Endothelial cell phagocytosis of senescent neutrophils decreases procoagulant activity

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Summary
Abundant senescent neutrophils traverse the vascular compartment and may contribute to pathologic conditions. For example, they become procoagulant when undergoing apoptosis and may contribute to thrombosis or inflammation. Our previous studies demonstrated a dominant clearance pathway in which the neutrophils can be phagocytosed by liver macrophages. The aim of this study was to explore an alternate pathway of neutrophil clearance by endothelial cells. Phagocytosis of the neutrophils by endothelial cells was performed using various experimental approaches including flow cytometry, confocal microscopy and electron microscopy assays in vitro and in vivo. Procoagulant activity of cultured neutrophils was evaluated by coagulation time, factor Xase and prothrombinase assays. Lactadherin functioned as a novel probe for the detection of phosphatidylserine on apoptotic cells, an opsonin (bridge) between apoptotic cell and phagocyte for promoting phagocytosis, and an efficient anticoagulant for inhibition of factor Xase and thrombin formation. When cultured, purified human neutrophils spontaneously entered apoptosis and developed procoagulant activity that was directly related to the degree of phosphatidylserine exposure. Co-culture of aged neutrophils and endothelial cells resulted in phagocytosis of the neutrophils and prolonged coagulation time. Lactadherin diminished the procoagulant activity and increased the rate of neutrophil clearance. In vivo, neutrophils were sequestered by endothelial cells after blockade of Kupffer cells, a process that was dependent upon both phosphatidylserine exposure and P-selectin expression. Thus, the ability of endothelial cells to clear senescent neutrophils may limit the procoagulant and/or inflammatory impact of these cells.

Keywords
Phagocytosis, endothelial cell, neutrophil, procoagulant activity, lactadherin

Introduction
Neutrophils are the most abundant white blood cells and provide a rapid response against pathogens. Their granules are packed with oxidative and inflammatory molecules essential for their antimicrobial function. In bacterial infections, and some other disease states, neutrophil production is increased and granule contents are released, contributing to inflammation and excessive procoagulant activity (PCA) (1). The inflammatory and procoagulant potential of neutrophils raises questions about the regulatory pathway(s) through which senescent cells are cleared to limit inflammatory injury or thrombosis.

Phosphatidylserine (PS), one of the four major phospholipids, is usually confined to the inner leaflet of the cell membrane. PS is exposed on the outer membrane during apoptosis occurs or cells are activated (2). Exposed PS on cells not only become a critical “eat-me” signal for phagocytes (3, 4) but also provides a catalytic surface for the assembly of tenase and prothrombinase complexes (5). Our prior study indicated that PS exposure is a major mechanism through which acute promyelocytic leukaemia (APL) blasts...
and amniotic fluid cell enhance PCA, activated or injured red blood cells (RBC) and platelets contributes to hypercoagulable state in patients with nephrotic syndrome (6-8). Physiologically, 10 million new neutrophils are released into the bloodstream from the bone marrow every minute, and infections may result in more rapid neutrophil production with accumulation in the blood stream and tissues. Externalised PS on senescent neutrophils would likely contribute to PCA (7, 9), meanwhile secondary necrosis of apoptotic neutrophils may release certain chemicals, leading to proinflammatory and autoimmune disorders (10, 11). Therefore, the same number of the cells must be cleared at the same time to maintain cell homeostasis and normal coagulation function as well as preserve the resident tissue cells and trigger the secretion of vascular endothelial growth factor for endothelial repair (12, 13), which triggered research efforts to elucidate the pathogenesis of neutrophils neutralisation and clearance.

Conventionally thinking, most apoptotic neutrophils are cleared by professional phagocytes such as macrophages, monocytes and immature dendritic cells (14-16). Our previous studies have demonstrated that Kupffer cells can promote apoptosis and engulfment of neutrophils (17-19) mediated through external translocation of PS on neutrophils and P-selectin expressed on hepatic sinusoids endothelial cells (HSECs). However, during infections, professional macrophages may become overwhelmed by the excessive amounts of neutrophils and may not always clear apoptotic neutrophils efficiently. Compared with professional phagocytes, the ECs have prominent advantages in ubiquitous distribution and cell number in circulation. We infer that this may lead to the clearance of these neutrophil corpses by amateur phagocytes such as ECs. So we assume that the ECs, which exist in a large amount on the walls of blood vessels and have potential phagocytic ability, may contribute to the clearance of excessive polymorphonuclear cells (PMNs) in circulation and maintain a normal and stable coagulation state.

It is well known that the removal of apoptotic cells by phagocytes could be greatly enhanced by lactadherin through its opsonin activity (20-22). Lactadherin is a kind of glycoprotein secreted by both professional and non-professional phagocytes (23, 24). It can bridge the PS on apoptotic-cell membranes and αvβ3/5 integrin on phagocyte membranes through arginine-glycine-aspartate (RGD) motifs (25). In the absence of lactadherin, engulfment of apoptotic cells by macrophages can be significantly restrained (26). In addition, the second C-terminal domain (C2) of lactadherin has sequence homology to the C-domain of factor (F)V and VIII (27), which evidenced competition of PS-binding sites (28). Thus lactadherin could be both an effective cofactor in phagocytosis and a specific anticoagulant through competing for PS binding sites with factors V and FVIII. Our previous report demonstrated that lactadherin and phagocytosis could cooperatively ameliorate the clotting disorders in APL (29). However, it remains unclear whether lactadherin could influence the interactions between PMNs and ECs and further elimination of PMNs by ECs. In this study, lactadherin was selected as a useful tool to detect the apoptosis, block PCA of PMNs, and opsonise phagocytosis. In the present study we explored the relationship between neutrophil senescence, procoagulant enzyme complex activity, and uptake of neutrophils by ECs. ECs phagocytosis of neutrophils was evaluated in vitro as well as in vivo. In addition, we investigated the role of lactadherin in phagocytosis and determined the contribution of lactadherin and phagocytosis cooperation to the modulation of PCA of neutrophil.

Materials and methods

Materials

Human FX, FXa and FIXa were obtained from Enzyme Research Laboratories (South Bend, IN, USA); human prothrombin, thrombin, and FVa and FVIIa were purchased from Haemalogistic Technologies Inc. (Burlington, VT, USA). Recombinant human FVIII was from American Diagnostica Inc (Stamford, CT, USA). A polyclonal antibody against human tissue factor (anti-TF) and fluorescein isothiocyanate (FITC)-conjugated anti-TF (clone VD8) were also obtained from American Diagnostica Inc. Purified annexin V, rabbit anti-human P-selectin polyclonal antibody (CD62P), PE-conjugated CD13 and matched isotype control antibody were from BD Biosciences Pharmingen (San Diego, CA, USA). FITC-labelled lactadherin (FITC-lact), anti-lactadherin (anti-lact), anti-lactadherin-C2 (anti-lactC2) antibodies, Alexa fluor 647 and FITC-labelled annexin V were prepared in our laboratory. Propidium iodide (PI) was obtained from Shanghai Dobio CO, LTD. (Shanghai, China). CellTracker™ Green CMFDA and CellTracker™ Red CMTPX were from Molecular Probes (Invitrogen, Eugene, OR, USA). The substrates Chromogenix S-2765 and S-2238 were purchased from DiaPharma Group (West Chester, OH, USA). Lipopolysaccharide (LPS) and GdCl3 were from Sigma (St Louis, MO, USA). Percoll were from GE Healthcare (Uppsala, Sweden). Male Wistar rats of 7- to 9-week-old were obtained from Charles River Laboratories International, Inc. (Wilmington, MA, USA).

Protein purification and labelling

Bovine lactadherin was purified as previously described, and was labelled with FITC or Alexa Fluor 647 according to the package instructions. The ratio of fluorescein to lactadherin was 1.2/1 or 1.1/1 (30, 31).

Cell preparation

Human neutrophils were isolated from the peripheral venous blood of healthy volunteers with informed content by a modified method described previously (32). In brief, 40 ml of fresh venous blood was drawn from each volunteer and blended lightly in four 15-ml polypropylene conical tubes containing 200 µl of 100 mM ethylenediaminetetraacetic acid (EDTA). After centrifuged at 300 x g for 15 minutes (min) at 20°C, platelet-rich plasma (PRP) was aspirated carefully and centrifuged again at 2,500 x g for 15 min, and platelet-poor plasma (PPP) was collected in a 15-ml tube. Subsequently, 3 ml of 6% dextran (500,000 mol wt; Amersham Bios-
ciences, Uppsala, Sweden) was added and the total volume was brought up to 15 ml with 0.9% sodium chloride solution. The mixture was laid aside steadily for erythrocyte sedimentation for 30 min at room temperature; simultaneously 2 ml of 42% Percoll was prepared on top of 2 ml of 51% Percoll. The leukocyte-rich and erythrocyte-poor upper layer was collected and centrifuged at 300 x g for 5 min. The pellet was resuspended with 2 ml of PPP and transferred to the tube containing layered Percoll. The sample was then centrifuged at 300 x g for 20 min at 20°C, and the neutrophils were aspirated from the interface between the 51% and 42% Percoll layers. After washing twice with Hanks balanced salt solution (HBSS, without Ca\(^{2+}\) and Mg\(^{2+}\)) (Thermo Fisher HyClone, Logan, UT, USA), the concentration was determined by manual counting with a hemacytometer, freshly harvested neutrophils were cultured in RPMI 1640 medium (Lonza, Walkersville, MD, USA) supplemented with 10% heat-inactivated fetal bovine serum (Thermo Fisher HyClone), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin solution (Thermo Fisher HyClone) at 1 x 10\(^8\) cells/well in a 6-well cell culture plate (Becton Dickinson, Franklin Lakes, NJ, USA). Incubation was performed at 37°C and 5% CO\(_2\) in an incubator (Thermo Fisher Scientific, Marietta, OH, USA), and cell apoptosis was determined in a dynamic mode for 24 hours (h). Human umbilical vein ECs were purchased from PromoCell (Heidelberg, Germany) and cultured in Endothelial Cell Growth Medium MV 2 supplemented with 10% fetal bovine serum at 37°C and 5% CO\(_2\).

### Flow cytometry assays

Neutrophils were incubated with Phycoerythrin (PE)-conjugated CD13 or IgG2a in dark for 15 min at room temperature prior to purity evaluation on a flow cytometer (FACSARia, Becton Dickinson). To detect cell apoptosis or TF expression, cultured neutrophils at various time points were washed twice with HBSS and incubated for 15 min at room temperature in dark with FITC-lactadherin/ Alexa Fluor 647-annexin V, FITC-lactadherin/ propidium iodide (PI), or Alexa Fluor 647-lactadherin/ FITC-CD142, separately. A total of 10,000 events per sample were acquired and analysed with BD FACSDiva Software.

For phagocytosis assay, neutrophils were precultured for 24 h and incubation with 1 µM CMFDA at 37°C and 5% CO\(_2\) for 30 min. Then the ECs labelled with CMTPX were overlaid and cocultured with CMFDA-labelled neutrophils in the presence or absence of anti-lact or anti-lactC2, and the percentage of phagocytosis was evaluated at various time points. At each time point, the mixture was collected from each well and the ECs were detached by 800 µl of 25% trypsin-EDTA solution for 3 min at 37°C. Phagocytosis was quantified by measuring the percentage of green fluorescence (CMFDA)-positive CMTPX (red) phagocytes by FACSARia flow cytometry (29).

### Confocal microscopy assays

Neutrophils cultured for 12 h were labelled with FITC-lact/Alexa Fluor 647-annexin V, FITC-lact/PI, FITC-annexin V/PI and FITC-CD142, respectively. After incubation in dark for 15 min at room temperature, the cells were centrifuged at 300 x g for 3 min and the supernatant, containing uncombined fluorescein, was removed. The pellet was gently resuspended with 200 µl of Tyrode’s buffer containing 1.5 mM CaCl\(_2\) and then observed immediately on the LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany). Through this method, PS exposure and TF expression of the apoptotic neutrophils could be visually detected.

Distinct staining of neutrophils and ECs was applied to definitively determine the phagocytosis of neutrophils by ECs. The neutrophils precultured for 24 h were resuspended and incubated in 1 µM CMFDA at 37°C for 30 min. Concomitantly, ECs were incubated in 0.5 µM CMTPX under the same conditions as neutrophils. Then the ECs monolayer was overlaid with 200 µl of neutrophils suspension for further coculture for 2 h at 37°C and 5% CO\(_2\). Aliquots of the cell mixture were collected at 30, 60, 90 and 120 min and each was washed twice with Tyrode’s buffer. Formaldehyde (3.7%) in phosphate-buffered saline (PBS) was then added for 15 min at room temperature to fix the cells. The samples were observed and imaged by confocal microscopy as above for apoptotic neutrophils. This method enabled us to roughly determine the phagocytosis by overlaying neutrophils and ECs.

### Scanning electron microscopy assays

Cocultured ECs and neutrophils were collected and fixed with Karnovsky fixative and washed with 0.1 M cacodylate buffer. Then the cells were treated with 2% OsO\(_4\), 1% tannine and 1% OsO\(_4\). After dehydration by acetone, critical drying of the samples was performed, and finally an approximate 10 nm film of platinum was sprayed on the samples. All images were digitally photographed at 5.00 kV on an S-3400N scanning electron microscope (Hitachi Ltd., Tokyo, Japan) using an ultra-high-resolution mode, and the interactions between neutrophils and ECs could be determined dynamically.

### Transmission electron microscopy assays

The cocultured cells as above were fixed in 2% glutaraldehyde and postfixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer following 4% OsO\(_4\) and 2% uranyl treatment. Subsequently, the samples were dehydrated and embedded in durecupan (Fluka, Munich, Germany). Ultrathin-sections prepared with Reichert ultracut S ultramicrotome (Leica) were observed on a JEM-200 transmission electron microscope (JEOL Ltd., Tokyo, Japan). Images were developed from negatives and digitalised by Heidelberg Linosan 1450 scanner (Heidelberg, Germany).

### Procoagulant activity of cultured neutrophils

The following assays were performed to test the PCA of apoptotic neutrophils with PS exposure in the absence or presence of ECs. Cultured neutrophils at various time points, and control cells were washed twice and resuspended at a concentration of 1.2 ± 0.1 x 10\(^5\)/ml. All reagents were preheated at 37°C prior to experiments.
unless stated otherwise. Lactadherin, annexin V and anti-TF were used as inhibitors in inhibition assays. All data were representative of at least three independent experiments and values were expressed as mean ± SD.

Coagulation time

Neutrophils cultured 24 h and fresh neutrophils at various time points were incubated with or without ECs in PPP for 5 min at 37°C. Then 25 mM CaCl₂ was added to each reaction system and coagulation time was immediately measured by a KC4A™ blood coagulometer (SIGMA-Amelung GmbH Company, Labcon, Heppenheim, Germany). The inhibition assays were started with the incubation of cells with inhibitors (final concentration: 128 nM-lactadherin, 128 nM- annexin V and 25.6 µg/ml- anti-TF) for 10 min at 25°C. The mixture was then incubated with PPP, and coagulation time was measured after the addition of Ca²⁺ as above (8).

Factor Xase and prothrombinase assays

The formation of intrinsic, extrinsic FXa and prothrombinase in the presence of 24 h-cultured or fresh neutrophils (incubated with or without ECs) at various time points was performed with a two-step amidolytic substrate assay using the following protocols. For the intrinsic FXa formation assay, each group was incubated with 1 nM FVa, 130 nM FX, 5 nM FVIII, 0.2 nM thrombin and 5 mM Ca²⁺ in FXa buffer (1 ml 10x TBS, 2 µM pück, 6 µg/ml- aprotinin) for 10 min at 25°C. The reaction was then stopped by the addition of EDTA to 7 mM final concentration. After the addition of 10 µl S-2765 (0.8 mM) to each reaction, the quantity of FXa formed was determined immediately at 405 nm in kinetic mode on a Universal Microplate Spectrophotometer (PowerWave XS; Bio-Tek, Winooski, VT, USA). Results were evaluated against the rate of substrate cleavage of a standard dilution of FXa. For the extrinsic FXa formation assay, each group was incubated with 130 nM FX, 1nM FVIIa and 5 mM Ca²⁺ for 5 min at 25°C. The reaction was quenched by EDTA, and the amount of FXa formation was determined as described above. To measure the production of prothrombinase, the incubation was performed with 1 nM FVa, 0.05 nM FXa, 1 µM prothrombin and 5 mM Ca²⁺ in prothrombinase buffer (1 ml 10x TBS, 50 µl 10% bovine serum albumin [BSA], 8.8 ml ddH₂O) for 5 min at 25°C. The production of thrombin was measured using chromogenic substrate S-2238 after the addition of EDTA. Inhibition of coagulation complexes by lactadherin or anti-TF was measured as follows. Neutrophils cultured for 24 h were incubated with varying concentrations of lactadherin (0–128 nM) and anti-TF (0–25.6 µg/ml) in Tyrode’s buffer for 10 min at 25°C. The mixture was then incubated with the specified clotting factors according to the above protocols. The quantity of thrombin or FXa formation was assessed as previously described (8).

Phagocytosis of neutrophils by ECs in vivo

To observe the distribution and phagocytosis of neutrophils in vivo, the following experiments were performed in a modified method as described previously (18). Briefly, 7- to 9-week-old male Wistar rats were injected with LPS (0.1 mg/kg intravenously [IV]) or sterile saline as control, and perfusion fixed at 0, 3, 6, 9, 12 and 24 h after injection. In another group, gadolinium trichloride (GdCl₃, 10 mg/kg, IV) was administered 6 h before the LPS injection. The organ-specific distribution and apoptosis of neutrophils could be evaluated through this method (17-19). Then 10 min later some animals were injected with annexin V, anti-P-selectin or lactadherin, and the liver of each animal was perfusion-fixed as above. Animals were killed 30 min after injections, and samples of the liver, spleen and lungs were taken for microscopic examinations. Paraffin sections (4 µm) stained using immunohistochemistry as well as semi-thin sections (0.2 µm) stained with toluidine blue were analysed by light microscopy and transmission electron microscopy. Cell number/mm³ was counted and each value was expressed as mean ± SD of 3-5 rats. Thus the influence of annexin V, anti-P-selectin and lactadherin on the interactions between neutrophils and ECs could be identified through counting the neutrophil numbers in the liver in a dynamic mode. Further, the phagocytosis of neutrophils by hepatic ECs could also be visually qualified by transmission electron microscopy. This study conformed to the guidelines for the care and use of laboratory animals established by the Animal Care Committee of Harvard Medical School.

Statistical analysis

Numerical variables were tested for normal distribution with the Kolmogorov-Smirnov test. Data are expressed as mean ± standard deviation (SD), and statistical analysis was made by t-test or ANOVA as appropriate.

Results

Purity and PS expression of neutrophils

Isolated neutrophils were obtained and depleted of monocyte contamination through the method described in “cells preparation”. We tested the purity of isolated neutrophils. Around 95% of total cells were neutrophils with stab and segmented neutrophils by morphological observation and 93.4% of isolated cells were CD13⁺ by flow cytometry in addition to a small amount of RBCs (Figure 1A, C). Results from the flow cytometry assays indicated that the apoptosis of neutrophils with PS exposure developed in a time-dependent manner, and after 24 h of culture almost all neutrophils were apoptotic. The proportion of early and advanced apoptotic cells labelled with lactadherin increased from 8.2% at 0 h to 96.8% at 24 h, and about half of the total cultured neutrophils were apoptotic after 9 h of culture (Figure 1B). The labelling rate of lactadherin was higher than that of annexin V, probably due to the higher specificity and affinity of lactadherin towards PS. For
example, the binding rates of lactadherin and annexin V at 9 h were 41.9% and 34.7%, respectively. PI-bound nuclei identified late apoptotic neutrophils without intact cell membranes, which occurred after longer culture times. Nearly no TF present on cell surfaces measured by flow cytometry using FITC-anti-TF (clone VD8) (data not shown).

The apoptosis of cultured neutrophils was confirmed by confocal microscopy (Figure 1C, D). Although both lactadherin and annexin V can bind to PS, most of the neutrophils displayed diffuse or annular green fluorescence, while red fluorescence was seen on only a few at 24 h. This is probably due to the different binding ability of lactadherin and annexin V to PS and the distinct parts of the cell membranes occupied by the two tags. Further, some advanced apoptotic neutrophils showed PI staining, indicating that cell membranes had lost integrity (Figure 1E, F).

**Figure 1: Purity assessment and PS analyses of cultured neutrophils.** A) Observation of freshly isolated neutrophils on a light microscope (40 x) with Wright-Giemsa staining and detection with PE-conjugated CD13 by flow cytometry. B) Neutrophils cultured at 9 and 24 h were collected and incubated with FITC-lactadherin and Alexa Fluor 647-annexin V or PI for 15 min in the dark before flow cytometric analysis. Fresh neutrophils were used as control. Lactadherin/annexin V-positive and PI-negative cells represented the early apoptotic neutrophils with intact cell membranes, while cells undergoing late apoptosis were both positive for lactadherin and PI. C-F) Confocal microscopic imaging of neutrophils cultured for 12 h. Cells were incubated with both FITC-lactadherin (green) and Alexa Fluor 647-annexin V (red) (C and D), and double-labelled cells are displayed in yellow. E and F) Neutrophils incubated with FITC-lactadherin or annexin V (green) plus PI (red). Scale bars: 10 μm. PI: propidium iodide.
vations at various timepoints showed the time-dependent apoptosis of cultured neutrophil.

**Procoagulant activity of cultured neutrophils**

Coagulation time of the cultured neutrophils was assessed by recalcification-time assays. Neutrophils cultured 6 h or more showed significantly shortened coagulation time compared to neutrophils cultured 3 h or less (p < 0.05), indicating the PCA was proportional to the number of apoptotic neutrophils (▶ Figure 2A). In order to explore the contribution of PS and TF to the PCA of apoptotic neutrophils (cultured for 24 h), we performed inhibition assays of coagulation with lactadherin, annexin V and anti-TF, respectively. The coagulation time of 24 h-cultured neutrophils was significantly prolonged in presence of anti-TF (p < 0.05) compared with the no treatment group, and reached the level of fresh and 3 h-groups in the presence of lactadherin and annexin V, respectively (▶ Figure 2A). The mechanism of lactadherin and annexin V inhibition was likely through competing for binding sites (PS) with coagulation factors.

We further investigated the PCA of cultured neutrophils using intrinsic, extrinsic FXa and prothrombinase assays. The results showed the positive effects of exposed PS on conversion of FX and prothrombin to FXa and thrombin, respectively. In the presence of FVIIa or FVIIIa, IXa and Ca²⁺, cultured neutrophils could promote the production of FXa through the extrinsic and intrinsic pathway, respectively. Similarly, these cells also enhanced the activation of prothrombin in the presence of FVa and Ca²⁺ (▶ Figure 2B). Because both the FXa and prothrombinase complexes require PS-containing membranes, these results indicated that PS was an important procoagulant source on cultured neutrophils (33). Both FXa and thrombin production were culture time- and cell number-dependent (data not shown). As shown in ▶ Figure 2B, the amounts of FXa and thrombin produced by the neutrophils cultured for 6 h or more were much higher than that for neutrophils cultured for 3 h or less (p < 0.05). Furthermore, the

![Figure 2: Procoagulant activity assays of cultured neutrophils. A) Coagulation time of cultured neutrophils (1.2 x 10⁴) at various timepoints (0, 3, 6, 12 and 24 h) was determined. * indicates statistical significance (p < 0.05) from fresh (0 h) and 3 h-cultured neutrophils. Coagulation times of neutrophils (cultured for 24 h) were detected in the absence or presence of 128 nM lactadherin, or annexin V or 25.6 µg/ml anti-TF. #p < 0.05 vs no treatment of 24 h-cultured neutrophils. Data are displayed as mean ± SD for triplicate samples of independent experiments; p-value < 0.05 was considered significant. B) The precultured neutrophils at various time points were incubated with different cofactors followed by determination of procoagulant enzyme complexes formation. *, # and + indicate significant (p < 0.05) differences from extrinsic FXa, intrinsic FXa and thrombin production of fresh and 3 h-cultured neutrophils, respectively. C and D) Inhibition assays of enzyme activation by lactadherin and anti-TF antibody. The extrinsic Xa (●), intrinsic Xa (○) and thrombin (▲) production of 24 h-cultured neutrophils were measured in the presence of indicated concentrations of lactadherin and anti-TF, respectively. Each point represents mean ± SD for triplicate samples of independent experiments. Lactadherin and anti-TF decreased production of procoagulant enzyme complexes to 90% and 20%, respectively. Data were normalised to the amount of formation without lactadherin for comparison of the extent of inhibition. PCA: procoagulant activity.](image-url)
PCA of cultured neutrophils could also be inhibited by lactadherin and anti-TF. The amount of FXa or thrombin was significantly reduced by lactadherin in a dose-dependent manner, with lactadherin ultimately inhibiting production of the three procoagulant enzyme complexes were up to 90%, while anti-TF did no more than 20% (Figure 2C, D). These results again demonstrated that the PCA of cultured neutrophils is majorly dependent on PS exposure.

Phagocytosis of neutrophils by ECs in vitro

Previous studies showed that ECs are able to phagocytose senescent and apoptotic RBCs (34). However, it is unknown whether ECs are also able to engulf apoptotic neutrophils. To answer this question, we performed microscopic assays on cocultured ECs with precultured neutrophils to observe the interactions between the two cells. Scanning electron microscopy sterically depicted the dynamic phagocytosis process during which the neutrophils were adhered to and then engulfed by ECs (Figure 3A, B). Further, transmission electron microscopy clearly displayed the ECs containing neutrophils as well as adhered neutrophils (C). Local magnification of the active endothelial cell in (A) depicted the neutrophil endocytosed by the endothelial cell (D). Karyopyknosis and apoptotic bodies could be seen within this neutrophil surrounded by some debris. E) After incubation for 30 min, images of representative confocal microscopy showed that CMFDA-stained neutrophils (green) were taken up by CMTPX-labelled ECs (red). F) After incubation for 2 h, neutrophils were undergoing degradation with dispersed apoptotic bodies or digested debris in ECs.
taining internalised neutrophils, implying the uptake of neutrophils by ECs (▶Figure 3C, D). Observations also revealed that one EC could often trap several neutrophils at the same time (▶Figure 3E), and the apoptotic corpses or digested debris of neutrophils in the ECs were observed by confocal microscopy (▶Figure 3F), indicating that some internalised neutrophils were “digested” by ECs. The phagocytosis process was time-dependent and significantly increased in the presence of lactadherin (data not shown). According to the distinct scatter characteristics of ECs and neutrophils labelled with specific fluorochromes, we differentiated the ECs containing neutrophils from other separate ECs and neutrophils by flow cytometry as described previously (35). ECs with internalised neutrophils were detected as a double-labelled cluster, which increased dynamically with coculture time (▶Figure 4A). This dynamic process of phagocytosis was promoted by lactadherin, causing much more double-labelled ECs than that without lactadherin (p < 0.05). Annexin V, anti-EGF, anti-lactC2 and especially anti-lact all dramatically suppressed neutrophil ingestion by ECs, compared with lactadherin (p < 0.05) (▶Figure 4B).

In addition, the clearance of neutrophils by ECs could decrease the PCA induced by neutrophils, which was demonstrated by further coagulation experiments on cocultured ECs and PMNs. With the coculture time extended, the coagulaton time of apoptotic PMNs and ECs gradually prolonged and reached statistical significance at 60 min. The PCA of control (fresh PMNs + ECs) at 120 min did not (▶Figure 5A). Accordingly, the ability of cocultured cells (apoptotic PMNs + ECs) to promote conversions of FX and prothrombin to FXa and thrombin, respectively, was also decreased with coculture time (▶Figure 5B). One hour increase in coculture time made a significant decrease in productions of active enzymes (p < 0.05). These results suggest that the elimination of apoptotic neutrophils by ECs led to alleviation of coagulant stress. However, the phagocytosis process tended to diminish beyond 2 h of coculture time, probably because the limited amount of lactadherin produced by ECs could not suffice further neutrophils phagocytosis (data not shown).

Phagocytosis of neutrophils by ECs in vivo

To observe in vivo the phagocytosis of neutrophils by ECs, experimental animals were injected with LPS with or without GdCl3 (which inhibits Kupffer cells), then followed by annexin V, anti-P-selectin or lactadherin in different groups. Consistent with previous studies (24), our results without GdCl3 showed the number of neutrophils was increased after LPS injection and peaked at 6 h (data not shown). Tissue-specific assays in distinct organs revealed that most of the neutrophils accumulated in the liver, followed by the spleen and the lungs. This distribution of neutrophils was re-

Figure 5: Coagulation assays for cocultured ECs and PMNs. Fresh and 24 h-cultured PMNs were cocultured with or without ECs (10 : 1 in cell number) for coagulation time determination at various coculture time points, respectively. A) There was significant prolonged coagulation time by 60 min of coculture compared with 0 and 30 min in apoptotic PMNs+ECs group. As control, fresh PMNs+ECs has no apparent change during 120 min. B) In a separate experiment, mixed cells were incubated with various cofactors to determine the production of FXa and thrombin at different coculture time points. Coculture of apoptotic PMNs and ECs decreased the production of FXa and thrombin by 60 min compared with 0 and 30 min (*#p < 0.05). All data were representative of at least three independent experiments and values were expressed as mean ± SD; p-value <0.05 was considered significant. EC, endothelial cell; PMNs, polymorphonuclear cells.

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related to the massive expression of P-selectin on hepatic ECs induced by LPS. Interestingly, the peak number of neutrophils occurred at 9 h in the liver, 3 h in the spleen and 6 h in lungs after injections of GdCl$_3$ and LPS (Figure 6A). This distribution induced by GdCl$_3$ and LPS was probably due to the venous blood flow from the spleen and the lungs to the liver. These results suggest that the abundant neutrophils induced by LPS were undergoing apoptosis and were then cleared by hepatic ECs.

After injections of either annexin V, anti-P-selectin or lactadherin, the liver sections of experimental animals were obtained to analyse the temporal distribution of neutrophils. Although the time-dependent distribution of neutrophils by different treatments was the same, the cell number was quite different (Figure 6B). Compared with sterile saline control, lactadherin treatment led to the decreased numbers of neutrophils at all timepoints (p < 0.05 vs control). However, annexin V and anti-P-selectin treatments resulted in more neutrophils than in the control (p < 0.05). These results indicate that lactadherin could promote neutrophil phagocytosis by ECs through its bridging role between the two cells, while annexin V and anti-P-selectin could block PS and P-selectin on the two cells. Imaging of the liver sections by transmission electron microscopy visually confirmed the above-mentioned results. Apoptotic neutrophils could be seen in the hepatic blood stream, some of which were already trapped or “swallowed” by hepatic vascular ECs (Figure 6C).

Discussion

In the current study we elucidate a novel pathway through which ECs remove aged and apoptotic neutrophils. This pathway is dependent upon exposed PS on neutrophils and expressed P-selectin on ECs. Both the anticoagulant effect and phagocytosis are enhanced by lactadherin. Thus, our studies provide the basis for the hypothesis that this pathway is a physiologic method for limiting coagulation and inflammation.

Neutrophils have a relatively short life-span and progress toward apoptosis in the circulating blood. Cultured neutrophils in vitro displayed a time-dependent dynamic process of apoptosis with most of them apoptotic at 24 h. Externalised PS on cultured neutrophils was detected using lactadherin and annexin V. Compared with annexin V, lactadherin showed a more sensitive probe for the detection of PS and a more effective inhibitor for the PCA of neutrophils. Our results showed that the cultured neutrophils support the assembly of prothrombinase, intrinsic and extrinsic FXa and shorten the time to fibrin strand formation. Moreover, blocking of PS with lactadherin markedly inhibits the PCA of neutrophils, whereas anti-TF affects the coagulation time mildly, which suggest that the PCA of neutrophils is PS dependent. TF, the primary initiator of coagulation in vivo, plays a major role in both thrombosis and haemostasis. The expression of TF in neutrophils has been disputed (36, 37), possibly due to different methodological approaches and usage of TF antibodies in different studies (38). Isolated neutrophils failed to express significant amounts of TF antigen, which may be due to the use of mouse anti-human TF.
antibodies (clone VD8) in our report. The VD8 clone is able to detect and efficiently inhibit TF expression and activity on LPS-stimulated monocytes/MPs but was low compared to CLB/TF-5 and VIC7 (38).

As non-professional phagocytes, ECs have potential phagocytic function, which has been previously identified in the phagocytosis of RBC (39). Herein we demonstrate for the first time the phagocytosis of neutrophils by ECs as well as the effects of lactadherin in enhancing this process. Because different ECs have a unique phenotype, we choose popular human umbilical vein ECs (HUVECs) in our experiments. We think further studies are necessary to culture other ECs (e.g. hepatic sinusoids ECs, splenic sinusoids ECs and pulmonary capillaries ECs) in vitro. Results from confocal and electron microscopy assays of cocultured ECs and neutrophils in vitro showed that the neutrophils could be trapped and endocytosed by ECs in a time-dependent manner. In this kinetic process, PS exposure may enable neutrophils to be recognised and sequestered by ECs. Longer coculture time led to more PS-exposing neutrophils and more ECs phagocytosis of neutrophils. Consistent with previous studies (40), our current data indicate that the interactions between ECs and neutrophils could be significantly augmented by lactadherin, while an anti-EGF, anti-LactC2 and especially anti-lact significantly inhibited this effect, which further confirmed that lactadherin can bridge the PS on apoptotic neutrophils and the integrins on ECs during phagocytosis. Our previous report that the expression of P-selectin on ECs and platelets can enhance the neutrophils phagocytosis by Kupffer cells (19), may also contribute to the adherence of neutrophils to ECs and their eventual sequestration. Although the precise mechanism of neutrophil phagocytosis by ECs remains unclear, the PS signalling and lactadherin enhancement as well as P-selectin expression may be collectively involved in this complex process.

What is known about this topic?

- Endothelial cells remove apoptotic neutrophils through a novel pathway, which is dependent upon exposed phosphatidylserine (PS) and P-selectin.
- Both uptake of neutrophils by ECs and anticoagulant function are enhanced by lactadherin.
- The studies provide the basis for the hypothesis that this pathway is a physiologic method for limiting coagulation and inflammation.

What does this paper add?

- Endothelial cells, a large cell population in circulation, can have a prominent phagocytic function that may contribute to anti-thrombotic homeostasis by removing apoptotic neutrophils.
- Through competing for PS binding sites with factor V and VIII, lactadherin functioned as a novel probe for the detection of PS (apoptotic marker) on apoptotic cells, an opsonin (bridge) between apoptotic cell and phagocyte for promoting phagocytosis, and an efficient anticoagulant for inhibition of intrinsic factor Xase, extrinsic factor Xase, and prothrombinase formation. The anti-coagulant and pro-phagocytic effects of lactadherin suggest that it may participate in the process.
- When cultured for 24 hours, purified human neutrophils spontaneously entered apoptosis and developed procoagulant activity that was directly related to the degree of PS exposure from 6 hours.
- Co-culture of apoptotic neutrophils and endothelial cells resulted in phagocytosis of the neutrophils and prolonged coagulation time from 60 min.
- Lactadherin diminished the procoagulant activity and increased the rate of neutrophil clearance.
- In vivo, neutrophils were sequestered by endothelial cells after blockade of macrophages, a process that was dependent upon both PS exposure and P-selectin expression.
- The ability of endothelial cells to clear senescent neutrophils may limit the procoagulant and/or inflammatory impact of these cells.
within 30 min of injection (46). We assumed that the resulting expression of P-selectin and excess of the PMNs occurred with little effect on PMN longevity. Total neutrophil number peaked at 6 h, with most of the accumulation found in the liver rather than the spleen or the lungs. LPS can induce and sustain the expression of P-selectin on hepatic ECs followed by accumulation of neutrophils mainly in the liver. LPS + GdCl$_3$ were administrated to inhibit Kupffer cells in another animal group, where the number of neutrophils peaked at 9 h, indicating that the neutrophils were apoptosised with time and removed by ECs other than Kupffer cells, though with a 3 h delay compared with LPS treatment alone. Data from transmission electron microscopy of liver sections visually displayed the phagocytosis of neutrophils by ECs. The PS exposed on apoptosed neutrophils may accelerate the clearance of neutrophils by ECs. When treated with lactadherin, the number of neutrophils was greatly reduced because lactadherin can promote the phagocytosis through the PS-integrin signalling pathway. However, total neutrophils were increased by treatment with annexin V and anti-P-selectin, due to blocking of PS on neutrophils and P-selectin on ECs, respectively. Although both lactadherin and annexin V are specific towards PS, the latter lacks RGD motif, which is an important ligand for integrins on phagocytes. These results demonstrate again in vivo that both PS and P-selectin participate in the mechanisms of neutrophil clearance by ECs. As the first step, the aggregation of abundant neutrophils in the liver is likely related to the massive P-selectin of hepatic ECs. Subsequently, the apoptotic neutrophils were endocytosed by ECs through recognising PS with the help of lactadherin. Such distribution heterogeneity guarantees the efficient and selective clearance by ECs in the liver, the most important detoxification and metabolism organ in the body.

Presumably, the ECs and Kupffer cells in the liver might be two key clearers of intravital neutrophils. As non-professional phagocytes, the ECs exist in vivo as a large cell population and possess potent phagocytic ability, such that their participation may greatly facilitate the clearance of excessive neutrophils. Physiologically, the homeostatic circumstance is a prerequisite for polycellular organisms to survive and fight effectively against exogenous invasions. Although heavy neutrophil burden may secure the clearance of pathogens, the great amount of senescent and dead neutrophils would leave a critical problem, i.e. hypercoagulation state, in addition to the infection itself. Under infectious conditions, however, ECs can be activated and respond with expression of adhesion molecules, resulting in increased adhesion and trafficking of neutrophils (47). Lactadherin secretion may also be upregulated to facilitate the engulfment of apoptotic cells under stress state-like acute infection or inflammation (48). It can be inferred that with the cooperation of ECs and professional phagocytes, the leukocyte count can be effectively sustained within or restored to normal range when the pathophysiological condition is under control. For example, disseminated intravascular coagulation, which may lead to severe or even vital bleeding, is often caused by severe infectious or inflammatory disorders, especially bacterial infections (49). We suggest that excessive neutrophils may be involved in this process possibly along with massive destruction of vascular endothelia. Thus the impaired ECs cannot perform their role effectively in the elimination of excessive neutrophils induced by primary diseases. However, the neutrophilia and associated hypercoagulopathy caused by certain pathophysiological conditions can usually be downregulated by professional and non-professional phagocytes.

Results from this study lead us to conclude that ECs have a prominent phagocytic function that may contribute to anti-thrombotic homeostasis by removing apoptotic neutrophils. The anti-coagulant and pro-phagocytic effects of lactadherin suggest that it may participate in the process. Further in vivo studies will be necessary to indicate the relative importance of these pathways for specific disease states.

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**Conflicts of interest**
None declared.

**References**


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**Abbreviations**

PS: phosphatidylserine; anti-TF: against human tissue factor; PCA: procoagulant activity; PMN: polymorphonuclear cell; APL: acute promyelocytic leukemia; EC: endothelial cell; HUVECs: human umbilical vein endothelial cell; HSECs: hepatic sinusoids endothelial cells, PI: propidium iodide.


