Constitutive Death of Platelets Leading to Scavenger Receptor-mediated Phagocytosis

A CASPASE-INDEPENDENT CELL CLEARANCE PROGRAM

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Apoptosis is a physiological program for the deletion of cells in which caspases govern events leading to safe clearance by phagocytes. However, a growing weight of evidence now suggests that not all forms of programmed cell death are caspase-dependent. We now report a complete and constitutive but caspase-independent program for the specific phagocytic clearance of intact effete platelets, anucleated blood cells of critical importance in health and disease. Platelets aged in vitro not only exhibited increased expression of proapoptotic Bak and Bax but also evidenced constitutive diminution of function such as decreased aggregation to ADP, which was accelerated by culture in the absence of plasma. This abrogation of cell function in plasma-deprived platelets was associated with morphological and biochemical features similar to those of granulocyte apoptosis, that is, cytoplasmic condensation, plasma membrane changes including exposure of phosphatidylserine and the granule protein P-selectin, and recognition by phagocyte scavenger receptors. However, and in contrast with constitutive death of other inflammatory blood cells by apoptosis, these events were not affected by caspase inhibitors, nor was there evidence of caspase-3 activation either by hydrolysis of analog peptide substrates or Western blot analysis, serving to emphasize that neither programmed cell death nor clearance by phagocytes need involve caspases.

Apoptosis has attracted intense scrutiny as a self-contained and physiological program for deletion of unwanted cells (1, 2). Caspase activation has emerged as a key effector mechanism responsible for many of the classical phenomena of apoptosis (3–5) including the first biochemical marker of this type of cell death, endonuclease activation (6, 7). Furthermore, caspases have been persuasively implicated in directing plasma membrane changes that lead to the key physiological outcome of apoptosis, the nonphlogistic recognition and uptake of the intact dying cell by phagocytes (8).

However, the study of caspase-mediated cell death by apoptosis has been dominated by experiments that have frequently relied on artificially induced death in transformed cells and that have rarely paid attention to their recognition and clearance by phagocytes. Nevertheless, these criticisms have been addressed in studies of primary blood cells freshly isolated from healthy human donors (9). For example, neutrophil granulocytes purified by methods designed to minimize artificial activation (10) undergo constitutive apoptosis that clearly directs specific recognition by phagocytes (11, 12). Furthermore, not only is there evidence that “machinery” caspases such as caspase-3 are activated as neutrophils aged in culture undergo constitutive apoptosis (13–15), but it is also possible to inhibit both apoptosis and recognition by phagocytes using broad spectrum caspase inhibitors such as zVAD-fmk (16, 17). Indeed, in this system, zVAD-fmk inhibits even the very earliest plasma membrane changes that lead to binding of aged neutrophils by phagocytes (17).

The platelet is another blood cell with a short in vivo half-life (18). Platelets are critical for normal hemostasis, but disorders of platelet number and function are common, giving rise to a range of bleeding or thrombotic disorders, including stroke and myocardial infarction. Given their importance, it is remarkable that there has been very little study of the constitutive death program that likely accounts for platelet deletion in vivo. An important preliminary study (19) suggested that platelets may undergo an apoptotic program because increases in the expression of proapoptotic members of the Bcl-2 family of death-regulating proteins were observed following treatment with ionomycin, a calcium ionophore that induces apoptosis in a range of cell types, most notably lymphocytic cells (20, 21). Interestingly, this study showed that ionomycin-induced increases in Bax and Bak, as well as the cell surface expression of PS, were unaffected by zVAD-fmk and were hence caspase-independent.

To investigate whether platelets exhibited a constitutive death program, freshly isolated platelets were cultured for up to 24 h at 37 °C. In the presence of plasma we observed an increase in levels of proapoptotic Bax and Bak, which, in keeping with an earlier report (19), was consistent with the suggestion that platelets might engage a death program. In support of this, we also observed a constitutive loss of aggregation and spreading functions. By depriving platelets of plasma, a puta-
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tive source of survival factors, not only was loss of function accelerated, but there was also revelation of a cell death program characterized by cytoplasmic condensation, retention of plasma membrane integrity, display at the cell surface of phosphatidylserine and P-selectin, and specific recognition by phagocyte scavenger receptors. However, there was no evidence of caspase-3 activation, emphasizing that constitutive cell death in platelets represents a complete but caspase-independent program leading to phagocyte clearance of intact effete cells.

EXPERIMENTAL PROCEDURES

Materials—All chemicals were of analytical reagent grade and purchased from Sigma unless stated otherwise. Percoll was obtained from Amersham Pharmacia Biotech; sodium citrate solution was from Pharmacia and Baker; HBSS without Ca2+ and Mg2+, pH 6.4, RPMI 1640, Iscove’s modified Dulbecco’s medium, and supplements (penicillin, streptomycin, glutamine, and fetal bovine serum) were from Life Technologies, Inc.; FITC and/or phycoerythrin-conjugated mAbs to CD61 (clone BL-E6) and CD62P (clone CRC81) were from Caltag Laboratories (TCS Biologicals Ltd., Botolph Clayton, UK); anti-CD42a (clone GR-P) was from Serotec Ltd (Kidlington, UK); unconjugated anti-CD62P clone CRC81 and G1–4 were from Caltag Laboratories and Ancell Corporation (Bayport, MN), respectively; FITC-conjugated annexin-V was from BioWhittaker UK Ltd (Wokingham, UK); pan interleukin-1 receptor antibody was from OrthoDiagnostics; and JC-1 (catalog number T3168) and CM-Orange (catalog number C2927) were from Molecular Probes (Leiden, The Netherlands).

Platelet Isolation and Culture—Freshly drawn venous blood was obtained from aspirin-free healthy donors, and PRP was prepared following citration and centrifugation at 300 × g for 20 min. PPP was prepared from PRP by centrifugation at 1200 × g. Washed platelets were prepared by diluting PRP with 5 volumes of HBSS, pH 6.4, containing EDTA (4 mM) into a round-bottom capped polystyrene centrifuge tube before centrifugation at 280 × g for 20 min. Platelets were resuspended in HBSS, pH 6.4, before fixation in ice-cold 10% PFA. Following fixation, washed platelets were resuspended in 10% sodium cacodylate buffer, pH 7.4, containing 2.5% glutaraldehyde and allowing overnight. Samples were centrifuged at 280 × g, the supernatant was removed, and the cells were overlaid with a minimal volume of PRP-derived serum, which in turn was overlaid with the caducy buffer for at least 1 h at room temperature. The pellet was then resuspended in fresh fixative and treated as normal resected tissue for TEM.

Monocyte Isolation and Culture—Human monocytes were isolated from freshly drawn venous blood following citration, dextran sedimentation, and plasma-Percoll density gradient centrifugation as described previously (10, 11). Human monocyte-derived macrophages (Mo) were obtained by the standard technique of culturing adherent monocytes for 5–7 days in Iscove’s Dulbecco’s modified Eagle’s medium plus 10% PRP-derived serum (11, 22).

Phagocytic Recognition of Aged Platelets—Platelets labeled with CM-Orange and aged in culture were washed free of conditioned media and resuspended in HBSS before addition to a prewashed monolayer of adherent phagocyte cell lines cultured in 24-well plates. Typically 5 × 10^7 platelets were incubated with 1 × 10^6 phagocytes at 37 °C for 10 min for platelets aged in the absence of plasma and 30 min for platelets aged in citrated plasma. Following the incubation period, the phagocyte monolayer was washed free of noninteracting platelets, and any adherent platelets were removed by treatment with trypsin at 37 °C for 5 min followed by 5 mM EDTA at 4 °C, to recover the human Mo, and trypsin/EDTA was used to remove platelets adherent to the platelet monolayer. Prior to flow cytometric and epifluorescent microscopic analysis. Immunofluorescence labeling of intact platelets for surface expression of CD42a, CD61, or CD62P was typically performed by resuspending 5 μl of cultured platelets with 40 μl of the appropriate FITC-conjugated mAb (diluted 1:1000 with 10% new born calf serum in PBS) for 10 min before adding 400 μl of fluorescence-activated cell sorter sheath fluid and sampling by flow cytometry using a single laser FACScan (Becton-Dickinson, Mountain View, CA).

Intracellular immunolabeling of the Bel family of proteins was performed following fixation and permeabilization of the platelets with PermeaFix (1 μl/5 × 10^8 platelets for 30 min on ice). Excess fixative was then removed with two washes of PBS before resuspending with 100 μl of fresh complete medium. Platelets were then incubated overnight with neat antibodies to Bak (1 μl), Bax (1 μl), Bcl-2 (1 μl), Bcl-x (1 μl), and Mcl-1 (1 μl) or their appropriate negative controls. All primary antibodies were detected with FITC-conjugated F(ab')2 fragments of sheep anti-rabbit polyclonal antibodies or goat anti-mouse polyclonal antibodies from Sigma.

Platelet Aggregation Experiments—Platelet aggregation experiments were performed in a PAP4 aggregometer (Bio-DATA Corporation) in which 0.5-ml aliquots of platelets, resuspended in PPP, were incubated for 2 min while stirring at 37 °C before the direct addition of agonist. Prior to aggregation experiments, washed platelets were harvested by centrifugation at 280 × g for 20 min and resuspended in autologous PPP. Agonists used were ADP (10 μM), thrombin (10 units/ml), platelet-activating factor (10 μM), and U46619 (10 μM). In some instances, platelets were preincubated for 1 min with the anti-aggregating reagent MK522 (10 μM) prior to the addition of agonist.

Platelet Adhesion and Cell Spreading on Collagen—Glass microscope slides were coated with either collagens I or IV (100 mg/ml solutions in PBS, pH 7.4). After 30 min the slides were washed exhaustively with PBS before applying a volume of suspended platelets in cultured media. Following a 20-min incubation, the slides were flicked free of media and nonadherent cells and snap-frozen with methanol and acetone (1:1). The slides were then air-dried before staining with phalloidin-FITC (10 μg/ml) in PBS (2 μg/ml). Slides were examined by epifluorescent microscopy (Nikon Diaphot 300).

Sample Preparation for Transmission Electron Microscopy—Cell suspensions were prepared for electron microscopy by first washing the platelets with PBS and fixing with 2.5% (v/v) glutaraldehyde in PBS for at least 1 h at 4 °C. Cells were then pelleted at 280 × g before resuspending in a sodium cadoclylate buffer, pH 7.4, containing 2.5% glutaraldehyde and leaving overnight. Samples were recenterfuged at 280 × g, the supernatant was removed, and the cells were overlaid with a minimal volume of PRP-derived serum, which in turn was overlaid with the caducy buffer for at least 1 h at room temperature. The pellet was then resuspended in fresh fixative and treated as normal resected tissue for TEM.
RESULTS

Cultured Platelets Exhibit Increased Levels of Proapoptotic Bak and Bax—An important determinant of whether a cell will undergo apoptosis is its intracellular balance between anti-apoptotic members of the Bcl-2 protein family such as Bcl-2 and Mcl-1 and proapoptotic members such as Bax and Bak (23, 24). The possibility that platelets might be able to undergo an apoptosis-like death has been raised by Vanags et al. (19) who reported that ionomycin, which triggers apoptosis in many cell types (20, 21, 25), caused an increase in the expression of proapoptotic Bak and Bax, but not Bcl-2. To extend the findings of Vanags et al. we decided to study a different model system in which apoptosis-like death might occur. Granulocytes “aged” in culture undergo constitutive death that is accelerated in the absence of survival factors (26–28) and is correlated both with increased levels of Bax (29, 30), but not Bak (31), and decreased levels of Mcl-1 (32). We therefore tested the hypothesis that prolonged culture of platelets might also lead to an increase in the expression of proapoptotic Bcl-2 family members and a susceptibility to apoptosis-like death.

By immunofluorescence flow cytometry we confirmed that freshly isolated platelets expressed Bax, Bak, and Mcl-1, but not Bcl-2 (Fig. 1). The ability of each antibody to recognize its antigen was verified both by flow cytometry and Western blot analysis with appropriate control cell lines.2 Interestingly, the levels of immunodetectable Bak and Bax increased by 3.4- and 2.4-fold, respectively, as platelets were aged for 18 h in citrated plasma (Fig. 1, B and C). No significant changes in Mcl-1 were apparent. In keeping with studies of ionomycin stimulation of platelets (19), these data suggest that platelets cultured for 18 h can adopt a more proapoptotic balance and further suggest that aging in culture, as originally reported for neutrophils (11), was likely to be a useful model for platelet cell death. Furthermore, a tendency to apoptosis-like death during culture was reinforced by the observations that after 18 h of culture, aged platelets exhibited diminished mitochondrial membrane potential as measured by JC-1 and release of cytochrome c into the cytoplasm (data not presented).

Because plasma is a potential source of exogenous survival factors, we went on to seek evidence that plasma deprivation, which resulted in increased levels of Bax and Bak comparable with those observed for platelets aged in the presence of plasma (data not shown), might accelerate and therefore reveal a constitutive death program overlooked in previous short term culture experiments on platelets.

Aged Platelets Exhibit Impaired Function—Platelets possess many of the functional responses exhibited by other inflammatory blood cells and a key feature of apoptosis in leukocytes that are cultured overnight in the presence of serum is loss of the ability to respond to external stimuli and mount pro-inflammatory responses (33). Interestingly, abrogation of cell function is characteristic of platelets that have been stored at 37 °C in the presence of plasma, especially the inability to respond to weak agonists such as ADP. With the use of an aggregometer, which measures the light transmittance of a stirred platelet suspension, we were able to confirm that platelets lost the ability to aggregate but not to undergo a shape change in response to ADP when cultured overnight in the presence of citrated plasma (Fig. 2).

Treatment of freshly isolated (viable) platelets with 10 μM ADP resulted in an immediate shape change, observed as a slight decrease in light transmittance (upward deflection in Fig. 2A), followed by an irreversible decrease in light transmittance that was indicative of a full aggregation response (Fig. 2A).

2 L. Magowan, unpublished observations.
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the presence of 1 μM ADP there was no evidence of aggregation, although shape change, which is the most sensitive response of platelets, persisted. Aggregation, but not shape change, could also be blocked by preincubating freshly isolated platelets with the anti-GPIIb reagent MK852 (data not presented). In comparison, platelets aged over a 24-h period in plasma lost the ability to aggregate (t½ = 12 ± 2 h) but not to undergo a shape change in response to ADP (Fig. 2B). The inability of aged platelets to respond was also observed with the agonist platelet-activating factor and the endoperoxide analog U46619 (data not presented).

In keeping with the hypothesis that plasma might contain survival factors that normally retard constitutive platelet death, we found that the loss of an aggregation response to ADP was markedly accelerated when platelets were washed and cultured in the absence of plasma (t½ = 1.5 ± 0.6 h). This rapid loss in ADP-induced aggregation by washed platelets was prevented and returned to rates comparable with those of unwashed platelets maintained in plasma by reconstituting the washed platelets with PPP (t½ = 12 ± 2 h) (Fig. 2C). This indicated that the loss in platelet response was not dependent on the washing procedure per se but rather on the absence of permissive factors within plasma. Culturing washed platelets in the presence of the megakaryocyte differentiation factor thrombopoietin and known survival factors in other cell systems such as platelet-derived growth factor, granulocyte-macrophage colony-stimulating factor, and insulin-like growth factor-1, had no effect. As a control for oncocytic effects we tested bovine serum albumin at 4 mg/ml and found no protective effect and could eliminate glucose as a confounding factor given that its concentration in HBSS (1 mg/ml) is equivalent to plasma concentrations (0.7–1.1 mg/ml). Initial investigations to characterize the putative soluble plasma survival factor(s) by dialysis have revealed the activity to be of 50 kDa or greater in molecular size and stable to long term storage at 4 °C and −20 °C.

Constitutive loss of platelet function on incubation at 37 °C was further confirmed by assessing the ability of aged platelets to adhere and spread on collagen coated surfaces with the formation of lamellipodia and filopodia. Microscopic examination of phalloidin-FITC-stained preparations revealed that freshly isolated platelets readily adhered to both collagen I- and IV-coated glass slides, whereas aged platelets, whether cultured in the presence or absence of plasma protein, did not (data not presented).

Aged Platelets Maintain Plasma Membrane Integrity—Down-regulation of cell function is a feature of programmed cell death in other blood cells (33, 35, 36) but might also have reflected necrosis. Assessing necrosis was not straightforward in platelets because their small size precluded the use of vital dyes such as Trypan Blue because admission of dye could not be confidently assessed by light microscopy. Similarly, the absence of a nucleus precluded the use of DNA staining vital dyes such as Hoechst and propidium iodide. Nevertheless, flow cytometry revealed that the forward and side scatter properties of fresh and aged platelets, whether cultured in HBSS or PPP, were virtually superimposable (data not presented). This contrasted with the deliberate impairment of plasma membrane integrity by hypotonic lysis, thermal treatment, or mild acid treatment that invariably resulted in the appearance of cellular debris and the loss of platelets as assessed by forward and side scatter (data not shown).

Further evidence against necrosis being a confounding factor in the constitutive loss of platelet function was the use of phalloidin-FITC as an actin-binding “vital dye” where greater than 99% of both fresh and aged platelets excluded the dye when assessed by flow cytometry (Fig. 3A). As a positive control to reveal the potential level of intracellular staining, aged platelets were permeabilized with the fixative PermeaFix prior to staining with phalloidin-FITC. Fluorescence microscopy confirmed that phalloidin-FITC had stained intracellular F-actin of permeabilized platelets (data not presented). These data were a strong indication that aged platelets were capable of maintaining plasma integrity under the conditions employed in this study.

As further confirmation of platelet integrity, we assessed the level of LDH activity, an abundant intracellular enzyme of platelets, in the supernatants of cultured platelets (Fig. 3B). Importantly, less than 4% of total LDH activity was found in the supernatant of platelets aged for 18 h in HBSS in the absence of serum. To confirm that soluble LDH maintained under comparable conditions was stable, we cultured whole cell lysates from fresh platelets either on its own or with erythrocytes. These experiments revealed that soluble LDH was stable in culture over a 24-h period (Fig. 3B). The 75% loss in LDH activity in the cytosol of intact aged platelets appeared likely to reflect intracellular catabolism of retained LDH or its inactivation by, for example, transglutaminases. Unfortunately, given the high levels of LDH in plasma, we were unable to reliably assess the release of LDH from platelets aged in...
plasma. Taken together with the phalloidin data, these results provide strong evidence against the possibility that aged platelets may have undergone necrosis but rather may have undergone a form of programmed cell death.

**Transmission Electron Microscopy of Aged Platelets Is Suggestive of a Cell Death Program**—To further eliminate the possibility of necrosis, we prepared samples for TEM. Freshly isolated platelets, whether washed or not, exhibited characteristic discoid-like features of nonactivated platelets (Fig. 4). This was confirmed by the absence of filopodia and cell surface protrusions or a centrally clumped body of organelles surrounded by a circumferential band of a constricting microtubular network. A cross-section through the platelets also revealed rounded by a circumferential band of a constricting microtubular network. A cross-section through the platelets also revealed rounded by a circumferential band of a constricting microtubular network.

**Aged Platelets Exhibit Cell Surface Changes of Apoptosis**—Although exposure of phosphatidylserine (PS) in the outer membrane of platelets was originally demonstrated to be a marker of platelet activation with procoagulant properties (39), PS exposure is also recognized as a reliable marker of cells undergoing caspase-dependent cell death (40–42). In granulocytes, PS exposure and caspase activation are tightly linked to nuclear changes typical of apoptosis (14, 16, 17). We therefore sought to determine by flow cytometry the level of PS exposure using FITC-conjugated annexin-V, a high affinity probe for PS (43). Flow cytometric analysis of control platelets aged for 24 h in citrated plasma and labeled with annexin-V-FITC revealed a bimodal distribution with typically no more than 8% found positive (Fig. 5A). Although a background level of 0.2–0.5% of fresh platelets bound annexin-V, any increases were not apparent until at least 8 h of culture where annexin-V binding increased steadily to 8% by 24 h with minimal increases seen thereafter. In contrast, washed platelets aged in the absence of plasma, which again contained few annexin-V binding cells in the first 6 h of culture, rapidly switched after 8 h to be >80% positive by 18 h (Fig. 5A). To address the confounding possibility that PS exposure was simply a result of the cell having insufficient energy to maintain the “flip-flop” activity, washed platelets were aged in the presence of varying concentrations of glucose (1 mg/ml to 10 mg/ml). Following assessment of the level of PS exposure by Annexin-V binding and flow cytometry, no significant differences were seen (data not presented). The inability of aged platelets to maintain an asymmetric distribution for PS was strong evidence of an apoptosis-like constitutive cell death program because activated platelets expressing PS possess a translocase that re-establishes an asymmetric distribution, unless platelets have undergone secondary events of aggregation (44).

Furthermore, we sought to confirm morphological evidence of granule fusion with the plasma membrane during constitutive platelet death seen by TEM (Fig. 4) because this is an important cell surface feature of later stages of constitutive apoptosis in neutrophils (38). To assess for this possibility we probed for the intracellular α and dense granule marker P-selectin (45) (Fig. 5B). Reassuringly we found that platelets maintained in citrated plasma did not express any cell surface P-selectin in the first 8 h of culture, evidence against platelet activation. Indeed, after aging for 24 h in the presence of plasma we found that only a small proportion (~10%) of platelets expressed P-selectin. However, washed platelets cultured in HBSS in the absence of serum rapidly mobilized intracellular stores of P-selectin following 6 h of in vitro culture so that all cells were positive by 7–10 h.

**Constitutive Platelet Death Is Caspase-independent—Activation of caspases is reported to be upstream of plasma membrane changes associated with apoptosis including PS exposure (8, 16, 17, 40, 42). Although Western blot analysis confirmed...**
that caspase-3 was present as a 32-kDa species in platelets that was reduced in aged preparations (Fig. 6), we were unable to identify caspase-mediated cleavage to an active fragment (17 kDa) as was observed with apoptotic Jurkat T cells. Interestingly, aged platelets did reveal the presence of proteolized species of molecular weights just less than the p32 parent band, which a very recent report (46) indicates is due to calpain-mediated processing to nonactivated species. In keeping with these and other results (19, 46), and in contrast to apoptotic Jurkat T cells, we were also unable to detect caspase activity using fluorogenic-AFC or chromogenic-pNA substrates for either caspase-1 or caspase-3 (data not presented).

In further agreement with these data we also found that a number of protease inhibitors, including the caspase inhibitors Asp-Glu-Val-Asp-fluoromethylketone and zVAD-fmk, had no effect on the rate of PS exposure (Table I). Additionally, we also found that the inhibitors had no effect on the refractiveness of aged platelets to ADP-induced aggregation (data not presented) whether cultured either in the presence or absence of plasma. Similarly, phagocyte recognition of washed platelets aged in the presence of caspase inhibitors was not different from control, confirming the caspase-independent nature of phagocyte clearance of aged platelets (Table I). Combined, these results suggest that caspases do not have a major role in the constitutive cell death of platelets and complement recent studies on apoptosis-like events associated with platelet activation (19, 46).

Aged Platelets Are Ingested by Phagocytes via Scavenger Receptors—In vivo, the most important feature of the apoptotic cell death program is that intact effete cells are recognized and rapidly ingested by professional and semi-professional phagocytes. In keeping with this, we found that platelets cultured for 18 h in the absence of plasma were readily ingested by 6-day-old human Mφ following a 30-min phagocytosis assay as evidenced by TEM (Fig. 7).

To quantitate platelet ingestion by a range of phagocytes, we developed a flow cytometric method (Fig. 8) that was dependent on incubating the phagocytes with platelets that had been prelabeled with an orange fluorescing reagent (CM-Orange) (Fig. 8A). Phagocytes were then sorted by flow cytometry where
they were readily resolved from platelets by forward and side scatter (Fig. 8B) with any shift in orange fluorescence (FL2) attributed to interacting platelets (Fig. 8C). To discriminate between adherence and ingestion we also labeled the phagocytes prior to flow cytometry with an FITC-conjugated anti-CD61 mAb. CD61, also known as glycoprotein IIIa, is a specific cell surface marker for platelets (Fig. 8A) in which its expression was not found to alter as platelets were aged in culture whether in the presence or absence of plasma. Interestingly, we observed that anti-CD61 mAb routinely failed to label our phagocytes, suggesting that platelets were ingested, in keeping with TEM (Fig. 7) and that any adherent platelets were removed prior to flow cytometry. Assessment of cytospin preparations by confocal microscopy also confirmed that platelets were contained within phagocytes (data not presented).

Moreover, time course experiments repeatedly showed that in a fixed time phagocytosis assay the maximal level of ingestion by human Mφ and Bowes melanoma cells was achieved with platelets aged for 12 h in the absence of plasma or 24 h for those cultured in citrated plasma.

The degree to which cell lines ingested aged platelets, whether cultured in the presence or absence of plasma, was found to be unaffected by various well characterized inhibitors of recognition (Table II) (48). These included the integrin inhibitor RGDS, the PS receptor-competitor phospho-l-serine, the cationic sugars glucosamine and galactosamine, the anti-CD36 mAb SMb, and the anti-CD14 mAb 61D3 (Table II). Conclusive evidence against a role for CD36 in phagocytosis was confirmed with the use of Bowes melanoma cells stably transfected with CD36, which exhibited no increase in phagocytosis when compared with control Bowes melanoma cell lines (49). However, greater than 75% inhibition was observed with fucoidan, a recognized inhibitor of the scavenger receptor pathway, in contrast to the lack of inhibition by dextran at the same concentration, which served as control. Polynositol, another inhibitor of the scavenger receptor pathway, but not its standard control polycytidine also inhibited recognition, although not as effectively as fucoidan (Table II). Nevertheless, further confirmation of a major role for the scavenger receptor was obtained with the anti-murine scavenger receptor mAb 2F8, which inhibited mouse peritoneal macrophage uptake of aged platelets.

Because our studies had also shown that aged platelets expressed P-selectin (Fig. 5B), which mediates adhesion of activated platelets to monocytes (50), and given that fucoidan is known to bind the lectin domain of P-selectin, we explored the role of P-selectin in platelet recognition. By using a function-blocking mAb (clone G1) to P-selectin, which recognizes the lectin domain, we found that the recognition of aged platelets by human Mφ and Bowes melanoma cells was only weakly affected, and no synergy with polynositol was observed (Table II). This suggests that although P-selectin had a minor role in the phagocytosis of aged platelets it was not primarily responsible for mediating phagocytic recognition and clearance, a conclusion in agreement with others (51).

DISCUSSION

Platelets play a crucial role in hemostasis and thrombosis, with the consequence that they are of central importance in common disorders such as myocardial infarction and stroke. However, little is known of candidate mechanisms for safe clearance of these anucleated blood elements. Prompted by earlier work on constitutive apoptosis in other key blood cells, granulocytes, we sought evidence for a constitutive death program available to platelets. In keeping with earlier work (19) we confirmed that platelets expressed members of the Bcl-2 family of cell death-regulating proteins and observed that there was an apparent proapoptotic shift in platelets aged for 18 h in citrated plasma. We also observed that aged platelets lost the

| Condition/inhibitor          | Concentration | PS-positive washed platelets (after 18 h) | n | Recognition of aged washed platelets (normalized) | n |
|------------------------------|---------------|------------------------------------------|---|-------------------------------------------------|---|
| Aged washed                  |               | 71.2                                     | 7 | 100.0                                           | 4 |
| Me2SO control                | [0.1%]        | 70.6 ± 3.8                               | 7 | 99.2 ± 4.7                                      | 4 |
| Acetyl-leucine-leucynorleucinal | 100          | 76.6 ± 3.5                               | 5 | 117.4 ± 17.0                                    | 4 |
| Calpeptin                    | 100           | 77.3 ± 0.9                               | 7 | ND                                              | 4 |
| zVAD-fmk                      | 100           | 84.2 ± 8.0                               | 5 | 99.5 ± 7.9                                      | 4 |
| Acetyl-leucine-leucynorleucinal | 100          | 71.8 ± 5.9                               | 7 | ND                                              | 4 |
| Acetyl-leucine-leucynorleucinal | 100          | 77.3 ± 0.9                               | 5 | ND                                              | 4 |

**a** n, number of separate experiments. **b** ND, not done.
ability to aggregate in response to weak agonists such as ADP and failed to adhere and spread on collagen coated surfaces.

Given that serum is a rich source of survival factors for granulocytes, with which platelets have much in common, we reasoned that the physiological milieu of platelets, plasma, was likely to also contain factors capable of retarding their programmed death. We therefore sought evidence of accelerated programmed cell death when platelets were cultured in the

**TABLE II**

**Platelet ingestion is mediated by the scavenger receptor**

The level of inhibition by the following compounds on the ingestion of platelets that had been aged in either the absence or presence of plasma protein were assessed by flow cytometry as follows: −, 0–5% inhibition; +, 5–20% inhibition; ++, 30–50% inhibition; ++++, 75–90% inhibition. A typical interaction assay resulted in 40 ± 8% of Mφ ingesting platelets aged in the absence of plasma after a 10-min assay and 38 ± 6% after 40 min for platelets aged in the presence of plasma, in contrast to BOWES where the level of ingestion was 30 ± 12% and 20 ± 6%, respectively.

| Platelets aged in plasma | Concentration | Mφ (human) | BOWES (human) | TEPM (mouse) | HUVEC (human) |
|--------------------------|---------------|------------|---------------|--------------|---------------|
| EGTA                     | 1 mm          | −          | −             | ND           | −             | −             |
| RGDS                     | 1 mm          | −          | −             | ND           | −             | −             |
| RGES                     | 1 mm          | −          | −             | ND           | −             | −             |
| Phospho-t-serine         | 1 mm          | −          | −             | −            | −             | −             |
| N-Acetyl glucosamine     | 20 mm         | −          | −             | −            | −             | −             |
| N-Acetyl galactosamine   | 20 mm         | −          | −             | −            | −             | −             |
| Poly-inositol            | 100 μg/ml     | ++         | ++            | ++           | ++           | ++           |
| Poly-cytidine            | 100 μg/ml     | −          | −             | −            | −             | −             |
| Fucoidan                 | 100 μg/ml     | +++        | +++           | +++          | +++          | +++          |
| Dextran                  | 100 μg/ml     | −          | ND            | −            | ND           | ND           |
| mAb 2F8 (anti-scavenger receptor) | 10 μg/ml | NA | NA | NA | NA | NA |
| mAb G-1 (P-selectin blocking Ab) | 10 μg/ml | + | ND | ++ | ND | ND |
| mAb G-1 + 100 μg/ml poly-inositol | 10 μg/ml | ++ | ND | ND | ND | ND |
| mAb G-1 + 100 μg/ml EGTA | 10 μg/ml | + | ND | ND | ND | ND |
| mAb CRC81 (anti-P-selectin) | 10 μg/ml | − | ND | ND | ND | ND |

*a* BOWES, Bowes melanoma cells.

*b* TEPM, thioglycollate-elicited peritoneal macrophages.

*c* HUVEC, human umbilical vein endothelial cells.

*d* ND, not done.

*e* NA, not applicable.
absence of plasma, finding that they not only exhibited an accelerated loss of function but also displayed many features in common with programmed death of granulocytes. Such changes included morphological evidence of cytoplasmic condensation and cell surface expression of the “eat me” signal phosphatidylserine (52) and granule components such as P-selectin. Importantly, however, there was strong evidence that such aged platelets retained plasma membrane integrity because there was no detectable release of the cytoplasmic marker enzyme lactate dehydrogenase, nor did the aged cells admit actin-binding phalloidin-FITC. Furthermore, aged platelets were recognized and ingested by all professional and semi-professional phagocytes tested by a mechanism in which phagocyte scavenger receptors predominated.

An important conclusion to be drawn from these studies is that constitutive death in plasma-deprived platelets represents a caspase-independent program for phagocyte clearance of effete cells. Not only did broad spectrum caspase inhibitors fail to affect all phenomena associated with platelet death, including recognition by phagocytes, but there was no evidence that caspase pseudo-substrates were cleaved. Given earlier studies in various models of apoptotic cell death, in which the display of cell surface “eat me” signals such as phosphatidylserine exposure appears to be mediated by caspases (8, 40, 42), we believe that demonstration of a complete caspase-independent program for cell death and clearance is an important addition to examples of caspase-independent cell death where recognition of such cells by phagocytes has not been previously studied (53–56).

With regard to platelet cell death, these studies also complement recent reports suggesting that ionomycin stimulation of platelets, a model of activation-induced cell death, can recapitulate many of the features found in apoptosis (19, 46). Although there is some evidence that caspases may participate in platelet activation (57), Wolf et al. (46) suggest that the effects of ionomycin on platelets are independent of caspases because calpains disable activation by partial cleavage of their pro-domains. Similar mechanisms could be at play in constitutive platelet cell death given its independence from caspases despite the presence of cytochrome c in supernatants from cell lysates, which can activate caspases (46), and the remarkably similar evidence of partial caspase-3 cleavage on Western blot. This possibility is now under active investigation, especially as further studies are clearly required to characterize the caspase-independent mechanisms by which aged platelets express PS because this was not affected by the calpain inhibitors acetyl-leucyl-leucyl-norleucinal and calpeptin.

It will also be important to define in more detail the molecular mechanisms by which phagocyte class A scavenger receptors mediate recognition and ingestion of aged platelets, as clearly indicated by the inhibitor studies presented in this report. Although the role of the scavenger receptor has already been implicated in the clearance of apoptotic thymocytes by mouse macrophages (58) the ligands displayed by dying cells, which lead to their recognition by scavenger receptors, are currently unknown. Furthermore, although PS is widely recognized as an “eat me” signal (52), it is not properly understood why various phagocyte types will apparently ignore PS in favor of other yet to be characterized signals for ingestion (59).

Because constitutive death in plasma-deprived platelets was caspase-independent and could not be assessed for typical nuclear changes because these cells have no nucleus, we feel it is not appropriate to label this form of cell death as “apoptosis.” However, we believe that the data indicate that platelets can undergo a form of programmed cell death that can be regulated by exogenous influences, in particular plasma-derived survival factors. In keeping with this, we observed that the return of plasma-deprived platelets to plasma slowed the phenomena of cell death, most notably returning the rate of loss of aggregation and levels of PS exposure to that observed for platelets cultured in plasma. Ongoing work is directed at the biochemical characterization of the survival activity present in plasma because a range of candidate cytokine survival factors could not substitute for plasma. Nevertheless it should be emphasized that plasma deprivation appeared merely to accelerate a constitutive death program that was already active in platelets at 37 °C and evident after 18–24 h of culture in plasma. However, study of constitutive death in plasma-replete platelets will be difficult because our preliminary work demonstrated progressive loss of platelets from populations cultured for > 24 h, presumably reflecting relatively rapid secondary necrosis of that proportion of cultured platelets undergoing programmed death each day.

In conclusion, we have provided in vitro evidence that human platelets can undergo a constitutive program of cell death that was caspase-independent and that resulted in the specific recognition of effete cells by phagocytes employing the scavenger receptor as a recognition mechanism. Although our findings have potentially important implications for understanding platelet kinetics and the related pathogenesis of thrombotic and bleeding disorders, no firm conclusions on in vivo relevance can be drawn from the current data. Nevertheless, these data raise the exciting prospect that platelet lifespan and clearance is amenable to exogenous regulation for therapeutic purposes.

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