Annexin-1 and Peptide Derivatives Are Released by Apoptotic Cells and Stimulate Phagocytosis of Apoptotic Neutrophils by Macrophages[1]

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The resolution of inflammation is a dynamically regulated process that may be subverted in many pathological conditions. Macrophage (Mϕ) phagocytic clearance of apoptotic leukocytes plays an important role in the resolution of inflammation as this process prevents the exposure of tissues at the inflammatory site to the noxious contents of lytic cells. It is increasingly appreciated that endogenously produced mediators, such as lipoxins, act as potent regulators (nanomolar range) of the phagocytic clearance of apoptotic cells. In this study, we have investigated the intriguing possibility that apoptotic cells release signals that promote their clearance by phagocytes. We report that conditioned medium from apoptotic human polymorphonuclear neutrophils (PMN), Jurkat T lymphocytes, and human mesangial cells promote phagocytosis of apoptotic PMN by Mϕ and THP-1 cells differentiated to a Mϕ-like phenotype. This prophagocytic activity appears to be dose dependent, sensitive to the caspase inhibitor zVAD-fmk, and is associated with actin rearrangement and release of TGF-β1, but not IL-8. The prophagocytic effect can be blocked by the formyl peptide receptor antagonist Boc2, suggesting that the prophagocytic factor(s) may interact with the lipoxin A_4 receptor, FPRL-1. Using nanoelectrospray liquid chromatography mass spectrometry and immunodepletion and immunoneutralization studies, we have ascertained that annexin-1 and peptide derivatives are putative prophagocytic factors released by apoptotic cells that promote phagocytosis of apoptotic PMN by M[phi] and differentiated THP-1 cells. These data highlight the role of annexin-1 and peptide derivatives in promoting the resolution of inflammation and expand on the therapeutic anti-inflammatory potential of annexin-1. *The Journal of Immunology,* **2007, 178: 4595–4605.**

Inflammation is a critical determinant of effective host defense. The resolution of inflammation is a tightly regulated process that may be subverted in many pathological conditions. Macrophage (Mϕ)[3] phagocytic clearance of apoptotic leukocytes plays a key role in the resolution of inflammation as this process prevents the exposure of tissues at the inflammatory site to the noxious and potentially immunogenic contents of lytic cells (1, 2). In addition, the uptake of apoptotic cells stimulates the release of anti-inflammatory mediators, such as TGF-β1, IL-10, and PGE_2, and can inhibit the secretion of proinflammatory mediators, such as TNF-α, from phagocytes (3–5).

Secreted "find-me," exposed "eat-me," and the absence of "don't-eat-me" signals are now familiar terms that are used to describe the myriad of signaling molecules involved in the phagocytic clearance of apoptotic cells. Eat-me signals include new molecules that appear on the apoptotic cell surface, such as phosphatidylserine (PtdSer) (6), modification of existing molecules such as ICAM-3 (7), and change in charge of the cell surface (8). Extracellular bridging molecules such as milk-fat-globule 8 (9), thrombospondin-1 (10), and serum protein S (11) can cover the apoptotic cell surface and link them to phagocytes by providing recognition sites for phagocyte receptors. Don't-eat-me signals include CD31 which on living leukocytes and Mϕ mediate the active, temperature-dependent detachment of leukocytes from Mϕ (12). During apoptosis, this function is disabled allowing the engulfment of the dying cell (12). More recently, a find-me signal was identified by Lauber et al. (13) who demonstrated that a lipid molecule, lysophosphatidylcholine (LPC), was released by apoptotic cells and was chemotactic for phagocytes.

There is a growing appreciation that phagocytic clearance can be regulated by endogenously produced mediators. Such molecules include lipoxins (LXs), eicosanoids produced via transcellular metabolism of arachidonic acid, whose biosynthesis is detectable after the initial proinflammatory response (14). The locus of the bioactions of mediators such as LXs appears to be the Mϕ where they prime the cell for phagocytosis of apoptotic leukocytes in vitro (15, 16) and in vivo (17). Evidence points to a role in phagocytosis for pleiotropic ligands (lipid, peptide, and protein

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Received for publication November 28, 2006. Accepted for publication January 4, 2007.

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[1] This work was supported by the Health Research Board, Ireland (to C.G.), Enterprise Ireland (to C.G.), The Wellcome Trust (to C.G.), European Union FP6 Funding (LSHM-CT-2004-005033) (to C.G.), Science Foundation Ireland (to M.B.F., A.d.S., K.J.W., G.C.; Grant 02/IN.1/B117), and The Irish Research Council for Science, Engineering, and Technology (to M.S.). This work was funded under the Programme for Research in Third Level Institutions, administered by the Higher Education Authority.

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[3] Abbreviations used in this paper: Mϕ, macrophage; PtdSer, phosphatidylserine; LPC, lysophosphatidylcholine; LX, lipoxin; PVDF, polyvinylidene difluoride; MS, mass spectrometry; LC, liquid chromatography; Nano-LC MS/MS, nanoelectrospray LC MS/MS; FPR, formyl peptide receptor; FPRL-1, FPR-like-1; PMN, polymorphonuclear neutrophil; hMC, human mesangial cell; 8-Br-cAMP, 8-bromoadenosine cAMP; Rp-cAMP, adenosine 3′,5′-cyclic monophosphorothioate; PKA, protein kinase A; PI, propidium iodide; MYH9, nonmuscle myosin II heavy chain isoform A; CF, cystic fibrosis.

(18)) of the lipoxin receptor (formyl-peptide receptor like-1 (FPRL-1)), including the glucocorticoid inducible protein, annexin-1 (19). We previously demonstrated that the supernatant of dexamethasone-treated Mϕ and the annexin-derived peptide Ac2-26 modulate Mϕ phagocytosis of apoptotic polymorphonuclear neutrophils (PMN) (20). A role for annexin-1 in phagocytic clearance of apoptotic cells is supported by recent data that have shown that during apoptosis, annexin-1 is recruited from the cytosol of Jurkat T lymphocytes and exported to the outer cell membrane in a caspase-dependent manner. There it colocalizes with PtdSer and mediates phagocytosis of the apoptotic targets by phagocytes (21). Furthermore, Fan et al. (22) have demonstrated that annexin-1 and annexin-2 act as bridging molecules, linking phagocyte and target cells, and promote phagocytosis. It has been demonstrated that the mitochondrial peptide fragment MYFI NILTL and the synthetic peptide MMK-1, LESIFRSLLFRVM, can stimulate PMN chemotaxis but that this effect can be attenuated by the aspirin triggered lipoxin, 15-epi-LXA_4 (23). We have previously demonstrated that these peptides stimulate Mϕ phagocytosis via FPRL-1 (17).

In the current study, we demonstrate that apoptotic cells actively promote their clearance by Mϕ via release of prophagocytic signals. We have investigated the underlying signaling mechanisms and have identified annexin-1 and its peptide derivatives as potential candidates that induce clearance of apoptotic cells by phagocytes.

Materials and Methods

Materials

RPMI 1640, MCDB-131, PBS, and Oregon Green phalloidin were purchased from Invitrogen Life Technologies. LXA_4 (5(*S*)-6(*R*)-15(*S*)-trihydroxy-7,9,13-*trans*-11-*cis*-eicosatetraenoic acid) was obtained from Biomol. PMA and 8-bromoadenosine cAMP (8-Br-cAMP) were purchased from Sigma-Aldrich. Adenosine 3′,5′-cyclic monophosphorothioate (Rp-cAMP), PHM-L LIPOSORB Absorbent, and anti-phosphoserine 16B4 Ab were obtained from Calbiochem. The antagonist Boc2 (*N*-*t*-Boc-Phe-D-Leu-Phe-D-Leu-Phe) that blocks both the formyl peptide receptor (FPR) and LX receptor (FPRL-1) activation (19) was purchased from MP Biomedicals. Proteinase K was purchased from Roche Diagnostics. zVAD-fmk, a pancaspase inhibitor, and TGF-β1 and IL-8 ELISA kits were obtained from R&D Systems. Mouse anti-human myosin IIA Ab was obtained from Covance. Rabbit anti-human annexin-1 Ab was purchased from Zymed Laboratories (Cambridge Biosciences). Vivaspin 500 polyethersulfone 10-kDa molecular mass cut-off spin columns were purchased from Vivascience.

Reagents were dissolved in DMSO or ethanol and further diluted in medium (final concentration, $\leq$0.1%). Equivalent concentrations of DMSO or ethanol were used as vehicle controls.

Human leukocyte isolation and culture

Human PMN and monocytes were isolated from peripheral venous blood drawn from healthy volunteers according to institutional ethical guidelines as previously described (15). Briefly, mononuclear cells were separated by centrifugation on Ficoll-Paque (Pharmacia) and plated at 2×10^6 cells/ml in RPMI 1640 supplemented with 10% autologous serum, 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies). Lymphocytes were removed after 1 h of culture and Mϕ were obtained by further culturing monocytes for 7 days.

PMN were isolated by dextran sedimentation (Dextran T500; Pharmacia) and contaminating erythrocytes were removed by hypotonic lysis. The PMN were then resuspended at 4×10^6 cells/ml. Spontaneous apoptosis was achieved by culturing the PMN in RPMI 1640 supplemented with 10% autologous serum, 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, for 20 h at 37°C in 5% CO_2 atmosphere (17).

For preparation of supernatant of apoptotic PMN, freshly isolated PMN were suspended at 4×10^6 cells/ml in RPMI 1640 without serum, supplemented with 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and incubated for 20 h at 37°C in 5% CO_2 atmosphere. The conditioned medium of apoptotic PMN was clarified by centrifugation and aliquots of supernatant were stored at −20°C. For preparation of supernatant of necrotic PMN, freshly isolated PMN were suspended at 4×10^6 cells/ml in RPMI 1640 (without serum), supplemented with 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin, and incubated at 58°C for 30 min. The conditioned medium of necrotic PMN was clarified by centrifugation and aliquots of supernatant were stored at −20°C. For preparation of supernatant of apoptotic PMN treated with the caspase inhibitor zVAD-fmk, freshly isolated PMN were suspended at 4×10^6 cells/ml in RPMI 1640 (without serum), supplemented with 2 mM/L glutamine, 100 IU/ml penicillin, 100 μg/ml streptomycin, 100 μM zVAD-fmk, and incubated for 20 h at 37°C in 5% CO_2 atmosphere. The conditioned medium was clarified by centrifugation and aliquots of supernatant were stored at −20°C.

Cell culture

THP-1 cells (leukemic, monocytic cell line) were obtained from the European Collection of Cell Cultures and cultured in RPMI 1640 with 10% FCS, 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. THP-1 cells were differentiated to a Mϕ-like phenotype by treatment with 10 nM PMA for 48 h at 37°C (24).

Primary human mesangial cells (hMC) were obtained from the European Collection of Cell Cultures and cultured in MCDB-131 supplemented with 10% FCS, 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. For induction of apoptosis, hMC were exposed to UV irradiation at 254 nm for 5 min followed by incubation for 24 h at 37°C in 5% CO_2 atmosphere. The conditioned medium were clarified by centrifugation and aliquots of supernatants were stored at −20°C.

The human leukemic T cell lymphoblast cell line (Jurkat) was obtained from the European Collection of Cell Cultures and cultured in RPMI 1640 with 10% FCS, 2 mM/L glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. The Jurkat T cells were induced to undergo apoptosis by culturing at a concentration of 4×10^6 cells/ml in serum free medium and exposing the cells to UV irradiation at 254 nm for 2 min followed by incubation for 24 h at 37°C in 5% CO_2 atmosphere. The conditioned medium were clarified by centrifugation and aliquots of supernatants were stored at −20°C.

Physical treatment of the supernatant of apoptotic PMN

Enzyme digestion of the protein content of the supernatant of apoptotic PMN was conducted by addition of proteinase K (100 μg/ml) to the supernatant and incubation for 1 h at 50°C, followed by boiling for 5 min to denature the enzyme. For heat inactivation of the supernatant of apoptotic PMN, the supernatant was heated at 90°C for 10 min. Lipids were removed from the supernatant of apoptotic PMN using PHM-L LIPOSORB Absorbent according to the manufacturer's instructions. To fractionate the supernatant of apoptotic PMN, the supernatant was passed through Vivaspin 500 polyethersulfone 10-kDa molecular mass cut-off spin columns from Vivascience by centrifuging at 13,000 rpm for 10 min at 4°C. Following treatments, the supernatants were stored at −20°C.

Mϕ/THP-1 cell phagocytosis of apoptotic PMN

Human Mϕ (or differentiated THP-1 cells) were treated with the appropriate stimuli as indicated for 15 min at 37°C. The treated cells were washed with RPMI 1640 before coincubation with apoptotic PMN (1×10^6 PMN/well) at 37°C for 30 min (Mϕ) or 2 h (THP-1 cells). Noningested cells were removed by washing three times with cold PBS. Phagocytosis was assayed by myeloperoxidase staining of coculture fixed with 2.5% glutaraldehyde as previously reported (15). For each experiment, the number of Mϕ (or differentiated THP-1 cells) containing one or more PMN in at least four fields (minimum of 400 cells) was expressed as a percentage of the total number of Mϕ (or differentiated THP-1 cells).

For inhibitor studies, differentiated THP-1 cells were treated with the stable cell-permeable cAMP analog 8-bromo-cAMP (2 mM), the protein kinase A (PKA) inhibitor Rp-cAMP (100 μM), Boc2 (100 μM), or the neutralizing anti-annexin-1 Ab (1/100) in the presence or absence of the supernatant of apoptotic PMN, for 15 min at 37°C before coincubation with apoptotic PMN as described above.

Actin staining

Mϕ were grown on glass coverslips and incubated with control, the supernatant of freshly isolated PMN or the supernatant of apoptotic PMN for 15 min at 37°C. Cells were rinsed with PBS and fixed with 3.8% paraformaldehyde-PBS for 20 min at room temperature. Cells were permeabilized with 0.1% Triton X-100 in PBS for 5 min and stained with Oregon Green-phalloidin (0.33 μM/L) for 30 min at room temperature. Coverslips were mounted on microscope glass slides with Probing Antifade medium (Molecular Probes). Cells were viewed on an Axiovert 200M fluorescent microscope (Zeiss) using Axiovision image analysis software (Imaging Associates).

Measurement of apoptosis

Freshly isolated PMN, aged PMN, normal Jurkat T cells, UV-treated Jurkat T cells, normal hMC and UV-treated hMC were incubated with Annexin V^{FITC} (IQ Products) and propidium iodide (PI; Sigma-Aldrich), and analyzed by flow cytometry (Coulter EPICS XL). Cells which were Annexin V^{FITC} positive and PI negative were identified as early apoptotic. Cells which were Annexin V^{FITC} positive and PI positive were identified as late apoptotic or necrotic.

Immunoprecipitation and immunoblotting

Differentiated THP-1 cells (2×10^6 cells/ml) were serum starved for 24 h before treatment with either the supernatant of freshly isolated PMN or the supernatant of apoptotic PMN. Lysates were harvested in radioimmunoprecipitation assay (RIPA) lysis buffer (20 mM Tris-HCl (pH 7.4), 50 mM NaCl, 5 mM ethylene diaminetetraacetic acid, 1% igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM NaF, 1 mM PMSF, 1 mM Na_3Vo_4, 1 μM leupeptin, 0.3 μM aprotinin). The lysates were clarified by centrifugation at 14,000 rpm for 12 min and samples were normalized for total protein. Phosphoserine proteins were immunopurified from 1 mg of precleared lysate using 4 μg of mouse anti-phosphoserine 16B4 Ab and rocked overnight at 4°C. A total of 10 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) was added to the protein-Ab mixture and samples were rocked for a further 2 h at 4°C. Precipitated immunocomplexes were washed three times in fresh lysis buffer and boiled in sample buffer. Samples were subsequently resolved by electrophoresis on 10% SDS polyacrylamide gels and transferred to Immobilon-P polyvinylidene diflouride (PVDF) membranes (Millipore). The membranes were incubated in PBS plus 0.1% Tween 20 plus 5% BSA before probing with a mouse anti-human myosin IIA Ab (1 in 1000 in PBS plus 0.1% Tween 20 plus 5% BSA; Covance). After incubation with a HRP-conjugated anti-mouse secondary Ab (1 in 2000 in PBS plus 0.1% Tween 20 plus 5% BSA; New England Biolabs), bound Ab was visualized with an ECL detection system (Santa Cruz Biotechnology).

Protein identification by mass spectrometry (MS)

Protein from 2 ml of conditioned medium of apoptotic PMN was concentrated by trichloroacetic acid/deoxycholate precipitation, fractionated using SDS-PAGE, and digested in-gel with trypsin according to the method of Shevchenko et al. (25). The resulting peptide mixtures were resuspended in 1% formic acid and analyzed using nanoelectrospray liquid chromatography MS (Nano-LC MS/MS). An HPLC instrument (Dionex) was interfaced with an LTQ ion trap mass spectrometer (ThermoFinnigan). One hundred fifty-micrometer inner diameter-fused silica capillary microcolumns (Polymicro Technologies) were pulled to a fine tip using a P-2000 laser puller (Sutter Instruments) and packed with 10 cm of 5-μm Zorbax Eclipse XDB-C18 resin (Agilent Technologies). Samples were loaded manually using a pressure vessel. Chromatography buffer solutions (buffer A, 5% acetonitrile and 0.1% formic acid; buffer B, 80% acetonitrile and 0.1% formic acid) were used to deliver a 60-min gradient (35 min to 45% buffer B, 10 min to 90%, hold 10 min, 3 min to 5%, hold for 15 min). A flow rate of 2 μl/minute was used at the electrospray source. The SEQUEST program (26) was used to search the Human IPI Protein Sequence Release (version 3.15) (ftp://ftp.ebi.ac.uk/pub/databases/IPI/current/). The probability based evaluation program, Protein Prophet, was used for filtering putative identifications based on a maximum *p* value threshold corresponding to 90% likelihood of correct identification (27).

Immunoblotting

Supernatants of freshly isolated PMN, apoptotic PMN, apoptotic Jurkat T cells, apoptotic hMC, and necrotic PMN were resolved by electrophoresis on 12% SDS polyacrylamide gels and transferred to Immobilon-P PVDF membranes (Millipore). The membranes were incubated in PBS plus 0.1% Tween 20 plus 5% milk before probing with a rabbit anti-annexin-1 Ab (1/500 dilution in PBS plus 0.1% Tween 20 plus 5% milk; Zymed Laboratories). After incubation with a HRP-conjugated anti-rabbit secondary Ab (1/2000 in PBS plus 0.1% Tween 20 plus 5% milk; New England Biolabs), bound Ab was visualized with an ECL detection system (Santa Cruz Biotechnology).

Annexin-1 neutralization and depletion

The neutralization of annexin-1 in the supernatant of apoptotic PMN was achieved by incubating the supernatant of apoptotic PMN with a polyclonal anti-annexin-1 Ab (1/100; Zymed Laboratories) during the stimulation of THP-1 cells and before coincubation with apoptotic PMN.

The immunodepletion of annexin-1 from the supernatant of apoptotic PMN was conducted by incubating 1-ml samples of supernatant of apoptotic PMN with 25 μl of the polyclonal Ab directed against annexin-1 (Zymed Laboratories) or a control rabbit IgG overnight at 4°C, followed by incubation with 50 μl of protein A/G-agarose beads (Santa Cruz Biotechnology) for 2 h at 4°C. The immune complexes were separated by centrifugation and the supernatants were collected. Immunoprecipitation was performed three more times. Annexin-1 depletion was verified by immunoblotting as described above.

Cytokine production

TGF-β1 and IL-8 were assayed in supernatants of cocultures of Mϕ (or THP-1 cells) and apoptotic PMN by ELISA according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was conducted using unpaired Student's *t* test with $p < 0.05$ for *n* independent samples being deemed statistically significant.

Results

Supernatants of apoptotic PMN stimulate nonphlogistic phagocytosis of apoptotic PMN and actin rearrangement in Mϕ

To investigate whether apoptotic cells release factors that stimulate their phagocytic clearance, the conditioned medium of apoptotic cells were used to stimulate monocyte-derived Mϕ and differentiated THP-1 cells to phagocytose apoptotic PMN. Apoptosis was measured by flow cytometry in the PMN samples from which the conditioned medium were derived and found to be 6.3 ± 1.5%, for freshly isolated PMN, and 44.0 ± 12.1%*, *, $p < 0.005$ vs viable PMN, $n = 4$ (Table I). Treatment of Mϕ with the supernatant of apoptotic PMN resulted in a significant increase in phagocytosis when compared with the supernatant of viable PMN (percentage of phagocytosis: control, 14.9 ± 2.7%; supernatant of freshly isolated PMN, 16.4 ± 3.2%; supernatant of apoptotic PMN, 23.7 ± 4.0%*, *, $p < 0.005$ vs control, $n = 3$). As illustrated in Fig. 1*A*, comparable results were found using the leukemic, monocytic cell line THP-1, differentiated to a Mϕ-like phenotype, as phagocyte (percent phagocytosis: control, 4.8 ± 1.6%; supernatant of freshly isolated PMN, 4.4 ± 1.0%; supernatant of apoptotic PMN, 8.2 ± 1.4%*, *, $p < 0.005$ vs control, $n = 3$). The prophagocytic effect could be progressively abrogated by diluting the supernatant of apoptotic PMN with fresh medium suggesting a concentration-dependent relationship between the putative prophagocytic factors and the prophagocytic effect (Fig. 1*B*).

The process of phagocytosis is highly dependent on the localized polymerization of actin filaments which facilitate the formation of filopodia that surround the cells to be engulfed. Therefore, it was interesting to observe that the supernatant of apoptotic PMN, but not the supernatant of freshly isolated PMN, induced actin reorganization in Mϕ as illustrated in Fig. 1*C*.

The interaction of apoptotic cells with phagocytes has been shown to deliver anti-inflammatory and anti-immunogenic signals to the surrounding environment (3, 4). To investigate whether the

Table I. *Levels of apoptosis before and after induction of apoptosis in PMN, Jurkat T cells, and hMC*[a]

Cell Type	% Apoptosis
Viable PMN	6.3 ± 1.5
Aged PMN	44.0 ± 12.1*
Viable Jurkat T cells	7.7 ± 2.3
UV-treated Jurkat T cells	78.3 ± 5.0#
Viable hMC	5.3 ± 2.3
UV-treated hMC	20.0 ± 5.6**

[a] Values are percentage of apoptosis and represent means ± SEM: *, $p < 0.005$ vs viable PMN ($n = 4$); #, $p < 0.001$ vs viable Jurkat T cells ($n = 3$); **, $p < 0.05$ vs viable hMC ($n = 3$).

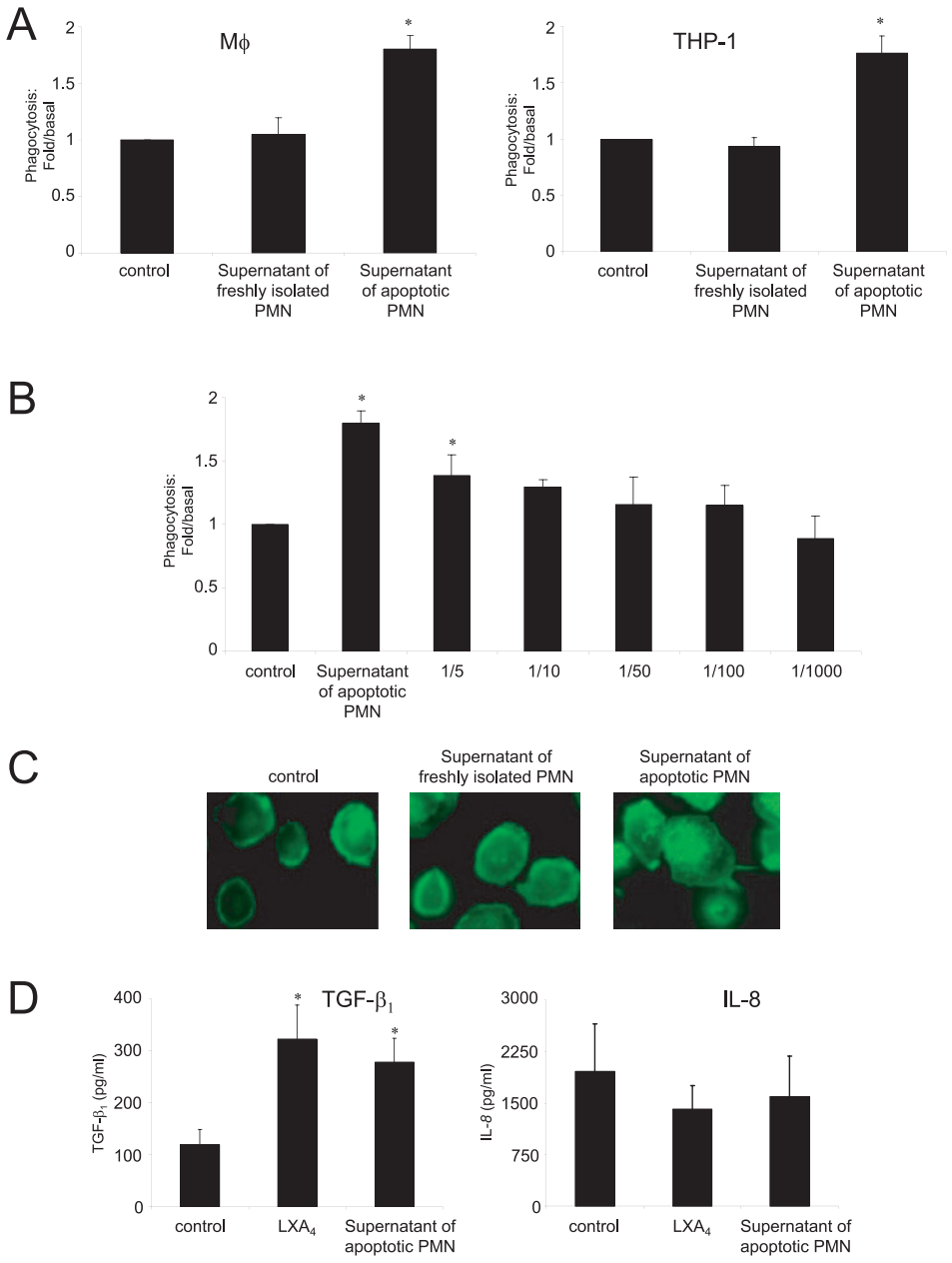

FIGURE 1. The supernatants of apoptotic PMN stimulate phagocytosis of apoptotic cells and actin rearrangement in Mϕ. *A*, Mϕ or differentiated THP-1 cells were treated with the supernatant of freshly isolated PMN or the supernatant of apoptotic PMN for 15 min before coincubation with apoptotic PMN for 30 min (Mϕ) or 2 h (THP-1 cells). Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 5$): ∗, $p < 0.005$ vs control. *B*, Supernatant of apoptotic PMN was diluted as indicated with fresh culture medium and used to stimulate differentiated THP-1 cells before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 3$): ∗, $p < 0.05$ vs control. *C*, For actin rearrangement studies, Mϕ were treated with appropriate stimuli for 15 min. The cells were fixed, stained with Oregon Green phalloidin, and viewed by conventional fluorescence microscopy. *D*, Mϕ were treated with vehicle (control), LXA_4 (1 nM), and the supernatant of apoptotic PMN before coincubation with apoptotic PMN. After 30 min, supernatants of the coincubations were collected and TGF-β1 and IL-8 production was measured by ELISA. Data are mean concentration of cytokine released (picograms per milliliter) ± SEM ($n = 3$): ∗, $p < 0.05$ vs control.

phagocytosis stimulated by the putative prophagocytic factors in the supernatant of apoptotic PMN was nonphlogistic, the release of the proinflammatory cytokine IL-8 and the anti-inflammatory cytokine TGF-β1 were measured by ELISA. The phagocytosis stimulated by the supernatant of apoptotic PMN was not associated with increased IL-8 release over basal levels but did provoke a significant release of TGF-β1 (Fig. 1*D*). No changes were observed with the supernatant of freshly isolated PMN. LXA_4 has previously been shown by our laboratory to have a nonphlogistic, prophagocytic effect in Mϕs (15) and was used as a positive control in these experiments (Fig. 1*D*). Stimulated Mϕ that were not exposed to apoptotic PMN did not show a significant difference in release of either cytokine compared with basal levels (data not shown).

Supernatant of apoptotic mesangial cells, apoptotic Jurkat T cells, but not necrotic PMN stimulate phagocytosis of apoptotic PMN

To investigate whether other apoptotic cell types may also release prophagocytic factors, we examined the supernatants of apoptotic hMC and apoptotic Jurkat T cells induced to undergo apoptosis via UV irradiation. The supernatant of apoptotic hMC and apoptotic Jurkat T cells increased phagocytosis to a similar extent as the supernatant of apoptotic PMN (Fig. 2). Apoptosis was found to be 78.3 ± 5.0% for Jurkat T cells ($n = 3$) and 20.0 ± 5.6% for hMC ($n = 3$) as measured by flow cytometry (Table I). Interestingly, the supernatant of necrotic PMN did not stimulate phagocytosis suggesting that the release of the putative prophagocytic signals is dependent on the apoptotic process (Fig. 2).

Characterization of the prophagocytic factors released by apoptotic PMN

Treatment of the supernatant of apoptotic PMN with proteinase K (100 μg/ml) for 1 h at 50°C, followed by boiling to inactivate the enzyme, abrogated the prophagocytic effect suggesting that the mediators were proteins (Fig. 3*A*). Moreover, we determined that the prophagocytic activity was unaltered by heating the supernatant to 90°C for 10 min before stimulation of the THP-1 cells to phagocytose apoptotic PMN (Fig. 3*B*).

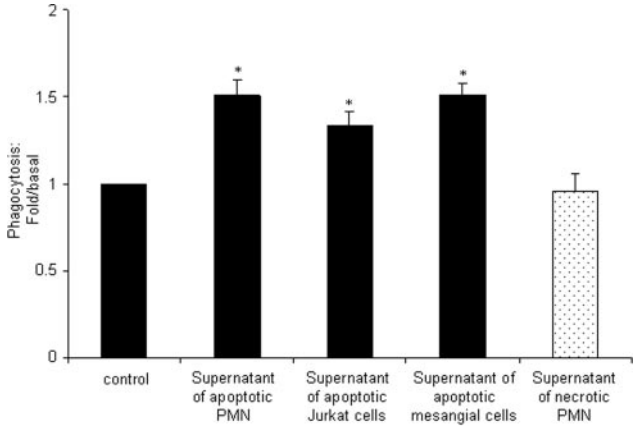

FIGURE 2. The supernatants of apoptotic PMN, apoptotic Jurkat T cells, apoptotic mesangial cells, but not necrotic PMN, stimulate phagocytosis of apoptotic cells. Differentiated THP-1 cells were exposed to the supernatant of apoptotic PMN, the supernatant of apoptotic Jurkat T cells, the supernatant of apoptotic hMC, or the supernatant of necrotic PMN for 15 min before coincubation with apoptotic PMN for 2 h. Data are means ± SEM ($n = 3$): *, $p < 0.05$ vs control.

Lauber et al. (13) demonstrated that a lipid molecule, LPC, was released by apoptotic cells and was chemotactic for phagocytes. Therefore, to investigate whether the prophagocytic activity in the supernatant of apoptotic PMN could also be due to a lipid, we removed lipid molecules from the supernatant using PHM-L LIPOSORB Absorbent. Removal of lipids failed to affect the prophagocytic activity of the supernatant of apoptotic PMN (Fig. 3*C*).

As caspases play a central role in the apoptotic process, the possibility that caspase activity was involved in the release of the prophagocytic factors from apoptotic cells was examined. The pancaspase inhibitor zVAD-fmk (100 μM) was added to the PMN culture immediately before induction of apoptosis by aging. The prophagocytic activity was no longer present in the supernatant of apoptotic PMN prepared in the presence of zVAD-fmk suggesting that the release of the prophagocytic factors is dependent on caspase activity during the apoptotic process (Fig. 3*D*).

Possible signaling mechanisms involved in the phagocytosis stimulated by the supernatant of apoptotic PMN

We have previously shown that LXs, endogenously produced eicosanoids with anti-inflammatory actions, can stimulate phagocytosis of apoptotic leukocytes in vitro (15, 16) and in vivo (17). Furthermore, we have demonstrated that peptide agonists of FPRL-1 can also stimulate phagocytosis (17, 20). Ligand binding to the fMLP receptor and FPRL-1 can be blocked by the antagonist, Boc2 (19). As illustrated in Fig. 4*A*, Boc2 also blocks the enhanced phagocytosis stimulated by the supernatant of apoptotic PMN suggesting that the putative prophagocytic factors may act via the FPRL-1 receptor. Interestingly, when used in concert, the prophagocytic effect of LXA_4 (1 nM) and the supernatant of apoptotic PMN are not additive, suggesting that that there is a common signaling mechanism involved (Fig. 4*B*). Elevation of intracellular cAMP by prior exposure of THP-1 cells to the cell permeant analog 8-bromo-cAMP, inhibited the phagocytosis stimulated by the supernatant of apoptotic PMN. Conversely, the protein kinase A inhibitor, Rp-cAMP, mimicked the effect of the supernatant of apoptotic PMN, but was not additive with these supernatants suggesting that they may act on a common target (Fig. 4*C*).

Nonmuscle myosin II H chain isoform A (MYH9) has been implicated as having a role in establishing cell polarity (28) and MYH9 activity has previously been demonstrated to play a role in particle internalization during both FcγR and complement receptor type 3-mediated phagocytosis (29). We have previously shown that LXA_4 and Ac2-26 dephosphorylate serine residues within MYH9 (16). To investigate whether the supernatant of apoptotic PMN had an effect on MYH9 phosphorylation, we prefractionated the phosphoserine proteins by immunoprecipitation from cell lysates of THP-1 cells treated with the supernatant of freshly isolated PMN or the supernatant of apoptotic PMN, before immunoblotting for MYH9. As shown in Fig. 4*D*, MYH9 undergoes significant serine dephosphorylation in response to stimulation with the supernatant of apoptotic PMN compared with the supernatant of freshly isolated PMN. Total cellular levels of MYH9 remain unchanged after treatments (Fig. 4*D*).

Supernatant of apoptotic PMN, apoptotic Jurkat T cells, apoptotic mesangial cells, but neither viable nor necrotic PMN, contain annexin-1 and are associated with enhanced phagocytosis

To identify the potential prophagocytic factors in the supernatant of apoptotic PMN, we used Nano-LC MS/MS to analyze the protein content of this conditioned medium. The supernatant of apoptotic PMN was concentrated, the proteins separated by SDS-PAGE, and individual bands prepared for MS as described in *Materials and Methods*. A list of 73 proteins present in the supernatant of apoptotic PMN was created (see Table II and supplemental Table I[4]) which represented a panel of potential prophagocytic factors. Annexin-1 was identified by 10 high-scoring peptide mass spectra (Fig. 5*A*) and stood out as a potential prophagocytic factor as we have recently reported that annexin-1 is present in the supernatant from dexamethasone-treated Mϕs and is responsible for the enhanced phagocytosis stimulated by this supernatant (20). We confirmed the presence of annexin-1 by Western blot in the supernatant of apoptotic PMN and also found that apoptotic hMC and apoptotic Jurkat T cells released this molecule (Fig. 5*B*). Annexin-1 was present in the supernatant of viable and necrotic PMN, but in amounts that were almost undetectable by Western blot and probably insufficient to stimulate phagocytosis (Fig. 5*B*).

To investigate whether annexin-1 played a role in the phagocytosis modulated by the supernatant of apoptotic PMN, the supernatant of apoptotic PMN was depleted of annexin-1 by four consecutive immunoprecipitations using a polyclonal anti-annexin-1 Ab. These immunodepleted supernatants failed to increase phagocytosis of apoptotic PMN by differentiated THP-1 cells, suggesting that annexin-1 contributes to the prophagocytic activity of the supernatant of apoptotic PMN (Fig. 5*C*). Immunodepletion using an IgG control Ab had no effect on the prophagocytic activity of the supernatant. The effectiveness of the immunoprecipitation protocol was investigated by Western blot analysis, probing for annexin-1 in the samples immunodepleted using an anti-annexin-1 Ab or IgG control (Fig. 5*C*).

It has been reported that the full-length annexin-1 molecule can be cleaved by a serine protease, neutrophil elastase (30, 31). We postulated that the resultant ~3-kDa cleavage product derived from the N-terminal end of the annexin-1 protein may be responsible for the prophagocytic effect of the supernatant of apoptotic PMN. To investigate this possibility, we fractionated the supernatant using 10-kDa molecular mass cut-off spin columns to separate the annexin-1 fragments and examine the prophagocytic activity of

[4] The online version of this article contains supplemental material.

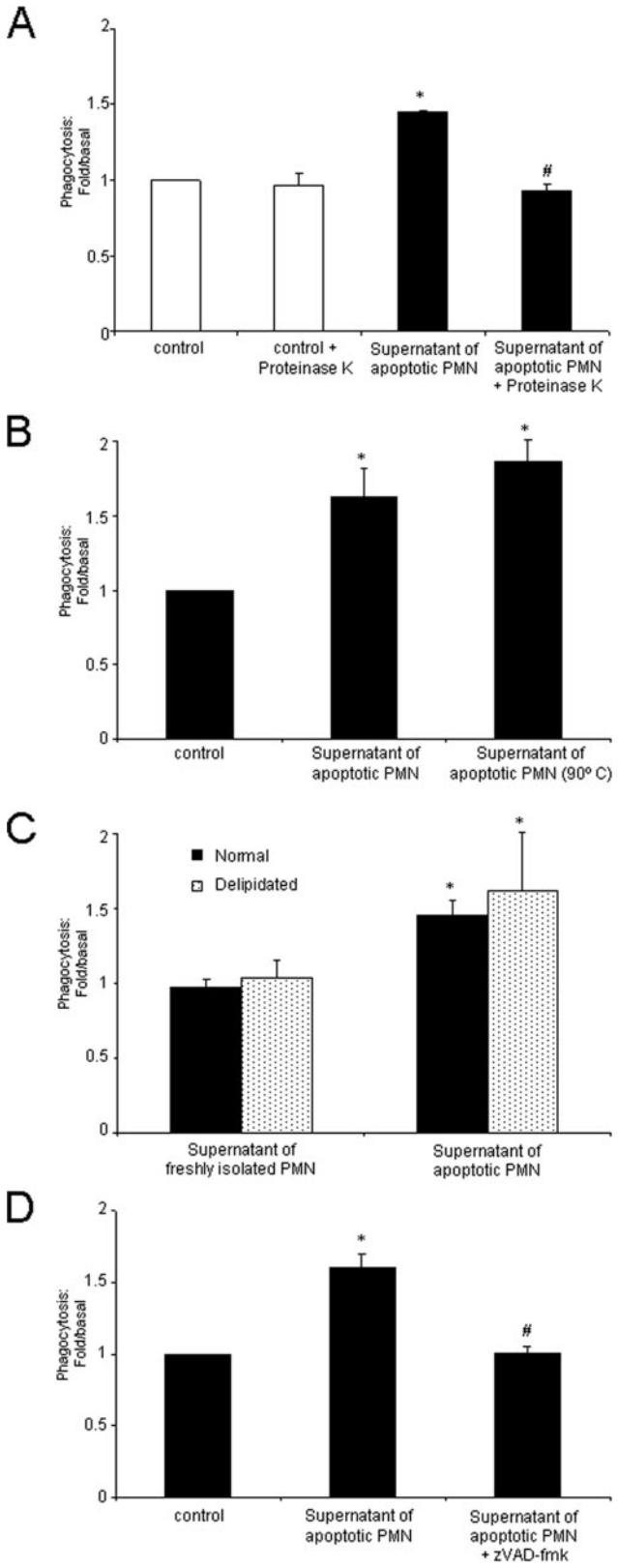

FIGURE 3. Characterization of the putative prophagocytic factors in the supernatant of apoptotic PMN. *A*, Differentiated THP-1 cells were exposed to control ± proteinase K (100 μg/ml) treatment or supernatant of apoptotic PMN ± proteinase K (100 μg/ml) treatment for 15 min before coincubation with apoptotic PMN for 2 h. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 3$): *, $p < 0.005$ vs control, #, $p < 0.005$ vs supernatant of apoptotic PMN. *B*, Differentiated THP-1 cells were treated with the supernatant of apoptotic PMN or the supernatant of apoptotic PMN that had been heated for 10 min at 90°C, for 15 min before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 4$): *, $p < 0.05$ vs control. *C*, Differentiated THP-1 cells were treated with the supernatant of freshly isolated PMN ± delipidation treatment or the supernatant of apoptotic PMN ± delipidation treatment for 15 min before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 5$): *, $p < 0.05$ vs appropriate control. *D*, Differentiated THP-1 cells were treated the supernatant of apoptotic PMN or supernatant of PMN allowed to become apoptotic in the presence of zVAD-fmk (100 μM) for 15 min before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 3$): *, $p < 0.05$ vs control, #, $p < 0.05$ vs supernatant of apoptotic PMN.

each fraction. Both fractions stimulated phagocytosis to a similar extent but this prophagocytic effect was abrogated by addition of an annexin-1-neutralizing Ab suggesting that both fractions contained annexin-1 or annexin-1 fragments with prophagocytic activity (Fig. 5*D*). As expected, the neutralizing Ab also abrogated the prophagocytic effect of the neat supernatant of apoptotic. Basal levels of phagocytosis nor the phagocytosis stimulated by LXA_4 were affected by the neutralizing Ab, precluding the possibility that the Abs were acting directly on the Mϕ cell surface annexin-1, previously shown to be involved in phagocytosis (22).

Discussion

In this study, we report that cells undergoing apoptosis release signals that stimulate nonphlogistic phagocytosis of apoptotic PMN by human monocyte-derived Mϕ and differentiated THP-1 cells. This effect is coupled to release of TGF-β1 and F-actin reorganization in Mϕ. Using Nano-LC MS/MS, we have ascertained that annexin-1 and possibly a low molecular mass fragment of annexin-1 are released by the apoptotic cells and are responsible for the prophagocytic activity observed in the conditioned medium of apoptotic PMN, hMC, and Jurkat T cells.

During the apoptotic process, cells undergo extensive macromolecule changes that encompass cleavage and translocation, including proteolytic cleavage by caspase proteases of key structural proteins, chromatin condensation, and membrane blebbing (32). It has been speculated that these changes may provide a mechanism for the safe removal of cellular debris, including signaling for uptake by phagocytes as well as elimination, modification, and detoxification of certain cellular constituents (33). In vivo, apoptotic cells are essentially always found inside phagocytes in healthy tissues suggesting that phagocytosis of apoptotic cells occurs very rapidly once the apoptotic process has begun (34). This observation points to the existence of an efficient and robust system that regulates the clearance of apoptotic cells. Therefore, it is reasonable to predict that dying cells may release signals to induce migration of phagocytes to sites of apoptosis and to facilitate their uptake.

Lauber et al. (13) demonstrated that apoptotic cells release attraction signals that induce the migration of professional phagocytes to sites of apoptosis. Through a series of experiments, the authors identified that a soluble factor, LPC, was released by apoptotic cells in a caspase-3-dependent manner. LPC was released into the supernatants of UV-treated cells, and attracted differentiated THP-1 cells as well as primary Mϕ. Earlier studies showed that other factors were released by apoptotic cells including tRNA synthetases (35) and a ribosomal protein dimer that mediates monocyte attraction (36, 37). We theorized that having attracted phagocytes to their location, apoptotic cells might release signals

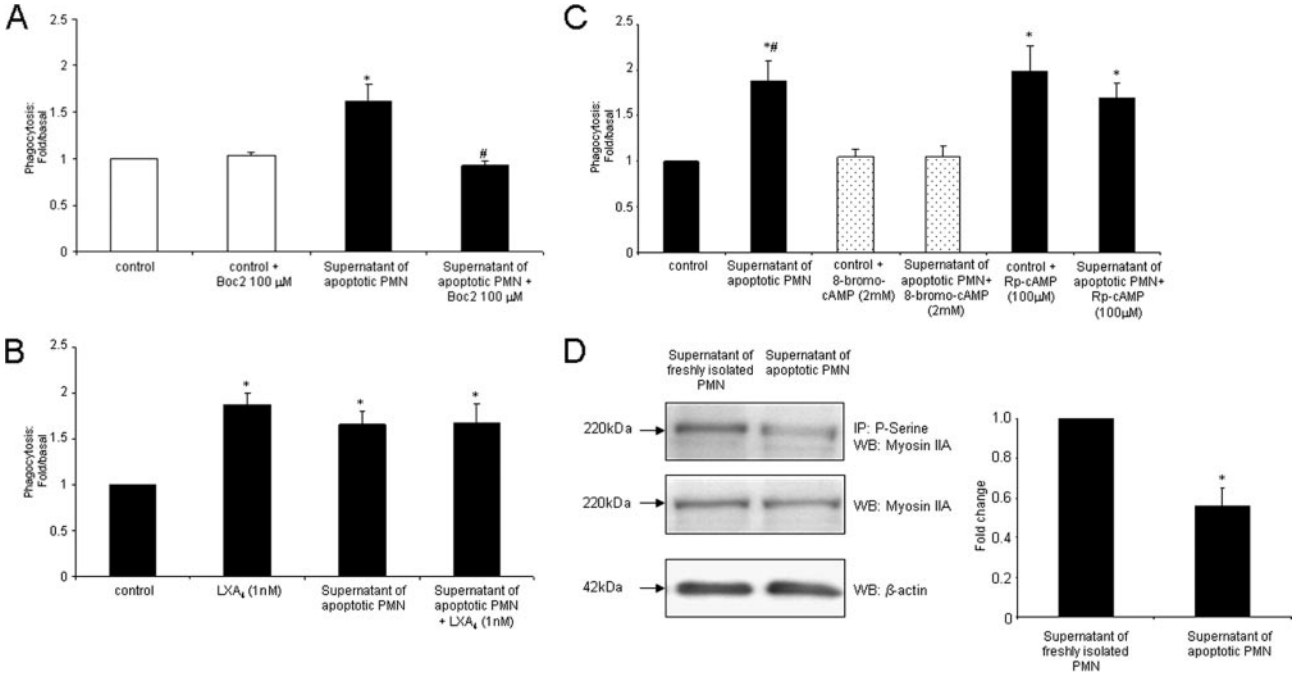

FIGURE 4. Signaling events associated with phagocytosis modulated by the supernatant of apoptotic PMN. *A*, Differentiated THP-1 cells were exposed to control ± Boc2 (100 μM) and the supernatant of apoptotic PMN ± Boc2 (100 μM) for 15 min before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 4$): ∗, $p < 0.05$ vs control, #, $p < 0.005$ vs supernatant of apoptotic PMN. *B*, Differentiated THP-1 cells were treated LXA_4 (1 nM) or the supernatant of apoptotic PMN ± LXA_4 (1 nM) for 15 min before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 4$): ∗, $p < 0.05$ vs control. *C*, Differentiated THP-1 cells were exposed to control ± 8-bromo-cAMP (2 mM) or Rp-cAMP (100 μM), or the supernatant of apoptotic PMN ± 8-bromo-cAMP (2 mM) or Rp-cAMP (100 μM) for 15 min before coincubation with apoptotic PMN. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 6$): ∗, $p < 0.02$ vs control, #, $p < 0.005$ vs the supernatant of apoptotic PMN plus 8-bromo-cAMP (2 mM). *D*, Differentiated THP-1 cells were treated with the supernatant of freshly isolated PMN or the supernatant of apoptotic PMN for 15 min at 37°C before lysis in RIPA buffer. Phosphoserine proteins were immunoprecipitated from 1 mg of whole cell lysate using an anti-phosphoserine 16B4 Ab. Immunocomplexes were denatured in sample buffer, separated by SDS page, transferred to PVDF membranes, and probed for myosin IIA. Total cellular myosin IIA was determined by probing samples of whole cell lysate for myosin IIA. Expression of β-actin was examined as a loading control. A representative blot of $n = 3$ is shown. Densitometry results are depicted graphically as fold/basal relative to β-actin compared with cells treated with the supernatant of freshly isolated PMN: ∗, $p < 0.05$ vs the supernatant of freshly isolated PMN.

to promote their phagocytic clearance and in doing so protect surrounding tissues from harmful exposure to the inflammatory or immunogenic contents released as the cells undergo secondary necrosis. We studied the effects of conditioned medium of apoptotic cells on the phagocytosis of apoptotic PMN by Mϕ and differentiated THP-1 cells. Here, we report that the supernatants of apoptotic PMN, hMC, and Jurkat T cells promote phagocytosis of apoptotic PMN by Mϕ and differentiated THP-1 cells. We found that supernatant from neither viable nor necrotic PMN-stimulated phagocytosis suggesting the prophagocytic effect was apoptosis specific.

Biochemical characterization of the supernatant of apoptotic PMN suggested that the putative prophagocytic factors were proteins and not lipids. We used tandem MS to create a list of the proteins present in the supernatant of apoptotic PMN. As the supernatants of apoptotic PMN were prepared from apoptotic PMN samples that contained a fraction of viable cells, we suggest that the resultant list of proteins contained potential prophagocytic molecules but also many proteins indiscriminately released due to normal cellular growth, cell lysis, and other cellular functions. Among the potential prophagocytic molecules was annexin-1. The presence of annexin-1 was confirmed by Western blot and was also detected in the supernatants of apoptotic hMC and Jurkat T cells. The role of annexin-1 in the observed prophagocytic effect of the supernatant of apoptotic PMN was confirmed by immunodepletion studies. When annexin-1 was immunodepleted from the supernatant of apoptotic PMN, the supernatant's prophagocytic activity was abrogated. An immunodepletion process conducted in parallel using an IgG control Ab had no effect on the level of stimulated phagocytosis. A role for annexin-1 in the phagocytosis of apoptotic cells is supported by our previous finding that annexin-1 released by dexamethasone-treated Mϕ, and the annexin-derived peptide Ac2–26 modulate Mϕ phagocytosis of apoptotic PMN (20). Furthermore, Arur et al. (21) have shown that annexin-1 is exported from the cytosol to the plasma membrane of anti-Fas IgM-treated apoptotic Jurkat T cells in a caspase-dependent manner where it colocalizes with PtdSer in discrete patches. It is now well-established that PtdSer is an important recognition and engulfment trigger on apoptotic cells (6). Lack of annexin-1 function in the Jurkat T cells caused by annexin-1 small interfering RNA or anti-annexin-1 Abs was shown to correlate with a decrease in tethering and internalization by endothelial cells supporting the idea of annexin-1 as an engulfment ligand. Moreover, soluble annexin-1 significantly enhanced tethering of annexin-1-silenced apoptotic cells to phagocytes to near normal wild-type levels (21). Fan et al. (22) have reported that mAbs against annexin-1 and -2 inhibited phagocytosis of apoptotic cells when directed against the apoptotic targets as well as the phagocyte. Additionally, many of the anti-inflammatory effects of glucocorticoids have been attributed to annexin-1 in experimental models of inflammation (38). However, not all cells express annexin-1, but when one looks at the extent of

Table II. *Proteins released by PMN aged in vitro for 20 h as identified by Nano-LC MS/MS*[a]

	Protein Identity	Uniprot ID	Accession No.	No. of Unique Peptides
1	α-Actinin-1	ACTN1	P12814	12
2	α-Actinin-4	ACTN4	O43707	7
3	Annexin-1	ANXA1	P04083	10
4	Annexin-3	ANXA3	P12429	12
5	Annexin-4	ANXA4	P09525	1
6	Annexin-5	ANXA5	P08758	2
7	Annexin-6	ANXA6	P08133	1
8	Actin-related protein 2/3 complex	ARPC4	P59998	2
9	Brain acid soluble protein 1	BASP	P80723	1
10	Bactericidal permeability-increasing protein	BPI	P17213	3
11	Calnexin	CALX	P27824	1
12	Adenylyl cyclise-associated protein 1	CAP1	Q01518	1
13	Catalase	CATA	P04040	10
14	Chitinase-3-like protein 1	CH3L1	P36222	2
15	Clip-associating protein 1	CLAP1	Q7Z460	1
16	Phosphatidylinositol 4;5-biphosphate (PIP2)-dependent arf1 GTPase-activating protein	DDEF1	Q9ULH1	4
17	Discs large homolog 5	DLG5	Q8TDM6	1
18	Leukocyte elastase	ELNE	P08246	12
19	α-Enolase	ENOA	P06733	14
20	Endoplasmin precursor	ENPL	P14625	1
21	Ezrin	EZRI	P15311	1
22	Antibacterial protein FALL-39	FAL39	P49913	5
23	Filamin-A	FLNA	P21333	3
24	Ferritin L chain	FRIL	P02792	1
25	Glyceraldehyde-3-phosphate dehydrogenase	G3P	P04406	1
26	Glucose-6-phosphate isomerase	G6PI	P06744	17
27	Rho GDP-dissociation inhibitor 2	GDIS	P52566	4
28	Glutathione *S*-transferase P	GSTP1	P09211	1
29	Core histone macro-H2A.2	H2AW	Q9P0M6	1
30	Hemoglobin β subunit	HBB	P68871	7
31	Hornerin	HORN	Q86YZ3	5
32	Leukocyte elastase inhibitor	ILEU	P30740	17
33	Integrin β-2	ITB2	P05107	1
34	Laminin β-1 chain	LAMB1	P07942	1
35	L-lactate dehydrogenase A-like 6B	LDH6B	Q9BYZ2	2
36	Eosinophil lysophospholipase	LPPL	Q05315	2
37	Lysozyme C precursor	LYSC	P61626	3
38	Methyltransferase-like protein 6	METL6	Q8TCB7	1
39	Matrix metalloproteinase-8	MMP8	P22894	7
40	Matrix metalloproteinase-9	MMP9	P14780	9
41	Eosinophil peroxidase	PERE	P11678	15
42	Myeloperoxidase	PERM	P05164	33
43	Phosphoglycerate mutase 1	PGAM1	P18669	5
44	Plastin-2	PLSL	P13796	22
45	Purine nucleoside phosphorylase	PNPH	P00491	1
46	Peptidyl-prolyl *cis*-transisomerase B	PPIB	P23284	3
47	Profilin-1	PROF1	P07737	4
48	Glycogen phosphorylase	PYGL	P06737	2
49	Fibrinogen	Q3KPF2	Q3KPF2	1
50	Skeletal muscle specific actinin; α3	Q4KKV2	Q4KKV2	1
51	Proteinase 3	Q4VB09	Q4VB09	2
52	Ribonuclease	Q4VBC1	Q4VBC1	6
53	Azurocidin	Q52LG4	Q52LG4	10
54	Full-length CDNA clone cs0de007yp21 of placenta of *Homo sapiens*	Q53XB8	Q53XB8	1
55	Benzodiazapine	Q53Y59	Q53Y59	1
56	Proteolysis-inducing factor	Q53YJ2	Q53YJ2	2
57	S100 calcium-binding protein A8	Q5SY70	Q5SY70	24
58	Lipocalin	Q5SYW0	Q5SYW0	13
59	Chaperone; ABC1 activity of BC1 complex like	Q5T7A5	Q5T7A5	1
60	Actin	Q5U032	Q5U032	3
61	Myeloid cell nuclear differentiation Ag	Q5VUU6	Q5VUU6	1
62	Serum albumin	Q645G4	Q645G4	12
63	Defensin; α3	Q6EZE9	Q6EZE9	4
64	S100A9 protein	Q6FGA1	Q6FGA1	9
65	CTSG protein	Q6IBJ6	Q6IBJ6	14
66	S-100P protein	S100P	P25815	1
67	Calgranulin C	S10AC	P80511	4
68	Solute carrier family 13 member 1	S13A1	Q9BZW2	1
69	Erythrocyte band 7 integral membrane protein	STOM	P27105	8
70	Transaldolase	TALDO	P37837	7
71	Transketolase	TKT	P29401	11
72	Triosephosphate isomerase	TPIS	P60174	6
73	Lactotransferrin	TRFL	P02788	39

[a] Protein identifications were generated using the SEQUEST program to search the Uniprot Human IPI Protein Sequence Database (version 3.15). The probability based evaluation program, Protein Prophet, was used for filtering putative identifications based on a maximum *p* value threshold corresponding to 90% likelihood of correct identification (see supplemental Table I data). The number of unique peptides found for each protein is indicated in the final column.

redundancy in apoptotic cell recognition and engulfment, it is reasonable to speculate these cells have similar mechanisms to stimulate their phagocytic clearance once apoptosis is initiated. Interestingly, some of the proteins which we detected as being released by aged PMN overlap with those described by Bannenberg et al. (39) which display temporal changes during the resolution phase

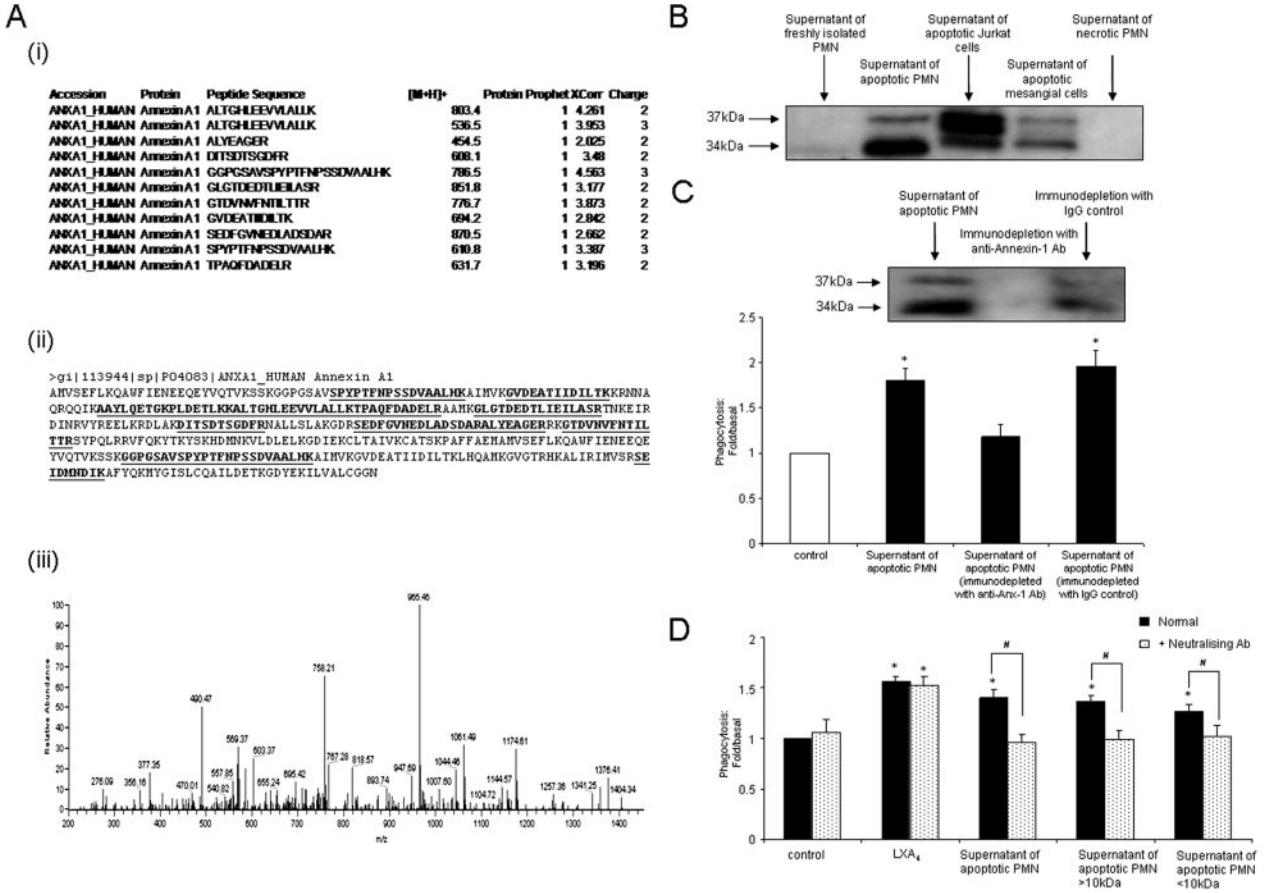

FIGURE 5. Annexin-1 is released by apoptotic PMN, apoptotic Jurkat T cells, apoptotic mesangial cells, but not freshly isolated nor necrotic PMN, and stimulates phagocytosis of apoptotic PMN. *A*, Annexin-1 (ANXA1) was identified in the supernatant of apoptotic PMN by LC MS. High-scoring peptides (*i*) were identified throughout the protein sequence (*ii*). A representative spectrum of the [M + 2H]2+ ion from one peptide is shown (*iii*). *B*, Samples of the supernatant of freshly isolated PMN, apoptotic PMN, apoptotic Jurkat T cells, apoptotic mesangial cells, and necrotic PMN were denatured in sample buffer, separated by SDS-PAGE, transferred to PVDF membrane and probed for annexin-1. A representative blot of $n = 3$ is shown. *C*, Samples of the supernatant of apoptotic PMN, the supernatant of apoptotic PMN immunodepleted of annexin-1 with a polyclonal anti-annexin-1 Ab, and the supernatant of apoptotic PMN immunodepleted using an IgG control were denatured in sample buffer, separated by SDS-PAGE, transferred to PVDF membrane and probed for annexin-1. A representative blot of $n = 3$ is shown. To examine the prophagocytic potential of the immunodepleted samples, differentiated THP-1 cells were treated the supernatant of apoptotic PMN, the supernatant of apoptotic PMN immunodepleted with a polyclonal anti-annexin-1 Ab or the supernatant of apoptotic PMN immunodepleted using an IgG control for 15 min before coincubation with apoptotic PMN for 2 h. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 3$): *, $p < 0.05$ vs control. *D*, Differentiated THP-1 cells were treated LXA_4 (1 nM), the supernatant of apoptotic PMN, the fraction of supernatant of apoptotic PMN containing molecules >10 kDa, and the fraction of supernatant of apoptotic PMN containing molecules <10 kDa in the presence or absence of a neutralizing Ab against annexin-1 for 15 min before coincubation with apoptotic PMN for 2 h. Data are expressed as fold of induction over basal (control) and represent means ± SEM ($n = 3$): *, $p < 0.05$ vs control, #, $p < 0.05$.

of murine peritoneal inflammation. These include fibrinogen and S100A9 protein.

It has been reported that cleaved annexin-1 is present in the bronchoalveolar lavage fluid obtained from patients with cystic fibrosis and appears to be cleaved by neutrophil elastase at the N-terminal portion between Val[36] and Ser[37] (31). Recently, Rescher et al. (40) have shown annexin-1 to be a substrate for human leukocyte elastase in vitro. We investigated whether the ~3-kDa N-terminal fragment of annexin-1 produced when annexin-1 is cleaved was present in the supernatant of apoptotic PMN and may be responsible for the observed prophagocytic effect. We fractionated the supernatant of apoptotic PMN using a 10-kDa molecular mass cut-off membrane and found that both fractions contained prophagocytic activity that could be abrogated using a neutralizing Ab for annexin-1. We verified by Western blot that the 34- and 37-kDa fragments of annexin-1 did not pass through the membrane (data not shown). We therefore suggest that a ~3-kDa fragment(s) of annexin-1 possessing prophagocytic activity, as well as full-length annexin-1 and the inactive 34-kDa fragment, are present in the supernatant of apoptotic PMN and that their prophagocytic effects can be negated by a neutralizing Ab against annexin-1.

We investigated the signaling pathways activated by the supernatant of apoptotic PMN and found that results further supported the theory that annexin-1 and its peptide derivatives stimulate phagocytosis of apoptotic cells by Mϕ. Perretti et al. (19) demonstrated that neutrophil-derived annexin-1 could be immunoprecipitated with the FPRL-1 selectively when the leukocytes were adhered to endothelial monolayers. In additional experiments, both the annexin-1-derived peptide Ac2-26 and a shorter peptide, denoted Ac2-12, competed with tritium-labeled LXA_4 for binding to the FPRL-1 (19). It has also been reported that annexin-1 interacts with FPRL-1 only, while Ac2-26 bound both FPR and FPRL-1 (41), supporting our hypothesis that annexin-1 and its peptide derivatives interact with FPRL-1 to stimulate phagocytosis of

apoptotic cells. Interestingly, an annexin-1 peptide, Ac1-25, has also been shown to bind FPRL2 (42). We have previously elaborated on the anti-inflammatory actions of LXA_4 and Ac2-26 by reporting that both molecules promote the phagocytosis of apoptotic cells by Mϕ and differentiated THP-1 cells and that this effect can be blocked by the FPR/FPRL-1 antagonist, Boc2 (15, 20). In this study, we have found that the prophagocytic effect of the supernatant of apoptotic PMN can also be blocked by Boc2. Furthermore, the prophagocytic activity of LXA_4 and the supernatant of apoptotic PMN are not additive suggesting a common receptor mechanism. We also suggest the involvement of the cAMP/PKA pathway which is known to be involved in the intracellular signaling that controls the uptake of apoptotic cells (43). We have previously shown that phagocytosis stimulated by LXA_4 (15), the peptide mimetic of annexin-1 Ac2-26, and the supernatant of dexamethasone-treated Mϕ (20) can be inhibited by the cell permeable cAMP analog 8-bromo-cAMP and mimicked by the PKA inhibitor Rp-cAMP. We now report similar results when using the supernatant of apoptotic PMN to stimulate phagocytosis, suggesting a modulatory role for cAMP.

MYH9 is a class IIA nonmuscle myosin H chain protein. Myosin IIA has been implicated as having a role in establishing cell polarity (28) and myosin IIA activity has previously been demonstrated to play a role in particle internalization during both FcγR and complement receptor type 3-mediated phagocytosis (29). Complement receptor-mediated phagocytosis specifically uses MYH9 to facilitate the formation of actin cups around particles (29). Our data demonstrate that the supernatant of apoptotic PMN stimulates serine dephosphorylation of MYH9 similarly to LXA_4 and Ac2-26 (16). These data, along with the observed effect on actin rearrangement, suggest that during apoptosis, cells release annexin-1 and perhaps peptide derivatives that induce cell polarization involving MYH9 which "primes" the THP-1 cell for phagocytosis.

The release of annexin-1 appears to be dependent on the apoptotic process. It is well-established that caspases play a central role in apoptosis (44), although it has been demonstrated that serine proteases mediate apoptosis-like cell death under caspase-inhibiting conditions (45). Arur et al. (21) demonstrated that annexin-1 recruitment to the cell surface of apoptotic Jurkat T cells is sensitive to the pancaspase inhibitor zVAD-fmk. When we incubated zVAD-fmk with freshly isolated PMN and allowed them to become apoptotic by aging in vitro, the subsequent conditioned medium failed to stimulate phagocytosis suggesting that caspase enzyme activity played a role in release of annexin-1. Incubation with the caspase inhibitor reduced the percentage of PMN undergoing apoptosis compared with vehicle control though not to a statistically significant degree (data not shown), so we cannot rule out the possibility that the general reduction in apoptosis as a result of inhibition of the caspase enzymes may have resulted in the reduced prophagocytic effect of the conditioned medium. Additionally, supernatants of PMN induced to die by necrosis failed to stimulate phagocytosis therefore ruling out the possibility that intracellular annexin-1 is released upon cell lysis rather than by an active process.

The finding that annexin-1 is released by apoptotic cells and promotes their nonphlogistic engulfment by phagocytes is particularly relevant in diseases exacerbated by impaired clearance of apoptotic cells. Impaired clearance of apoptotic cells has been demonstrated in cystic fibrosis (CF) airways (46) and has been implicated in the pathogenesis of systemic lupus erythematosus (47). Interestingly, Bensalem et al. (48) recently reported that annexin-1 was down-regulated in CF knockout mice lacking the CF transmembrane regulator gene and in CF patients a decreased level of annexin-1 correlated with a more severe phenotype. Anti-annexin-1 IgM and IgG Abs have been found in patients with systemic lupus erythematosus (49).

In conclusion, our results indicate the annexin-1 and its peptide derivatives are released by apoptotic cells and promote efficient phagocytosis of apoptotic cells. Having been released by apoptotic PMN, annexin-1 may contribute in a number of ways to resolve inflammation, not only promoting clearance of apoptotic cells by phagocytes but also promoting apoptosis in surrounding PMN (50) and preventing PMN transendothelial extravasation (51). These findings add to the growing body of evidence that supports the significance of annexin-1 and its peptidomimetics (19, 20) in promoting the resolution of inflammation.

Disclosures

The authors have no financial conflict of interest.

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