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ORIGINAL ARTICLE

Platelet microparticles contain active caspase 3

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Abstract
During storage, platelets undergo processes resembling apoptosis, including microparticle release, aminophospholipid exposure, and procaspase 3 processing. Recently, we showed that microparticles from endothelial cells contain caspase 3, one of the executioner enzymes of apoptosis. In this study we determined whether platelet-derived microparticles (PMP) contain caspase 3 *in vitro* (stored platelet concentrate) and *ex vivo* (plasma from healthy humans). In addition, we studied the underlying mechanism of caspase 3 formation in PMP, and the ability of such PMP to induce apoptosis in human macrophages (THP-1 cells). The presence of caspase 3 (antigen) was studied by Western blot and flowcytometry, and activity was determined by Ac-DEVD-pNA and ROCK I cleavage. *In vitro*, PMP numbers increased during storage. From day one onwards, PMP contained procaspase 3, whereas caspase 3 (antigen and activity) was detectable after 5–7 days of storage. PMP contained caspase 9 but not caspase 8, and the time course of caspase 9 formation paralleled procaspase 3 disappearance and caspase 3 appearance. In addition, PMP in human plasma also contained detectable quantities of caspase 3. Incubation of THP-1 cells with PMP induced apoptosis. Taken together, PMP contain caspase 3 *in vitro* and *ex vivo*. Our data implicate that procaspase 3 is likely to be processed by caspase 9 in PMP during storage. PMP induce apoptosis of human macrophages, but whether this induction is due to the transfer of caspase 3 remains to be determined.

Keywords: *Caspase 3, microparticles, platelets, procaspase 3, apoptosis, macrophage*

Introduction

Platelet-derived microparticles (PMP) are by far the most abundant microparticles (MP) in the human circulation. PMP expose negatively charged phospholipids and promote coagulation. Elevated numbers have been reported in a variety of diseases associated with arterial thrombosis [1–7].

Although human platelets are anucleated cells, they contain several components essential for apoptosis. For instance, upon addition of cytochrome C, caspase 9 and caspase 3 are formed in platelet lysates, indicating a functional intrinsic apoptosis pathway [8]. In addition, procaspase 3 is processed and/or disappears during storage of platelet concentrates [9–14], and PMP are released [15–18].

Recently, we showed that microparticles from endothelial cells contain caspase 3 *in vitro* and *ex vivo* [19]. Although in these experiments we also observed that microparticles from platelets and erythrocytes weakly stained for caspase 3, we concluded that ''additional studies will be required to substantiate our present findings''. Therefore, in the present study, we determined the presence of procaspase 3 and caspase 3 in PMP and platelets during storage *in vitro*, and in MP from plasma samples of healthy human individuals. Furthermore, we also addressed the question whether such PMP may have a biological function.

Methods

Platelet concentrates

Two fresh platelet concentrates (<1 leukocyte/3×10^8 platelets) from five donors each were obtained from Sanquin (Amsterdam, The Netherlands) and stored for 17 days without agitation at room temperature. MP derived from monocytes (CD14), granulocytes (CD66e) or erythrocytes (CD234; glycophorin A) were not detectable in both platelet concentrates by flow cytometry.

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Platelet isolation and western blotting

Every other day, 20 mL platelet concentrate was collected sterile, from which 10 mL was used for platelet isolation. Before centrifugation (20 minutes at 800 *g*, 20°C), 2 mL acid citrate dextrose (ACD; trisodium citrate, 0.85 mol/L; D-glucose, 0.11 mol/L; citric acid, 0.071 mol/L; pH 4.4) was added. Platelets were washed in 10 mL bicarbonate buffer (NaCl, 137 mmol/L; $NaHCO_3$, 11.9 mmol/L; D-glucose, 5.6 mmol/L; $MgCl_2$, 1.0 mmol/L; KCl, 2.6 mmol/L; and EDTA, 1.0 mmol/L; pH 6.5), and resuspended in 15 mL bicarbonate buffer. For Western blot, 100 μL aliquots were diluted 10-fold with phosphate-buffered saline (PBS, pH 7.4). Platelets were pelleted and dissolved in 30 μL reducing sample buffer, from which 10 μL was transferred to gradient gels (8–16%, BioRad; Hercules, CA, USA) and blotted to nitrocellulose (BioRad). Western blots of stored platelets were labelled with antibodies against procaspase 3 (Transduction Laboratories; San Diego, CA, USA) or caspase 3 (Cell Signaling; Danvers, MA, USA). Secondary horseradish peroxidase-labelled antibodies were from BioRad (goat-anti-mouse) or Promega (Madison, WI, USA; goat-anti-rabbit). The latter antibody was used in combination with the caspase 3 antibody. For detection, a Lumi-light plus conjugate was used according to the manufacturers' instructions (Roche; Indianapolis, IN, USA).

PMP isolation

Phosphate buffered saline (PBS; NaCl, 154 mmol/L; phosphate, 1.4 mmol/L; pH 7.5; 10 mL) and ACD (4 mL) were added to 10 mL platelet concentrate. To remove platelets, this platelet-rich suspension was centrifuged for 20 minutes at 800 *g* and 20°C. Supernatant was collected and centrifuged twice at similar conditions to remove remaining platelets. From this final platelet-free supernatant (<0.2% platelets of the initial plateletcount), fixed volumes of 250 μL aliquots were used to isolate PMP for flow cytometric analysis. For *ex vivo* experiments, citrate-anticoagulated blood (0.32% v/v) was used as described earlier [20], from which 250 μL plasma aliquots were used for flow cytometry.

Flow cytometric analysis

PMP were isolated by centrifugation (30 minutes, 18,890 *g* and 20°C) from two aliquots of 250 μL platelet-free supernatant. Microparticle-free supernatant (225 μL from each aliquot) was removed. The two remaining PMP pellets were diluted in either 225 μL PBS containing (0.32% w/v) trisodium-citrate for PMP counting or 225 μL of 0.1% (v/v) perm wash (Becton Dickinson, San Jose, CA, USA) for caspase 3 labeling. Both PMP suspensions were re-centrifuged. After centrifugation, the supernatants were removed and the PMP pellets were resupended in either citrate-containing PBS (75 μL) or 0.1% (v/v) perm wash (75 μL). The PBS/citrate resuspended PMP pellet (5 μL) was diluted in 35 μL PBS/$CaCl_2$ and stained with phycoerythrin (PE)-labelled anti-CD61 (Pharmingen, San Diego, CA, USA; 5 μL; 1 : 10 pre-diluted in PBS/$CaCl_2$). The other PMP pellet (5 μL) was diluted in 35 μL 0.1% (v/v) perm wash containing $CaCl_2$ (2.5 mmol/L) and doublestained with fluorescein isothiocyanate (FITC)-labeled anti-caspase 3 (Becton Dickinson, San Jose, CA, USA; 5 μL) and PE-labeled anti-CD61 (5 μL, 1:10 pre-diluted in 0.1% (v/v) perm wash/$CaCl_2$). Ig-FITC (Immuno Quality Products, Groningen, The Netherlands) was used as control antibody for caspase 3 labeling and IgG1-PE (Becton Dickinson) as control antibody for anti-CD61-PE. After 15 minutes incubation at room temperature, PBS/calcium or 0.1% (v/v) perm wash/calcium (900 μL) was added. Samples were analysed for 1 minute on a FACS Calibur (Becton Dickinson) and calculations were performed with Cellquest Pro (version 4.0.2; Becton Dickinson).

Caspase 3 activity assay

To determine whether PMP contain caspase 3 activity, PMP were isolated from fixed volumes platelet-free supernatant (2 mL, divided in two aliquots of 1 mL) by centrifugation for 1 hour at 18,890 *g* and 20°C. The MP-free supernatant was removed and the pellet was washed once with PBS. The PMP-containing pellet was resuspended in Cell Lysis Buffer (Calbiochem, San Diego, CA, USA; 100 μL) and subjected to three freeze (liquid nitrogen)/thaw (37°C) cycles. Caspase 3 activity was determined with the substrate Ac-DEVD-pNA (caspase 3 activity and caspase 3-like activity) in the presence and absence of the caspase 3 inhibitor Ac-DEVD-CHO (Calbiochem) as described by the manufacturer. The caspase 3 (-like) activity was calculated by subtracting the activity in the presence of Ac-DEVD-CHO from the activity in its absence.

Western blotting

For Western blotting, PMP were isolated from fixed volumes platelet-free supernatant (5 mL, aliquots of 1 mL) and washed in PBS (1 hour at 18,890 *g* and 20°C). The pellet was dissolved in reducing sample buffer (final volume 60 μL), transferred (10 μL) to gradient gels (8–16%) and blotted to nitrocellulose. MP from three healthy individuals, who gave their informed consent, were isolated from

2.5 mL platelet-free plasma and washed in PBS as described before. The pellet was dissolved in 30 μL reducing sample buffer and 10 μL was used for gelelectroforesis and Western blot. Western blots of PMP and MP from healthy individuals were labelled with antibodies against procaspase 3 or caspase 3. Western blots of stored PMP were also stained for procaspase 8/caspase 8, procaspase 9/caspase 9 (both antibodies from Cell Signaling; Danvers, MA, USA), and Rho-associated Coiled-coil-containing protein Kinase I (ROCK I, Transduction Laboratories). Anti-α-tubulin (Sigma, St Louise, MO, USA) was used as a lane loading control. Secondary horseradish peroxidase-labelled antibodies were from BioRad (goat-anti-mouse) or Promega (goat-anti-rabbit). For detection, a Lumi-light plus conjugate was used.

Culture of THP-1 cells

THP-1 monocytes were cultured as described by Bejta et al. [21]. THP-1 monocytes (0.5×10^6 cells) were differentiated into macrophages by incubation with PMA (200 ng/mL) for 72 hours. After incubation, cells were washed three times with culture medium. Subsequently, 1 mL fresh culture medium was added with either PMP (50 μL) or PMP-free supernatant (50 μL; control). After 24 hours, culture supernatant and cells were collected. The culture supernatant was centrifuged for 7 minutes at 500 *g* to remove detached cells. Cells were stained with either annexin V (Immuno Quality Products, Groningen, The Netherlands) plus propidium iodide (PI; gift Eric Reits, Department of Cell Biology and Histology, AMC, Amsterdam, The Netherlands) or anti-caspase 3 (Becton Dickinson, San Jose, CA, USA) as described previously [22]. To estimate the uptake of PMP by THP-1 cells, PMP numbers were estimated by flow cytometry before and after addition to the THP-1 cells.

PMP were isolated from platelet-free supernatant (2 mL; two platelet concentrates) after 11 days of storage. Aliquots (1 mL) were centrifuged for 1 hour at 18,890 *g* to obtain PMP. After centrifugation, PMP-free supernatant (975 μL) was removed from each aliquot. PMP pellets were resuspended in the remaining 25 μL and pooled. To estimate the number of PMP before addition to THP-1 cells, 1.0 μL of the PMP suspension was used. To estimate the PMP count after incubation with THP-1 cells, cell-free culture supernatant (250 μL) was used to estimate remaining PMP numbers. The culture supernatant was centrifuged (30 minutes at 18 890 *g*). In addition, MP-free supernatant (225 μL) was removed and the pellet was resuspended in the remaining 25 μL, from which 5 μL was used to estimate PMP numbers.

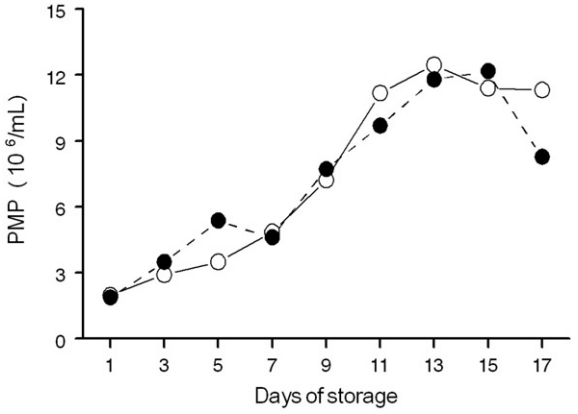

Figure 1. Numbers of PMP during storage. Two platelet concentrates (A and B) were stored at room temperature for 17 days without shaking. On uneven days, fixed volumes of sample were collected as described in Methods and analysed by flow cytometry. Anti-CD61 positive PMP are shown for both concentrates (open and filled symbols, concentrate A and B respectively).

Results

PMP numbers during storage

In both platelet concentrates, PMP numbers increased approximately six-fold between day 1 and 17 of storage (Figure 1).

Occurrence of (pro) caspase 3 in PMP

From day 1 onwards, PMP contained procaspase 3 (32 kDa; Figure 2A). On days 7 and 9, the highest quantities of procaspase 3 were present, which gradually disappeared from day 9 onwards. Minor quantities of caspase 3 (17 kDa) were detectable in PMP from day 7 onwards. Caspase 3 was most prominent at days 11–13, and became less thereafter (Figure 2B). To confirm the presence of caspase 3 in PMP, we also directly determined its occurrence in individual PMP by flow cytometric analysis (overall data are summarized in Figure 2C). The numbers of PMP containing caspase 3 increased in time, reaching maximum levels on days 11–13. Figures 2D–F (day 1) and 2G-I (day 11) show representative examples of staining for caspase 3 (Figures 2D, 2G), CD61 (Figures 2E, 2H) or their combinations (Figures 2F, 2I). From Figures 2G and 2I it is apparent that most PMP contain caspase 3 on day 11. Nevertheless, already on day 1 low numbers of caspase 3-positive PMP were present (Figures 2D and 2F). Not only the numbers of caspase 3-containing PMP increased, but also the fraction of caspase 3-positive PMP (of total events) increased in time. This fraction increased from 41% (day 1) to 77% (day 11).

To investigate whether or not caspase 3 also occurs in PMP *in vivo*, we determined the occurrence of both procaspase 3 and caspase 3 in MP fractions,

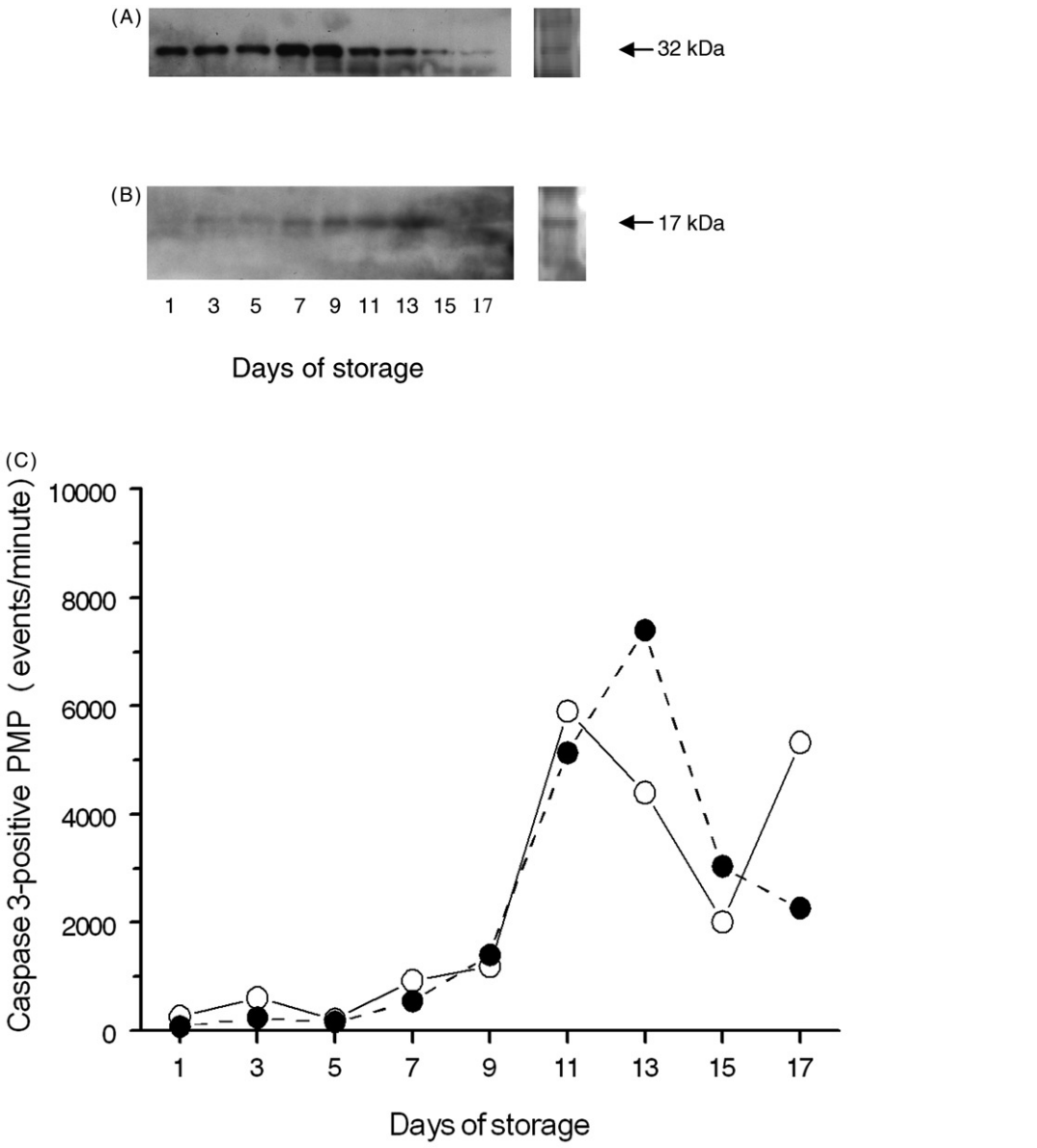

Figure 2. Procaspase 3 and caspase 3 in PMP. PMP during storage and MP from a healthy human individual (Control, Co) were analysed for the presence of procaspase 3 (32 kDa) and caspase 3 (17 kDa) by Western blot (Figures 2A and 2B, respectively). To study the presence of caspase 3 in individual PMP, intra-vesicular caspase 3 was determined by flow cytometry (Figure 2C, open and filled symbols, concentrates A and B, respectively). Examples of staining for caspase 3 (Figures 2D and 2G), CD61 (Figures 2E and 2H), or their combination (Figures 2F and 2I) are shown for day 1 and 11, respectively (unfilled, control; filled, (anti) caspase 3 or (anti) CD61). All blots, histograms and dotplots shown are from a single platelet concentrate (concentrate B). The two concentrates gave comparable data. MP from a healthy individual were also studied for the presence of caspase 3 (Figure 2J) or CD61 (Figure 2K) or their combination (Figure 2L).

containing mainly PMP, isolated from plasma samples of three healthy individuals. Weak bands of both procaspase 3 (Figure 2A, Co) and caspase 3 (Figure 2B, Co) were visible. Flow cytometry confirmed that CD61-positive (i.e. platelet-derived) MP from healthy individuals indeed contain caspase 3 (Figure 2J and 2L). Taken together, PMP contain procaspase 3 and caspase 3 during storage.

Caspase 3 activity in PMP

To determine the activity of caspase 3 in PMP, we used two approaches. First, a caspase 3 activity assay (with the chromogenic substrate Ac-DEVD-pNA for caspase 3 and caspase 3-like activity) was used. Second, we studied the occurrence of the (intravesicular) caspase 3-specific cleavage product of Rho-associated Coiled-coil-containing protein Kinase I (ROCK I). ROCK I is a 160 kDa protein, that either is reversibly activated by G proteins, or irreversibly activated by caspase 3. Caspase 3 cleaves ROCK I, which results in formation of a unique and constitutively active 130 kDa fragment. Caspase 3-mediated cleavage of ROCK I can be inhibited by both the pan caspase inhibitor z-VAD-fmk and the caspase 3-specific inhibitor z-DEVD-fmk [23].

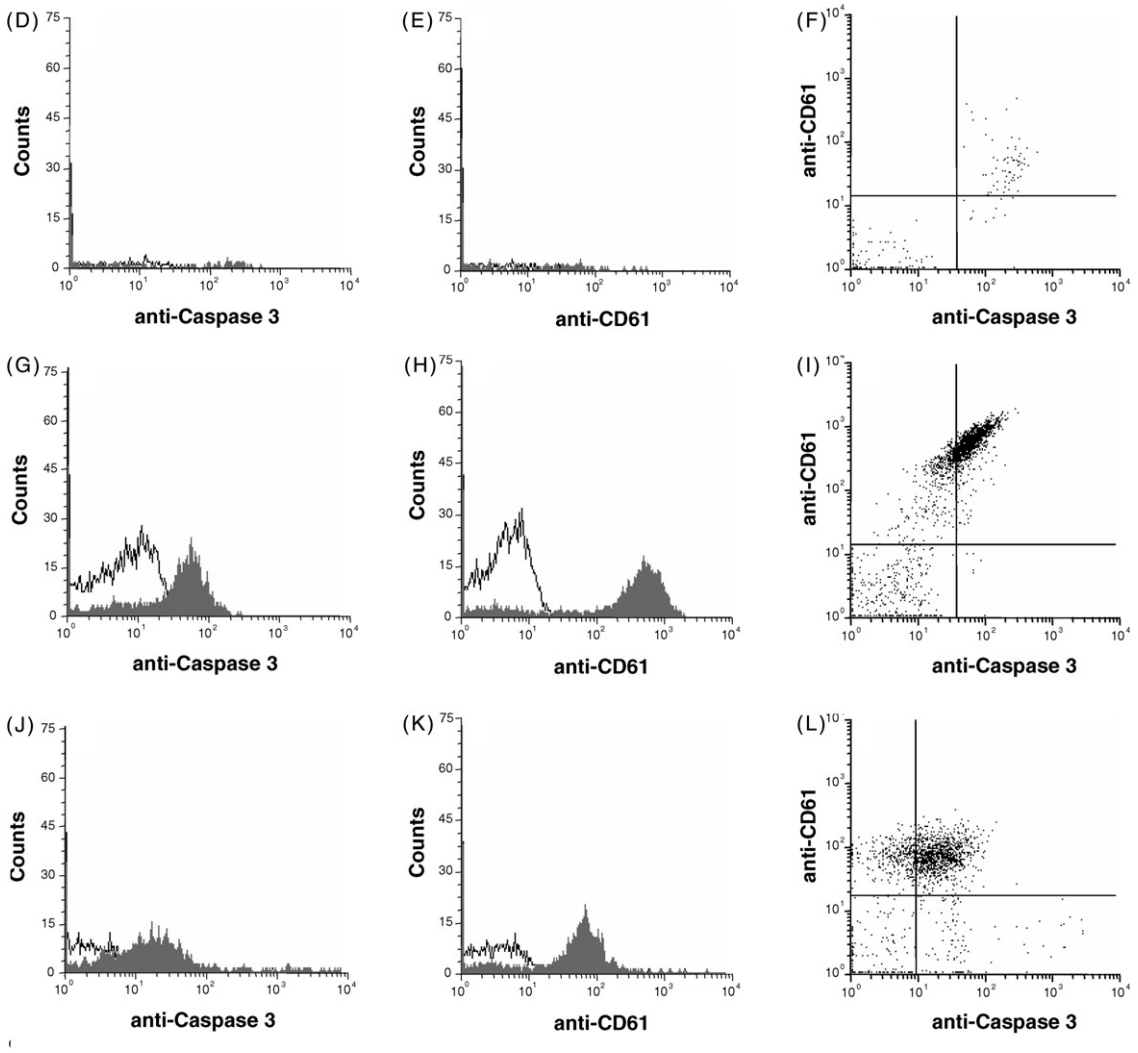

Figure 2. Continued.

PMP lysates from both platelet concentrates contained detectable levels of caspase 3 activity, which reached a maximum at day 9–11 (Figure 3A). The 160 kDa full-length form of ROCK I was detectable in PMP from day one onwards (Figure 3B), but disappeared after day 9. The caspase 3-specific cleavage fragment of ROCK I (130 kDa) was detectable from day 7 onwards, indicating that intravesicular caspase 3 cleaves ROCK I. In summary, both lines of evidence show that functionally active caspase 3 occurs in PMP.

Underlying mechanism of caspase 3 formation in PMP

Procaspase 3 is processed by either caspase 8 or caspase 9 into its active form (17 kDa and 12 kDa). Neither procaspase 8 nor caspase 8 could be detected in PMP (data not shown), but procaspase 9 (47 kDa) was present from day 1 onwards. Caspase 9 (37 kDa) was detectable from day 7 onwards (Figure 4a). Anti-α-tubulin labelling was used as a lane loading control (Figure 4b). Taken together, PMP contain caspase 9 and not caspase 8, indicating that procaspase 3 may be processed by caspase 9 in PMP. From these data, however, we could not exclude the possibility that caspase 3 within PMP originates from platelets, in which procaspase 3 was already processed to caspase 3. Therefore, we also determined the presence of caspase 3 and its precursor in the stored platelets (Figure 5). Whereas the 17 kDa fragment of (active) caspase 3 was not detectable in platelets, a smaller—approximately 14 kDa fragment—was detectable (Figure 5B; days 3–9), which may be due to rapid autodegradation of caspase 3 as described for other cell types [24]. Procaspase 3 was present during the first nine days of storage (Figure 5A). Thereafter, procaspase 3 disappeared rapidly, and after day 11 procaspase 3 was no longer detectable. Thus, the appearance of caspase 3 in PMP occurred especially after the complete disappearance of procaspase 3 within the platelets. Since hardly any procaspase 3 was detectable in platelets after day 9, the total amount of procaspase 3 in PMP is expected to remain constant from day 9 onwards. The time-dependent decrease of procaspase 3 and concurrent

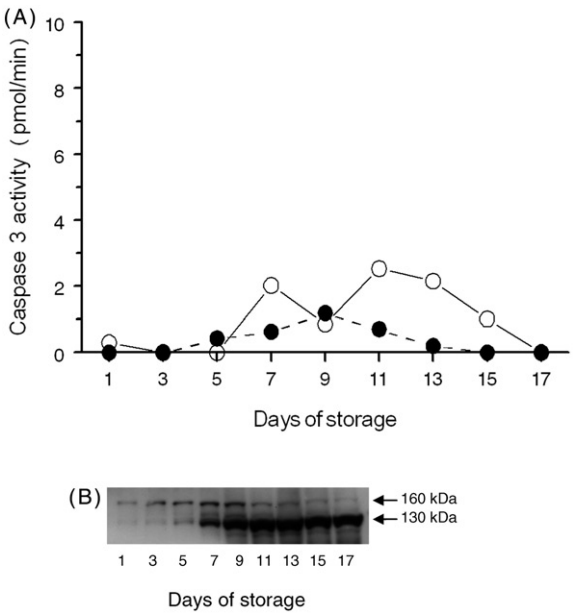

Figure 3. Caspase 3 activity in PMP. PMP were analysed for functional caspase 3 activity by (i) a commercial caspase 3 activity assay (Figure 3A) and (ii) cleavage of a specific intravesicular caspase 3 substrate, ROCK I (130 kDa; Figure 3B). Data from the activity assay are shown separately for both platelet concentrates.

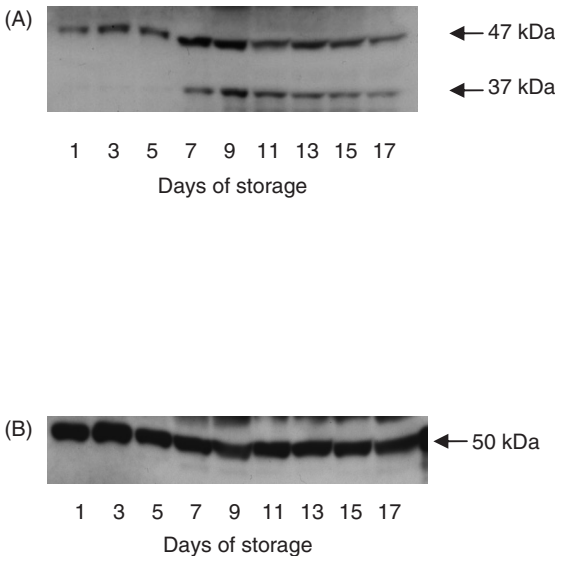

Figure 4. Procaspase 9 cleavage in PMP. PMP were analysed for the presence of procaspase 9 (47 kDa) and caspase 9 (37 kDa) during storage (Figure 4A). Anti-α-tubulin labelling was used as a lane loading control (Figure 4B).

formation of caspase 3 in PMP after day 9, however, implicate that caspase 3 is likely to have been formed from its precursor *within* the PMP.

Induction of apoptosis by PMP

The biological relevance of caspase 3 in PMP is unknown. One of the main functions of caspase 3 is the execution of apoptosis. Therefore, we studied the effects of caspase 3-containing PMP on human

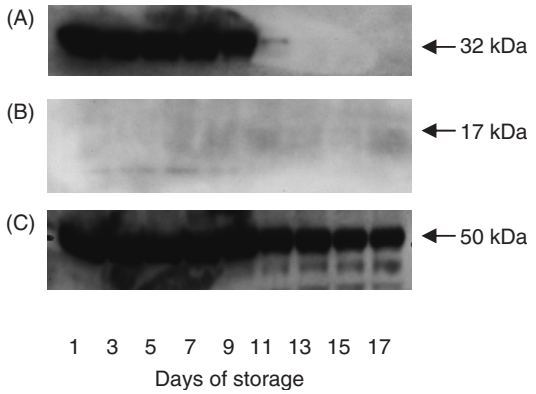

Figure 5. Procaspase 3 and caspase 3 in platelets. Platelets, during storage, were analysed for the presence of procaspase 3 (32 kDa) and caspase 3 (17 kDa) by Western blot (Figures 5A and 5B, respectively). Anti-α-tubulin labelling was used as a lane loading control (Figure 5C).

macrophages *in vitro*. THP-1 cells were incubated with PMP (estimated ratio 25 PMP/THP-1 cell). PMP, isolated from two platelet concentrates (A and B), were tested for their ability to induce apoptosis in two independent experiments. PMP were taken up by THP-1 cells (Figure 6A), although a large difference in uptake was observed (range <10% to >80%). Subsequently, we studied the apoptotic state of THP-1 cells after incubation with PMP (Figure 6B). After correction for incubation of THP-1 cells with PMP-free supernatant, increases were observed in the fractions (%) of THP-1 cells staining for annexin V (0.4% – 6.7%), PI (0.7% –38.3%) or annexin V and PI (0.2% – 6.7%). In contrast, intracellular caspase 3 was hardly detectable. In summary, when PMP were taken up by THP-1 cells variable fractions of cells showed signs of apoptosis. The extent of apoptosis induction, however, was not directly related to the numbers of PMP taken up.

Discussion

In line with earlier studies, we observed an increase in PMP numbers during platelet storage. Our present study demonstrates that such PMP contain procaspase 3 and subsequently functionally active caspase 3. Since also PMP contain caspase 3 *in vivo*, we believe that the occurrence of caspase 3 in PMP is a general phenomenon not strictly limited to platelet aging during storage *in vitro*.

So far, it was unclear whether or not procaspase 3 is converted into caspase 3 within human platelets [8–14, 25, 26]. In our present study, platelets were directly dissolved in reducing sample buffer, thereby preventing artificial proteolysis of procaspase 3 in platelet lysates. Our data indicate that procaspase 3 disappears in platelets without the concurrent formation of caspase 3. These data are completely

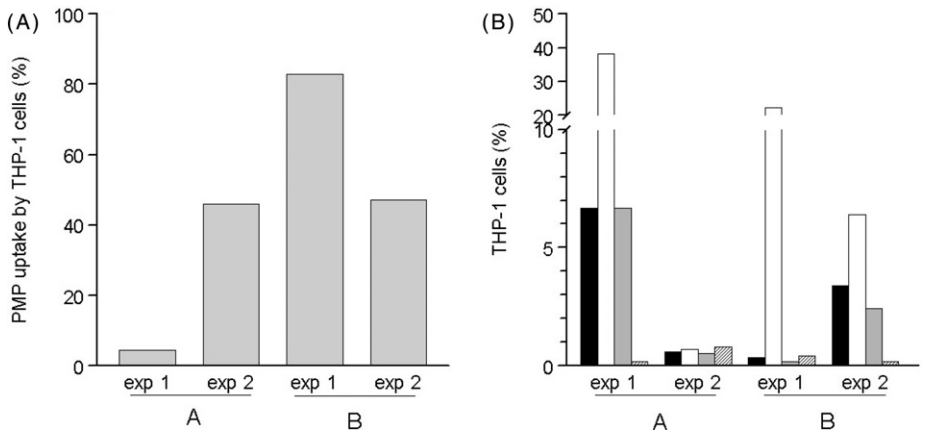

Figure 6. Induction of apoptosis by PMP. THP-1 cells were incubated with PMP from two platelet concentrates (A and B) for 24 hours in two experiments (indicated exp. 1 or 2 in Figure 6). The percentages of PMP taken up by THP-1 cells are shown in Figure 6A. Fractions of THP-1 cells staining for annexin V, PI, or annexin V/PI, or caspase 3 after incubation with PMP are shown in Figure 6B. The percentages were corrected for incubation of THP-1 cells with PMP-free supernatant. Annexin V staining: black bar, PI: white bar, annexin V/PI: gray bar, and caspase 3: striped bar.

in line with earlier studies from Shcherbina, Brown and Wadhawan [10, 14, 25].

Recently, we demonstrated that endothelial cell-derived microparticles (EMP) contain caspase 3 without detectable quantities of its precursor procaspase 3 [19]. The present study shows that PMP, in contrast to EMP, contain substantial amounts of procaspase 3. Since the time course of caspase 9 formation in PMP was paralleled by (i) disappearance of procaspase 3, (ii) appearance of caspase 3 antigen (17 kDa), (iii) caspase 3 activity (Ac-DEVD-pNA), and (iv) cleavage of intravesicular ROCK I, our data suggests that procaspase 3 is processed by caspase 9 *within* the PMP. Since both procaspase 3 and caspase 3 were not or no longer detectable in platelets during the before mentioned caspase 3 formation in PMP, it is unlikely that caspase 3 in PMP originates from platelets.

Caspase 3 activity has also been reported in exosomes [27]. Exosomes are small vesicles stored in multivesicular bodies, which become secreted into the cellular environment during secretion. It was suggested that 'packaging activated caspase 3 within exosomes might be a mechanism developed by cells to ensure survival', since the exosome-releasing cells were viable and showed no signs of apoptosis. In that study, caspase 3 was shown to process an intra-exosomal protein (Lyn). Thus, processing of proteins within vesicles may be a more general phenomenon.

Since caspase 3 or a caspase 3-like activity has been reported to occur in both exosomes and MP in cultures of viable cells, one may hypothesize that cells remove a potentially dangerous molecule by the release of vesicles into their environment [27] (Abid Hussein et al; Thromb Haemost 2007, in press). To which extent such vesicles possess any biological activity, however, is unknown. Our present study shows that a fraction of human THP-1 cells showed signs of underlying apoptosis or possibly even necrosis upon incubation with (caspase 3-containing) PMP. Strikingly, hardly any THP-1 cells stained for caspase 3. In these preliminary experiments, relatively large subpopulations of THP-1 cells stained for PI, a marker of necrosis, suggesting that the execution phase of apoptosis may have been completed already. Since we used a relatively large excess of PMP per cell (ratio approximately 25 : 1) and we only determined the effects of PMP at a single time interval, additional experiments are essential to gain further insight into this process. Furthermore, to which extent the observed induction of apoptosis is due to the transfer of caspase 3 also remains to be determined.

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