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Glucocorticoid Augmentation of Macrophage Capacity for Phagocytosis of Apoptotic Cells Is Associated with Reduced p130Cas Expression, Loss of Paxillin/pyk2 Phosphorylation, and High Levels of Active Rac

Katherine M. Giles,* Katherine Ross,* Adriano G. Rossi,* Neil A. Hotchin,† Christopher Haslett,* and Ian Dransfield2*

Phagocytic clearance of apoptotic granulocytes has a pivotal role in determining an inflammatory outcome, resolution or progression to a chronic state associated with development of fibrotic repair mechanisms, and/or autoimmune responses. In this study, we describe reprogramming of monocyte to macrophage differentiation by glucocorticoids, resulting in a marked augmentation of their capacity for phagocytosis of apoptotic neutrophils. This monocyte/macrophage phenotype was characterized by decreased phosphorylation, and therefore recruitment of paxillin and pyk2 to focal contacts and a down-regulation of p130Cas, a key adaptor molecule in integrin adhesion signaling. Glucocorticoid-treated cells also displayed higher levels of active Rac and cytoskeletal activity, which were mirrored by increases in phagocytic capability for apoptotic neutrophils. We propose that changes in the capacity for reorganization of cytoskeletal elements induced by glucocorticoids are essential for efficient phagocytic uptake of apoptotic cells. The Journal of Immunology, 2001, 167: 976–986.

The acute inflammatory response provides a defense mechanism against microbial infection or tissue injury involving the rapid and coordinated recruitment of granulocytes and other inflammatory cells in response to chemokines and other inflammatory mediators. This response is normally self-resolving, but the pathogenesis of a number of diseases such as asthma, emphysema, and rheumatoid arthritis is characterized by a persistent accumulation of inflammatory cells presumably as a result of failure of the natural resolution process (1–3). During normal inflammatory resolution, clearance of extravasated granulocytes requires the induction of apoptosis and consequent uptake by macrophages (4). Apoptotic cell death, in contrast to necrosis, is associated with maintenance of cell membrane integrity (4) and down-regulation of granulocyte secretory function (5), thereby inhibiting potential exacerbation of the inflammatory response through the release of cytotoxic granule contents and proinflammatory cytokines. Additionally, macrophage ingestion of apoptotic cells, unlike phagocytosis of necrotic cells or opsonized particles, does not induce proinflammatory mediator production (6) and can functionally down-regulate cytokine release induced by LPS or opsonized cells (7). Failure to clear apoptotic cells may result in secondary necrosis with predictable consequences in terms of tissue injury and/or initiation of autoimmune processes when phagocyte clearance processes in vivo are overwhelmed by excess apoptotic cell load (8–10). Clearly, if induction of apoptotic pathways is to be considered as a potential therapy for cancer or inflammatory disease, a parallel strategy to maximize phagocytic clearance is likely to be required to avoid the deleterious consequences of necrotic cell death.

A number of cell surface molecules have been proposed to mediate the uptake of apoptotic cells; these include lectins (11), αβ3 integrin/CD36/thrombospondin complex (12, 13), phosphatidylinerine receptors (14), scavenger receptors (15), receptors for oxidized lipids (16), CD14 (17), CD29 (18), the ABC1 transporter (19), and receptors for complement components C3bi (CR3/CR4) (20) and C1q (21). The lack of complete inhibition of phagocytosis by soluble ligands or blocking mAb points to functional redundancy, and we have suggested that a more effective strategy for altering clearance of apoptotic cells would be manipulation of macrophage phagocytic potential (22). Although cytokines such as TNF, GM-CSF, TGF-β, or IL-1 may be used to augment phagocytic clearance (23), the effects have been small and the pleiotropic consequences of some of these agents may restrict their potential therapeutic value. Our previous studies have revealed that disruption of cytoskeletal and adhesion contacts in monocye-derived macrophages by elevation of intracellular cAMP inhibits phagocytosis of apoptotic cells (24). In contrast, ligation of CD44 (25) or adhesion to fibronectin (26) results in a rapid and dramatic augmentation of apoptotic cell uptake. For example, CD44 induces a 400% increase in phagocytic index within a 30-min assay period. If similar augmentation of phagocytic activity were attainable in vivo, the potential for clearance of apoptotic cells over the course of an inflammatory response would be considerable. Recent genetic studies in Caenorhabditis elegans have further suggested a role for adhesion signaling in the control of phagocytosis. Ced-5, -2, and -10, members of a family of genes required for clearance of cellular corpses during development, are highly homologous to mammalian proteins DOCK180 (27) (myoblast city protein in Drosophila) (28), Crk, and Rac (29), involved in mediating integrin signaling in mammalian cells. Integrin ligation induces the
formation of a multiprotein complex involving DOCK180 and Crk, the adapter protein p130Cas, and the guanine nucleotide exchange factor C3G (30–33). Assembly and membrane localization of this complex activates the GTPases Rac (34, 35) and Rho (36), inducing membrane ruffling and lamellipodia formation (37) required for spreading, adhesion, and cell migration. Ced-5 and ced-2 have been shown to interact and activate ced-10 GTPase activity in vitro (29), and ced-5, –2, and –10-deficient animals have defects in both cell migration and phagocytosis (27, 29).

Glucocorticoids represent a powerful antiinflammatory treatment due to their capacity for inhibition of inflammatory cell recruitment and down-regulation of production and responsiveness of cells to proinflammatory cytokines (38). We have recently described a novel glucocorticoid receptor-dependent promotion of macrophage capacity for phagocytosis of apoptotic cells following short-term exposure of macrophages to glucocorticoids (39). In the present study, we show that long-term exposure of monocytes to the synthetic glucocorticoid dexamethasone (DX) reprograms monocyte differentiation toward a proresolution phenotype, exhibiting increased phagocytosis of apoptotic cells. Monocytes treated with DX represented a homogeneous cell population characterized by a more rounded appearance. Marked down-regulation of expression of p130Cas, which is required for integrin adhesion signaling through the DOCK180/Crk/C3G complex, together with reduced phosphorylation and recruitment of paxillin and pyk2 to sites of adhesion, may account for this phenotypic alteration. We therefore propose that the dramatic increase in phagocytic potential in DX-treated monocyte-derived macrophages results from changes in the capacity for adhesion-dependent reorganization of cytoskeletal elements that are then available for coordinated phagocytic uptake of apoptotic cells.

Materials and Methods

Abs and other reagents

Reagents were obtained from Sigma (Poole, U.K.), unless otherwise stated. Iscove’s DMEM (IDMEM) was from Life Technologies (Paisley, U.K.). DX and percoll were from Amersham Pharma Biotech (Buckingham, U.K.). DX was obtained from David Bull Laboratories (Warwick, U.K.). Primary Abs were from the following sources: p130Cas, paxillin, Pyk2, Rac, and RC-20 (anti-phosphotyrosine) mAb were from Transduction Laboratories (supplied by Becton Dickinson, Oxford, U.K.); CrkL and Pyk2, Rac, and RC-20 (anti-phosphotyrosine) mAb were from Transduction Laboratories (supplied by Becton Dickinson, Oxford, U.K.); Crk, the adapter protein p130Cas, and the guanine nucleotide exchange factor C3G (35), β1 (12G10), and class II (WR18) were from Serotec (Oxford, U.K.). Control mouse IgGs (IgG1 and IgG2a), BerMac3 (CD163) and rabbit Igs, and F(ab')2 goat anti-mouse Ig FITC and HRP conjugates were from Dako (Ely, U.K.). The following monoclonals were generously provided as gifts: 5A4 (CD44; G. Dougherty, University of California, San Francisco, CA), SMb and 15.2 (CD36 and CD54, respectively; N. Hogg, Imperial Cancer Research Fund (London, U.K.), 23C6 (CD51/61; M. Horton, UCL, London, U.K.), PM6/13 (CD61; M. Wilkinson, Wellcome Trust, London, U.K.), 3G8 (CD16; J. Unkeless, Mount Sinai Medical School, New York, NY), UCHM1 (CD14; P. Beverley, UCL), 6D3 (CD14; C. Gregory, Nottingham, U.K.). Secondary anti-rabbit HRP was from Amersham Pharma Biotech.

Cell isolation and culture

Mononuclear and polymorphonuclear leukocytes were isolated as previously described (25). In brief, erythrocytes were sedimented from freshly drawn peripheral blood, with 0.6% (w/v) dextran T500, followed by fractionation of leukocytes on a discontinuous Percoll gradient (prepared in 0.6% (w/v) dextran T500, followed by fractionation of neutrophils from the 63/73% interface, and washed 3 times in PBS (without Ca²⁺/Mg²⁺) before culture. Neutrophils (re-suspended at 4 x 10⁶ cells/ml in IDMEM containing 10% autologous se-

<sup>7</sup> Abbreviations used in this paper: DX, dexamethasone; IDMEM, Iscove’s DMEM; PAK, p21-activated kinase.

Macrophage phagocytosis assay

Monocyte-derived macrophages cultured in 48-well tissue culture plates, as described above, were cultured in the presence or absence of DX, or 10 μM RU38486 for varying periods of time. For experiments using inhibition of phagocytosis, macrophages were washed once, then incubated with phagocytosis inhibitors (at the concentrations described in figure legends) for 15 min before the phagocytosis assay. The macrophage monolayer was then overlaid with apoptotic neutrophils (washed and resuspended at a final concentration of 4 x 10⁶/ml in IDMEM) and incubated at 37°C, 5% CO₂ for 20 min. Noningested neutrophils were removed by washing in IDMEM, and monolayers were then fixed in 2.5% glutaraldehyde. The percentage of phagocytosis of neutrophils stained for myeloperoxidase activity with 0.1 mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxide was quantified microscopically by counting at least 500 cells in randomly selected fields per well, and an average between the duplicate wells was calculated. Phagocytic index was calculated as: (average number of neutrophils phagocytozed per macrophage) x (x% macrophages that had phagocytozed one or more neutrophil). Controls for inhibitors used were as follows: All mAb used have previously been shown to inhibit apoptotic cell phagocytosis and were used at concentrations that were deemed to be saturating by flow cytometry. The following inhibitors were found to be functionally active at concentrations used in this study. RGDS (integrin inhibition peptide) was shown to inhibit αvβ₃ integrin-mediated adhesion of T lymphocytes to fibronectin. Phospho-lat-eri-serine was shown to inhibit binding of FITC annexin V to apoptotic neutrophils in flow cytometric analysis. Dextran sulfate inhibited uptake of acetylated low density lipoprotein by monocyte-derived macrophages. Other reagents (glyburide and glucosamine) were used at concentrations that have previously been shown to exert inhibitory effects.

Flow cytometry

Flow cytometry was performed essentially as described (25), with all incubations conducted on ice to prevent internalization of Ab. Macrophages were detached from tissue culture plastic using PBS containing 2 mM EDTA and 0.5% serum. After washing with ice-cold PBS containing 0.2% (w/v) BSA and 0.1% (w/v) sodium azide cells (10⁷ assay) were preincubated for 30 min with various concentrations used in this study. RGDS (integrin inhibition peptide) was shown to inhibit αvβ₃ integrin-mediated adhesion of T lymphocytes to fibronectin. Phospho-lat-eri-serine was shown to inhibit binding of FITC annexin V to apoptotic neutrophils in flow cytometric analysis. Dextran sulfate inhibited uptake of acetylated low density lipoprotein by monocyte-derived macrophages. Other reagents (glyburide and glucosamine) were used at concentrations that have previously been shown to exert inhibitory effects.

Electron microscopy

Macrophages cultured on glass coverslips in the presence or absence of 1 μM DX for 5 days were fixed with 3% glacial acetic acid in 0.1 M sodium cacodylate buffer, pH 7.4, for 3 h, and postfixed with 1% osmium tetroxide in 0.1 M cacodylate buffer for 2 h. After dehydration in an ascending acetone series, and critical point drying with CO₂, samples were sputter coated with 20 nm gold/palladium and examined using a Phillips 505 scanning electron microscope.

Immunoprecipitation and Western blotting

Adherent macrophage cultures were washed with PBS containing 0.1 mM NaVO₃ plus protease inhibitor cocktail (Boehringer Mannheim, Mannheim, Germany), and were lysed by incubation with PBS containing 1% Nonidet P-40, 0.1 mM NaN₃, and protease inhibitor cocktail, 10 μM on ice. Membrane and nuclear material were removed by centrifugation at 14,000 x g, 4°C, 30 min. Lysates were preclariﬁed by incubation with protein A agarose-coupled rabbit anti-mouse IgG, 4°C, 30 min. The resulting lysates were tested for protein concentration using a detergent-compatible protein estimation kit (Pierce, Rockford, IL), and equilibrated to contain equivalent levels of protein. A total of 100 μl lystate (100–150 μg total protein) was incubated with 1 μg of either mouse IgG control, anti-paxillin, or pyk2 mAb, 4°C, 30 min, shaking. Immunoprecipitation was achieved by incubation for 30 min with protein A-coupled rabbit anti-
mouse IgG (Sigma), and washed twice in TBS containing 0.1% Triton X-100, and once in 25 mM Tris, plus 0.05% SDS. Samples were resolved using a 9% reducing polyacrylamide gel and transferred electrophoretically (50 V for 1 h) onto nitrocellulose (Amersham Pharmacia Biotech). For detection of phosphotyrosines, membranes were blocked with TBS plus 0.05% Tween 20 (TBS-T) and all other blots with TBS-T plus 10% nonfat dried milk powder (w/v).

Assay for detection of activated Rac

Adherent macrophage cultures were lysed in radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Boehringer Mannheim) plus 1 mM PMSF. Lysates were cleared of membrane and nuclear material by centrifugation, total protein was estimated, and levels were equilibrated as described for immunoprecipitation. A total of 20 µl of refreshed medium was removed for estimation of total Rac protein, and the remaining (~300 µg) was incubated with GST-p21-activated kinase (PAK) (cdc42 or Rac interacting binding domain) fusion protein coupled to Sepharose beads, 4°C, 1 h, shaking. Beads were washed four times in ice-cold Tris buffer (50 mM Tris, 150 mM NaCl, 10 mM MgCl2, protease inhibitor mixture, 1 mM PMSF), and the amount of active Rac bound to the PAK cdc or Rac interacting binding domain quantified by SDS PAGE and Western blotting, as described for immunoprecipitation.

RNA isolation and RT-PCR

Macrophages were washed once in ice-cold PBS, lysed, and RNA extracted using TRIzol (Life Technologies; protocol as manufacturer’s instructions). RNA was DNase treated to remove genomic DNA for 1 h, 37°C, and the resulting RNA was used in RT-PCR reaction using Life Technologies One Step RT-PCR kit (protocol as manufacturer’s instructions). Primers used (35 cycles, annealing 53°C) were as follows: DOCK180, 5'-GGACGAGGAGACGAACAG, 3'-AAAGCGATTCG GTGTAGTTA; M-DOCK, 5'-TGCTGAAGTGGCGTATGAAG, 3'-CCTGACGACAAATGAACT; actin, 5'-CCACCAACTGGGAGCA CATTG, 3'-GTCTCAAACATGATCTGGGTCACT.

Results

Glucocorticoids augment phagocytosis of apoptotic cells in a time-dependent manner

Monocytes isolated from peripheral blood were cultured in vitro for up to 5 days in the presence of the synthetic glucocorticoid DX, and macrophage potential for phagocytosis of apoptotic cells was determined. Preliminary experiments revealed that macrophages treated with DX for >24 h were highly phagocytic, leading to some cell detachment during our standard assay (data not shown). We therefore reduced the assay time from 30 to 20 min to minimize cell loss and reveal differences between treated cells. DX treatment consistently increased monocyte-derived macrophage phagocytosis of apoptotic neutrophils in a manner that was related to the duration of treatment (Fig. 1A), and blocked by addition of the steroid receptor antagonist RU38486 (10 µM), indicating that a glucocorticoid receptor-dependent process mediated these effects (results not shown). In particular, we noted a striking effect of exposure of freshly isolated monocytes to DX for 120 h upon phagocytic potential. We therefore investigated whether a time window for steroid exposure early in maturation was sufficient to generate a highly phagocytic phenotype. Monocytes were incubated with DX for periods of 24 h (0–24, 24–48, 48–72, 72–96, and 96–120). To ensure that observed changes were the result of DX exposure exclusively during the stated time window, the steroid receptor antagonist RU38486 was added following removal of DX-containing media. The addition of the antagonist alone had no significant effect (data not shown). Augmentation of apoptotic cell phagocytosis was much less pronounced when DX was added later in the culture period, and that exposure of monocytes to DX within the first 24 h was sufficient to induce highly phagocytic macrophages (Fig. 1B). Comparison of phagocytic indices revealed that 5-day DX-treated monocytes/macrophages had a 9-fold higher capacity for apoptotic cell clearance in a 20-min assay (Fig. 1, C–E). Thus, glucocorticoids represent the most potent stimulus for augmentation of macrophage capacity for phagocytosis of apoptotic cells described to date. Moreover, glucocorticoid modulation of phagocytosis could have profound effects on clearance of apoptotic cells during the resolution of inflammation.

Glucocorticoids reprogram macrophage phenotype

Examination of macrophages by phase-contrast microscopy revealed that augmented phagocytic potential following 5-day treatment with DX was accompanied by distinct morphological changes consistent with the suggestion that DX influences in vitro differentiation of monocytes. Under our standard culture conditions (IDMEM containing 10% autologous serum) at 5 days, macrophages were composed of a heterogeneous population of cells with different morphologies: multinucleated giant cells, spread apparently motile cells with evidence of membrane ruffling, rounded less spread cells, and occasional dendritic-like cells (Fig. 2A). Microscopy-based quantitation revealed that large macrophages (>25 µm diameter) represented 29.5 ± 5.8% of cells present in 5-day monocyte/macrophage preparations (n = 4 separate experiments). Treatment of freshly isolated monocytes with DX for 5 days resulted in consistent morphological changes, with the absence of large multinucleated cell populations and reduced numbers of spread macrophages (large macrophages representing less than 2% of the cells present). In contrast, the proportion of rounded cells was increased (Fig. 2B). Further examination of macrophage morphology using scanning electron microscopy demonstrated that the rounded DX-treated cells were attached, with ruffled membranes and filopodial processes (Fig. 2D) when compared with untreated cells (Fig. 2C). An increase in the morphological homogeneity of DX-treated monocyte-derived macrophages was also reflected in the laser scatter properties of DX-treated cells and more uniform expression of a number of surface molecules, including CD14, when analyzed by flow cytometry (Fig. 2E). Analysis of the coefficient of variation for the fluorescence peaks (untreated vs DX-treated ± SEM) for CD14 (108 ± 14; 60 ± 5, n = 13), CD16 (96 ± 8; 62 ± 4, n = 11), HLA-DR (122 ± 11; 96 ± 6, n = 10), CD44 (99 ± 15; 66 ± 7, n = 6), and CD51 (81 ± 5; 53 ± 4, n = 11) confirmed this impression and would be consistent with reprogramming of monocyte differentiation by DX during in vitro culture.

Increased phagocytosis of apoptotic cells involves multiple phagocytic receptors

Flow cytometric analysis was further used to determine whether DX augmentation of phagocytosis was associated with increased expression of receptors previously implicated in the recognition process (see Ref. 22 for review and Table I). Although most receptors examined exhibited more uniform levels of surface expression, as described above, we did not observe changes in the percentages of positive cells following DX treatment. However, comparison of mean fluorescence intensity of binding revealed some consistent alterations in the levels of surface expression of certain receptors. HLA-DR and the macrophage differentiation markers FcyRIII (CD16) and BerMac (CD163) were expressed at slightly elevated levels on DX-treated macrophages (Table I). In contrast, we found significantly reduced expression of CD44, CD44v3, ICAM-1 (CD54), and integrin β3 subunit (CD61) (p < 0.05 using Student’s t test). Binding of the CD36 mAb Smød was always found to be lower than that of the IVC7 CD36 mAb, even when both Abs are used at saturating concentrations, suggesting that Smød may recognize an epitope that is not present on all CD36 molecules. Interestingly, although overall levels of CD36 expression (IVC7 staining) were decreased with DX treatment, binding of the Smød mAb was not reduced. Together these results indicated
that augmented phagocytic capacity was not associated with increased expression of putative apoptotic cell recognition receptors. However, since surface expression does not necessarily indicate the presence of functionally active receptors, we used specific mAb/soluble ligand inhibitors of apoptotic recognition pathways to define their contribution to DX-treated macrophage phagocytosis. Inhibitors of CD36 (SM∞ mAb, 1:50 ascites) and αvβ3 (0.5 mM RGDS peptide) did not prevent phagocytosis of apoptotic neutrophils by DX-treated macrophages (Table I), suggesting that this pathway does not play a major role in DX-augmented phagocytosis. In addition, 10 mM glucosamine exhibited only partial inhibition of DX-augmented phagocytosis, further suggesting that integrin-mediated recognition is not the dominant pathway utilized. However, it should be noted that although we have validated that RGDS is functionally active in preventing αvβ3-mediated T cell adhesion to fibronectin, in our experimental system RGDS does not inhibit untreated macrophage phagocytosis either (percentage of phagocytosis control 28.5 ± 4%, RGDS 27.5 ± 4%; mean ± SEM, n = 7). Therefore, these data cannot be taken to indicate that treatment of monocytes with DX induces a switch to αvβ3-independent recognition of apoptotic cells. Inhibition of CD14 with the 61D3 mAb also failed to inhibit DX-treated macrophage phagocytosis of apoptotic neutrophils, suggesting that glucocorticoid up-regulated phagocytosis was not CD14 dependent (data not shown). We observed a small inhibitory effect of 2 mM phospho-L-serine upon DX-induced phagocytosis of apoptotic neutrophils, implying that the phosphatidylserine receptor does not play a major role. However, it is possible that phospho-L-serine is not the most effective inhibitor of phosphatidylserine receptor function. Phagocytosis was markedly inhibited in the presence of 500 μg/ml fucoidan (31% of control, p < 0.01), implicating scavenger receptor involvement. However, other scavenger receptor ligands, including dextran sulfate (Table I), only partially inhibited phagocytosis. These data suggest broader effects of fucoidan, which may also inhibit other carbohydrate recognition molecules (for example, selectins). One interpretation of these data is that DX augmentation may involve multiple receptor pathways acting cooperatively. Alternatively, DX might promote engagement of a novel receptor pathway to provide more efficient apoptotic cell recognition.

Glucocorticoids alter macrophage cytoskeletal organization

The distinct morphological appearance of DX-treated macrophages shown in Fig. 2 suggested that control of adhesion was
altered following DX treatment. In untreated cells, visualization of focal contacts within macrophages cultured for 5 days on glass slides showed punctate (podosome-like) staining of actin (Fig. 3C) with concentric association of talin (Ref. 24 and our unpublished data) and paxillin (Fig. 3D), probably representing sites of cell-substratum contact. Paxillin and other proteins associated with adhesion (vinculin and tyrosine-phosphorylated proteins (data not shown)) were also observed toward the cell periphery (see Fig. 3D), consistent with the adherent phenotype of macrophages. In contrast, DX-treated cells showed an absence of actin- and paxillin-containing podosomes (Fig. 3, E and F), suggesting an altered organization of adhesion structures.

Altered cytoskeletal protein phosphorylation and organization in DX-treated macrophages

Since paxillin recruitment to sites of adhesion is regulated by phosphorylation, we next examined levels of expression and the tyrosine phosphorylation status of paxillin and pyk2 by Western blot analysis of immunoprecipitated proteins. Although paxillin and pyk2 are still expressed at equivalent levels in DX-treated macrophages (not shown), phosphorylation was found to be reduced in adherent DX-treated macrophages when compared with untreated macrophages (Fig. 4). The observed decrease in phosphorylation of paxillin and pyk2 was consistent with the altered distribution of paxillin in macrophages and raised the possibility that DX treatment disrupted adhesion-dependent signaling. The p130Cas/Crk/DOCK180 complex is a major mediator of adhesion signaling. We therefore examined the levels of expression of CrKL, CrkII, and p130Cas by Western blot analysis, and since we were unable to reproducibly immunoblot DOCK180 (data not shown), we used RT-PCR to test for mRNA for DOCK180 and M-DOCK. Although we did not see changes in levels of CrKL (Fig. 5A) or CrkII (data not shown), p130Cas expression was markedly reduced in DX-treated macrophages (Fig. 5B). No differences in the levels of mRNA for DOCK180 or M-DOCK were noted in PCR analysis (Fig. 5C), although differences may be apparent at the level of protein. Despite these marked changes in cytoskeletal organization and phosphorylation, we were surprised to find that DX-treated cells were extremely active in formation of lamellipodia and cellular extensions necessary for phagocytosis in time lapse video
microscopy analysis (data not shown). To test whether the actin-regulatory machinery remained functional in DX-treated macrophages, we assessed the levels of activity of the Rho family GTPase Rac, which is involved in membrane ruffling and extension of cellular processes. Using pull-down assays with p21-activated kinase-GST agarose, we found that DX caused a marked increase in the amount of active Rac detectable within macrophage lysates (Fig. 5D). The pronounced morphological alteration observed in DX-treated cells may reflect reprogramming of the capacity for adhesion-dependent signal transduction via down-regulation of p130Cas, reduced paxillin, and pyk2 phosphorylation, and failure to form podosome-like adhesion structures. However, DX-treated macrophages were found to have high levels of active Rac that might contribute to the increased capacity for cytoskeletal reorganization necessary for phagocytosis.

Discussion

In this study, we present evidence that a macrophage phenotype with augmented phagocytic potential for clearance of apoptotic cells is induced following exposure to DX. We believe that DX exerts distinct regulatory mechanisms upon macrophage behavior, depending upon the stage of monocyte maturation. In our previously published work, monocytes/macrophages were treated with DX from 96 to 120 h, leading to a relatively modest increase in phagocytic potential (39). In contrast with the morphological changes described in this work for cells treated with DX immediately following isolation, no gross changes in morphology are observed following exposure of 96-h monocytes/macrophages to DX (data not shown). Importantly, we demonstrate that treatment of monocytes with DX for the first 24 h of the 5-day culture period following isolation from peripheral blood is critical for induction of this phenotype, suggesting that glucocorticoids, acting via glucocorticoid receptors, have the potential to reprogram monocyte differentiation. In support of our suggestion, we observe that DX-treated monocytes mature into macrophages that exhibit a uniform morphological appearance consisting of smaller, more rounded cells with more homogeneous laser scatter properties in flow cytometric analysis. Although a number of macrophage surface receptors are expressed at slightly reduced levels in DX-treated cells when compared with untreated cells, the range of expression within the population was found to be less in DX-treated monocytes/macrophages, indicating that functional homogeneity is matched by cell surface phenotype. In addition, the specific increase in BerMac (CD163) expression following DX treatment lends further support to the suggestion of a reprogramming event. However, our analysis failed to reveal any single surface molecular change that would define a prophagocytic phenotype.

In preliminary experiments, we noted that highly phagocytic macrophages were prone to detach during washing following a 30-min assay. One speculation would be that internalization of plasma membrane during phagocytosis of apoptotic cells may compromise cell adhesion. In addition, the marked functional alterations that we observe following glucocorticoid treatment of monocytes further illustrate a close association between control of monocyte/macrophage adhesion and clearance of apoptotic cells. Many of the receptors implicated in phagocytosis also have key roles in macrophage adhesion and migration (22). In this study, we demonstrate that augmentation of phagocytic potential is associated with decreased tyrosine phosphorylation of paxillin and pyk2, proteins that represent important components of adhesion contacts (for review, see Ref. 40), and decreased expression of p130Cas, a mediator of adhesion signaling (32). Immunofluorescence analysis revealed that adhesion structures containing localized paxillin and actin were altered in DX-treated macrophages. Reduced expression of p130Cas would be predicted to disrupt Crk/DOCK180

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<th>Expression</th>
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*“Control” receptor expression represents expression on 5-day DX-treated monocyte-derived macrophages relative to untreated 5-day monocyte-derived macrophages. **Control” phagocytosis represents phagocytosis of apoptotic neutrophils by 5-day DX-treated monocyte-derived macrophages in the absence of inhibitor. **NA, Not available.
complexes, which together with reduced phosphorylation of paxillin and pyk2 may have implications for control of the turnover of adhesion structures in macrophages (shown schematically in Fig. 6). Since decreased p130Cas expression in DX-treated macrophages is associated with augmentation of phagocytic capacity, specific recruitment of p130Cas to focal contacts following adhesion to matrix may mimic loss of p130Cas observed in DX-treated cells and influence the availability of other components to drive cytoskeletal reorganization necessary for phagocytosis. We have tried to define whether cytoskeletal components we have examined contribute directly to phagocytosis. However, these studies are made difficult by the morphological and functional heterogeneity of macrophage preparations. Furthermore, cytoskeletal elements present within internalized apoptotic neutrophils complicate interpretation of observed staining patterns. Both Crk and p130Cas have previously been shown to influence the capacity for actin reorganization in rat-1 fibroblast cells (41, 42); therefore, down-regulation of the central signaling molecule p130Cas is likely to have important implications for the control of adhesion and migration in DX-treated monocytes. We propose that the repertoire of adhesion receptors that are engaged on the macrophage surface might control phagocytic potential indirectly by releasing or sequestering key regulatory molecules such as p130Cas from focal adhesion complexes.

Time-lapse video microscopy reveals that despite the small rounded appearance of DX monocytes/macrophages, these cells remain extremely membrane active, rapidly extending and retracting cellular processes (data not shown). Increased levels of active Rac in DX-treated macrophages lend support to the suggestion that although recruitment of proteins such as paxillin to podosome adhesion-signaling complexes does not occur in the absence of p130Cas, Rac may still drive the extension and retraction of processes observed in DX-treated cells. One possibility is that other p130Cas-like adapters such as HEF1 and Efts/Sin (Ref. 32 and}

**FIGURE 3.** Effects of DX on localization of actin and paxillin in macrophages. Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1 μM DX. Localization of actin and paxillin was determined using either rhodamine phallolidin (C and E) or anti-paxillin mAb (D and F) together with fluorescence microscopy. Staining observed with control mAb is shown in A and B. These representative micrographs illustrate typical punctate actin staining of contact sites similar to podosomes in control macrophages (C and D) that are absent in DX-treated macrophages (E and F). Paxillin is also localized to smaller focal adhesion-like structures near the cell periphery in control macrophages that are less well defined in DX-treated macrophages.

**FIGURE 4.** DX down-regulates phosphorylation of pyk2 and paxillin. Adherent peripheral blood monocyte-derived macrophages were cultured for 5 days in the presence or absence of 1 μM DX. Pyk2 and paxillin were immunoprecipitated from cell lysates, and protein phosphorylation of immunoprecipitated proteins was analyzed by SDS-PAGE together with Western blotting using the anti-phosphotyrosine mAb RC-20 and ECL detection. In this gel, IgG denotes protein phosphorylation patterns associated with a nonbinding IgG1 control mAb. The band at ~85 kDa, present to some extent in all immunoprecipitates, probably represents a nonspecific component. C lanes are from untreated control macrophages, and D lanes are from DX-treated macrophages.
references therein), present in macrophages, may allow the recruitment of Rac/Crk/DOCK180 specifically to membranes in a manner that facilitates phagocytosis of apoptotic cells and possibly other particles (43). Indeed, in preliminary experiments using low density IgG-opsonized particles, we have found that DX-treated monocytes/macrophages are more efficient phagocytes (untreated, 24.5% phagocytosis; DX treated, 53.5% phagocytosis, n = 4).

Importantly, these data suggest that expression or phosphorylation of p130Cas may have a negative regulatory role upon macrophage phagocytic potential. We would speculate that decreased expression/phosphorylation of p130Cas together with augmented Rac activity defines a subpopulation of macrophages highly competent for phagocytosis of apoptotic cells.

Interestingly, recent data suggest that dendritic cell capacity for presentation of apoptotic cell-derived material via MHC class I or class II may depend on αβ- or β2-mediated internalization mechanisms (44). Our data suggest that in addition to differences in integrin usage, DX-treated macrophages lack p130Cas, a molecule that is recruited following apoptotic cell binding to dendritic cells (45). One possibility is that p130Cas expression may determine the cellular consequences of apoptotic cell handling in macrophages and other cell types. Recent studies have shown that immature dendritic cells treated with glucocorticoids down-regulate the capacity for production of IL-12, and consequently induce a regulatory phenotype (46–48). Changes in the adhesion status of macrophages described in this work may also have important consequences for other macrophage functions that influence the progression of inflammation. Loss of podosome adhesion structures observed in Wiskott-Aldrich syndrome macrophages leads to defective chemotaxis responses as a result of loss of the capacity for polarization (49). Whether DX-treated macrophages show increased potential for directed migration necessary for recruitment or emigration of cells to and from the inflamed site has not been assessed and may also be dependent on the effects of DX upon expression of chemokine receptors and activation of kinases such as extracellular signal-related kinase 1 and 2. One speculation would be that in addition to augmented capacity for clearance of apoptotic cells, emigration of DX-treated macrophages from inflamed sites to draining lymph nodes might be altered. Formation of podosome structures in osteoclasts has also been shown to require p130Cas/
Crk/DOCK180 (50). One interesting possibility is that the detrimental effects of prolonged steroid treatment on bone homeostasis may be mediated through disruption of important adhesion events associated with bone homeostasis.

A striking feature of DX-treated macrophage phenotype was the lack of large multinucleated cells observed in culture when compared with untreated cells. Preliminary data indicate that formation of multinucleated macrophages promoted by treatment with IFN-γ (51), or following stimulation with CD98 (52), is inhibited by DX, consistent with data on alveolar macrophages (53), and suggests a dominant regulatory effect of glucocorticoids upon macrophage differentiation. Although previous reports have suggested that glucocorticoids drive monocyte apoptosis (54), we believe that these apparently discrepant results are accounted for by differences in the culture media used. In this present study, monocytes were cultured in 10% autologous serum with no differences in the numbers of cells recovered with or without DX (control, 152 ± 16 cells/field; DX, 140 ± 16 cells/field; average counts ± SEM from 10 separate experiments). Moreover, the phenotype we observe is unlikely to represent selection of a subpopulation of monocytes that
are resistant to glucocorticoid-induced death. The percentage of monocyte-derived macrophages that phagocytose apoptotic cells is increased at least 3-fold by DX treatment, requiring that most of the cells in the initial population be lost if selection of a phagocytic phenotype occurred. Although production of modulatory cytokines (e.g., TGFβ) following DX treatment might influence monocyte differentiation and phagocytic potential, exposure of monocytes to TGFβ or IL-10 does not induce the phagocytic functional phenotype described in this study (not shown).

In conclusion, we report the novel finding that early exposure of monocytes to glucocorticoids induces a proresolution phenotype. The proportion of monocytes/macrophages capable of phagocytosis of apoptotic cells and the phagocytic index are increased dramatically following DX treatment. The combined effect would give rise to a markedly enhanced potential for clearance of apoptotic cells from an inflammatory site following DX treatment, with considerable implications for therapeutic strategies for manipulation of inflammatory processes in vivo. The phagocytic phenotype of DX-treated monocytes/macrophages was characterized by profound morphological changes, down-regulation of phosphorylation of paxillin and pyk2, and loss of p130Cas expression. We propose that the increased Rac activity we observe together with these changes in cytoskeletal changes may define a prophagocytic macrophage phenotype. These data further emphasize the importance of understanding the contribution of adhesion-related signaling pathways in the regulation of macrophage phagocytosis.

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References


