Engulfment of apoptotic cells by microvascular endothelial cells
induces proinflammatory responses

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Abstract

Circulating endothelial cells (CEC) have been detected in a variety of vascular disorders but their interactions with healthy endothelium remain unknown. The aim of this study was to evaluate the response of human endothelial cells (EC) to apoptotic or necrotic endothelial cells in an in vitro model and to delineate pathogenetic pathways. Here we show that incubation of human microvascular endothelial cells (HMEC-1) with apoptotic EC resulted in increased expression of chemokines and enhanced binding of leukocytes to HMEC-1 whereas exposure of HMEC-1 to necrotic EC led to no changes in leukocyte binding affinity. Both, apoptotic and necrotic cells were bound and engulfed by HMEC-1 and primary human umbilical vein endothelial cells (HUVEC). We therefore suggest that exposure to apoptotic and necrotic EC induce different patterns of chemokine synthesis and leukocyte adhesion in healthy EC. These data indicate that CEC are not only markers of vascular damage but may induce pro-inflammatory signals in the endothelium.
Introduction

Apoptosis is essential to maintain homeostasis in multi-cellular organisms and apoptotic cells are rapidly and effectively cleared by professional phagocytes before they undergo secondary necrosis and release their noxious cytoplasmic content into the environment.\(^1\) Ineffective clearance of apoptotic cells contributes to disease pathogenesis.\(^2,3\) This may be true especially for autoimmune diseases like systemic lupus erythematosus (SLE) since apoptotic cells are thought to be a potential source of autoantigens and disturbed clearance of apoptotic corpses may initiate and drive autoimmunity.\(^4,6\) This concept of acquired autoimmunity has also been confirmed in several animal models that showed disturbed engulfment of apoptotic cells.\(^7-10\)

Vascular endothelial cells serve as a crucial barrier between tissues and the circulation. They secrete a variety of substances, regulate coagulation and take part in the immune response.\(^11\) Under physiological conditions CEC are almost not traceable whereas in a variety of vascular diseases such as myocardial infarction, small-vessel vasculitis, transplantation or cancer high numbers of CEC are detectable in the peripheral circulation.\(^12-17\) Consequentially, accumulation of CEC may affect the homeostasis of the vessel wall by interfering with the endothelial cell layer both in the vicinity of endothelial lesions or even distant from the site of injury.

Clearance of dying cells or cellular debris has been mostly ascribed to professional phagocytes, e.g. antigen-presenting macrophages, neutrophils or dendritic cells, but it is conceivable that other cell types such as epithelial or endothelial cells may also partake in this process.\(^18,19\) Endothelial cells are no professional phagocytes although specific sub-populations, such as liver endothelial cells or cells from high endothelial venules,
are able to phagocytose apoptotic cells.\textsuperscript{20,21} Phagocytosis of circulating endothelial
debris by healthy endothelium thus appears to be an intriguing concept.

The role of endothelial- or platelet-derived microparticles in the circulation is subject of
several recently published studies but few, if any, data, shed light on the impact of
apoptotic endothelial corpses on the adjacent endothelium.\textsuperscript{22-31} The aim of the present
study was to establish an \textit{in vitro} model to study the interaction of apoptotic or necrotic
EC with a healthy endothelial cell layer. We here demonstrated that microvascular EC,
when exposed to apoptotic cells, react with the release of proinflammatory chemotactic
cytokines and that this response triggers enhanced adhesion of primary neutrophils and
macrophages.
Materials and Methods

The study was approved by the Hannover Medical School Ethics Committee and conducted in accordance with the Declaration of Helsinki. Written informed consent was obtained from all patients included in this study.

Endothelial cell culture

HUVEC were isolated from umbilical cords by exposure of the vein to chymotrypsin for 30 min at 37°C. Cells were collected and cultured in endothelial cell medium (ECM+, Promocell, Heidelberg, Germany) and gentamycin. After 24 hours HUVEC were intensively washed with phosphate-buffered saline (PBS), sub-cultured in ECM+ medium and used up to passage four. The human microvascular endothelial cell line HMEC-1 was obtained from the Centers of Disease Control (Atlanta, GA, USA) and maintained in MCDB-131 medium supplemented with 10% fetal calf serum (FCS), gentamycin, 10 mM L-glutamine, 10 ng/ml EGF and 1 µg/ml hydrocortisone (BD Biosciences, Heidelberg, Germany).

Isolation of neutrophils

Blood was obtained from healthy volunteers. Granulocytes were isolated by density-gradient centrifugation over isotonic Biocoll (Biochrom, Berlin, Germany). After hypertonic lysis of the erythrocytes with ice-cold lysis buffer [155 mM NH₄Cl, 10 mM NaHCO₃, and 0.5 mM ethylenediamine tetraacetate (EDTA), pH 7.4], the granulocytes (with a neutrophil content of 95%) were washed in PBS, re-suspended in Hank’s
Balanced Salt Solution (HBSS) and labelled with 5-chloromethylfluorescein diacetate (CMFDA, Invitrogen, Karlsruhe, Germany) for 30 minutes.

**Enrichment of circulating endothelial cells**

Circulating endothelial cells were isolated as described previously. Briefly, 7 mL EDTA-blood was incubated with Dynabeads (Invitrogen) coated with anti-human CD146 (Biocytex, Marseille, France) and CEC were isolated using a magnetic device (Invitrogen). From each sample an aliquot was stained with FITC-coupled UEA-1 and examined under a fluorescence microscope.

**Induction of apoptosis or necrosis**

HMEC-1 were exposed to ultraviolet (UV) light at a wavelength of 254 nm at various doses. Generation of reactive oxygen species (ROS) was measured by dihydroxyethidium (DHE) staining, cell viability was assessed by measuring internal ATP concentration and integrity of the cell membrane was confirmed by measuring lactate dehydrogenase (LDH) in the supernatant using the Celltiter-Glo® luminescence and the Cytotox 96® cytotoxicity assay according to the manufacturer's instructions (Promega). Induction of apoptosis was confirmed by determing activity of Caspases 3 and 7 using the luminescence based Caspase-Glo® 3/7 assay and fragmented nuclear DNA was stained with the Dead-End® Fluorometric TUNEL system according to the manufacturer’s protocols (Promega, Mannheim, Germany). The percentage of apoptotic cells was assessed by FACS analysis with FITC-labelled activated caspase ligand CaspACE® FITC-VAD-FMK (Promega).
As a second approach to induce apoptosis, HMEC-1 were exposed to TNF-α (3 nmol/L, BD Biosciences) and camptothecin (CPT, 0.15 µmol/L, Sigma-Aldrich, München, Germany) for 24 hours. At these concentrations endothelial cells were positive for active caspases 3 and 7 and showed positive staining for FITC-VAD-FMK.

To obtain necrotic cell lysates HMEC-1 were resuspended in PBS and subjected to repeated freeze-thaw cycles at –80°C.

Co-incubation of HMEC-1 and apoptotic or necrotic endothelial cells

HMEC-1 were plated in 100 mm² dishes at 4 x 10⁵ cells/ml in MCDB-131 full medium. After 4 hours the medium was replaced with PBS and cells were exposed to UV light. PBS was again replaced with MCDB-131 full medium and HMEC-1 were incubated for another 16 hours. In some experiments TNF-α/CPT treated HMEC-1 were used as controls for apoptotic cells. Lysed necrotic cells were obtained as described above. The irradiated cells were harvested, washed in PBS and co-incubated with untreated HMEC-1 for different time points. The HMEC-1 layer was then washed five times with PBS, harvested by trypsination and resuspended in RNA lysis buffer (Qiagen, Hilden, Germany). For the measurement of protein content supernatant of the co-cultures was collected, cleared from cell debris by centrifugation at 13000 x G, aliquoted and stored at –80°C until the ELISA assay was performed.

Transwell experiments

HMEC-1 were seeded onto collagen-coated membranes in transwell chambers (12 mm diameter, 3 µm pore size; Corning, Schiphol-Rijk, Netherlands) at 1 x 10⁶ cells/ml. In the
meantime HMEC-1 were seeded in six-well plates at a density of $1 \times 10^6$ cells/ml for 4 hours. Apoptosis or necrosis was induced as described above. After an incubation time of 16 hours the transwell chambers with the untreated HMEC-1 were inserted into the six well plates containing the damaged cells and both were co-incubated for different time points. In a second set of experiments both untreated and damaged cells were co-incubated for three and six hours directly after induction of apoptosis or necrosis. HMEC-1 were removed from the membrane und subjected to RNA extraction and real-time qPCR analysis.

**RNA isolation, quantification and real-time qPCR**

To obtain total RNA we used the RNeasy™ miniprep system in combination with an on-column DNase digest according to the manufacturer’s protocol (Qiagen). Quality of the RNA was determined using a Bioanalyser and the RNA 6000 Nano LabChip (Agilent Technologies, Waldbronn, Germany). For real-time qPCR, 2 µg of total RNA was subjected to reverse transcription using a mix of random hexamers and oligo(dT)$_{12-15}$ oligonucleotides (Stratagene, Amsterdam, Netherlands) and MMLV RNase H$^-$ point mutant reverse transcriptase (Promega). qPCR was performed on a SDS 7700 system (Applied Biosystems, Darmstadt, Germany) with Rox dye as internal control (Invitrogen), FastStart taq Polymerase (Roche Diagnostics, Penzberg, Germany) and gene-specific primers in combination with SYBR-Green chemistry (Invitrogen) or with Fam-Tamra labelled TaqMan® probes (BioTez, Berlin, Germany). Data were analyzed using the Q-gene software. Primers were designed with Primer Express 2.0 software.
(Applied Biosystems). Sequence information of the oligonucleotides used for real-time qPCR is supplied as supplemental data.

**Enzyme-linked immunosorbent assay (ELISA)**

Conditioned medium was collected from HMEC-1 before and after co-incubation with apoptotic or necrotic HMEC-1, centrifuged to remove particulates and frozen at -80°C. IL-8 and MCP1 protein content was measured using the IL-8 and the MCP1 Quantikine™ kit according to the manufacturer's protocol (R&D Systems, Wiesbaden, Germany).

**Leukocyte adhesion assay**

Endothelial cells were seeded in 96 well plates at 8 x 10^4 cells/well. After adherence the cells were exposed to apoptotic or necrotic HMEC-1 (1 x 10^5 cells/well) or were left untreated. After 4 hours the supernatant was cleared of cells and cellular debris by centrifugation at 1000 x G. CMFDA labelled neutrophils or macrophages were added to the ECs at a concentration of 5 x 10^4 cells/well. After 30 minutes the supernatant containing unbound cells was removed, ECs were washed five times with ice-cold PBS, fixed with 4 % paraformaldehyde (PFA) in PBS and embedded with Vectashield™ (Vector Laboratories, Burlingame, USA). The number of adherent neutrophils and macrophages was counted in six visual fields per well and averaged.

**Adhesion of neutrophils to HMEC-1 exposed to human CEC**
HMEC-1 were seeded on collagen-coated coverslips (Sigma-Aldrich, München, Germany) at a concentration of $1 \times 10^5$ cells/well. After reaching confluence HMEC-1 were exposed to CEC from patients with acute ANCA-associated vasculitis or from healthy controls. As control experiments the HMEC-1 were incubated with the same number of CD146-coated Dynabeads or left untreated. After three hours the supernatant was cleared from Dynabeads and cell debris and CMFDA-stained neutrophils were added to the cells at a concentration of $1 \times 10^5$ cells/well. After 30 minutes the supernatant was removed and the coverslips were washed five times with ice-cold PBS, fixed with methanol at -20°C for 15 minutes and examined under a fluorescence microscope.

Phagocytosis of apoptotic or necrotic HMEC-1

HMEC-1 were seeded on collagen-coated glass coverslips (12 mm in diameter) at a density of $4 \times 10^5$ cells. For some experiments HMEC-1 were labeled with the Chloromethylbenzamido Celltracker CM-Dil (Invitrogen) for 30 minutes. When the cells reached confluence they were incubated with equal numbers of CMFDA labelled apoptotic or necrotic HMEC-1 or with FITC-UEA-1-stained cell fragments for different time points. Non-phagocytosed cells and fragments were then removed by intensive washing with ice-cold PBS and the cells were fixed in 4% PFA and embedded with Vectashield. Phagocytosis was determined using an Axioplan microscope equipped for epifluorescence (ZEISS, Goettingen, Germany) or a TCS SP2 AOBS confocal microscope equipped with argon and krypton laser beams (Leica, Heidelberg, Germany).
Statistical analysis

Results were expressed as the mean ± SD of at least three independent experiments. Where human donors were used, cells were from different donors. Results were analyzed for statistical significance using the two-tailed Mann-Whitney U test.
Results

Dose-dependent induction of apoptosis or necrosis in HMEC-1 by UV-light

UV irradiation was chosen to induce apoptosis in HMEC-1 to avoid effects of chemical compounds on the recipient cells in subsequent co-culture experiments. Exposure of HMEC-1 to UV light induces reactive oxygen species (ROS), leading to a dose-dependent pattern of reversible cell damage, apoptosis and finally necrosis. Exposure of HMEC-1 to low doses of UV light (40 J/cm²) led to markedly elevated levels of intracellular ROS (Figure 1A) and decreased intracellular ATP content but low levels of active Caspases 3/7 and only few cells with fragmented nuclear DNA were observed, suggesting absence of apoptosis (Figure 1B, C). Increasing doses of UV irradiation (85-170 J/cm²) led to a further decline of intracellular ATP, enhanced ROS generation and marked activation of Caspases 3/7. TUNEL staining demonstrated degradation of nuclear DNA and FACS analysis of the active caspase ligand FITC-VAD-FMK demonstrated positivity in 66 % ± 8.4 (n=3, p<0.05 at 85 J/cm²) and 75 % ± 3.5 (n=3, p<0.05 at 170 J/cm²) whereas in untreated cells only 1.7 % ± 3.5 (n=3, p<0.05) were positive for FITC-VAD-FMK. Light microscopy of the cells showed condensed cytoplasm, blebbing of apoptotic bodies and shrinkage of the nucleus, in keeping with apoptosis (data not shown). Higher doses of UV light (≥ 250 J/cm²) led to a dramatic decline of intra-cellular ATP and caspase activity fell to levels below those of untreated cells, indicating necrotic cell death (Figure 1B,C). Necrosis was corroborated by release of lactate dehydrogenase and propidium iodide staining (data not shown). These data gave us confidence that UV light at doses between 85 and 170 J/cm² induced apoptosis while higher doses led to necrosis.
Exposure of apoptotic or necrotic HMEC-1 to untreated endothelial cells

HMEC-1 were exposed to UV light at different doses and incubated overnight to obtain apoptotic or necrotic cells. Necrotic HMEC-1 material was also obtained by lysis of HMEC-1 during repeated freeze/thaw cycles. Exposure of apoptotic or necrotic HMEC-1 to untreated HMEC-1 did not induce any distinct changes in interleukin-6 (IL-6), interleukin-10 (IL-10), vascular endothelial growth factor (VEGF) or intracellular adhesion molecule-1 (ICAM-1) transcript at any time points (data not shown). In contrast, mRNA levels of IL-8 were clearly increased in HMEC-1 exposed for three hours to apoptotic or necrotic cells (∼2-fold increase, n=5). Longer exposure times did not lead to further increase of IL-8 transcript and 16 hours of co-incubation led to IL-8 levels below those of untreated HMEC-1 (Figure 2).

MCP1 transcript levels peaked (∼2-fold up-regulation compared to untreated HMEC-1, n=5) after three hours exposure to apoptotic cells. Longer co-incubation of HMEC-1 and apoptotic cells for up to 16 hours resulted in a decrease of MCP1 mRNA to almost half the amount seen in untreated HMEC-1. In contrast to IL-8, co-incubation with necrotic cells did not induce an increase of MCP1 expression in HMEC-1 (Figure 2). TRAIL expression in HMEC-1 did not change during the first six hours of co-incubation with apoptotic or necrotic ECs. After 16 hours, however, TRAIL mRNA levels declined to less than half of those in untreated HMEC-1. There was no difference between apoptotic and necrotic cells (Figure 2).

To prove that enhanced expression of IL-8 and MCP1 transcript was indeed mediated by the apoptotic conditions of the cells or whether it was due to cellular modifications
caused by UV irradiation we conducted a second approach with HMEC-1 exposed to a combination of TNF-α (3 nmol/L) and CPT (0.15 µmol/L). The combination of the two agents was necessary because neither TNF-α, nor CPT alone induced apoptosis in HMEC-1. TNF-α/CPT-treated apoptotic HMEC-1 induced up-regulation of IL-8 transcript in the recipient cells after three hours of co-incubation (> 2-fold increase, n=5), too. The impact of TNF-α/CPT-treated HMEC-1 on MCP1 expression was much weaker (1.5-fold increase, n=5; Figure 3).

Further experiments were conducted to rule out that contaminating RNA from irradiated cells was responsible for any of these effects. Total RNA was extracted from HMEC-1 16 hours after exposure to UV light. Already low UV doses of 40 J/cm² caused slight RNA degradation and UV doses in excess of ≥ 85 J/cm² led to a total degradation of RNA (Supplemental Figure 1). These results showed that all mRNA results reflected altered expression in healthy endothelium and not contamination from irradiated EC.

To study the importance of cell-cell contact for the differences in cytokine synthesis, apoptotic or necrotic cells and healthy HMEC-1 were grown separately on opposite sides of a permeable membrane in transwell chambers. Untreated HMEC-1 from the upper chamber were removed and subjected to RNA extraction and qPCR after different periods of co-incubation. In these experiments IL-8 and MCP1 mRNA amounts did not differ from controls, suggesting that any increase in cytokine synthesis after co-incubation is mediated by direct cell-cell contact and not by soluble markers that may be released by the irradiated cells (data not shown).

Next we measured the release of IL-8 and MCP1 protein into the supernatant of HMEC-1 after exposure for three hours to apoptotic or necrotic cells. In accordance with RNA
results, apoptotic and necrotic cells induced a significant increase in IL-8 protein release from HMEC-1. In contrast, only exposure of HMEC-1 to EC that were exposed to UV light at a dose of 85 J/cm² showed a significant increase in protein synthesis (Fig. 4). We also measured the release of IL-8 and MCP1 from cells either exposed to UV-light or treated with TNF-α/CPT. Both conditions did not cause any significant increase in IL-8 or MCP1 protein amount compared to untreated controls (data not shown).

**Phagocytosis of apoptotic or necrotic cells by HMEC-1 and HUVEC**

In the following experiments we tried to determine the fate of apoptotic or necrotic cells that were co-incubated with HMEC-1 or HUVEC. Both cell types rapidly bound and engulfed apoptotic cell corpses or necrotic cell fragments. After three hours exposure of necrotic cells to HMEC-1 fluorescent vesicles containing endothelial fragments were clearly visible inside the cell (Figure 5A). Prolonged co-incubation led to the appearance of fluorescent dye in lysosomes, indicating lysosomal digestion of endothelial cell fragments (Figure 5B). Also apoptotic HMEC-1 were bound and engulfed by untreated HMEC-1 (Figure 5C). Confocal microscopy on HUVEC demonstrated that apoptotic HMEC-1 not only bound to the surface of HUVEC but were rather ingested (Figure 5D). Since both HUVEC and HMEC-1 were able to engulf damaged cells phagocytosis of endothelial debris by healthy endothelial cells seems to represent a general concept in clearance of dead cells.

To further study the effect of engulfment of apoptotic cells on expression of cytokines HMEC-1 were treated with cytochalasin D prior to exposure to damaged cells. Cytochalasin D inhibits polymerization of the cytoskeleton and prevents uptake, but not
the binding, of damaged cells or cell fragments. Pretreatment of HMEC-1 with cytochalasin D (4 µmol/L) did not have any effects on basal mRNA synthesis of HMEC-1, but it reversed the enhanced synthesis of IL-8 and MCP1 transcript in HMEC-1 exposed to apoptotic cells (Figure 6A). We also studied whether different temperatures have any influence on cytokine expression. HMEC-1 were exposed to apoptotic cells for three hours at 4°C, 21°C and 37°C. At low temperatures mRNA expression of IL-8 was reduced compared to 37°C, but there were no differences between controls and cells that bound apoptotic cells. At 21°C a slight increase in IL-8 expression was seen in HMEC-1 exposed to apoptotic cells. The same pattern was seen in MCP1 expression. These data implicate, that the binding of apoptotic cell fragments to the surface of the recipient cell is not sufficient and that functional internalization of apoptotic cells may be required for the induction of inflammatory gene synthesis (Figure 6B).

**Exposure of apoptotic cells to HMEC-1 altered adhesion properties of leucocytes**

Adhesion of neutrophils (>3.9 fold increase, p<0.001) and macrophages (>2.5 fold increase, p<0.001) to HMEC-1 increased significantly after contact with apoptotic cells for 4 hours whereas exposure of HMEC-1 to necrotic or untreated cells did not have any significant effect on neutrophil or macrophage adhesion. We repeated this assay with macrovascular HUVEC and could confirm this finding in that pre-exposure of HUVEC to apoptotic HMEC-1 but not to necrotic or control cells resulted in elevated binding of neutrophils (>2-fold increase, p<0.01) and macrophages (>2.3-fold increase, p<0.001, Figure 7A). Since we could not measure any significant changes in ICAM-1 expression of HMEC-1 exposed to damaged cells, we wondered whether the enhanced binding of...
neutrophils was dependent on the supernatant of the recipient cells or on the cells itself. Therefore, we transferred the supernatant of HMEC-1 that were exposed to apoptotic cells to untreated HMEC-1 and measured adhesion of neutrophils. Surprisingly, the numbers of bound neutrophils on the pretreated EC was much lower compared to the number of neutrophils that adhere to HMEC-1 receiving the preconditioned supernatant. Moreover, the number of neutrophils that bound to the pretreated cells did not differ between controls and HMEC-1 that were exposed to apoptotic cells. In contrast, supernatant of HMEC-1 exposed to apoptotic cells caused an increase in neutrophil-binding as compared to supernatant from controls (Figure 7B).

**CEC from patients increased adhesion of neutrophils to HMEC-1**

To test whether the data we obtained from the *in vitro* experiments may reflect the conditions *in vivo* we incubated HMEC-1 with circulating endothelial cells isolated from patients with ANCA-associated vasculitis or from healthy controls. Adhesion of healthy neutrophils significantly increased (>7.5 fold increase; p<0.001, n=4) under conditions where HMEC-1 were exposed to CEC from patients, whereas CEC from healthy subjects (n=4) or incubation with CD146-coated Dynabeads (n=4) induced a slight and insignificant rise in the number of bound neutrophils (Figure 7C).
Discussion

Since their first description CEC have been used as markers of vascular damage across an ever-widening variety of diseases.\textsuperscript{12} The clinical use of this marker has been demonstrated in a broad range of vascular disorders and technical consensus is currently discussed to permit a more widespread use.\textsuperscript{33} However, the accumulation of CEC in the blood not only serves as an easily accessible diagnostic marker but also represents a high risk factor for the onset of inflammation in the vasculature.

Up to now, we could only speculate about the phenotype of the CEC. It is conceivable that these circulating cells undergo anoikis, a specific type of apoptosis that is caused by detachment of the cell from its supportive matrix.\textsuperscript{34} Apoptosis is a naturally occurring process during normal growth or development and is necessary to remove excess cells from tissue by neighbouring cells or macrophages. The complete removal of apoptotic cells is important because only effective clearance of apoptotic cells prevents proinflammatory responses.\textsuperscript{2,35} In vasculitis, however, markedly elevated numbers of CEC seem to exhaust the existing clearance mechanisms. Few data, if any, shed light on the impact of apoptotic or necrotic endothelial corpses on other cell subsets. In this study, we provide, for the first time, proof of such interactions between apoptotic and necrotic EC and healthy endothelium in vitro. We observed an inflammatory phenotype with elevated level of proinflammatory IL-8 and MCP1 transcripts after exposure to apoptotic cells. These results differ from previous findings where engulfment of apoptotic cells by phagocytes suppresses the onset of inflammation and immune responses through release of anti-inflammatory cytokines.\textsuperscript{36-39} Similarly, non–professional phagocytes like epithelial cells release a set of growth and survival factors.
and promote EC proliferation when exposed to apoptotic cells.\textsuperscript{40} Others, however, have described that engulfment of apoptotic cells by phagocytes results in secretion of pro-inflammatory cytokines.\textsuperscript{41,42} Khan and colleagues studied the influence of oxidised lipoproteins on the release of pro-inflammatory cytokines in macrophages (MΦ) that were exposed to apoptotic cells. Under normal conditions engulfment of apoptotic cells by MΦ suppressed the release of pro-inflammatory cytokines. In contrast, pre-treatment with oxidised lipoprotein prevented this suppression.\textsuperscript{43} Our work lends further support to the hypothesis that apoptotic cells can induce pro-inflammatory reactions under certain conditions. Ongoing apoptosis is a key feature of multi-cellular organisms and a sustained pro-inflammatory reaction would be detrimental. We speculate that anti-inflammatory effects dominate when apoptosis occurs under normal conditions. However, in disorders where high amounts of apoptotic cells are present non-professional phagocytes may trigger pro-inflammatory signals, but these signals are overridden by anti-inflammatory effects in phagocytes. The biological importance of an endothelial pro-inflammatory response after exposure to apoptotic cells certainly deserves further study.

It is tempting to speculate about the effects of IL-8 and MCP1 synthesis by endothelial cells in our model, since both IL-8 and MCP1 are involved in inflammatory diseases. IL-8 plays a role in the activation of monocytes. Induction of IL-8 in healthy endothelium by apoptotic endothelial cells may recruit monocytes to vascular lesions.\textsuperscript{44} MCP1 has been implicated in vasculogenesis, the development of atherosclerotic lesions and in thrombosis.\textsuperscript{45,46} Both chemokines have previously been detected in endothelial cells under various conditions.\textsuperscript{47,48} In our model, exposure of HMEC-1 to apoptotic cells
markedly increased the mRNA expression of IL-8 and MCP1. We also demonstrated that direct contact of apoptotic cells to HMEC-1 is essential for the elevated release of IL-8 and MCP1. The proinflammatory response was accompanied by enhanced binding of neutrophils and macrophages to HMEC-1 and to primary macrovascular HUVEC. However, we were not able to detect any significant changes in expression of the adhesion molecules ICAM-1. This could be explained by the fact that the inflammatory responses in our in vitro model took place in between the first three hours and that transcriptional regulation of ICAM-1 may occur later on. Another possibility may be that the main factor that drives the increased binding of neutrophils is not the endothelial cell itself but its release of chemotactic factors. This hypothesis is supported by our findings that the preconditioned supernatants of HMEC-1 exposed to apoptotic cells triggered enhanced binding of neutrophils to untreated ECs.

Taken together, these results indicate that apoptotic endothelial cells can be involved in leukocyte recruitment and adhesion to healthy endothelium. Surprisingly, exposure of HMEC-1 to necrotic HMEC-1 induced neither increased binding of leukocytes nor release of enhanced amounts of MCP1.

Recent data by Secchiero and colleagues suggest a role for TRAIL and its receptors in mediating cytokine-induced adherence of leukocytes to endothelial cells by selective down-regulation of CCL8 and CXCL10 chemokines and promoting the survival/proliferation of endothelial cells.49,50 TRAIL is structurally related to the TNF-family of cytokines and was described as a major factor for propagating pro-apoptotic signals.51,52 Other studies suggest a role for TRAIL and its receptors in mediating pro-survival signals, possibly via activation of the extracellular-regulated kinase/mitogen-
activated protein kinase (ERK/MAPK) and nuclear factor κB pathways. We were interested to note that prolonged exposure to apoptotic or necrotic EC led to a marked decline in TRAIL mRNA. These data suggest that prolonged exposure provides an anti-inflammatory signal as a negative regulatory mechanism.

Previous studies have demonstrated that endothelial cells phagocytose latex beads, crystals and bacteria but these cells have not been implicated in clearance of apoptotic cells. Here we show, that HMEC-1 as well as HUVEC engulf necrotic and apoptotic endothelial material. Under physiological conditions engulfment of apoptotic cells by healthy endothelium may be of minor importance because this task is performed by macrophages and granulocytes. The situation may be different in vascular diseases, such as ANCA-associated vasculitis or active atherosclerosis, where engulfment of apoptotic cells is delayed or the larger numbers of apoptotic cells may overwhelm the clearance by professional phagocytes. Under these conditions, engulfment of apoptotic endothelial cells by healthy endothelium may represent a last resort to clear apoptotic material from peripheral blood. Finally, the pro-inflammatory response observed here could serve to recruit immune-competent cells to sites of ongoing vascular damage. Our results may well have considerable importance in vascular diseases although this hypothesis needs to be confirmed by further studies.

It may be argued that our findings have limited relevance to the situation in vivo since we incubated healthy endothelium with much higher cell numbers of damaged EC than were normally found in the blood of patients. It must be appreciated, however, that CEC have only been detected in peripheral blood and that cell numbers within vascular lesions may be much higher. Finally, CEC isolated from patients with vasculitis caused
a much higher increase in neutrophil adhesion to co-cultivated HMEC-1 than do apoptotic HMEC-1, although they were applied in much lower concentrations. Therefore, the proinflammatory potential of CEC seems to be much greater than that of experimentally damaged HMEC-1. In this regard, our findings also underscore the need for further analysis of CEC in terms of phenotype and expression of surface markers. Unfortunately, CEC are few in number and it has been difficult to study these cells in detail. We have described mainly necrotic cells in systemic ANCA-associated vasculitis although the distinction between apoptosis and necrosis remains difficult in this setting. Moreover, it cannot be excluded that apoptotic circulating cells undergo secondary necrosis. The lack of markers that permit information as to the origin of CEC (microvascular vs. macrovascular) is also troubling. In summary, we do not have any information about the original condition of the detached cells at the site of damage. Finally, different techniques used for the enrichment of CEC may yield different results. For instance, a recent study described a subpopulation of inflammatory circulating endothelial cells in patients with PR3-ANCA-associated vasculitis that were able to re-adhere to fibronectin-coated wells and to form a monolayer, demonstrating that this specific subpopulation of CEC seems to be neither of an apoptotic nor a necrotic phenotype.

In conclusion, we demonstrate that apoptotic endothelial cells induce IL-8 and MCP1 synthesis in healthy human microvascular endothelial cells and lead to enhanced adherence of leukocytes. Direct cell-cell contact is mandatory for the induction of this response. Finally, we demonstrate engulfment of apoptotic and necrotic endothelial material by healthy human endothelium and that engulfment, rather than binding of the
damaged cells, is a prerequisite for the inflammatory responses. These findings may provide an important mechanism by which inflammatory circulating endothelial cells gain pathogenetic importance locally or distant from sites of injury. Interactions of circulating endothelial cells with healthy endothelium warrant further studies and the importance of our findings in vivo remains to be elucidated.
References


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Figure Legends

Figure 1. UV light induces ROS generation and apoptosis in HMEC-1. A, exposure to different doses of UV light enhances generation of ROS as determined by DHE staining. B, UV light induces a dose-dependent activation of caspase 3/7 and a decrease in internal ATP content. Both parameters were measured three hours after irradiation. C, increasing amount of UV light triggers fragmentation of nuclear DNA as demonstrated by TUNEL staining 24 hours after irradiation. Necrosis occurs at high doses of UV light as seen by annexinV/propidiumiodid-staining in the lower right micrograph. Bars represent 50 µm.

Figure 2. HMEC-1 exposed to apoptotic or necrotic EC show altered expression of IL-8, MCP1 and TRAIL transcripts. HMEC-1 were incubated with apoptotic or necrotic EC for different time periods and mRNA level of IL-8, MCP1 and TRAIL were determined by real-time qPCR. Data are given as mean ± SD of five independent experiments.

Figure 3. Enhanced expression of IL-8 and MCP1 is independent of the method of apoptosis induction. Apoptosis was induced in HMEC-1 either by UV irradiation or by treatment with a combination of TNF-α (3 nmol/L) and CPT (0.15 µmol/L) for 24 hours. Healthy HMEC-1 were exposed to the apoptotic cells for three hours and mRNA level of IL-8 and MCP1 were determined by real-time qPCR. Data are given as mean ± SD of five independent experiments.
Figure 4. Exposure to apoptotic EC resulted in enhanced release of IL-8 and MCP1 protein. HMEC-1 were incubated with apoptotic or necrotic EC for three hours and the release of IL-8 and MCP1 protein into the supernatant was measured by ELISA. Data are given as mean ± SD of five independent experiments (* p< 0.01; ** p<0.001).

Figure 5. Engulfment of apoptotic or necrotic EC by HMEC-1 and HUVEC. A, exposure of HMEC-1 to necrotic HMEC-1 for three hours resulted in engulfment of labelled cell fragments. B, after six hours of co-incubation phagocytosed particles appear in the lysosomes around the nucleus (arrows). C, CM-Dil labelled HUVEC were incubated with CMFDA-labelled apoptotic HMEC-1. After 1 hour apoptotic cells were engulfed by HUVEC (white arrows). The yellow arrow indicates an apoptotic cell that binds to the surface of a HUVE cell. D, CM-Dil stained HMEC-1 were exposed for 2.5 hours to apoptotic HMEC-1. The micrograph shows a confocal image of a cell engulfing an apoptotic (green) cell. A cross section of the whole cell is shown in the micrograph at the bottom. Bars represent 50 µm (A-C) or 20 µm (D).

Figure 6. Engulfment of apoptotic cells by HMEC-1 is necessary for enhanced synthesis of IL-8 and MCP1 mRNA. A, HMEC-1 were exposed to untreated or apoptotic cells for three hours and IL-8 and MCP1 expression was measured by real-time qPCR. Pretreatment with Cytochalasin D (4 µmol/L) reversed the enhanced synthesis of IL-8 and MCP1 to the level of the controls. B, at temperatures where the internalization of bound cells is prevented, apoptotic cells failed to induce enhanced expression of IL-8 and MCP1 transcript. Data are given as mean ± SD of three independent experiments.
Figure 7. Apoptotic cells trigger enhanced binding of leukocytes to endothelial cells. A, exposure of HMEC-1 and HUVEC to apoptotic HMEC-1 for four hours increased adhesion of PMN and MΦ whereas exposure to necrotic cells failed to induce enhanced binding of leukocytes to HMEC-1. HUVEC co-incubated with necrotic HMEC-1 showed enhanced binding of MΦ but not of PMN. B, the supernatant of HMEC-1 that were exposed to apoptotic EC is sufficient to promote increased binding of neutrophils to HMEC-1 whereas the pretreated cells alone do not show enhanced binding of neutrophils. C, incubation of HMEC-1 with CEC isolated from patients with PR3-ANCA-associated vasculitis leads to a significant increase in adhesion of neutrophils. MΦ: primary blood mononuclear cells (monocytes/macrophages), PMN: polymorphonuclear cells (neutrophils). Data represent mean ± SEM of five (A,C) or three (B) independent experiments. Mann-Whitney U test was performed to compare differences in leukocyte adhesion (*p< 0.01; **p<0.001).
Fig. 2

IL-8

MCP1

TRAIL
Fig. 3
Fig. 4

**IL-8**

**MCP1**
Fig. 6

A

IL-8

MCP1

 Cytochalasin D

untreated

+ HMEC

+ apopt. cells

 B

IL-8

MCP1

4°C

21°C

37°C

4°C

21°C

37°C
Fig. 7

A

HMEC-1

H U V E C

B

C

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Engulfment of apoptotic cells by microvascular endothelial cells induces proinflammatory responses

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