Macromolecular Activators of Phagocytosis from Platelets (MAPPs) and Their Activator Peptide, HATKTAK

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Abstract

Small and large macromolecular activators of phagocytosis from platelets (S-MAPP and L-MAPP, respectively) are released from activated platelets and enhance Fcγ receptor-mediated phagocytosis by neutrophils. Platelets stored in the form of platelet concentrates lose their MAPP-producing activity within 48 h, but recover it when incubated in dialyzed plasma against PBS and supplemented with Ca^{++}. Experiments using expired platelet concentrates revealed that dimer and tetramer holo-transferrin (TF2 and TF4, respectively) function as precursors of MAPPs, while those using platelet lysates showed that MAPPs are activated by the peptide, HATKTAK, which is cleaved from apolipoprotein CIII on high-density lipoproteins (HDL). Experiments on the production of artificial MAPPs by incubating TF2 and TF4 rich solutions with HATKTAK suggested that the binding of HATKTAK and transferrin occurs between the C-terminal K of HATKTAK and sialic acid at the end of the sugar residue of transferrin. Native MAPPs function in the presence of plasma, whereas artificial MAPPs do not. Differences between native and artificial MAPPs may result in artificial MAPPs being more vulnerable to damage by plasma. An immunohistochemical study using an antibody against HATKTAK revealed the wide spread distribution of positive reactions in the human body; therefore, HATKTAK appears to play some roles in biological reactions other than lipid metabolism. Since the amino acid sequence HATKTAK has only been found in primates, the reaction of HATKTAK may be specific to these animals.

Keywords: Platelets; Neutrophils; Phagocytosis; Apolipoprotein CIII; Artificial MAPPs; Native MAPPs

Introduction

Platelets have been suggested to affect leukocytic functions via various mechanisms [1-11]. We previously reported that phagocytosis by neutrophils and monocytes was enhanced by platelets or the products released by them [3,4]. Small and large macromolecular activators of phagocytosis from platelets (S-MAPP and L-MAPP, respectively), which were discovered in the products released from human platelets, have been shown to enhance the phagocytosis of neutrophils via Fcγ receptors [12]. We herein investigated the characterization of MAPPs as well as their activation mechanisms. We reviewed studies on the activation of MAPPs by the apolipoprotein CIII (apo CIII) - derived peptide, HATKTAK in platelets [13] in addition to perspectives on these studies.

Preparation of Platelet Release Products (PRPr) and Phagocytosis Assays by Microplate and Flow Cytometric Methods

Platelets and neutrophils were separated from the fresh heparinized venous blood of healthy adults by centrifugation on two layers of mixtures of Percoll and Ficoll-Paque (Pharmacia, Uppsala, Sweden) [4] or Mono-Poly resolving medium (ICN Bio-chemicals Japan, Tokyo, Japan) [14].

In order to prepare PRPr, washed platelets were stimulated by incubation at 37°C in the presence of Ca^{++} in a glass tube followed by centrifugation [4], or by stimulation with 0.1 unit/mL of thrombin [12].

In order to assess phagocytic activity, neutrophils were attached to Terasaki microplates (Nunk, Roskilde, Denmark) (2,000 neutrophils/well), incubated with PRPr or other stimulants, and then incubated with Sheep Red Blood Cells (SRBCs) sensitized with anti-SRBC rabbit IgG. After the removal of SRBCs not ingested and fixation, ingested SRBCs were counted under a light microscope.

In most experiments, the phagocytosis assay was performed using this microplate method.

In some experiments, a flow cytometric study of whole blood using FITC-labeled *E. coli* (Sigma, St Louis, MO, USA) as a foreign material was performed to assess neutrophilic phagocytosis [6].

Effects of PRPr and MAPPs on Neutrophilic Phagocytosis

The number of SRBCs ingested by neutrophils treated with PRPr was more than two-fold that by control neutrophils [12].

In the gel filtration of PRPr on Superdex 200 (GE Healthcare UK Ltd, Buckinghamshire, UK), two peaks each of phagocytosis enhancement (S-MAPP, 150 kDa; L-MAPP, 300 kDa) (native MAPPs) and suppression (S-MSPP, 60 kDa to 100 kDa; L-MSPP, 400 kDa to 500 kDa) [15-17] were observed.

Similar to phagocytosis experiments using Terasaki microplates, PRPr and native MAPPs showed the activation of phagocytosis in the flow cytometric analysis [6].

In Vitro Production of MAPPs

Expired platelet concentrates kindly supplied by the Red Cross Blood Centers of Wakayama City and Takamatsu city were used to investigate MAPPs and MSPPs. When this study started, platelet concentrates were expired three hours after blood drawing and supplied to us as soon as possible.

The activation of phagocytosis was induced by PRPr prepared from platelets obtained from platelet concentrates stored for 3 h. The cut-off time for the storage of platelet concentrates was extended to 48 h, a time at which platelets lose their MAPP-releasing function. In contrast, plasma from platelet concentrates obtained their MAPP function [17]. We purified MAPPs biochemically from this plasma, but failed to purify MSPPs [15].

Since we prepared monoclonal antibodies against MAPPs using biochemically purified S-MAPP and L-MAPP as antigens, we were able to obtain a sufficient amount of purified MAPPs by affinity chromatography of plasma from platelet concentrates stored for 48 h [18]. Peptides obtained by the digestion of affinity-purified S-MAPP with lysyl endopeptidase had an identical amino acid sequence to transferrin (V1-V8, K434-K455, and K545-K552) [14].

The expiration time of platelet concentrates was extended to 72 h. We obtained platelet concentrates one day later when MAPP activity had disappeared from plasma. Therefore, we hypothesized that stored platelets that have lost their MAPP-releasing function may recover it when the anticoagulant is removed and Ca^{++} is supplied [19]. In experiments in which platelets and plasma were prepared from platelet concentrates expired at 72 h and platelets were incubated with plasma dialyzed against PBS, supplemented with Ca^{++}, and stimulated by thrombin, the results obtained showed that stored platelets recovered their ability to produce MAPPs and precursors of MAPPs were present in the fractions of gel filtration of plasma corresponding to S-MAPP and L-MAPP (native precursors) [19].

Since the molecular sizes of MAPPs are nearly two- and four-fold that of transferrin, we hypothesized that the precursors of MAPPs may be dimers and tetramers of holo-transferrin.

Therefore, we treated holo-transferrin (Sigma) with glutaraldehyde to produce multimers of transferrin. Dimer and tetramer holo-transferrin (TF2 and TF4, respectively) were found to function as artificial precursors of S-MAPP and L-MAPP. TF2 and TF4-rich materials were obtained in the same fractions as S-MAPP and L-MAPP in Superdex 200 gel filtration. In a study of affinity chromatography, all native MAPPs and precursors were adsorbed to the anti-transferrin antibody [14].

Since stored platelets failed to produce MAPPs if the peptide, DYYPEEDTEGD (amino acid sequence of the high-affinity thrombin receptor, GPIbα) or monoclonal antibody PM 6/40 against the high-affinity thrombin receptor (Serotec, England) was present, thrombin bound to the high-affinity thrombin receptor GPIbα appeared to induce the production of MAPPs [20].

We then attempted to produce MAPPs using platelet lysates. Platelets from platelet concentrates expired at 72 h were freeze-thawed and the supernatant (platelet lysate) was incubated with native or artificial precursors of MAPPs and thrombin, MAPP function was obtained. The experiment on MAPP production using the platelet lysate with antthrombin III suggested that thrombin reacts with platelet lysate-derived substances [21].

Gel-filtration studies revealed that the macromolecular fraction of the platelet lysate exhibited MAPP-producing activity (High-Molecular-Weight (HMW) activator), and that a platelet lysate-derived substance with a molecular size of approximately 800 Da obtained by the thrombin treatment directly exhibited MAPP forming activity with the native and artificial precursors (Low-Molecular-Weight (LMW) activator).

Ultracentrifugation of the platelet lysate revealed that HMW activator activity was separated in the fraction with a density of 1.063 to 1.21 (High Density Lipoprotein (HDL) – rich fraction). Since the anti-apo A1 antibody negated the action of the HDL fraction of the platelet lysate to produce a LMW activator by thrombin, the HMW activator appears to belong to HDL. The HDL fraction of the platelet lysate was separated into lipids and proteins by extraction using ether-alcohol. The protein fraction revealed MAPP-forming functions. The MAPP-producing activity of the protein fraction was adsorbed to an anti-Apo CIII column [22].

Characterization of the LMW Activator

In order to characterize the LMW activator, commercially available apo CIII (Chemicon International Inc, Temecula, CA, USA) was treated with thrombin, and cation exchange chromatography using the MONO S column (GE Healthcare UK Ltd) and gel filtration using the Superdex peptide column were performed. In addition, the production of the LMW activator using trypsin (Sigma) instead of thrombin was tested.

These experiments suggested that the LMW activator is a cationic peptide included in apo CIII with a molecular size of approximately 800 Da, and that the N-terminal of the LMW activator is an amino acid after the K or R of apo CIII and the C-terminal is K or R because trypsin produced the LMW activator from apo CIII with the same characteristics as thrombin. The only peptide that satisfies these requirements is HATKTAK (H18-K24). The incubation of HATKTAK with the native and artificial precursors of MAPPs resulted in the production of MAPPs.

Binding of HATKTAK with Transferrin

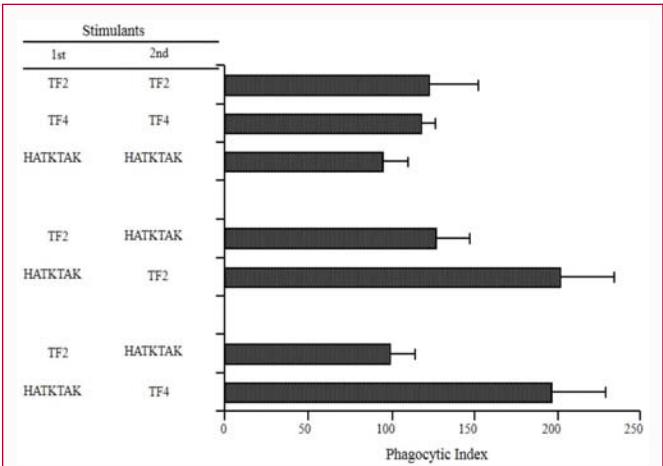In order to identify the binding site of HATKTAK with transferrin,

Figure 1: Effects of lysine (K), histidine (H), and sialic acid (SA) on the production of artificial MAPPs. [A] Effects of K and H: artificial MAPPs were produced by incubating 2.5 ml of TF2-rich or TF4-rich materials (optical density at 280 nm, 0.01) with 62.5 ng HATKTAK in the presence of 1M K or H at 37°C for 60 min. [B] Effects of SA: 1.25 ml of 25 μg/ml SA was incubated with 1.25 ml of 50 ng/ml HATKTAK at 37°C for 60 min and 1.25 ml of TF2 or TF4 (optical density at 280 nm, 0.02) was then added and incubated at 37°C for 60 min. The artificial MAPP samples shown in [A] and [B] were applied on a PD10 column (GE Healthcare UK Ltd.) to obtain a high-molecular-weight fraction (MAPP-rich fraction) before use.

Figure 2: Separate and serial stimulations of neutrophils with precursors of MAPPs and HATKTAK. Neutrophils seeded and adhered on a Terasaki microplate were incubated with TF2 or TF4 (optical density at 280 nm, 0.01) and 10 μl of 25 ng/ml HATKTAK separately and serially.

HATKTAK and TF2 or TF4 were incubated with a high concentration of lysine (K), histidine (H), or sialic acid (SA). MAPP activity was observed in TF2 or TF4 incubated with HATKTAK in the presence of H (Figure 1A), but not in those in the presence of K or SA (Figure 1A and 1B). These results suggest that HATKTAK binds SA at the end of the sugar residue of transferrin via the C-terminal K (lysine). In a phagocytosis experiment, neutrophils were initially incubated with HATKTAK and then with artificial precursors of MAPP, and an increase in phagocytosis were observed. If the stimulation was performed in the reverse order, the activation of phagocytosis was not observed (Figure 2).

Differences between Native MAPPs and Artificially Produced MAPPs

The effects of native and artificially produced MAPPs on neutrophilic phagocytosis were compared. The microplate and flow cytometric methods were both used. In the study using the microplate method, in which plasma was not involved when neutrophils were stimulated, all PRPr, native S-MAPP and L-MAPP, and artificially
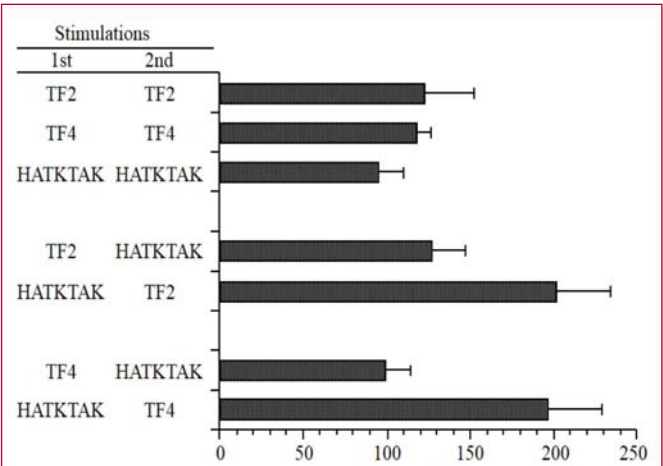
produced S-MAPP and L-MAPP enhanced phagocytosis by neutrophils. However, in the flow cytometric study in which whole blood was used, PRPr and native MAPPs enhanced phagocytosis by neutrophils, whereas artificially produced MAPPs did not. When a 20% volume of plasma was added to artificially produced MAPPs in the microplate method, the action of MAPPs disappeared (Table 1).

Discussion

We found that activated platelets produce and release MAPPs along with their activator peptide, HATKTAK [13], which is involved in Apo CIII (H18-K24).

Apo CIII moves from one lipoprotein to another and is not equally distributed between lipoproteins. In normolipidemic individuals, most apo CIII is in HDL [23]. In patients with triglyceridemia, more Apo CIII is in triglyceride-rich lipoproteins [24]. To the best of our knowledge, the digestion of apo CIII has not yet been examined in detail, except in some *in vitro* studies [25]. Our results indicated that peptides cleaved from apo CIII function in biological reactions other than lipid metabolism. The binding of HATKTAK to transferrin in the precursors of MAPPs was inhibited by K, but not by H, suggesting that HATKTAK reacts with transferrin via C-terminal K and with neutrophils via H at the N terminus. SA inhibited the production

Table 1: Comparison of the effects of PRPr, native MAPPs and artificial MAPPs on neutrophilic phagocytic activity assessed by the microplate method and flow cytometric method.

Method	PRPr	S-MAPP From PRPr	L-MAPP From PRPr	Produced S-MAPP	Produced L-MAPP
Microplate (PBS-BSA)	217 ± 15	206 ± 14	192 ± 21	203 ± 23	203 ± 32
(examined cases)*	(9)	(6)	(6)	(9)	(9)
(positive cases)**	(9)	(6)	(6)	(9)	(9)
Microplate (20% plasma)	290 ± 94	184 ± 32	201 ± 17	89 ± 16	82 ± 25
(examined cases)*	(3)	(3)	(3)	(3)	(3)
(positive cases)**	(3)	(3)	(3)	(0)	(0)
Flow cytometry	204 ± 19	239 ± 43	241 ± 22	113 ± *56*	138 ± 86
(examined cases)*	(4)	(4)	(4)	(7)	(8)
(positive cases)**	(4)	(4)	(4)	(2)	(2)

*: number of examined cases; **: number of positive cases showing a phagocytic index >150.

of artificial MAPPs, which indicated that HATKTAK binds with transferrin through the SA of the sugar residue. Neutrophilic phagocytosis was accelerated by separate, but serial stimulations by HATKTAK and artificial precursors, suggesting that the location of HATKTAK binding on neutrophils is in the transferrin receptor or close to it. Evidence for native MAPPs reacting with neutrophils through transferrin receptors is provided by some antibodies against transferrin receptors inhibiting the phagocytosis-activating function of native MAPPs [10]. Artificially produced MAPPs do not function in the plasma, whereas native MAPPs do. This fact suggests that artificial MAPPs are more vulnerable to damage by plasma components. Since only primates have HATKTAK in apo CIII (the National Center for Biotechnology Information database) [26], investigating HATKTAK and MAPPs using typical experimental animals may be difficult. We raised a rabbit antibody against HATKTAK and showed a positive reaction in, for example, platelets in blood coagula and thrombi [27], the cytoplasm and fibers of some nerve cells, and the glomerular filtrates and proximal tubules of kidneys [28]. In addition to immunohistochemistry using an anti-HATKTAK antibody to investigate the distribution of HATKTAK, the measurement of HATKTAK in liquid test samples such as urine, plasma, serum, and bile is possible and necessary for the further investigation of HATKTAK in order to clarify its biological and pathological significance.

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