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Induction of Apoptosis in Circulating Angiogenic Cells by Microparticles

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Objective. **Systemic sclerosis (SSc) is an autoimmune disease marked by aberrant activation and apoptosis of endothelial cells (ECs) and decreased numbers of circulating angiogenic cells (CACs). The aim of this study was to analyze whether microparticles might link pathologic activation and apoptosis of ECs with reduced numbers of CACs.**

Methods. **Apoptosis was quantified by staining for annexin V and measurement of caspase 3 activity. The uptake of microparticles by CACs was determined by fluorescence-activated cell sorting and by fluorescence microscopy. Tritiated arachidonic acid and phosphatidylinositol 3,5-bisphosphate were used to demonstrate the transfer of arachidonic acid and highlight the role of the acid sphingomyelinase in microparticle-induced apoptosis of endothelial progenitor cells.**

Results. **Microparticles derived from activated or apoptotic ECs, the expression of which is strongly increased in the blood of patients with SSc, induce apoptosis in CACs in a dose-dependent manner. Microparticles, which are rich in arachidonic acid, are phagocytosed by CACs. Inhibition of phagocytosis prevents the induction of apoptosis in CACs by microparticles. Microparticles can transport arachidonic acid from ECs to CACs, and purified arachidonic acid mimics the proapoptotic effects of microparticles. Arachidonic acid activates the acid sphingomyelinase, and inhibition of acid sphingomyelinase prevents microparticle-induced apoptosis of CACs. Thus, phagocytosis of microparticles might stimulate the activity of acid sphingomyelinase and activate the apoptotic machinery.**

Conclusion. **The induction of apoptosis in CACs by microparticles derived from ECs provides a novel link between aberrant activation or apoptosis of ECs, decreased numbers of CACs, and impaired formation of new vessels in SSc.**

Microangiopathy is a prominent pathologic event in a variety of clinical conditions and can result from endothelial cell (EC) death as well as impairment in the number or function of circulating angiogenic cells. Among conditions in which microangiopathy promotes pathogenesis, systemic sclerosis (SSc) is an autoimmune disease of unknown etiology that affects the skin and a variety of internal organs such as the heart, lungs, and gastrointestinal tract. Apoptosis of microvascular ECs is one of the first pathologic changes in SSc (1) and is followed by progressive vasculopathy of the small arteries and capillaries (2,3).

Despite several proangiogenic stimuli such as hypoxia (4), vascular endothelial growth factor (VEGF) (5–7), and monocyte chemotactic protein 1 (8), angiogenesis is insufficient in patients with SSc. Instead, a progressive loss of capillaries occurs, resulting in reduced blood flow and tissue ischemia. Clinically, the progressive vasculopathy frequently results in fingertip

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ulcers and strongly contributes to the morbidity of patients with SSc (9,10).

Angiogenesis requires the interplay of various cell types, among which circulating angiogenic cells (CACs) appear to be of particular interest. These cells were formerly considered as a subpopulation of endothelial progenitor cells (EPCs) named "early outgrowth EPCs" and are now regarded as a distinct (progenitor) cell type (11). In contrast to "true" late outgrowth EPCs, it is controversial whether CACs differentiate into mature ECs and thus contribute directly to vasculogenesis (12). However, there is robust evidence that CACs are potent circulating regulators of angiogenic responses in ischemic tissues, and they also contribute to the repair of damaged preexisting vessels (13,14).

Consistent with the roles of CACs in vessel formation and vascular repair, the number of CACs in the blood of healthy individuals is relatively low under normal circumstances but increases in response to tissue ischemia or vascular damage (14,15). In patients with SSc, in whom prominent vascular damage and tissue hypoxia are present, an increase number of CACs would be expected. However, although the number of "true" EPCs in the blood of patients with SSc appears to be increased (16,17), the number of CACs in the blood of these patients seems to be significantly decreased (7,18). Recent data showed that sera from patients with SSc are able to induce profound apoptosis in CACs via the Akt/forkhead transcription factor FKHRL1/Bim pathway (18). However, the mediators inducing apoptosis of CACs in SSc sera as well as the link between EC damage and reduced numbers of CACs in SSc are currently unknown.

Microparticles are small, membrane-bound structures that are released from cells by exocytic budding of the membrane during activation or apoptosis (19). During the budding process, the normal membrane asymmetry is lost, and phosphatidylserine appears on the outer leaflet of the microparticle membrane (20). In addition to displaying altered surface lipids, microparticles display cell surface markers from the parental cell from which they originate. Microparticles have important functions in intercellular communication (21). Microparticles not only represent a concentrated source of immunologically active molecules but also have been shown to transport cell surface receptors and lipids between different cell types (19). Via these mechanisms, microparticles have a profound impact on cells to regulate diverse processes such as inflammation, coagulation, antigen presentation, and apoptosis (19).

We recently showed that microparticles derived from ECs are significantly up-regulated in the blood of patients with SSc (22). Given the potential of microparticles to induce apoptosis (23), we investigated in the present study whether microparticles might contribute to reduced numbers of CACs, as may occur in SSc. The data presented herein indicate that microparticles can induce apoptosis in CACs by a mechanism involving arachidonic acid. Taken together with previous results, these findings provide evidence for a link between EC death and reduced numbers of CACs in SSc and possibly other vascular diseases.

PATIENTS AND METHODS

Isolation and culture of CACs. Peripheral blood mononuclear cells were isolated from patients with SSc (n = 10) and healthy volunteers (n = 13) by the Ficoll density-gradient method, as previously described (24). All patients fulfilled the criteria for SSc as proposed by LeRoy and Medsger (25). The clinical characteristics of the patients were as follows: 8 women and 2 men, mean age 42 years (range 22–56 years), 7 patients with limited cutaneous SSc, 3 patients with diffuse cutaneous SSc, mean disease duration 6 years (range 1–15 years), no treatment with disease-modifying antirheumatic drugs or vasoactive drugs, and no history of smoking, arterial hypertension, or hyperlipidemia. After isolation, cells were cultured on fibronectin-coated culture dishes (10 μg/ml; Sigma-Aldrich) in endothelial basal medium 2 (Cambrex) supplemented with 5% fetal bovine serum, VEGF-A, human fibroblast growth factor 2, human epidermal growth factor, insulin-like growth factor 1, ascorbic acid, heparin, and hydrocortisone to promote endothelial differentiation. After 36 hours, nonadherent cells were replated on fibronectin-coated culture dishes and cultured in EGM-2 medium for an additional 4 days before analysis.

Staining of CACs. The phenotype of CACs was confirmed by double-staining with Dil-labeled acetylated low-density lipoprotein (Ac-LDL) and fluorescein isothiocyanate (FITC)–labeled *Ulex europaeus* agglutinin type I (lectin) (26). CACs were incubated with Ac-LDL (2.4 gm/ml; Invitrogen) for 3 hours and subsequently fixed with 4% paraformaldehyde for 20 minutes. The cells were incubated for 1 hour with lectin (10 μg/ml; Sigma-Aldrich). Three randomly selected high-power fields were evaluated by fluorescence microscopy using a Nikon Eclipse 80i microscope equipped with a digital camera. Only cells with double-positive staining for Ac-LDL and lectin were counted as CACs.

Stimulation of the release of microparticles. Apoptosis in primary human microvascular ECs (HMVECs; Invitrogen), immortalized HMVECs (27), and immortalized murine glomerular ECs (28) was induced by incubation with staurosporine (Sigma-Aldrich) at a concentration of 10 μM. After 12 hours, supernatants were collected, and microparticles were isolated by differential centrifugation as previously described (23,29,30). For coculture experiments, CACs were incubated for 4 days with freshly isolated microparticles (1.0×10^4 to 1.0×10^5) derived from apoptotic ECs. After 2 days, the supernatant was removed, and fresh medium containing the same number of microparticles was added.

Quantification of microparticles. Microparticles derived from ECs were identified by fluorescence-activated cell sorting (FACS) after staining for annexin V and the EC markers CD144 (Serotec) and CD31 (BD PharMingen). Staining with isotype antibodies and annexin V in the absence of calcium was used as control. Microparticles were defined as vesicles positive for annexin V and surface markers of ECs. The numbers of microparticles were determined using a FACSCalibur flow cytometer (BD PharMingen).

Caspase 3 activity assay. The activities of caspase 3–like proteases were determined using the EnzChek Caspase-3 Assay Kit (Invitrogen) as previously described (23).

Quantification of apoptotic cells. The number of apoptotic and necrotic CACs upon incubation with microparticles was analyzed by FACS after staining for annexin V (31). After trypsinization, CACs were washed twice with ice-cold phosphate buffered saline (PBS) containing 5 m*M* $CaCl_2$ and incubated with FITC–labeled annexin V (Becton Dickinson). Cells incubated with FITC-labeled annexin V in PBS in the absence of calcium served as controls. The cells were analyzed using a BD FACSCalibur flow cytometer (Becton Dickinson).

Analysis of microparticle phagocytosis. The uptake of microparticles by CACs was analyzed by 2 different techniques, FACS and fluorescence microscopy. For both approaches, microparticles were labeled with the fluorescent membrane cell linker PKH26 (Sigma-Aldrich). Labeled microparticles were coincubated with CACs for 2 hours at 37°C. For FACS analysis, the percentage of CACs containing microparticles was determined as the percentage of PKH26-positive cells in the FL2 channel. For fluorescence microscopy, CACs were labeled with Ac-LDL and lectin and incubated with PKH26-stained microparticles. In a subset of experiments, coincubation of CACs with microparticles was performed at 4°C. In other experiments, CACs were preincubated with cytochalasin D (Sigma-Aldrich) at a concentration of 20 μM. CACs cultured at 37°C without cytochalasin D and microparticles were used as controls.

Stimulation of CACs with arachidonic acid. CACs were stimulated with arachidonic acid (Sigma-Aldrich) in concentrations from 25 n*M* to 250 n*M* for 24 hours, 48 hours, or 4 days. The concentrations used in our study represent pathophysiologically relevant concentrations, because they cover the mean levels of arachidonic acid stored within microparticles in the blood of patients with SSc and healthy volunteers (22,30).

Labeling of ECs and microparticles derived from ECs and analysis of the transport of tritiated arachidonic acid. ECs were labeled with tritiated arachidonic acid. ECs were incubated with 1 μCi tritiated arachidonic acid for 24 hours at 37°C. Extensive washing was performed to remove unincorporated tritiated arachidonic acid. The radioactivity of unstimulated cells labeled with tritiated arachidonic acid was defined as the total radioactivity incorporated into ECs and was measured using a Canberra Packard 2500 TR Liquid Scintillation Analyzer and Rotiszint scintillation fluid (Roth).

Inhibition of acid sphingomyelinase. The activity of acid sphingomyelinase was inhibited using phosphatidylinositol 3,5-bisphosphate, a specific inhibitor of acid sphingomyelinase (32,33). CACs were incubated with phosphatidylinositol 3,5-bisphosphate in concentrations of 10 μM for 1 hour.

Statistical analysis. Data are expressed as the mean ± SEM. Wilcoxon's signed rank test for related samples was used for statistical analyses. *P* values less than 0.05 were considered significant.

RESULTS

Microparticle-mediated decrease in the numbers of CACs. Apoptosis of ECs is the initiating step in the vascular pathogenesis of SSc, and the number of microparticles from apoptotic ECs is strongly increased in the blood of patients with SSc (1,22). To analyze whether microparticles derived from apoptotic ECs affect the number of CACs, CACs from patients with SSc and healthy volunteers were coincubated with microparticles for 4 days. The number of microparticles used for these experiments was based on the numbers of microparticles in the blood of patients with SSc and has also been used for other studies (22,23,29,30).

At baseline, the mean ± SEM number of CACs was reduced by 33 ± 10% in patients with SSc compared with healthy volunteers ($P = 0.04$). In the presence of microparticles, a significant dose-dependent decrease in the number of CACs was observed. Coincubation of CACs from patients with SSc with 1.0×10^4 microparticles derived from apoptotic ECs decreased the mean ± SEM number of LDL/lectin double-positive cells to 56 ± 14% compared with controls ($P < 0.05$) (Figure 1A). Upon coincubation with 1.0×10^5 microparticles, a further reduction in the mean ± SEM number of CACs to 35 ± 12% was observed ($P < 0.05$). Similarly, coincubation with microparticles dose-dependently decreased the numbers of CACs derived from healthy individuals. In the presence of 1.0×10^4 or 1.0×10^5 microparticles from apoptotic ECs, the mean ± SEM percentages of CACs were reduced to 56 ± 9% and 33 ± 13%, respectively (both $P < 0.05$) (Figure 1A). No differences were observed in the activity between microparticles derived from immortalized murine glomerular ECs, immortalized HMVECs, and primary HMVECs (results not shown).

Microparticle-induced apoptosis in CACs. To investigate whether the decreased number of CACs upon coincubation with microparticles is attributable to apoptosis, the activity of caspase 3 was determined. In CACs cultured in the absence of exogenous microparticles, caspase 3 activity was measured as 711 ± 89 (mean ± SEM) arbitrary fluorescence units (AFUs). The activity of caspase 3 in cellular extracts of CACs cocultured with 1.0×10^5 microparticles for 4 days was significantly increased to 1,528 ± 137 AFUs ($P < 0.05$),

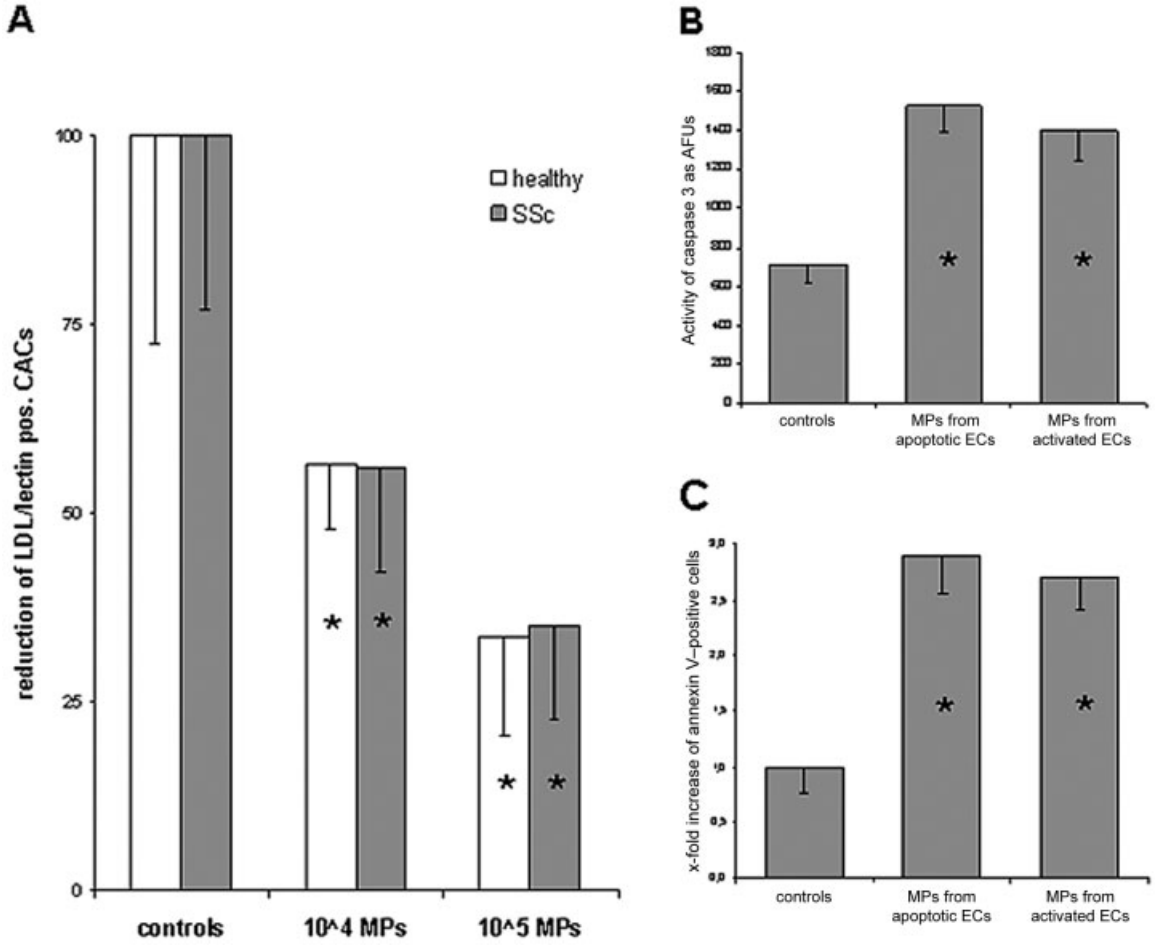


Figure 1. Incubation with microparticles (MPs) decreases the numbers of circulating angiogenic cells (CACs) and induces apoptosis. **A,** Incubation of CACs with microparticles derived from apoptotic endothelial cells (ECs) resulted in a dose-dependent decrease in the number of low-density lipoprotein (LDL)/lectin double-positive (pos.) CACs in patients with systemic sclerosis (SSc) and healthy individuals. Bars show the mean ± SEM results from 10 patients with SSc and 13 controls. **B,** Incubation of CACs with microparticles derived from apoptotic ECs resulted in a significant increase in the activity of caspase 3, measured as arbitrary fluorescence units (AFUs). Bars show the mean ± SEM results from 5 independent experiments. **C,** Incubation of CACs with microparticles derived from apoptotic ECs increased the numbers of annexin V–positive apoptotic CACs, as analyzed by fluorescence-activated cell sorting (n = 5). Bars show the mean ± SEM results from 5 independent experiments. $* = P < 0.05$ versus control.

suggesting that microparticles from apoptotic ECs induce apoptosis in CACs (Figure 1B). To confirm the induction of apoptosis in CACs by microparticles on an additional experimental level, the number of annexin V–positive CACs was determined upon incubation with 1.0×10^5 microparticles. Consistent with the increased activity of caspase 3, incubation with microparticles dose-dependently increased the number of annexin V–positive cells by a mean ± SEM of 293 ± 35% compared with controls ($P < 0.05$) (Figure 1C). Significant increases in the activity of caspase 3 and in the number of annexin V–positive cells were also observed after 48 hours. Thus, incubation of CACs with microparticles significantly increased the numbers of primary apoptotic CACs. Microparticle-induced apoptosis in CACs in turn resulted in the release of annexin V–positive particles from CACs. The number of microparticles released from apoptotic CACs was ~30% lower than the number released from U937 cells.

Besides apoptosis, activation of ECs is also prominent in SSc. We therefore investigated whether microparticles derived from activated ECs are also able to

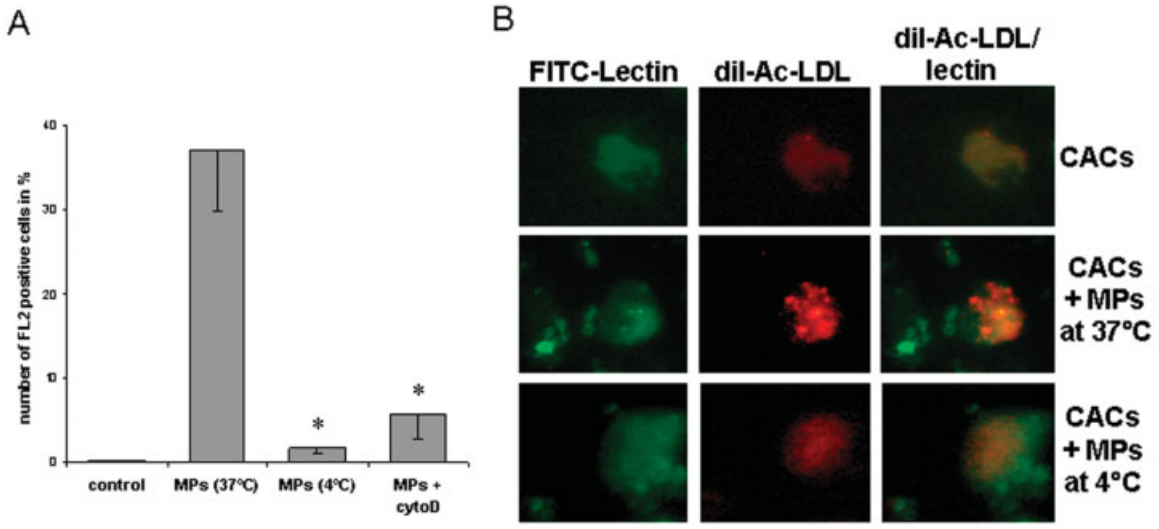


Figure 2. Phagocytosis of microparticles (MPs) by circulating angiogenic cells (CACs). **A,** Direct interaction of microparticles with CACs. To analyze whether microparticles interact physically with microparticles and whether microparticles are taken up by CACs, CACs were incubated with labeled microparticles at 37°C or at 4°C, the temperature at which active ingestion is inhibited. Microparticles were prelabeled with PKH26, a red fluorescent membrane dye, which can be detected in the FL2 scatter by fluorescence-activated cell sorting analysis. Free microparticles in the supernatant were removed by extensive washing. The uptake of microparticles observed at 37°C was almost completely blocked by incubation at 4°C. Cytochalasin D (cytoD), an inhibitor of phagocytosis, also significantly reduced the ingestion of microparticles. Bars show the mean ± SEM results from 6 independent experiments. $* = P < 0.05$ versus control. **B,** Uptake of microparticles by CACs, as demonstrated by fluorescence microscopy. Microparticles were stained with the membrane marker PKH26 (bright red). CACs were identified by double-staining with fluorescein isothiocyanate (FITC)/lectin (green) and Dil-labeled acetylated low-density lipoprotein (Ac-LDL) (light red). Microparticles that were not phagocytosed by CACs were removed by extensive washing. At 37°C, bright red signals for microparticles were detected in Ac-LDL/lectin double-positive CACs. In contrast, no microparticles were detected in CACs when incubation was performed at 4°C, suggesting that the uptake of microparticles by CACs occurs by active phagocytosis.

induce apoptosis in CACs. Indeed, the induction of apoptosis in CACs was not restricted to microparticles derived from apoptotic ECs but was also observed to a comparable degree with microparticles derived from tumor necrosis factor α–stimulated ECs (Figures 1B and C). In contrast to CACs, no induction of apoptosis was observed in ECs incubated with up to 10^5 microparticles.

We next analyzed whether EC-derived microparticles purified from the peripheral blood of patients with SSc can cause apoptosis of CACs, and whether EC-derived microparticles from patients with SSc are functionally different from other EC-derived microparticles. We isolated microparticles derived from the ECs of SSc patients with fingertip ulcers, SSc patients without fingertip ulcers who had clinically mild vascular disease, patients with primary Raynaud's phenomenon, and healthy control subjects (n = 8 per group). Consistent with previous reports, the number of microparticles was increased in patients with SSc, with a trend toward higher numbers in patients with fingertip ulcers (22). No differences were observed between patients with primary Raynaud's phenomenon and healthy control subjects. However, after adjusting for the different number of microparticles, the potency to induce apoptosis in CACs did not differ between the groups. Comparable, dose-dependent induction of apoptosis was observed with microparticles from SSc patients with fingertip ulcers, SSc patients without fingertip ulcers and clinically mild vascular disease, patients with primary Raynaud's phenomenon, and healthy control subjects.

Phagocytosis of microparticles by CACs. We previously showed that in contrast to fibroblasts, macrophages take up microparticles (23,29,32). To investigate whether CACs can take up microparticles as well, and whether this is an active process, CACs were incubated with PKH26-labeled microparticles at 37°C or at 4°C for 2 hours. Then, the percentage of CACs containing PKH26-labeled microparticles was determined by FACS analysis. After extensive washing to remove free microparticles, 37 ± 7% (mean ± SEM) of all LDL/lectin-positive CACs incubated at 37°C stained positive for PKH26 ($P < 0.05$) (Figure 2A). When CACs were

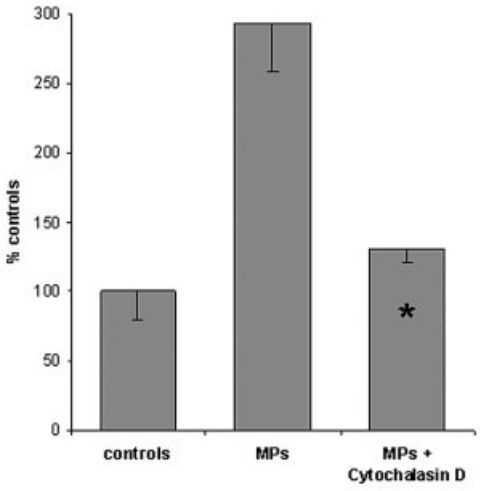


Figure 3. Inhibition of microparticle phagocytosis prevents the induction of apoptosis. When CACs were preincubated with cytochalasin D, an inhibitor of phagocytosis, the induction of apoptosis by microparticles in CACs was significantly blunted, suggesting that phagocytosis of microparticles is crucial for their proapoptotic effects. Bars show the mean ± SEM results from 5 independent experiments. * = $P < 0.05$ versus CACs incubated with microparticles in the absence of cytochalasin D. See Figure 2 for definitions.

cocultured with microparticles from apoptotic ECs at 4°C, the temperature at which active uptake but not passive attachment is inhibited (34), the number of CACs that stained positively for PKH26 was strongly reduced to 2 ± 1% ($P < 0.05$ versus 37°C). Similar to the results obtained at 4°C, the number of PKH26-positive cells was much lower when CACs were preincubated with cytochalasin D, an inhibitor of phagocytosis. In the presence of cytochalasin D at 37°C, the number of PKH26-positive CACs was reduced to only 6 ± 3%, confirming that microparticles are taken up by phagocytosis (Figure 2A).

To directly visualize the uptake of microparticles by CACs, fluorescence microscopy was performed using CACs stained with LDL and lectin and microparticles labeled with PKH26. After 60 minutes of coincubation at 37°C, microparticles were incorporated into CACs, as shown by intense localized PKH26 staining inside of LDL/lectin double-positive cells (Figure 2B). Consistent with the data obtained by FACS analysis, no PKH26 staining of microparticles was detectable in CACs at 4°C (Figure 2B). Similar results were obtained when CACs were preincubated with cytochalasin D at 37°C. Consistent with this observation, no microparticles were observed to be associated with CACs in the presence of cytochalasin D (data not shown), suggesting the microparticles are taken up by CACs via phagocytosis.

Prevention of apoptosis in CACs by inhibiting phagocytosis of microparticles. We observed that CACs undergo apoptosis upon cocultivation with microparticles, and that microparticles are phagocytosed by CACs. To investigate whether phagocytosis of microparticles is essential for the induction of apoptosis, phagocytosis of microparticles was inhibited by cytochalasin D. Cytochalasin D did not alter the basal rate of apoptosis (data not shown) but strongly reduced the induction of apoptosis in CACs by microparticles. When CACs were cocultured with microparticles in the presence of cytochalasin D, the number of annexin V–positive apoptotic cells decreased from a mean ± SEM of 293 ± 35% to 131 ± 10% ($P < 0.05$ versus control) (Figure 3).

Arachidonic acid mimics the proapoptotic effects of microparticles. Microparticles have been shown to contain high concentrations of arachidonic acid (19,35). To investigate whether microparticles might activate the apoptosis in CACs via the arachidonic acid pathway, CACs were incubated with purified arachidonic acid for 48 hours. Arachidonic acid induced apoptosis in CACs in a dose-dependent manner (Figure 4). At concentrations of 25 n*M* and 50 n*M*, arachidonic acid increased the numbers of CACs positive for annexin V by a mean ± SEM of 1.7 ± 0.2-fold and 4.2 ± 0.9-fold, respectively, compared with controls ($P < 0.05$ for 50 n*M*). The most

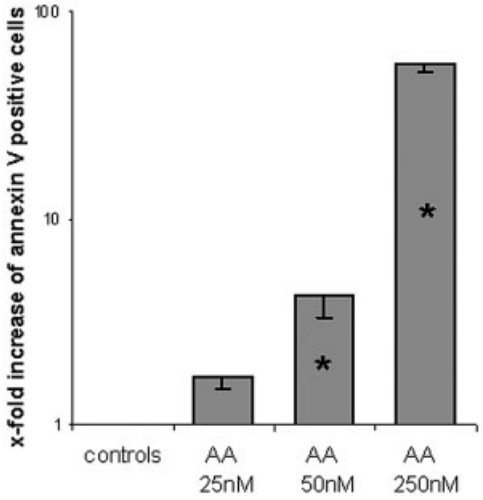
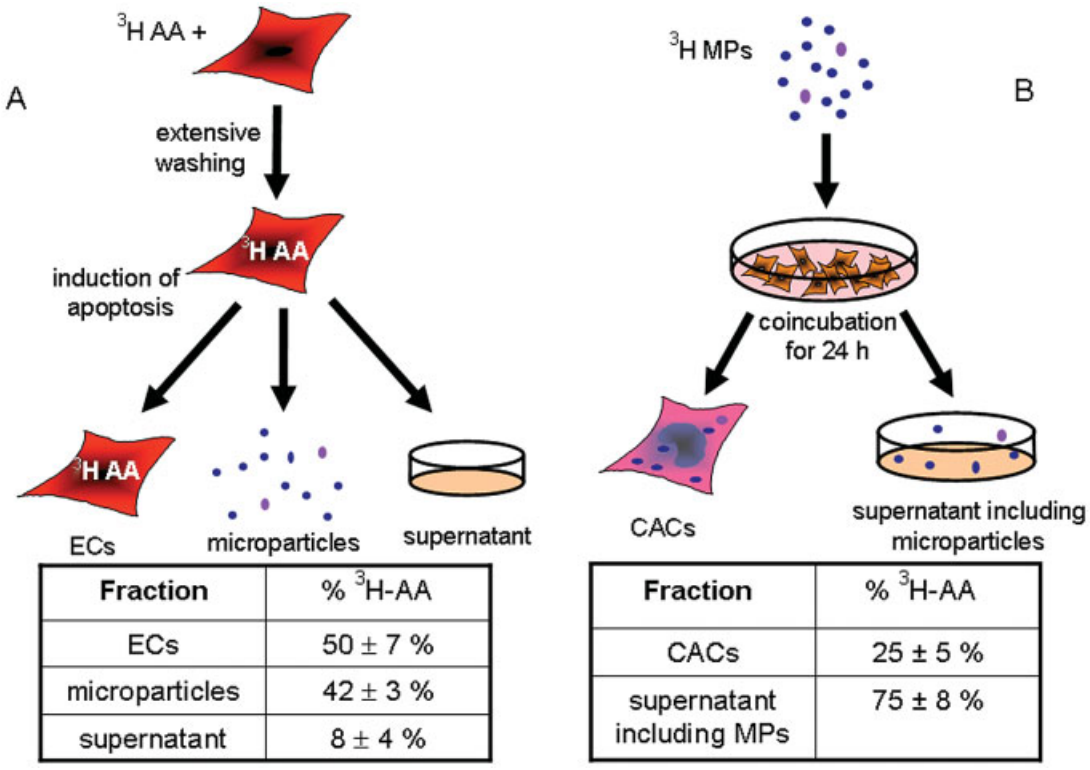


Figure 4. Dose-dependent induction of apoptosis in circulating angiogenic cells (CACs) by arachidonic acid (AA). To investigate whether the induction of apoptosis by microparticles occurs via arachidonic acid–dependent pathways, CACs were incubated with purified arachidonic acid. Bars show the mean ± SEM results from 5 independent experiments. * = $P < 0.05$ versus control.



Fraction	% ^{3}H-AA
ECs	50 ± 7 %
microparticles	42 ± 3 %
supernatant	8 ± 4 %

Fraction	% ^{3}H-AA
CACs	25 ± 5 %
supernatant including MPs	75 ± 8 %

Figure 5. Microparticles transport arachidonic acid from endothelial cells (ECs) to circulating angiogenic cells (CACs). ECs were labeled with tritiated arachidonic acid (^{3}H AA) and then incubated with staurosporine to stimulate the release of microparticles. Microparticles were isolated by differential centrifugation, and radioactivity was determined in ECs, microparticles, and the supernatant. **A,** A large amount of the total radioactivity was observed in the microparticle fraction. **B,** When microparticles labeled with tritiated arachidonic acid (^{3}H MPs) were incubated with CACs, a significant portion of tritiated arachidonic acid was observed in CACs. Values are the mean ± SEM results from 4 independent experiments. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131.

pronounced effects were observed at a concentration of 250 n*M*, with a mean ± SEM increase in the numbers of apoptotic CACs of 55.8 ± 4.6-fold ($P < 0.05$) (Figure 4). These concentrations of arachidonic acid are in the range of arachidonic acid stored in microparticles in the blood of patients with SSc and control subjects (22,30). A dose-dependent induction of apoptosis in CACs by arachidonic acid was also observed after 24 hours and after 4 days.

Transport of arachidonic acid from ECs to CACs by microparticles. Following the observation that arachidonic acid mimics the proapoptotic effects of microparticles on CACs, we investigated whether microparticles can transfer arachidonic acid to CACs. When immortalized murine glomerular ECs labeled with tritiated arachidonic acid were stimulated with staurosporine to induce the release of microparticles, a mean ± SEM of 42 ± 3% of the total radioactivity was observed within the microparticle fraction (Figure 5A). This finding confirmed that a significant proportion of cellular arachidonic acid is incorporated into microparticles. Of the remaining tritiated arachidonic acid, 50 ± 7% was observed in the cellular fraction, and 8 ± 4% of the total tritiated arachidonic acid was detected in the microparticle-free supernatant (Figure 5A).

To demonstrate the transfer of arachidonic acid from microparticles to CACs, microparticles labeled with tritiated arachidonic acid were incubated with CACs. After 24 hours, a mean ± SEM of 25 ± 5% of the initial radioactivity from tritiated arachidonic acid–labeled microparticles was detected in CACs, and 75 ± 8% was detected in the supernatant (containing both microparticle-associated tritiated arachidonic acid and free tritiated arachidonic acid) (Figure 5B). These findings demonstrated that microparticles derived from ECs can deliver arachidonic acid to CACs. Similar results

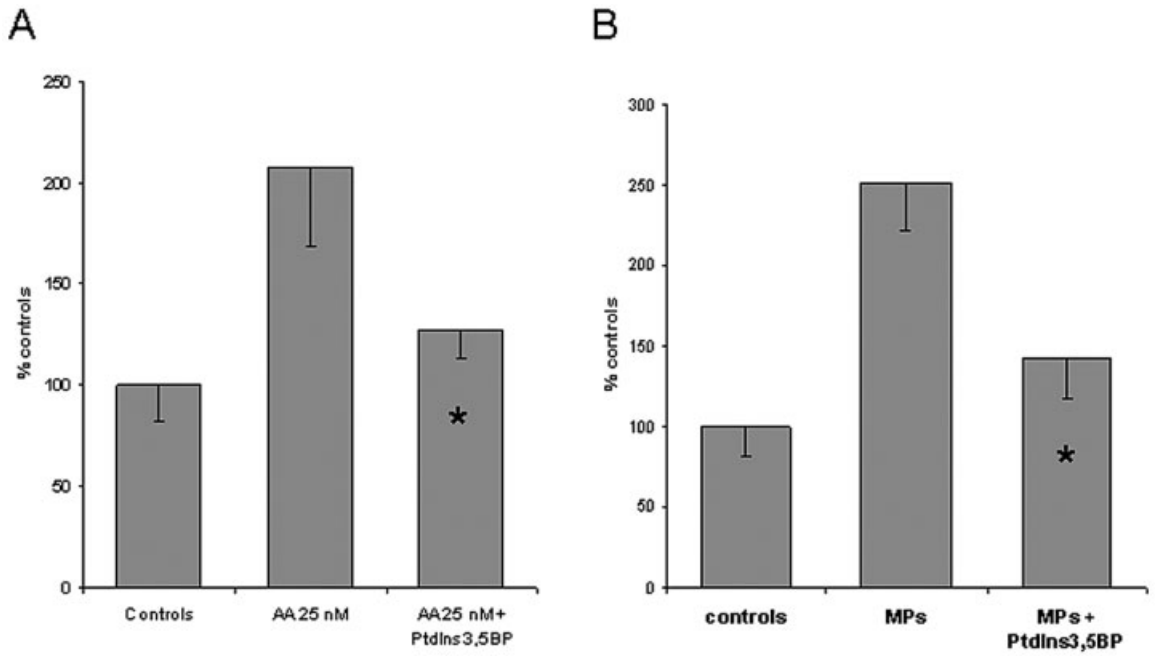


Figure 6. Inhibition of acid sphingomyelinase reduces microparticle-induced apoptosis in CACs. Preincubation with phosphatidylinositol 3,5-bisphosphate (PtdIns3,5BP), a specific inhibitor of acid sphingomyelinase, significantly decreased the proapoptotic effects of **A,** purified arachidonic acid (AA) and **B,** microparticles. Bars show the mean ± SEM results from 5 experiments. $* = P < 0.05$ versus controls. See Figure 2 for other definitions.

were obtained using microparticles derived from immortalized HMVECs and primary HMVECs.

Prevention of microparticle-induced apoptosis by inhibition of acid sphingomyelinase. Acid sphingomyelinase catalyzes the metabolism of sphingomyelin into proapoptotic ceramides (36). Incubation with microparticles increased the messenger RNA levels of acid sphingomyelinase in CACs by a mean ± SEM of 2.3 ± 0.3-fold ($P = 0.03$). Consistently, the activity of acid sphingomyelinase was also stimulated by 5.2 ± 0.3-fold ($P = 0.05$).

To further investigate the role of acid sphingomyelinase for the induction of apoptosis in CACs, we used phosphatidylinositol 3,5-bisphosphate, an inhibitor of acid sphingomyelinase (33). To demonstrate that phosphatidylinositol 3,5-bisphosphate can prevent the induction of apoptosis by arachidonic acid, CACs were preincubated with 10 μM phosphatidylinositol 3,5-bisphosphate before treatment with arachidonic acid. Phosphatidylinositol 3,5-bisphosphate did not affect the basal rate of apoptosis in untreated CACs. However, phosphatidylinositol 3,5-bisphosphate significantly reduced the induction of apoptosis upon treatment with arachidonic acid. Pretreatment with phosphatidylinositol 3,5-bisphosphate completely blocked the induction of apoptosis upon treatment with 25 n*M* arachidonic acid and reduced the mean ± SEM numbers of annexin V–positive CACs from 2.1 ± 0.4-fold to 1.3 ± 0.2-fold compared with controls ($P < 0.05$) (Figure 6A). Accordingly, phosphatidylinositol 3,5-bisphosphate strongly inhibited the proapoptotic effects of 250 n*M* arachidonic acid and decreased the mean ± SEM number of apoptotic cells from 51.6 ± 8.9-fold to 16.7 ± 1.6-fold as compared with controls ($P < 0.05$). Inhibition of acid sphingomyelinase by phosphatidylinositol 3,5-bisphosphate also decreased the number of secondary necrotic, annexin V/phosphatidylinositol double-positive cells from 6.5 ± 1.8-fold to 1.0 ± 0.1-fold ($P < 0.05$) at a concentration of 25 n*M* arachidonic acid (data not shown) and from 419 ± 100-fold to 165 ± 76-fold ($P < 0.05$) at a concentration of 250 n*M* arachidonic acid.

To investigate whether activation of acid sphingomyelinase plays a role in the induction of apoptosis by microparticles, CACs were pretreated with phosphatidylinositol 3,5-bisphosphate before coincubation with microparticles. Pretreatment of CACs with phosphatidylinositol 3,5-bisphosphate significantly reduced the induction of apoptosis in CACs by microparticles from a

mean ± SEM of 2.5 ± 0.3 to 1.4 ± 0.3 ($P < 0.05$) (Figure 6B). Similarly, preincubation with phosphatidylinositol 3,5-bisphosphate reduced the number of annexin V/phosphatidylinositol double-positive CACs from 5.3 ± 0.7 to 2.9 ± 0.3 ($P < 0.05$) (data not shown). These data suggest that conversion of arachidonic acid to ceramides by acid sphingomyelinase plays an important role in the induction of apoptosis by microparticles in CACs. In contrast to the inhibition of acid sphingomyelinase, inhibition of cyclooxygenase 2 (COX-2) by NS-398 in concentrations up to 10 μM or inhibition of 5-lipoxygenase by MK-886 in concentrations up to 1 μM did not prevent the proapoptotic effects of microparticles on CACs.

DISCUSSION

The results of the current study provide new insights into the interplay of microparticles and CACs and provide a potential mechanism to account for the decreased number of CACs in patients with SSc. Our data indicate that microparticles derived from apoptotic or activated ECs can induce apoptosis of CACs. In view of their potency in inducing CAC death, microparticles might link the apoptosis of ECs in SSc to the decreased numbers of CACs in SSc.

Kuwana and coworkers demonstrated that the number of CACs, which are important for angiogenic responses in ischemic tissues and contribute to vascular remodeling, is severely reduced in patients with SSc (7). In the present study, we provide a potential explanation for this reduction and present evidence for a novel mechanism by which CACs might be destroyed in the peripheral blood of patients with SSc. The number of microparticles derived from ECs is highly increased in the blood of patients with SSc (22). We demonstrated that these microparticles induce apoptosis in CACs and might thereby decrease the number of CACs in patients with SSc. However, although microparticles from ECs are likely most important due to their high numbers, the induction of apoptosis in CACs by microparticles is not restricted to microparticles derived from ECs. Consistent with the proposed common mechanism of arachidonic acid–dependent apoptosis, microparticles derived from monocytes and T cells can also induce apoptosis in CACs.

Arachidonic acid is a major mediator of the cellular effects of microparticles (32,37–39). We previously demonstrated that microparticles shuttle arachidonic acid from leukocytes to fibroblasts (30). In the present study, we showed that microparticles also transport arachidonic acid from ECs to CACs. Thus, the transport of arachidonic acid seems to be a general mechanism of intercellular communication by microparticles. However, the effects of microparticle-derived arachidonic acid are diverse and are highly dependent on cell type. In fibroblasts, arachidonic acid stimulated the synthesis of prostaglandin E_2 via expression of COX-2 and microsomal prostaglandin E synthase 1. Effects of microparticle-derived arachidonic acid on the viability of fibroblasts (23) and also mature ECs were not detectable. In contrast, arachidonic acid potently induced apoptosis in CACs. The proapoptotic effects of arachidonic acid observed in our study are consistent with previous observations. HL-60 cells depleted of arachidonic acid are more resistant to apoptosis (40). Furthermore, a potential therapeutic effect of nonsteroidal antiinflammatory drugs on colorectal cancer has been explained by an intracellular accumulation of arachidonic acid upon inhibition of COX (41).

Arachidonic acid stimulates the activity of acid sphingomyelinase and enhances the conversion of sphingomyelin into C_2/C_{16} ceramides (42,43). Elevated levels of C_2/C_{16} ceramides in turn activate caspases to initiate the apoptotic machinery (44). Incubation with phosphatidylinositol 3,5-bisphosphate, a highly specific inhibitor of acid sphingomyelinase (33), prevented the induction of apoptosis by microparticles in CACs, suggesting that microparticles induce apoptosis in CACs by increasing the intracellular levels of C_2/C_{16} ceramides.

The different effects of microparticles and microparticle-derived arachidonic acid on fibroblasts and CACs might be attributable to distinct interactions of different cell types with microparticles. Fibroblasts do not take up microparticles (23,32). However, when microparticles interact with fibroblasts, they form pseudopodia-like processes; after prolonged contact, they are re-released from the cell membrane of fibroblasts without detectable morphologic changes (Distler JHW, et al: unpublished observations). In contrast, microparticles are phagocytosed by CACs. Similar to CACs, macrophages take up microparticles and subsequently undergo apoptosis (23). Based on the results presented herein, we propose the following model for the induction of apoptosis in CACs by microparticles: the ingestion of microparticles by CACs increases the intracellular concentration of arachidonic acid; arachidonic acid activates acid sphingomyelinase to metabolize sphingomyelin to C_2/C_{16} ceramides; increased levels of C_2/C_{16} ceramides in turn initiate apoptosis in CACs by activating caspases. However, further experiments are needed to confirm this model. Moreover, our data do

not exclude the possibility that components of microparticles other than arachidonic acid also contribute to the induction of apoptosis by microparticles and may mediate ceramide-induced apoptosis (45).

In summary, this study provides evidence for a novel link between apoptosis of ECs and reduced numbers of CACs in patients with SSc. Microparticles derived from ECs, the numbers of which are highly increased in the blood of patients with SSc, induce apoptosis of CACs via sphingomyelinase/ceramide-dependent pathways. Thus, microparticles might augment a vicious circle of increased EC damage and insufficient vascular repair.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. J. H. W. Distler had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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REFERENCES

1. Sgonc R, Gruschwitz MS, Dietrich H, Recheis H, Gershwin ME, Wick G. Endothelial cell apoptosis is a primary pathogenetic event underlying skin lesions in avian and human scleroderma. J Clin Invest 1996;98:785–92.
2. Guiducci S, Distler O, Distler JH, Matucci-Cerinic M. Mechanisms of vascular damage in SSc: implications for vascular treatment strategies. Rheumatology (Oxford) 2008;47 Suppl 5:v18–20.
3. Maurer B, Busch N, Jungel A, Pileckyte M, Gay RE, Michel BA, et al. Transcription factor fos-related antigen-2 induces progressive peripheral vasculopathy in mice closely resembling human systemic sclerosis. Circulation 2009;120:2367–-76.
4. Beyer C, Schett G, Gay S, Distler O, Distler JH. Hypoxia: hypoxia in the pathogenesis of systemic sclerosis. Arthritis Res Ther 2009;11:220.
5. Distler O, Del Rosso A, Giacomelli R, Cipriani P, Conforti ML, Guiducci S, et al. Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. Arthritis Res 2002;4:R11.
6. Distler O, Distler JH, Scheid A, Acker T, Hirth A, Rethage J, et al. Uncontrolled expression of vascular endothelial growth factor and its receptors leads to insufficient skin angiogenesis in patients with systemic sclerosis. Circ Res 2004;95:109–16.
7. Kuwana M, Okazaki Y, Yasuoka H, Kawakami Y, Ikeda Y. Defective vasculogenesis in systemic sclerosis. Lancet 2004;364: 603–10.
8. Distler JH, Akhmetshina A, Schett G, Distler O. Monocyte chemoattractant proteins in the pathogenesis of systemic sclerosis. Rheumatology (Oxford) 2009;48:98–103.
9. Distler JH, Gay S, Distler O. Angiogenesis and vasculogenesis in systemic sclerosis. Rheumatology (Oxford) 2006;45 Suppl 3:iii26–7.
10. Gabrielli A, Avvedimento EV, Krieg T. Scleroderma. N Engl J Med 2009;360:1989–2003.
11. Hirschi KK, Ingram DA, Yoder MC. Assessing identity, phenotype, and fate of endothelial progenitor cells. Arterioscler Thromb Vasc Biol 2008;28:1584–95.
12. Mukai N, Akahori T, Komaki M, Li Q, Kanayasu-Toyoda T, Ishii-Watabe A, et al. A comparison of the tube forming potentials of early and late endothelial progenitor cells. Exp Cell Res 2008;314:430–40.
13. Distler JH, Beyer C, Schett G, Luscher TF, Gay S, Distler O. Endothelial progenitor cells: novel players in the pathogenesis of rheumatic diseases [review]. Arthritis Rheum 2009;60:3168–79.
14. Urbich C, Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. Circ Res 2004;95:343–53.
15. Zammaretti P, Zisch AH. Adult 'endothelial progenitor cells': renewing vasculature. Int J Biochem Cell Biol 2005;37:493–503.
16. Avouac J, Juin F, Wipff J, Couraud PO, Chiocchia G, Kahan A, et al. Circulating endothelial progenitor cells in systemic sclerosis: association with disease severity. Ann Rheum Dis 2008;67: 1455–60.
17. Del Papa N, Quirici N, Soligo D, Scavullo C, Cortiana M, Borsotti C, et al. Bone marrow endothelial progenitors are defective in systemic sclerosis. Arthritis Rheum 2006;54:2605–15.
18. Zhu S, Evans S, Yan B, Povsic TJ, Tapson V, Goldschmidt-Clermont PJ, et al. Transcriptional regulation of Bim by FOXO3a and Akt mediates scleroderma serum-induced apoptosis in endothelial progenitor cells. Circulation 2008;118:2156–65.
19. Distler JH, Pisetsky DS, Huber LC, Kalden JR, Gay S, Distler O. Microparticles as regulators of inflammation: novel players of cellular crosstalk in the rheumatic diseases [review]. Arthritis Rheum 2005;52:3337–48.
20. Zwaal RF, Schroit AJ. Pathophysiologic implications of membrane phospholipid asymmetry in blood cells. Blood 1997;89: 1121–32.
21. Distler JH, Huber LC, Gay S, Distler O, Pisetsky DS. Microparticles as mediators of cellular cross-talk in inflammatory disease. Autoimmunity 2006;39:683–90.
22. Guiducci S, Distler JH, Jungel A, Huscher D, Huber LC, Michel BA, et al. The relationship between plasma microparticles and disease manifestations in patients with systemic sclerosis. Arthritis Rheum 2008;58:2845–53.
23. Distler JH, Huber LC, Hueber AJ, Reich CF III, Gay S, Distler O, et al. The release of microparticles by apoptotic cells and their effects on macrophages. Apoptosis 2005;10:731–41.
24. Distler JH, Jungel A, Caretto D, Schulze-Horsel U, Kowal-Bielecka O, Gay RE, et al. Monocyte chemoattractant protein 1 released from glycosaminoglycans mediates its profibrotic effects in systemic sclerosis via the release of interleukin-4 from T cells. Arthritis Rheum 2006;54:214–25.
25. LeRoy EC, Medsger TA Jr. Criteria for the classification of early systemic sclerosis. J Rheumatol 2001;28:1573–6.
26. Distler JH, Allanore Y, Avouac J, Giacomelli R, Guiducci S, Moritz F, et al. EULAR Scleroderma Trials and Research group statement and recommendations on endothelial precursor cells. Ann Rheum Dis 2009;68:163–8.
27. Ades EW, Candal FJ, Swerlick RA, George VG, Summers S, Bosse DC, et al. HMEC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 1992;99:683–90.
28. Li ZD, Bork JP, Krueger B, Patsenker E, Schulze-Krebs A, Hahn EG, et al. VEGF induces proliferation, migration, and TGF-β1 expression in mouse glomerular endothelial cells via mitogen-

activated protein kinase and phosphatidylinositol 3-kinase. Biochem Biophys Res Commun 2005;334:1049–60.
29. Distler JH, Jungel A, Huber LC, Seemayer CA, Reich CF III, Gay RE, et al. The induction of matrix metalloproteinase and cytokine expression in synovial fibroblasts stimulated with immune cell microparticles. Proc Natl Acad Sci U S A 2005;102:2892–7.
30. Jungel A, Distler O, Schulze-Horsel U, Huber LC, Ha HR, Simmen B, et al. Microparticles stimulate the synthesis of prostaglandin E_2 via induction of cyclooxygenase 2 and microsomal prostaglandin E synthase 1. Arthritis Rheum 2007;56:3564–74.
31. Akhmetshina A, Dees C, Pileckyte M, Maurer B, Axmann R, Jungel A, et al. Dual inhibition of c-abl and PDGF receptor signaling by dasatinib and nilotinib for the treatment of dermal fibrosis. FASEB J 2008;22:2214–22.
32. Huber LC, Jungel A, Distler JH, Moritz F, Gay RE, Michel BA, et al. The role of membrane lipids in the induction of macrophage apoptosis by microparticles. Apoptosis 2007;12:363–74.
33. Kolzer M, Arenz C, Ferlinz K, Werth N, Schulze H, Klingenstein R, et al. Phosphatidylinositol-3,5-bisphosphate is a potent and selective inhibitor of acid sphingomyelinase. Biol Chem 2003;384: 1293–8.
34. Wang E, Michl J, Pfeffer LM, Silverstein SC, Tamm I. Interferon suppresses pinocytosis but stimulates phagocytosis in mouse peritoneal macrophages: related changes in cytoskeletal organization. J Cell Biol 1984;98:1328–41.
35. Weerheim AM, Kolb AM, Sturk A, Nieuwland R. Phospholipid composition of cell-derived microparticles determined by one-dimensional high-performance thin-layer chromatography. Anal Biochem 2002;302:191–8.
36. Obeid LM, Linardic CM, Karolak LA, Hannun YA. Programmed cell death induced by ceramide. Science 1993;259:1769–71.
37. Barry OP, Kazanietz MG, Pratico D, FitzGerald GA. Arachidonic acid in platelet microparticles up-regulates cyclooxygenase-2-dependent prostaglandin formation via a protein kinase C/mitogen-activated protein kinase-dependent pathway. J Biol Chem 1999;274:7545–56.
38. Barry OP, Pratico D, Savani RC, FitzGerald GA. Modulation of monocyte-endothelial cell interactions by platelet microparticles. J Clin Invest 1998;102:136–44.
39. Pfister SL. Role of platelet microparticles in the production of thromboxane by rabbit pulmonary artery. Hypertension 2004;43: 428–33.
40. Surette ME, Fonteh AN, Bernatchez C, Chilton FH. Perturbations in the control of cellular arachidonic acid levels block cell growth and induce apoptosis in HL-60 cells. Carcinogenesis 1999;20: 757–63.
41. Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell 1995;83:493–501.
42. Jayadev S, Linardic CM, Hannun YA. Identification of arachidonic acid as a mediator of sphingomyelin hydrolysis in response to tumor necrosis factor α. J Biol Chem 1994;269:5757–63.
43. Marchesini N, Hannun YA. Acid and neutral sphingomyelinases: roles and mechanisms of regulation. Biochem Cell Biol 2004;82: 27–44.
44. Thevissen K, Francois IE, Winderickx J, Pannecouque C, Cammue BP. Ceramide involvement in apoptosis and apoptotic diseases. Mini Rev Med Chem 2006;6:699–709.
45. Zeidan YH, Hannun YA. The acid sphingomyelinase/ceramide pathway: biomedical significance and mechanisms of regulation. Curr Mol Med 2010;10:454–66.