Down-Regulation of Monocyte Apoptosis by Phagocytosis of Platelets: Involvement of a Caspase-9, Caspase-3, and Heat Shock Protein 70-Dependent Pathway

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Monocytes interact and cross-talk with platelets in many settings including inflammation, hemostasis, or vascular disorders. During inflammatory diseases, there is a rapid targeting of monocytes and platelets to points of inflammation and endothelial injury, where they lie side-by-side. In this in vitro study, we investigated different interactions between monocytes and platelets and elucidated whether platelets might affect monocyte apoptosis. Freshly isolated human monocytes were rendered apoptotic by serum deprivation or CD95 ligation and cocultured with platelets. Monocyte apoptosis was determined by flow cytometry, TUNEL staining, DNA electrophoresis, and transmission electron microscopy imaging. We could show that monocyte apoptosis was highly suppressed when platelets were added to the cultures. Transmission electron microscopy depicted that monocytes completely ingested thrombocytes by phagocytosis. Blocking thrombocyte uptake by the phagocytosis inhibitor cytochalasin D abrogated the enhanced monocyte survival and led to high apoptosis levels. Monocyte survival was paralleled by down-regulation of caspase-9 and -3 and up-regulation of heat shock protein 70 during uptake of platelets. Platelet supernatants and contents of platelet granules were ineffective in altering monocyte senescence. Also, ingestion of latex beads or zymosan by monocytes was ineffective to mimic platelet-dependent rescue from apoptosis. In conclusion, this study shows that platelets can suppress apoptosis of monocytes by a specific phagocytosis-dependent process with further consequences for atherosclerotic or inflammatory conditions.


**Materials and Methods**

**Abs and reagents**

PE-conjugated mAb against activated caspase-3, anti-caspase-8 polyclonal Ab, and anti-caspase-9 mAb (clone 2.22) was purchased from BD PharMingen (San Diego, CA). Anti-CD95/FAS mAb (clone CH-11, 500 ng/ml) and FITC-labeled anti-CD62P mAb (20–40 μg/ml) were obtained from Coulter Immunotech (Krefeld, Germany). Anti-heat shock protein (hsp-70)3 mAb was purchased from StressGen (Victoria, Canada), reverse transcriptase from Stratagene (Heidelberg, Germany), Taq DNA polymerase from Life Technologies (Karlsruhe, Germany), dNTPs from New England Biolabs (Beverly, MA), pdN6 from Boehringer Mannheim (Mannheim, Germany).

Abbreviations used in this paper: hsp, heat shock protein; PCD, programmed cell death; PI, propidium iodide; TEM, transmission electron microscopy; MFC, mean fluorescent channel.
Monocytes and cell culture

Mononuclear cells were obtained from Ficoll-Hypaque density centrifugation (400 x g, 20 min), washed, and further purified by centrifugation on a hypotonic Percoll density gradient (57% in PBS; 400 x g, 30 min). Two interphases were found in which the upper phase contained the enriched monocytes. Cells were collected, washed three times in cold PBS, and seeded out in 24-well culture plates (Greiner, Nürtingen, Germany) in RPMI 1640 cell culture medium containing 2 mM L-glutamine, 50 μg/ml penicillin/streptomycin, 5 mM HEPES, and 10 μM 2-ME at 37°C in a 5% CO2/95% air atmosphere. Monocytes were further purified by the adherence to the culture plates, which finally gave a purity of >85%. This was assessed by flow cytometry on a FACScan flow cytometer (BD Biosciences, Mountain View, CA) defined by forward and side light scatter properties, as well as by detection of the CD14 surface molecule and staining for nonspecific esterase. Monocytes (0.5 x 10^7/ml) were incubated in culture medium with 0.2-5% FCS (endotoxin content, < 0.01 ng/ml) or were rendered apoptotic with a CD95 mAb (0.5 μg/ml) applied over the 60-h culture time.

Preparation of platelets, determination of surface receptors, and coculture with monocytes

Platelets were prepared according to standard methods from 20 ml blood from healthy donors anticoagulated with 0.01 M sodium citrate. Centrifugation was performed at 200 x g for 10 min to get platelet-rich plasma. Platelets were washed first in an apprise (0.5 U/ml) containing EDTA (5 mM) buffer, then twice in 5 ml HEPES/Tyrode buffer, each with centrifugation at 800 x g for 10 min. Finally, platelets were resuspended in RPMI 1640 containing 0.2% FCS and immediately transferred to monocyte culture plates. Platelets (5 x 10^6-2 x 10^7/ml) were added to monocytes after 20 h for 4 h at a monocyte:platelet ratio of 1:1-1:4. Platelets kept in culture over this short time period exerted normal shape and function as determined by flow cytometry. Monocytes were additionally labeled with FITC-conjugated dUTP in the presence of TdT for 1 h at 37°C. DNA was labeled with FITC-conjugated dUTP in the presence of TdT for 1 h at 37°C. Cells were washed off the cocultures after staining of nuclei using propidium iodide (PI) staining. Flow cytometric analysis was performed with a FACScan flow cytometer (BD Biosciences) for a total of 10,000 events. In brief, platelets were resuspended in cell diluent and mixed with PKH67 dye in equal volumes at room temperature for 2 min. Staining reaction was blocked by adding 2 ml pure FCS for 1 min. After centrifugation of thrombocytes, they were washed extensively and resuspended in RPMI 1640 containing 0.2% FCS. Labeled platelets were used for coculture experiments. Parallel detection of PI-stained nuclei (red fluorescence) and green fluorescence of labeled platelets by flow cytometry allowed recognition of apoptotic vs intact monocytes with and without ingested platelets. Latex beads (5 x 10^6/ml) or zymosan particles (10 x 10^6/ml) boiled for 30 min and osmopsonized with human AB serum were added to monocytes after 20 h and were washed off the cocultures after 4 h. For inhibition of phagocytosis, cytochalasin D (30 μM) was added to monocytes 30 min before adding platelets and washed off carefully after 1.5 h.

Detection of apoptosis

Quantification of apoptosis by flow cytometry. Monocyte apoptosis was determined by propidium iodide (PI) staining of nuclei using flow cytometry. Mononuclear cells were obtained by forward and side light scatter properties, as well as by detection of the CD14 surface molecule and staining for nonspecific esterase. Monocytes (0.5 x 10^7/ml) were incubated in culture medium with 0.2-5% FCS (endotoxin content, < 0.01 ng/ml) or were rendered apoptotic with a CD95 mAb (0.5 μg/ml) applied over the 60-h culture time.

DNA electrophoresis. DNA extraction and electrophoresis were performed as described previously, with slight modifications (15). DNA extraction was performed with GenomicPrep Cells and a Tissue DNA Isolation kit (Aldrich, St. Louis, Missouri), unless otherwise indicated.

Transmission electron microscopy (TEM)

Typical morphologic alterations indicative for apoptosis were evaluated by electron microscopy as described previously (15). Cells were washed off the culture plates, centrifuged, fixed in 1% glutaraldehyde/0.1 M sodium cacodylate-HCl (pH 7.4), and postfixed in 1% OsO4/0.15 M sodium cacodylate-HCl (pH 7.4). Samples were dehydrated in an ascending ethanol series and embedded in epoxy resin (Epon 8122). Ultrathin sections were mounted on 150-mesh Formvar-coated copper grids and poststained with aqueous-saturated uranyl acetate and 2% lead citrate before being examined on a Philips CM 10 electron microscope (Philips Electronic Instruments, Mahwah, NJ) at an accelerating voltage of 60 kV.

Evaluation of cell necrosis

Viability of monocytes after different treatments was determined by trypan blue exclusion or PI uptake of nonpermeabilized cells using flow cytometry.

Flow cytometric caspase-3 and hsp-70 detection

Monocyte expression of activated caspase-3 was determined by flow cytometry. Monocytes were washed in PBS, fixed with 4% paraformaldehyde, and permeabilized with 0.1% saponin. For staining, a mAb against activated caspase-3 or hsp-70 (5 μg/ml, respectively) was applied for 20 min.

Detection, induction, and inhibition of phagocytosis

Phagocytosis of platelets was shown by TEM and specific membrane linking of platelets with a PKH67 Green Fluorescent Cell Linker kit (Sigma-Aldrich) according to the manufacturer’s instructions. In brief, platelets were resuspended in cell diluent and mixed with PKH67 dye in equal volumes at room temperature for 2 min. Staining reaction was blocked by adding 2 ml pure FCS for 1 min. After centrifugation of thrombocytes by 2000 x g for 10 min, they were washed extensively and resuspended in RPMI 1640 containing 0.2% FCS. Labeled platelets were used for coculture experiments. Parallel detection of PI-stained nuclei (red fluorescence) and green fluorescence of labeled platelets by flow cytometry allowed recognition of apoptotic vs intact monocytes with and without ingested platelets. Latex beads (5 x 10^6/ml) or zymosan particles (10 x 10^6/ml) boiled for 30 min and osmopsonized with human AB serum were added to monocytes after 20 h and were washed off the cocultures after 4 h.

For inhibition of phagocytosis, cytochalasin D (30 μM) was added to monocytes 30 min before adding platelets and washed off carefully after 1.5 h.

Semiquantitative RT-PCR

RNA isolation from monocytes after stimulation was conducted using an RNasefree kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Before transcribing into cDNA, DNase (DNase I, RNase free; Boehringer Mannheim) digestion was performed. cDNA was synthesized after the addition of 5 μM random primers (pd(N)6; Roche Diagnostics, Mannheim, Germany), 1 mM dNTPs (New England Biolabs), and incubation at 37°C with Moloney murine leukemia virus reverse transcriptase (Stratagene, Heidelberg, Germany). Contamination with DNA was excluded by performing PCR from templates incubated without reverse transcriptase. The primers used for PCR amplification were 5′-ATG GAT GAT GAT ATC GCC GCG-3′ and 5′-TCT CCA TGT CGT CCC GAT TG-3′ (human β-acid, 248 bp), as well as 5′-CAG CAC CTA CTC CGA CAA CCA CC-3′ and 5′-GCC CCT AAT CTA CTT CAA TGG-3′ (human hemoglobin A2, 44 bp). The PCR mixture (40 μl) contained 2 mM MgCl2, 0.2 mM dNTP, 1 μM primer, and 1 U Taq DNA polymerase. Samples were amplified during 30 cycles by 60-s denaturation at 94°C, 30 s annealing at 62°C (hsp70), or 55°C (β-acid) and 60-s elongation in a Peltier thermal cycler (Biometra Uno II Thermocycler; Biometra, Göttingen, Germany).

For semiquantitative PCR, the ratio between the expression of β-acid and hsp70 was analyzed. Signal intensity as measured by PCR products was analyzed on a 1.5% agarose gel and visualized by ethidium bromide staining. Densitometric quantification of PCR signals was performed by the BioImage Intelligent Quantiﬁer program (BioImage, Ann Arbor, MI).
Western blotting

Cells were washed in PBS and resuspended at 10⁶ cells/100 µl of sample buffer containing 2% SDS, 62.5 mM Tris-HCl (pH 6, 8), 10% glycerol, 5% 2-ME, and bromphenol blue, heated at 95°C for 10 min, and stored at -20°C until analysis. Samples, each containing 20 µg protein, were separated by a NuPAGE Gel 4–12% BT (Invitrogen, Groningen, The Netherlands) and transferred to nitrocellulose membranes (BA83 (0.2 µm); Schleicher & Schuell, Dassel, Germany) by a Western blot modul (Invitrogen). Membranes were washed three times with potassium phosphate buffer (0.05 M K₂HPO₄, pH 8.5) and incubated with digoxigenin-3-0-methylcarbonyl-e-aminocaproic acid-N-hydroxysuccinimide ester) and Nonidet P-40 (0.01% v/v) for 2 h. Then membranes were blocked with 5% fat-free milk powder in TTBS buffer (0.01% Tween 20, 0.05 M Tris-HCl, and 0.15 M NaCl, pH 7.5) and afterward incubated with the indicated Abs. Reactive bands were visualized for detection of caspase-8 after incubation with anti-rabbit IgG peroxidase-labeled Fab fragments, for caspase-9 after incubation with anti-mouse IgG peroxidase-labeled Fab fragments and staining with BM Teton (Roche, Mannheim, Germany). As an isotype-matched control for the primary Ab, mouse IgG2b was used. BioImager master 3D was used for analysis of visualized bands.

Statistical analysis

Results are given as means ± SEM. For statistical analysis, the Mann-Whitney U test and for paired comparisons the Wilcoxon-signed rank test or Student’s t test was performed. A p < 0.05 was considered to be statistically significant. All experiments were performed at least five times with different buffy coats.

Results

Down-regulation of monocyte apoptosis by platelets

Coculture with platelets highly enhanced the life span of monocytes. As shown in Fig. 1A, 54 ± 5% of monocytes were apoptotic when cultured for 60 h in medium containing 0.2% FCS. Apoptosis was quantified by PI staining of permeabilized cells. When platelets were added, apoptosis levels of monocytes decreased in parallel with the number of added platelets and reached at a monocyte:platelet ratio of 1:40 baseline levels of 12 ± 4%. A coincubation time of 4 h was sufficient to exert this protective effect. Similarly, when monocytes were rendered apoptotic by a CD95 mAb (Fig. 1B), platelets reduced the monocyte apoptosis level from 48 ± 7% to 25 ± 11% (p < 0.05; monocyte:platelet ratio: 1:10). This down-regulation of monocyte apoptosis by platelets was confirmed when apoptosis was determined by the TUNEL method (data not shown) or by DNA electrophoresis. Fig. 2 shows that internucleosomal DNA fragmentation of monocytes during constitutive apoptosis (0.2% FCS) as well as anti-CD95-induced apoptosis was highly reduced after the addition of platelets. Also, TEM revealed that >50% of monocytes cultured in 0.2% FCS-containing medium for 60 h exerted nuclear and cytoplasmic alterations that were typical for apoptosis. In Fig. 3A, three intact monocytes after a culture in 5% FCS-containing medium are depicted; in Fig. 3B, as a representative illustration, two apoptotic

FIGURE 1. A, Inhibition of constitutive monocyte apoptosis by platelets. Constitutive monocyte apoptosis was induced by culturing cells in medium supplemented with 0.2% FCS. It was determined by PI staining of permeabilized cells using flow cytometry. Different amounts of platelets were added for 4 h. A significant inhibition of apoptosis was determined beginning with a monocyte:platelet ratio of 1:10; a maximum protective effect was noted at a ratio of 1:40. Means ± SEM are given and were obtained from 12 independent experiments. *, p < 0.05. B, Down-regulation of monocyte apoptosis induced by CD95 stimulation by platelets. Platelets at a monocyte:platelet ratio of 1:10 significantly inhibited CD95 mAb-induced apoptosis of monocytes detected by flow cytometry. Data are means ± SEM from eight independent experiments. *, p < 0.05.

FIGURE 2. Oligonucleosomal DNA cleavage of monocytes is suppressed by the coculture with platelets. DNA electrophoresis proved oligonucleosomal DNA fragmentation when monocytes were cultured in 0.2% FCS-containing medium or were treated with a mAb against CD95. In both settings, the coculture with platelets (monocyte:platelet ratio of 1:10) markedly reduced DNA fragmentation. No DNA cleavage was found in control monocytes cultured in 5% FCS-containing medium. Lane 6 (M) shows a molecular mass marker of a 123-bp DNA ladder. A representative DNA electrophoresis of four performed is depicted.

FIGURE 3. TEM reveals complete ingestion of platelets. A, Three monocytes that maintained normal morphology after a 60-h culture in 5% FCS-containing medium. B, Typical changes of an apoptotic monocyte after serum deprivation for 60 h. Dense nuclear condensation, cytoplasmic vacuolization, shrinkage, and rounding is visible. Investigating monocytes after coculture with platelets showed that monocytes ingested platelets (arrows) in huge amounts and, despite culturing them in 0.2% FCS-containing medium, <8% of analyzed monocytes showed apoptotic features (C). In contrast, monocytes exerted typical signs of apoptosis after ingestion of latex beads (arrow, D). Magnification: ×7200, A–C; ×9600, D.
monocytes after a culture in 0.2% FCS-containing medium are shown. When monocyte morphology was studied after the coculture with platelets, uptake of these cells and platelet ghosts into monocyctic lysosomes became visible (Fig. 3C). Despite the culture of monocytes in 0.2% FCS-containing medium, <8% of studied monocytes revealed nuclear alterations indicative for apoptosis. Trypsinization of monocytes without consecutive disruption of platelets from monocytes excluded plasma membrane invaginations and confirmed true phagolysosome formation.

**Monocyte phagocytosis of platelets and implications for apoptosis**

We reasoned that phagocytosis of platelets might be the background and prerequisite for enhanced monocyte survival. To confirm this, we labeled platelets with a fluorescent linker to demonstrate thrombocyte adherence and phagocytosis and studied the effects of the phagocytosis inhibitor cytochalasin D (30 μM). As shown in Fig. 4, where apoptosis was detected by reduced PI staining of permeabilized monocytes, 54.7% of monocytes were apoptotic after the culture in 0.2% FCS-containing medium for 60 h (upper left panel). Administration of cytochalasin D for 1 h did not affect the viability of cells (upper right panel). Addition of platelets exhibiting green fluorescence caused a fluorescence shift to the right, indicating that >70% of monocytes had ingested platelets or that adherence between both the cell types took place. Low PI staining of apoptotic cells was demonstrable in 16.2% (7.8 + 8.4%; Fig. 4, lower left panel). When cytochalasin D was added, monocytes with ingested platelets significantly decreased in number (Fig. 4, lower right panel, upper right quadrant, 39.1%) and increasingly became apoptotic (26.2 + 13.8%).

Experiments using EDTA/trypsin to disrupt adherent platelets from monocytes 2 h after the start of the coculture, a period over which effective phagocytosis occurred, did not reduce the degree of cytoprotection. Similarly, when platelets were allowed to adhere to monocytes over 1 h (a time too short for effective phagocytosis) and then washed off, protection from apoptosis was lacking (data not shown). These findings underline that adherence of platelets to monocytes was insufficient to rescue monocytes from apoptosis.

We further tested whether phagocytosis per se was sufficient to enhance the life span of monocytes and administered latex beads or zymosan particles to monocytes. As shown by TEM for latex beads, a significant number of monocytes became apoptotic in 0.2% FCS-containing medium (>50%), although most monocytes had phagoxytosed latex beads (Fig. 3D). In Fig. 5, it is shown that neither latex beads nor zymosan particles influenced monocyte apoptosis levels and lacked the protective effect shown for platelets.

Platelet granule contents obtained after sonication of platelets were not effective to mimic the cytoprotective capacity of platelets. Also, when monocytes and platelets were cocultured but separated from a direct contact by a permeable mesh, the life span of monocytes was unaffected (data not shown).

**Down-regulation of caspase-9 and -3 is involved in protection from growth factor-dependent apoptosis by platelets**

Caspases-3 is one of the essential mediators of monocyte programmed cell death (PCD). We looked for expression of active caspase-3 by flow cytometry (16) with respect to platelet-dependent down-regulation of monocyte apoptosis. Table I shows that constitutive apoptosis induced by serum deprivation was characterized by a significant expression of activated caspase-3, which

**Table I. Comparison of expression of activated caspase-3 in monocytes after serum starvation or in control medium treated with anti-CD95 mAb with or without platelets**

<table>
<thead>
<tr>
<th>Treatment of Monocytes</th>
<th>Monocytes Expressing Activated Caspase-3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5% FCS, control</td>
<td>24.5 ± 4.3</td>
</tr>
<tr>
<td>0.2% FCS</td>
<td>50.0 ± 5.7</td>
</tr>
<tr>
<td>0.2% FCS and platelets</td>
<td>22.1 ± 3.9†</td>
</tr>
<tr>
<td>0.2% FCS, anti-CD95 mAb</td>
<td>42.3 ± 5.2</td>
</tr>
<tr>
<td>5% FCS, anti-CD95 mAb and platelets</td>
<td>37.3 ± 3.4**</td>
</tr>
</tbody>
</table>

*† Caspase-3 was determined by flow cytometry.

+, p < 0.05 as compared to 0.2% FCS without platelets; **, not significantly different (p > 0.05) from 5% FCS, anti-CD95 mAb without platelets.
was down-regulated to baseline levels by addition of platelets. By studying the proximal regulatory caspases-8 and -9 by immunoblotting, we could show that caspase-8 was not activated by serum reduction and not affected by addition of platelets (Fig. 6A). However, when we performed Western blotting for caspase-9, a significant reduction of pro-caspase-9 with the concomitant appearance of cleaved fragments and subunits was demonstrable in monocytes under serum starvation (Fig. 6B). Involvement and activation of caspase-9 was reverted by the coculture with platelets. When apoptosis was induced by CD95, caspase-3 stimulation occurred, but now platelets could not block caspase-3 activation significantly (Table I).

Inhibition of platelet-dependent monocyte apoptosis and hsp-70 expression

hsp-70 has been shown to exert antiapoptotic properties (17). Thus, we determined hsp-70 expression on a mRNA level by RT-PCR. Addition of platelets to monocytes highly increased hsp-70-specific mRNA (Fig. 7). Phagocytosis of latex beads could not stimulate hsp-70 expression. Heat shock (47°C for 30 min) as a control led to an increase of hsp-70. Serum starvation (0.2% FCS) significantly reduced hsp-70 expression as compared with medium containing 5% FCS, underlining the importance of hsp-70 for survival of monocytes. Similar results were obtained when hsp-70 was detected at a protein level by flow cytometry (data not shown).

Activation of platelets increased their ability to protect monocytes from apoptosis

We further wanted to clarify whether activation of platelets had any influence on their protective effects on monocytes. Platelets were activated by preparation in hypotonic buffer and analyzed for CD62P expression for mean fluorescent channel (MFC) using flow cytometry. As shown in Fig. 8, reduction of apoptosis of monocytes after addition of platelets was significantly dependent from the grade of activation of platelets.

Discussion

The development and progression of atherosclerotic, thrombotic, or inflammatory vascular diseases depend on the cells involved in these conditions, their destructive or protective activity and life span within the lesion. The latter factors are determined by the apoptosis program of the involved cells which is regulated physiologically and highly influenced by environmental factors. Monocytes/macrophages but also platelets adjacent to the vessel wall belong to the predominant cells leading to initiation of vascular diseases. This has been extensively described, e.g., for early atherosclerotic alterations (1, 18).

![Figure 6](image1.png)

**Figure 6.** Regulation of caspase-8 and caspase-9 after serum deprivation and uptake of platelets shown by immunoblotting. A, Protein expression of caspase-8 (activated and unactivated (55kDa)) was neither affected by serum starvation (0.2% FCS) nor changed after adding platelets (0.2% Th) as compared with control (5% FCS). B, Immunoblotting for caspase-9 was performed. Pro-caspase-9 (47 kDa) was significantly reduced after starvation (0.2% FCS) and cleaved caspase-9 subunits (37 kDa) appeared (0.2% FCS) in contrast to control (5% FCS). Coculturing with platelets reversed this effect (0.2% Th). M, molecular mass marker. Th, Thrombocytes. A representative immunoblotting of three performed is depicted.

![Figure 7](image2.png)

**Figure 7.** RT-PCR shows up-regulation of hsp-70 after phagocytosis of platelets but not after uptake of latex beads. There was a basal hsp-70 mRNA expression in monocytes cultured in 5% FCS-containing medium that was reduced after culture in 0.2% FCS-containing medium. When thrombocytes were added to monocytes cultured in 0.2% FCS-containing medium, a marked up-regulation of hsp-70 mRNA expression was demonstrable (lane Th). Addition of latex beads to monocytes in 0.2% FCS-containing medium had no stimulatory effect. As a positive control, monocytes were also heat stressed with 47°C for 30 min. M, marker dX174 HaeIII digest. Densitometric determination of hsp-70-specific RT-PCR signals of differently treated monocytes is given in B.

![Figure 8](image3.png)

**Figure 8.** Activation of platelets up-regulates protection of monocyte apoptosis. Platelets were activated by preparation in hypotonic buffer as described in Materials and Methods, which was determined by CD62P expression. The antiapoptotic effects were significantly higher for activated platelets expressing a MFC of 241 ± 25 for CD62P as compared with unstimulated platelets (MFC, 27 ± 4). *, p < 0.05.
Our data show that platelets are able to down-regulate the apoptosis program of cultured monocytes. This effect was drastic since already at a ratio between 1:20 and 1:40 (monocytes:platelets), which is exceeded by far in the blood, constitutive monocyte apoptosis induced by serum starvation was completely abrogated to baseline by suppressing caspase-9 and consecutively caspase-3. It has been shown by different groups that platelets themselves are equipped with a whole set of caspases, non-caspase proteinases, and substrates for these enzymes which all depend on the activation status of thrombocytes (19–21). It is conceivable that some accesso ries on the platelet side interfere with the counterparts on the monocyte side after phagocytosis and digestion. Since platelets down-regulated monocyte caspase-9 and -3, more a contribution of substrates rather than caspas es by platelets for monocy topathic enzymes is supposed. Immunoblotting of platelet lysates excluded a significant amount of caspases supplied by these cells. Perhaps platelet compounds reactivate the energy household of monocytic mitochondria which is injured by serum deprivation (22–24). We could show that in the case of serum factor-dependent apoptosis platelets inhibited caspase-9 and the distal executive caspase-3. In the case of CD95-mediated PCD, platelets rescued monocytes from apoptosis without significant down-regulation of caspase-3. Our experiments could not completely elucidate at what stage platelets interfered here with the apoptosis pathway of monocytes. Probably, hsp are active and protective in this apoptotic pathway (25). We conclude that caspase-3 inhibition is not generally and probably only indirectly involved in the cytoprotective actions of platelets (26).

Monocyte apoptosis was studied and quantified by different methods, such as flow cytometry using PI staining of nuclei after cell permeabilization, TUNEL staining, DNA electrophoresis, or electron microscopy, which all gave similar results. All of the nuclear alterations indicative for PCD were suppressed by the co culture with platelets. When we tried to determine monocyte apoptosis on the cell membrane by annexin V staining, it soon became obvious that freshly isolated platelets expressed significant amounts of phosphatidylserine at their surface binding annexin V. This is in accordance with earlier findings describing phosphatidylserine exposure of platelets as an activation marker indicating procoagulant properties (27). A recent study by Brown et al. (28) also demonstrated annexin V binding by cultured platelets which increased by aging. It was concluded that platelets cultured in vitro undergo constitutive apoptosis by a caspase-independent pathway which was typically followed by recognition and uptake by phagocytes. Taking these data into account, we think that platelets mimic apoptotic cells and cross-talk with monocytes/macrophages or other phagocytes in a fashion as apoptotic cells do. In our study, platelets were fully viable as determined by morphology (TEM), preserved expression of surface markers, and ability of stimulation by thrombin. TEM demonstrated that monocytes in culture rapidly captured platelets which first adhered at the surface and consecutively were phagocytosed. Thus, intact platelets may share properties of apoptotic cells and can be used to study the interaction between apoptotic cells and phagocytes. We could show that the uptake not merely adherence of platelets was the essential step for the enhanced life expectancy of monocytes which was also paralleled by hsp-70 induction. Inhibition of the uptake of thrombocytes by cytochalasin D abrogated the protective effects of platelets. True phagolysosome formation not merely platelet invagination was essential for cytoprotection because trypsinization of monocytes had no influence on ingestion rates. Adherence of platelets on the surface of monocytes was insufficient for down-regulation of apoptosis, because short coculture times of 1 h with consecutive washing off thrombocytes not allowing significant uptake could not modulate cell senescence. Platelet lysates were ineffective in suppressing monocyte PCD. In contrast, phagocytosis of particles such as latex beads or zymosan did not alter monocyte senescence, suggesting that the uptake process per se is not sufficient to modulate PCD and that platelet surface receptors or intracellular compounds released in phagolysosomes are needed. Activation of platelets enhanced the protective, antiapoptotic effects on monocytes. As the background and explanation for this, activation of platelets induced higher uptake rates as detected by TEM. Higher numbers of ingested platelets are able to provide bigger amounts of protective compounds. The important conclusion can be drawn by our data that phagocytosis of platelets by monocytes is not an inert process leaving the phagocyte unaffected but evokes a whole cascade of processes in the phagocyte shown here by the induction of survival signals. Previously, it has been suggested that the disposal of apoptotic cells was not accompanied by secretary or inflammatory responses until we and others described that active and mainly immunosuppressive pathways were evoked by apoptotic cell phagocytosis, leading to enhanced production of anti-inflammatory IL-10, TGF-β, prostanoids or IL-6, or even to down-regulation of proinflammatory cytokines (29–31). In our view, better survival of the phagocyte due to platelet disposal fits in this scheme of anti-inflammation since more surrounding cells in a vascular lesion, including platelets or apoptotic inflammatory cells, can be taken up and removed.

Further studies will elucidate which receptors on monocytes and platelets are primarily engaged in uptake and downstream processes and which signaling pathways are involved. Although aggregation between monocytes and platelets has been observed in the blood and called platelet satellitism, we do not think that this phenomenon is of major importance within the bloodstream under physiological conditions. Shear stress might disrupt the clots. However, in the vessel wall or at inflammatory sites when platelets are activated and the contact time is long enough these aggregates might become relevant.

In conclusion, our study shows that ingestion of platelets by monocytes suppresses their constitutive or induced apoptosis program primarily by affecting caspase-9, -3, and hsp-70 expression. These findings may have important implications for the understanding of progression and resolution of thrombotic and inflammatory vascular disorders or atherosclerosis and may offer therapeutic options.

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References


