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Inhibition of microparticle release triggers endothelial cell apoptosis and detachment

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Summary
Endothelial cell cultures contain caspase 3-containing microparticles (EMP), which are reported to form during or after cell detachment. We hypothesize that also adherent endothelial cells release EMP, thus protecting these cells from caspase 3 accumulation, detachment and apoptosis. Human umbilical vein endothelial cells (HUVEC) were incubated with and without inhibitors of microparticle release (Y-27632, calpeptin), both in the absence or presence of additional "external stress", i.e. the apoptotic agent staurosporin (200 nM) or the activating cytokine interleukin (IL)-1α (5 ng/ml). Control cultures contained mainly viable adherent cells and minor fractions of apoptotic detached cells and microparticles in the *absence* of inhibitors. In the *presence* of inhibitors, caspase 3 accumulated in adherent cells and detachment tended to increase. During incubation with either staurosporin or IL-1α in the *absence* of inhibitors of microparticle release, adherent cells remained viable, and detachment and EMP release increased slightly. In the *presence* of inhibitors, dramatic changes occurred in staurosporin-treated cultures. Caspase 3 accumulated in adherent cells and >90% of the cells detached within 48 hours. In IL-1α-treated cultures no accumulation of caspase 3 was observed in adherent cells, although detachment increased. Scanning electron microscopy studies confirmed the presence of EMP on both adherent and detached cells. Prolonged culture of detached cells indicated a rapid EMP formation as well as some EMP formation at longer culture periods. Inhibition of EMP release causes accumulation of caspase 3 and promotes cell detachment, although the extent depends on the kind of "external stress". Thus, the release of caspase 3-containing microparticles may contribute to endothelial cell survival.

Keywords
Apoptosis, microparticles, endothelial cells

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Introduction

Like other eukaryotic cells, endothelial cells release microparticles (MP; EMP: endothelial microparticles) *in vitro* (1–3) and *in vivo* (4–7). To which extent EMP originate from adherent or from detached endothelial cells, however, is a still unanswered question. Previously, we reported a correlation between the numbers of detached cells and EMP *in vitro* (8). Other investigators provided indications that EMP are released from adherent endothelial cells during detachment, and that endothelial cells "rapidly lost adhesion" immediately after release of EMP (9, 10). Thus, EMP are presumed to originate from detaching and detached endothelial cells. However, Hamilton et al. showed that endothelial cells escape from complement-induced lysis by releasing C5b-9-enriched EMP (11), suggesting that EMP release may contribute to survival by eliminating *externally* imposed stress.

Recently, we demonstrated that EMP from endothelial cell cultures contain substantial quantities of active (17 kDa) caspase 3 (8). These data prompted us to hypothesize that adherent endothelial cells may also release caspase 3-containing EMP, and thus escape from *internally* imposed stress, detachment and apoptosis. If true, then inhibition of EMP release is expected to result in intracellular accumulation of caspase 3 in adherent cells, with increased cell detachment and apoptosis. To test this hypothesis, we treated endothelial cells with a sub-lethal concentration of the apoptotic agent staurosporin or the activator interleukin-1α (IL-1α), without or with widely used inhibitors of microparticle release, i.e. Y-27632 and calpeptin (9, 12, 13).

Materials and methods

Reagents and assays

Medium M199, penicillin, streptomycin and L-glutamin were from GibcoBRL (Life Technologies, Paisley, UK). Human

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serum and fetal calf serum (both heat inactivated during 30 minutes [min] at 56ºC; HuSi and FCSi, respectively) were from BioWhittaker (Walkersville, MD, USA). Human serum albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Recombinant human interleukin (IL)-1α was from Sigma (St. Louis, MO, USA). Human recombinant basic fibroblast growth factor and epidermal growth factor were from Invitrogen life technologies (Carlsbad, CA, USA). Collagenase (type 1A) and staurosporin were from Sigma (St. Louis, MO, USA). Heparin (400 U/ml) was obtained from Leo Pharma BV (Breda, The Netherlands), trypsin from Difco Laboratories (Detroit, MI, USA), calpeptin from Calbiochem (La Jolla, CA, USA), and Y-27632 from Tocris (Ellisville, MO, USA). Y-27632 is a specific inhibitor of Rho-associated serine/threonine kinases I and II [i.e. ROCK I (p160ROCK, ROKβ) and ROCK II (Rho-kinase, ROKα)], enzymes which are directly involved in the release of apoptotic blebs (9, 12). Calpeptin inhibits calpain, a Ca^{2+}-dependent protease, that plays a role in (E)MP formation (13). For Western blot analysis, anti-human caspase 3 monoclonal antibody from Alexis Biochemicals (San Diego, CA, USA) and polyclonal goat-anti-mouse HRP conjugate (DAKO; Glostrup, Denmark) were used. Tissue culture flasks were from Greiner Labortechnik (Frickenhausen, Germany) and gelatin was from Difco Laboratories (Sparks, MD, USA).

Isolation, culture and treatment of human umbilical vein endothelial cells (HUVEC)

HUVEC were collected as described previously (3). Upon confluency at passage 3 in 25 cm^2 culture flasks, HUVEC were kept for two days in a resting state. Culture supernatant was refreshed and cultures were treated without or with staurosporin (200 nM, a sub-lethal concentration in the culture conditions used), or IL-1α (5 ng/ml, a concentration providing extensive endothelial cell activation such as cell surface exposure of E-selectin). Where indicated, cultures were co-incubated with Y-27632 (30 μM; two hours preincubation) and/or calpeptin (200 μM; 1 hour [h] preincubation, or, when used in combination with Y-27632, added after 1 h of incubation with Y-27632). Stock solutions of staurosporin, IL-1α , Y-27632 and calpeptin were prepared in ethanol, medium M199, PBS and DMSO (dimethylsulfoxide), respectively. Control cultures were incubated with DMSO and ethanol.

In three experiments, we studied whether detached cells release EMP. Detached cells were harvested from 10 ml culture medium from HUVEC cultures treated without or with staurosporin or IL-1α (24 h). Detached cells were resuspended in 10 ml fresh culture medium (without staurosporin or IL-1α), and numbers of detached endothelial cells and EMP were determined at fixed time intervals (3–48 h) by flow cytometry.

Flow cytometric analysis of HUVEC

Conditioned media were collected and centrifuged (10 min, 180 *g* and 20°C) to isolate detached cells. Pellets were resuspended in PBS containing 1% (v/v) FCSi (pH 7.4). Adherent cells were detached by trypsinization. After 4 min, trypsin was neutralized by PBS/FCSi (10% v/v). Both cell suspensions were centrifuged (10 min, 180 *g* and 4°C) and pellets were resuspended in PBS/FCSi (1% v/v), and then again centrifuged (10 min, 180 *g* and 4°C). Detached cells were resuspended in 0.5 ml PBS/FCSi (1% v/v) and adherent cells in 1.0 ml PBS/FCSi (1% v/v). Cells were labeled with annexin V-FITC (IQP; Groningen, The Netherlands) and propidium iodide (PI; a gift from Dr. E. Reits, Department of Cell Biology and Histology, AMC, The Netherlands) as described previously (8). Intracellular caspase 3 was detected using the Active Caspase-3 mAb Apoptosis Kit I from BD Pharmingen (San Diego, CA, USA). Samples were analyzed in a FACSCalibur flowcytometer (Becton Dickenson, San Jose, CA, USA). The cell number was estimated per culture flask using flow cytometry.

Isolation of EMP

Aliquots (1 ml) of the cell-free culture supernatants were snap-frozen in liquid nitrogen and stored at – 80°C. Before use, samples were thawed on melting ice for 1.5 h, and then centrifuged (1 h, 17,570 *g* and 20°C). Then, 975 μl of supernatant was removed and the pellet was resuspended in 225 μl PBS (154 mM NaCl, 1.4 mM phosphate) containing 10.9 mM trisodium citrate, or in perm/wash (0.1% v/v) for intravesicular caspase 3 staining. MP were resuspended and again centrifuged (30 min, 17,570 *g* and 20°C), 225 μl supernatant was removed and MP were diluted and resuspended by adding 75 μl PBS/citrate or perm/wash (0.1% v/v).

Flow cytometric analysis of EMP

EMP were analyzed in a FACSCalibur flow cytometer as described previously (3). To detect intravesicular caspase 3, MP (5 μl) were diluted with 35 μl 0.1% perm/wash solution containing 2.5 mM $CaCl_2$ plus either anti-caspase 3-FITC (BD) or control antibody, Ig-FITC (IQP, Groningen, The Netherlands). For annexin V staining, MP (5 μl) were diluted with 35 μl PBS containing 2.5 mM $CaCl_2$ (pH 7.4). Annexin V-APC (Caltag Laboratories, Carlsbad, CA, USA; 5 μl 20-fold prediluted) was added. To remove the excess of unbound annexin V, 200 μl PBS/calcium buffer (or 200 μl 0.1% perm/wash containing $CaCl_2$ for intravesicular staining of caspase 3) 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 EMP were resuspended with 300 μl PBS/calcium or 300 μl 0.1% perm/wash containing $CaCl_2$. Previously, we demonstrated that numbers of EMP (N) and detached cells highly correlate (8). Therefore, the efficacy of inhibitors to inhibit EMP release was expressed either as ratio per detached cell, i.e. $N_{EMP}/N_{detached\ cell}$, or, where indicated, as percentage from the control of that particular condition, i.e. untreated, or staurosporin or IL-1α without inhibitors: $([(N_{EMP,\ control}/N_{detached\ cells,\ control}) - (N_{EMP,\ inhibitor}/N_{detached\ cells,\ inhibitor})] / (N_{EMP,\ control}/N_{detached\ cells,\ control})) \times 100\%$.

Western blotting

Detached and adherent endothelial cells were separately isolated, washed and collected in PBS/FCSi (0.5 and 1.0 ml, respectively). From these suspensions, 300 μl (detached cells) and 800 μl (adherent cells) were used to isolate cells. Subsequently, two-fold concentrated reducing sample buffer was used to dissolve the pellets of the detached cells (final volume 20 μl) and adherent cells (final volume 40 μl). From the detached cell lysate, 10 μl was applied to SDS-PAGE, and from the adherent cell

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lysates, volumes were adjusted to 5 x 10^4 cells per lane. After removal of detached cells, EMP were isolated from the cell-free culture supernatants by centrifugation (1 h, 17,570 g and 20°C) and resuspended in 10 µl phosphate-buffered saline (PBS) plus 10 µl two-fold concentrated reducing sample buffer. Per EMP sample, 10 µl was applied to SDS-PAGE. Prior to electrophoresis, all samples were preheated (5 min at 100°C). Electrophoresis was carried out in 8–16% gradient SDS-PAGE gels (BioRad, Hercules, CA, USA). Proteins were transferred to PVDF membrane (BioRad). Blots were incubated for 1 h at room temperature with blocking buffer [Tris-buffered saline-Tween (TBST); 10 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween-20; pH 7.4), containing 5% (w/v) dry milk powder (Protifar; Nutricia, Vienna, Austria)]. The blots were incubated with monoclonal anti-human caspase 3 (1:1,000; v/v) overnight at 4°C, followed by incubation with polyclonal goat-anti-mouse HRP conjugate (1:30,000; v/v) for 1 h at room temperature. Between incubation steps, blots were washed three times with TBST for 5–10 min. All antibodies were diluted with 2.5% (w/v) blocking buffer. The bands were visualized on Fuji Medical X-ray film by using Lumi-Light Plus Western Blotting Substrate (Roche, Mannheim, Germany).

Previously we showed that detached cell lysates from control and IL-1α-treated cultures contain 17 kDa caspase 3 (8). The absence of detectable amounts of caspase 3 in detached cell lysates by Western blot in our present experiments should be interpreted as "below detection level" rather than being completely absent, since lesser numbers of detached cells were available due to the necessity of downscaling of the culture conditions compared to our previous studies as a consequence of the number of experimental conditions to be tested simultaneously.

Scanning electron microscopy (SEM)

HUVEC (third passage) were cultured on gelatin-coated coverslips. At 90% confluence, cells were incubated overnight without or with staurosporin (200 nM) or IL-1α (5 ng/ml). Specimens were prepared essentially as described by van Berkel et al. (14) Briefly, cells were fixed in McDowell's fixative for 45 min, washed (phosphate buffer), dehydrated and dried with hexamethyldisilazane. Detached cells were captured on poly-L-lysin-coated coverslips (30 min) and then treated as described above. Dried coverslips were mounted on stubs and coated with 10 nm gold, and imaged with a Philips SEM 525.

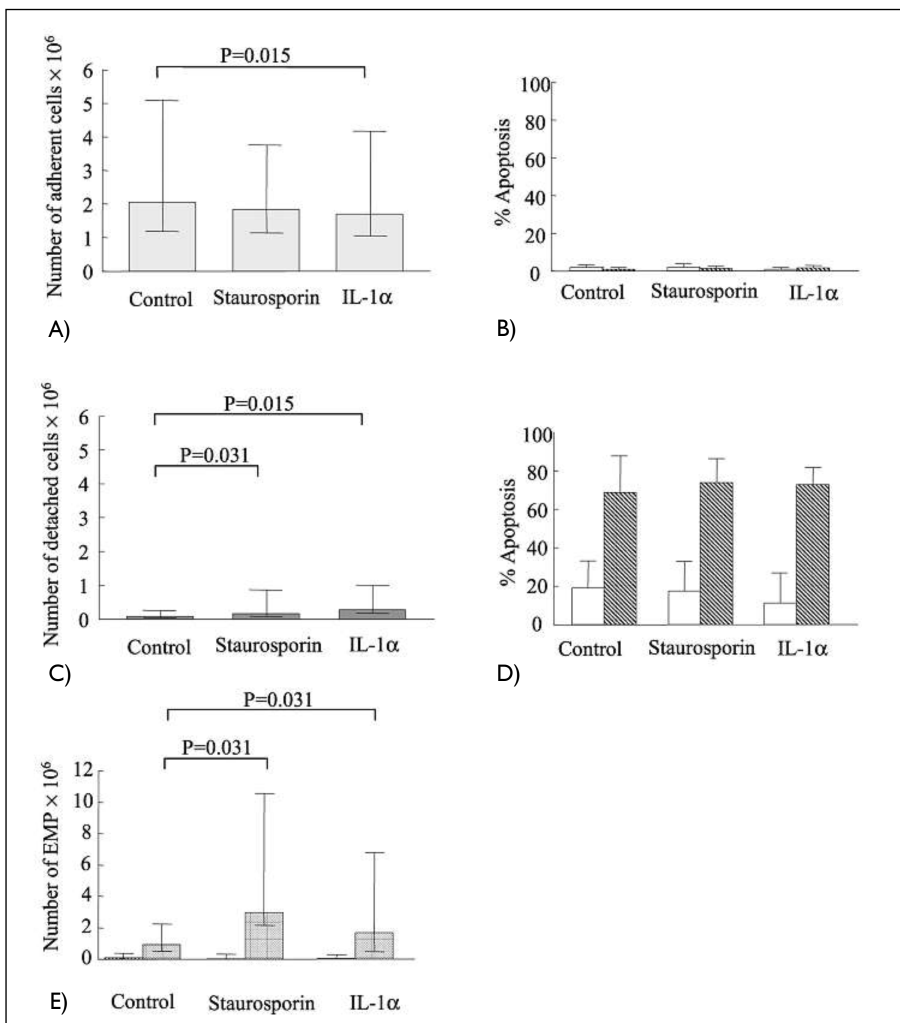

Figure 1: Basal conditions: endothelial cell cultures in the *absence* of inhibitors of microparticle release. Endothelial cells were incubated without (control) or with staurosporin (200 nM) or IL-1α (5 ng/ml) for 24 hours. Adherent cells (A, B), detached cells (C, D) and EMP (E) were isolated and analyzed as described in *Methods*, and their numbers were estimated by flow cytometry. Bars indicate median (range). Adherent cells (B) and detached cells (D) were analyzed for their apoptotic status by annexin V binding (open bars; 'early apoptosis') or by staining for both annexin V and PI (dashed bars; 'late apoptosis'). Bars indicate mean and SD (n=3). In E, both annexin V-positive (dotted bars) and annexin V-negative EMP (lower bars) are shown (n=6).

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Table 1: Effect of inhibitors of micro partical release and detachment and release at 24 hours. Endothelial cells were incubated in the absence (control) or presence of staurosporin (200 nM) or IL-1α (5 ng/ml), with or without Y-27632, calpeptin or both (n=3). After 24 hours, detached cells and EMP were isolated as described in *Materials and methods*. Detachment is expressed as % of the total number of cells, i.e. the number of adherent and detached cells present. Differences between conditions with and without inhibitor(s) were analyzed by paired t-test as described in the *Statistical analysis* section of *Materials and methods*. Data are presented as mean ± SD.

	Control	P	Staurosporin	P	IL-1α	P
Number of EMP x 10^6						
No inhibitors	1.1 ± 0.2	-	4.5 ± 2.7	-	3.1 ± 1.7	-
Y27632	1.4 ± 0.4	P>0.05	2.8 ± 0.7	P>0.05	2.5 ± 1.2	P>0.05
Calpeptin	1.4 ± 0.6	P>0.05	3.6 ± 0.4	P>0.05	2.4 ± 1.8	P>0.05
Y-27632 + Calpeptin	1.4 ± 0.5	P>0.05	5.1 ± 0.8	P>0.05	2.6 ± 1.5	P>0.05
Detached cells (% from total)						
No inhibitors	5.7 ± 2.2		15.4 ± 10.1		19.5 ± 13.6	
Y27632	15.7 ± 1.3	P<0.05	29.1 ± 4.6	P>0.05	37.7 ± 8.2	P>0.05
Calpeptin	17.7 ± 8.2	P<0.05	71.2 ± 8.0	P<0.001	43.6 ± 16.6	P>0.05
Y-27632 + Calpeptin	26.9 ± 2.1	P<0.001	81.4 ± 7.2	P<0.001	56.8 ± 10.3	P<0.05

Statistical analysis

All data were analyzed with GraphPad Prism for Windows, release 3.02 (San Diego, CA, USA). Data from preliminary experiments regarding differences in numbers of adherent cells, detached cells or EMP between control, staurosporin and IL-1α conditions were analyzed by Wilcoxon matched pairs test (one-tailed analysis). Values are expressed as median (range). Data regarding annexin V/PI labeling, i.e. the extent of apoptosis, of adherent cells and detached cells were analyzed by paired t-test (one-tailed analysis). Differences in the percentages of adherent cells or detached cells upon incubation with inhibitors in the absence or presence of staurosporin or IL-1α were analyzed with one-way analysis of variance (ANOVA). The method of Dunnett or Bonferroni was used to correct for multiple comparisons. Correlations were determined using Pearson's correlation test (two-tailed analysis). For the time dependent experiments (0–48 h), the areas under curve per condition (control, staurosporin or IL-1α) were calculated in the absence or presence of inhibitors of EMP release, and these data were compared using paired t-test (one-tailed). Differences were considered statistically significant at $p<0.05$.

Results

Basal conditions: endothelial cell cultures in the *absence* of inhibitors of microparticle release

Figure 1 shows that in response to external stress, i.e. incubation with either staurosporin or IL-1α, numbers of detached cells (Fig. 1C) and annexin V-binding EMP (Fig. 1E) increased (n=6). Neither staurosporin nor IL-1α affected the exposure of aminophospholipids (binding of annexin V: early apoptosis, open bars) or nuclear fragmentation (PI staining and annexin V binding: late apoptosis, dashed bars) of adherent (Fig. 1B) or detached (Fig. 1D) cells. Whereas minor fractions of adherent cells stained for annexin V or PI (Fig. 1B), approximately 90% of detached cells were undergoing early or late apoptosis at this 24-h culture period (Fig. 1D). In sum, in the three conditions studied, endothelial cell cultures contain mainly viable adherent cells, low numbers of apoptotic detached cells and some EMP. Detachment and EMP release increased in response to external stress, but the apoptotic status of the adherent and detached cells was unaffected.

Effects of inhibitors of microparticle release on endothelial detachment and EMP release

The effects of two widely used inhibitors of microparticle release (Y-27632, calpeptin) on cell detachment and EMP release were tested in endothelial cell cultures in the absence (control, untreated) or presence of external stress (staurosporin, IL-1α) for 24 h (Table 1). In control cultures, the numbers of EMP were unaffected by either Y-27632, calpeptin or their combination. In contrast, detached endothelial cell fractions increased from 5.7% ± 2.2 to 26.9% ± 2.1. Also in staurosporin- or IL-1α-treated cultures, only minor effects of Y-2763 and/or calpeptin were observed on EMP release, but detachment increased dramatically from 15.1% ± 5.1 to 81.4% ± 7.2 with staurosporin and 56.8% ± 10.3 with IL-1α. When we assume that most EMP originate from detaching or detached endothelial cells (Fig. 3A), then it can be calculated from the data shown in Table 1 that in the presence of the combination of inhibitors, the ratio of EMP/detached cell decreases from 8.3 ± 0.7 to 2.9 ± 1.1 in control cultures ($p<0.05$), from 15.1 ± 5.1 to 1.9 ± 1.0 in staurosporin-treated cultures ($p<0.001$), and from 7.3 ± 0.5 to 2.0 ± 1.0 in IL-1α-treated cultures ($p<0.001$). Evidently, the inhibitors do inhibit EMP formation but not below a certain basal level.

To gain a more detailed insight into the complex relationship between EMP release, induction of apoptosis and cell detachment, we determined the time dependence of the effects of inhibitors of microparticle release both in the absence (Fig. 2) and presence of additional external stress (staurosporin or IL-1α, Figs. 3 and 4, respectively), as well as the presence of caspase 3.

Effects of inhibitors of microparticle release in endothelial cell cultures *without* additional external stress

In control cultures in the *absence* of inhibitors of EMP release (open symbols throughout Figs. 2–4), adherent cell fractions binding annexin V (Fig. 2B) or containing caspase 3 (Fig. 2C) remained constant in time, and caspase 3 was not detectable on Western blot (Fig. 2D, left). Detachment increased slightly (Fig. 2A) and from 12 h onwards > 80% of detached cells bound annexin V (Fig. 2E), but due to the low numbers of detached cells at 3 and 6 h these fractions varied considerably. Detached cell fractions staining for caspase 3 ranged between 10–30% (Fig. 2F),

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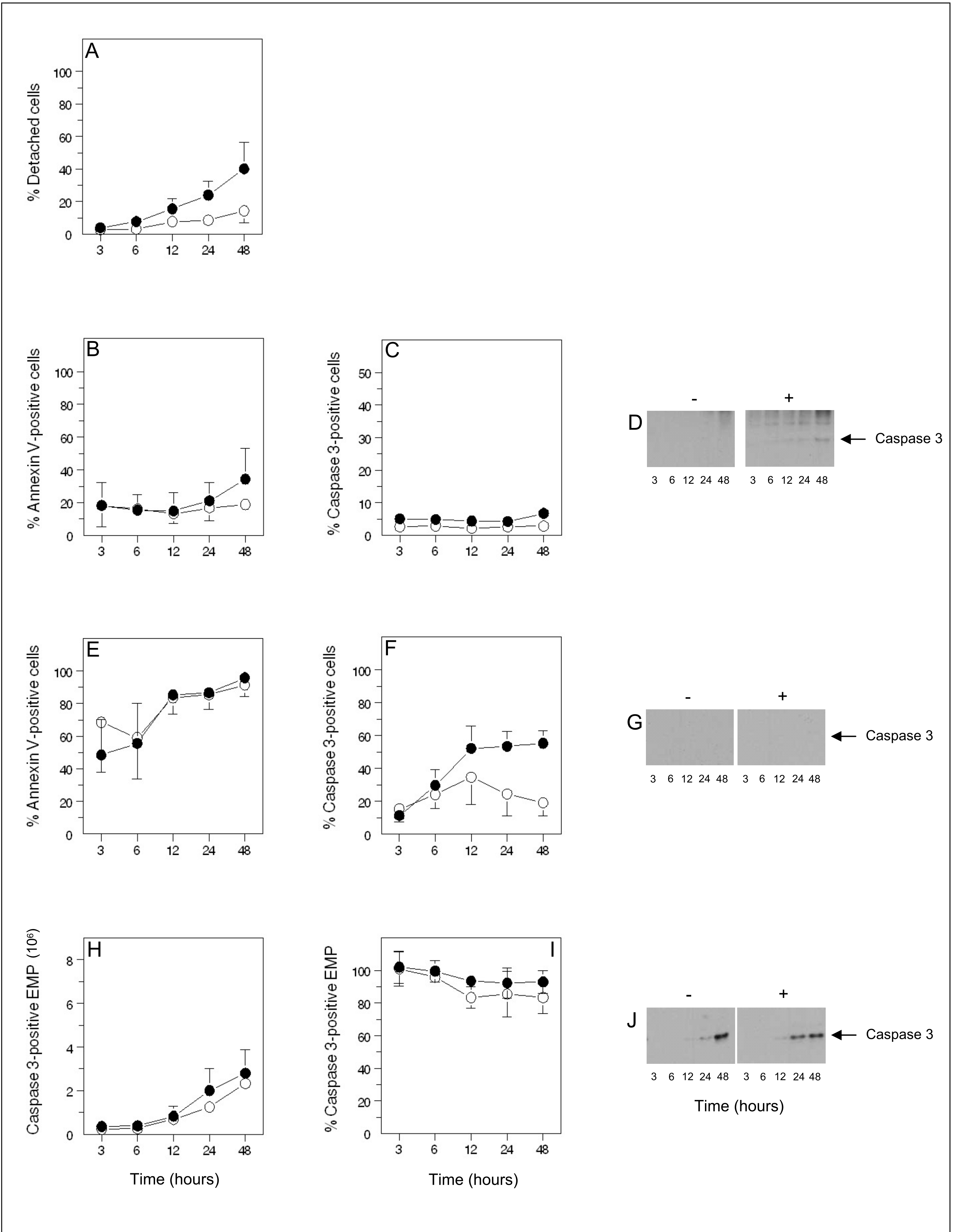

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and caspase 3 was not detectable on blot (Fig. 2G, left). Numbers of caspase 3-containing EMP increased in time (2H) and virtually all EMP contained caspase 3 (Fig. 2I). The occurrence of caspase 3 in EMP was confirmed at 24 and 48 h by Western blot (Fig. 2J, left). The annexin V findings indicate that some 50% of detached cells are not yet apoptotic in the first few hours after detachment.

In the *presence* of inhibitors (closed symbols throughout Figs. 2–4), adherent cell fractions binding annexin V (Fig. 2B) tended to increase (P=0.186). Fractions staining for caspase 3, however, increased slightly (Fig. 2C; P=0.03) and a faint (17 kDa) caspase 3 band became visible at 48 h (Fig. 2D, right panel). Detachment tended to increase (Fig. 2A; P=0.07). Similar to adherent cells, detached cell fractions binding annexin V were unaffected (Fig. 2E; P=0.377), but those containing caspase 3 increased (Fig. 2F; P=0.003). The latter could not be confirmed on blot (Fig. 2G, right), which probably is due to the low numbers of detached cells. The total EMP numbers increased similar to the cultures without inhibitor treatment (Fig. 2H; P=0.497), and fractions of caspase 3-containing EMP (Fig. 2I; P=0.096) were unaffected by the inhibition treatment. Caspase 3 in EMP was visible at both 24 and 48 h (Fig. 2J, right).

In sum, adherent endothelial cells in control cultures, i.e. without external stress, showed a modest accumulation of caspase 3 in the presence of inhibitors of microparticle release. Cell detachment tended to increase, evidence was obtained for the presence of caspase 3 in detached cells, and detached cells were not immediately apoptotic upon their detachment. Finally, the numbers of EMP were comparable in the absence and presence of these inhibitors.

Effects of inhibitors of microparticle release in endothelial cell cultures in the presence of additional external stress: staurosporin

In the absence of inhibitors of microparticle release, detached cell fractions increased compared to the cultures without external stress (Fig. 3A vs. 2A; P=0.041; open symbols). Compared to cultures without external stress (Fig. 2B and C), adherent cell fractions in staurosporin-treated cultures staining for annexin V or caspase 3 (Fig. 3B and C, respectively) were not increased (P=0.390 and p=0.199, respectively). Also on blot, caspase 3 was not detectable (Fig. 3D, left). At prolonged culture periods, detached cell fractions binding annexin V (Fig. 3E) increased (P=0.016) compared to control cultures (Fig. 2E), but caspase 3-containing fractions were unaffected (Fig. 3F vs. 2F; P=0.461). Depending on the experiment, in some lysates a weak caspase 3 band was visible at 12 h (Fig. 3G, left). The numbers of caspase 3-containing EMP increased compared to untreated cultures (Fig. 3H vs. 2H; P=0.017), and virtually all EMP contained caspase 3 (Fig. 3I). On blot, already from 12 h onwards, caspase 3 was clearly visible in EMP lysates (Fig. 3J, left), which evidently is earlier than in control cultures (Fig. 3J, left).

In the presence of inhibitors, more than 90% of endothelial cells detached within 48 h (Fig. 3A; P=0.01, compared to staurosporin alone; closed symbols). After 48 h, >80% of the few remaining adherent cells stained for annexin V (Fig. 3B; P=0.02 vs. staurosporin alone), whereas 20% contained caspase 3 (Fig. 3C; P=0.04). Accumulation of caspase 3 in adherent cell fractions was confirmed on blot (Fig. 3D, right). The absence of caspase 3 on Western blot at 48 h is most likely explained by the insufficient numbers of adherent cells due to the extensive cell detachment. Detached cell fractions staining for annexin V were unaffected (Fig. 3E; P=0.241), but the fractions of caspase 3-containing detached cells strongly increased (Fig. 3F; P<0.001). The latter was confirmed by Western blot (Fig. 3G, right). The absolute numbers of caspase 3-containing EMP increased slightly (Fig. 3H; P=0.02), and again virtually all EMP contained caspase 3 (Fig. 3I). Again, the presence of caspase 3 in EMP could be confirmed on Western blot (Fig. 3J, right).

Thus, exposure of endothelial cell cultures to mild external stress, i.e. a low concentration of the apoptosis-inducer staurosporin, triggered accumulation of caspase 3 in adherent cells and massive detachment in the presence of inhibitors of microparticle release. Also, the inhibitors of EMP release caused caspase 3 accumulation in detached cells.

Effects of inhibitors of microparticle release in endothelial cell cultures in the presence of additional external stress: IL-1α

The IL-1α-induced increase in detachment was comparable to control cultures in the *absence* of inhibitors of microparticle release (Fig. 4A vs. 2A; P=0.146; open symbols). Compared to the control cultures, i.e. the endothelial cell cultures without external stress, fractions of annexin V-binding adherent cells were lower (Fig. 4B vs. 2B; P=0.03) and those of caspase 3-containing adherent cells were comparable (Fig. 4C vs. 2C; P=0.145). On blot no caspase 3 was detectable (Fig. 4D, left). Detached cell fractions staining for annexin V or caspase 3 were both comparable to untreated cultures (Fig. 4E vs. 2E and 4F vs. 2F, respectively; P=0.359 and P=0.448, respectively). No caspase 3 was detectable in detached cell lysates (Fig. 4G, left). EMP release was comparable to untreated cultures (Fig. 4H vs. 2H; P=0.407), and again most if not all EMP contained caspase 3 (Fig. 4I). Faint caspase 3 bands were visible after 24 and 48 h on Western blot (Fig. 4J, left).

In the *presence* of inhibitors of microparticle release, detachment increased (Fig. 4A vs. 2A; P=0.02). Fractions of adherent cells staining for annexin V increased (Fig. 4B; P=0.04), but those staining for caspase 3 remained low and were unchanged compared to IL-1α alone (Fig. 4C; P=0.115). On Western blot, a faint caspase 3 band became visible in some lysates (Fig. 4D, right panel). Detached cell fractions staining for annexin V were

Figure 2: Effects of inhibitors of microparticle release in endothelial cell cultures *without* external stress. Endothelial cell cultures (n=3) were incubated *without* external stress up to 48 hours, in the absence (open symbols) or presence (closed symbols) of Y-27632 (30 μM) plus calpeptin (200 μM). A) Fractions of detached cells. B and E) show adherent (B) and detached (E) endothelial cell fractions binding annexin V as an indicator of apoptosis. C and F) show adherent (C) and detached (F) endothelial cell fractions staining for intracellular caspase 3. whereas D and G show the 'total amounts' of 17 kDa caspase 3 detectable in lysates of adherent- (D) and detached (G) endothelial cells in the absence (-) or presence (+) of Y-27632 plus calpeptin. H-J show the absolute numbers of caspase 3-containing EMP (H), the fractions of caspase 3-containing EMP (I) and Western blots of EMP lysates of 17 kDa caspase 3 (J).

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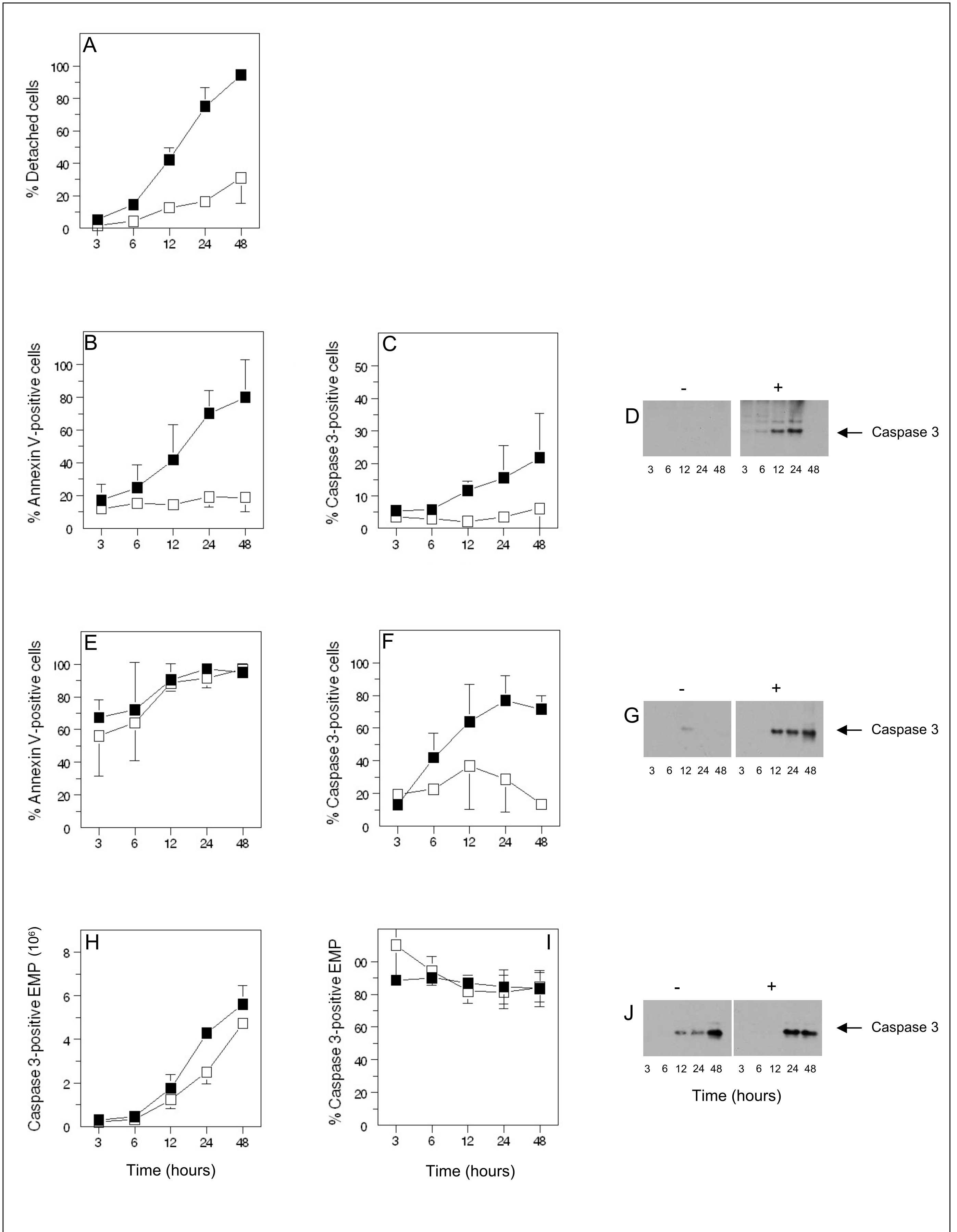

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unaffected (Fig. 4E; P=0.157). Although caspase 3-containing detached cell fractions increased (Fig. 4F; P=0.02), no or hardly any caspase 3 was detectable on blot (Fig. 4G, right). The numbers of caspase 3-containing EMP increased insignificantly (Fig. 4H; P=0.139). Again, most EMP contained caspase 3 (Fig. 4I). The presence of caspase 3 was confirmed by blot at 24 and 48 h (Fig. 4J, right).

Taken together, the overall responses induced by IL-1α, both in the presence and absence of inhibitors, closely paralleled the changes occurring in control cultures in time (Fig. 2), with the exception of some increased cell detachment.

The origin of EMP, attached or detached cells?

To further study the complex relationship between adherent cells, cell detachment and release of EMP, we performed SEM on adherent and detached endothelial cells in the three conditions studied (Fig. 5). Adherent cells showed confluent, apparently healthy monolayers in the three conditions studied (not shown). At higher magnifications, however, some differences became apparent. Although adherent cells from control cultures showed an intact monolayer (Fig. 5A), some (adherent) cells showed extensive blebbing (Fig. 5E). In IL-1α-treated cultures, a few adherent cells showed signs of retraction and showed extensive blebbing (Fig. 5B). Futhermore, some adherent cells were undergoing detachment (Fig. 5F). In staurosporin-treated cultures, several adherent cells showed a very different, 'spongy', morphology and again rather 'local' blebbing (Fig. 5C). Detached cells (Fig. 5D and H) showed the 'spongy' appearance. No apparent differences were present between detached cells in the three conditions studied (data not shown). Some but not all detached cells showed extensive blebbing (Fig. 5H).

The SEM findings indicate EMP formation to occur on the surface of adherent as well as detached endothelial cells in the three conditions studied. When the data on the number of EMP and the extent of cell detachment were combined (Fig. 6A), a very strong correlation was observed (r=0.825, P<0.0001), suggesting that the majority of EMP originates from detaching and/or detached cells. To further investigate this issue, we cultured isolated detached cells from control, IL-1α- or staurosporin-treated cultures, and determined EMP formation in time (Fig. 6B). In all three conditions, 50% of the EMP had already been formed within 3 h, and after 24–48 h EMP formation reached a plateau.

Figure 3: Effects of inhibitors of microparticle release in endothelial cell cultures in the presence of external stress: staurosporin. Endothelial cell cultures (n=3) were incubated *with* additional external stress (staurosporin) up to 48 hours, in the absence (open symbols) or presence (closed symbols) of Y-27632 (30 μM) plus calpeptin (200 μM). A) shows the fractions of detached cells. Figures 3B and 3E show adherent (3B) and detached (3E) endothelial cell fractions binding annexin V. C and F) show adherent (C) and detached (F) endothelial cell fractions staining for intracellular caspase 3. D and G) show the 'total amounts' of 17 kDa caspase 3 detectable in lysates of adherent- (D) and detached (G) endothelial cells in the absence (-) or presence (+) of Y-27632 plus calpeptin. H-J) show the absolute numbers of caspase 3-containing EMP (3H), the fractions of caspase 3-containing EMP (3I) and Western blots of EMP lysates of 17 kDa caspase 3 (J).

Discussion

Our present study shows that there is a highly complex relationship between adherent endothelial cells, detached endothelial cells and EMP formation. In both control cultures and those treated with staurosporin or IL-1α, the SEM studies indicated low numbers of adherent as well as detached cells to be invariably present with extensive signs of blebbing. The correlation we observed between the numbers of EMP and detached cells indicates the close relation between these two processes. The culture of detached cells indicates that most EMP are formed from detached cells immediately upon or within a few hours after detachment, with more EMP formation after prolonged detachment. These data fit with the observation that a substantial fraction of the detached cells only becomes apoptotic (annexin V-positive) after 6–12 h (Figs. 2E, 3E and 4E).

In the present study, we hypothesized that if indeed some of the EMP are released directly from adherent cells to dispose of caspase 3 and thus prevent cell detachment, then inhibition of this release should lead to intracellular accumulation of caspase 3 in adherent endothelial cells. As a consequence, such an accumulation would cause an increased tendency of adherent cells to detach. All experimental data in the present study can be incorporated in the model presented in Figure 7. In the control condition (top), adherent cells (green; center) show a basal formation of some active caspase 3 (C3, red) from its inactive precursor procaspase 3 (PC3, black). Once sufficient caspase 3 is formed, caspase 3 is released into EMP, resulting in a cell solely containing procaspase 3 (adherent cell, left). Other adherent cells in which caspase 3 is formed (arrow 1), release no or insufficient EMP (adherent cell, right), resulting in accumulation of caspase 3, cell detachment and release of microparticles or apoptotic bodies either (arrow 2) during or after detachment. In the presence of Y-27632 and calpeptin (second panel), the release of EMP from adherent cells becomes disturbed, and caspase 3 accumulates in adherent cells. As a consequence the equilibrium (arrow 3) shifts towards accumulation of caspase 3, detachment and release of microparticles of apoptotic bodies during or after detachment. In cultures treated with staurosporin (third panel), the conversion of procaspase 3 into caspase 3 is promoted. Increased formation of caspase 3 in adherent cells is expected to trigger an increased release of (caspase 3-containing) EMP, but also (arrow 4) an increased detachment and formation of microparticles of apoptotic bodies during or after detachment is to be expected. In cultures treated with staurosporin, Y-27632 and calpeptin, the adherent cells become unable to release microparticles, expected to result in massive accumulation of caspase 3 in adherent cells and a very strong shift to the right, i.e. massive cell detachment and release of microparticles of apoptotic bodies during or after detachment. Confirmation of this model is provided by the finding that the inhibitors of EMP formation, e.g. from 8.3 to 2.9 EMP/detached cell in control cultures, cause accumulation of caspase 3 even in the detached cells (Figs. 2F, 3F and 4F).

Our hypothesis of cell survival by EMP formation is substantiated by two other findings. First, cells deficient in functional caspase 3 activity, including hepatocytes, thymocytes or MCF-7 cells, do not or hardly release any MP, suggesting that

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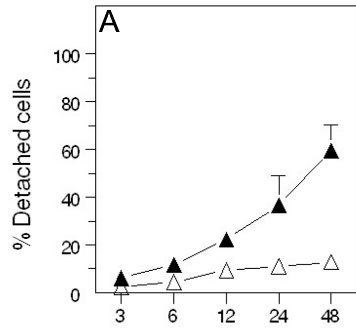

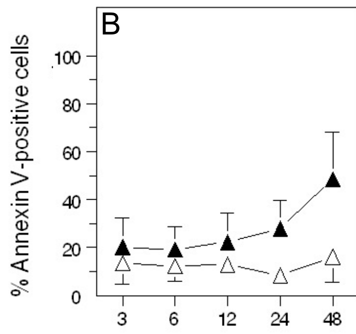

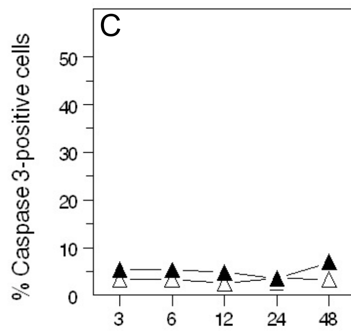

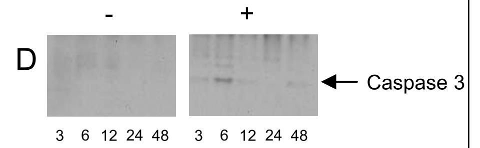

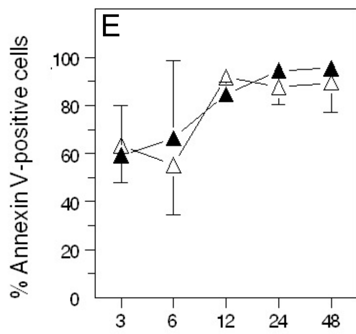

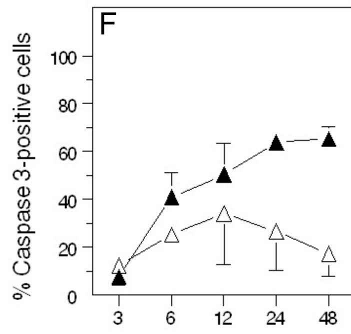

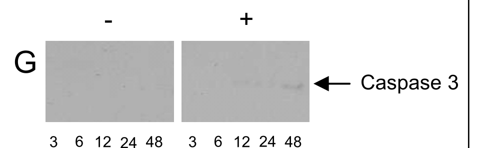

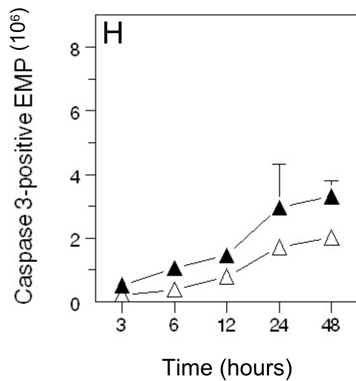

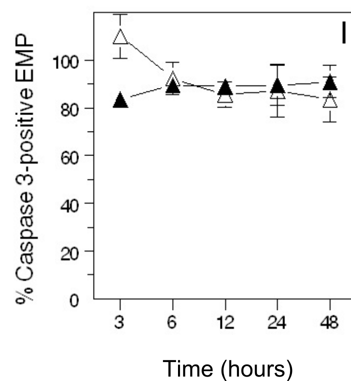

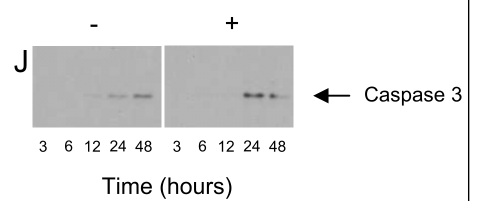

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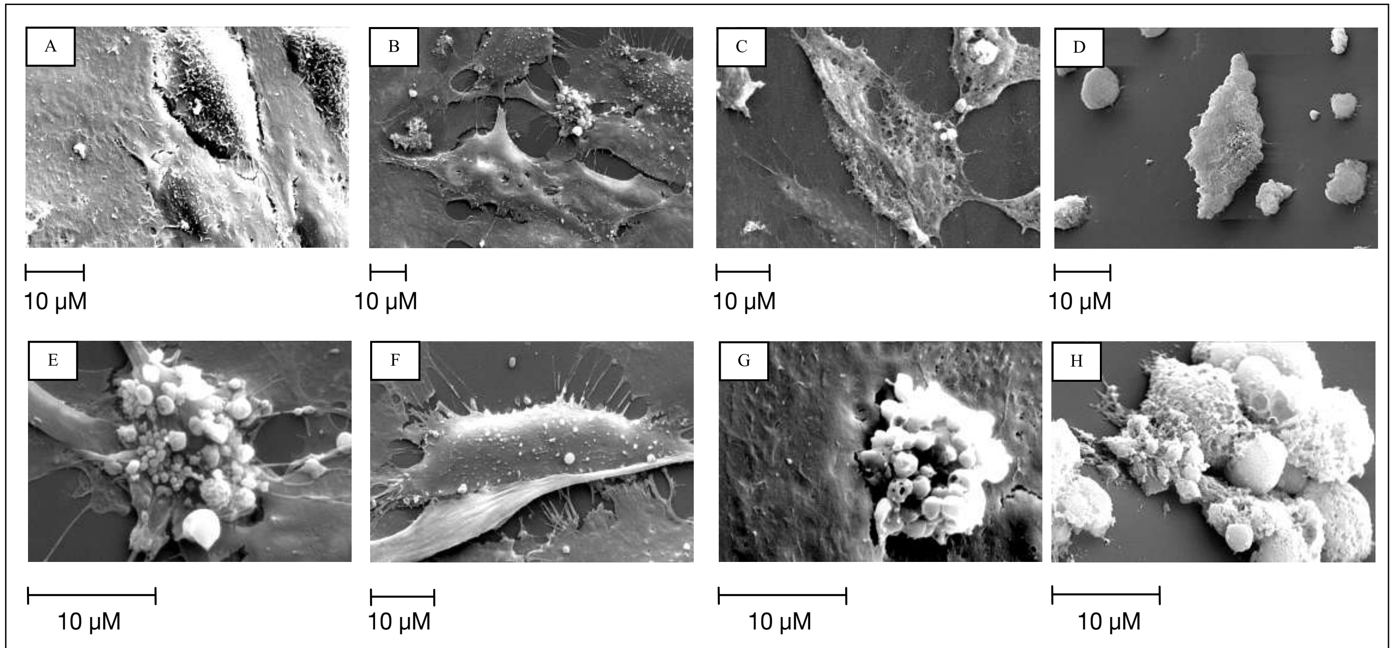

Figure 5: Scanning electron microscopy (SEM) of adherent and detached endothelial cells. SEM was performed as described in *Materials and methods* of adherent endothelial cells from control cultures (A, E), and IL-1α-treated (B, F) or staurosporin-treated (C, G) cultures. Detached cells are shown from control cultures (D, H).

caspase 3 contributes to or facilitates its own removal via formation of MP (15, 16). Second, when we incubated endothelial cells with z-VAD, a general inhibitor of caspases, a 20 kDa inactive form of caspase 3 accumulated in adherent endothelial cells. Concurrently, EMP release became less, and 17 kDa caspase 3 could no longer be detected in EMP fractions and detachment decreased (data not shown).

Interestingly, not only MP but also exosomes from non-apoptotic cells contain a caspase 3-like activity (17). These authors postulated that packaging active caspase in exosomes may be a mechanism to ensure cell survival. Additional supportive evidence that EMP formation/shedding contributes to cellular survival comes from an earlier study showing that various cell types, including endothelial cells, escape from complement-induced lysis by releasing complement C5b-9-enriched EMP (11). Thus, two different types of cell-derived vesicles, microparticles and exosomes, both facilitate removal of potentially dangerous biomolecules, and thus act as 'garbage sacs'.

Figure 4: Effects of inhibitors of microparticle release in endothelial cell cultures in the presence of external stress: IL-1α. Endothelial cell cultures (n=3) were incubated with additional external stress (IL-1α) up to 48 hours, in the absence (open symbols) or presence (closed symbols) of Y-27632 (30 μM) plus calpeptin (200 μM) A) Fractions of detached cells. B and E) Adherent (B) and detached (E) endothelial cell fractions binding annexin V. C and F) Adherent (C) and detached (F) endothelial cell fractions staining for intracellular caspase 3. D and G) 'Total amounts' of 17 kDa caspase 3 detectable in lysates of adherent- (D) and detached (G) endothelial cells in the absence (-) or presence (+) of Y-27632 plus calpeptin. H-J) Absolute numbers of caspase 3-containing EMP (H), the fractions of caspase 3-containing EMP (I) and Western blots of EMP lysates of 17 kDa caspase 3 (J).

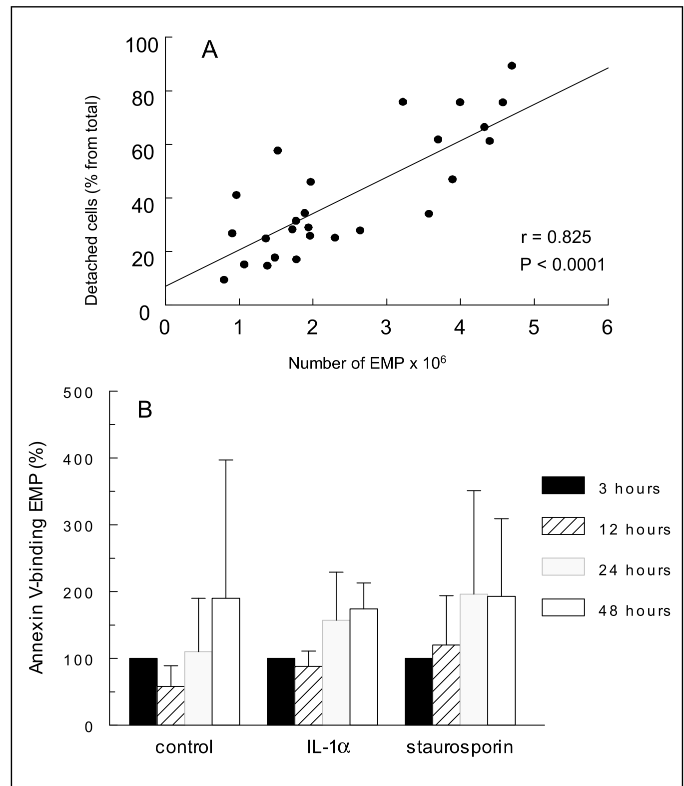

Figure 6: The origin of EMP, attached or detached cells? The numbers of EMP and % of detached cells, as presented in Table 1, show the strong correlation between EMP release and detachment (A). B) shows EMP release from detached cells isolated from control cultures and from cultures treated with either IL-1α or staurosporin (n=3 for each condition). EMP data are expressed as % of the EMP count determined at 3 hours (for each condition).

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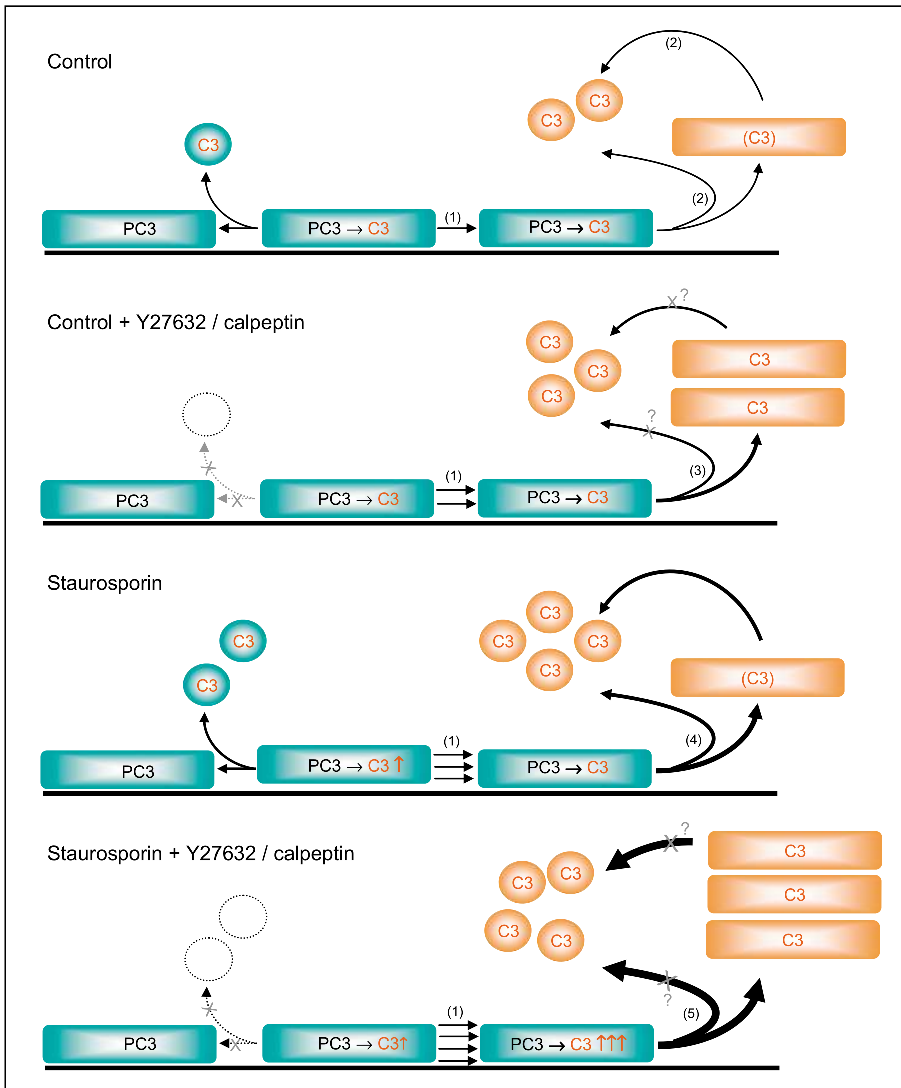

Figure 7: Schematic model of the complex relationship between adherent endothelial cells, detachment and release of EMP. This model shows the hypothesis that attached cells escape from accumulation of caspase 3, cell detachment and apoptosis by EMP formation. The model incorporates the data presented in Figs. 1–4 and Table 1. Shown are four conditions, i.e. control and staurosporin-treated cultures in the absence or presence of Y-27632 plus calpeptin. Adherent cells are green, detached cells are orange, EMP from adherent cells are green and from detached cells orange. In the presence of Y-27632 plus calpeptin, release of caspase 3-containing EMP becomes inhibited per cell, resulting in accumulation of caspase 3 in adherent cells, cell detachment and accumulation of caspase 3 in detached cells. The number of arrows (1) indicate the extent of shift towards accumulation of caspase 3 and detachment, (2) shows the release of EMP during detachment and/or from detached cells, (3), (4) and (5) represent increased detachment (depicted by the thickness of the arrows) as well as release of EMP during detachment or from detached cells.

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It should be noted that the inhibitors used in the present study, i.e. the ROCK inhibitor Y-27632 and the calpain inhibitor calpeptin, exert various effects on (endothelial) cells. For instance, O'Connell et al. showed that PIP_2 incubated in platelet membranes inhibited activation-induced microparticle formation >90%, presumably by interaction of PIP_2 with various membrane proteins. Incubation of permeabilized platelets with purified calpain reduced PIP_2 levels, whereas in the presence of calpeptin the PIP_2 levels increased (18). This indicates that calpeptin may influence PIP_2 levels by calpain independent from its calcium-dependent protease activity.

Endothelial cells can also detach by caspase-independent mechanisms, and cell death of detached cells is then a consequence of detachment (anoikis) of originally viable endothelial cells. For example, Hasmim and coworkers showed that expression of β integrin cytoplasmic domains in endothelial cells induced caspase-independent detachment that was followed by anoikis (19). Our present data also indicate that detached cells only become apoptotic some time after detachment.

Taken together, we postulate that the release of EMP is a general mechanism to enable cells to dispose potentially harmful and redundant compounds, thereby supporting cellular survival. Unfortunately, this hypothesis can not presently be tested directly, because we have no specific markers available to distinguish EMP originating from adherent cells and detaching/detached cells.

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