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Apoptotic Cells Induce Migration of Phagocytes via Caspase-3-Mediated Release of a Lipid Attraction Signal

Kirsten Lauber,[1] Erwin Bohn,[2] Stefan Martin Kröber,[3] Yi-jin Xiao,[4] Sibylle G. Blumenthal,[1] Ralph K. Lindemann,[1] Patrizia Marini,[5] Carolin Wiedig,[2] Anke Zobywalski,[6] Shairaz Baksh,[7] Yan Xu,[4] Ingo B. Autenrieth,[2] Klaus Schulze-Osthoff,[8] Claus Belka,[5] Gernot Stuhler,[6] and Sebastian Wesselborg[1,]*

[1]Department of Internal Medicine I
[2]Institute of Medical Microbiology
[3]Institute of Pathology
University of Tübingen
Germany
[4]Department of Cancer Biology and Department of Gynecology and Obstetrics
Cleveland Clinic Foundation
Cleveland, Ohio 44195
[5]Department of Radiation Oncology
[6]Department of Internal Medicine II
University of Tübingen
Germany
[7]Department of Medicine
Beth Israel Deaconess Hospital
Harvard Medical School
Boston, Massachusetts 02115
[8]Institute of Molecular Medicine
University of Düsseldorf
Germany

Summary

Efficient engulfment of the intact cell corpse is a critical end point of apoptosis, required to prevent secondary necrosis and inflammation. The presentation of "eat-me" signals on the dying cell is an important part of this process of recognition and engulfment by professional phagocytes. Here, we present evidence that apoptotic cells secrete chemotactic factor(s) that stimulate the attraction of monocytic cells and primary macrophages. The activation of caspase-3 in the apoptotic cell was found to be required for the release of this chemotactic factor(s). The putative chemoattractant was identified as the phospholipid, lysophosphatidylcholine. Further analysis showed that lysophosphatidylcholine was released from apoptotic cells due to the caspase-3 mediated activation of the calcium-independent phospholipase A_2. These data suggest that in addition to eat-me signals, apoptotic cells display attraction signals to ensure the efficient removal of apoptotic cells and prevent postapoptotic necrosis.

Introduction

Under physiologic conditions, unwanted cells die by apoptosis, and the dying cells are quickly and efficiently engulfed as an intact cell corpse. If cells die by necrosis, or when apoptotic cells fail to be cleared and undergo postapoptotic (secondary) necrosis, the intracellular contents are released and thus cause harmful inflammatory responses. Therefore, apoptosis has to be complemented by a timely removal of the apoptotic cell (Rosen and Casciola-Rosen, 1999).

The clearance of apoptotic cells by phagocytes or neighboring cells contains two central elements: (1) recognition and (2) subsequent engulfment of the apoptotic cell. This process is initiated by the display of so-called "eat-me" signals on the surface of apoptotic cells. The most important eat-me signal appears to be the display of phosphatidylserine (PS) on the surface of the dying cell. PS, in turn, is recognized by a recently cloned PS receptor whose blockade abrogates the engulfment of apoptotic cells by macrophages (Fadok et al., 2000; Savill and Fadok, 2000).

Recognition and phagocytosis on the part of the engulfing cell is mediated by two partially redundant signaling pathways that were first identified in *C. elegans*: (1) the CED-2/CED-5/CED-10/CED-12-pathway with the respective mammalian homologous Crk II/DOCK-180/Rac1/ELMO-pathway (Gumienny et al., 2001; Reddien and Horvitz, 2000) and (2) the signaling module comprised of CED-1, CED-6, and CED-7, where CED-1 and CED-7 are membrane proteins either directly or indirectly involved in corpse recognition, while CED-6 represents an adaptor protein (Hengartner, 2001).

In a whole organism, it is likely that recognition and engulfment alone might not suffice in order to guarantee the efficient removal of apoptotic cells, if the dying cell and the phagocyte are not next to each other (as they are in *C. elegans*). Whether in mammals migration of professional scavengers to the apoptotic cell might precede the subsequent recognition and engulfment was not known. Interestingly, all genes within the CED-2/CED-5/CED-10/CED-12 signaling cassette have been reported to be involved in cell migration (Gumienny et al., 2001; Reddien and Horvitz, 2000). This prompted us to investigate whether apoptotic cells might display attraction signals in addition to eat-me signals in order to induce the migration of phagocytes to the site of apoptotic cell death.

Here, we present evidence that apoptotic cells secrete a chemotactic signal that induces attraction of monocytic cells in a caspase-3-dependent fashion. Further studies identified the phospholipid lysophosphatidylcholine (LPC) as a potential candidate mediating the attraction of monocytic cells to apoptotic cells. In addition, we identified the calcium-independent phospholipase A_2 as one potential player in the caspase-3-mediated generation of LPC. Thus, apoptotic cells most likely instigate their disposal by displaying attraction signals that induce the recruitment of phagocytes and subsequent recognition and engulfment of the apoptotic remains.

*Correspondence: snwessel@med.uni-tuebingen.de

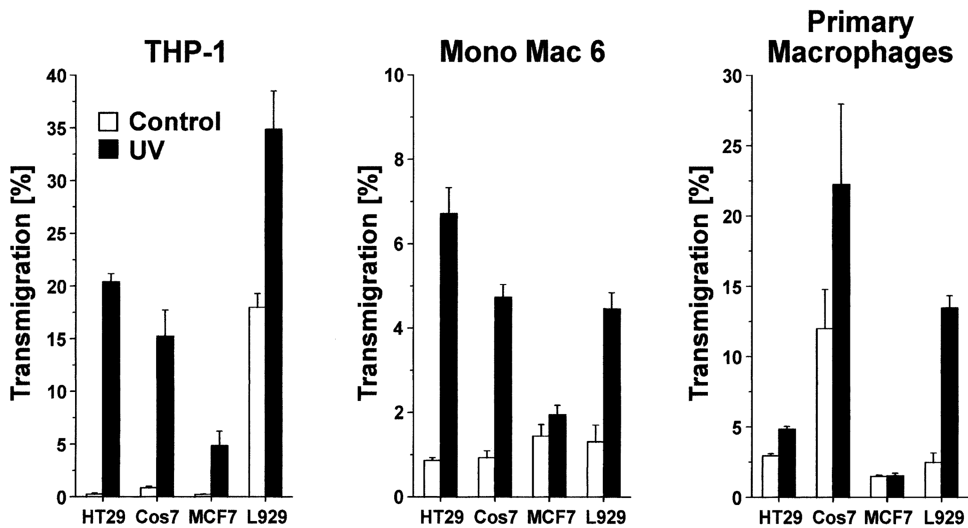

Figure 1. Attraction of Monocytic Cells to Supernatants of Apoptotic Cells

The different tumor cell lines HT29, Cos7, MCF7, and L929 were left untreated or induced to undergo apoptosis by UV irradiation (10 mJ/cm^2). After 13 hr, cell-free supernatants were prepared and migration of THP-1, Mono Mac 6 cells, or primary human macrophages was determined as described in Experimental Procedures.

Results

Supernatants of Different Apoptotic Cell Lines Attract Monocytic THP-1, Mono Mac 6 Cells, and Primary Macrophages

To investigate the involvement of chemotaxis in phagocytic clearance of apoptotic cells we tested supernatants of apoptotic cells for their ability to induce migration of monocytic cells. Therefore, we induced apoptosis via UV irradiation in different cell lines such as the human colorectal adenocarcinoma cell line HT29, human breast carcinoma MCF7 cells, the African green monkey kidney cell line COS7, and murine fibroblast L929 cells. After 13 hr, supernatants were collected and tested for their ability to induce the transmigration of the human monocytic cell lines THP-1, Mono Mac 6, and primary human macrophages. As shown in Figure 1, supernatants of almost all UV-irradiated apoptotic cell lines were able to induce monocyte migration. In the case of L929 cells, supernatants of unstimulated control cells also induced transmigration of THP-1, which was further enhanced upon apoptosis induction. Interestingly, supernatants of apoptotic human breast carcinoma MCF7 cells instigated only minimal transmigration activity of the monocytic cell lines and primary macrophages.

Caspase-3 Is Essential for Chemotaxis Induction by Apoptotic Cells

Since MCF7 cells are deficient for the expression of caspase-3, we tested whether the activation of caspase-3 might be required for the production of chemotactic factors. As shown in Figure 2A, supernatants of apoptotic caspase-3-deficient vector control cells ($MCF7_{vector}$) displayed almost no chemoattractive activity on THP-1 cells. Conversely, supernatants of MCF7 cells stably expressing caspase-3 ($MCF7_{casp3}$) (Jänicke et al., 1998) induced migration as early as 11 hr after UV irradiation and reached plateau level after 15 to 16 hr. The generation of chemotactic activity in the supernatants of $MCF7_{casp3}$ cells was due to caspase activation, since addition of the broad-spectrum caspase inhibitor zVAD-fmk to $MCF7_{casp3}$ cells completely abolished the migration activity (Figure 2B). In order to exclude that the absence of chemotactic activity of caspase-3 deficient cells was due to a decreased apoptosis induction, we analyzed the cleavage of caspase-3 and -7 and of the caspase substrate poly(ADP-ribose) polymerase (PARP). In addition, we measured apoptosis via the induction of cell shrinkage, since analysis of DNA fragmentation cannot be determined in caspase-3-deficient cells (Jänicke et al., 1998). As shown in Figure 2C, the caspase substrate PARP was cleaved to a similar extent in $MCF7_{vector}$ and $MCF7_{casp3}$ cells, though the processing of procaspase-7 was slightly decreased in $MCF7_{vector}$ cells. Caspase-3 was only moderately cleaved in $MCF7_{casp3}$ cells and, as expected, it was completely absent in caspase-3-deficient cells. Addition of zVAD-fmk blocked the processing of all caspases and of PARP. Induction of apoptosis was mediated to a similar extent in $MCF7_{vector}$ and $MCF7_{casp3}$ cells (Figure 2D). Thus, activation of caspase-3 seemed to be crucial for the release of the chemotactic factor by apoptotic cells, but not for the induction of apoptosis.

Other Apoptotic Stimuli Can also Induce the Release of Chemotactic Factors

In order to test whether apoptotic stimuli other than UV can also evoke the release of chemotactic factors, we treated $MCF7_{vector}$ and $MCF7_{casp3}$ cells with the potent apoptosis inducer staurosporine and the anticancer drug mitomycin C. As shown in Figures 3A and 3C, staurosporine and mitomycin C induced to a similar extent as UV irradiation apoptosis and the release of chemotactic factors in $MCF7_{casp3}$ cells that was completely blocked upon addition of zVAD-fmk. Again, there was no detectable chemotactic activity in supernatants of

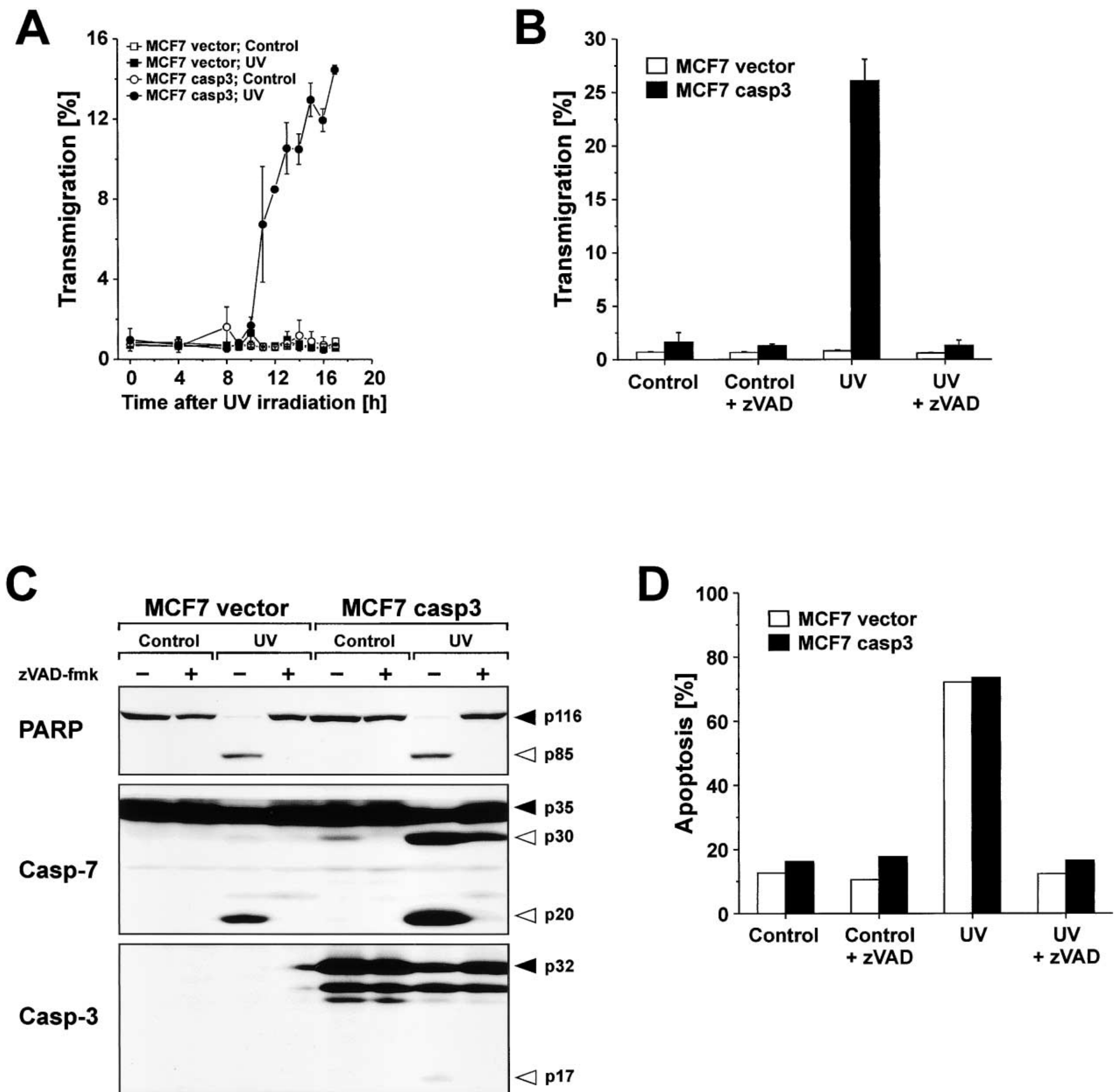

Figure 2. Caspase-3 Is Essential for Attraction of THP-1 Cells to Apoptotic MCF7 Cells

(A) Supernatants of apoptotic caspase-3-positive MCF7 cells induce migration of THP-1 cells. Caspase-3-deficient vector control cells and caspase-3 transfected MCF7 cells were left untreated or UV irradiated and incubated for the indicated amount of time. Subsequently, transmigration of THP-1 cells was assessed.

(B) Effect of caspase inhibition on the release of chemotactic activity by apoptotic cells. $MCF7_{vector}$ or $MCF7_{casp3}$ cells were left untreated or UV-irradiated and then incubated for 13 hr in the presence or absence of the pancaspase inhibitor zVAD-fmk (100 μM). Subsequently, transmigration of THP-1 cells was determined.

(C) Immunoblot analysis of processing of caspases and PARP. $MCF7_{vector}$ or $MCF7_{casp3}$ cells were treated as described in Figure 2B. After 12 hr, proteolytic processing of caspase-3, -7, and PARP was detected by immunoblotting. Filled arrowheads indicate the uncleaved and open arrowheads the cleaved form of the indicated proteins.

(D) Induction of apoptosis. $MCF7_{vector}$ or $MCF7_{casp3}$ cells were treated as described in Figure 2B and after 13 hr apoptosis was assessed by cell shrinkage.

$MCF7_{vector}$ cells (Figure 3A). Immunoblot analysis revealed that staurosporine, mitomycin C, and UV irradiation induced the cleavage of PARP and caspase-7 to a similar extent in $MCF7_{vector}$ and $MCF7_{casp3}$ cells. As expected, caspase-3 was only processed in caspase-3-transfected MCF7 cells (Figure 3B). The production of chemotactic factors by mitomycin-C-treated $MCF7_{casp3}$ cells followed similar kinetics as UV-irradiated cells (Figures 3D and 2A). In contrast, staurosporine treatment caused the release of chemoattractants at an earlier time point in apoptotic $MCF7_{casp3}$ cells (Figure 3D).

Recent reports have shown that intracellular factors such as heat shock protein gp96 (Binder et al., 2000) and ATP (Honda et al., 2001) can induce chemotaxis.

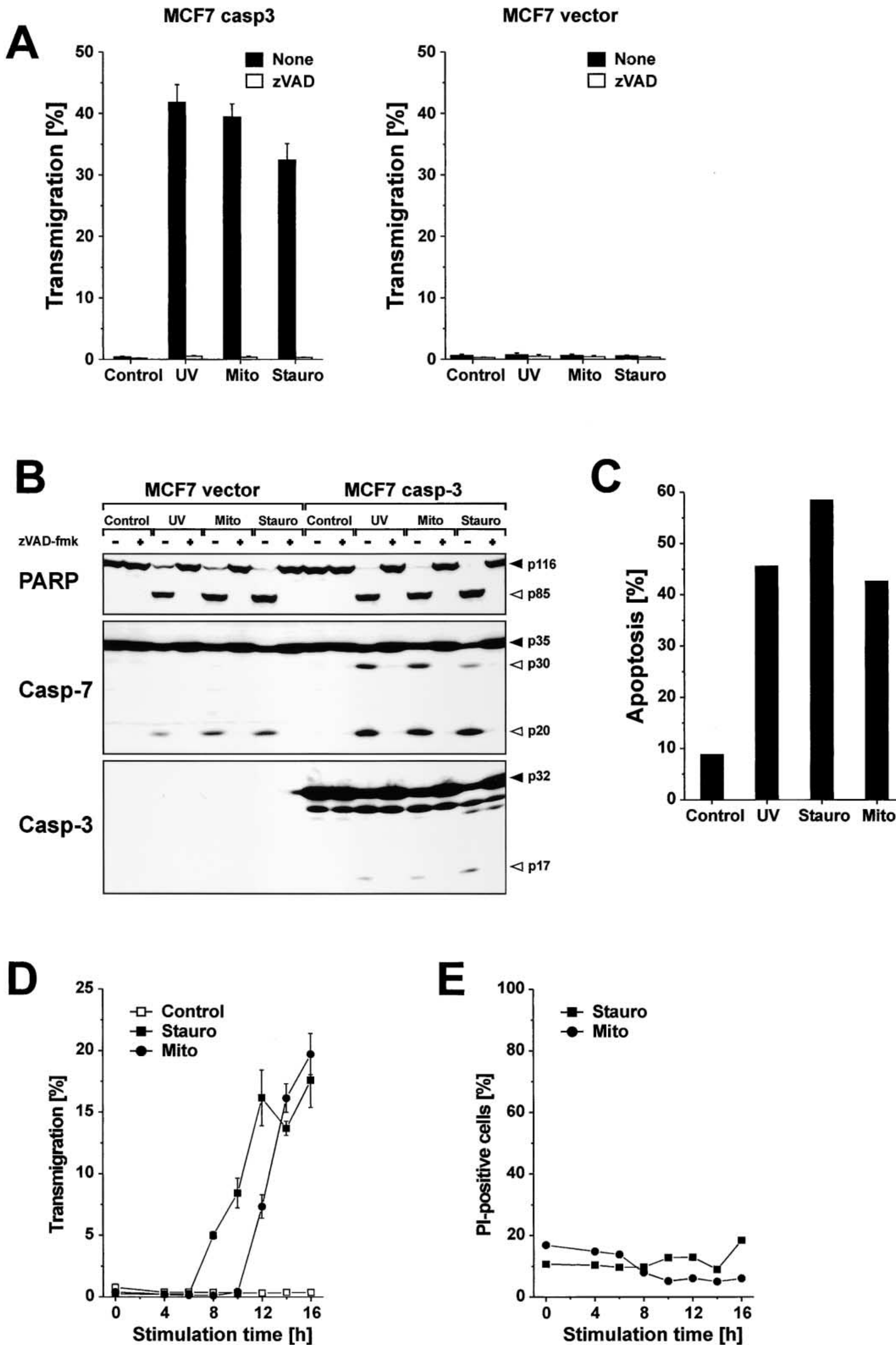

Figure 3. Induction of Apoptosis by Staurosporine and Mitomycin C Mediates the Release of the Chemotactic Factor

(A) Effect of apoptosis induction via UV-irradiation, mitomycin C, and staurosporine on the release of chemotactic activity from MCF7 cells. $MCF7_{vector}$ or $MCF7_{casp3}$ cells were UV irradiated (UV), incubated with medium (Control), mitomycin C (25 μg/ml; Mito), or staurosporine (2.5 μM; Stauro) and incubated with or without 100 μM zVAD-fmk for 13 hr. Cell-free supernatants were prepared and filtered on Sephadex G15 columns and applied to migration assay with THP-1 cells.

(B) Immunoblot analysis of processing of caspases and PARP. $MCF7_{vector}$ or $MCF7_{casp3}$ cells were UV irradiated (10 hr), incubated with medium (5 hr), mitomycin C (10 hr), or staurosporine (5 hr) in the absence or presence of 100 μM zVAD-fmk.

Since caspase-3 is the major caspase involved in the degradation of a number of vital substrates, it was conceivable that apoptotic stimuli might induce a faster and more pronounced disintegration of caspase-3 positive MCF7 cells. However, using the exclusion of propidium iodide as a parameter for membrane integrity we could demonstrate that the membrane of the apoptotic cells treated with staurosporine, mitomycin C (Figure 3E) or UV (data not shown) was intact at all time points tested for migration activity. Thus, apoptotic cells release a chemotactic factor at a time point when the plasma membrane is still intact and thus before commitment to postapoptotic necrosis.

Physical and Biochemical Characterization of the Chemotactic Factor

Next, we wished to identify the substance class of the chemoattractant. Interestingly, neither inhibition of protein synthesis nor of protein release affected the release of chemotactic activity by apoptotic $MCF7_{casp3}$ cells nor UV-induced apoptosis. Again, inhibition of caspases abrogated both apoptosis and chemotactic activity (Figures 4A and 4B). As shown in Figure 4C, the chemotactic factor could not be inactivated by heat treatment. Treatment of the apoptotic supernatants with proteinase K, Dnase, or RNase could further exclude proteins, DNA, and RNA as potential candidates (Figure 4D). Extraction of the apoptotic supernatants with chloroform or diethyl ether completely abrogated all chemotactic activity, suggesting that the chemoattractant was most likely of a lipid nature (Figure 4E).

Chemotactic Activity Is Not Mediated by Membrane Blebs

A recent report demonstrated that the release of apoptotic vesicles of about 0.2 μm in diameter derived from B cells induced chemotactic attraction of THP-1 cells (Segundo et al., 1999). In addition, it has been shown that caspase-3-mediated activation of the Rho-associated kinase ROCK I (Sebbagh et al., 2001) or of p21-activated kinase 2 (PAK2) (Rudel and Bokoch, 1997) are involved in apoptotic membrane blebbing. Thus, it was conceivable that during the course of apoptosis, caspase-3 activates ROCK-I or PAK2, which in turn induce membrane blebbing and subsequent attraction of phagocytes. However, neither 0.2 μm-filtration nor ultracentrifugation could deplete any chemotactic activity of the supernatants of apoptotic $MCF7_{casp3}$ cells (Figures 4F and 4G). Accordingly, we could not detect any chemotactic activity in the membranous precipitate after centrifugation (Figure 4G). Therefore, we concluded that soluble lipids rather than membrane blebs were responsible for the chemotactic attraction of THP-1 cells.

Chemotaxis Induction by Apoptotic Cells Is Mediated via Lysophosphatidylcholine

Next, we investigated whether different biologically active phospholipids could neutralize the chemotactic activity of supernatants from apoptotic cells in the transmigration assay. To this end, we added phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), lysophosphatic acid (LPA), platelet-activating factor (PAF), or sphingosine-1-phosphate (S1P) to THP-1 cells on the upper side of the transmigration membrane to test whether these phospholipids could neutralize the chemotactic activity of supernatants of apoptotic cells in the lower chamber. Among these phospholipids, LPA, LPC, S1P, and PAF have been shown to induce chemotaxis in different cell systems, whereas PS can act as an eat-me signal. As shown in Figure 5A, only LPC and PAF were able to block the transmigration of THP-1 cells to supernatants of apoptotic $MCF7_{casp3}$ cells in a dose-dependent manner. To make sure that this inhibitory effect was not due to cytotoxic effects of the phospholipids on THP-1 cells, we performed an MTT viability assay. In contrast to LPA, most of the lipids used (including LPC and PAF) had only a moderate effect on the viability of the THP-1 cells (Figure 5B).

Since inhibition of chemotaxis could be due to a direct inhibitory effect on the migration activity of THP-1 cells rather than to a neutralizing effect on the chemotactic gradient, we tested whether LPC and PAF could induce migration of THP-1 cells on their own. Therefore, different phospholipids were added to the lower chamber and assayed for their chemotactic activity. Only LPC but neither PAF, LPA, nor S1P were able to attract THP-1 cells (Figure 5C). Maximal chemotactic activity of LPC was observed at a concentration of 20 to 30 μM, whereas higher concentrations reduced the migration activity of THP-1 cells that might be attributed to a decrease in viability as observed in Figure 5B.

Inhibition of $iPLA_2$ but Not of $cPLA_2$ in Apoptotic Cells Abrogates the Release of Chemotactic Activity

LPC is generated upon phospholipase A_2 (PLA_2) mediated hydrolysis of membraneous phosphatidylcholine into LPC and arachidonic acid. Recent reports demonstrate that the cytosolic, calcium-dependent PLA_2 ($cPLA_2$) and the cytosolic, calcium-independent PLA_2 ($iPLA_2$) are cleaved by caspase-3 during apoptosis (Atsumi et al., 1998, 2000). Interestingly, the release of arachidonic acid during apoptosis is accomplished by the caspase-mediated activation of $iPLA_2$, whereas $cPLA_2$ is inactivated instead. Therefore, we investigated whether the caspase-3-mediated activation of $iPLA_2$ was responsible for the release of chemotactic LPC from

(C) Induction of apoptosis. $MCF7_{casp3}$ cells were left untreated, UV irradiated, stimulated with mitomycin C, or staurosporine. After 24 hr, induction of apoptosis was determined by flow cytometric evaluation of hypodiploid nuclei.

(D) Kinetics of chemoattractant release. $MCF7_{casp3}$ cells were incubated with medium, staurosporine, or mitomycin C. After indicated time points, cell-free supernatants were prepared, filtered on Sephadex G15 columns and migration of THP-1 cells was assessed.

(E) Kinetics of membrane integrity. Cells were treated with staurosporine, or mitomycin C for the indicated amount of time. Membrane dysfunction was determined by the uptake of propidium iodide.

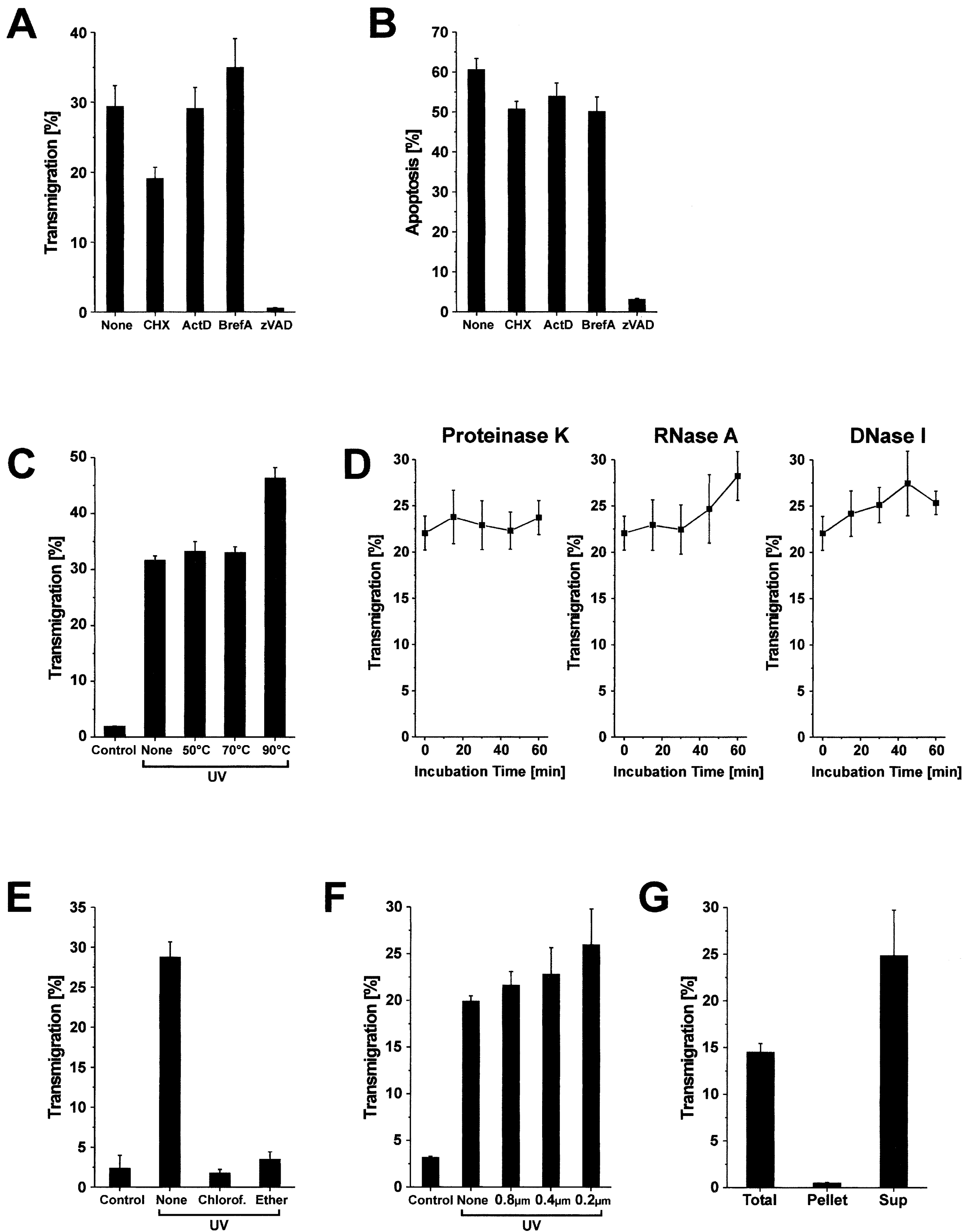

Figure 4. Physical and Biochemical Characterization of Chemoattractant

(A) Effect of the inhibition of protein synthesis, protein secretion, and caspases on the release of chemoattractant from apoptotic cells. $MCF7_{casp3}$ cells were UV irradiated and then incubated with or without actinomycin D (100 ng/ml), cycloheximide (1 μg/ml), brefeldin A (1 μg/ml), or zVAD-fmk (100 μM). After 13 hr, cell-free supernatants were prepared and Sephadex G15 filtered.

(B) Apoptosis induction. $MCF7_{casp3}$ cells were treated as in Figure 4A and induction of apoptosis was measured after 24 hr by flow cytometric evaluation of hypodiploid nuclei.

(C) Heat treatment of supernatants of apoptotic cells. $MCF7_{casp3}$ cells were untreated or UV irradiated. After 13 hr, cell-free supernatants were prepared and heated for 10 min at 50°C, 70°C, or 90°C, or left at room temperature.

apoptotic cells by specific inhibition of $iPLA_2$ in $MCF7_{casp3}$ cells via bromoenol lactone (BEL) (Balsinde and Dennis, 1996) and $cPLA_2$ by addition of arachidonoyl trifluoromethyl ketone ($AACOCF_3$) (Street et al., 1993) prior to induction of apoptosis via UV irradiation. As shown in Figure 6A, inhibition of $cPLA_2$ in $MCF7_{casp3}$ cells only moderately decreased attraction of THP-1 cells, whereas addition of the $iPLA_2$ inhibitor BEL to $MCF7_{casp3}$ cells completely abrogated the release of chemotactic activity. Since inhibition of PLA_2s has been demonstrated to interfere with apoptosis induction, we investigated whether inhibition of PLA_2 reduced the chemotactic activity secondarily by reducing the amount of UV-induced apoptosis in $MCF7_{casp3}$ cells. When $MCF7_{casp3}$ cells were treated with the highest concentrations used in the transmigration assay, inhibition of $iPLA_2$ slightly decreased apoptosis whereas inhibition of $cPLA_2$ had no effect on UV-mediated apoptosis (Figure 6B). Since inhibition of $iPLA_2$ only partially reduced UV-mediated apoptosis but completely blocked the induction of chemotactic activity, we concluded that $iPLA_2$ was involved in the caspase-3-dependent release of the chemoattractant LPC in apoptotic cells. In addition, inhibition of $iPLA_2$ via BEL not only blocked the release of chemotactic activity from apoptotic $MCF7_{casp3}$ cells but also from other apoptotic cell lines such as L929 and Cos7 (data not shown).

Atsumi et al. recently demonstrated that activation of $iPLA_2$ during apoptosis mediates the release of arachidonic acid (Atsumi et al., 1998). Therefore, in order to investigate whether the second $iPLA_2$-mediated cleavage product LPC was also released during the course of apoptosis, we analyzed the culture supernatants for LPC using electrospray ionization mass spectrometry (ESI-MS) analyses. As shown in Figure 6C, induction of apoptosis in $MCF7_{casp3}$ cells mediated an increased release of total LPC as well as of 16:0-LPC and 18:0-LPC. Inhibition of caspases or $iPLA_2$ abrogated the enhanced release of LPC from apoptotic $MCF7_{casp3}$ cells whereas apoptotic $MCF7_{vector}$ cells revealed no increased LPC-release. The amount of LPC measured in the supernatants of apoptotic $MCF7_{casp3}$ cells was about 100-fold lower than the amount of purified LPC required to induce migration of THP-1 cells (Figure 5C). This might be due to incomplete dissolution of purified phospholipids or the fact that the chemotactically active LPC might consist of micelles that do not readily form after dissolution of purified LPC. Thus, the LPC in the supernatants might be in a form that reveals a higher chemotactic activity than commercially purified LPC.

In order to demonstrate that inhibition of caspases or $iPLA_2$ in apoptotic $MCF7_{casp3}$ cells abrogates the attraction of monocytic cells other than THP-1 cells, we tested Mono Mac 6 cells and primary human macrophages. Thus, we could show that inhibition of caspases or $iPLA_2$ in UV-irradiated $MCF7_{casp3}$ cells also blocked the attraction of Mono Mac 6 and THP-1 cells and of primary macrophages (Figure 6D).

Caspase-3 Induces the Processing of $iPLA_2$ In Vitro and In Vivo

During apoptosis $iPLA_2$ is processed from an 85 kDa full-length form into a 70 kDa fragment. A putative caspase-3 cleavage site (DVTD[183]) was located near the C-terminal end of the first ankyrin repeat that rendered a 70 kDa N-terminal fragment (Atsumi et al., 2000). Although the overexpression of caspase-3 induced processing of $iPLA_2$ in HEK293 cells, the authors did not exclude that other caspases might be involved in the processing of $iPLA_2$. Therefore, we investigated whether caspase-3 was a prerequisite for the processing of $iPLA_2$ in vivo and in vitro. As shown in Figure 6E, $iPLA_2$ was cleaved only in $MCF7_{casp3}$ cells upon treatment with UV, mitomycin C, or staurosporine whereas virtually no processing occurred in caspase-3-deficient cells. Surprisingly, and in contrast to the reported C-terminal 70 kDa fragment, we observed two fragments of 25 and 26 kDa. Incubation of purified histidine-tagged $iPLA_2$ with purified caspase-3, -6, -7, and -8 in vitro revealed that only caspase-3 was able to induce the processing of full-length $iPLA_2$ into a 26 kDa fragment (Figure 6F). Additionally, we observed two fragments of 52 and 42 kDa upon treatment with caspase-3. Sequence analysis of $iPLA_2$ revealed a further potential caspase cleavage site at DLFD[513] which would produce a 32 kDa fragment. Since we observed the 25/26 kDa fragments only in immunoblots using an antiserum against amino acids 557–576 but not against amino acids 735–745 (data not shown; see also Figure 7A), we presume that additional cleavage sites are located near the C terminus such as MVVD[733], DCTD[737], or RAVD[744] that would generate fragments of about 25–26 kDa (see Figure 7A).

Removal of Ankyrin Repeats Increases $iPLA_2$-Mediated Release of Chemotactic Activity

Cleavage at DLFD[513] would remove all ankyrin repeats and truncate $iPLA_2$ near its catalytic center at aa 517–520 (see Figure 7A). Since the ankyrin repeats have been suggested to function as a negative regulator of $iPLA_2$ (Larsson et al., 1998), their complete removal might enhance the catalytic activity of $iPLA_2$. Conversely, processing at Asp[513] in close proximity to the catalytic center (GTST[520]) might abolish $iPLA_2$ activity. In order to investigate whether the caspase-3 mediated cleavage

(D) Treatment of supernatants of apoptotic cells with proteinase K, RNase A, or DNase I. Cell-free supernatants were generated as in Figure 4C and then incubated for 0 to 60 min with proteinase K, RNase A, or DNase I.

(E) Depletion of chemotactic activity from supernatants by lipid extraction. Cell-free supernatants were prepared as in Figure 4C and subsequently extracted with chloroform or diethyl ether.

(F) Effect of filtration of supernatants on chemotactic activity. Cell-free supernatants were generated as described in Figure 4C and subsequently filtered through 0.2–0.8 μm pore filters.

(G) Effect of ultracentrifugation of supernatants on chemotactic potential. Cell-free supernatants of UV-treated $MCF7_{casp3}$ cells were ultracentrifuged at 1×10^5g for 2 hr and the supernatant applied to migration assay. The pellet was redissolved in the same volume of fresh medium and applied together with noncentrifuged supernatant (Total) to migration assay. (A and C–G) All transmigration assays were performed with THP-1 cells.

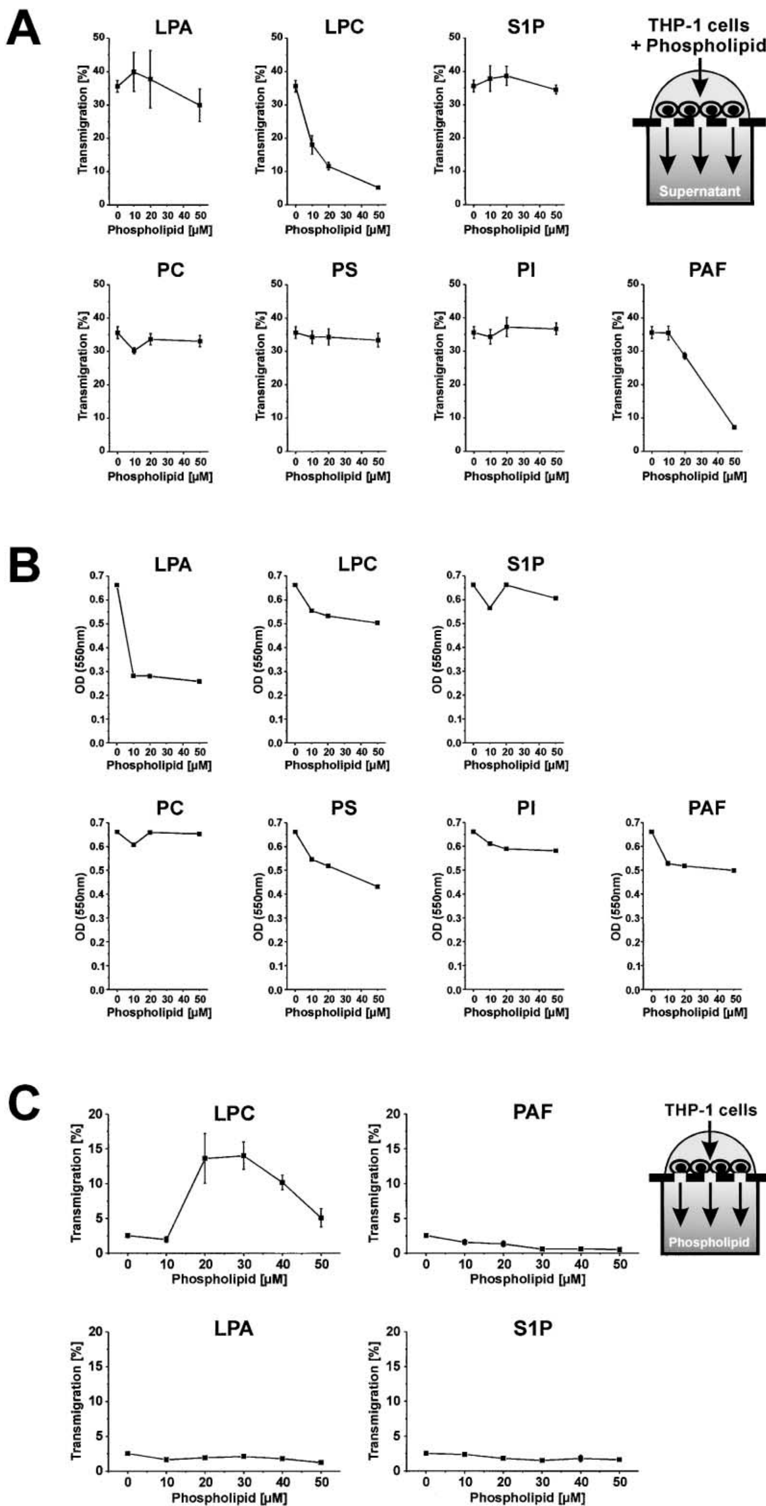

Figure 5. Lysophosphatidylcholine Mediates Attraction of THP-1 Cells to Apoptotic cells

(A) Effect of different phospholipids in neutralizing the chemotactic factor in supernatants of apoptotic cells. Respective phospholipids were added at indicated concentrations directly to THP-1 cell suspension on the upper side of the transmigration membrane (see diagram) and their potential to neutralize the chemotactic gradient of supernatants of apoptotic UV-treated $MCF7_{casp3}$ cells was determined.

(B) Effect of different phospholipids on cell viability. THP-1 cells were incubated with the indicated concentration of respective phospholipid and cell viability was determined by an MTT assay.

(C) Effect of different phospholipids to induce migration of THP-1 cells. LPC, PAF, LPA, or S1P were added at the indicated concentrations directly to the lower chamber (see diagram) and transmigration of THP-1 cells was assessed.

at Asp^{513} would affect the $iPLA_2$ mediated release of chemotactic activity, we overexpressed C-terminal Flag-tagged mutant (aa 514–806) or full-length N-terminal Flag-tagged $iPLA_2$ in $MCF7_{casp3}$ cells (see Figure 7A). Additionally, we overexpressed the C-terminal Flag-tagged mutant (aa 514–733) in order to investigate the effect of the fully caspase-truncated p26 form of $iPLA_2$. As shown in Figure 7B, full-length and mutant $iPLA_2$ (aa 514–806) were expressed in $MCF7_{casp3}$ cells and further degraded upon apoptosis induction by UV-irradiation. Interestingly, both overexpressed mutant forms of $iPLA_2$ (aa 514–733 and 514–806) could no longer be detected after UV irradiation in the immunoblot using anti-Flag antibodies, thus confirming additional caspase-medi-

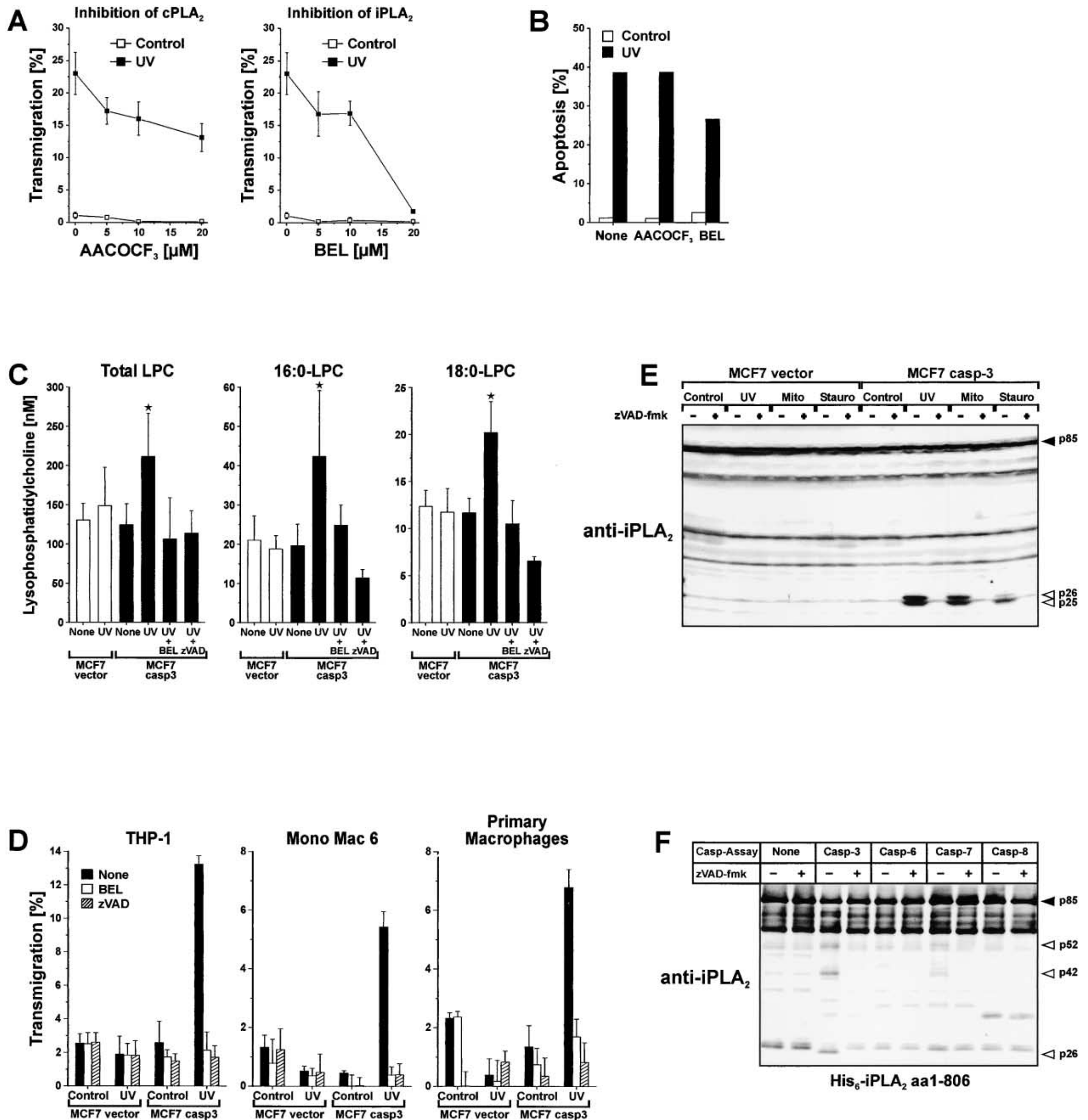

Figure 6. Effect of the Inhibition of $iPLA_2$ on the Release of Chemotactic Activity, LPC, and the Role of Caspase-3 on $iPLA_2$ Processing

(A) Inhibition of $iPLA_2$ but not of $cPLA_2$ in apoptotic cells prevents the release of chemoattractant. $MCF7_{casp3}$ cells were left untreated or UV irradiated and then incubated with the indicated concentrations of the $cPLA_2$ inhibitor $AACOCF_3$ or of the $iPLA_2$ inhibitor BEL for 13 hr. Migration assay was performed with THP-1 cells.

(B) Effect of inhibition of $cPLA_2$ and $iPLA_2$ on apoptosis induction. $MCF7_{casp3}$ cells were left untreated or UV-irradiated and then incubated with 20 µM $AACOCF_3$ or BEL. After 24 hr, induction of apoptosis was assessed by flow cytometric evaluation of hypodiploid nuclei.

(C) Mass spectrometrical detection of LPC in culture supernatants. $MCF7_{vector}$ and $MCF7_{casp3}$ cells were left untreated or UV irradiated. $MCF7_{casp3}$ cells were additionally incubated in the absence or presence of 100 µM zVAD-fmk or 20 µM BEL. After 13 hr, supernatants were collected and the concentration of total LPC, 16:0-LPC and 18:0-LPC was detected by electrospray ionization mass spectrometry analysis as described in Experimental Procedures.

(D) Inhibition of caspases or $iPLA_2$ abrogates the chemotactic attraction of THP-1, Mono Mac 6 cells, and primary human macrophages to UV-irradiated $MCF7_{casp3}$ cells. $MCF7_{vector}$ and $MCF7_{casp3}$ cells were left untreated or UV irradiated and then incubated with or without 100 µM zVAD-fmk or 20 µM BEL for 13 hr. Migration assays were performed with THP-1, Mono Mac 6 cells, and primary human macrophages (day 5).

(E) Cleavage of $iPLA_2$ during apoptosis. $MCF7_{vector}$ and $MCF7_{casp3}$ cells were stimulated as described in Figure 3B. Cleavage of $iPLA_2$ was detected by immunoblotting with an antiserum against aa 557–576 of $iPLA_2$.

(F) Caspase-mediated cleavage of $iPLA_2$ in vitro. Purified histidine-tagged $iPLA_2$ was incubated with purified caspase-3, -6, -7, and -8 with or without 100 µM zVAD-fmk. Processing of $iPLA_2$ was detected by immunoblotting with an antiserum against aa 557–576 of $iPLA_2$.

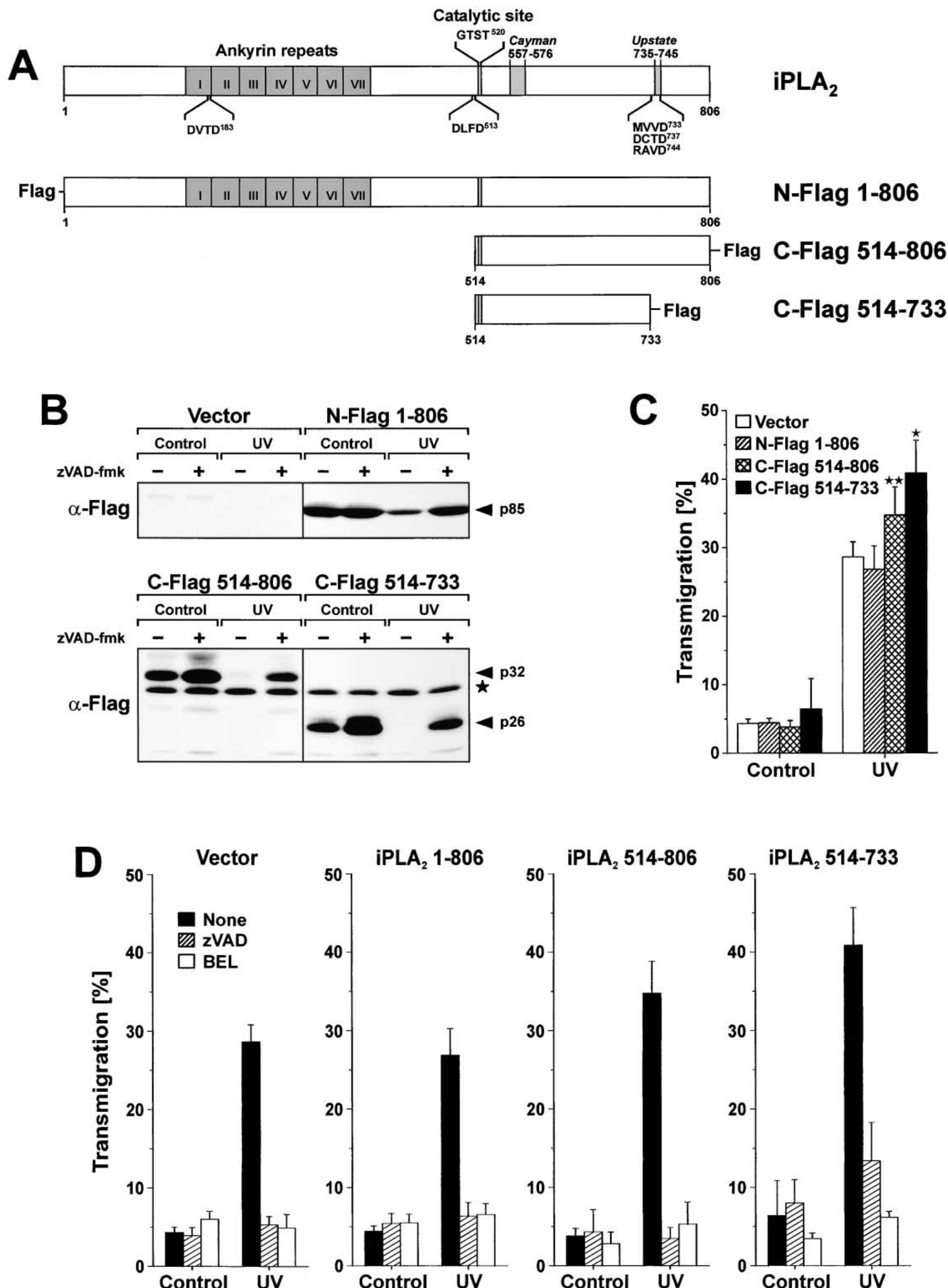

Figure 7. Effect of the Removal of the Ankyrin Repeats of $iPLA_2$ on the Release of Chemotactic Activity

(A) Schematic structure of wild-type and mutant human $iPLA_2$. Catalytic center, putative caspase cleavage sites and positions of immunizing peptides for antiserum generation are shown.

(B) Overexpression of full-length and truncated $iPLA_2$. $MCF7_{casp3}$ cells were transiently transfected with N terminally Flag-tagged full-length (aa 1–806), C terminally Flag-tagged mutated (aa 514–806) or C terminally Flag-tagged mutated $iPLA_2$ (aa 514–733). Then, cells were left untreated or UV-irradiated in the presence or absence of 100 μM zVAD-fmk. Processing of transfected $iPLA_2$ was detected with an anti-Flag antibody. Nonspecific band is indicated with an asterisk.

(C) Effect of overexpressed full-length and truncated $iPLA_2$ on the release of chemotactic activity. $MCF7_{casp3}$ cells expressing vector alone (Vector) or the indicated Flag-tagged versions of full-length and mutant $iPLA_2$ were left untreated or UV irradiated for 13 hr. Asterisk indicates $p < 0.01$ and double asterisk $p < 0.05$ in comparison to "Vector/UV" (independent t test).

(D) Effect of inhibition of $iPLA_2$ and caspases on the release of chemoattractant. $MCF7_{casp3}$ cells expressing vector alone or the indicated Flag-tagged versions of full-length and mutant $iPLA_2$ were left untreated or UV irradiated and then incubated in the absence or presence of 100 μM zVAD-fmk or 20 μM BEL for 13 hr. (C and D) Transmigration assays were performed with THP-1 cells.

ated cleavage of $iPLA_2$ at the C terminus (Figure 7B). When we analyzed the effect of native and both truncated forms of $iPLA_2$ on the release of chemotactic activity, we observed that overexpression of full-length $iPLA_2$ did not further augment the chemotactic activity in supernatants of apoptotic $MCF7_{casp3}$ cells, whereas overexpression of both truncated forms of $iPLA_2$ increased the release of chemotactic activity (Figure 7C). Interestingly, overexpression of the fully caspase-truncated p26 form (aa 514–733) displayed an even more pronounced effect than the partially truncated p32-form (aa 514–806) of $iPLA_2$. However, overexpression of either form of mutant $iPLA_2$ (aa 514–806 and aa 514–733) could not induce significant attraction in supernatants of nonapoptotic $MCF7_{casp3}$ cells (Figure 7C). As in previous experiments, inhibition of $iPLA_2$ by addition of BEL completely abrogated the release of chemotactic activity in all apoptotic $MCF7_{casp3}$ cells overexpressing different constructs of $iPLA_2$ (Figure 7D). Remarkably, caspase inhibition by the addition of zVAD-fmk only partially blocked the release of chemotactic activity from apoptotic $MCF7_{casp3}$ cells overexpressing the truncated p26 form. This was consistently observed in several experiments but never found in cells overexpressing the empty vector alone, full-length $iPLA_2$, or mutant $iPLA_2$ (aa 514–806). The activity retained in the presence of zVAD corresponded to the increase of chemotactic activity in p26-transfected cells compared to vector-transfected cells. Since the completely truncated p26 form (aa 514–733) does not require further processing, this might explain why apoptotic $MCF7_{casp3}$ cells overexpressing the p26 fragment still released chemotactic activity in the presence of zVAD-fmk.

We also tested the ability of the apoptotic supernatants to recruit macrophages in two in vivo model systems including a peritonitis and a subcutaneous infiltration model. To this end, supernatants of apoptotic and nonapoptotic cells were injected into the peritoneum or subcutaneously, and then histologically evaluated in a time course for their effect on macrophage infiltration. Supporting our previous results, in both models there was a considerably more pronounced (2-fold and higher) recruitment of macrophages after injection of the supernatants from UV-treated MCF-7_{casp3} cells compared to supernatants from untreated cells (see Supplemental Figures S1 and S2 available at http://www.cell.com/cgi/content/full/113/6/717/DC1). The chemotactic activity was comparable to the effects seen after injection of purified LPC or proteose peptone and LPS that were used as positive controls of potent proinflammatory stimuli. Thus, these data underline a biological relevance of our findings and suggest that attraction signals could play a role in macrophage recruitment in vivo.

Discussion

To date relatively little is known about what triggers migration of professional phagocytes to the site of apoptotic cell death. One can envision that the sole display of eat-me signals might not suffice in order to guarantee a timely removal of the apoptotic cell before commitment to postapoptotic necrosis. Therefore, the display of long-range attraction signals by the dying cell might represent a prerequisite for its efficient removal by professional scavengers.

In the present study, we investigated this hypothesis and observed that supernatants of apoptotic cells induced migration of monocytic THP-1 cells, Mono Mac 6 cells, and primary macrophages. The major release of the chemotactic factor was mediated via caspase-3 since supernatants of apoptotic caspase-3-deficient MCF7 cells, in contrast to caspase-3 expressing MCF7 cells, showed almost no attraction of THP-1 cells. In addition to the attraction of monocytic cells in vitro, supernatants of apoptotic $MCF7_{casp3}$ cells appeared to induce also the attraction of macrophages upon intraperitoneal or subcutaneous injection in vivo (see Supplemental Figures S1, and S2 at URL above).

Biochemical characterization of the supernatants of apoptotic cells revealed that the putative chemoattractant was not a protein, DNA, or RNA but most likely a lipid instead. Potential chemotactic lipid factors were arachidonic acid and its derivates as well as apoptotic membrane blebs and phospholipids. However, we could rule out arachidonic acid and most of its derivates (prostaglandins, leukotrienes, and thromboxanes), because they are not extractable with diethyl ether or chloroform. Apoptotic vesicles could be excluded since neither filtration nor ultracentrifugation of the supernatants of apoptotic cells abrogated the attraction of monocytic THP-1 cells. Therefore, we focused on phospholipids such as LPA, LPC, S1P, and PAF that have been shown to induce chemotaxis. Of all the phospholipids tested only LPC and PAF could neutralize the chemotactic capacity in supernatants of apoptotic cells when added directly to the responding THP-1 cells. However, only LPC could induce migration on its own whereas PAF was ineffective. Therefore, LPC that has been known for a long time as a chemoattractant for monocytes (Hoffman et al., 1982) seemed to be a crucial factor for the attraction of monocytes to apoptotic cells.

LPC is generated via PLA_2-mediated processing of the membranous phosphatidylcholine into LPC and arachidonic acid. Mammalian PLA_2 isoenzymes are subdivided into four major families, including cytosolic, calcium-dependent ($cPLA_2$), secretory ($sPLA_2$), cytosolic, calcium-independent PLA_2 ($iPLA_2$), and PAF acetyl hydrolases (Murakami et al., 2000). It was shown that processing by caspases inactivates $cPLA_2$, whereas $iPLA_2$ is activated instead (Atsumi et al., 2000) and mediates the release of arachidonic acid from apoptotic cells (Atsumi et al., 1998). In addition to arachidonic acid, we could now demonstrate that the second $iPLA_2$-mediated cleavage product LPC was also released from apoptotic cells.

Because ankyrin repeats function as a negative regulator of $iPLA_2$ (Larsson et al., 1998) their caspase mediated removal might contribute to the activation of $iPLA_2$. Using a partially truncated p32-form (aa 514–806) or the completely truncated p26 form (aa 514–733) of $iPLA_2$ we could show that the cleavage of $iPLA_2$ near the catalytic site and the complete removal of ankyrin repeats did not affect the release of chemotactic activity but rather increased the attraction of THP-1 cells. Nevertheless, it is conceivable that caspases might activate $iPLA_2$ in a rather indirect way. Thus, $iPLA_2$ might be retained within the cytosol via an anchoring protein that obstructs the

access of $iPLA_2$ to its membrane-associated substrate phosphatidylcholine. Caspase-mediated processing of $iPLA_2$ might release $iPLA_2$ from its inhibitor, thus increasing its access to endogenous substrate. Conversely, caspases might directly cleave the putative inhibitor and thereby release $iPLA_2$. Accordingly, $iPLA_2$ has been reported to exist as a multimeric complex of 270–350 kDa in the cell that might contain a putative cytosolic inhibitor (Larsson et al., 1998). The observation that overexpression of the completely truncated p26 form of $iPLA_2$ does not induce significant chemotactic activity in nonapoptotic cells, yet increases the release of chemotactic activity in cells undergoing apoptosis indicates that caspases might exert a direct *and* an indirect effect on the activation of $iPLA_2$.

Whether or not under physiologic conditions $iPLA_2$ and LPC will be the sole elements required for inducing the attraction of phagocytes to the apoptotic cell has to be proven. It is noteworthy that the perturbed membrane asymmetry of cells undergoing apoptosis enables the access of secretory PLA_2 ($sPLA_2$) (Atsumi et al., 1997; Fourcade et al., 1995; Murakami et al., 2000). Therefore, $sPLA_2$ might also contribute to the generation of LPC via hydrolysis of phosphatidylcholine from the outer membrane leaflet of apoptotic cells. Conversely, $iPLA_2$ and LPC might not only be involved in attraction of phagocytes but also contribute to the recognition of apoptotic cells. In this context, a recent report demonstrated that the $iPLA_2$-mediated generation of cell-bound LPC might opsonize the dying cell for phagocytosis by binding of natural IgM antibodies to LPC and subsequent complement activation (Kim et al., 2002). As in the case of phosphatidylserine regarding recognition and engulfment, there might as well exist factors other than LPC that contribute to the display of attraction signals by apoptotic cells.

Current reports show that a family of G protein-coupled receptors (GPRs) mediates the binding and signal transduction of extracellular phospholipids. Thus, LPA and S1P bind to receptors of the Edg subfamily of GPRs, while the PAF receptor as well as the receptors for LPC and SPC also constitute members of the GPR group. SPC has been shown to bind to the ovarian cancer GPR (OGR1) and to GPR4 (Xu et al., 2000; Zhu et al., 2001). LPC has recently been demonstrated to bind to the orphan GPR G2A and, with lower affinity than SPC, to GPR4 (Kabarowski et al., 2001; Zhu et al., 2001). Triggering of G2A via LPC-induced migration in addition to an increase in intracellular calcium concentration, G2A-receptor internalization and ERK protein kinase activation (Kabarowski et al., 2001). Furthermore, G2A-deficient mice have been reported to develop a late-onset multiorgan inflammation with striking similarity to the human autoimmune disease systemic lupus erythematosus (Le et al., 2001). In this context it is of interest to note that THP-1 cells, Mono Mac 6 cells, primary human monocytes, and macrophages expressed the known LPC receptors G2A and GPR4 (see Supplemental Figure S3 available at http://www.cell.com/cgi/content/full/113/6/717/DC1).

In general, autoimmunity is thought to be the result of the inefficient elimination of autoreactive lymphocytes by apoptosis. However, there is evidence that inefficient removal of apoptotic cells might also contribute to proinflammatory responses and autoimmune diseases (Rosen and Casciola-Rosen, 1999). Usually, internalization of an apoptotic cell by macrophages is accompanied by the expression of anti-inflammatory cytokines such as transforming growth factor-β (Fadok et al., 1998). Conversely, uptake of necrotic cells by specialized phagocytes as the dendritic cells (DCs) has rather an adverse effect in activation of the DC to express costimulatory molecules that are necessary for T cell activation and induction of an inflammatory response (Galluci et al., 1999; Sauter et al., 2000). Therefore, one might speculate that if apoptotic cells are not engulfed in time by phagocytes, postapoptotic necrosis occurs, initiating inflammation and autoimmunity instead of self-tolerance.

Taken together, we conclude that the clearance of apoptotic cells is a complex multistep mechanism. During the course of apoptosis cells display "eat-me" signals such as PS and attraction signals such as LPC. Attraction signals initiate the migration of professional phagocytes to the apoptotic cell. Phagocytes eventually bind to the dying cell and due to the lack of exposed "detachment" signals, they are not repulsed (Brown et al., 2002). Display of eat-me signals ensures the recognition and subsequent engulfment of the apoptotic cell. Further studies will be required to understand how defects in exposing attraction or eat-me signals on the part of the apoptotic cell as well as defects in chemotaxis, recognition, and engulfment on the part of the phagocyte might contribute to the generation of pathological conditions such as autoimmunity.

Experimental Procedures

Cells

MCF7 and THP-1 cells were cultured in RPMI-1640 and HT29, COS7 and L929 cells in DMEM supplemented with 10% fetal calf serum. Mono Mac 6 cells were kindly provided by Dr. Ziegler-Heitbrock. MCF7 cells stably transfected with caspase-3 and the caspase-3-deficient vector control cells were a kind gift of R.U. Jänicke and A.G. Porter (Jänicke et al., 1998). Human monocyte-derived macrophages were generated from peripheral blood mononuclear cells of healthy donors. Monocytes were obtained using a negative-selection-based magnetic cell separation system (Miltenyi Biotec). Monocytes were then cultured in macrophage-serum-free medium (Invitrogen) supplemented with 2.5% autologous serum and 25 ng/ml human GM-CSF (Schering-Plough) in teflon dishes for 5 days yielding about 80% of CD14 and CD206 double-positive macrophages.

Induction of Apoptosis; Production and Treatment of Chemoattractive Cell Culture Supernatants

2×10^6 cells cultured in 6-well plates were UV-irradiated with 10 mJ/cm^2 with the UV Stratalinker 2400 (Stratagene). Alternatively, apoptosis was induced with staurosporine (2.5 μM) or mitomycin C (25 $\mu g/ml$) for the indicated time. Supernatants were prepared by centrifugation at $1 \times 10^4 g$ for 5 min and stored at -70°C. In experiments where different inhibitors, mitomycin C, or staurosporine were used, the cell-free supernatants were additionally passed through a 2 ml gel bed of Sephadex G15. For heat-inactivation, supernatants were heated at 50°C, 70°C, 90°C or left at room temperature for 10 min. Enzyme digestion of supernatants was carried out by adding 50 $\mu g/ml$ proteinase K, 50 $\mu g/ml$ RNase A, or 8 $\mu g/ml$ DNase I and incubating for 0 to 60 min at 37°C. Chloroform and diethyl ether extraction was performed with 1 volume of chloroform or diethyl ether, and was repeated twice. Filtration of the culture supernatants was carried out with Millex sterile filters of 0.2 to 0.8 μm pore size. Ultracentrifugation of the cell culture supernatants was performed at $1 \times 10^5 g$ for 2 hr. The resulting supernatant was transferred to a

fresh tube and the pellet was redissolved in the same volume of RPMI-1640 medium.

Transmigration Assay

Transmigration assays were performed with 8 μm pore size ChemoTX plates (Neuroprobe). Three-hundred microliters of respective culture supernatant was placed into the lower chamber of ChemoTX plate and 50 μl of calcein-stained THP-1 or Mono Mac 6 cells (2×10^6/ml) added on top of the filter-membrane and the assay was incubated for 90 min at 37°C. Migrated cells were lysed and green fluorescence was analyzed using an excitation wavelength of 480 nm and an emission wavelength of 508 nm. Transmigration was assessed in percentage of total cells deployed and mean values ± SD from triplicate experiments are given.

Measurement of Apoptosis and Cell Viability

For determination of apoptosis, the leakage of fragmented DNA from apoptotic nuclei was measured by flow cytometry as described (Engels et al., 2000). Alternatively, apoptosis induction was measured by cell shrinkage of trypsinized MCF7 cells by flow cytometric analysis using the FSC profile as a parameter for cell size. Membrane permeability of adherent MCF7 cells was assessed after trypsinization by the uptake of propidium iodide (2 μg/ml) into nonfixed cells and subsequent flow cytometric analysis using FSC/FL2 profile. For determination of cell viability, 1×10^5 THP-1 cells were incubated with the respective phospholipids for 2 hr at 37°C. Subsequently, cells were incubated for 5 hr at 37°C in the presence of MTT (450 μg/ml). Resulting formazan crystals were dissolved in 4% SDS and measured at 550 nm. Results were shown as mean values of duplicates.

In Vitro Cleavage of $iPLA_2$

For in vitro processing of purified $iPLA_2$ 500 ng of histidine-tagged full-length $iPLA_2$ was incubated with 5 μg of purified caspase-3, caspase-6, caspase-7, or caspase-8 in the presence or absence of 100 μM zVAD-fmk in a total volume of 50 μl of a buffer containing 50 mM HEPES (pH 7.3), 100 mM NaCl, 10% sucrose, 0.1% CHAPS, and 10 mM DTT at 37°C for 4 hr.

Western Blot Analysis

Western blot analysis was performed as described (Engels et al., 2000) with monoclonal antibodies against PARP (Qbiogene-Alexis), caspase-7, caspase-3 (BD Biosciences), or Flag (Eastman Kodak). $iPLA_2$ was detected with a rabbit antiserum generated by immunization with a peptide spanning amino acids 557–576 of $iPLA_2$ (Cayman Chemicals) that detected the 85 kDa full-length form and the 25/26 kDa fragments. Alternatively, $iPLA_2$ was detected with a rabbit antiserum against amino acids 735–745 (Upstate Biotechnology) that detected only the p85 form of $iPLA_2$.

Expression of Caspases and $iPLA_2$

E. coli BL21 (DE3) expression clones of histidine-tagged human caspase-3 and caspase-6 in the plasmid pET23 were a generous gift from V.M. Dixit (Genentech, South San Francisco, CA). Histidine-tagged human caspase-7, caspase-8, and human full-length $iPLA_2$ were cloned into pET15b according to standard procedures and purified as described (Lauber et al., 2001). N terminally Flag-tagged full-length $iPLA_2$ (aa 1–806) and C terminally Flag-tagged truncated $iPLA_2$ (aa 514–806) were cloned into pEGFP-N1 (without fusion to EGFP). 3×10^5 $MCF7_{casp3}$ cells per well were seeded into 6-well plates and incubated for 8 hr. Subsequently, cells were transfected with 1 μg of expression vector per well using FuGENE ™ 6 (Roche).

Electrospray Ionization Mass Spectrometry (ESI-MS) Analyses

Lipids were prepared from serum-free culture supernatants and analyzed by mass spectrometry using a Quattro Ultima triple quadrupole ESI-MS (Micromass Inc.) as described previously (Xiao et al., 2000, 2001).

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