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Macrophage-Induced Neutrophil Apoptosis

Adriana J. Meszaros, Jonathan S. Reichner, and Jorge E. Albina

Macrophages (Mφ) contribute to the resolution of early inflammation by recognizing and ingesting apoptotic polymorphonuclear neutrophils (PMN). In addition, experiments reported here demonstrated that Mφ can actively induce PMN apoptosis. Coculture of cells from 2- or 5-day-old wounds in rats, or of Mφ purified from such preparations, with PMN-rich wound cell populations obtained 1 day after wounding increased PMN apoptosis by >3-fold. Neither resident- nor Propionibacterium acnes-elicted peritoneal Mφ-induced PMN apoptosis. Apoptosis was not mediated by a soluble factor and required E:T contact. Fixed wound-Mφ and membrane isolates from viable Mφ were as effective as intact cells in inducing PMN apoptosis. Mφ-induced apoptosis was inhibited by peptide Arg-Gly-Asp-Ser, anti-β3 (CD61) Ab, CD36 peptide, or anti-TNF-α Ab. Soluble TNF-α did not induce PMN apoptosis. In additional studies, K562 cells (negative for β3, TNF-α, and Fas ligand) transfected to express either αβ3 integrin, an uncleavable membrane form of TNF-α, or both were used in cocultures with wound PMN. Only the double transfectants were able to induce PMN apoptosis, an effect inhibited by anti-β3 (CD61) or anti-TNF-α Abs. These results demonstrate that wound Mφ induce PMN apoptosis through a constitutive effector mechanism requiring both intercellular binding through integrin-ligand interactions and membrane-bound TNF-α.


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Materials and Methods

**Animals**

Male Fischer rats (150–200 g; VAF-plus; Charles River Laboratories, Wilmington, MA) were housed in barrier cages, fed rat chow and water ad libitum, and were monitored by Brown University/Rhode Island Hospital veterinary personnel.

**Wound model, cells, and culture conditions**

The s.c. polyvinyl alcohol sponge wound model using rats was described previously (20). Single wound cell suspensions were generated from sponges retrieved 1, 2, and 5 days after implantation and used immediately.

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Polymorphonuclear leukocytes (PMN) are the first blood-borne nucleated cells to populate a site of acute inflammation. PMNs isolated from the blood are normally short-lived and undergo spontaneous apoptosis upon in vitro culture (1, 2). However, entry into an inflammatory locus extends PMN lifespan (3–7). In this regard, work from this laboratory demonstrated that >90% PMN harvested within 24 h after injury from sterile experimental wounds in rats survive overnight incubation in vitro (8). In contrast, ~60% of circulating PMN and those retrieved from the wounds 2–3 days after wounding are apoptotic by the end of identical cultures (8). Thus, while early inflammatory PMN have a survival advantage over their circulating counterparts, PMN longevity appears to be reduced as the wound progresses. The current paradigm indicates that infiltrating PMN undergo spontaneous apoptosis at the site of inflammation, where they are subsequently recognized and ingested by neighboring macrophages (Mφ) (reviewed in Ref. 9). In support of this paradigm, Mφ isolated from the aforementioned wounds, where the disappearance of PMN coincides temporally with the establishment of a Mφ-dominant infiltrate, were able to ingest apoptotic, but not viable, wound PMN (8).

Apolipoproteins of inflammatory PMN and their clearance by Mφ has been interpreted as beneficial because the recognition and ingestion of apoptotic PMN, or of their shed apoptotic bodies, prevents the lytic release of potentially noxious contents into the microenvironment (10). In addition, it has been proposed that the ingestion of apoptotic PMN triggers the production of antiinflammatory mediators by the Mφ (11, 12). Moreover, persistent PMN-rich inflammatory infiltrates have been associated with the increased tissue destruction associated with unresolved inflammatory reactions, such as those found in adult respiratory distress syndrome, rheumatoid arthritis, and gout (reviewed in Refs. 13 and 14). It appears, then, that efficient PMN removal is required for the resolution of acute inflammation and probably for the progression of wound healing.

Experiments reported here tested the hypothesis that, beyond ingesting already apoptotic PMN, Mφ can actively induce apoptosis of PMN. Results to be presented, obtained using an experimental wound model in rats, support this hypothesis and demonstrate that the capacity to induce apoptosis in wound PMN is restricted to Mφ isolated from the wound. In contrast with other models where PMN apoptosis is induced by Mφ secretory products (15–19), findings indicate that the induction of PMN apoptosis by wound Mφ requires cell-to-cell contact and the constitutive presentation on Mφ effectors of β3 integrin subunits, CD36, and membrane-bound, but not soluble, TNF-α. Further confirming evidence was provided by transfection of αβ3 integrin and an uncleavable form of membrane-bound TNF-α (mTNFdm) into otherwise noncytotoxic cells, which endowed them with the ability to induce wound PMN apoptosis.

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3 Abbreviations used in this paper: PMN, polymorphonuclear leukocyte; Mφ, macrophage; mTNFdm, uncleavable membrane-bound TNF-α by deletion and mutation; RGDS, peptide Arg-Gly-Asp-Ser; RGES, peptide Arg-Gly-Glu-Ser; IOD, integrated OD; FasL, Fas ligand.
Where indicated, Møs in day 2 and 5 wound cell preparations were enriched by adherence to plastic. Cell counts were performed on LeukoStat-stained (Fisher Scientific, Pittsburgh, PA) cytopsin (Shandon, Pittsburgh, PA). Resident- and Propionibacterium acnes-elicited peritoneal Møs were collected as previously described (8). The human chronic myelogenous leukemia cell line, K562 (American Type Culture Collection, Manassas, VA), was maintained in RPMI 1640 (Life Technologies, Grand Island, NY), 10% FBS (HyClone, Logan, UT), 2 mM glutamine, 100 U/mI penicillin-streptomycin and propagated at 37°C in a 7% CO2 in air humidified incubator. K562 cells stably transfected with αβ3 integrin subunits were generously provided by Eric Brown (University of California, San Francisco, CA) (21) and maintained in media containing 1.2 mg/ml G418 (Sigma, St. Louis, MO). Expression of αβ3 was confirmed by flow cytometry. In coculture experiments, 1 x 105 cells from 2- or 5-day-old wounds, peritoneal Møs, or K562 cells were coincubated with 2 x 105 cells isolated from 1-day-old wounds in RPMI 1640, 1% FBS, 10 mM MOPS, 5 x 10-5 M-2-ME, and 100 U/ml penicillin-streptomycin for 24 h at 37°C in 7% CO2 in air. Control cultures contained day 1 wound cells. In additional coculture experiments, day 5 wound cells were separated from day 1 wound cells in 0.2 μM transwell 24-well plates (Costar, Cambridge, MA). Day 5 wound cells were also fixed (1% parafomaldehyde, 1 h at room temperature) and washed with PBS before coculture with day 1 wound cells at a 5:1 ratio. Membranes from the day 5 wound cells were isolated as previously described (22) and used similarly in cocultures.

Where indicated, day 5 wound cells were treated with 1 mM peptide Arg-Gly-Asp-Ser (RGDS) (Sigma, 20 min, 37°C), 25 μg/ml anti-rat CD61 (β3 chain) mAb (PharMingen, San Diego, CA), or 25 μg/ml anti-rat TNF-α polyclonal Ab (Endogen, Woburn, PA) before exposure to day 1 wound cells. Additionally, day 1 wound cells were treated with 25 μg/ml anti-rat TNFR-1 (s55) or (p55) polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA) before exposure to day 5 wound cells. Ab treatments were performed as indicated in the text. Control cultures were treated with 1 mM peptide Arg-Gly-Glu-Ser (RGES) (Sigma), 25 μg/ml species-matched nonspecific IgG control Ab (Jackson ImmunoResearch, West Grove, PA), or left untreated. Day 5 and 1 wound cells were also cocultured in the presence of 20 μg/ml CD36 peptide (18.0 mg/ml American Peptide Company, Sunnyvale, CA). In addition, day 1 wound cells were treated for 24 h with 10–100 ng/ml rat TNF-α (Biosource International, Camarillo, CA) alone or in cocultures containing K562 cells transfected with αβ3.

Where indicated, K562 cells were treated with 25 μg/ml anti-human-β3 (AP-3) polyclonal Ab (American Type Culture Collection), 25 μg/ml anti-human CD36 mAb (Ab-3) (NeoMarkers, Union City, CA), or 25 μg/ml anti-mouse TNF-α polyclonal Ab (Biosource International) for 1 h at room temperature before exposure to day 1 wound cells. Control K562 cells were treated with 25 μg/ml species-matched nonspecific IgG control Ab (Jackson ImmunoResearch) or left untreated.

Assessment of apoptosis

PMN (≥300) were evaluated for apoptosis through morphological characterization on LeukoStat-stained cytoktons using bright field microscopy. Criteria used to diagnose apoptosis included chromatin aggregation, cytoplasmatic vacuolation, and/or cell shrinkage (2). Results obtained by morphological examination were directly compared with those from flow cytometry with propidium iodide analysis of DNA content (23). Results obtained with either method were not different (i.e., PMN apoptosis in cultured day 1 wound cells = 5.4 ± 1.9% by morphology vs 6.6 ± 1.4% by propidium iodide, p > 0.05, χ2; PMN apoptosis in day 5 wound cells cocultured with day 1 wound cells = 17.9 ± 0.8% by morphology vs 20.1 ± 4.2% by propidium iodide, p > 0.05, χ2). Data shown are from morphological examination. Results reported in Figs. 1 and 2 are total apoptotic PMN at the end of culture. Thereafter, results are expressed as induced apoptosis (%), which equals apoptosis in cocultures minus apoptosis in day 1 wound cells cultured alone.

Cell transfection

Self-flanked cDNA was isolated from pSV23SmTNFdif-9K11E (LMBP 3404) (Belgian coordinated collections of microorganisms/Laboratorium voor Kreftvervagingen Biologische Plasmiden, Biological-Physiological Institute, Gent, Belgium) and transfected to Human fibroblasts (NIH3T3) with Lipofectin (American Type Culture Collection). Transformation products were screened initially by PCR using synthesized T7 and BGH primer pairs (Genosys, The Woodlands, TX) as per Invitrogen’s sequence specifications. Proper orientation of the insert was confirmed by restriction enzyme mapping using SpeI and DNA sequencing (Yale University, New Haven, CT). K562 cells were cotransfected with mTNFdm and pEYFP-N1 plasmid for yellow-green fluorescence protein (Clontech, San Francisco, CA) by electroporation at 220 V, 1000 μF using a Gene Pulsar II (Bio-Rad, Richmond, VA). Populations of transfectants expressing green fluorescence protein were purified to >90% positive by fluorescence cell sorting on a FACScan (Becton Dickinson, Franklin Lakes, NJ). Control K562 cells were cotransfected with pcDNA3.1 vector backbone and pEYFP-N1.

Analysis of protein expression

Expression of β3 integrin subunit was analyzed by fluorescent flow cytometry as previously described (26) using anti-human β3 (AP-3) polyclonal Ab (American Type Culture Collection) or species-matched nonspecific IgG control Ab (Jackson ImmunoResearch) and quantified by mean channel fluorescence.

Expression of mTNFdm protein after transfection of wild-type K562 cells and K562 cells stably transfected with αβ3 integrin subunits was confirmed by mean fluorescence. Transfected K562 cells were fixed in 4% parafomaldehyde in PBS for 30 min at room temperature, cytoplasm onto glass slides, and stained. Fixed K562 cells cytopsin was blocked with 10% normal goat serum for 30 min at room temperature and then stained with either 1 μg/ml anti-mouse TNF-α polyclonal Ab (Biosource International), 1 μg/ml anti-human TNF-α polyclonal Ab (R&D Systems, Minneapolis, MN), or 1 μg/ml mouse (Sigma) or rabbit (Jackson ImmunoResearch) IgG isotype controls for 1 h at room temperature. Slides were washed and incubated with 1 μg/ml Texas Red-conjugated goat F(ab)2, anti-rabbit IgG (Accurate, Westbury, NY) for 30 min.

Positive cells were quantified using a Nikon Microphot-FXA (Microvideo Instruments, Avon, MA), images were captured using a SenSys CCD fitted with PVCM acquisition software (Photometrics, Tucson, AZ), and integrated OD per cell (IOD) was determined for 40 cells using NIH Image 1.61 (National Institute of Health, Bethesda, MD) (in wild-type K562, IOD = 0.0 for species-matched nonspecific IgG vs IOD = 17.3 for anti-TNF-α; in K562 cells transfected with mTNFdm, IOD = 24.1 for species-matched nonspecific IgG vs IOD = 301.1 for anti-TNF-α, p < 0.05, Student’s t test).

Data presentation and analysis

Experiments were repeated at least twice. Throughout the manuscript, n represents the number of independent experiments each including at least triplicate samples. Unless otherwise stated, data are means ± SD from triplicate samples from a representative experiment. Statistical analysis was performed as indicated in the text.

Results and Discussion

PMN apoptosis in cultured day 1 wound cells correlates with the number of Mø

Comparable to previous results (8), 87.4 ± 3.4% of the cells retrieved from 1-day-old wounds were PMN, with ≤3% of the PMN being apoptotic upon harvest (8). Mø represented 4–21% of the remaining cells. The frequency of apoptotic PMN in the preparations after 24 h in culture ranged between 2 and 16% (n = 58) and correlated linearly with the number of Mø present (p < 0.05, r2 = 0.91) (Fig. 1).

Coculture of day 1 and day 5 wound cells increases PMN apoptosis

The strong correlation between the frequency of PMN apoptosis in cultured day 1 wound cells and the number of Mø present suggested the hypothesis that PMN apoptosis could be actively induced by Mø. To test this hypothesis, day 1 wound cell preparations were exposed to excess Mø in coculture experiments (n = 24) where day 5 wound cells (90.2 ± 2.6% Mø, 6.0 ± 2.2% PMN) were added at a 5:1 ratio to day 1 wound cells and incubated for 24 h. The frequency of apoptotic PMN by the end of culture (18.0 ± 0.8%) was higher than in cultures of day 1 wound cells alone (6.2 ± 0.5%, p < 0.05, Student’s t test) (Fig. 2).
results were obtained when Mφ from day 5 wound cell preparations were purified by adherence ($\geq 98\%$ Mφ) and used in coculture experiments ($6.6 \pm 0.8\%$ apoptosis in day 1 wound cells cultured alone vs $14.4 \pm 1.1\%$ in cocultures with day 5 wound Mφ, $p < 0.05$, Student’s $t$ test).

Excess PMN apoptosis was detectable by 4 h coculture and increased with time through 24 h (data not shown). Addition of day 5 wound cells at a 1:1 ratio was sufficient to increase PMN apoptosis in cocultures, with maximal PMN killing found at a 25:1 E:T ratio (induced apoptosis = $4.3 \pm 0.4\%$ for 1:1 cocultures, $11.1 \pm 0.8\%$ for 5:1, and $19.6 \pm 2.3\%$ for 25:1 cocultures, where induced apoptosis is the frequency of PMN apoptosis in cocultures minus that found in cultures of day 1 wound cells alone, $p < 0.05$, ANOVA). An E:T ratio of 5:1 was selected for subsequent experiments because previous work demonstrated this to be the maximal ratio of Mφ to PMN found in the wounds during the PMN dominant phase of inflammation (days 1–3 after wounding) (8).

As mentioned above, unpurified day 5 wound cell preparations used as effectors contained $\sim 6.0\%$ PMN. These cells did not account for the excess PMN apoptosis observed in cocultures. This conclusion is supported by findings in cocultures of day 1 wound cells with adherence-purified Mφ, fixed day 5 wound cells, or isolated cell membranes (see below) where PMN apoptosis was the same as in cocultures with unpurified day 5 wound cells.

The frequency of PMN apoptosis determined at the end of culture probably underestimates its actual rate because PMN undergoing apoptosis during the overnight coculture are likely to be rapidly ingested by Mφ (10). In this regard, 3–6% of Mφ contained myeloperoxidase-positive vacuoles by the end of culture.

The ability to induce PMN apoptosis was not restricted to Mφ from 5-day-old wounds because adherence-purified Mφ from 2-day-old wounds ($\geq 95\%$ Mφ) were equally competent in inducing PMN apoptosis (induced apoptosis = $13.4 \pm 1.6\%$ for day 2 wound Mφ cocultured with day 1 wound cells at a 5:1 ratio). In contrast, resident- and $P. acnes$-elicited peritoneal Mφ, which were previously shown to recognize and ingest apoptotic wound PMN (8), were incapable of inducing PMN apoptosis (induced apoptosis = $-1.1 \pm 1.6\%$ and $-0.4 \pm 0.7\%$, respectively).

Recent work from this laboratory demonstrated that inducible NO synthase is expressed exclusively by Mφ in target cells (28), its potential role in Mφ-dependent PMN apoptosis was investigated. Wound PMN proved to be remarkably resistant to NO. Culture of these cells for 24 h with NO donors in concentrations shown in this laboratory to kill different cell lines (5-nitroso-N-acetyl-d,L-penicillamine up to 200 $\mu M$, and 3-morpholinosydnonimine up to 100 $\mu M$) did not induce PMN apoptosis. Moreover, day 5 adherence-purified wound Mφ treated with the inducible NO synthase inhibitor, $N^\text{G}$-monomethyl-L-arginine (500 $\mu M$), were as capable of inducing PMN apoptosis as untreated Mφ (data not shown).

**Induction of PMN apoptosis by Mφ requires cell-to-cell contact**

The induction of PMN apoptosis by Mφ was determined to be mediated by constitutive effectors on the Mφ membrane. In this regard, data in Fig. 3 demonstrate that: 1) separating day 5 and day 1 wound cells with transwell inserts. In addition, day 1 wound cells were cultured with: 1) supernatants from overnight incubations of day 5 adherence-purified wound Mφ; 2) paraformaldehydefixed day 5 wound cells (fixed cells) at a 5:1 ratio to day 1 wound cells; or 3) day 5 wound cell membrane isolates (cell membranes) prepared from 10$^6$ fresh day 5 wound cells as indicated in Materials and Methods. Induced apoptosis (%) was calculated from the frequency of apoptotic PMN in cocultures minus its frequency in day 1 wound cell cultures. Induced apoptosis in cocultures using live, fixed, or membrane isolates from day 5 wound cells were not different from each other ($p > 0.05$, ANOVA).
1 wound cells in a transwell culture system prevents PMN apoptosis; 2) addition of culture supernatants from day 5 adherence-purified wound Mφ at 50% v:v to day 1 wound cells failed to induce PMN apoptosis; 3) fixed day 5 wound cells and cell membrane fractions from day 5 wound cells were as competent as live cells in inducing PMN apoptosis.

Mφ induction of PMN apoptosis requires Mφ expression of β3 integrin and CD36

The current paradigm indicates that the ingestion of apoptotic PMN by Mφ requires intercellular binding where a Mφ αβ3 integrin/CD36 complex binds to extracellular thrombospondin, which in turn acts as a “bridge” to PMN thrombospondin receptors (reviewed in Ref. 29). Indeed, previous work in this laboratory demonstrated that phagocytosis of apoptotic wound PMN by wound Mφ was partially inhibitable by the integrin-binding site peptide RGDS (8). As shown in Fig. 4, pretreatment of day 5 wound cells with RGDS (Fig. 4A) or with an anti-rat β3 Ab (Fig. 4B) suppressed Mφ induction of PMN apoptosis. Integrin involvement was restricted to the Mφ because treatment of day 1 wound cells with RGDS before coculture did not alter induction of PMN apoptosis (data not shown). Moreover, addition to cocultures of a synthetic partial CD36 peptide known to block CD36 binding to thrombospondin (30) also reduced Mφ induction of PMN apoptosis (Fig. 4C).

Integrin-ligand interactions may explain the “tethering” of viable PMN to Mφ reported by this laboratory (8) and by others (31) but are unlikely to mediate the induction of PMN apoptosis. While engagement of β3 integrins has been shown to trigger PMN apoptosis (32), a similar capacity has not been reported for β1 subunits. Present results and those by others (33) demonstrate that integrin presentation in Mφ-to-PMN binding occurs on the effector Mφ and not on the PMN target.

Membrane-bound TNF-α mediates Mφ induction of PMN apoptosis

Based on reports of the susceptibility of blood PMN to TNF-α (18), experiments were conducted to examine the potential role of this cytokine as the effector of Mφ-induced PMN apoptosis. Pretreatment of day 5 wound cells with Ab directed against rat TNF-α markedly reduced the ability of wound Mφ to induce PMN apoptosis in cocultures (Fig. 5). Similar results were obtained when paraformaldehyde-fixed day 5 wound cells were pretreated with anti-TNF-α Ab (induced apoptosis = 10.6 ± 1.4% for anti-TNF-α vs 4.9 ± 1.2% for anti-TNF-α , p < 0.05, Student’s t test). Interestingly, day 1 wound PMN tolerated overnight treatment with soluble rat rTNF-α up to 100 ng/ml (5.1 ± 0.7% PMN apoptosis in day 1 wound cells alone vs 5.0 ± 0.6% in day 1 wound cells with 100 ng/ml rTNF-α). This observation agrees with reports by others that, unlike blood PMN, inflammatory PMN do not undergo apoptosis in response to soluble TNF-α (19).

The absolute requirement for cell-to-cell contact in the induction of PMN apoptosis by Mφ, the suppressive effects of the anti-TNF-α Ab, and the ineffectiveness of soluble TNF-α suggested that the membrane-bound form of TNF-α was cytotoxic to the PMN. In this regard, TNF-α is synthesized as an integral transmembrane 26-kDa protein that is cleaved to generate a 17-kDa soluble secretory component (34). The membrane-bound form of TNF-α is known to mediate tumor cell killing (35–37) inclusive of some target cells known to resist soluble TNF-α (38).

To further investigate the requirement for membrane-bound vs soluble TNF-α in the induction of PMN apoptosis, day 5 wound cells were treated with anti-TNF-α Ab and cocultured with day 1 wound cells in the presence of excess soluble rTNF-α (100 ng/ml).

Mφ-induced PMN apoptosis was not reestablished by this treatment (induced apoptosis = 9.8 ± 1.4% for wound Mφ; 0.8 ± 0.7% for anti-TNF-α-pretreated wound Mφ; 1.2 ± 1.0% for anti-TNF-α-pretreated wound Mφ plus rTNF-α).

Pretreatment of day 1 wound cells with Ab directed against rat TNFR-I decreased the capacity of day 5 wound cells to induce PMN apoptosis in cocultures by 41% (induced apoptosis = 14.6 ± 1.9% for species-matched nonspecific IgG-treated cells vs 6.9 ± 1.3% for anti-TNF-α-pretreated day 1 wound cells, p < 0.05, Student’s t test). While TNFR-I is thought to be the main mediator of cellular responses to soluble TNF-α (39), engagement of both TNFR-I and -II has been shown to be involved in blood PMN apoptosis (18). Moreover, Grell et al. (39) demonstrated that membrane-bound TNF-α cooperatively signals through both receptors.
Engagement of both PMN TNF-α receptors may, thus, be necessary for MΦ-induced PMN apoptosis through membrane-bound TNF-α. In this regard, pretreatment of day 1 wound cells with Ab directed against rat TNFR-II abolished the capacity of day 5 wound cells to induce PMN apoptosis in cocultures (induced apoptosis = 14.6 ± 1.1% for species-matched nonspecific IgG-treated cells vs 1.4 ± 1.1% for anti-TNFR-II-pretreated day 1 wound cells, p < 0.05, Student’s t test). Pretreatment with both anti-TNFFR-I and -II Ab did not differ from results obtained with each Ab alone (induced apoptosis = 13.4 ± 0.7% for species-matched nonspecific IgG-treated cells vs 4.7 ± 0.4% for anti-TNFR-I- and -II-pretreated day 1 wound cells, p < 0.05, Student’s t test).

Gene transfer of αβ3 and membrane-bound TNF-α endow noncytotoxic effectors with the ability to induce PMN apoptosis

Results shown so far confirmed roles for MΦ β3, CD36, and membrane-bound TNF-α in mediating PMN apoptosis. However, they did not exclude Fas ligand (FasL) as a component of the effector mechanism. Indeed, the Fas (CD95)-FasL system has been reported to induce PMN apoptosis in vitro (16, 40). Because Abs capable of neutralizing rat FasL are not available, an alternative approach was taken to investigate both the potential involvement of this effector molecule and to further dissect the roles of β3, CD36, and membrane-bound TNF-α in the induction of wound PMN apoptosis by wound MΦ. For this purpose, the β3-negative, FasL-negative, TNF-α-negative, and CD36-positive (41–44) K562 cell line was used in subsequent experiments. Coculture of wild-type K562 cells with day 1 wound cells did not increase PMN apoptosis (induced apoptosis = 0.3 ± 0.2%, mean ± SEM, n = 8).

To more precisely define the role of β3 integrin in the induction of PMN apoptosis, stable αβ3 K562 cell transfectants (21) were used in cocultures with day 1 wound cells. These cells, like wild-type K562 cells, are TNF-α negative by immunofluorescence staining and noncytotoxic to actinomycin D-treated TNF-α-sensitive WEHI cells or FasL-sensitive Jurkat cells (data not shown). As indicated in Fig. 6, neither TNF-α/β3– wild-type K562 cells nor TNF-α/β3+ K562 transfectants were able to induce PMN apoptosis. In addition, αβ3 K562 cell transfectants were cocultured with day 1 wound cells in the presence of excess soluble rTNF-α (100 ng/ml). In agreement with coculture experiments with day 5 wound cells, PMN apoptosis was not induced by this treatment (induced apoptosis = 0.8 ± 1.4% for TNF-α/β3+ K562 cells vs 0.9 ± 0.8% for TNF-α/β3+ K562 cells plus rTNF-α).

Wild-type K562 cells transiently transfected with the mTNFdm expression vector, which produces unleavable membrane-bound TNF-α (TNF-α/β3 in Fig. 6) were equally ineffective inducing PMN apoptosis (Fig. 6).

Double transfectants, αβ3 and mTNFdm, of K562 cells (TNF-α/β3+ in Fig. 6) induced 16.3 ± 1.0% PMN apoptosis (Fig. 6). Transfection of β3-positive K562 cells with the pcDNA3.1 vector backbone alone did not induce PMN apoptosis (data not shown). Demonstrating further that β3 integrin and membrane-bound TNF-α expression on effector cells are necessary to induce wound-PMN apoptosis, treatment of the double K562 cell transfectants with anti-β3 or anti-TNF-α Abs before coculture with day 1 wound cells suppressed their ability to induce PMN apoptosis (Fig. 7).

It has been proposed that αβ3 and CD36 cooperatively bind thrombospondin, which in turn tethers apoptotic PMN to MΦ before their phagocytic removal (45). However, pretreatment of double K562 cell transfectants (TNF-α/β3+) with anti-CD36 blocking Ab (46) did not suppress the induction of PMN apoptosis (induced apoptosis = 18.0 ± 1.3% for TNF-α/β3+ K562 cells vs 18.0 ± 2.0% for TNF-α/β3+ K562 cells pretreated with CD36 Ab). These data contrast findings with day 1 and day 5 wound cell cocultures where a partial CD36 peptide reduced PMN apoptosis by 47.2 ± 8.5%. The dramatic overexpression of β3 in the transfected cells (mean channel fluorescence with anti-β3 Ab staining, wild-type K562 cells = 8.9 vs K562 cells stably transfected with β3 integrin = 827.6) may provide sufficient anchoring for thrombospondin to allow PMN tethering to effector cells regardless of the availability of CD36.

As mentioned in the Introduction, the current paradigm in regard to the resolution of acute inflammation indicates that PMN undergo spontaneous apoptosis and are then engaged and engulfed by MΦ. Previous work from this and other laboratories indicate that the putative MΦ integrin-thrombospondin-PMN extracellular matrix receptor complex is relevant to the recognition and phagocytosis of apoptotic PMN by MΦ (8, 33, 45). This mechanism may serve simply to intimately associate PMN with MΦ and
lead to phagocytosis if the PMN is apoptotic or to PMN demise through membrane-bound TNF-α if viable. Alternatively, engagement of the recognition complex may trigger intracellular signaling in either or both cell types. Results with fixed Mφ and with Mφ membranes rule out intracellular signaling at the Mφ level. In regard to the PMN, current results allow the conclusion that the association of viable PMN with Mφ through RGDs- or anti-β3-inhibitable means is not sufficient to trigger their apoptosis. Moreover, as shown for both Mφ and αβ3-positive K562 cells transfected, the availability of the binding mechanism does not sensitize the cells to soluble-TNF-α.

The resistance of early PMN to soluble TNF-α and their sensitivity to the membrane-bound form of this cytokine in the context of the wound microenvironment is worthy of comment. Extracellular fluid obtained from the wounds contains ~2400 U/ml and 600 U/ml soluble TNF-α 12 and 24 h postinjury, respectively (activity represents ~12 and 3 ng/ml of bioactive TNF-α, respectively) (our unpublished observations). In contrast, day 5 wound Mφ contain 2.7 ± 0.4 U TNF-α/106 (data not shown). The differential sensitivity of PMN to soluble vs membrane-bound TNF-α together with the requirement for integrin-mediated association of PMN to Mφ may serve to assure that PMN survive in the hostile environment of the wound and only undergo apoptosis when specifically instructed by the Mφ.

Findings presented here extend the current model explaining the disappearance of PMN from an inflammatory site by demonstrating that wound Mφ, but not other Mφ populations, can use an integrin- and membrane-bound TNF-α-dependent effector mechanism to actively induce PMN apoptotic death. The relative quantitative contribution of spontaneous vs Mφ-induced PMN apoptosis to the resolution of the neutrophil infiltrate of wounds and other sites of sterile inflammation remains to be determined.

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