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Active caspase-3 is removed from cells by release of caspase-3-enriched vesicles

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ABSTRACT

Cleavage of Rho associated Coiled Coil kinase I (ROCK I) by caspase-3 contributes to membrane blebbing. Whether caspase-3 and ROCK I also play a role in the release of membrane vesicles is unknown. Therefore, we transfected a human breast cancer cell line (MCF-7) that is caspase-3 deficient, lacks membrane blebbing, and does not release membrane vesicles, with caspase-3. Cells expressing caspase-3 demonstrate both ROCK I-mediated membrane blebbing, and release of small (400–600 nm) membrane vesicles in a ROCK I-independent manner. These membrane vesicles contain caspase-3, and are enriched in caspase-3 activity compared to the releasing cells. Caspase-3-containing vesicles are taken up by untransfected cells but the cells do not show any sign of apoptosis. In conclusion, we show that the release of caspase-3-enriched membrane vesicles and membrane blebbing are two differentially regulated processes. Furthermore, we hypothesize that packaging of caspase-3 into membrane vesicles contributes to cellular homeostasis by the removal of caspase-3, and concurrently, protects the cells' environment from direct exposure to caspase-3 activity.

1. Introduction

Membrane (extracellular) vesicles (further mentioned as vesicles) are 30–1000 nm in diameter, and such vesicles are ubiquitously present in conditioned culture media and body fluids including blood, saliva, liquor cerebrospinalis, and synovial fluid. Vesicles are present in physiological and pathological circumstances, and their numbers, cellular origin, composition, and function, are disease (state) dependent. Vesicles are known for their coagulant phenotype [8,17,29–31,39,46], but they can also affect inflammation [4–7,18,23] and angiogenesis [10,22,28], and contribute to intercellular signaling [20,37,40,41].

Vesicles from human blood cells and endothelial cells contain caspase-3 [2]. Because release of caspase-3-containing vesicles seems to counteract the induction of apoptosis [15], and because inhibition of vesicle release results in the intracellular accumulation of caspase-3 and apoptosis [1], we and others hypothesize that the release of such caspase-3-containing vesicles may be a general mechanism that contributes to cellular homeostasis by counteracting cellular stress [1,14,15].

Whether membrane blebbing is a process that results in the release of vesicles, or whether membrane blebbing and vesicle release are two independent processes, is unknown. Membrane blebbing, which is a characteristic feature of cells undergoing apoptosis, can be induced by activation of Rho associated Coiled Coil kinase I (ROCK I). ROCK I is activated by either caspase-3-induced cleavage, or via Rho GTPase. Activated ROCK I induces an increase in myosin light chain phosphorylation, which in turn contributes to membrane blebbing [12,34].

Although caspase-3 induces membrane blebbing by cleavage of ROCK I [12,34], and vesicles contain caspase-3, it is unknown whether caspase-3 plays any role in vesicle release, whether vesicle release is mediated by ROCK I, and whether vesicles are enriched in caspase-3 activity compared to the cells. To address these questions, we used a human breast cancer cell line MCF-7, which lacks membrane blebbing and is deficient of caspase-3, to study the role of caspase-3 and ROCK I in the release of vesicles.

2. Material and methods

2.1. Human caspase-3 expression vectors

Twenty nine kDa caspase-3 was cloned untagged (caspase-3) or tagged with the EGFP sequence at the 3′end (caspase-3*). Twenty nine

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kDa caspase-3 is known to activate itself by autocleavage, resulting in 17 kDa and 12 kDa caspase-3 fragments [27].

For cloning details please see supplementary data.

2.2. Transfection of MCF-7 cells

MCF-7 cells were a gift from the Hubrecht Laboratory (Utrecht, The Netherlands). MCF-7 cells were selected since they lack the procaspase-3 protein due to a 125 bp deletion in exon 3 of the caspase-3 gene. This deletion leads to a frameshift starting at codon 18 which results in a stop codon at codon 41 [21]. Cells were cultured in DMEM F12 (Invitrogen, Carlsbad, CA) supplemented with 7.5% fetal calf serum (FCS; PAA, Pasching, Austria), non-essential amino acids (Invitrogen), penicillin and streptomycin (10 units/mL and 10 μg/mL, respectively; Invitrogen) for 2 days before transfection. Transfection was performed with 1 μg DNA encoding caspase-3, caspase-3* or EGFP, using Fugene-6 (3 μL; Roche, Mannheim, Germany) according to the manufacturer's instructions. In all experiments untransfected cells were used as control unless stated otherwise.

2.3. Release of vesicles and membrane blebbing from caspase-3-expressing cells by live cell imaging

To visualize the release of vesicles and membrane blebbing, live cell imaging was performed as described previously [38]. MCF-7 cells (15,000) were plated in glass-bottom dishes (Lab-Tek II chambered coverglass; Nunc, Roskilde, Denmark). Cells were incubated for 2 days at 37 °C in an atmosphere containing 5% CO_2. Thereafter, cells were transfected with the expression construct encoding the caspase-3* protein. In parallel experiments, cells were either transfected with EGFP or not transfected. Transfection with caspase-3 (without EGFP) was not performed because expression of this construct cannot be monitored.

After transfection (4 h), the conditioned culture medium was covered with mineral oil (M3516; Sigma, St. Louis, MO) to prevent evaporation of the medium. The glass-bottom dish was placed under an inverted fluorescence DM IRBE microscope (Leica Microsystems, Wetzlar, Germany), equipped with a Plan Apo 63×/1.40 oil objective and a cooled CCD camera (type 2000s; PCO AG, Kelheim, Germany). Cells were imaged for 48 h at 37 °C in an atmosphere containing 5% CO_2. Phase contrast images were acquired at time intervals of 5 min and fluorescence images at time intervals of 60 min to monitor expression of caspase-3*. Live cell images were analyzed using custom-made software. In the live cell images, vesicles were transiently visible as little black dots near the cell membrane.

2.4. Detailed visualization of the release of vesicles by live cell imaging

MCF-7 cells were cultured in glass-bottom dishes as described above, and subsequently transfected with the expression vector encoding the caspase-3* protein. After transfection for 24 h, the conditioned culture medium was covered with mineral oil and cells were imaged for 5 h. A phase contrast image was made every 5 or 20 s, depending on the experiment. Fluorescence images were made only at the start of the experiment to monitor expression of caspase-3* to circumvent the risk of apoptosis from phototoxicity. Live cell images were analyzed using custom-made software and vesicles were visible as moving little black dots.

2.5. Quantification of vesicles released from caspase-3-expressing cells

2.5.1. Isolation of vesicles from cell-conditioned culture supernatant

MCF-7 cells (45,000) were cultured in 6-well plates and transfected as described. At 48 h after transfection, culture supernatants were harvested and centrifuged for 10 min at 180 *g* to remove detached cells. Vesicles were isolated from cell-free supernatant by centrifugation at 18,890 *g*. Vesicles were washed once and used for the various assays.

2.5.2. Flow cytometry of vesicles

To estimate the number of released vesicles, the vesicles were isolated from cell-free culture supernatant (250 μL) by centrifugation (30 min at 18,890 *g*). After centrifugation, vesicle-free supernatant was removed (225 μL) and vesicles were resuspended in PBS-containing citrate 0.32% (225 μL) to avoid clumping. After washing at 18,890 *g* for 30 min, vesicle-free supernatant was removed (225 μL) and vesicles were resuspended in the remaining fluid. Thereafter, vesicles (5 μL) were added to a mixture of PBS-containing 2.5 mmol/L calcium chloride (35 μL) and allophycocyanin (APC)-labeled annexin V (5 μL; Caltag Medsystems, Buckingham, UK) and were incubated for 15 min at room temperature. Thereafter, PBS-containing calcium chloride (300 μL) was added and vesicles were counted on a FACSCalibur (Becton Dickinson, San Jose, CA) for 1 min and analyzed with CellQuest™ pro software (version 4.02; Becton Dickinson). To estimate the number of released vesicles, we measured the flow rate in μL/min for each experiment and used the following formula: numbers of vesicles/well = counted numbers annexin V-positive vesicles × (350 μL/flow rate in μL per min) × (25 μL/5 μL) × (2000 μL culture medium per well/250 μL).

2.5.3. Resistive pulse sensing

MCF-7 cells (45,000) were cultured in 6-well plates and transfected as described. At 48 h after transfection, culture supernatants were harvested and centrifuged for 10 min at 180 *g* to remove detached cells. The concentration and size distribution of vesicles were measured with resistive pulse sensing (qNano; Izon, Christchurch, New Zealand) using a NP200A nanopore and a NP400A nanopore. Samples were measured with a pressure of 7 mbar, stretch of 45.00 mm, and voltage of 0.40 V. Samples were analyzed for 10 min or until 1000 vesicles were counted. Results obtained with the NP200A pore were used for vesicles with a diameter of less than 400 nm (the NP200A pore is optimized for the detection of vesicles of 100 nm–400 nm and the NP400A pore is optimized for the detection of vesicles of 200–800 nm; personal communication with Izon). Results obtained with the NP400A pore were used for vesicles with a diameter of 400 nm to 1000 nm. The concentration of vesicles present in the culture supernatant of untransfected cells was subtracted from the concentration of vesicles present after transfection with EGFP, caspase-3 or caspase-3*.

2.6. Inhibition of vesicle release

To investigate whether the release of vesicles is ROCK I mediated, we transfected MCF-7 cells as described in the presence or absence of the ROCK I inhibitor Y27632 (30 μM in PBS, 2 h preincubation; Tocris, Ellisville, MO) and analyzed the number of generated vesicles by flow cytometry and resistive pulse sensing as described before.

2.7. Detection of intravesicular active caspase-3

For detection of intravesicular active caspase-3, vesicles were isolated from cell-free culture supernatant (250 μL) by centrifugation (30 min 18,890 *g*). After centrifugation, vesicle-free supernatant was removed (225 μL) and vesicles were washed in 0.1% permwash (225 μL; Becton Dickinson) by centrifugation at 18,890 *g* for 30 min. Then, the vesicle-free supernatant was removed and vesicles were resuspended in the remaining fluid. Vesicles (5 μL) were added to a mixture of permwash-containing 2.5 mmol/L calcium chloride (35 μL), APC-labeled annexin V (5 μL) and phycoerythrin (PE)-labeled anti-caspase-3 (recognizes only the active form of caspase-3; Becton Dickinson), and incubated for 30 min at room temperature. After incubation, permwash (300 μL)-containing 2.5 mmol/L calcium chloride was added and samples were analyzed on a FACSCalibur for 1 min.

2.8. Caspase-3 activity assay

To measure intracellular caspase-3 activity, MCF-7 cells were transfected as described, and after 48 h, cells were harvested after trypsinization and washed (10 min and 180 *g*) using PBS containing 1% FCS. Then, cells (600,000) were washed with PBS and lysed in cell lysis buffer (Calbiochem, San Diego, CA; 60 μL) for 5 min at 4 °C before 3 freeze/thaw cycles in liquid nitrogen and at 37 °C, respectively. Then, lysates were centrifuged for 10 min at 10,000 *g* and supernatants were used to measure caspase-3 activity. Caspase-3 activity was determined by using the chromogenic substrate Ac-DEVD-pNA (Calbiochem) in the presence or absence of the caspase-3 inhibitor Ac-DEVD-CHO (Calbiochem). The absorbance at 405 nm was measured during 90 min with time intervals of 5 min, as developed by Barrett and Cathepsin [3] and Du et al. [16], and as described by the manufacturer. To calculate the caspase-3 activity in the samples, a conversion factor of the plate reader was used. The conversion factor was determined by measuring the absorbance of 50 μM ρ-nitroaniline at 405 nm, and calculated as ratio of the concentration of ρ-nitroaniline and absorbance. To calculate the caspase-3 activity in samples in pmol/min, first the absorbance generated in time in the presence of Ac-DEVD-CHO was subtracted from the absorbance generated in its absence. Second, the net increase in absorbance was divided by the measuring time (90 min). Thereafter, the formula described by the manufacturer was used: net increase in absorbance/min × conversion factor × assay volume (μL). The caspase-3 activity in pmol/min was expressed per μg protein as determined by the Coomassie brilliant blue assay (Pierce, Rockford, IL).

To measure intravesicular caspase-3 activity, 2 × 1 mL cell-free culture supernatant was centrifuged for 1 h at 18,890 *g*. Vesicles were pooled and washed once in 1 mL PBS. Thereafter, 990 μL supernatant was removed and vesicles were resuspended in 50 μL cell lysis buffer followed by measurement of caspase-3 activity using the same procedure as described for cells.

2.9. Western blot

Vesicles were isolated from 2 × 1 mL cell-free culture supernatant by centrifugation at 18,890 *g* for 1 h, pooled and washed once in PBS. Then vesicles were resuspended in 25 μL reducing sample buffer. All samples were boiled for 5 min. Samples (15 μL) were loaded on an 8–16% gradient gel (Biorad, Hercules, CA) and blotted to polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). Membranes were incubated with 5% protifar (Nutricia, Vienna, Austria) in Tris-buffered saline with 0.005% Tween (Merck, Darmstadt, Germany) for 1 h to reduce non-specific staining, followed by incubation with a mouse anti-human caspase-3 antibody (clone 31A1067 recognizing 29 kDa- and 17 kDa caspase-3; Alexis, San Diego, CA) or a mouse anti-human-GFP-horseradish peroxidase-labeled antibody (Miltenyi Biotec, Bergisch Gladbach, Germany). For blots incubated with caspase-3 antibody, a secondary antibody goat anti-mouse-horseradish peroxidase (1:30,000; Dako, Glostrup, Denmark) was used. To visualize the bands, membranes were incubated with a 5-fold diluted peroxidase substrate (LumiLight; Roche Diagnostics, Almere, The Netherlands) for 5 min, followed by analysis of luminescence using a LAS3000 luminescence image analyzer (Fuji, Valhalla, NY).

2.10. Transfer of caspase-3 to MCF-7 cells by vesicles

MCF-7 cells were cultured and transfected as described, except for the removal of the transfection medium after 5 h of transfection and washing the cells once with PBS before addition of fresh culture medium to ensure that the fugene/DNA mixture was completely removed. After 42 h, the conditioned culture supernatant was harvested and detached cells were removed by centrifugation (180 *g* 10 min, 4 °C). Vesicles (5 aliquots of 1 mL) were isolated from cell-free culture supernatant by centrifugation (18,890 *g*, 1 h, 4 °C). Vesicle-free supernatant (975 μL) was removed and used as control. Vesicle pellets were resuspended in the remaining 25 μL. Vesicles of one pellet were used directly and the other vesicle-pellets (4 × 25 μL) were pooled and concentrated to 25 μL by centrifugation (18,890 *g*, 30 min, 4 °C).

Two days before addition of vesicles, MCF-7 cells were cultured in a 12-well plate. Before addition of vesicles, culture medium was replaced by fresh culture medium and thereafter, the indicated vesicle-free supernatants (25 μL), or vesicles (25 μL) were added. After 48 h, adherent cells and detached cells were harvested. Cell numbers were counted and EGFP fluorescence was measured using a FACSCalibur (Becton Dickinson). Cells were labeled with annexin V-APC and propidium iodide (Invitrogen) as described before [1]. Cells were also labeled with anti-caspase-3-PE (Becton Dickinson) as described before [1].

2.11. Statistical analysis

All data were analyzed with GraphPad Prism for Windows, release 5 (Prism, San Diego, CA). Data from all experiments were analyzed with unpaired t tests (one tailed) and values are expressed as mean ± SD.

3. Results

3.1. No release of vesicles from untransfected MCF-7 cells

MCF-7 cells are caspase-3 deficient and lack membrane blebbing. Whether this deficiency of caspase-3 impairs the release of vesicles is unknown. Therefore, we determined whether untransfected MCF-7 cells release vesicles. As shown in Fig. 1A, such cells hardly release any vesicles. Furthermore, MCF-7 cells do not show the typical morphological features of cells undergoing apoptosis, such as cell shrinkage or membrane blebbing (live cell imaging, data not shown).

3.2. Release of vesicles from caspase-3-expressing MCF-7 cells

After transfection, MCF-7 cells express both caspase-3 and caspase-3-EGFP (caspase-3*). Both forms of caspase-3 show autocleavage resulting in the characteristic 17 kDa and 12 kDa/12 kDa-EGFP fragments (data not shown). The release of vesicles increases approximately 5-fold and 3-fold, respectively, in cells expressing either caspase-3 or caspase-3* compared to control (EGFP-expressing) cells (Fig. 1A; $P = 0.005$ and $P = 0.003$, respectively).

Because flow cytometry (Fig. 1A) underestimates the concentration of vesicles and gives hardly any information about their size and size distribution [42,43], we also analyzed the vesicles using resistive pulse sensing in an independent series of experiments. Fig. 1B confirms that the release of vesicles increases when cells express either caspase-3 or caspase-3*. This increase is significant for relatively large vesicles with a diameter between 400–500 and 500–600 nm released from cells expressing caspase-3* as compared to these sizes of vesicles released from control (EGFP) cells ($P = 0.019$ and $P = 0.008$, respectively), but not significant for vesicles with a diameter between 100–200 and 200–300 nm ($P = 0.4041$ and $P = 0.0617$, respectively). Taken together, the release of relatively large membrane vesicles by MCF-7 cells increases when the cells express caspase-3.

To visualize the release of the vesicles, live cell imaging experiments were performed with MCF-7 cells expressing caspase-3*. In the first experiments, we made phase contrast images every 5 min. In this film, the released vesicles are visible as little black dots (Suppl. film). To obtain a more detailed insight into the release of vesicles, we also performed experiments where images were made every 5 or 20 s of cells expressing caspase-3*. Fig. 1C–F and G–I show the release of single vesicles from 2 different cells. In Fig. 1C–F, a phase contrast image was made every 20 s, showing the formation of a vesicle within a membrane ruffle (C), the vesicle just before release (D), the release of the vesicle (E) and the released vesicle (F). In Fig. 1G–I,

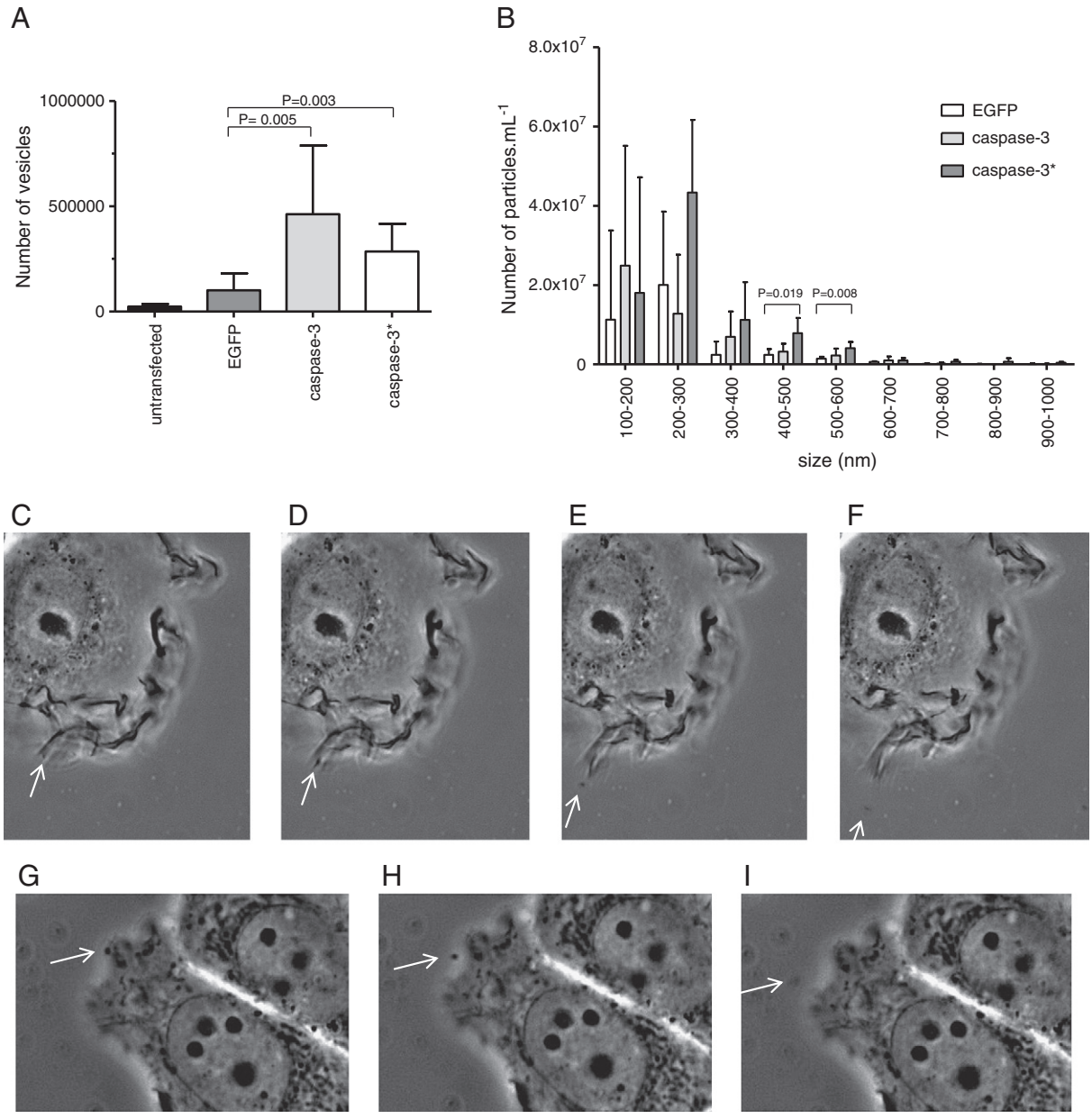

Fig. 1. Release of vesicles from MCF-7 cells expressing caspase-3. A. The number of vesicles released from caspase-3-expressing- and caspase-3-EGFP (caspase-3*)-expressing MCF-7 cells (n = 5 and n = 6, respectively) was determined by annexin V fluorescence using flow cytometry. Untransfected (n = 4) and EGFP-transfected cells (n = 8) were used as controls. B. The concentration of particles released from MCF-7 cells expressing caspase-3 or caspase-3* (n = 4) was determined by resistive pulse sensing. Untransfected (n = 4) and EGFP-transfected cells (n = 4) were used as controls. C–I. Vesicle release (arrows) from caspase-3*-expressing cells (C–F and G–I) was visualized and studied with live cell imaging. A phase contrast image was made every 20 s (C–F) or every 5 s (G–I). C. A vesicle is formed in a membrane ruffle. D. The vesicle is almost released from the membrane. E. Release of the vesicle. F. The released vesicle has moved out of focus. G. A vesicle is formed at the cell membrane. H. Release of the vesicle. I. The vesicle is released.

a phase contrast image was made every 5 s, showing a vesicle at the cell membrane (G), the release of the vesicle (H) and the released vesicle (I). These live cell images also show that cells releasing the vesicles do not yet show the characteristic morphological features of apoptosis, including cell shrinkage and membrane blebbing.

3.3. No involvement of ROCK I in vesicle release

When MCF-7 cells express caspase-3, they show the characteristic membrane blebbing, which is absent when the cells are pre-treated with an inhibitor of ROCK I. Thus, also in our model the caspase-3-induced membrane blebbing strictly depends on activation of ROCK I (see Suppl. Fig. 1) [12,34]. In contrast, the release of vesicles is unaffected by inhibition of ROCK I (Fig. 2), showing that membrane blebbing and the release of vesicles are 2 different processes.

Then the question was raised whether the active form of caspase-3 was required for the release of vesicles. To address this question, we preincubated cells expressing caspase-3* with the caspase-3 inhibitor Z-DEVD-FMK, and we observed an approximately 70% reduction in the release of vesicles (data not shown), strongly indicating that caspase-3 activity is required for the release of vesicles.

3.4. Vesicles contain caspase-3

Previously, we and others have shown that vesicles contain caspase-3 or a caspase-3-like activity [1,2,9,14,15]. Because caspase-3 induces the release of vesicles (this study), we hypothesized that cells

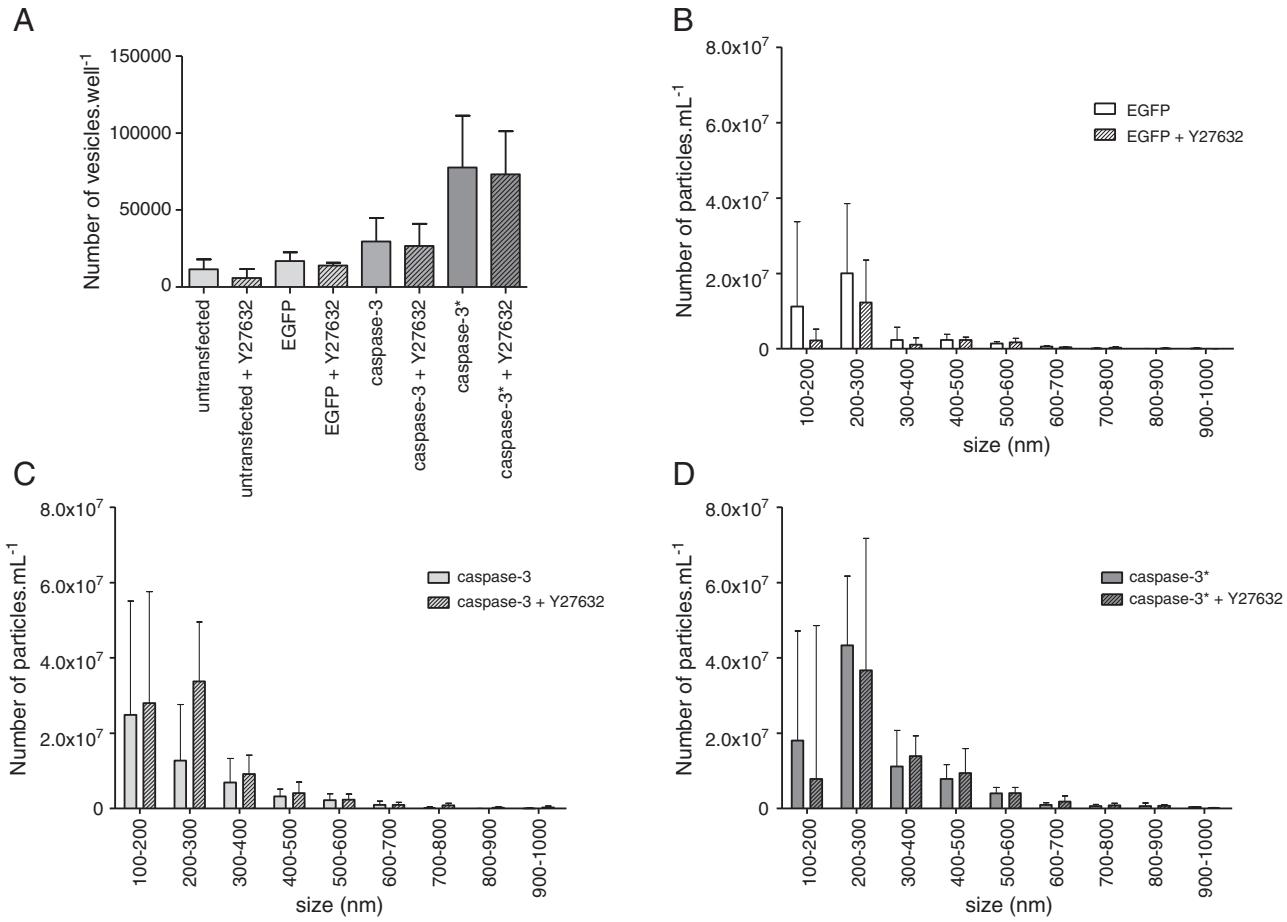

Fig. 2. No involvement of ROCK I in vesicle release. A. The number of vesicles released from caspase-3-expressing- and caspase-3*-expressing MCF-7 cells in the absence or presence of the ROCK I inhibitor Y27632 (n = 3) was determined by annexin V fluorescence using flow cytometry. Untransfected (n = 3) and EGFP-transfected cells (n = 3) were used as controls. B–D. The concentration of particles released by caspase-3-expressing (C, n = 4) and caspase-3*-expressing MCF-7 cells (D, n = 4) in the absence or presence of the ROCK I inhibitor Y27632 was determined by resistive pulse sensing. Untransfected (n = 4) and EGFP-transfected cells (B, n = 4) were used as controls.

may try to escape from caspase-3-induced cell death by sorting caspase-3 into vesicles, which are then released.

The presence of caspase-3 was confirmed by Western blot (Fig. 3A). Vesicles contain the 17 kDa cleavage product of caspase-3, and vesicles from cells expressing caspase-3* also contain detectable quantities of the 12 kDa-EGFP cleavage product (40 kDa; anti-GFP blot) and uncleaved caspase-3*(57 kDa; both anti-caspase-3 blot and anti-GFP blot). Western blots do not provide any information on the presence of caspase-3 in single vesicles, and therefore, we also used flow cytometry. On average, caspase-3 is detectable in 55% and 51% of the vesicles from cells expressing caspase-3 or caspase-3*, as compared to 3% and 11% of vesicles from untransfected cells or EGFP-expressing cells, respectively (Fig. 3B).

Vesicles from cells expressing caspase-3 or caspase-3* contain the active (17 kDa) form of caspase-3, and thus we measured caspase-3 activity in both vesicles and the corresponding cells. Vesicles from cells expressing caspase-3 or caspase-3* contain 9 to 10-fold higher caspase-3 activity than the corresponding cells (Fig. 4; $P = 0.009$ and $P = 0.0004$, respectively), and caspase-3 activity was low or absent in control cells and vesicles. Thus, vesicles are enriched in caspase-3 activity compared to cells, revealing that active caspase-3 is indeed sorted into vesicles.

3.5. Transfer of caspase-3 to MCF-7 cells by vesicles

It is tempting to compare caspase-3-enriched vesicles to small cargo containers which contain waste. The obvious question then is, are such waste-containing containers harmful to their environment? To address this issue, we incubated untransfected MCF-7 cells with vesicles, isolated from different volumes (i.e. 1 and 4 mL) of conditioned culture medium containing vesicles from cells transfected with caspase-3(*). Within 48 h, approx. 60% of the added vesicles disappeared from the culture medium in the presence of target cells, whereas approx. 30% of the vesicles disappeared in the absence of target cells (data not shown). Thus, we assume that the net disappearance of approx. 30% of the vesicles was due to their uptake by cells.

After uptake of vesicles by untransfected cells, fractions of MCF-7 cells were positive for EGFP (Fig. 5A). Also minute fractions of cells stained for caspase-3 (Fig. 5B), and even smaller fractions of cells showed signs of early apoptosis (annexin V; Fig. 5C), or detachment (Fig. 5D). Thus, caspase-3-containing vesicles did bind to or fused with cells, but that did not lead to a marked increase in apoptosis of the untransfected cells, showing that the packaging and removal of caspase-3 by vesicles seem an efficient and safe way to protect both the releasing cells and the environment from active caspase-3.

4. Discussion

Our present findings are summarized in a model shown in Fig. 6. We show that (untransfected) MCF-7 cells, which lack caspase-3 and do not show membrane blebbing, release hardly any vesicles (Fig. 6A, top). When cells express caspase-3, the cells release vesicles (Fig. 6B, left) and show (from left to right) different stages of cell

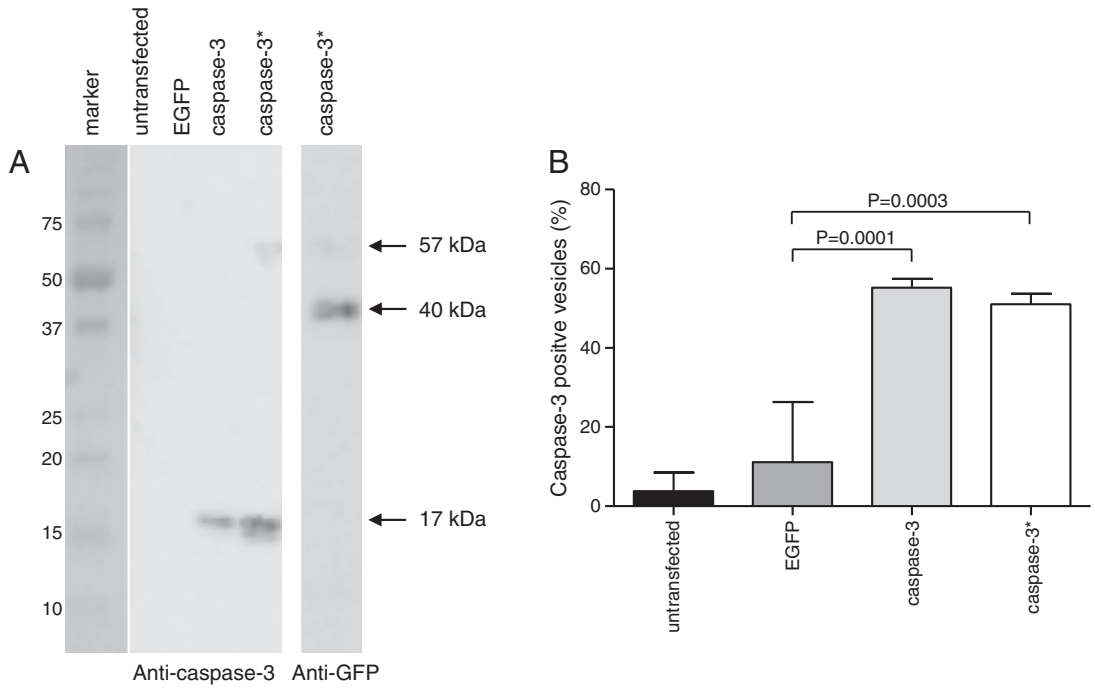

Fig. 3. Presence of active caspase-3 in vesicles. A. The presence of active caspase-3 in vesicles released from untransfected cells, EGFP-, caspase-3- or caspase-3*-expressing cells was determined by Western blot. Blots were incubated with anti-caspase-3 or anti-GFP. B. Active caspase-3 in individual vesicles released from untransfected cells (n = 5), EGFP- (n = 5), caspase-3- (n = 5) and caspase-3*-expressing cells (n = 5) was analyzed by flow cytometry using anti-active-caspase-3 antibody, and expressed as percentage positive vesicles of total analyzed vesicles.

shrinkage (rounded cells), membrane blebbing, release of apoptotic bodies, cell fragmentation, and cell detachment. In the presence of a ROCK I inhibitor, membrane blebbing is absent in cells expressing caspase-3, which confirms findings in earlier studies [12,34]. However, the release of vesicles is not inhibited and thus independent of activation of ROCK I (Fig. 6C, left). These results show that release of membrane vesicles and membrane blebbing are 2 processes that are differentially regulated. Furthermore, we also observed that in the presence of an inhibitor of ROCK I, cells expressing caspase-3 still show cell shrinkage, release of apoptotic bodies, cell fragmentation, and cell detachment (Fig. 6C, from left to right).

Membrane blebbing and the release of vesicles are poorly defined processes, and it is unknown whether membrane blebbing precedes or is a prerequisite for formation and release of vesicles. Although membrane blebbing resembles vesicle formation, both processes may be independent. In our study, it is shown that the release of vesicles is not preceded by membrane blebbing (Suppl. film and Fig. 1C–I). Vesicles are released from the cell during membrane ruffling, but in the absence of membrane blebbing. Furthermore, we also show that membrane blebbing is not a prerequisite for the release of vesicles, since inhibition of membrane blebbing by inhibition of ROCK I (shown in Suppl. Fig. 1C) does not interfere with the release of vesicles (Suppl. Fig. 1D and 2). Therefore, we suggest that membrane blebbing and the release of vesicles are 2 distinct and perhaps even independent processes.

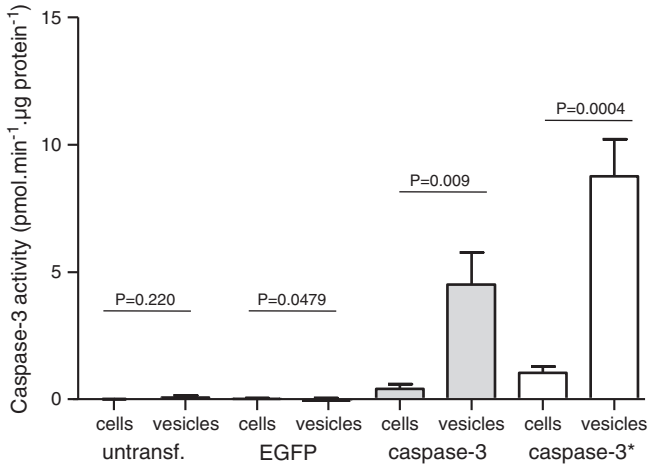

Fig. 4. Enrichment of active caspase-3 in vesicles. Caspase-3 activity was determined in untransfected cells (untransf. n = 3), EGFP- (n = 5), caspase-3 (n = 4) and caspase-3*-expressing cells (n = 5) and their vesicles and was expressed as pmol·min^{-1}·µg protein^{-1}.

To exclude the possibility that our results depend on an overexpression system, we also studied the release of vesicles from a caspase-3-containing cell line, the human pancreatic adenocarcinoma BxPC3. Similar to the MCF-7 model, the release of vesicles from BxPC3 cells was unaffected by Y27632, the inhibitor of ROCK I, thus showing that also in cells expressing normal levels of caspase-3, the inhibition of ROCK I is unable to prevent the release of vesicles. The release of vesicles from BxPC3 cells is inhibited by an inhibitor of caspase-3 activity, ZDEVD-FMK, showing that caspase-3 activity is necessary for vesicle formation (data not shown). Thus, the release of vesicles depends on caspase-3 activity but is independent of ROCK I in both types of cancer cells.

Previously, we have shown that control, interleukin-1α-stimulated as well as staurosporin-treated human umbilical vein endothelial cells (HUVEC) release caspase-3-containing vesicles, which were present after 3 h when a marker of early apoptosis, i.e. the binding of annexin V to cells, was virtually absent [1]. Thus, vesicles are released before the onset of apoptosis, and thus release of vesicles precedes and/or occurs during apoptosis rather than vesicles being formed by membrane blebbing in the final stage of apoptosis. This observation is confirmed by the data shown in the Suppl. film and Fig. 1C–G, in which we show that vesicles are released in the absence of membrane blebbing.

In the previous endothelial cell study, we also showed that caspase-3 accumulates in cells when the release of vesicles is inhibited by combined inhibition of ROCK I and calpain [1]. The knowledge gained from our recent study provides an alternative explanation of our earlier

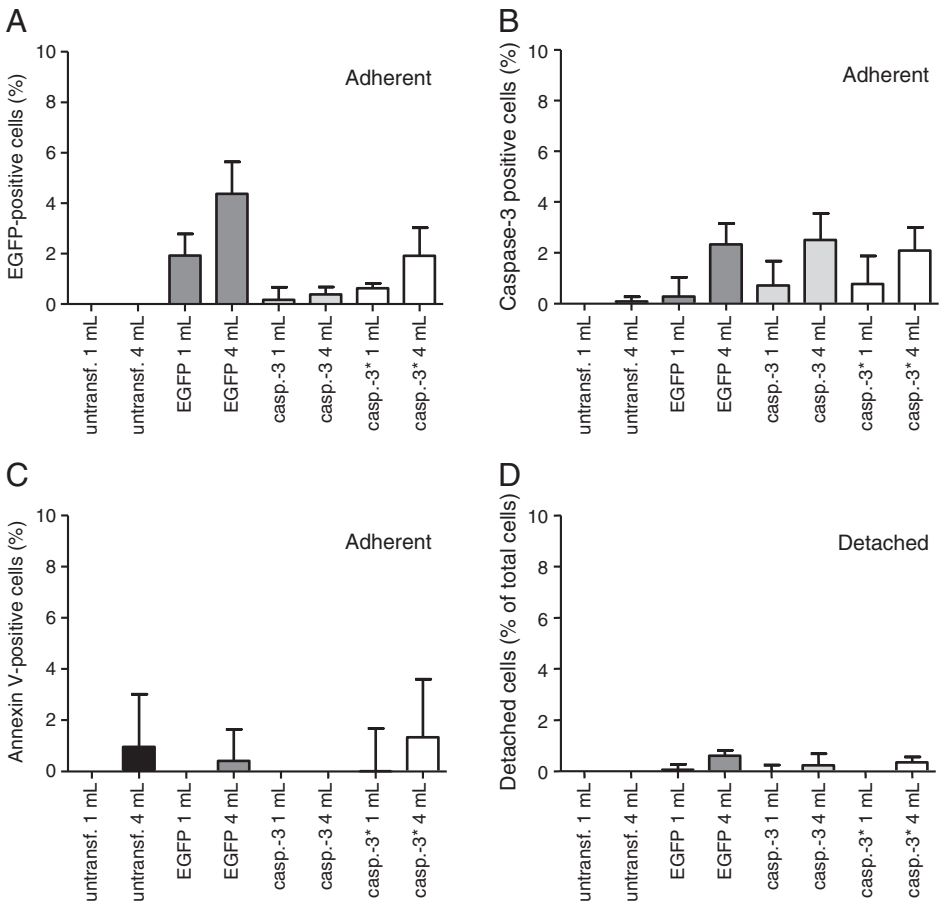

Fig. 5. Transfer of caspase-3 to MCF-7 cells by vesicles. The effect of caspase-3-enriched vesicles on MCF-7 cells was studied by addition of vesicles released from untransfected- (untransf.), EGFP-, caspase-3 (casp.-3)-, and caspase-3*(casp.-3*)-expressing cells to MCF-7 cells. Two increasing concentrations of vesicles, isolated from 1 mL and 4 mL culture supernatant, respectively, were used. In all figures shown, vesicle-free supernatant was used as negative control and subtracted from the signal obtained by vesicles isolated from 1 mL or 4 mL. A. EGFP positivity in adherent cells was studied and expressed as percentage of total adherent cells. B. Caspase-3 positivity in cells was studied and expressed as percentage of total adherent cells. C. Annexin V positivity in cells was studied and expressed as percentage of total adherent cells. D. The numbers of detached cells were studied and expressed as percentage of total cells.

results, assuming that the molecular mechanism underlying the release of vesicles is conserved. Since we now know that ROCK I is not involved in the release of vesicles, at least not in MCF-7 cells expressing caspase-3 and in BxPC3 cells, we may only conclude from our earlier experiments that the intracellular accumulation of caspase-3 is hardly dependent on inhibition of ROCK I. Whether the observed intracellular accumulation of caspase-3 is explained by inhibition of calpain alone, however, is unclear, because inhibition of ROCK I in combination with inhibition of calpain may have an additional effect than inhibition of calpain alone.

In contrast to our studies, Li et al. reported a ROCK I-mediated vesicle release [26]. There may be several explanations for differences in outcome of our study and that of Li et al. [26], such as the use of different cell types and the fact that Li et al. [26] do not provide evidence that the release of vesicles is inhibited by the ROCK inhibitor, but merely show that lower amounts of transglutaminase and flotillin are present in the isolated vesicles after inhibition of ROCK I. From other studies, we know that components affecting the biochemical composition of cell membranes, may also affect the content of vesicles. For example, the intravesicular amount of caspase-3 increased when the cholesterol-lowering component simvastatin was used, compared to vesicles released from control cells [15]. Furthermore, Li et al. [26] show images of blebs at the cell surface, but do not show that these blebs/vesicles are released. In addition, it is demonstrated that blebbing is inhibited by a ROCK I inhibitor, as shown previously [12,34], a phenomenon which we also observed in our present study. For their Western blots, Li et al. [26] first removed cell debris at 12.000 *g*, followed by isolation of the vesicles at 100,000 *g*, a condition which isolates also smaller vesicles such as exosomes. It is unknown whether the (released) blebs/vesicles, which are shown in their images, are removed in the first centrifugation step. Therefore, it is unclear whether the vesicles shown in their images, or the vesicles isolated by ultracentrifugation are responsible for the lower amounts of transglutaminase and flotillin.

The molecular mechanisms underlying vesicle release have only been partially elucidated. For example, platelet-derived vesicles are released when the actin cytoskeleton becomes destabilized [11], requiring the involvement of phosphoinositide 3-kinase [45]. Also calpain plays a role because inhibition of calpain blocks the release of vesicles [13,35]. In addition, ROCK II is involved in the release of vesicles from endothelial cells [33]. However, to which extent these mechanisms play any role in the release of vesicles in our model has to be investigated.

We show that the vesicles are enriched in active caspase-3 compared to the releasing cells. Because addition of such vesicles to untransfected MCF-7 does not induce apoptosis, caspase-3 that has been sorted into vesicles does not seem to be harmful to neighboring cells, at least in vitro. Caspase-3 is not the only protein that is selectively sorted into vesicles. Well-known examples of sorting of dangerous proteins and other harmful molecules into vesicles are e.g. the complement

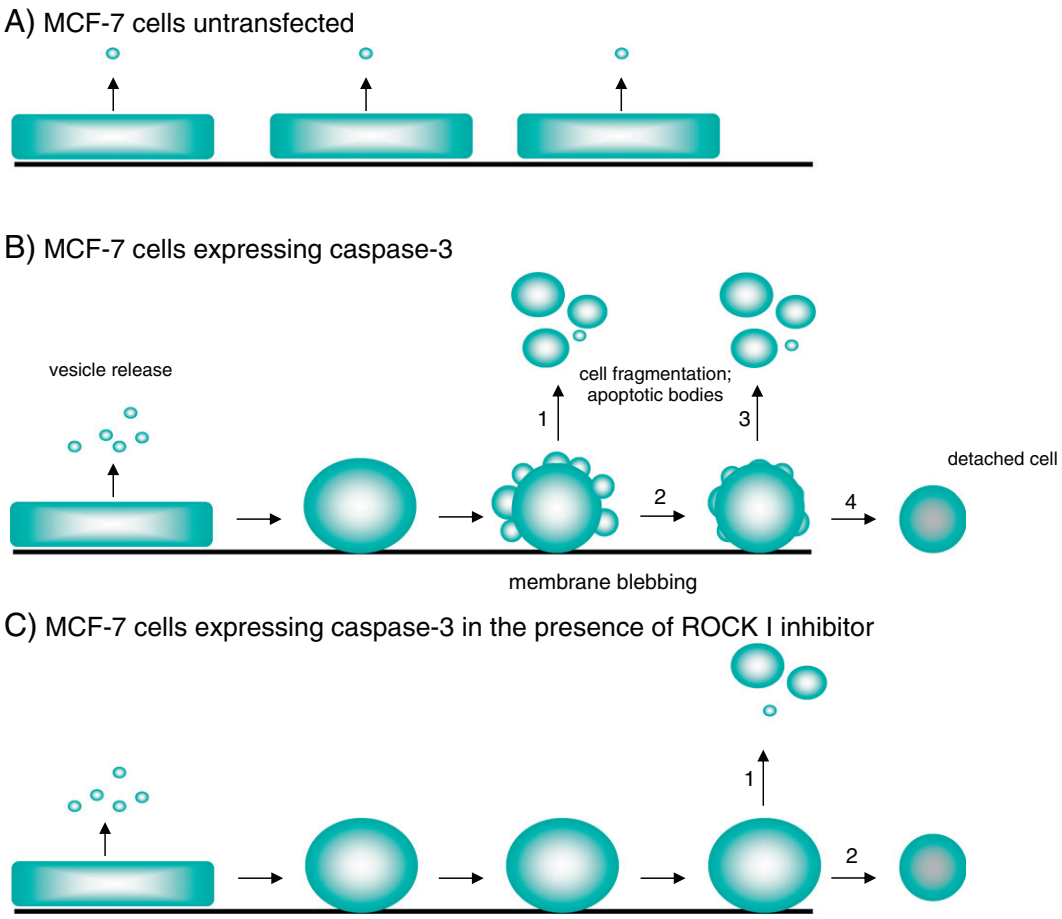

Fig. 6. Model of vesicle release, membrane blebbing and cell fragmentation of MCF-7 cells. This figure summarizes our results. A. Untransfected cells hardly release any vesicles. Cell shrinkage, membrane blebbing and cell fragmentation are absent. B. MCF-7 cells expressing caspase-3 release more vesicles than untransfected cells (left), show cell shrinkage (indicated by the rounded cell), which is followed by membrane blebbing. This membrane blebbing can (1) directly lead to the release of apoptotic bodies and cell fragmentation (in approximately 50% of the caspase-3* -expressing cells), or (2) result in the retraction of membrane blebbing, which can be followed by (3) release of apoptotic bodies and cell fragmentation (in approximately 20% of the caspase-3* -expressing cells) or (4) detachment of the cell (in approximately 30% of the caspase-3* -expressing cells). C. In the presence of the ROCK I inhibitor Y27632, cells expressing caspase-3 release vesicles and show cell shrinkage, but lack membrane blebbing. Although no membrane blebbing is observed, the rounded cell (1) releases apoptotic bodies and fragments, or (2) detaches directly without the release of either apoptotic bodies or cell fragments.

C5b-9 complex [19], cytostatics [32,36], the proapoptotic ceramide and prostate apoptosis response 4 [44]. We and others suggest that cells use vesicles to remove dangerous or potentially dangerous molecules that may threaten the cells' viability and survival. By efficient packaging of such molecules into vesicles, the cells can remain healthy and viable [1,14,19,32,36]. In this manner, vesicles contribute to cellular homeostasis by functioning as garbage bags which can then be phagocytosed and degraded by other cells.

In the present study, we demonstrate that the release of vesicles and membrane blebbing are 2 different and perhaps even independent processes. Furthermore, we show that active caspase-3 is enriched in vesicles and is not harmful to neighboring cells. We propose that the sorting of active caspase-3 into vesicles contributes to cellular defense against apoptosis.

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Appendix A. Supplementary data

The caspase-3 open reading frame was amplified from the preprocaspase-3 clone IOH11204 (Invitrogen) using a 5′primer GGGGACAAGTTTGTACAAAAAAGCAGGCTCCACCATGTCTGGAATATC CCTGGACAAC, and either a 3′primer GGGGACCACTTTGTACAAGA AAGCTGGGTTCTAGTGATAAAAATAGAGTTCTTTTGT (with stop codon) or GGGGACCACTTTGTACAAGAAAGCTGGGTTGTAGTGATAAA AATAGAGTTCTTTTGT (without stop codon). Using Gateway technology (Invitrogen) [24,25], the resulting PCR fragments were shuttled via pDON/Zeo (Invitrogen), into pEGFP-N3 (Clontech, Mountain View, CA). The resulting transfection vectors express the caspase-3 protein, or the caspase-3-protein tagged with EGFP at the C-terminus (caspase-3*) under control of the CMV promoter. Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbamcr.2013.03.013.

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