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Cell-derived microparticles contain caspase 3 *in vitro* and *in vivo*

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Summary. *Background*: Microparticles (MP) from endothelial cells (endothelial microparticles; EMP) circulate in disease states, but the processes such as apoptosis or cell activation underlying their release are unclear. *Objectives*: We investigated whether adherent (viable) or detached (apoptotic) endothelial cells are the possible source of EMP *in vitro*, i.e. under control and interleukin (IL)-1α activation conditions, and *in vivo*. *Methods*: Adherent and detached endothelial cells, and EMP, were isolated from human umbilical vein endothelial cell cultures ($n = 6$), treated without or with IL-1α (5 ng mL^{-1}; 24 h). Cell fractions were analyzed by flow cytometry for annexin V binding, propidium iodide (PI) and caspase 3 staining ($n = 3$). Caspase 3 in EMP was studied using Western blot ($n = 6$) and flow cytometry ($n = 6$). Plasma from healthy subjects and systemic lupus erythematosus patients (both $n = 3$) were analyzed for caspase 3-containing (E)MP. *Results*: Detached but not adherent cells double-stained for annexin V and PI, confirming the apoptotic conditions of the detached cells and the viable nature of the adherent cells. Caspase 3 was solely present in the detached cells and procaspase 3 in the adherent cells. Caspase 3 was present in EMP from both control and IL-1α-treated cultures. Counts of EMP and detached cells, but not adherent cells, highly correlated ($r = 0.959$, $P < 0.0001$). *In vivo* circulating MP from nucleated (endothelial cells, monocytes) and anucleated cells (platelets, erythrocytes) contained caspase 3. *Conclusions*: EMP contain caspase 3 and may be mainly derived from detached (apoptotic) endothelial cells *in vitro*. The presence of caspase 3 in MP from anucleated cell types, however, suggests that its presence may not necessarily be related to apoptosis *in vivo* but may be associated with caspase 3 activation unrelated to apoptosis.

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Introduction

The physiological status of the endothelium is one of the important factors in maintaining a proper hemostasis. Dysfunction of endothelial cells may result from excessive activation or programmed cell death (apoptosis) induced by a host of factors. In various pathologies, dysfunction of the endothelial cells may play a role, such as hypertension [1], cardiovascular diseases [2], diabetes [3], systemic lupus erythematosus (SLE) [4], renal failure [5] and vasculitis [6], and it is thought to play a role in the development of coronary atherosclerosis [7]. Thus, monitoring of the endothelial cells status is clinically pivotal, but so far only soluble E-selectin and von Willebrand factor are thought to be specific biochemical markers that reflect the activation/dysfunction status of the endothelial cells [8–10].

Recently, the presence of microparticles (MP) from endothelial cells (EMP) has been reported in plasma from patients suffering from, for example, lupus anticoagulant [11], multiple sclerosis [12], preeclampsia [13], acute coronary syndromes [14], thrombotic thrombocytopenic purpura [15], paroxysmal nocturnal hemoglobinuria [16], severe systemic inflammatory response syndrome [17] and severe malaria complicated with coma [18]. Recently, we showed that E-selectin identifies approximately 50% of EMP from activated endothelial cells *in vitro* and that such vesicles do also occur *in vivo* [19]. Others also demonstrated the occurrence of such E-selectin-positive EMP in plasma from patients suffering from active systemic vasculitis [20]. The presence of EMP in the circulation as a marker of endothelial dysfunction has very recently been reviewed by Horstman *et al.* [21].

In vitro, small fractions of endothelial cells appear as 'floaters' that display typical biochemical and morphological features of apoptosis [22–25]. This is a well-known phenomenon, called anoikis (Greek for 'homelessness'), i.e. the induction of apoptosis when a cell loses contact with the underlying matrix [26]. As a matter of fact, resistance to apoptosis in spite of the loss of

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this matrix contact may be involved in successful metastasis of cancer cells [27]. Circulating, i.e. detached, endothelial cells have also been reported in various disease states [28–31]. Whether or not there is indeed a relationship between the detached, apoptotic endothelial cells and EMP, however, is unknown. Therefore, in the present study we collected and analyzed adherent and detached endothelial cells separately in order to investigate their possible relationship to EMP formation.

Materials and methods

Reagents and assays

Medium M199, penicillin, streptomycin, and L-glutamin were obtained from GibcoBRL, Life Technologies (Paisley, UK). IgG1–FITC, IgG1–PE (clone X40), CD14–PE (clone MϕP9[8,9], IgG2b), CD61–PE (clone VI-PL2, IgG1) and CD31–PE (clone WM-59, IgG1) were obtained from Becton Dickinson [(BD), San Jose, CA, USA]. In some experiments, CD61–PE (clone Y2/51, IgG1) from Miltenyi Biotec (Bergisch Gladbach, Germany) was used. No differences were observed between these two MoAbs. Anti-glycophorin A–PE (clone JC159, IgG1) was from Dako A/S (Glostrup, Denmark). Human serum and fetal calf serum (both heat inactivated during 30 min at 56 °C; HuSi and FCSi, respectively) were from BioWhittaker (Walkersville, MD, USA). Human serum albumin (HSA) was obtained from the Central Laboratory of the Netherlands Red Cross Blood-transfusion Service (CLB; Amsterdam, the Netherlands), CD54–PE (clone 84H10, IgG1) from Immunotech (Marseille, France), and CD62E–PE (clone HAE-1f, IgG1) from Ancell (Lausen, Switzerland). Recombinant human interleukin (IL)-1α was either from GibcoBRL (Gaithersburg, MD, USA) or Sigma (St Louis, MO, USA). Human recombinant basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Collagenase (type 1A), and propidium iodide (PI) were from Sigma. Annexin V-allophycocyanin (APC) was from Caltag Laboratories (Burlingame, CA, USA), heparin (400 U mL^{-1}) from Leo Pharma BV (Breda, the Netherlands) and trypsin from Difco Laboratories (Detroit, MI, USA). The following antibodies were used for Western blot analysis: antihuman procaspase 3 MoAb from Transduction Laboratories (Lexington, KY, USA), antihuman caspase 3 polyclonal antibody from Cell Signaling Technology (Beverly, MA, USA), antihuman α-tubulin MoAb and antimouse horseradish peroxidase (HRP) conjugate from BioRad (Hercules, CA, USA), and antirabbit IgG HRP conjugate from Promega (Madison, WI, USA). Tissue culture flasks were from Greiner Labortechnik (Frickenhausen, Germany) and gelatin from Difco Laboratories (Sparks, MD, USA).

Isolation, culture and treatment of human umbilical vein endothelial cells

Human umbilical vein endothelial cells (HUVEC) were collected from human umbilical cord veins as described previously [19]. Briefly, umbilical cords were digested with collagenase for 20 min at 37 °C. Detached cells were perfused with medium M199 supplemented with 10% HuSi. The cell suspension was centrifuged for 10 min at 180 × g and 20 °C, and cells were resuspended in culture medium. HUVEC were cultured in tissue culture flasks coated with 0.75% gelatin (passage 0). Upon confluency at passage 3, HUVEC were kept for 3–4 days in a resting state. Afterward, the culture supernatant was refreshed and the cells were either untreated (control) or incubated with IL-1α (5 ng mL^{-1}) for 24 h.

Conditioned media (10 mL) were harvested and centrifuged for 10 min at 180 × g and 20 °C to isolate detached cells. The pellets containing the detached cells were carefully resuspended in 1% FCSi in PBS (pH 7.4). In parallel, the adherent endothelial cells were harvested by trypsinization. After 4 min, trypsin was neutralized by PBS/FCSi. Both cell suspensions were separately centrifuged for 10 min at 180 × g and 4 °C, resuspended in PBS/FCSi, kept on melting ice for 15 min, and then again centrifuged for 10 min at 180 × g and 4 °C. Because adherent cells outnumbered detached cells in all experiments, detached cells were resuspended in 0.5 mL PBS/FCSi and the adherent cells in 1 mL PBS/FCSi. The absolute number of cells (N) was estimated using the following formula: N = (events counted by flow cytometry/aspiration volume) × 60 (dilution factor) × cell suspension volume.

Labeling of endothelial cells with annexin V and PI

Upon incubation without or with IL-1α, detached and adherent endothelial cells were collected separately in PBS/FCSi as described in the previous section. The cell suspensions were centrifuged for 10 min at 180 × g and 20 °C and pellets were resuspended in ice-cold binding buffer (10 mmol L^{-1} HEPES, 150 mmol L^{-1} NaCl, 8 mmol L^{-1} KCl, 1.4 mmol L^{-1} $CaCl_2$, 1.0 mmol L^{-1} $MgCl_2$; pH 7.4). The cells were washed twice with the ice-cold buffer. The pellets were then carefully resuspended in the buffer (100 μL). Annexin V–FITC (diluted 1 : 200 v/v) was added and the mixture was incubated in the dark for at least 10 min at 4 °C. The excess of unbound annexin V was removed by addition of 1 mL binding buffer and centrifugation of the mixture for 10 min at 180 × g and 20 °C. The pellets were resuspended in 300 μL binding buffer and 5 μL PI (5 μg mL^{-1} final concentration) was added to each sample immediately prior to flow cytometric measurement. The fluorescence thresholds were set in terms of binding of annexin V and PI to adherent cells harvested from untreated (control) HUVEC. In one preliminary experiment, 5000 events were analyzed by flow cytometry for the adherent cells, and detached cells were analyzed for 1 min. In two other experiments, 1500 events were analyzed for both detached and adherent cells.

Flow cytometric analysis of caspase 3 in endothelial cells

Both detached and adherent endothelial cells were analyzed by flow cytometry for the presence of caspase 3 using the apoptosis kit I (BD). Detached and adherent cells were collected, washed twice with cold PBS, resuspended in fixation

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and permeabilization solution and subsequently incubated for 20 min on ice in this solution. The cells were then pelleted (10 min at 180 × *g* and 20 °C) and washed twice with detergent buffer [perm/wash; 1 : 10 (v/v)]. The pellet was resuspended with the detergent buffer (100 μL) and incubated with either control antibody or rabbit anticaspase 3 MoAb (5 μL) for 30 min at room temperature. Afterwards, the cells were washed with detergent buffer (1 mL) and finally resuspended in 500 μL detergent buffer before analysis. Detached cells were analyzed for 1 min and for adherent cells 1500 events were analyzed.

Isolation of (E)MP

Aliquots (1 mL) of the cell-free culture supernatant were snapfrozen in liquid nitrogen and stored at − 80 °C. Before use, samples were thawed on melting ice for 1 h, then centrifuged for 1 h at 17 570 × *g* and 20 °C. Then, 900 μL of (MP-free) supernatant was removed. The remaining 100 μL (MP-enriched) suspension was diluted with 900 μL PBS (154 mmol L^{-1} NaCl, 1.4 mmol L^{-1} phosphate) containing 10.9 mmol L^{-1} trisodium citrate. MP were resuspended and again centrifuged for 1 h at 17 570 × *g* and 20 °C. Again, 900 μL of supernatant was removed and MP were resuspended in the remaining 100 μL. Plasma samples from citrate-anticoagulated venous blood of SLE patients and healthy controls (with their informed consent) were collected and handled as described previously [19]. For flow cytometry detection of (E)MP from SLE patients and healthy individuals, the MP suspension was diluted 4-fold with PBS/citrate (pH 7.4).

Flow cytometric analysis of (E)MP

MP samples were analyzed in a FACSCalibur flow cytometer (BD). Forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain and MP were identified as described previously by their FSC and SSC characteristics and binding of annexin V [19]. MP (5 μL) were diluted with 35 μL PBS containing 2.5 mmol L^{-1} $CaCl_2$ (pH 7.4). Then, 5 μL annexin V–APC was added (0.66 or 0.5 μg mL^{-1} final concentration; two different batches of APC-labeled annexin V were used, and both batches were titrated for optimal staining). In the control samples of the MP, annexin V-positive events were identified by placing a threshold in a MP sample (5 μL) diluted with PBS containing 10.9 mmol L^{-1} trisodium citrate (40 μL; pH 7.4) and 5 μL of annexin V, i.e. without Ca^{2+}. The mixture of MP and annexin V was then incubated for 15 min in the dark at room temperature. To remove the excess of free annexin V, 200 μL PBS/calcium buffer was added and the suspension was centrifuged for 30 min at 17 570 × *g* and 20 °C. Finally, 200 μL of supernatant was removed, and MP were resuspended with 300 μL PBS/calcium. All samples were analyzed for 1 min in the flow cytometer.

Western blotting

Cell-free culture supernatants (5 mL) were collected after 24 h. After removal of detached cells, EMP were isolated by centrifugation for 1 h at 17 570 × *g* and 20 °C and resuspended in PBS/citrate. Subsequently, EMP were pelleted (1 h at 17 570 × *g* and 20 °C) and resuspended in 24 μL PBS. To this EMP suspension, 6 μL of 5-fold concentrated sample buffer containing β-mercaptoethanol (12.5% v/v), bromophenol blue (0.025% v/v), glycerol (25% v/v), SDS (10% w/v) and Tris base (312.5 mM; pH 6.8), was added. Detached and adherent endothelial cells were separately isolated, washed and collected in PBS/FCSi (0.5 and 1.0 mL, respectively). In two experiments, 330 μL of detached cells suspension and 830 μL of adherent cell suspension were used to pellet the cells. In the other four experiments the amount of detached and adherent cell suspensions used to pellet the cells were 290 μL and 790 μL, respectively. Subsequently, sample buffer was used to dissolve the pellets of the detached cells (final volume 30 μL) and adherent cells (final volume 60 μL). Fixed volumes (30 μL) of cell lysates in sample buffer were applied for electrophoresis. Before electrophoresis, all samples were heated for 5 min at 100 °C. Electrophoresis was carried out in 15% polyacrylamide gel. The proteins were transferred to nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany). Blots were incubated for 60 min at room temperature with blocking buffer [Tris-buffered saline–Tween (TBS–T); 10 mmol L^{-1} Tris–HCl, 150 mmol L^{-1} NaCl, 0.05% (v/v) Tween-20; pH 7.4], containing 5% (w/v) dry milk powder (Protifar; Nutricia, Vienna, Austria). The blots were subsequently incubated with (rabbit) antihuman caspase 3 polyclonal antibody (1 : 1000) for 24 h at 4 °C, followed by antirabbit IgG–HRP conjugate (1 : 7500) for at least 45 min at room temperature. In addition, the same blots were incubated with (mouse) antihuman procaspase 3 MoAb (1 : 1000) for 2 h at room temperature and (goat) antimouse IgG–HRP conjugate (1 : 3000) for 60 min at room temperature. Finally, the blots were also incubated with anti-α-tubulin MoAb for 60 min at room temperature and (goat) antimouse IgG–HRP conjugate (1 : 3000) for 60 min at room temperature. After each incubation step, the blots were washed three times with TBS–T for 5–10 min. All antibodies were diluted with blocking buffer. The bands were detected using an enhanced chemiluminescence kit (ECL; Amersham Biosciences, Buckinghamshire, UK) and exposed to Fuji Medical X-ray film.

Flow cytometric analysis of caspase 3 in (E)MP

The presence of caspase 3 in subpopulations of MP was studied by flow cytometry using apoptosis kit I (BD) with slight modification of the manufacturer's protocol. Briefly, EMP were isolated as described in the previous section. The EMP suspension (25 μL) was diluted with 500 μL detergent (diluted 1 : 10 in distilled water prior to use) and centrifuged for 30 min at 17 570 × *g* and 20 °C. Then, 500 μL of (MP-free) supernatant was removed and EMP were resuspended with 75 μL PBS/citrate (pH 7.4). EMP (5 μL) were diluted with 35 μL detergent. Rabbit anticaspase 3 antibody plus CD31–PE (PECAM-1), CD54–PE (ICAM-1) or CD62E–PE (E-selectin) (5 μL each) was added. Isotype-matched control antibodies

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(IgG1) were also used to set fluorescence thresholds. The mixture was then incubated for 30 min in the dark at room temperature. To remove the excess of unbound MoAb, 500 μL detergent (1 : 1000 diluted in distilled water) was added and the suspension was centrifuged for 30 min at 17 570 × *g* and 20 °C. Finally, 500 μL of supernatant was removed, and MP were resuspended with additional 300 μL detergent (1 : 1000). For flow cytometric analysis of MP from SLE patients and healthy controls, the MoAb concentrations used were 0.16 μg mL^{-1} for glycophorin A–PE and 0.25 μg mL^{-1} for CD14–PE (LPS-receptor). Dilution of CD61–PE (β_3) was 1 : 100 (v/v).

Patients and controls

SLE patients ($n = 3$; women) were included in this study who fulfillled the revised criteria of the American College of Rheumatology for the diagnosis of SLE [32]. Their age was 45, 51 and 55 years. The SLE Disease Activity Index (SLEDAI) [33] was 22, 20 and 4, respectively. As controls, three age-matched women were included. The study fulfilled the guidelines of the Medical Ethical Committee of the Slotervaart Hospital (Amsterdam, the Netherlands).

Statistical analysis

Data were analyzed with Prism (3.0) for Windows. All data were analyzed with paired *t*-test (two-tailed analysis). Data were considered statistically significant at $P \leq 0.05$. Correlations were determined using Pearson's correlation test (two-tailed analysis).

Results

Numbers of EMP and endothelial cells

EMP were isolated from control or IL-1α-treated endothelial cells ($n = 6$) and identified by their characteristic FSC and SSC, and their binding of annexin V. Figure 1A shows that treatment with IL-1α resulted in a double but statistically insignificant increase in EMP numbers ($P = 0.118$). It should be mentioned that in five out of the six experiments the numbers of EMP were elevated upon IL-1α-treatment compared with control.

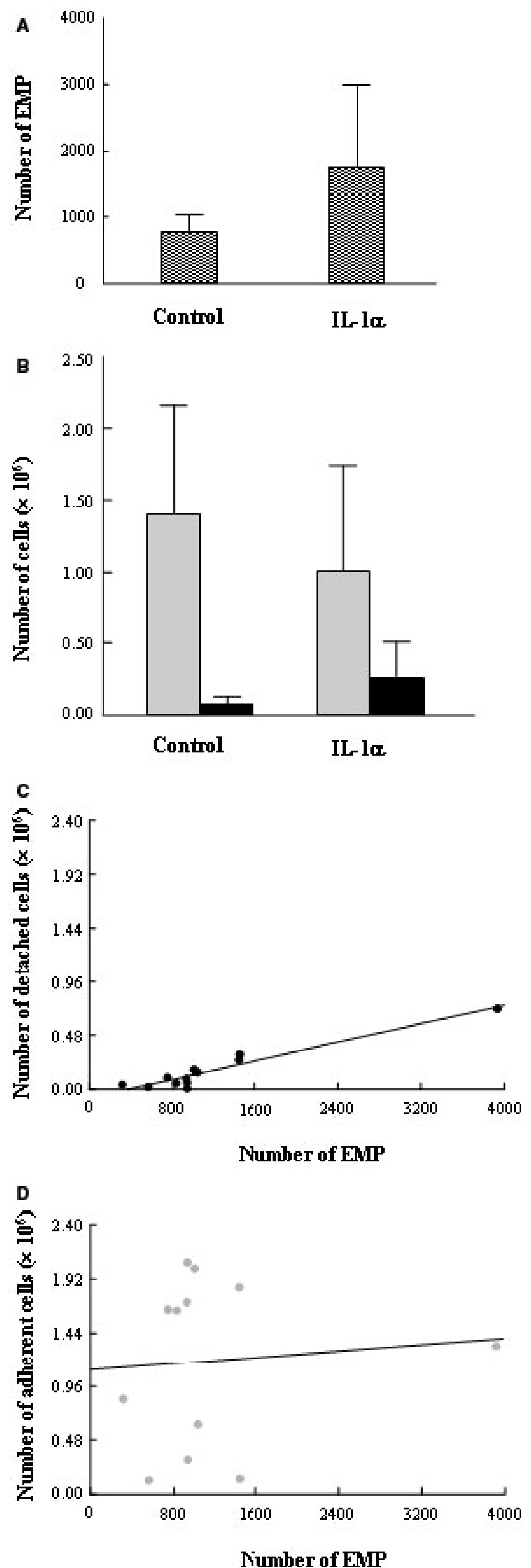

Fig. 1. Analysis of endothelial microparticles (EMP) and endothelial cell numbers. Endothelial cells were incubated without (control) or with interleukin (IL)-1α (5 ng mL^{-1}) for 24 h. Adherent and detached cells as well as EMP were isolated as described ($n = 6$). (A) Numbers of EMP, identified on their characteristic FSC and SSC, and binding of annexin V ($P > 0.05$). (B) Numbers of adherent cells (gray bars; $P > 0.05$) or detached cells (black bars; $P > 0.05$). The correlation between the numbers of EMP and the numbers of detached cells is shown in C ($r = 0.959$, $P < 0.0001$). The correlation between the numbers of EMP and the numbers of adherent cells is shown in D ($r = 0.087$, $P = 0.787$). For panels C and D, data from control and IL-1α-treated cultures are included.

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In control cultures, 6.0% ± 3.5 (mean ± SD) of the total endothelial cell number occurred as detached cells (Fig. 1B). These percentages were calculated by setting the total number of adherent plus detached cells per experiment at 100%. Upon treatment with IL-1α, this fraction increased to 23.1% ± 23.7 ($P = 0.162$ compared with control). Again, similar to EMP, in five out of the six experiments the percentages of detached cells were increased upon IL-1α treatment. In one of the experiments, however, the number of detached cells was exceptionally high (65.2%) compared with control (3.2%), and this number deviated more than 3-fold from the average. When this number was omitted from the statistical analysis, there was still no significant difference ($P = 0.282$).

The overall correlations for control ($n = 6$) plus IL-1α treatment ($n = 6$) between the numbers of EMP and detached as well as adherent cells are provided in Fig. 1C and D, respectively. The numbers of EMP correlated highly with the numbers of detached cells (Fig. 1C; $r = 0.959$, $P < 0.0001$), but not with adherent cells (Fig. 1D; $r = 0.087$, $P = 0.787$). Again, in one sample an exceptionally high number of EMP was observed. When this sample was omitted, correlations were $r = 0.825$ ($P = 0.001$) and $r = 0.104$ ($P = 0.760$), respectively. This sample was different from the one in which we observed an exceptionally elevated number of detached cells.

Annexin V and PI staining of endothelial cells

Adherent and detached endothelial cells were isolated from control (Fig. 2A,B) and from IL-1α (Fig. 2C,D)-treated cells (5 ng mL^{-1}; 24 h), and analyzed for binding of annexin V in combination with PI staining. Cells that stain for both annexin V and PI (i.e. events in the upper right quadrant of Fig. 2A–D) are generally considered to be in the late stage of apoptosis [34]. Representative dot plots are shown. Most of the detached endothelial cells (Fig. 2B,D) double-stained for annexin V and PI (control vs. IL-1α: 68.6% ± 19.1 and 72.8% ± 8.9, respectively; $P = 0.621$). In contrast, only a minor fraction of the adherent cells (Fig. 2A and C; upper right quadrant) were positive for annexin V and PI (control vs. IL-1α: 1.0% ± 0.0 and 1.6% ± 1.2, respectively; $P = 0.422$). Thus, adherent cells remained viable after treatment with IL-1α. Therefore, under both control and IL-1α conditions, viable and apoptotic cells coexist in endothelial cell cultures. IL-1α, however, did not affect either cell detachment or the apoptosis status of adherent and detached endothelial cell fractions.

Presence of caspase 3 in endothelial cells

To confirm the presence of apoptotic cells even under control conditions, both detached and adherent endothelial cells were analyzed flow cytometrically for the presence of caspase 3. The majority of the detached cells, both under control conditions [Fig. 3B; 60.0% ± 23.3 ($n = 3$)] and after treatment with IL-1α for 24 h [Fig. 3D; 55.4% ± 17.1 ($n = 3$)] stained for caspase 3. Treatment with IL-1α did not affect the fraction of detached cells that stained for caspase 3 ($P = 0.728$). In contrast, only minor fractions of adherent cells stained for caspase 3 under control conditions (Fig. 3A; 0.3% ± 0.3) or after treatment with IL-1α (Fig. 3C; 2.7% ± 2.9, $P = 0.314$

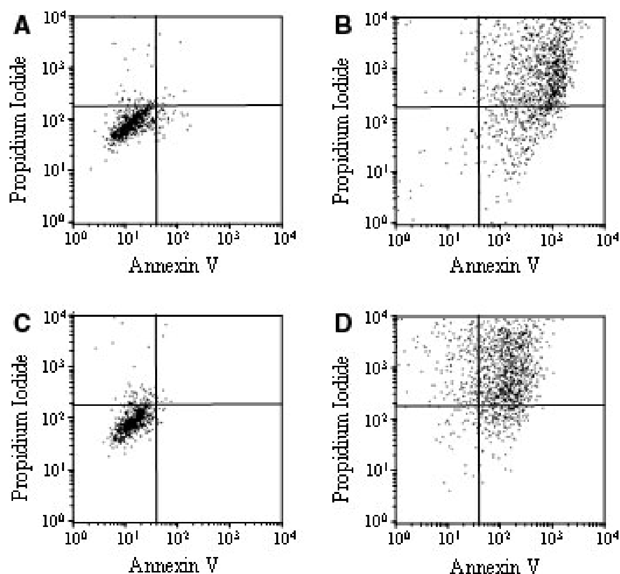

Fig. 2. Annexin V and propidium iodide (PI) staining of endothelial cells. Endothelial cells were incubated without (control; A,B) or with interleukin (IL)-1α (5 ng mL^{-1}; C,D) for 24 h. Detached (B,D) and adherent endothelial cells (A,C) were isolated, labeled with annexin V and PI, and analyzed by flow cytometry ($n = 3$). The dot plots shown were obtained within one representative experiment. Whereas most of the detached endothelial cells stained for both annexin V and PI, only a minor fraction of the adherent cells was positive for both markers.

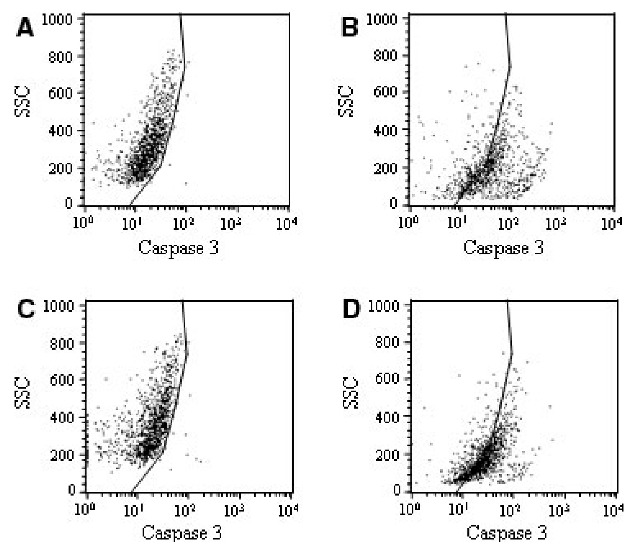

Fig. 3. Intracellular detection of caspase 3 by flow cytometry. Endothelial cells were incubated without (control; A,B) or with interleukin (IL)-1α (5 ng mL^{-1}; C,D) for 24 h. The detached (B,D) and adherent cells (A,C) were collected separately, labeled with anticaspase 3 MoAb, and analyzed by flow cytometry ($n = 3$). The dot plots shown were obtained within one representative experiment. Both in the absence and presence of IL-1α, the majority of the detached cells stained for caspase 3, whereas only minor fractions of the adherent cells stained positive.

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compared with control). Thus, cellular fractions of adherent or detached endothelial cells that stain for caspase 3 were not affected by treatment with IL-1α.

Detection of caspase 3 in EMP by Western blot

Because the numbers of EMP highly correlated with the numbers of detached endothelial cells, which contained caspase 3, we hypothesized that EMP may also contain caspase 3. Therefore, the presence of caspase 3 and its precursor, procaspase 3, were studied by Western blot (Fig. 4). EMP from control as well as IL-1α-treated endothelial cells contained substantial amounts of the 17-kDa (and to a lesser extent the 19-kDa) form of caspase 3. In contrast, procaspase 3 (32 kDa) was not detectable. EMP were isolated from fixed volumes of conditioned medium (5 mL) and the absolute numbers of EMP were determined for the conditions studied to be approximately 2-fold different (Fig. 1A), which was reflected in the intensity of staining of α-tubulin as a marker to indicate the amounts of EMP loaded per lane. In four out of the six experiments, the 17-kDa caspase 3 band was clearly more pronounced in EMP lysates from IL-1α-treated cells compared with control. In the other two experiments, these bands showed similar intensities. These data are roughly in line with the observed increase in EMP numbers upon IL-1α treatment (Fig. 1A). Whether or not this treatment with IL-1α affects the quantity of caspase 3 in EMP, however, remains to be established.

To confirm the presence of caspase 3 in detached endothelial cells and absence in adherent cells, cell lysates were also subjected to Western blotting. Detached endothelial cells from control as well as IL-1α-treated cell cultures contained the 17-kDa form of caspase 3 but not its 32-kDa proform. In contrast, adherent cells contained only procaspase 3. Because only a minor fraction of the endothelial cells was detached (Fig. 1B), the higher number of cells in the lysates from adherent cells compared with the detached cells explains why the tubulin band of the adherent cells is much more pronounced than that of the detached cells on the Western blots shown in Fig. 4. Treatment with IL-1α did not affect the caspase 3 positivity of the EMP or the detached cells, nor the procaspase positivity and absence of caspase 3 in the adherent cells.

Detection of caspase 3 in subpopulations of EMP

To investigate whether the presence of caspase 3 is restricted to particular subpopulations of EMP, we labeled EMP with anticaspase 3 MoAb in the absence or presence of saponin (Fig. 5A and B, respectively). This figure confirms that EMP contain caspase 3 and illustrates that permeabilization is essential for the detection of this intravesicular protein. Because permeabilization impaired the binding of annexin V, we omitted annexin V from the experiments described below. From the areas under the curve it is apparent that saponin

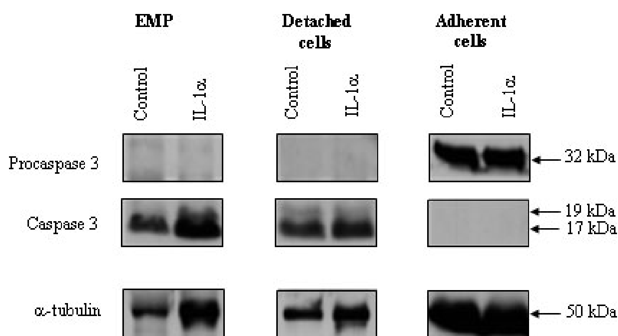

Fig. 4. Western blot of (pro)caspase 3 in endothelial microparticles (EMP). EMP were isolated from culture supernatants of endothelial cells incubated in the absence (control) or presence of interleukin (IL)-1α (5 ng mL^{-1}) for 24 h. EMP were isolated from fixed quantities of volume and tubulin was used as an additional marker to estimate the quantities of protein loaded per lane. EMP contained the 17- and 19-kDa forms of caspase 3 but not the 32-kDa proform. In parallel, adherent and detached endothelial cell lysates were also analyzed for the presence of (pro) caspase 3. The blots shown represent one out of six separate experiments and were all obtained within one representative experiment.

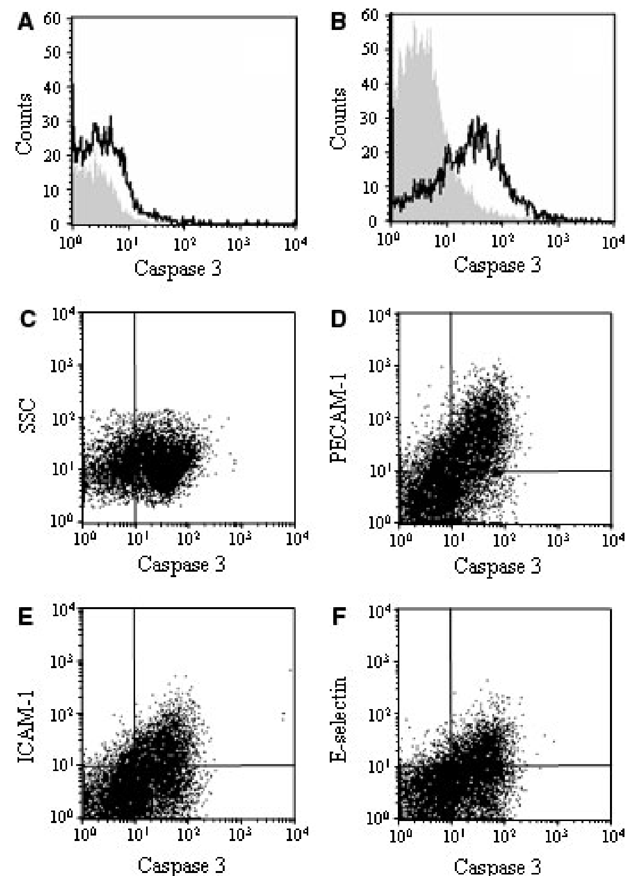

Fig. 5. Flow cytometric detection of caspase 3 in endothelial microparticles (EMP). EMP were isolated and labeled with either IgG1 control antibody (gray) or anticaspase 3 MoAb (dark line) in the absence (A) or presence (B) of saponin. (C–F) Dot plots, obtained within one experiment representative of five similar experiments, from EMP obtained after treatment of endothelial cells with interleukin (IL)-1α, that were incubated with anticaspase 3 MoAb alone (C) or in combination with PECAM-1 (CD31, D), ICAM-1 (CD54, E) or E-selectin (CD62E, F).

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affected the numbers of events analyzed by the flow cytometer, particularly in the (IgG1) control condition, but not caspase 3 positivity itself.

Previously, we showed that ICAM-1 (CD54) and E-selectin (CD62E) are exposed only on EMP from IL-1α-treated but not control cells, whereas PECAM-1 (CD31)-exposing EMP occur in both conditions [19]. EMP from IL-1α-treated cells ($n = 6$) were incubated with anticaspase 3 MoAb without (Fig. 5C) or in combination with MoAbs directed against either PECAM-1 (Fig. 5D), ICAM-1 (Fig. 5E) or E-selectin (Fig. 5F). The (representative) dot plots show that virtually all PECAM-1+ EMP contain caspase 3 (87.2% ± 5.0; $n = 6$). ICAM-1- and E-selectin-exposing EMP also contained caspase 3 (89.2% ± 2.4 and 88.4% ± 3.9, respectively).

Detection of microparticle-associated caspase 3 in human plasma

Recently, we and others demonstrated that E-selectin specifically detects EMP in human plasma samples [19,20]. To study the possible occurrence of caspase 3 in such vesicles *in vivo*, total MP fractions were isolated from plasma samples of three SLE patients and three healthy individuals, and stained with anticaspase 3 plus MoAbs directed against either E-selectin (Fig. 6, top row), platelet glycoprotein IIIa (β_3; CD61; second row), erythrocyte glycophorin A (third row) or monocyte LPS-receptor (CD14; bottom row). MP from all plasma samples stained for caspase 3. Plasma samples from two SLE patients also contained a subpopulation of E-selectin-exposing EMP, which strongly double-stained for caspase 3. Most by far of the caspase 3-containing MP originated from platelets and to a lesser extent from erythrocytes. Plasma samples of the two 'E-selectin-positive' SLE patients also contained monocyte-derived MP that double stained for caspase 3.

Discussion

In the present study we investigated the possible relationship between adherent and detached endothelial cells and the formation of EMP. In line with previous studies, we found that detached endothelial cells are apoptotic, similar to other cells losing their association with the matrix [22–26], i.e. most of the detached cells stained for annexin V and PI, and contained caspase 3. Also, most of the EMP contained caspase 3, *in vitro* and in particular *in vivo*. In addition, EMP numbers correlated highly with the numbers of detached cells, suggesting that the majority of EMP originate from these cells. *In vivo*, the presence of caspase 3 was not only restricted to MP originating from nucleated cells, since significant fractions of MP from platelets and erythrocytes also contained detectable amounts of

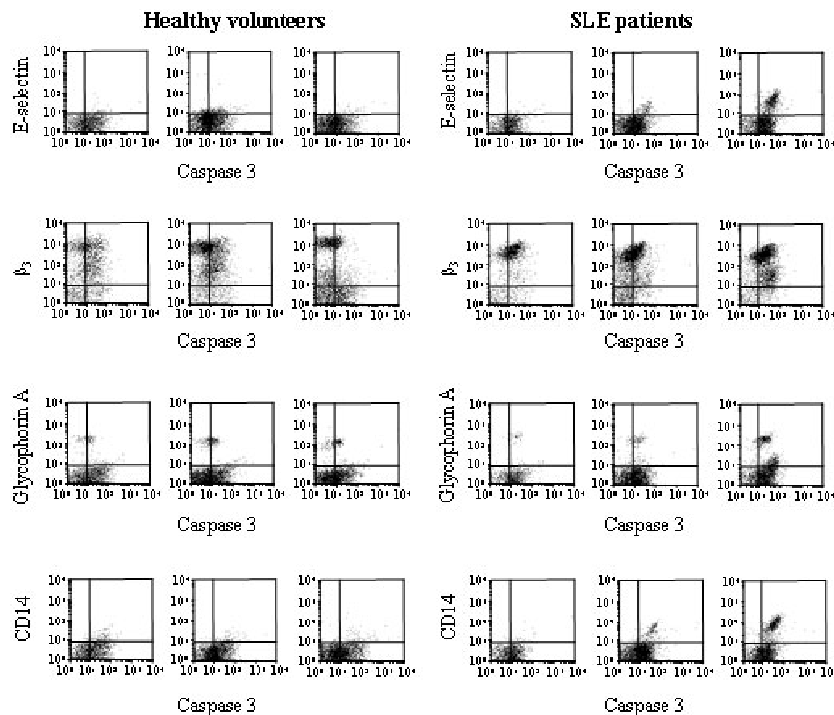

Fig. 6. The presence of caspase 3 in microparticles (MP) *in vivo*. MP were isolated from plasma samples of three systemic lupus erythematosus (SLE) patients (shown in columns 4, 5 and 6) and three healthy individuals (shown in columns 1, 2 and 3), labeled with MoAbs and analyzed by flow cytometry. Each column represents dot plots from a single person, labeled with anticaspase 3 in combination with either (i) anti-E-selectin (top row), (ii) anti-β_3 (CD61) (second row), (iii) antiglycophorin A (third row) or (iv) anti-CD14 (bottom row). All analyzed plasma samples contained MP that stained for caspase 3. Most of these vesicles were derived from platelets (β_3) and to a lesser extent from erythrocytes (glycophorin A). Plasma from two SLE patients contained a population of E-selectin-exposing (E)MP that strongly double-stained for caspase 3. Plasma from these two patients also contained MP from monocytes that also double-stained for caspase 3 (CD14).

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caspase 3. Evidently, MP formation is not linked to full-blown apoptosis, i.e. nuclear fragmentation, cell death and disintegration. Nevertheless, the present findings are based only on a limited number of *in vitro* and *ex vivo* experiments. Also, compared with age- and sex-matched healthy controls, only plasma from two of the three SLE patients studied contained significantly different (sub)populations of MP. Therefore, additional studies will be required to substantiate our present findings.

Platelets are known to contain procaspase 3 and other proteins involved in the apoptosis process [35]. The occurrence of caspase 3 in MP from platelets may suggest that this enzyme is somehow involved in the process of membrane vesiculation. Caspase 3 elicits other cellular functions than solely apoptosis, such as maintaining the cellular morphology [36]. Thus, the presence of caspase 3 in these MP may not be necessarily linked to apoptosis at all, but simply coincide with caspase 3 activation without ongoing apoptosis.

Hamilton *et al.* demonstrated that endothelial cells are protected from complement-induced lysis by shedding EMP containing the C5b-9 complex [37]. In other words, the release of EMP protected the cells against (extracellular) stress. It has been reported that (cultured) cells also undergo constitutive apoptosis [38,39]. If so, one may hypothesize that vesiculation protects cells not only against extracellular stress but also against intracellular stress, e.g. by releasing caspase 3-containing EMP. Thus, at present we cannot exclude that a fraction of the caspase 3-containing EMP originates from adherent endothelial cells. Alternatively, a careful examination of the dot plots presented in Fig. 5 (upper left in panels Fig. 5D–F) suggests that a small fraction of EMP is also present that did not contain caspase 3. Such EMP may have originated from adherent cells. Such a small fraction, however, was absent in E-selectin-exposing MP *in vivo*.

Clancy *et al.* demonstrated elevated levels of activated circulating endothelial cells in SLE patients [40]. They suggested that these levels may represent a marker of endothelial injury. In the light of their findings, our *in vivo* observation of caspase 3 in E-selectin-exposing MP suggests that most of such vesicles may have originated from circulating, apoptotic endothelial cells which had also become activated as evidenced by the exposure of E-selectin on the EMP.

The present findings show that MP may contain caspase 3. Whether or not these cell-derived MP subsequently transfer caspase 3 to other cells and thus contribute to the induction of endothelial cell dysfunction (as recently shown for MP from cultured endothelial cells [41], women with preeclampsia [42], patients with myocardial infarction [43], or T lymphocytes [44]), remains to be demonstrated.

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