hongkong/33982/2009(H9N2) and internal genes of PR8, was named as H9N2RG. The reassortant $H7N1_{AH1\ HA+PR8\ NA}$ was with HA of A/Anhui/1/2013 and seven genes of PR8, which is rescued as previously reported [18]. H7N9Vac, H3N2WT and $H7N1_{AH1\ HA+PR8\ NA}$ were propagated in 9–11-day-old embryonated chicken eggs, H9N2RG was grown in Madin-Darby canine kidney (MDCK) cells (ATCC, USA) with Modified Eagle's Medium (invitrogen, USA)containing 2 μg/mL N-tosyl-L-phenylalanine chloromethyl ketone (TPCK)–treated trypsin (Sigma, USA). Virus stocks were purified by adsorption to and elution from turkey red blood cells (TRBCs) and stored at −80°Cuntil use [19]. Virus titer was determined by titration in MDCK cells and the tissue culture infectious dose affecting 50% of the cultures ($TCID_{50}$) is calculated by the Reed–Muench formula [20].

2.2. Detection of MBL binding to influenza virus

Recombinant human MBL (rhMBL) was purchased from Sino Biological Inc (Beijing, China). Ninety-six-well plates were coated with 2×10^5 $TCID_{50}$ influenza virus at a volume of 100 μl/well for overnight at 4 °C, then were blocked for 1 h with 1% Bovine Serum Albumin (BSA, Roche, Switzerland) at 37 °C. Different concentrations of rhMBL (0, 1, 3, 5, 7 μg/mL) were added and incubated for 1 h at 37 °C. The virus-dose dependent binding assay was conducted as that wells were precoated with 2×10^2, 2×10^3, 2×10^4 and 2×10^5 $TCID_{50}$ influenza viruses per well. Then 3 μg/mL rhMBL was added and incubated for 1 h at 37 °C. The binding was detected by the biotinylated human MBL pAb (0.2 μg/mL) (R&D, USA), followed by streptavidin-horseradish peroxidase (HRP) (1:200) (R&D, USA) and tetramethylbenzidine substrate solution (BD, USA), the reaction was stopped by 2 M H_2SO_4 and the Optical Density (OD) at 450 nm was measured by ELISA reader (Perkin-Elmer, USA). The wells coated with 10 μg/mL mannan from *Saccharomyces cerevisiae* (Sigma, USA) or coating buffer (Kirkegaard & Perry Laboratories, USA) were used as positive control and negative control respectively. The test was performed in duplicates and in three independent experiments, absorbance from negative control was subtracted and results were normalized to positive control, data was expressed as a relative absorbance value using mean ± SEM (%).

2.3. Hemagglutination inhibition (HI) assays

HI assay was performed in V-bottom 96-well plates as previously described [20]. Briefly, 25 μL influenza virus (4HAU) was mixed with 25 μL rhMBL of different concentrations diluted in Hank's Balanced Salt Solution (HBSS) containing 1.26 mM Ca^{2+} for 1 h at 37 °C, then 50 μL 1% TRBC was added to the mixture and incubate at room temperature for 30 min. For HI reverse assay: rhMBL was diluted in HBSS containing 10 mM EDTA or 10 mg/mL mannan, then incubated with 4 HAU of influenza virus. The results were expressed as the minimum inhibitory concentration (MIC) of rhMBL that exhibited HI effect.

2.4. Neuraminidase activity inhibition (NAI) assays

Influenza virus NA activity was measured by ELISA in which peanut agglutinin conjugated with HRP was used to detect β-D-galactose-N-acetylglucosamine exposed after removal of sialic acid from fetuin [21]. Appropriate amounts of virus in Dulbecco's 1X PBS with $CaCl_2$ and $MgCl_2$ (Life Technologies, USA) were used to perform the NAI assays. Different concentrations of rhMBL were diluted in HBSS containing Ca^{2+} and mixed with influenza virus in a total volume of 100 μL and preincubated at 37 °C for 1 h, and then transferred to wells precoated with fetuin (Sigma, USA) and incubated at 37 °C for 4 h, After washing, 100 μL of HRP-labeled peanut lectin (3 μg/mL) was added and after 1 h at room temperature, the wells were washed and o-phenylenediamine dihydrochloride in citrate buffer was added, reaction was stopped by 2 M H_2SO_4, and the OD at 492 nm was measured. The wells only with virus were used as the positive control, the OD of wells with HBSS used as a negative control was subtracted. Results were expressed as relative NA activity (%) calculated as the OD of the tested wells with virus and rhMBL divided by the OD of the wells with only virus.

Table 1
Distances from the potential N-linked glycosylation sites (NGS) to receptor binding domain or NA activity region (Å).

NGS	Protein	Distances from the NGS to functional region (Å)		
		H3N2WT	H9N2RG	H7N9Vac
63	HA	27.3	–	–
95	HA	–	23.2	–
122	HA	26.9	–	–
128	HA	–	17.6	–
126	HA	24.3	–	–
133	HA	16	–	–
144	HA	18.9	–	–
165	HA	24.1	–	–
198	HA	–	16.2	–
240	HA	–	–	37.3
246	HA	22.2	–	–
86	NA	29.4	29.4	30
146	NA	20.8	21.2	28.9
200	NA	18.1	18.4	18.4
234	NA	29.4	29.5	–
329	NA	26.5	–	–
402	NA	22.4	22.7	–

–: Denotes the absence of NGS in the corresponding virus.