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Vitamin K-Dependent Protein S Localizing Complement Regulator C4b-Binding Protein to the Surface of Apoptotic Cells

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Apoptosis is characterized by a lack of inflammatory reaction in surrounding tissues, suggesting local control of complement activation. During the initial stage of apoptosis, cells expose negatively charged phospholipid phosphatidylserine on their surfaces. The vitamin K-dependent protein S has a high affinity for this type of phospholipid. In human plasma, 60–70% of protein S circulates in complex with C4b-binding protein (C4BP). The reason why protein S and C4BP form a high-affinity complex in plasma is not known. However, C4BP is an important regulator of the classical pathway of the complement system where it acts as a cofactor in degradation of complement protein C4b. Using Jurkat cells as a model system for apoptosis, we now show protein S to bind to apoptotic cells. We further demonstrate protein S-mediated binding of C4BP to apoptotic cells. Binding of the C4BP-protein S complex to apoptotic cells was calcium-dependent and could be blocked with Abs directed against the phospholipid-binding domain in protein S. Annexin V, which binds to exposed phosphatidylserine on the apoptotic cell surface, could inhibit the binding of protein S. The C4BP that was bound via protein S to the apoptotic cells was able to interact with the complement protein C4b, supporting a physiological role of the C4BP/protein S complex in regulation of complement on the surface of apoptotic cells. The Journal of Immunology, 2002, 169: 2580–2586.

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apoptosis is under physiological conditions a well-regulated process, characterized by a lack of induction of inflammatory responses in the surrounding tissue (1). In viable cells, an asymmetric distribution of phospholipids between the outer and inner leaflet of the membrane is maintained, the negatively charged phosphatidylserine being predominantly localized in the inner leaflet (2). Erythrocytes and platelets were the first cells where this asymmetry was described (3, 4), but it was later also found to be true for nucleated cells (5). During one of the first stages of apoptosis, phosphatidylserine is transferred from the inner leaflet of the cell membrane to the outer leaflet (6, 7), resulting in the exposure of negatively charged phospholipid on the cell surface. This has been suggested to be a phagocytosis-stimulating signal to neighboring cells, resulting in the elimination of the apoptotic cell remnants.

Negatively charged phospholipid membranes, e.g., exposed on activated platelets, bind vitamin K-dependent coagulation factors, thus supporting the assembly of procoagulant enzyme-cofactor complexes (8–12). Several studies have also shown apoptotic cells to have procoagulant properties (13–15). However, there are also anticoagulant vitamin K-dependent proteins that bind negatively charged phospholipids, e.g., activated protein C (apc)3 and protein S (16). Protein S functions as cofactor to apc in the degradation of factors Va and VIIIa (17, 18). The importance of protein C and protein S is demonstrated by the fact that deficiency of either is associated with increased risk of thrombosis (19, 20). Because anionic membrane surfaces (such as that provided by activated platelets) have been shown to be equally competent in promoting pro- and anticoagulant reactions (21, 22), it is likely that the surface of apoptotic cells is also able to promote anticoagulant reactions.

In plasma, protein S exists in two forms; ~30–40% circulates as free protein S, the remainder forming a 1:1 high-affinity (Kd in a range of 0.1–0.6 nM) complex with C4b-binding protein (C4BP). The C4BP that was bound via protein S to the apoptotic cells was able to interact with the complement protein C4b, supporting a physiological role of the C4BP/protein S complex in regulation of complement on the surface of apoptotic cells.
hydrophobic patch (36, 37). CCP2 also seems to contribute slightly to the interaction (38).

The physiological role of the complex between C4BP and protein S has remained an enigma. It has been suggested that protein S, due to its high affinity for negatively charged phospholipids, localizes C4BP to areas where such phospholipids are exposed (39). The complex binds to synthetic phospholipid vesicles, but not to platelet-derived microparticles, even though free protein S efficiently binds to these particles (40).

It has been demonstrated that complement components attach to apoptotic cells, C1 arguably being the most important. C1q deficiency may predispose to autoimmunity in systemic lupus erythematosus as a consequence of an impaired clearance of apoptotic cells (41–44). Binding of C1 to apoptotic cells could result in activation of the early classical pathway unless efficient control mechanisms exist. A possible regulation could be achieved by the localization of C4BP to the apoptotic cell surface. The aim of the present investigation was to elucidate whether protein S has the capacity to localize C4BP to the apoptotic cell surface. Using Jurkat cells, a human T lymphocyte line, as a model system for apoptotic cells, we demonstrate protein S-mediated binding of C4BP to apoptotic cells. The surface-bound complex could bind C4b, the main target for C4BP in the complement system.

Materials and Methods

Cells

Human Jurkat cells were cultured in RPMI, with 10% heat-inactivated FCS, complemented with l-glutamine and antibiotics (growth medium). For the induction of apoptosis, cells were collected and resuspended at a concentration of 2 × 10⁶ cells/ml. Apoptosis was induced by addition of anti-Fas Ab CD95 (CH-11 clone; Immunotech, Marseille, France), 200 ng/ml (for gating details, see Analysis). For the induction of apoptosis, cells were collected and resuspended at a concentration of 5 × 10⁶ cells/ml. Apoptosis was induced by addition of anti-Fas Ab CD95 (CH-11 clone; Immunotech, Marseille, France), 200 ng/ml, to the growth medium and culture was continued for 5 h. This gave an ~50% apoptotic population and 50% live population of the cells gated (for gating details, see Analysis). After apoptosis induction, cells were harvested by centrifugation and washed in binding buffer (10 mM HEPES, 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂). Cells were then resuspended in binding buffer to a final concentration of 5 × 10⁶ cells/ml.

Proteins

Purified recombinant annexin V was purchased from BD PharMingen (San Diego, CA). Human protein S (45), C4BP, and the C4BP/protein S complex (46) and C4 (47) were purified from plasma as described in their respective references. To obtain C4BP without protein S, the purified C4BP/protein S complex was dialyzed against 80% ethylenglycole, 10 mM HEPES, 140 mM NaCl. For the purification, the respective references. To obtain C4BP without protein S, the purified C4BP/protein S complex was dialyzed against 80% ethylenglycole, 10 mM HEPES, 140 mM NaCl. For the purification, C4BP was dialyzed against 5 mM MOPS, 0.15 M NaCl, and then diluted to 0.5–2.0 mg/ml in 5 mM MOPS, 0.15 M NaCl, 0.1 mM sodium carbonate (pH 9.0), and finally applied on a gel with coupled MK 104 as described previously (48). In this fashion, β-chain-containing C4BP can be separated from protein S without losing its ability to bind protein S. The concentrations were determined by measuring absorbance at 280 nm, and FITC-labeling was confirmed by measuring absorbance at 495 nm. Rates of FITC to protein were within recommended range (0.3–1.0; Ref. 51).

Binding experiments

A total of 75 µl of cells were incubated with protein S, C4BP, plasma protein S/C4BP/protein S complex, or protein S preincubated with C4BP (at 37°C for 30 min, in the presence of 2 mM CaCl₂) in binding buffer at a final volume of 100 µl. In time course studies, binding of protein S leveled of at 25 min at room temperature, and remained stable at least until 60 min (data not shown). Therefore, 25 min was chosen as incubation time. Cells were then pelleted by centrifugation for 2 min at 350 × g, and washed in binding buffer. Following a second centrifugation, 15 µg/ml FITC-labeled Abs, HPS 54 for protein S, and MK 104 for all protein containing C4BP, annexin V labeled with PE (annexin V-PE; BD Biosciences, Mountain View, CA), 5 µl 10× diluted, and 5 µl 7-amino actinomycin D (7-AAA; ViaProbe; BD Biosciences) were added in binding buffer at a final volume of 100 µl. In control experiments, binding of FITC-labeled HPS 54 and 104 to corresponding proteins leveled off around 8 µg/ml and remained stable up to 100 µg/ml (highest concentration tested). Following a 30-min incubation in the dark at room temperature, samples were diluted with 100 µl of ice-cold binding buffer and immediately analyzed in the flow cytometer (FACSort; BD Biosciences). We also tested binding of the C4BP-protein S complex in the presence of human serum. The serum had been depleted of protein S and C4BP using HiTrap columns (5 ml; Amersham Pharmacia Biotech) coupled with HPS 54 and MK 104. Amounts of remaining protein S and C4BP were tested using ELISAs for protein S and C4BP and were below detection levels. We then added back the C4BP-protein S complex (final concentration in serum 200 µg/ml) and incubated apoptotic cells for 30 min at 37°C with 20% human serum where C4BP-protein S had been added back. Cells were washed and binding detected in the flow cytometer using FITC-labeled MK 104 as described above.

Annexin V inhibition

Apoptotic Jurkat cells, 75 µl, were incubated for 25 min at room temperature with protein S, 100 nM, and recombinant annexin V, 0, 3, or 100 nM in binding buffer at a final volume of 100 µl. Cells were then washed as described above and incubated with FITC-labeled HPS 54, 15 µg/ml, for 30 min at room temperature in the dark. Samples were then diluted in 100 µl of ice-cold binding buffer and immediately analyzed in the flow cytometer.

Binding of C4b

For the C4b-binding, the same protocol was used as for binding experiments, except that protein S concentration was kept to a final concentration of 250 nM, FITC-labeled C4b at increasing concentrations was added instead of MK 104-FITC, and annexin V-PE was not diluted. To see whether binding of C4b to C4BP/protein S on the apoptotic cell could be inhibited by a blocking Ab, unlabeled MK 104 (3.3-fold molar excess over C4b added) was added at the same time as C4b-FITC (300 nM) in a separate experiment.

Analysis

Flow cytometric results were analyzed using CellQuest software (BD Biosciences). Fluorescence was measured on a log scale and is expressed as

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geometric mean fluorescence, or percentage of maximal binding seen. A gate was set around cells where no dramatic size changes were discernible, i.e., forward and side scatter were unchanged as compared with cells where no anti-Fas Ab had been added. Early apoptotic cells were defined as cells staining single positive for annexin V-PE. Late apoptotic and necrotic cells were defined as cells staining double positive for annexin V-PE and 7-AAD; these cells were excluded from analysis. Live cells were defined as cells staining double negative for annexin V-PE and 7-AAD. There was no difference observed in the various binding studies between this live cell population and control cells, cultured without anti-Fas Ab. In all experiments, at least 10,000 cells were collected.

Results

Protein S binds to apoptotic cells

Jurkat cells, induced to undergo apoptosis with anti-Fas Ab, were mixed with protein S. Following incubation, cells were washed with ice-cold binding buffer and mixed with FITC-labeled mAb HPS 54, directed against the first EGF-like domain on protein S. Annexin V-PE, which binds to negatively charged phospholipid and thus was used for early detection of apoptosis, and 7-AAD were also added. Samples were diluted in ice-cold binding buffer and FACS analysis was immediately performed. Early apoptotic cells were defined as the population staining positive for annexin V-PE (binding negatively charged phospholipid) and negative for 7-AAD (nucleic acid dye), live cells were defined as the population that was negative for both. As shown in Fig. 1A, apoptotic cells bound protein S in a dose-dependent manner, whereas there was virtually no binding of protein S to live cells. Due to variation between experiments with regard to fluorescence intensity/cell, we have chosen to plot binding as mean percentage of maximum binding seen in each different experiment. The actual fluorescence intensity/cell (geometric mean ± SD) seen at highest concentration of protein S was 2285 ± 291. Binding was saturable, which enabled us to calculate an apparent $K_d$ of the protein S association to the apoptotic cell. Free protein S demonstrated a $K_d$ of $\sim$220 nM. For reference, the concentration of free protein S in human plasma is $\sim$100 nM, which constitutes $\sim$30% of total protein S (45, 46).

Because our hypothesis was that protein S binds to the negatively charged phospholipids exposed early in apoptosis, which is what annexin V also binds to, the annexin V-PE was diluted 10 times to minimize competition between the two proteins. In control experiments, annexin V reagent could be diluted 25-fold even at the highest concentration of protein S without loss of the signal, i.e., the apoptotic population could still be distinguished from the live population. As control, experiments were done in the same manner but with the exclusion of annexin V-PE and 7-AAD. Similar protein S binding was observed, which ensures that protein S did not bind to the annexin V. Furthermore, there was no binding in the absence of protein S between mAbs and cells, in the presence or absence of annexin V-PE/7-AAD (data not shown).

C4BP binds to apoptotic Jurkat cells in the presence of protein S

To test whether C4BP binds to apoptotic cells and if protein S has any influence on the interaction, FITC-labeled MK 104, directed against the $\alpha$-chains of C4BP, was used for detection. Three different forms of C4BP were tested. The first variant was the C4BP/protein S complex purified from plasma. The second variant was C4BP isolated from the purified C4BP/protein S complex on a heparin Sepharose column run in ethylene glycol. This breaks the strong hydrophobic bond between C4BP and protein S, and allows C4BP to be separated from protein S. This C4BP retains the ability to bind protein S. The third variant tested was C4BP incubated in vitro with protein S, to reconstitute the complex. All three forms of C4BP, i.e., in complex with protein S, free, and recomplexed with protein S, were tested for their ability to bind apoptotic cells. The results presented in Fig. 1B clearly show that C4BP/protein S complexes binds to apoptotic, but not to live Jurkat cells. Binding was dose-dependent, and C4BP by itself demonstrated almost no binding. For reference, the concentration of C4BP in human plasma is $\sim$300 nM, the majority being $\beta$-chain containing and thus circulating in complex with protein S (52). Due to variation between experiments with regard to fluorescence intensity/cell, we have chosen to plot binding as mean percentage of maximum binding seen in each different experiment. The actual fluorescence intensity/cell (geometric mean ± SD) seen at the highest concentration of C4BP/protein S complex was 465 ± 175. To investigate whether the very slight binding observed by C4BP alone was due to small amounts of contaminating protein S (although not detected by Western blot), we repeated the experiment using FITC-labeled HPS 54 instead of the anti-C4BP Ab. The results were very similar to those obtained with the FITC-labeled MK 104, showing that indeed the binding of C4BP was due to trace amounts of protein S in the sample (data not shown). We also tested binding of the C4BP-protein S complex to apoptotic cells in the presence of serum, using detection with FITC-labeled MK 104. Also in the presence of serum there was a clear binding of the C4BP-protein S complex as shown in Fig. 2.

Binding of protein S and C4BP-protein S is dependent on the phospholipid binding Gla-domain of protein S

FITC-labeled protein S and unlabeled C4BP/protein S (plasma purified complex) were preincubated with mAbs HPS 21 and HPS 54. HPS 21 is directed against the Gla domain of protein S and blocks binding of protein S to phospholipid, whereas HPS 54 is
directed against the first EGF-like domain in protein S (49). The proteins were then mixed with Jurkat cells induced to undergo apoptosis with anti-Fas Ab as before. Following incubation, cells incubated with FITC-labeled protein S were immediately analyzed in the flow cytometer while the cells mixed with the C4BP/protein S complex were washed and incubated with FITC-labeled MK 104 before analysis. Preincubation with HPS 21 blocked binding of both protein S and C4BP/protein S, strongly suggesting that the Gla domain of protein S is what attaches both free protein S and the C4BP/protein S complex to the apoptotic cell (Fig. 3, A and B). In contrast, HPS 54 did not block binding. Rather, it seemed to enhance the binding of FITC-labeled protein S (when the molar ratio between protein and Ab was 1:1) as compared with binding of protein S without preincubation with the mAb HPS 54 (data not shown). We have previously reported a similar phenomenon when studying protein S binding to synthetic phospholipid vesicles. The explanation was then found to be cross-linking of two protein S molecules on the phospholipid surface by the divalent Ab (49). In our binding experiments using detection of cell-bound protein S using FITC-labeled HPS 54, saturating concentrations of HPS 54 were used (see Materials and Methods), thus, no cross-linking was to be expected in those experiments. The binding of protein S to the apoptotic cell surface was Ca²⁺-dependent, and addition of EDTA at the end of the incubation of FITC-labeled protein S and cells completely abrogated the binding (data not shown).

**High concentrations of annexin V diminish the binding of protein S to apoptotic cells**

As annexin V binds with a high affinity to the negatively charged phospholipid exposed early in apoptosis (53), we tested whether an equimolar concentration of annexin V could affect the binding of protein S to the apoptotic cell. Annexin V, 100 nM was added to cells together with protein S, 100 nM. In addition, we also tested the effect of 3 nM unlabeled annexin V, which corresponds to the concentration of PE-labeled annexin V used in our binding experiments. Cells were washed and then incubated with FITC-labeled HPS 54 and 7-AAD. Cells were transferred to tubes containing ice-cold binding buffer and immediately analyzed in the flow cytometer. In Fig. 4 we demonstrate that in the presence of annexin V, protein S binding to the apoptotic cells is diminished, strongly suggesting that the interaction is between phospholipid and the protein S Gla domain. As annexin V-PE was not included in this experiment, it was not possible to selectively gate for the apoptotic population. Fig. 4B shows the same experiment where cells had not been induced to undergo apoptosis. However, it was clearly shown that annexin V is able to impair the protein S binding to apoptotic cells, demonstrating that the binding sites for protein S and annexin V on the apoptotic cells are the same.

**C4b binds to apoptotic cells in the presence of the C4BP/protein S complex**

To investigate whether the C4BP that was localized to the apoptotic cells could function to bind C4b, we preincubated cells induced to undergo apoptosis with the C4BP/protein S complex. The

**FIGURE 2.** Binding of the C4BP-protein S complex in the presence of serum. Apoptotic cells were incubated with serum with or without the C4BP-protein S complex and binding of the complex was detected with FITC-labeled MK 104. Filled line, cells incubated with serum depleted of C4BP and protein S. Dotted line, cells incubated with depleted serum where C4BP-protein S had been added back.

**FIGURE 3.** Inhibition of binding using mAbs. Protein S and the C4BP/protein S complex were preincubated with mAbs HPS 21 (directed against the Gla domain of protein S) and HPS 54 (directed against EGF1 of protein S) before being tested for binding to Jurkat cells. The protein S used was labeled with FITC and could be directly detected in the flow cytometer. For detection of binding of the C4BP/protein S complex, FITC-labeled MK 104 was used in a second step. Cell count is plotted against fluorescence intensity on a logarithmic scale. A and B, Apoptotic cells. C and D, Live cells. Black line, HPS 54 was used for preincubation; gray line, HPS 21 was used for preincubation.
cells were washed, and FITC-labeled C4b was added in the presence of annexin V-PE and 7-AAD. C4b-FITC was found to bind to apoptotic cells preincubated with the C4BP/protein S complex as compared with the apoptotic cells that were not preincubated with the C4BP/protein S complex (Fig. 5). Clearly, C4BP is able to bind C4b even when it is bound via protein S to apoptotic cells, demonstrating the physiological relevance of the C4BP/protein S complex binding. The mAb MK 104 directed against CCP1–2 of the C4BP α-chain (50), when added at the same time as C4b-FITC, could block the binding between C4BP and C4b. We have previously described the binding site of C4b on C4BP to be localized to the interface of α-chain CCP1–2 (35).

**FIGURE 4.** Annexin V diminishes binding of protein S to apoptotic cells. Apoptotic Jurkat cells were incubated with 100 nM protein S and 0, 3, and 100 nM annexin V. Cells were washed and bound protein detected using FITC-labeled HPS 54. Cell count is plotted against HPS 54-FITC fluorescence intensity on a logarithmic scale. Protein S binding: A. Apoptosis-induced cells. Black line, in the presence of 100 nM annexin V; dotted line, in the presence of 3 nM annexin V; gray line, in the absence of annexin V. B. Noninduced (live) cells. Black line, in the presence of 100 nM annexin V; gray line, in the absence of annexin V.

**FIGURE 5.** The C4BP/protein S complex increases binding of C4b to apoptotic cells. Increasing amounts of FITC-labeled C4b was mixed with apoptotic Jurkat cells, either preincubated with the C4BP/protein S complex or just with buffer. Cells were then analyzed in the flow cytometer. Increase in geometric mean fluorescent intensity/cell of cells preincubated with the C4BP/protein S complex, as compared with the intensity seen where cells were preincubated with just buffer, is plotted against the amount of FITC-labeled C4b added (∙). Each point represents the average of three experiments ± SD. When MK 104 was added to the cells at the same time as 300 nM C4b-FITC, the signal returned to that without C4BP/protein S (∙, indicated with an arrow).

**Discussion**

Recently, there has been increased attention on the role of the complement system in apoptosis. Lack of C1q has been linked to the pathogenesis of systemic lupus erythematosus, as C1q deficiency might predispose to autoimmunity due to impaired clearance of apoptotic cells (41, 43). Assembly of complement proteins has been observed on apoptotic HUVEC cells (44), and complement has been suggested to take part in the clearance of apoptotic cells by macrophages (54). The C5b-9 membrane attack complex has been implied to play a role both as protector from apoptosis (sublytic complex; Refs. 55–57) and as an inducer of apoptosis (58–60). Thus, the complement system seems involved in many aspects of apoptosis.

Our results demonstrate binding of C4BP/protein S complexes to apoptotic cells and provide new insight into the regulation of complement on apoptotic cells. In addition, binding of C4BP was totally dependent on protein S, because C4BP by itself did not display any binding. Protein S is shown to use the same binding site as annexin V, and binds to the cell surface exposed phosphatidylinerine via its Gla domain. Furthermore, C4BP localized via protein S to the cell surface could still bind C4b, implying that C4BP retains its physiological role also when attached to an apoptotic cell surface, because C4b will be cleaved by factor I when C4b is associated with C4BP (61, 62). This is similar to our previous results, where we saw that phospholipid vesicle-associated C4BP/protein S complex still could bind C4b (63). In addition, we could still detect binding of the protein S-C4BP complex to apoptotic cells when they were incubated with physiological concentration of C4BP-protein S in the presence of human serum, demonstrating that the affinity is sufficiently high for the complex to associate specifically with the apoptotic cells also when serum proteins are present. We were interested to study the physiological consequences of the binding of the protein S-C4BP complex to apoptotic cells. However, our efforts to reliably and reproducibly induce and follow complement activation on these cells have been unsuccessful. Nonetheless, the present study demonstrates protein S-mediated binding of C4BP to apoptotic cells and suggests a potential physiological role for the complex in vivo, in localizing C4BP, via protein S, to apoptotic cells where C4BP can down-regulate complement activation.

From a number of studies (41–44, 54), it appears that early complement components are important for the rapid clearance of apoptotic cells, but that the cell must be protected from assembly of later components in order not to provoke an inflammatory response triggered by the complement system. Apart from forming the cell lytic membrane attack complex, activation of complement yields an inflammatory response as anaphylatoxins (mainly C5a and C3a) and opsonins (mainly C3b) are generated. Thus, the presence of C4BP, a major inhibitor of the classical pathway, may be of utmost importance to inhibit inflammation close to apoptotic cells. As we have shown in the present study that C4BP cannot bind in the absence of protein S, our results point toward the physiological relevance of the C4BP/protein S complex formation. Although it has been suggested before, this is the first time we show that protein S is crucial to localize C4BP to an area where complement should be prevented.

Serum amyloid P component (SAP) has been shown to bind apoptotic cells (64). It is conceivable that SAP may modulate the function of apoptotic cell surface-bound C4BP, because SAP has been shown to have an effect on C4b-binding/factor I cofactor function of C4BP (65). However, the interaction between C4BP and SAP is inhibited by the presence of phosphoethanolamine-containing compounds (63, 65). Because SAP was shown to bind...
to the apoptotic cells via phosphatidylethanolamine, it is possible that SAP does not interact with C4BP bound to the apoptotic cell surface, but rather binds directly to the cell surface. In the present investigation, we observed that the C4BP-protein S complex could bind to apoptotic cells in the presence of serum, indicating that the complex can bind in the presence of SAP. Moreover, addition of serum concentrations of SAP in the binding studies did not affect binding of the C4BP-protein S complex to apoptotic cells (data not shown).

It has also been suggested that the death of T lymphocytes in atherosclerotic plaques may be considered beneficial if apoptosis is not accompanied by an inflammatory reaction (66). Our results and previous studies on Jurkat cells (42, 43) imply that strictly regulated complement could be essential in the clearance of such cells. C4BP by itself did not bind apoptotic cells, while protein S did. As apoptotic cells expose negatively charged phospholipids, as do activated platelets, the apoptotic cell surface has been considered, and also shown, to be procoagulant (13–15). Given our results and the fact that protein S has among the highest affinities of the vitamin K-dependent proteins for negatively charged phospholipids (67), the surface of the apoptotic cell may not be solely procoagulant, but also anticoagulant. In short, where there is coagulation, there is also anticoagulation. Casciola-Rosen et al. (15) showed that surface blebs of apoptotic cells are sites of enhanced procoagulant activity. These surface blebs resemble microparticles formed by activated platelets (68) to which we have shown protein S to bind. We saw no binding of the C4BP/protein S complex to these platelet-derived microparticles. The reason why these microparticles differed from apoptotic cells in this regard is unclear. Considering that they both expose negatively charged phospholipids, one could expect that they would display similar binding of not only free, but also C4BP-bound protein S. The explanation to this may lie in the physiological role of the platelet microparticles, foremost providing an area for coagulation or anticoagulation rather than complement activation. Because protein S loses its anticoagulant cofactor function when in complex with C4BP, binding of the complex to platelet microparticles would be without gain. In contrast, on the apoptotic cell surface where complement components have associated, binding of the protein S can be of great importance. It is conceivable that this surface may harbor both free protein S, performing its anticoagulant function, and protein S with C4BP, regulating complement action. The potential role of protein S in controlling coagulation on apoptotic cells is an important and interesting area of investigation, especially as patients suffering from protein S deficiency are at an increased risk of thrombosis.

In conclusion, we show that apoptotic cells bind protein S, and that protein S can localize the complement regulatory protein C4BP to the surface of apoptotic cells, thus providing the potential for local regulation of complement activation. In addition, the cell-bound C4BP could still associate with C4b, strongly suggesting a physiological role for the C4BP/protein S complex in vivo.

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