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Glucocorticoids Induce Protein S-Dependent Phagocytosis of Apoptotic Neutrophils by Human Macrophages

Aisleen McColl,* Stylianos Bournazos,* Sandra Franz,* Mauro Perretti, † B. Paul Morgan, ‡ Christopher Haslett,* and Ian Dransfield*‡*

During resolution of an inflammatory response, recruited neutrophil granulocytes undergo apoptosis and are removed by tissue phagocytes before induction of secondary necrosis without provoking proinflammatory cytokine production and release. Promotion of physiological neutrophil clearance mechanisms may represent a viable therapeutic strategy for the treatment of inflammatory or autoimmune diseases in which removal of apoptotic cells is impaired. The mechanism underlying enhancement of macrophage capacity for phagocytosis of apoptotic cells by the powerful anti-inflammatory drugs of the glucocorticoid family has remained elusive. In this study, we report that human monocyte-derived macrophages cultured in the presence of dexamethasone exhibit augmented capacity for phagocytosis of membrane-intact, early apoptotic cells only in the presence of a serum factor. Our results eliminate a role for a number of potential opsonins, including complement, pentraxin-3, and fibronectin. Using ion-exchange and gel filtration chromatography, we identified a high molecular mass serum fraction containing C4-binding protein and protein S responsible for the augmentation of phagocytosis of apoptotic neutrophils. Because the apoptotic neutrophils used in this study specifically bind protein S, we suggest that glucocorticoid treatment of macrophages induces a switch to a protein S-dependent apoptotic cell recognition mechanism. Consistent with this suggestion, pretreatment of macrophages with Abs to Mer tyrosine kinase, a member of the Tyro3/Axl/Mer family of receptor tyrosine kinases, prevented glucocorticoid augmentation of phagocytosis. Induction of a protein S/Mer tyrosine kinase-dependent apoptotic cell clearance pathway may contribute to the potent anti-inflammatory effects of glucocorticoids, representing a potential target for promoting resolution of inflammatory responses. The Journal of Immunology, 2009, 183: 2167–2175.

Successful restoration of a tissue to its original state after an inflammatory insult requires that large numbers of extravasated neutrophil granulocytes are cleared from the inflamed site. During this resolution phase of inflammation, recruited neutrophils undergo apoptosis and are subsequently removed by phagocytes (1), a rapid and efficient process that does not stimulate proinflammatory macrophage responses (2). Conversely, inefficient or defective clearance of membrane-intact apoptotic neutrophils may result in release of their histotoxic intracellular contents as a consequence of secondary necrosis, potentially causing local tissue damage and contributing to pathogenesis of inflammatory disease (3). An attractive approach for therapeutic intervention in inflammatory diseases would therefore be to manipulate the processes involved in physiological clearance of neutrophils from inflamed sites. Although promotion of neutrophil apoptosis may be achievable pharmacologically (4), under some circumstances in vivo it will be important to ensure that the capacity for apoptotic cell clearance within tissues is matched to avoid potential deleterious consequences of the presence of non-phagocytosed apoptotic cells (5).

We have previously reported that the powerful anti-inflammatory drugs of the glucocorticoid family (methylprednisolone, hydrocortisone, or dexamethasone (Dex)) specifically enhance noninflammatory phagocytosis of apoptotic cells by human and murine macrophages (6, 7). Glucocorticoids have been shown to modulate the expression of over 100 genes, including those known to be associated with apoptotic cell phagocytosis, such as CD163, FPR1, and Mer tyrosine kinase (Merkt) receptors and MFG-E8 and C1q serum proteins (8). Furthermore, we have shown that human monocytes differentiated for 5 days in the presence of glucocorticoids exhibit a more homogeneous phenotype with reduced phosphorylation of molecules involved in integrin signaling and cytoskeletal rearrangement (7). However, the precise mechanism(s) by which glucocorticoids augment phagocytosis of apoptotic cells has remained elusive.

In this study, we have examined the mechanism underlying augmentation of human monocyte-derived macrophage (MDMϕ) capacity for phagocytosis of early membrane-intact apoptotic human neutrophils following exposure to glucocorticoids. Apoptotic neutrophils display a distinct surface molecular phenotype important for attenuation of functional responses (9) with additional surface changes that target dying cells for removal by phagocytes (10). A number of soluble factors present in serum, including complement C1q and C3b, properdin, collectins, long pentraxin-3, MFG-E8, galectin-3, and α2-macroglobulin, have been reported to bind to apoptotic human cells (11–18) and consequently modulate their...

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*Medical Research Council Centre for Inflammation Research, Queen’s Medical Research Institute, Edinburgh, United Kingdom; †William Harvey Research Institute Barts and London Queen Mary’s School of Medicine and Dentistry, London, United Kingdom; and ‡Department of Medical Biochemistry and Immunology, School of Medicine, Cardiff University, Cardiff, United Kingdom.

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2 Address correspondence and reprint requests to Dr. Ian Dransfield, Medical Research Council Centre for Inflammation Research, Queen’s Medical Research Institute, 47 Little France Crescent, Edinburgh, United Kingdom. E-mail address: i.dransfield@ed.ac.uk

3 Abbreviations used in this paper: Dex, dexamethasone; C4BP, C4-binding protein; CMFDA, 5-chloromethylfluorescein diacetate; MDMϕ, monocyte-derived macrophage; Merkt, Mer tyrosine kinase.
recognition and uptake by macrophages via a number of different surface receptors, including scavenger receptors, complement receptors, receptors for phosphatidylserine, and Merk (19–22). However, it is important to note that some of these opsonization events occur relatively late during the apoptotic process and accompany loss of membrane integrity (23).

In this study, we demonstrate that glucocorticoid augmentation of MDMΦ phagocytosis is associated with a switch from a serum-independent to a serum-dependent apoptotic cell recognition mechanism, which can be recapitulated with purified protein S, a 75-kDa vitamin K-dependent anticoagulation factor that is present in plasma at a relatively high concentration of ~25 μg/ml (24), and involves macrophage Merk, a member of the Tyro3/Axl/Mer family of immunoregulatory receptor tyrosine kinases (25). Our data strongly suggest that glucocorticoids critically regulate a switch in apoptotic cell clearance mechanisms used by macrophages, potentially contributing to their potent anti-inflammatory effects and thus representing a target for promoting inflammatory resolution.

Materials and Methods

Sera, serum proteins, and other reagents
All chemicals were purchased from Sigma-Aldrich, unless otherwise stated. Culture medium (IMDM), buffers (HBSS and PBS without divalent cations), and trypsin-EDTA were from PAA Laboratories. Percoll was from GE Healthcare. Dextran T500 was from Pharmacosmos. Dextran was obtained from Organon. Roscovitine was from Merck. Serum, from coagulated whole blood, was obtained by cardiac puncture from wild-type, 75-kDa vitamin K-dependent anticoagulation factor that is present in plasma at a relatively high concentration of ~25 μg/ml (24), and involves macrophage Merk, a member of the Tyro3/Axl/Mer family of immunoregulatory receptor tyrosine kinases (25). Our data strongly suggest that glucocorticoids critically regulate a switch in apoptotic cell clearance mechanisms used by macrophages, potentially contributing to their potent anti-inflammatory effects and thus representing a target for promoting inflammatory resolution.

Antibodies
Primary Abs were from the following sources: polyclonal rabbit anti-protein S Ab (1:5000; DakoCytomation), human anti-Mer mAb (clone D25508, murine IgG2b, 1:50; R&D Systems), and CD44 mAb (clone SA4; IgG1, 1:50; provided by G. Dougherty, University of California, San Francisco, CA). Control mouse Igs (IgGl, IgG2b, 1:50) were from Serotec. HRP-conjugated goat anti-rabbit IgG (1:2500) and FITC-conjugated F(ab′)2 goat anti-mouse Igs (1:50) were from DakoCytomation. PE-conjugated anti-CD16 mAb (clone 3G8, IgG1) was obtained from BD Biosciences. Agarose-coupled goat anti-rabbit IgG was obtained from Sigma-Aldrich.

Cell isolation
Mononuclear and polymorphonuclear leukocytes were isolated from freshly drawn, citrated human blood by dextran sedimentation and centrifugation over a discontinuous Percoll gradient (final concentrations of 55, 70, and 81% Percoll), as previously described (29). Mononuclear cells were aspirated from the 55/70% interface, and neutrophils from the 70/81% interface. Autolysosomes were separated by preparation of platelet-rich plasma (final CaCl2 concentration: 22 mM), as previously described (24).

In vitro culture of human MDMΦ
Mononuclear cells were resuspended at 4 × 106/ml in IMDM and adhered to 48-well tissue culture plates for 1 h at 37°C in 5% CO2. Nonadherent lymphocytes were removed by washing with IMDM, and adherent monocytes were cultured for 5 days in IMDM containing 10% autologous serum ± 1 μM DEX. These cells are >90% CD14+/CD16− at 5 days with functional and phenotypic characteristics of macrophages (Dex-DMMDΦ) (7).
Results are presented as mean ± SEM, and n = number of independent experiments using macrophages from different donors. Results were analyzed by repeated measures one-way ANOVA with a Bonferroni posttest.

Results
Glucocorticoid-augmented clearance of apoptotic neutrophils by macrophages is serum dependent

Previous studies relating to glucocorticoid-enhanced phagocytosis of apoptotic cells used monocyte-derived macrophages and apoptotic cell targets that had been cultured in the presence of serum (6, 7). To evaluate the potential role of serum opsonization in promoting apoptotic neutrophil clearance, human blood monocytes were cultured for 5 days in the absence or presence of Dex, and neutrophils were rendered apoptotic by overnight culture in serum-free conditions (Fig. 1A). Neutrophil populations cultured in serum-free conditions for 20 h exhibit a slightly higher percentage (63–70%, n = 35, 95% confidence limit) of annexin V+/propidium iodide− (apoptotic) cells when compared with neutrophils cultured in the presence of serum (50–60%, n = 10), consistent with the presence of a survival factor(s) in human serum. In addition, there were significantly higher percentages of annexin V+/propidium iodide− (secondarily necrotic) neutrophils in serum-free cultures (18–26%, n = 35) when compared with neutrophils cultured in the presence of serum (8–17%, n = 10).

When we determined the proportion of untreated MDM and Dex-MDM that were capable of phagocytosis of neutrophils, we were surprised to find that there was no significant augmentation of phagocytosis of serum-free apoptotic neutrophils observed for Dex-MDM (Fig. 1, C and E). In contrast, in the presence of 10% autologous serum, we observed increased phagocytic capacity for Dex-MDM (Fig. 1, D and E). The presence or absence of serum also had a small, but statistically significant stimulatory effect upon phagocytosis of apoptotic neutrophils by untreated MDM (Fig. 1E). The effect of serum on phagocytosis by Dex-MDM was concentration dependent and reached significance at 1% (data not shown). The possibility that the presence of serum acts to promote phagocytic activity of MDM directly was excluded in a series of experiments in which preincubation of apoptotic neutrophils with serum was found to confer augmentation of Dex-MDM phagocytic capacity (data not shown), raising the possibility that a serum factor binds to the apoptotic neutrophil surface to promote phagocytosis by Dex-MDM.

Augmentation of phagocytosis by serum is independent of the presence of necrotic neutrophils

Neutrophils undergo apoptosis in a relatively heterogeneous manner during in vitro culture (Fig. 1A), making it difficult to determine whether enhanced phagocytosis following opsonization depends upon the presence of apoptotic or secondarily necrotic cells. We therefore treated neutrophils with roscovitine, a cyclin-dependent kinase inhibitor that induces neutrophil apoptosis rapidly and uniformly without induction of secondary necrosis (4). Neutrophil populations cultured with 20 μM roscovitine in serum-free conditions for 4 h exhibit a high percentage (80–90%) of annexin V+/propidium iodide− (apoptotic) cells with less than 3% annexin V+/propidium iodide− (secondarily necrotic) cells (Fig. 1B). Serum-dependent enhancement of phagocytosis of roscovitine-treated apoptotic neutrophils by Dex-MDM confirmed that opsonization of early apoptotic cells was required (33.7 ± 9.3% and 51.8 ± 6.3% for Dex-MDM in the absence and presence of serum, respectively). Data are mean percentage phagocytosis ± SEM, n = 3 (*, p < 0.01).

Serum-dependent enhancement of phagocytosis of apoptotic neutrophils does not require complement activation

Down-regulation of complement regulatory molecules CD55 (decay-accelerating factor), CD46 (membrane cofactor protein), and CD35 (CR1) on the surface of human apoptotic neutrophils (31) may allow complement proteins present in serum to bind, and hence promote their removal by phagocytes (11). We found that addition of commercially available C1q-depleted human serum...
Cytosis by Dex-MDM or to C1q-depleted serum (data not shown) did not restore phagocytosis of apoptotic cells by untreated MDMs (Fig. 2A) and Dex-MDMs (Fig. 2B), suggesting that C1q was the serum opsonin binding to apoptotic neutrophils. However, addition of 70 μg/ml human C1q alone (Fig. 2A) or to C1q-depleted serum (data not shown) did not restore phagocytosis by Dex-MDMs to levels observed in the presence of autologous serum. In a series of experiments examining the effects of sera from different species, we noted that augmentation of phagocytosis by Dex-MDMs was also observed when apoptotic neutrophils were incubated in serum obtained from mice, allowing us to use specific knockouts to define serum components (data not shown). We found that serum derived from either male or female C1q-deficient mice restored serum-dependent augmentation of phagocytosis by Dex-MDMs (n = 3; ***, p < 0.001 compared with Dex-MDMs in the presence of serum). Serum derived from either male (M) or female (F) C1q-deficient mice restored serum-dependent enhancement of Dex-MDM effects of C3 inhibitor-treated serum were indistinguishable from control PBS-treated serum in enhancement of Dex-MDM effects of serum. We found that serum derived from either male or female C1q-deficient mice was able to significantly augment phagocytosis of apoptotic neutrophils (80.4 ± 3.23% for Dex-MDMs) and 42.5 ± 6.3% for Dex-MDMs in the presence of wild-type and annexin I knockout serum, respectively; mean percentage phagocytosis ± SD, n = 3). Similarly, comparison of phagocytosis of apoptotic cells in the presence of either control or annexin I-deficient mouse serum demonstrated that this pathway is not used by Dex-MDMs for recognition of apoptotic neutrophils (80.4 ± 6.3% and 79.3 ± 3.23% for Dex-MDMs in the presence of wild-type and annexin I knockout serum, respectively; mean percentage phagocytosis ± SD, n = 3).

Identification of a serum fraction with phagocytic activity
Preliminary experiments showed that the serum factor could be bound to Q Sepharose in a 50 mM HEPES buffer at pH 7.0 or above and eluted with 0.2 M NaCl (data not shown). Because fewer proteins would bind at pH 7.0, we ran subsequent separations at this pH to facilitate identification of the factor. Further fractionation of the 0.2 M NaCl eluate using Sephacryl S-300 column yielded two partially overlapping peaks of protein with descending size (Fig. 3, A and B), as might be expected for a crude protein fraction with the first peak, representing high molecular mass proteins (>300 kDa), able to confer augmentation of phagocytosis (Fig. 3C).

Identification of the serum component using mass spectrometry
Mass spectrometry analysis of the major proteins present in the high molecular mass fraction revealed that the principal proteins present were IgM, α2-macroglobulin, and C4-binding protein (C4BP), most likely in complex with protein S (33). The presence of protein S in the high molecular mass fractions isolated from gel filtration chromatography was confirmed by immunoblotting analysis (Fig. 3D). Previous work had eliminated a role for IgM in the augmentation of phagocytosis of apoptotic cells by Dex-MDMs (data not shown). Phagocytosis of apoptotic neutrophils by Dex-MDMs in the presence of 20 μg/ml α2-macroglobulin was not augmented, suggesting that α2-macroglobulin was not involved either (Fig. 4A), whereas addition of purified protein S during the

### Table I. Effect of sera and serum proteins on Dex-MDM phagocytosis

<table>
<thead>
<tr>
<th>Add Back (during phagocytosis)</th>
<th>Dex-MDM phagocytosis (%)</th>
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<tbody>
<tr>
<td>Autologous serum</td>
<td>301.4</td>
</tr>
<tr>
<td>Ultracentrifuged autologous</td>
<td>293.8</td>
</tr>
<tr>
<td>Murine serum</td>
<td>491.1</td>
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<tr>
<td>Bovine serum</td>
<td>241.8</td>
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<tr>
<td>IgG</td>
<td>109.8</td>
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<tr>
<td>Pentraxin-3</td>
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<tr>
<td>Fibronectin</td>
<td>97.9</td>
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<tr>
<td>Platelet releasate</td>
<td>84.7</td>
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* Data are shown as percentage of phagocytosis relative to that recorded for Dex-MDMs in the absence of serum (equivalent to 100%) for at least three independent experiments.
assay restored phagocytosis of apoptotic neutrophils by Dex-MDMφ to levels similar to those observed in the presence of 10% serum (Fig. 4B). We therefore tested whether immunodepletion of protein S from the 0.2 M NaCl eluate affected Dex-MDMφ phagocytosis of apoptotic neutrophils. As shown in Fig. 5A, immunodepletion did not result in the nonspecific removal of proteins from the 0.2 M NaCl eluate as assessed by total protein staining. Confirmation of the depletion of protein S from the 0.2 M NaCl eluate containing the prophagocytic activity was made by immunoblotting (Fig. 5B). In contrast to a mock Ab depletion of the 0.2 M NaCl eluate, protein S-depleted 0.2 M NaCl eluate failed to confer augmentation of phagocytosis by Dex-MDMφ (Fig. 5C). Together with data presented in Fig. 3C, these data suggested that protein S, possibly complexed with C4BP, was required to confer full phagocytic capacity of Dex-MDMφ. Interestingly, addition of 250 ng/ml purified human protein S (equivalent to the concentration of protein S present in 1% serum) to protein S-depleted 0.2 M NaCl eluate from ion-exchange chromatography fully restored Dex-MDMφ phagocytosis (Fig. 5C). These data raised the possibility that protein S acts as an opsonin, binding to the surface of apoptotic neutrophils to specifically promote clearance by Dex-MDMφ.

Protein S binds to apoptotic neutrophils in a calcium-dependent manner to mediate their removal by Dex-MDMφ.

To confirm that protein S was able to opsonize apoptotic neutrophils, we preincubated serum-free apoptotic neutrophils with either the high molecular mass fraction from gel filtration chromatography or 2.5 μg/ml human protein S before washing in IMDM with or without the addition of 5 mM EDTA. Binding of protein S to neutrophils in a Ca2+-dependent manner could be detected by flow cytometry using anti-protein S Ab together with CD16 staining to define apoptotic and nonapoptotic cells (29). The possibility that anti-protein S Abs were binding nonspecifically through FcR-mediated interactions was excluded by use of a rabbit Ig control (Fig. 6A) and by mAb blockade of FcγRIIA (data not shown). In the presence of Ca2+, protein S binds to apoptotic (CD16 low-expressing) and also nonapoptotic (CD16 high-expressing) cells, but the...
level of binding to apoptotic cells was 2.3-fold higher relative to that observed for nonapoptotic cells (Fig. 6B). In contrast, protein S binds poorly to cells in the absence of divalent cations (Fig. 6C). Interestingly, high levels of protein S binding to secondarily necrotic cells was observed (CD16 intermediate cells; Fig. 6A), as demonstrated for many other opsonins, including C-reactive protein and C1q (23, 34). Apoptotic neutrophils bind protein S when washed in divalent cation-containing medium, but not when

FIGURE 5. Protein S depletion from a 0.2 M NaCl elution fraction from Q-Sepharose abolished augmentation of phagocytosis by Dex-MDMφ. Immunodepletion of protein S was achieved by three rounds of depletion using a polyclonal rabbit anti-protein S Ab and agarose-coupled goat anti-rabbit IgG, as detailed in Materials and Methods. A mock depletion was performed using agarose-coupled goat anti-rabbit IgG alone. Samples were separated by SDS-PAGE on a 9% gel under nonreducing conditions and then transferred to nitrocellulose. A. The presence of protein S (~85 kDa) in the 0.2 M NaCl eluate from anion-exchange chromatography and in depleted fractions was confirmed by colloidal gold labeling of transferred protein (lane 1, 0.2 M NaCl eluate; lane 2, protein S-depleted 0.2 M NaCl eluate; lane 3, mock depletion of 0.2 M eluate; lane 4, anti-protein S-immunodepleted material, to confirm the presence of protein S). B. Specific depletion of protein S in the samples shown in A was confirmed by immunoblotting, as described in Materials and Methods. In view of the presence of Ab in the immunodepleted sample (lane 2), only the outlined section of the membrane in A is shown. C, The effects of protein S depletion upon phagocytosis of apoptotic neutrophils by Dex-MDMφ were assessed in a 30-min assay. Phagocytosis of apoptotic neutrophils by untreated MDMφ (□) is shown for comparison. In contrast to a mock depletion of protein S (PSmd), protein S depletion from a 0.2 M NaCl fraction (PSd) abolished augmentation of phagocytosis by Dex-MDMφ, an effect that was restored by addition of 250 ng/ml human protein S (equivalent to level in 1% autologous serum). Data are mean ± SEM; n = 3. * p < 0.05 compared with Dex-MDMφ in the presence of 0.2 M NaCl eluate.

FIGURE 6. Protein S binds to neutrophils in a calcium-dependent manner and confers augmentation of phagocytosis by Dex-MDMφ. Neutrophils cultured for 20 h in serum-free conditions were preincubated with 1% autologous serum for 30 min in the presence of 1.5 mM CaCl2. During subsequent steps, incubations were performed in TBS in either the presence or absence of 1.5 mM CaCl2 throughout. Neutrophils were washed before labeling with either A, rabbit Igs (as control), or B and C, rabbit anti-human protein S for 30 min. Neutrophils were then washed twice, labeled with PE-conjugated CD16 mAb for 20 min, and washed before flow cytometric analysis. Levels of CD16 expression can be used to define apoptotic (low), secondarily necrotic (intermediate), and nonapoptotic (high) neutrophils. Representative histograms for binding in either the presence (A and B) or absence (C) of 1.5 mM CaCl2 are shown. D, Calcium-dependent effects of protein S upon phagocytosis of neutrophils cultured for 20 h in serum-free conditions. Preincubation of neutrophils with either 10% autologous serum or 250 ng/ml purified protein S for 1 h, followed by washing in either the presence (IMDM) or absence (5 mM EDTA) of divalent cations before assessment of phagocytosis of apoptotic neutrophils in a 30-min assay by untreated MDMφ (□) and Dex-MDMφ (●). Augmentation of phagocytosis of apoptotic neutrophils by Dex-MDMφ following protein S preincubation was lost when neutrophils were washed in EDTA-containing IMDM before assessment of phagocytosis. Data are mean ± SEM; n = 3. ***, p < 0.001 compared with Dex-MDMφ in the presence of protein S.
Protein S-enhanced phagocytosis by Dex-MDMϕ is dependent on Merk. **A**, Surface expression of Mer on MDMϕ was assessed by indirect immunofluorescence together with flow cytometry. Representative overlay histograms show expression of Mer and CD44 for untreated MDMϕ (dotted lines) and Dex-DMϕ (solid lines) compared with binding of control IgG1 mAb. Expression of Mer (as determined by mean fluorescence intensity) was increased 1.6-fold in five separate comparisons that were made. In contrast, CD44 expression was down-regulated on the surface of Dex-DMϕ, as previously reported (7). Preincubation of Dex-DMϕ (■) with 10 μg/ml anti-Mer Ab for 10 min inhibited subsequent phagocytosis of apoptotic neutrophils in the presence of either 2.5 μg/ml protein S (B) or 10% autologous serum (C). Pretreatment with anti-Mer alone had no effect on phagocytosis by Dex-DMϕ in the absence of protein S or serum. Data are mean ± SEM; n = 3, ***p < 0.001, and *p < 0.05.

We also tested the effects of preincubation of neutrophils with or without protein S and then anti-protein S Ab before assessment of phagocytosis. In two experiments that were performed, the results for glucocorticoid-treated macrophages were as follows: no serum (18%), 1% serum (56%), and 1% serum plus anti-protein S (68%). One possibility is that binding of anti-protein S Ab to neutrophils (as shown in Fig. 6, A–C) may lead to their opsonization with IgG leading to phagocytosis by FcγR-mediated pathways. In the absence of commercially available Fab’ preparations of anti-protein S Ab to test this possibility directly, we used function-blocking mAb, IV.3, to block the interaction of IgG bound to the neutrophil surface with macrophage FcγRII (CD32). Treatment with IV.3 did not influence phagocytosis (65% phagocytosis for Dex DMϕ with 1% serum plus anti-protein S; 62% phagocytosis for DexDMϕ with 1% serum plus anti-protein S in the presence of IV.3; n = 2). These data may indicate either that the polyclonal Ab to protein S used in this study does not neutralize the phagocytotic activity or that multiple FcγRs (FcγRII and/or FcγRI) expressed by DMϕ mediate the uptake of anti-protein S-opsonized neutrophils.

Protein S-enhanced phagocytosis by Dex-DMϕ is dependent on Merk

Surface expression of Merk, a potential receptor for protein S (35), was increased (1.6-fold) on Dex-DMϕ compared with untreated DMϕ (Fig. 7A), consistent with previous reports using oligonucleotide arrays (8). The observed up-regulation of Merk expression was not due to a nonspecific increase in receptor expression because CD44 was decreased on the surface of Dex-DMϕ relative to untreated MDMϕ (Fig. 7A). To assess the contribution of Merk to protein S-dependent phagocytosis, Dex-DMϕ were pretreated with an anti-human Mer Ab for 10 min before phagocytosis. Although anti-Merk had no effect on phagocytosis in the absence of protein S, anti-Merk significantly inhibited phagocytosis by Dex-DMϕ in the presence of 2.5 μg/ml protein S (Fig. 7B). Similar experiments were undertaken to determine whether Abs to protein S would exert similar inhibitory effects on phagocytosis. However, pretreatment of neutrophils with anti-protein S resulted in an augmentation of macrophage phagocytosis, possibly through an opsonization event (see Fig. 6) leading to FcγR-mediated phagocytosis. In contrast, blockade of Mer also significantly inhibited phagocytosis in the presence of 10% autologous serum, implying that the Merk pathway is critical for glucocorticoid stimulation of phagocytosis of apoptotic neutrophils (Fig. 7C). We also examined the effects of short-term treatment of DMϕ with Dex upon the protein S dependency of phagocytosis of apoptotic neutrophils. DMϕ that had been cultured in the absence of Dex for 96 h were then treated for 24 h with Dex. Compared with untreated DMϕ (18 ± 5% phagocytosis in the absence of protein S), 96- to 120-h Dex-treated DMϕ had slightly higher basal levels of phagocytosis of apoptotic cells in the absence of protein S (25 ± 6%), but exhibited increased phagocytosis in the presence of protein S (60 ± 8%).

Discussion

In this study, we have examined the mechanisms that underlie the requirement for serum in augmentation of human macrophage phagocytosis of apoptotic neutrophils following treatment with glucocorticoids. We demonstrated that protein S opsonizes early apoptotic neutrophils (induced by treatment with roscovitine) to promote their internalization by Dex-DMϕ, and that the presence of cells that had undergone secondary necrosis was not necessary. This is an important observation because a number of serum opsonins have been reported to bind to late apoptotic or secondary necrotic neutrophils, including C1q, the pentraxins, C-reactive protein and pentraxin-3 (18, 23, 34). Restoration of phagocytic capacity of Dex-DMϕ by a high molecular mass serum fraction raised the possibility of a requirement for a C4BP-protein S complex, which has been reported to inhibit phagocytosis of apoptotic lymphocyte cell lines (36). Our data showing the presence of protein S in the high molecular mass fraction would imply that the C4BP-protein S complex can augment phagocytosis under some circumstances. Because protein S binding can be demonstrated following incubation of neutrophils cultured in the absence of serum with either the high molecular mass fraction from gel filtration or purified protein S, one possibility is that under certain conditions, protein S can dissociate from C4BP and subsequently oligomerize at the apoptotic neutrophil surface (35). Our data clearly demonstrate that protein S alone is able to confer the augmentation of phagocytosis of apoptotic neutrophils that we observe.

The importance of complement proteins in apoptotic cell opsonization has been highlighted in studies of complement deficiency. In C1q deficiency, impaired clearance of apoptotic cells is thought to contribute to the development of an systemic lupus erythematosus-like autoimmune disease (27). For Dex-DMϕ, C1q did not restore levels of phagocytosis to those observed in the presence of serum even when C1q-binding proteins such as pentraxin-3 or fibronectin (37, 38) were present. Moreover, C1q-deficient mouse serum was able to confer phagocytic activity, demonstrating that
CLq was not required for efficient phagocytosis of apoptotic neutrophils by Dex-MDMφ. We were also unable to demonstrate a role for opsonization of apoptotic neutrophils with C3bi for removal through CR3 and CR4, as reported by Elkon and colleagues (11). Furthermore, the data presented in this study argue against a role for IgG, pentraxin-3, fibronectin, annexin I, platelet-derived factors, and immune complexes in phagocytosis of apoptotic neutrophils by Dex-MDMφ.

We believe that this is the first report demonstrating a switch in the molecular mechanism used by human MDMφ for apoptotic cell clearance. Our observations are clearly different from the induction of phosphatidylserine-dependent recognition of apoptotic murine thymocytes by bone marrow-derived murine macrophages treated with β1,3 glucan reported by Fadok et al. (39). Treatment with β1,3 glucan did not increase phagocytic potential when compared with untreated bone marrow-derived macrophages, but did alter the molecular mechanism used. In contrast, our findings demonstrate that phagocytosis of apoptotic cells by Dex-MDMφ is profoundly augmented by glucocorticoids, promoting a critical switch from a protein S-independent to a protein S-dependent recognition pathway.

The tissue microenvironment has the potential to influence the mechanisms involved in apoptotic cell removal and thus apoptotic cell clearance capacity. The cytokine and matrix composition will determine the differentiation status of phagocyte populations during progression of an inflammatory response. Interestingly, protein S-dependent recognition of an apoptotic B cell line was previously characterized in MDMφ generated by differentiation in M-CSF (35), which promotes the development of M2 macrophages that have anti-inflammatory phenotype properties and respond to TLR stimulation by producing IL-10 (40). We find that when MDMφ were cultured in the presence of autologous serum, apoptotic cell recognition pathways that are used are predominantly protein S dependent-independent (as shown in Fig. 1). In contrast with dextreated MDMφ, these MDMφ have a more proinflammatory phenotype and release IL-12 in response to TLR stimulation, suggesting that protein S-dependent recognition pathways may be restricted to macrophase phenotypes associated with resolution of inflammation.

The production and release of potential opsonins (complement components, pentraxins, annexins, protein S, etc) are also regulated during inflammation. A number of reports indicate that inflammation and the coagulation cascade are closely regulated, particularly during the acute-phase response. Protein S is produced in the liver and by endothelial cells (41). Production of both protein S and C4BP in the liver appears to be controlled by inflammatory mediators, including IL-6 (42, 43). Levels of protein S are reduced in patients with ischemic stroke (44) and in patients with sepsis (45), possibly via the effects of TNF on endothelial cells (46). In contrast, glucocorticoids have been reported to elevate levels of protein S (47). Based upon data presented in this work, we propose that a major effect of glucocorticoids on macrophase differentiation is the induction of the capacity to recognize a distinct set of molecular cues that are presented on the apoptotic cell surface. Apoptotic cells display a complex surface molecular signature as a consequence of cell death with altered expression of receptors together with binding (or opsonization) of a number of different proteins. One implication of our observation is that the surface molecular signature of an apoptotic cell may be interpreted differently by different phagocyte populations.

We have also examined the effects of treatment of differentiated MDMφ with Dex for 24 h (6) upon acquisition of the capacity for protein S-dependent phagocytosis of apoptotic cells. Our data suggest that augmentation of phagocytosis observed following short-term treatment is also associated with use of a protein S-dependent pathway for recognition of apoptotic cells. Because both untreated MDMφ and Dex-MDMφ populations examined in this study express Mertk, the reason that Dex-MDMφ are enabled to use a protein S-dependent clearance pathway is not clear. One possibility is that the observed up-regulation of Mertk expression on the surface of Dex-MDMφ may be sufficient to confer phagocytic potential. Alternatively, Mertk may interact with other receptors on the cell membrane following glucocorticoid treatment. Ligand-activated Mertk forms dimers in the membrane, resulting in Mertk autophosphorylation and activation (48), and may heterodimerize with other Tyro3/Axl/Mer family receptors or cooperate with other receptors involved in the phagocytic process, such as scavenger receptor A (49) or αvβ3 (50). Induction of cooperative action of receptors may allow regulation of phagocytosis of apoptotic cells in response to different environmental cues encountered during the inflammatory response.

Alternatively, glucocorticoids may influence engagement of downstream signaling pathways critical for Mertk-dependent phagocytosis. We have previously demonstrated that glucocorticoid-treated MDMφ exhibited reduced phosphorylation and localization of paxillin and pyk2 to podosome-like adhesion structures, together with increased Rac activity (7). Interestingly, Rac guanine nucleotide exchange factor Vav1 is activated downstream of Mertk (51). Mertk has also been reported to induce FAK phosphorylation and recruitment to αvβ3 and formation of p130Cas/CrkII/Dock180 complex (50, 52). One possibility is that phosphorylation of Mertk at Tyr667 in the absence of assembly of adhesion structures promotes MDMφ phagocytic activity (52).

Induced expression of Mertk and protein S by glucocorticoids may promote acquisition of a negative-feedback pathway to both switch off proinflammatory cytokine production (53) and enhance phagocytic capacity for apoptotic cells. The efficacy of glucocorticoids in treatment of autoimmune diseases such as systemic lupus erythematosus that are characterized by impairment of apoptotic cell clearance may be due, in part, to engagement of these pro-resolution mechanisms. Manipulation of the Mertk pathway may represent a novel approach to engage aspects of glucocorticoid action that favor resolution of inflammation without promoting deleterious side effects.

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Disclosures

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References


