The C4b-binding protein - protein S complex inhibits the phagocytosis of apoptotic cells

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Abbreviations: C4BP; C4b-binding protein, CCP; complement control protein, HBSS; Hank’s balanced salt solution, MBL; mannose binding lectin, PE; phycoerythrin PS; protein S, GM-CSF; granulocyte macrophage-colony stimulating factor
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Abstract

The phagocytosis of apoptotic cells is a complex process involving numerous interactions between the target cell and the macrophage. We have examined a role of the major soluble inhibitor of the classical and lectin complement pathways, C4b-binding protein (C4BP), in the clearance of apoptotic cells. The major form of C4BP present in blood is composed of seven α-chains and one β-chain, which binds protein S (PS). Approximately 70% of all PS in human plasma is trapped in such complex and is able to localise C4BP to the surface of apoptotic cells due to the high affinity to phosphatidylinerine. Free PS has recently been shown to enhance phagocytosis of apoptotic cells by macrophages. We observed a stimulatory effect of free PS on the engulfment of apoptotic cells (BL-41 and Jurkat) by primary human macrophages or THP-1 cells and a decrease of activity in serum depleted of PS in agreement with previous results. However, we also show that the process is strongly inhibited in the presence of the C4BP-PS complex. Addition of the C4BP-PS complex to serum deficient in both molecules abolished the enhancing effect of serum on phagocytosis. The effect of both free PS and the C4BP-PS complex could be inhibited with monoclonal antibody directed against the Gla-domain of PS. Although the presence of the C4BP-PS complex on apoptotic cells may lead to decreased phagocytosis it may still be beneficial to the host since it could prevent secondary necrosis because it inhibits further complement attack.
Introduction

The process of apoptosis marks superfluous cells with signals that direct recognition, engulfment and degradation by phagocytes in a very complex process involving a number of molecules present on the apoptotic cell and the macrophage as well as molecules recruited from serum. The macrophages silently engulf the apoptotic cells and prevent leakage of contents from the dying cells that would otherwise lead to inflammation. Cells undergoing apoptosis expose many marker molecules that are recognised by the macrophages such as phosphatidylserine (normally restricted to inner-leaflet of cell membrane), changes in glycosylation, pathogen-like apoptotic-cell associated molecular patterns (ACAMPs) that are recognized by e.g. C1q (1) and mannose-binding lectin (MBL) (2) leading to deposition of complement factors C3b and iC3b (3), thrombospondin binding sites, oxidised LDL-like sites and intercellular adhesion molecule 3 (ICAM-3). Receptors on the phagocyte include scavenger receptors, CD14 (4), β2 integrins (3), complement receptor 1 (CR1), β3/β1/β5 integrins, and still poorly characterized receptors for C1q and phosphatidylinerine. Furthermore, a number of soluble molecules bridging the apoptotic target and phagocytes are known such as thrombospondin, C1q, MBL, C3b/iC3b, β2-glycoprotein I and the recently identified vitamin K-dependent anticoagulant protein S (PS) (5). PS is a 75 kDa blood glycoprotein that acts as a non-enzymatic cofactor to activated protein C in the degradation of activated coagulation factors Va and VIIIa (6), (7). PS is composed of a N-terminal Gla-domain followed by a thrombin sensitive region, four epidermal growth factor (EGF)-like domains and a sex hormone binding globulin (SHBG)-like region (schematically shown in Figure 5). The SHBG-like region consists of two laminin G-like domains (LG domains) both of which are involved in a high affinity binding to the β-chain of an abundant complement inhibitor C4b-binding protein (C4BP) (8). The Gla-
domain, binds with high affinity (K_D 100 nM) to negatively charged phosphatidylserine, which is exposed in the early stages of apoptosis. We have shown previously that PS localises C4BP to the surface of apoptotic cells (9). The most common form of C4BP consists of seven identical α-chains and one β-chain (schematically shown in Figure 5), consisting of 8 and 3 complement control protein (CCP) domains, respectively, but forms with six α-chains and one β-chain (α6β1) and seven α-chains only (α7β0) are also present (10). C4BP inhibits the classical and lectin pathway of complement by binding activated complement factor C4b and thereby inhibiting the formation of the crucial complement enzymatic complex C3-convertase and accelerating its natural decay. C4BP also acts as a cofactor to serine protease factor I in the degradation and inactivation of C4b (11) and C3b (12). C4BP is an acute phase protein (13) but only the α7β0 form increases in expression during inflammation (14), meaning that PS (the β-chain ligand) will not be depleted from plasma, which could otherwise lead to severe thrombotic disorders.

It has been shown that purified PS was equivalent to serum in its ability to stimulate macrophage phagocytosis of apoptotic lymphoma cells (5). Immunodepletion of PS with polyclonal antibody eliminated the phagocytic activity of serum. However, this previous study did not address a role of the C4BP-PS complex. Only 30% of all PS is free in plasma (7.5 µg/ml), the other 70% is in complex with C4BP. Binding to C4BP heavily modulates the function of PS. PS fully looses its anticoagulant property when bound to C4BP and once in complex it may also loose the possibility to interact with its putative receptor on the phagocyte as PS is known to interact with tyrosine kinase receptor Sky via SHBG domain (15). In the present study we have analysed the role of free PS versus the C4BP-PS complex in the phagocytosis of apoptotic cells. We reproduce the observation that free PS stimulates phagocytosis but strikingly we show that PS in complex with C4BP, by far the most common form of PS in serum, strongly inhibits phagocytic uptake of apoptotic cells. These data may indicate that PS will
target the C4BP-PS complex to the apoptotic cell surface where it functions as a complement regulator in order to prevent secondary necrosis following complement attack.

**Material and methods**

**Cells**

Human BL-41 cells (B cells, DSMZ, Germany) were cultured in RPMI supplemented with 10% heat-inactivated FCS, 3.4 mM L-glutamine, 100 units/ml of penicillin and streptomycin and 50 µM 2-mercaptoethanol. Jurkat cells (T cells, ATCC) were cultured in the same medium without 2-mercaptoethanol.

Human monocytes were prepared from buffy coat by a standard method. Briefly, the fresh buffy coat (Lund University Hospital) was mixed with equal amount of phosphate buffer saline (PBS) and dextran was added to 0.6 % (v/v). The mixture was left in room temperature for 1 hour and then the upper phase was withdrawn and centrifuged for 10 minutes at 1000 rpm. After two washes of the cells in PBS, Ficoll was layered under the cell suspension and the gradient was centrifuged for 35 minutes at 1460 rpm. The white blood cells were transferred to a new tube and centrifuged again at 1460 rpm for 35 minutes. The pellet of white blood cells was suspended in PBS and the cell concentration was determined. The cells were centrifuged (1000 rpm, 10 minutes) and resuspended at 2 \(10^6\) cells/ml. Two million cells were seeded in each well of a 24-well plate in RPMI supplemented with transferrin (Sigma, 25 µg/ml), gentamycin (Invitrogen, 50 µg/ml), L-glutamine (Invitrogen, 2 mM) and GM-CSF (R&D systems, 5 ng/ml). The cells were allowed to differentiate for 7 days before the phagocytosis assay was performed.

Human THP-1 cells (monocytes, ATCC number: TIB-202) were grown in RPMI supplemented with 10% heat-inactivated FCS, 3.4 mM L-glutamine, 100 units/ml of penicillin and streptomycin (growth medium). To differentiate the THP-1 cells
they were grown in 24 well plates in growth medium supplemented with 100 nM phorbol 12-myristate 13-acetate (PMA) for 72 h.

Apoptosis

Human BL-41 cells (0.5 $10^6$ cells/ml) were treated with 200 µg/ml etoposide (Sigma) in growth medium for 3 hours at 37°C. To monitor the amount of apoptotic and necrotic cells the cells were labelled with annexin-V-phycoerythrin (PE, Molecular probes) and Viaprobe (7-AAD, Molecular probes) and analysed by flow cytometry. Around 50% of the cells were positive for annexin-V after treatment of etoposide for 3h, 40% were double negative and 10% were double positive for annexin-V and Viaprobe. Human Jurkat cells (1 $10^6$ cells/ml) were treated with 0.5 µM staurosporine (Sigma) for 3 hours at 37°C and the relative amount of annexin-V and Viaprobe positive cells was in the same range as for the BL-41 cells.

Phagocytosis assay

After induction of apoptosis of the BL-41 cells or Jurkat cells, they were washed two times in PBS and then labelled with 5-(and-6)-carboxyfluorescein diacetate succinimidyl ester (CFSE, 9 ng/ml) for 20 minutes in 37°C at a cell concentration of 110$^6$ cells/ml. Both labelled apoptotic cells and macrophages were washed three times with Hank’s balanced salt solution (HBSS). The labelled apoptotic cells were incubated with the macrophages at a ratio of 2:1 in HBSS, supplemented with 0.75 mM CaCl$_2$ (final concentration 2.5 mM), for 1 h at 37°C. The wells were then washed two times with HBSS, to get rid of most non-phagocytosed apoptotic cells, before the macrophages were labelled with anti-CD11b and anti-CD14, conjugated with PE, at room temperature for 45 minutes. The cells were detached from the plate with 1% lidocaine (Sigma) in FACS buffer (PBS, 30 mM NaN$_3$, 1% BSA) and analysed by flow cytometry.
Proteins and sera

The C4BP-PS complex was purified from human plasma as described before (16). Human recombinant PS was expressed in human embryonic kidney (HEK) 293 cells (ATCC: 1573-CRL) and purified by essentially as described before (17,18). C4BP with β-chain but without PS was prepared by incubation of C4BP-PS in 4 M guanidium chloride, subsequent gel filtration and dialysis. Human serum deficient from the C4BP-PS complex and PS (PS/C4BP deficient) was prepared by passing fresh serum consecutively through two HiTrap columns (Amersham Biosciences), one coupled with MK104, a mouse mAb directed against CCP1 of the α-chain of C4BP, the other with MK21, a mouse mAb directed against the Gla-domain of PS. The flow through was collected and the depleted serum was stored in aliquots at –70°C.

C1q was purified by ion exchange on Biorex 70 followed by gel filtration as described before (19) and the remaining serum was used as serum deficient of C1q.

ELISA for determination of C4BP and PS concentrations

The concentration of C4BP and PS in normal human serum and serum deficient of both proteins was determined by ELISA. Plates were coated with antibody, rabbit PK 9008 (for detection of C4BP) and PK 7909 (for detection of PS) over night at 4°C at 10 µg/ml in 75 mM sodium carbonate pH 9.6. The plates were quenched with 50 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween, pH 7.5 (washing buffer) supplemented with 3% fish gelatin for 1 hour. In the detection of PS, 2 mM of CaCl₂ was used in all steps. After quenching, the plates were washed three times with washing buffer and then the serum was added for 4 hours. After another three washes the MK 104 (for detection of C4BP, 10 µg/ml) and MK 54 (for detection of PS, 10 µg/ml) were added and incubated for 1 hour. Goat anti-mouse antibody, conjugated with horseradish peroxidase (HRP, Dako), was added after
another three washes and incubated for 1 h. After the last three washes the plates were developed using OPD as substrate (Dako) and the absorbance at 490 nm was measured spectrophotometrically.

Haemolytic activity of human serum

Haemolytic assays of normal human serum and sera deficient in C1q, C4BP and PS were performed. Sheep erythrocytes were washed three times with DGVB++ (2.5 mM veronal buffer, pH 7.3, containing 70 mM NaCl, 140 mM glucose, 0.1 % gelatin, 1 mM MgCl₂ and 0.15 mM CaCl₂). The cells were incubated with a complement-fixing antibody (Amboceptor diluted 1:3000, Boehringerverke) at a concentration of 0.5×10⁹ cells/ml for 20 min at 37°C. After two washes with DGVB++ the cells were incubated for 1 h at 37°C with various concentrations of serum diluted in DGVB++. The samples were centrifuged and the amount of lysed erythrocytes was determined by spectrophotometric measurement of the amount of released hemoglobin at 405 nm.

Preparation of Fab fragments of monoclonal antibodies

Fab fragments of mouse mAb MK 21, directed against the Gla-domain, MK 54, directed against EGF-domain 1 and MK 34, directed against the SHBG-like domain of PS and MK 104, directed against CCP1 of the α-chain of C4BP, were prepared by papain digestion using the ImmunoPure Fab preparation Kit (Pierce) according to the manufactures instructions.
Results and discussion

The disposal of unwanted cells via apoptosis is a tightly controlled process in order to prevent inflammation and autoimmunity. A large number of factors are involved both on the apoptotic cell and the phagocyte as well as bridging molecules derived from serum. Among these proteins that bind to apoptotic cells there are several complement components (20). Binding of the early components of the classical pathway is thought to be very important in the clearance since deficiencies in these components although rare are the strongest risk factors for the development of systemic lupus erythematosus (SLE). SLE is characterized by the presence of autoantibodies against cell components present on apoptotic cells that normally are not exposed for prolonged time to the immune system (21). The potential role of C1q in clearance of apoptotic cells was supported by the observation that C1q and MBL stimulate uptake of apoptotic cells by macrophages (22). In addition, the collagenous tail of C1q and MBL binds to calreticulin on the macrophage, which will then give a signal via surface molecule CD91 for ingestion of the apoptotic cell by macropinocytosis (22). Furthermore, both C1q-deficient humans and C1q-deficient mice develop SLE-like disease (23). CR1 and CR3 are also involved as they recognize C3b/iC3b deposited on the surface of apoptotic cells. The complexity of phagocytosis of apoptotic cells may reflect redundancy necessary for health but it may also be simplified in particular tissues and circumstances in vivo.

Recently, PS was proposed to act as the major phagocytic factor in the uptake of apoptotic cells present in serum (5). This study, however, did not consider that 70% of all expressed PS in man is irreversibly bound to C4BP and this study did not address the effect of the complex. Activity of PS was first identified in bovine serum that does not contain C4BP-PS complex. The reason why these two proteins form a high affinity complex in human blood has long been an unsolved question. PS bound to C4BP cannot exert its anticoagulant function in the protein C pathway of inhibition of coagulation.
Since the SHBG-like domain of PS binds to the β-chain of C4BP this only leaves the Gla-domain free to exert any additional function. Gla-domains are present in a number of proteins, mostly coagulation factors and can bind to negatively charged phospholipids such as phosphatidylserine in the presence of calcium ions (24,25). Previously we showed that the Gla-domain of PS localises both free PS and C4BP-PS to the surface of apoptotic cells (9,26).

In the present study we have investigate the role of free PS versus the C4BP-PS complex in the phagocytosis of apoptotic cells. The presented results were obtained in a phagocytosis assay in which BL-41 cells were made apoptotic with etoposide (50% of the whole population) and presented to primary human macrophages for engulfment. Furthermore, similar results were obtained with using both Jurkat T cells and BL-41 cells and both the monocytic cell line THP-1 and primary macrophages. First we confirmed that in our assay human serum stimulated phagocytosis of apoptotic cells. When live and apoptotic BL-41 cells were added to macrophages in the presence or absence of 10% human serum we found that the phagocytosis of apoptotic cells increased significantly when serum was added (Fig. 1) and that the effect was dose-dependent (not shown). To allow comparison of data obtained in different experiments, the amount of engulfed live cells was set to zero for each experiment. The phagocytic index for live cells increased in the presence of 10% human serum but to a much lower extent than for the apoptotic cells (Fig. 1).

In order to study the role of C4BP, PS, C4BP-PS and C1q we have purified these proteins and prepared corresponding deficient sera. C4BP, C4BP-PS and C1q were purified from human plasma whereas PS was expressed recombinantly. The proteins were more than 95% pure as shown in Fig. 2D (10% SDS-PAGE under reducing conditions followed by a Coomassie staining). The C4BP-PS complex appears as a 70 kDa band (α-chains that are predominant polypeptide in the complex). PS appears as
a 75 kDa band and C1q as a 30 kDa band (Fig. 2D). Serum depleted from both PS and C4BP (PS/C4BP deficient serum) was prepared by passing freshly prepared normal human serum through two affinity columns coupled with monoclonal antibodies against the two proteins. Using ELISA we show this preparation to be completely depleted of both C4BP and PS (Fig. 2A and B). Another batch of human serum was depleted from C1q by ion exchange chromatography. A haemolytic assay measuring complement activation (classical pathway) on surface of sheep erythrocytes was used to ascertain that normal human serum and PS/C4BP deficient serum were highly active while C1q-deficient serum lost its ability to be activated via the classical pathway (Fig. 2C).

In the present study we confirm that free PS is one of several serum factors required for phagocytosis of apoptotic cells (Fig. 3A). Purified PS increased phagocytosis in a dose dependent manner while PS/C4BP deficient serum had lower but not entirely abolished stimulatory effect on phagocytosis (Fig 3A). This could be due to the fact that intact C1q was still present in our serum as shown by the haemolytic assay. Great care has to be exercised while depleting sera with antibodies since C1q can easily be co-depleted when using for example a polyclonal antibody that will form immune complexes with its target. We have used monoclonal antibodies to prepare deficient sera and we have shown that C1q was intact in our serum lacking C4BP and PS. Interestingly, we have observed that C1q-deficient serum entirely lost its ability to stimulate phagocytosis (Fig. 3B). Furthermore, purified C1q showed an increase of the phagocytic index when present in the assay (Fig. 3B).

Interestingly, we have found that addition of the C4BP-PS complex in the assay resulted in a drastically reduced amount of phagocytosed apoptotic cells (Fig. 3A). To further confirm this effect, the C4BP-PS complex or free PS were added to PS/C4BP deficient serum. PS added to deficient serum yielded a phagocytic index similar to intact serum, while addition of the complex again showed strong inhibitory effect (Fig. 3A).
When C4BP containing β-chain but no PS was added together with 10% normal serum, the phagocytic effect of serum decreased (Fig. 3A), which can be explained by the formation of the complex between C4BP and the free PS in the serum. The inhibitory effect of the complex is not surprising as it has been shown recently that phagocytosis of *Streptococcus pyogenes* is inhibited by the binding of C4BP to the M proteins on the surface of the bacteria (27). C4BP bound to the bacteria is able to strongly inhibit the activation of complement at least via the classical and lectin pathways and the bacteria can therefore avoid elimination and survive in its host. Apart from regulating complement activation on the surface of the bacteria it now appears that the binding of C4BP also inhibits the phagocytosis by macrophages. The effect of C4BP observed in the present study could be due to inhibition of C3b deposition on the surface of apoptotic cell. Also the size of the C4BP-PS complex could sterically block interactions between other phagocytic stimulators and their receptors.

To further characterize mechanisms by which PS and C4BP-PS exert their functions we have prepared a set of Fab fragments, to avoid activation of C1q and phagocytosis via Fc receptor. Fab fragments of several monoclonal antibodies directed against various parts of PS were used. The Fab fragments were added in the assay together with either the C4BP-PS complex or PS alone. MK 21, directed against the Gla-domain of PS, decreased the phagocytosis degree seen for free PS (Fig. 4). The most probable explanation for this observation is that MK 21 blocks binding of PS to phosphatidylserine. MK 34, directed against the SHBG-like domain on PS, did not significantly decrease the phagocytic index although there was a tendency towards inhibition of the PS effect. MK 34 abrogates the binding between PS and C4BP only partially (28), meaning that PS could possibly still interact with a receptor on the phagocyte even if MK 34 is bound to it. PS is known to interact with members of the Axl/Sky/Mer receptor family and they may be the target on the macrophage (29).
When adding the Fab fragments from MK54, directed against EGF 1, together with free PS there was no effect on the phagocytosis (Fig. 4). The inhibitory effect of the C4BP-PS complex was abolished when the Fab fragments from MK 21 was added (Fig. 4) while MK 34 could not block the activity of the complex (Fig. 4). Apparently, to exert its inhibitory function C4BP must be localized to the surface of apoptotic cell via the Gla-domain of PS. MK 104, directed against CCP1 on the α-chain of C4BP, did interfere with the inhibitory effect of the complex to some extent but could not entirely block it (Fig. 4).

Taken together, free PS stimulates phagocytosis of apoptotic cells while the same protein in complex with C4BP has inhibitory effect on this process. It appears that PS and C4BP are a part of a intriguing network of signals that stimulate and inhibit the process of phagocytosis of apoptotic cells in order to fine tune it to particular situations in different in vivo conditions. The current data indicate that the degree by which PS contributes to phagocytosis is mainly determined by the C4BP–PS complex. Although the presence of the C4BP-PS on apoptotic cells may lead to decreased phagocytosis it may, importantly, prevent secondary necrosis because it inhibits further complement attack.
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Figure legends

Figure 1 Phagocytic index of apoptotic and live cells in the presence and absence of human serum.

A dot plot of phagocytosed apoptotic cells in the presence of 10% human serum is shown in A. The channel FL-1 detects CFSE-stained cells and FL-2 detects PE-labelled anti-CD11b/CD14. The quadrant upper left (UL) shows stained primary macrophages, upper right (UR) shows macrophages with engulfed cells and lower right (LR) shows stained apoptotic or live BL-41 cells. The phagocytic index is defined as the percentage of macrophages that have engulfed apoptotic BL-41 cells and the number of phagocytosed live cells in the absence of serum is set to zero. The phagocytic indexes of apoptotic and live cells in the absence or presence of 10% human serum are shown in B. Mean values of 3-6 experiments are shown with indicated standard deviation (SD).

Figure 2 Quality and purity of the reagents used.

Quality of depleted sera is shown by comparing the presence of C4BP (A) and PS (B) in
normal human serum and serum depleted for both C4BP and PS in an ELISA. The C1q deficient serum is compared with normal human serum in their ability to activate complement leading to lysis in the haemolytic assay (C). The C4BP-PS complex, PS and C1q were separated on a 10% gel under reducing conditions and visualised by Coomassie staining to show purity (D).

**Figure 3 Effect of the C4BP-PS complex, PS and C1q on the phagocytic index.**
The effect of PS/C4BP deficient serum, PS, C4BP-PS and C4BP without PS as well as in PS/C4BP deficient serum restored with the C4BP-PS complex or free PS in the phagocytosis of apoptotic cells is shown in A. Phagocytic assay in C1q deficient serum and in the presence of purified C1q (B) is also shown. * < 0.05 ** < 0.01 *** < 0.001

**Figure 4 Blocking the site of PS responsible for the effect on phagocytic index.**
Fab fragments of monoclonal antibodies directed against various domains of PS were added in the phagocytosis assay together with either PS or the C4BP-PS complex. Fab fragments of MK 104, directed against CCP1 of the α-chain of C4BP, was also added together with the complex. * < 0.05

**Figure 5 Proposed mechanism of action of C4BP vs free PS in apoptotic cell clearance.**
A schematic diagram of putative roles of free PS and the C4BP-PS complex in the phagocytosis of apoptotic cells is shown. PS stimulate the uptake of apoptotic cells, and Fab fragment of MK 21 decrease this uptake, while MK 54 and MK 34 does not. The binding of PS to C4BP inhibits the phagocytic effect of PS. C4BP, seven α-chains and one β-chain, may work as a cofactor to FI in the degradation of C4b bound to the surface of the apoptotic cell and C4b in solution. C4BP may also accelerate the decay of the C3-convertase (C2aC4b) on the apoptotic cell, which releases C2a.
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Figure 1 Kask et al
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