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Regulation of Macrophage Phagocytosis of Apoptotic Cells by cAMP¹

Adriano G. Rossi,² Judith C. McCutcheon, Noémi Roy, Edwin R. Chilvers, Christopher Haslett, and Ian Dransfield

Regulation of macrophage capacity to remove apoptotic cells may control the balance of apoptotic and necrotic leukocytes at inflamed foci and the extent of leukocyte-mediated tissue damage. Although the molecules involved in the phagocytic process are beginning to be defined, little is known about the underlying regulatory and signaling mechanisms controlling this process. In this paper, we have investigated the effects of treatment of human monocyte-derived macrophages with PGs and other agents that elevate intracellular cAMP on phagocytosis. PGE₂ and PGD₂ specifically reduced the proportion of macrophages that phagocytosed apoptotic cells. Similar results were obtained with the membrane-permeable cAMP analogues dibutyryl-cAMP and 8-bromo-cAMP but not with the cGMP analogue dibutyryl-GMP. Consistent with the observation that phagocytosis was inhibited by cAMP elevation, treatment of monocyte-derived macrophages with PGE₂ resulted in rapid, transient increase in levels of intracellular cAMP. These effects were not due to nonspecific inhibition of monocyte-derived macrophage phagocytosis given that ingestion of Ig-opsonized erythrocytes was unaffected. Elevation of cAMP induced morphologic alterations indicative of changes in the adhesive status of the macrophage, including cell rounding and disassembly of structures that represent points of contact with substrate containing actin and talin. These results strongly suggest that rapid activation of cAMP signaling pathways by inflammatory mediators regulates processes that limit tissue injury and that modulation of cAMP levels represents an additional therapeutic target in the control of resolution of inflammation. *The Journal of Immunology*, 1998, 160: 3562–3568.

The ultimate fate of the large numbers of recruited neutrophil granulocytes during inflammatory responses has been widely assumed to involve necrosis and disintegration in situ before cellular fragments are cleared by local phagocytes (1). However, the capacity of many granule contents to cause cellular injury (2) and cleave tissue matrix proteins into chemotactic fragments (3) implies that necrotic cell death would damage healthy tissues and exacerbate inflammatory processes. There is now compelling evidence that neutrophil apoptosis, which is associated with preservation of granule contents and down-regulation of effector function, including granule secretion (4, 5), limits the potential for neutrophil-mediated tissue damage during progression of inflammatory responses (reviewed in Ref. 6). An important consequence of the apoptotic process is cell surface alterations that lead to rapid recognition and phagocytosis of apoptotic neutrophils (4, 7). In contrast to macrophage phagocytosis of particles opsonized with C or Ig, this does not provoke the release of proinflammatory macrophage mediators (thromboxane B₂ or *N*-acetyl- β -D-glucosaminidase) (8), contributing to limitation of tissue injury and promotion of resolution of inflammation. There is clear histologic evidence for the involvement of this process in acute inflammation (9), and necrotic

neutrophils are observed at chronically inflamed sites; thus, the tissue load of apoptotic cells is likely to be a critical factor in determining the outcome of inflammation.

Potential control mechanisms for regulation of the recognition and phagocytic pathways by which apoptotic cells are removed include prolongation of myeloid cell functional longevity, such as phagocytic and secretory responses by inflammatory mediators and growth factors (10–12). Inhibition of the rate of apoptosis in these cell populations at inflamed sites would serve a dual purpose of regulating the potential for neutrophil-mediated damage at these sites and the numbers of neutrophils that are available for recognition by macrophages. Inflammatory mediators also act to regulate phagocytosis of apoptotic cells by macrophages. Multiple receptor pathways are implicated in the phagocytosis of apoptotic cells, although specific pathways may be dominant in certain environmental conditions (13, 14). For human monocyte-derived macrophages, we have proposed a model in which macrophage $\alpha_v\beta_3$ and CD36 act in concert to allow apoptotic cell recognition (7, 13). Modulation of receptor expression during monocyte differentiation may determine the phagocytic ability of macrophage populations at different anatomic sites. Induction of $\alpha_v\beta_3$ occurs concomitantly with the acquisition of the capacity to phagocytose apoptotic cells during in vitro culture of monocytes (15). For murine macrophages, different phenotypes may be induced by local microenvironmental stimuli. Bone marrow-derived macrophages, in contrast to those isolated from the peritoneal cavity which utilize a phosphatidylinositol 3-kinase based pathway, use an $\alpha_v\beta_3$ integrin-based recognition mechanism (16). In addition, phagocytosis of β -1,3-glucan particles can induce a switch between these two pathways (17). There is also clear evidence that macrophage phagocytic responses are also influenced by integrin-mediated signal transduction pathways (18). Thus, control of apoptotic cell removal may be achieved by regulation of macrophage phagocytic potential.

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In this study, we have investigated whether modulation of protein kinase activity had a regulatory role in the process of macrophage recognition of apoptotic cells. In particular, we have examined the effects of short term treatment of human monocyte-derived macrophages with PGs, which elevate intracellular cAMP. Our results suggest that specific inhibition of phagocytosis of apoptotic neutrophils following cAMP elevation may involve disassembly of cytoskeletal elements involved in cell-substratum interaction without inducing cell detachment. These data support a model in which the matrix microenvironment exerts control over the macrophage capacity for clearance of apoptotic cells. Thus, pharmacologic targeting of elements of the PKA pathway may represent a novel strategy for the modulation of macrophage capacity for the clearance of apoptotic cells.

Materials and Methods

Abs and other reagents

Iscove's DMEM, HBSS, and culture supplements were from Life Technologies, Paisley, U.K., and Falcon tissue culture plastic was from A. & J. Beveridge, Edinburgh, U.K. Rhodamine-phalloidin was obtained from Molecular Probes (Eugene, OR). Dextran T500 was from Pharmacia, Milton Keynes, U.K. Normal rabbit serum was from the Scottish Ab Production Unit, Lanarkshire, U.K. Rabbit anti-human erythrocyte membrane Ags and FITC-conjugated F(ab')₂ fragments of goat anti-mouse Ig were from Dako (High Wycombe, U.K.), MAb all of IgG1 isotype, 23C6 (CD51/CD61 complex) and 13C2 (CD51), were a gift from Dr. Mike Horton, Imperial Cancer Research Fund, London, U.K. PM6/13 (CD61) was a gift from Dr. Mike Wilkinson, Royal College of Surgeons, London, U.K. FA6-152 (CD36) was selected from Ab panels obtained from the Vth International Workshop on Leukocyte Differentiation Ags. PGs were purchased from Cascade Biochem., Reading, U.K. All other chemicals were obtained from Sigma Chemical, Poole, U.K.

Cell isolation

Mononuclear cells and neutrophils were isolated from peripheral blood as described (4, 19). Freshly citrated blood was centrifuged at $400 \times g$ for 20 min, and the platelet-rich plasma supernatant was used to prepare autologous serum by addition of calcium chloride (10 mM final concentration). Leukocytes were isolated after removal of erythrocytes by sedimentation using 6% (w/v) dextran T500 in saline by fractionation on a discontinuous gradient of isotonic Percoll solutions made in $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free PBS (CMF-PBS) Percoll concentrations of 49.5, 63, and 72.9% at $700 \times g$ for 20 min. Mononuclear cells were aspirated from the 49.5%/63% interface and neutrophils from the 63%/72.9% interface and washed three times in HBSS before culture. Neutrophils were cultured at $4 \times 10^6/\text{ml}$ in Iscove's DMEM containing 10% autologous serum at 37°C in a 5% CO_2 atmosphere for 18 to 24 h. Neutrophils used in the phagocytosis assay were >50% apoptotic as determined by morphologic appearance, CD16 "low" expression, annexin V positivity and >98% of cells retain membrane integrity as assessed by the vital dye trypan blue. Monocytes were enriched from the mononuclear cells by selectively attaching them to 24-well plates for 1 h at 37°C . The nonadherent lymphocytes were removed and the adherent monocytes were washed twice in PBS. Monocytes were then cultured for 5 to 7 days in Iscove's DMEM containing 10% autologous serum at 37°C in a 5% CO_2 atmosphere. Their maturation into macrophages was assessed by flow cytometry using a panel of myeloid-specific and activation markers including CD16 and CD51/CD61 (data not shown).

Assay for macrophage phagocytosis of apoptotic neutrophils

Phagocytosis of apoptotic neutrophils was assayed by minor modifications of previously described methods (4, 7). Neutrophils that had been cultured *in vitro* were washed once with Iscove's DMEM and resuspended at 4×10^6 cells/ml in Iscove's DMEM alone. Adherent macrophages were washed in Iscove's DMEM before addition of 1 ml of the above neutrophil suspension to each well. After 30 min of incubation at 37°C , the wells were washed four times with ice-cold CMF-PBS, fixed in 2.5% glutaraldehyde/PBS for 10 min, and then stained for myeloperoxidase at 37°C using 0.1 mg/ml dimethoxybenzidine and 0.03% (v/v) hydrogen peroxide in PBS. The percentage of macrophages that had phagocytosed myeloperoxidase-positive apoptotic neutrophils was quantified microscopically by examination of randomly selected fields and counting at least 500 cells/well. Results from each experiment were expressed either as the mean percentage

of phagocytic macrophages of triplicate wells or as the percentage phagocytosis relative to untreated controls.

Macrophage phagocytosis of IgG-opsonized erythrocytes

Erythrocytes were washed and resuspended at $2.5 \times 10^7/\text{ml}$ in Iscove's DMEM and incubated at 4°C with rabbit polyclonal anti-human erythrocyte Ab (1:100) for 30 min. Opsonized erythrocytes were then washed and resuspended at $1 \times 10^9/\text{ml}$ in Iscove's DMEM and incubated with macrophages at a macrophage-erythrocyte ratio of 1:2.

Flow cytometry and immunocytochemistry

Flow cytometry was performed as described previously (19, 20), with all incubations conducted on ice. Six-day-old monocyte-derived macrophages were detached from cell culture plates by vigorous pipetting after incubation on ice with PBS for 30 min. Macrophages (1×10^5) were washed with ice-cold PBS containing 0.2% (w/v) BSA and 0.1% (w/v) sodium azide and preincubated with 20% (v/v) normal rabbit serum to block "nonspecific" binding to Ig Fc receptors. Cells were then incubated with saturating concentrations of mAb for 30 min. After washing, cells were incubated with FITC-conjugated F(ab')₂ goat anti-mouse Ig (1:25) for 30 min and washed twice before flow cytometric analysis using an EPICS Profile II flow cytometer (Coulter Electronics, Luton, U.K.) (20). For indirect immunofluorescence analysis, macrophages were resuspended at $1 \times 10^6/\text{ml}$ of Iscove's DMEM and $10^5/\text{well}$ added to multispot microscope slides and allowed to adhere by incubation for 1 h at 37°C . After rinsing with Iscove's DMEM, cells were fixed in 1% formaldehyde in PBS for 10 min. Cells were permeabilized using 1% Triton X-100 in PBS for 5 min and then washed thoroughly in PBS. Slides were blocked in PBS containing 20% normal rabbit serum for 10 min before incubation with primary Ab optimally diluted in PBS containing normal rabbit serum. Bound primary Ab was detected using F(ab')₂ FITC-conjugated rabbit anti-mouse Ig Ab. For visualization of actin, rhodamine-phalloidin (0.15 μM) was included. Slides were mounted in 50% glycerol in PBS and examined using an Olympus BH-2 microscope with a fluorescent lamp attachment.

Treatment of monocyte-derived macrophages with agents that elevate intracellular cAMP

Macrophages were washed once with Iscove's DMEM and incubated for various times as stated in text at 37°C with dbcAMP,³ 8-Br-cAMP, or PGs diluted in Iscove's DMEM. For cAMP determinations, PGE₂ was added in the presence of a 2.5 mM concentration of the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). The treated cells were then washed once with Iscove's DMEM before assessment of macrophage phagocytosis or immunocytochemistry.

Determination of cAMP concentration

Macrophages were cultured adherent to six-well tissue culture plates for 6 days as described above. Macrophages were washed once with Iscove's DMEM and incubated with Iscove's DMEM alone or Iscove's DMEM containing 0.5 mM IBMX and 3 μM PGE₂ for 0.5, 2, 5, 10, 15, or 45 min. The medium was then aspirate and 600 μl of ice-cold 0.5 M trichloroacetic acid were added. Macrophages were extracted on ice for 20 min and then scraped off, and material was transferred to Eppendorf tubes. The samples were then vortexed and centrifuged ($10,000 \times g$, 5 min), and 500 μl of the supernatant were added to 125 μl of 10 mM EDTA (pH 7.0) and 500 μl of freshly prepared 1,1,2-trichlorotrifluoroethane-tri-*n*-octylamine (50:50, v/v) (21). After further vigorous mixing, the samples were centrifuged ($10,000 \times g$, 2 min), and 500 μl of the aqueous layer were removed and neutralized with 100 μl of 6 M sodium hydrogen carbonate. Cyclic AMP measurements were made on triplicate 50- μl aliquots from each sample. Each tube contained 50 μl of buffer (50 mM Tris-HCl, 4 M EDTA, pH 7.5), 50 μl of sample, or standard containing known amounts of cAMP (doubling dilutions from 0.125 to 16 pmol with 250 pmol of cAMP used to define nonspecific binding) and 100 μl [³H]cAMP (88,000 dpm; DuPont (U.K.), Stevenage, U.K.). The reaction was initiated by addition of 150 μl -cAMP-binding protein (final concentration, 0.85 μg protein/ml) and incubated overnight at 4°C . The reaction was terminated by the addition of 250 μl of 0.5% activated charcoal. After 3 min, the samples were centrifuged ($10,000 \times g$, 4 min) and 200 μl of the supernatant were transferred to 5-ml scintillation vials. Five milliliters of Flo-Scint IV scintillation fluid (Canberra Packard, Pangbourne, U.K.) were added to each vial, and the samples were analyzed on a liquid scintillation counter.

³ Abbreviations used in this paper: dbcAMP, dibutyryl-cAMP; dbcGMP, dibutyryl-cAMP; IBMX, 3-isobutyl-1-methylxanthine; 8-Br-cAMP, 8-bromo-cAMP; PKA, protein kinase A.

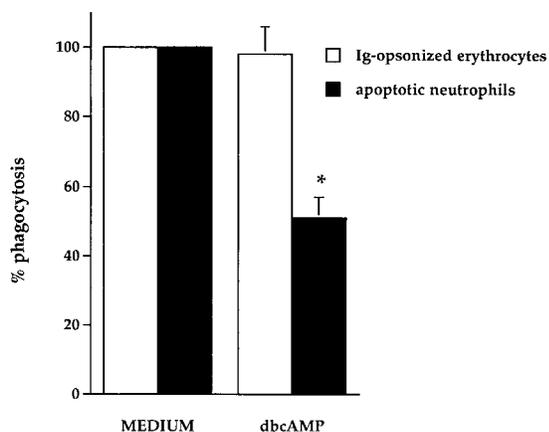


FIGURE 1. Effect of dbcAMP on macrophage recognition of apoptotic neutrophils and opsonized erythrocytes. Human monocyte-derived macrophages pretreated with dbcAMP (2 mM) for 15 min were assessed for their ability to phagocytose in vitro cultured neutrophils (■) or Ig-opsonized erythrocytes (□). Relative percentage phagocytosis is expressed compared with control medium pretreatment cells representing mean \pm SEM of 14 separate experiments. In these experiments, the percentage of cells ingesting apoptotic neutrophils under control conditions was $20 \pm 4\%$ and following dbcAMP treatment was $10 \pm 3\%$. The percentage of control macrophages ingesting opsonized erythrocytes was $30 \pm 3\%$, whereas in dbcAMP-treated macrophages the value was unchanged ($31 \pm 4\%$). * $p < 0.05$ compared with control.

Analysis of results

Results are expressed as the mean \pm SEM and n = number of independent experiments using macrophages from different donors. Results were analyzed by one-way analysis of variance followed by the Newman-Keuls procedure to determine significant differences between groups. p values < 0.05 were considered to be significant where * represents $p < 0.05$.

Results

Elevation of cAMP specifically inhibits macrophage phagocytosis of apoptotic neutrophils

Human monocyte-derived macrophages were pretreated with the membrane-permeable analogue of cAMP, dbcAMP, to mimic elevation of intracellular cAMP levels, and phagocytosis of apoptotic neutrophils was assessed. When compared with preincubation in medium alone, 2 mM dbcAMP decreased the percentage of monocyte-derived macrophages that phagocytosed apoptotic neutrophils (Fig. 1). The low proportion of phagocytic macrophages following dbcAMP treatment made assessment of the effects of dbcAMP on the number of apoptotic neutrophils phagocytosed per macrophage (phagocytic index) difficult, particularly since under control conditions the phagocytic index was ~ 1.4 . In a series of experiments, maximal effects of 2 mM dbcAMP treatment were apparent following 15 min of pretreatment with no further inhibition observed with longer incubation times (data not shown). All subsequent experiments were therefore performed using 15-min pretreatment times. Since elevation of cAMP might result in functional alterations downstream of the receptors mediating macrophage recognition of apoptotic neutrophils that would affect all phagocytic processes, we assessed whether dbcAMP had similar effects on Fc γ R-mediated phagocytosis. In contrast to the observed inhibition of monocyte-derived macrophage phagocytosis of apoptotic cells, pretreatment of macrophages with dbcAMP did not inhibit recognition of Ig-opsonized erythrocytes (Fig. 1). Thus, treatment of macrophages with an activator of PKA does not non-specifically inhibit all phagocytic pathways. Furthermore, modulation of phagocytosis of apoptotic neutrophils following cAMP

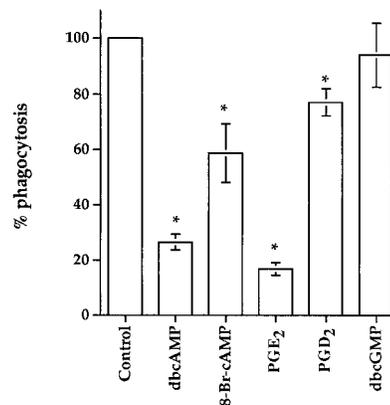


FIGURE 2. Effect of prostanooids, dbcAMP, 8-Br-cAMP, and dbcGMP on macrophage recognition of apoptotic cells. Human monocyte-derived macrophages pretreated with PGE₂ (10 μ M), PGD₂ (10 μ M), dbcAMP (2 mM), 8-Br-cAMP (2 mM), and dbcGMP (2 mM) for 15 min were assessed for their ability to phagocytose in vitro cultured neutrophils. The percentage of macrophages ingesting apoptotic neutrophils is expressed as the mean \pm SEM of 3 to 12 separate experiments. In this series of experiments, 24 ± 2 of control macrophages phagocytosed apoptotic neutrophils. * $p < 0.05$ compared with control.

elevation suggests that the potential of monocyte-derived macrophages to phagocytose apoptotic cells can be rapidly and dynamically regulated.

Effect of inflammatory mediators that elevate cAMP

Arachidonic acid metabolites of the PG series are key inflammatory mediators that interact with specific cell surface receptors to activate adenylate cyclase resulting in an elevation of intracellular cAMP. We therefore assessed the effects of a number of different PGs for their ability to inhibit monocyte-derived macrophage phagocytosis of apoptotic neutrophils. Macrophages were pretreated with either PGE₂ (10 μ M) or PGD₂ (10 μ M) for 15 min before testing their phagocytic ability. We also compared the effects of elevation of cGMP using dbcGMP (2 mM) in comparison with the positive controls, dbcAMP (2 mM) and 8 Br-cAMP (2 mM), as described above. We found that all the PGs tested were capable of inhibition of macrophage phagocytosis of apoptotic neutrophils (Fig. 2). In contrast to the effects of elevation of intracellular cAMP, raising cGMP levels did not inhibit monocyte-derived macrophage phagocytosis. These results suggest that macrophage phagocytosis can be regulated by intracellular signals and that the levels of intracellular cAMP may have a role in determining macrophage capacity for phagocytosis of apoptotic neutrophils.

Concentration-response effects of PGE₂, PGD₂, and dbcAMP on macrophage phagocytosis of apoptotic neutrophils

Since the above data suggest that macrophages express multiple receptors for different PG species, we next determined the relative potency and efficacy of PGE₂ and PGD₂ in comparison with dbcAMP for inhibition of phagocytosis of apoptotic neutrophils. Results shown in Figure 3 indicate that half-maximal inhibition (IC₅₀) is observed at ≈ 0.5 mM for dbcAMP treatment. Maximal inhibition of macrophage phagocytosis was observed following pretreatment with 10 μ M concentrations of both PGE₂ and PGD₂. However, PGE₂ (to IC₅₀ ≈ 1 μ M) was much more potent than PGD₂ (10 μ M inhibited phagocytosis only by $\approx 25\%$).

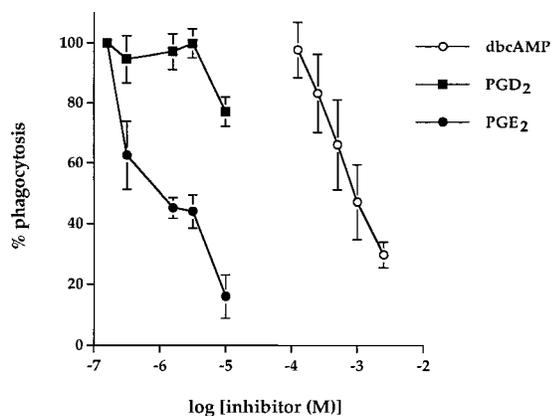


FIGURE 3. Concentration-response effects of dbcAMP, PGE₂, and PGD₂ on macrophage recognition of apoptotic cells. Human monocyte-derived macrophages pretreated with varying concentrations of dbcAMP (○) PGE₂ (●), and PGD₂ (■) for 15 min were assessed for their ability to phagocytose in vitro cultured neutrophils. The percentage of macrophages ingesting apoptotic neutrophils is expressed as the mean ± SEM of 5 to 12 separate experiments.

Effect of PGE₂ on intracellular cAMP concentrations in macrophages

To determine whether pretreatment of monocyte-derived macrophages with PGs exerted inhibitory effects via elevation of cAMP, we next measured intracellular concentrations of cAMP in macrophages following treatment with PGE₂ using a radioreceptor assay. As shown in Figure 4, PGE₂ acted rapidly to elevate cAMP, increasing cAMP levels 10-fold after 5 min. Interestingly, observed elevation was transient, returning to basal levels within 30 min. In contrast, inhibition of phagocytosis of apoptotic neutrophils following PGE₂ treatment was prolonged with inhibitory effects observed after 1 h (data not shown), implying that elevation of cAMP initiates signaling pathways that ultimately determine phagocytic responses.

Effects of cAMP elevation on macrophage adhesion

One possible mechanism for the rapid regulatory effects of cAMP elevation would be altered expression of surface receptors involved in macrophage recognition of apoptotic cells. However,

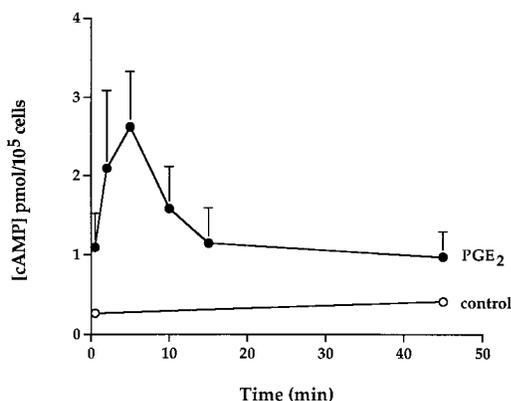


FIGURE 4. Effect of PGE₂ on intracellular levels of macrophage cAMP. The intracellular concentrations of cAMP in human monocyte-derived macrophages pretreated with PGE₂ (3 μM) in the presence of IBMX (2.5 mM) for the indicated times were determined. The results are expressed as picomols of cAMP/10⁵ cells representing mean ± SEM of four separate experiments.

flow cytometric analysis indicated that there was no significant alteration in surface expression of CD51, CD61, and the 61D3 Ag, as assessed by changes in mean fluorescence intensity, following dbcAMP pretreatment (data not shown), suggesting that the observed effects did not involve modulation of surface receptor expression. We therefore examined the effects of cAMP elevation on the distribution of receptors involved in the recognition process. Indirect immunofluorescence analysis of β₃ integrins and CD36 on macrophages pretreated with dbcAMP for 15 min revealed that the cellular distribution of CD36 was similar on untreated macrophages and those pretreated with dbcAMP. Although heterogeneity in terms of the pattern of distribution of the integrin β₃ subunit was observed in both untreated and treated macrophage populations and treatment of macrophages with dbcAMP resulted in an increase in the proportion of macrophages with a uniform β₃ distribution (data not shown), no clear association between β₃ distribution patterns and phagocytic capacity was apparent.

It has previously been demonstrated that elevation of cAMP induces phenotypic alterations in macrophages and other cell types. Microscopic examination of human monocyte-derived macrophages treated with dbcAMP (Fig. 5, A and B) and PGE₂ (data not shown) revealed clear morphologic alterations that were suggestive of altered adhesion. However, quantitative analysis revealed that elevation of cAMP did not alter the numbers of adherent macrophages (104 ± 4% of adhesion of untreated macrophages, *n* = 3 separate experiments). These data also demonstrate that observed inhibition of phagocytosis is not due to selective detachment of phagocytic macrophages. Moreover, additional experiments indicated that pretreatment of macrophages with dbcAMP did not alter the ability of macrophages to adhere to substrate (data not shown). Although dbcAMP did not affect the percentage of adherent cells, when we examined the intracellular distribution of cytoskeletal elements, we found that colocalization of actin and talin into discrete structures (representing contact points with substrate) was altered by dbcAMP treatment (Fig. 5, C–F). Similar results were found when we examined vinculin, paxillin, and tyrosine phosphorylation patterns (data not shown), suggesting that dbcAMP induces morphologic alterations by uncoupling adhesion receptors from cytoplasmic cytoskeletal elements, including potential signal transduction pathways (22).

Discussion

The regulatory mechanisms that underlie macrophage recognition, phagocytosis, and removal of apoptotic cells from inflamed sites are central to our understanding of the control and resolution of inflammation. Perturbation of the balance between neutrophil recruitment from the circulation, phagocytic removal of apoptotic neutrophils by macrophages (or other phagocytes), and neutrophil disintegration or necrosis may be critical determinants of whether inflammation ultimately resolves (6). A proportion of human monocyte-derived macrophages constitutively recognize and phagocytose apoptotic, but not freshly isolated, neutrophils (13), indicating that the apoptotic cell provides a phagocytic signal to the macrophage. However, since peripheral blood monocytes and some macrophage populations (e.g., alveolar macrophages; our unpublished observations) are relatively inefficient at phagocytosis of apoptotic cells, differentiation- or microenvironment-dependent phenotypic alterations may also regulate this process. Evidence for further regulatory mechanisms include the finding that treatment of macrophages with proinflammatory cytokines, notably GM-CSF, augments macrophage phagocytosis independently of altered receptor expression (23). More recently, we have demonstrated that

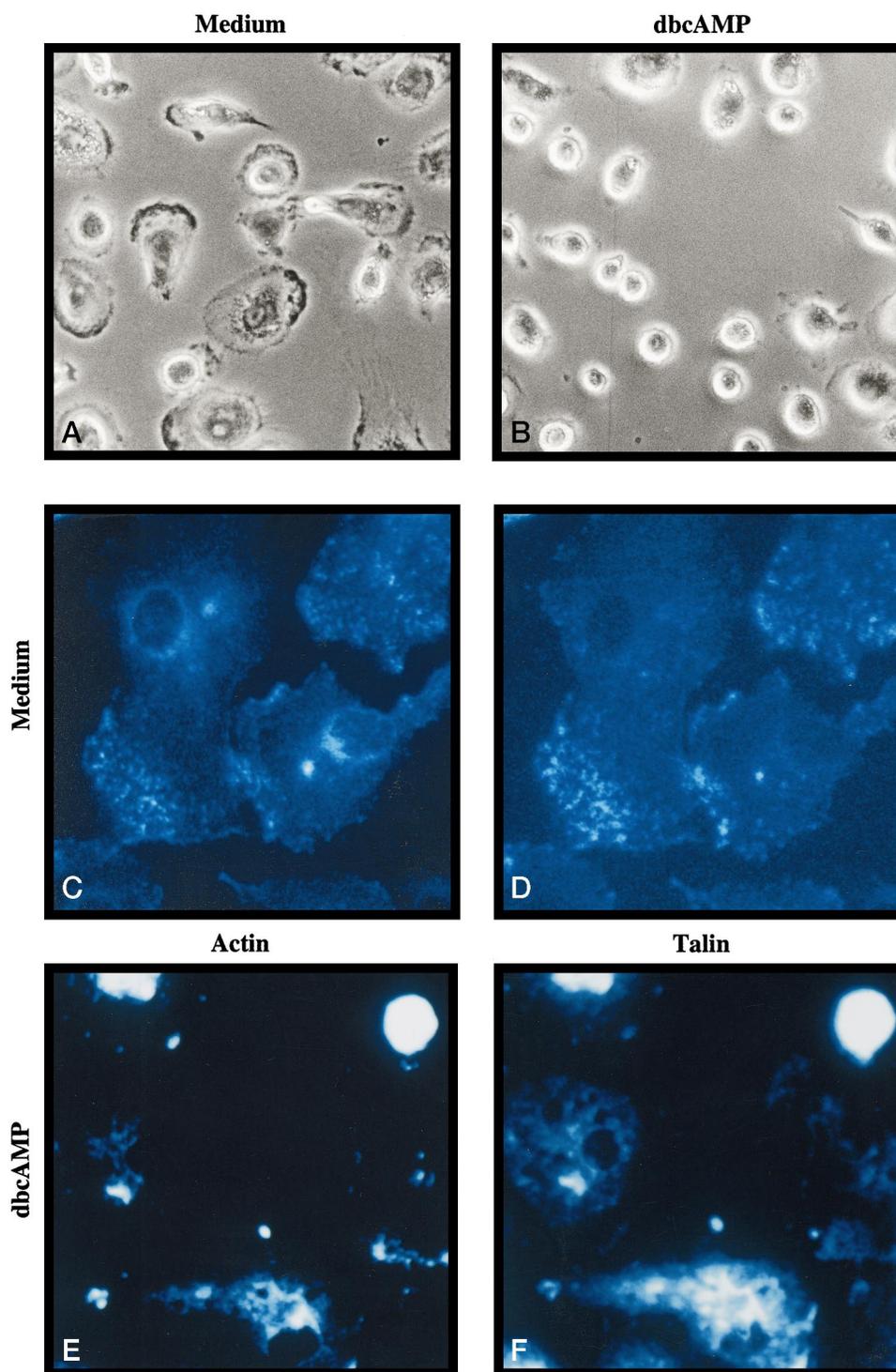


FIGURE 5. Effect of dbcAMP treatment on macrophage cytoskeleton. Untreated or dbcAMP-treated human monocyte-derived macrophages were washed twice before fixation and (A and B) examined using an inverted microscope ($\times 20$ objective) or stained for actin (C and E) using rhodamine phalloidin and talin (D and F) using dual labeling techniques. Scanning confocal laser microscopy ($\times 60$ objective) was then used to examine intracellular distributions of these proteins. Untreated macrophages (Medium) show morphologic features typical of motile cells (A). In contrast, following dbcAMP treatment, many more macrophages appear rounded (B). There is clear colocalization of actin and talin in untreated macrophages (C and D) possibly representing areas of contact with substrate. In dbcAMP-treated macrophages, actin staining is more disordered (E and F).

cross-linking of CD44 rapidly and specifically augments phagocytosis of apoptotic neutrophils, suggesting that macrophage phagocytic capacity can be dynamically regulated (20).

In this study, we demonstrate that elevation of cAMP within monocyte-derived macrophages specifically reduces macrophage potential to recognize and phagocytose apoptotic neutrophils but

not Ig-opsonized erythrocytes, indicating that cAMP does not block all phagocytic pathways in macrophages. Elevation of cAMP and activation of PKA may signal rapid, subtle responses to microenvironmental stimuli, resulting in reduced capacity for phagocytosis of apoptotic cells by macrophages. The prostanoids PGE₂ or PGD₂ have antiinflammatory effects on neutrophil

function in terms of granule secretion (24–26). However, our observation that macrophage removal of apoptotic cells is inhibited by these agents raises the possibility that they may increase the tissue load of apoptotic cells at inflamed sites. Inflammatory mediators may therefore have complex effects on processes involved in resolution of inflammation. One possibility is that PG-induced cAMP elevation may provide counterregulatory signals to proinflammatory mediators or CD44 cross-linking. Preliminary experiments indicate that dbcAMP treatment inhibits CD44 augmented monocyte-derived macrophage phagocytosis of apoptotic neutrophils (CD44 treated (20), 50.1 + 9% phagocytosis; CD44 + 2 mM dbcAMP treated, 18.6 + 8%; $n = 5$). However, since dbcAMP also inhibits phagocytosis under control conditions (see Figs. 1–3), it is difficult to determine the precise relationship between cAMP elevation and CD44-mediated signals in control of phagocytic capacity. We are currently further investigating the effects of PGs and other antiinflammatory agents on macrophage phagocytic responses in combination with GM-CSF or other proinflammatory cytokines.

The observations reported here may be of great significance for removal of apoptotic neutrophils in situ. A variety of inflammatory mediators (including PGs) that stimulate adenylate cyclase activity and activate PKA (27) are likely to decrease apoptotic cell removal. All monocyte-derived macrophages may be capable of phagocytosis of apoptotic cells, providing that a certain activation “threshold” is reached, with PKA playing a regulatory role in limiting phagocytic responses. For instance, some monocyte-derived macrophage subpopulations, e.g., multinucleated giant cells, show low levels of phagocytosis of apoptotic cells but are readily able to phagocytose Ig-opsonized erythrocytes, suggesting that phagocytic competence is not the basis for the lack of apoptotic cell uptake. One possibility is that differential PKA activity in giant cells accounts for observed low level responsiveness to apoptotic cell stimuli.

Data presented in this article indicate that there are multiple levels of control for macrophage removal of apoptotic cells from inflamed sites. Although the mechanism by which cAMP elevation down-regulates macrophage phagocytosis of apoptotic cells remains to be determined, we have observed that there are rapid alterations in the distribution of cytoskeletal components (actin, talin, vinculin, and paxillin) in macrophages following dbcAMP treatment. These changes are accompanied by cell “rounding” when macrophages are examined by light microscopy and show parallels with the reported effects of PGs on murine macrophage adhesion, spreading, and motility (28, 29). Membrane ruffling and filopodia extension were inhibited by prolonged PG treatment in a reversible manner, resulting in altered macrophage adhesion. Together with recent evidence that in cytotoxic T cells PKA is able to phosphorylate Rho (30), a key regulator of cytoskeletal organization, these data indicate that intracellular cAMP levels may also have a pivotal role.

We have investigated the effects of agents that disrupt microfilament and microtubule organization (cytochalasin B and nocodazole). Although we did find inhibition of recognition of apoptotic cells when macrophage were treated with nocodazole (2.5 $\mu\text{g/ml}$), it was difficult to assess the effects of cytochalasin B (5 $\mu\text{g/ml}$) because the washing procedure used for assessment of macrophage phagocytosis resulted in significant detachment of cells in three of five experiments. These findings suggest that treatment of macrophage with dbcAMP differs from disruption of microfilaments in two ways. First, unlike cytochalasin B treatment, dbcAMP does not inhibit phagocytosis of Ig-opsonized erythrocytes (Fig. 1) (31, 32). Indeed, Newman et al. (32) have previously demonstrated that cAMP does not inhibit FcR-mediated phagocytosis.

Second, although we have noted disassembly of structures that may represent areas of contact with substrate and cell “rounding,” treatment of macrophages with dbcAMP does not result in detachment, and cells remain firmly adherent through multiple washes that are necessary for assessment of phagocytic capacity. Adhesion of macrophages to extracellular matrix components, e.g., fibronectin, has previously been shown to modulate C or Ig receptor-mediated phagocytosis (33, 34), and it is tempting to speculate that the extracellular matrix composition of inflamed sites exerts indirect regulatory effects on the process of removal of apoptotic cells. One possibility is that intracellular levels of cAMP are altered following integrin-mediated adhesion to extracellular matrix components as found for human neutrophils (35).

In summary, elevation of cAMP, using cell permeable analogues of cAMP (dbcAMP, 8-Br-cAMP) or by receptor-directed stimuli (PGE_2 , PGD_2), in macrophages specifically inhibits phagocytosis of apoptotic cells but not Ig-opsonized erythrocytes. Modulation is independent of changes in the percentage of adherent macrophages, although a specific, rapid redistribution of cytoskeletal elements was observed, suggestive of changes in the adhesive status of macrophages. These data indicate that intracellular cAMP may have a key role in controlling the capacity for removal of apoptotic cells by macrophages at inflamed sites during the resolution of inflammatory processes.

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