Endothelial-to-mesenchymal transition in lipopolysaccharide-induced acute lung injury drives a progenitor cell-like phenotype

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Suzuki T, Tada Y, Nishimura R, Kawasaki T, Sekine A, Urushibara T, Kato F, Kinoshita T, Ikari J, West J, Tatsumi K. Endothelial-to-mesenchymal transition in lipopolysaccharide-induced acute lung injury drives a progenitor cell-like phenotype. Am J Physiol Lung Cell Mol Physiol 310: L1185–L1198, 2016. First published April 22, 2016; doi:10.1152/ajplung.00074.2016.——Pulmonary vascular endothelial function may be impaired by oxidative stress in endotoxemia-derived acute lung injury. Growing evidence suggests that endothelial-to-mesenchymal transition (EndMT) could play a pivotal role in various respiratory diseases; however, it remains unclear whether EndMT participates in the injury/repair process of septic acute lung injury. Here, we analyzed lipopolysaccharide (LPS)-treated mice whose total number of pulmonary vascular endothelial cells (PVECs) transiently decreased after production of reactive oxygen species (ROS), while the population of EndMT-PVECs significantly increased. NAD(P)H oxidase inhibition suppressed EndMT of PVECs. Most EndMT-PVECs derived from tissue-resident cells, not from bone marrow, as assessed by mice with chimeric bone marrow. Bromodeoxyuridine-incorporation assays revealed higher proliferation of capillary EndMT-PVECs. In addition, chimeric bone marrow. Bromodeoxyuridine-incorporation assays revealed higher proliferation of capillary EndMT-PVECs. In addition, EndMT-PVECs strongly expressed c-kit and CD133. LPS loading to human lung microvascular endothelial cells (HMVEC-Ls) induced reversible EndMT, as evidenced by phenotypic recovery observed after removal of LPS. LPS-induced EndMT-HMVEC-Ls had increased vasculogenic ability, aldehyde dehydrogenase activity, and expression of drug resistance genes, which are also fundamental properties of progenitor cells. Taken together, our results demonstrate that LPS induces EndMT of tissue-resident PVECs during the early phase of acute lung injury, partly mediated by ROS, contributing to increased proliferation of PVECs.

acute lung injury; cell transformation; endothelial-to-mesenchymal transition; progenitor cells; reactive oxygen species

ACUTE LUNG INJURY and its most severe manifestation, acute respiratory distress syndrome, are life-threatening complications in critically ill patients (48, 51). The morbidity and mortality of moderate and severe acute respiratory distress syndrome remain high, ranging between 30 and 50%, despite improvements in critical care patients’ management and new technological developments (43). The leading cause of acute lung injury is sepsis, and converging data suggest that endothelial activation and damage are hallmarks of acute lung injury pathophysiology (39, 40, 50). There have been several clinical trials targeting aggressive factors correlated with the development of acute lung injury, such as inflammatory cells and proinflammatory cytokines, but limited reports indicating validity and efficacy (35). Thus a paradigm shift in treatment strategy is needed. A treatment strategy designed to protect vascular endothelial cells and pulmonary epithelial cells against injury, and/or enhancement of the tissue repair would be among the most promising.

Endotoxia, a major cause of acute lung injury, is produced by deposition of large amounts of LPS, a gram-negative bacteria endotoxin (20, 41, 45). During this process, although some of the endothelial cells undergo apoptosis, most of them survive (16). There have been many studies focused on endothelial cell death in endotoxia, but little is known about the functional and molecular changes that occur in the surviving endothelial cells. We hypothesize that one of these innate strategies is endothelial-to-mesenchymal transition (EndMT).

EndMT is a process in which endothelial cells lose cell-to-cell contacts among them with polarity and undergo a dramatic remodeling of the cytoskeleton. EndMT is characterized by the following features: 1) loss of intracellular junctions and endothelial markers, 2) acquisition of mesenchymal markers, and 3) increased migratory and invasive properties (19). Growing evidence suggests that EndMT is enhanced or triggered by various factors including transforming growth factor-β (TGF-β), hepatocyte growth factor, fibroblast growth factor, and so on. TGF-β is likely to be the most potent among factors that induce EndMT (17, 19, 28).

EndMT has been studied mainly in the context of pathological tissue fibrosis in organs such as lung, heart, and kidney (22, 32, 52, 53). EndMT can also be adaptive and important in development. It contributes to cardiac development and angiogenesis (3–5, 49). For instance, embryonic endothelial cells undergo EndMT, and the transition to mesenchymal cells expressing α-smooth muscle actin (α-SMA) contributes to the maturation of pulmonary veins and arteries (3, 4, 12). In angiogenesis, EndMT may be required for the vessel-guiding tip cells to lose intercellular junctions that allow them to delaminate from the existing vessel and acquire the migratory phenotype necessary for angiogenic sprouting (49).

Considering that activation and damage of pulmonary vascular endothelial cells (PVECs) are pathophysiological hallmarks of acute lung injury, EndMT may contribute to the repair of vascular injury in acute lung injury.

Indeed, a recent study demonstrated that LPS induces a fibrotic-like phenotype in human umbilical vein endothelial cells (HUVECs) (16). This process was explained by EndMT, and it was reported to depend on NAD(P)H oxidase-dependent reactive oxygen species (ROS) production (16). Nevertheless, it has not been clearly demonstrated whether endotoxin evokes pulmonary vascular EndMT in acute lung injury. There are no good in vivo experimental models that prove the pathophysiological role of EndMT in septic acute lung injury.
In addition to these, we recently reported that lung tissue-resident endothelial progenitor cells (EPCs) contribute to pulmonary vascular repair after endotoxin-induced injury (27). We hypothesized that, in the process of tissue repair, LPS-induced EndMT-cells may dedifferentiate into a more progenitor cell-like phenotype.

In the present study, we examined how LPS-induced pulmonary vascular EndMT occurs in vitro and in vivo. Then we elucidated the possible role of ROS, one of the key factors of ARDS, in the process of EndMT. We also identified the origin of EndMT cells in bone marrow (BM) chimeras generated using green fluorescent protein (GFP)-95 transgenic mice and investigated its possible progenitor cell-like role.

**Materials and Methods**

**Mouse Model of Septic Lung Injury**

Seven- to 8-wk-old female C57BL/6 mice (Clea Japan, Tokyo, Japan) were intraperitoneally administered 10 mg/kg body wt LPS derived from *Escherichia coli* (O55:B5 Sigma, St. Louis, MO) dissolved in PBS. All animal experiments were conducted under protocols approved by the Review Board for animal experiments of Chiba University.

**Lung Wet/Dry Weight Ratio**

After mice were euthanized, lungs were removed, and wet weight was determined. Then the lung tissue was placed in an oven at 60°C for 24 h to obtain dry weight. The ratio of wet to dry lung was calculated to assess the degree of tissue edema.

**Measurement of Pulmonary Vascular Leakage**

Leakage of protein from the pulmonary vasculature was assessed by measuring the accumulation of Evans blue dye in the right lung. Evans blue dye (30 mg/kg; Sigma-Aldrich) in sterile saline was injected via the tail vein. After 1 h, the lungs were flushed with PBS and removed, and Evans blue was extracted. Evans blue was quantitated in a spectrophotometer by measuring absorbance at 620 nm as previously described (36). Evans blue dye extravasation was expressed as nanograms Evans blue per milligram dry tissue (9).

**Lung Histological Analyses**

Lungs were formalin-fixed and embedded in paraaffin. Sections (5 µm) were stained with hematoxylin and eosin (HE) for histological evaluation.

**Fluorescent Immunohistochemistry**

Lungs were embedded in OCT (Sakura Finetek, Tokyo, Japan) and frozen in liquid nitrogen to prepare the cryosections. Frozen lung tissues were cut into 6-µm-thick sections, immunostained, and visualized by confocal microscopy (Fluoview FV 10i, Olympus, Tokyo, Japan). The sections were fixed in acetone for 10 min, blocked with Block Ace (Dainippon Sumitomo Pharma, Tokyo, Japan) for 10 min, and incubated with the primary and secondary antibodies for 60–120 min. The following antibodies were used for immunostaining: anti-CD31-Alexa488 (BioLegend, San Diego, CA), anti-Ki67 (BioLegend), anti-α-SMA (Thermo Scientific, Waltham, MA), anti-CD133 (eBioscience, San Diego, CA), and anti-CD117 (eBioscience). We separated vessels by diameter and morphology to determine the location of EndMT-PVECs; those less than 5 µm in diameter and flattened were defined as "capillary" vessels.

**Human Pulmonary Vascular Endothelial Cell Injury Model**

Human lung microvascular endothelial cells (HMVEC-L) were purchased from Clonetics (Walkersville, MD) and were cultured in endothelial cell basal medium-2 (EBM-2, Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum and endothelial cell growth medium 2 (EGM-2 SingleQuots, Invitrogen, Carlsbad, CA). All cells were maintained at 37°C in a 5% CO₂ humidified incubator. Cells were cultured to 90% confluence and then transfected to starvation medium including EGM-2 supplemented with 1% fetal bovine serum, 0.1% gentamicin sulfate, and amphotericin-B; heparin; and ascorbic acid for 24 h. Cells were exposed to vehicle (PBS) or LPS (10 µg/ml) in fresh starvation medium at 37°C for 1, 2, 4, 6, 8, 96, or 144 h.

**Lung Single Cell Suspension**

At the time of harvest, the lungs were perfused blood free with 30 ml PBS containing 10 U/ml heparin (Novo-Heparin, Mochida, Tokyo, Japan) from the right ventricle, then minced and digested in an enzyme cocktail of DMEM (Sigma) containing 1% of BSA (Wako, Osaka, Japan), 2 mg/ml of collagenase (Wako), 100 µg/ml of DNase (Wako), and 2.5 mg of Dispase II (Roche Diagnostics, Mannheim, Germany) at 37°C for 60 min, then meshed through a 100-µm nylon cell strainer.

**Flow Cytometry of Lung Cells**

Mouse lung cells were pretreated with anti-CD16/32 antibody (BioLegend) to block Fc receptors and then incubated with specific antibodies at 4°C in the dark. The following antibodies were used for cell surface staining: anti-CD31-PE/Cy7 (BioLegend), anti-CD45-Alexa700 (BioLegend), anti-CD11b-FITC (BioLegend), anti-Ly-6G/Ly-6C (Gr-1)-Alexa647 (BioLegend), anti-CD34 (BioLegend), anti-CD133 (eBioscience), anti-CD117/c-kit-R-phyceroerythrin (PE) (BioLegend), and streptavidin-PE antibodies (BioLegend). To assess DNA degradation, the cells were incubated with 10 µg/ml of Hoechst 34580 (Life Technologies, Grand Island, NY) for 30 min at 37°C in the dark. To detect proliferating cells, bromodeoxyuridine (BrdU) APC flow kit (BD Pharmingen, San Diego, CA) was used for staining intracellular BrdU according to the manufacturer’s protocol. Regarding measurement of α-SMA and S100 calcium-binding protein A4 (S100A4), after surface staining the cells were incubated with anti-α-SMA (Thermo Scientific) and anti-S100A4 (Abcam, Cambridge, UK) for 35 min at 22°C. The secondary antibody used was a donkey anti-rabbit IgG-Alexa 488 (IgG; H+L) (Life Technologies) for 25 min at 22°C.

HMVEC-Ls were pretreated with anti-CD16/32 antibody and then incubated with the following reagents: anti-CD31, -CD45, and -α-SMA. Cell fluorescence was measured with the FACSCanto II instrument (Becton Dickinson, San Jose, CA) and analyzed by employing FlowJo software (Tree Star, San Carlos, CA).

**Isolation of Mouse PVECs**

Mouse PVECs were defined as CD31+/CD45- cells. Lung cells were incubated with anti-CD16/32 antibody (BioLegend) and then with anti-CD31-FITC (BioLegend) and anti-CD45-PE (BioLegend). For sorting CD31+/CD45- cells, the cells were incubated with anti-PE microbeads (Miltenyi Biotec, Auburn, CA) for 15 min at 4°C in the dark, and CD45+ cells were depleted through once in deplete mode of autoMACS or autoMACS Pro Separator (Miltenyi Biotec). Then the remaining cells were incubated with anti-FITC microbeads (Miltenyi Biotec) for 15 min at 4°C in the dark, and CD31+ cells were sorted through twice in positive selection mode.

**Fluorescent Immunocytochemistry**

Isolated mouse PVECs were fixed in a 1:1 mixture of methanol and acetone for 2 min followed by blocking with normal goat serum for 30 min as described previously (44). The cells were incubated with primary antibodies (anti-α-SMA, anti-S100A4, and anti-CD31) for 1 h at room temperature, and then with secondary antibodies for 1 h at room temperature. Finally, Hoechst 34580 (Sigma) was used to identify the cell nuclei, and the cells were examined by confocal...
microscopy (Fluoview FV 10i, Olympus). HMVEC-Ls cultured with or without LPS for 144 h were immunostained by the same method.

Quantitative RT-PCR Analysis

Total RNA from CD31+/CD45− cells was isolated with Nucleospin RNA XS (MACHEREY NAGEL, Düren, Germany) according to the manufacturer’s instructions. RNA was subjected to RT-PCR with SuperScript VILO (Life Technologies) according to the manufacturer’s protocol and single-stranded cDNA was synthesized. The resultant cDNA samples were subjected to PCR for amplification by using an ABI Prism 7300 Sequence Detection System (Applied Biosystems, Carlsbad, CA). Specific primers and probes were designed by using the web software of Universal ProbeLibrary Assay Design Center (Roche Applied Science). The Ct value of each sample was normalized with Hprt1 as the endogenous control gene and the relative expression level was calculated by the $2^{-\Delta\DeltaCT}$ method.

ROS Generation Assay

After surface staining, the cells were incubated in PBS containing 40 μM of dichlorofluorescein diacetate (DCFDA; Life Technologies) or 5 μM CellRox Deep Red (Life Technologies) for 30 min at 37°C, to measure intracellular ROS.

Tube Formation Assay

Isolated PVECs suspended in EBM-2 (Lonza) supplemented with single aliquots of 10% FBS EGM-2 MV (Lonza) were seeded at a cell density of 4 × 10^5 cells/well into Matrigel (BD)-coated chamber slides. These were examined for network formation under a microscope.

BM Reconstitution

Donor BM cells were obtained from the femoral and peroneal bones of transgenic mice that expressed enhanced GFP under control of CAG promoter (GFP-transgenic mice; Japan SLC, Shizuoka). On the same day, recipient wild-type female mice (5 wk old) were irradiated with 5 and 4.5 Gy with an interval of 3 h and transplanted by injection of the donor BM cells (1.0 × 10^7 cells in 200 μl PBS) through the tail vein.

NAD(P)H Oxidase Inhibition

Apocynin (10 mg·kg$^{-1}$·day$^{-1}$) ip) (Sigma) or diphenyleneiodonium sulfate (DPI; Toronto Research Chemicals, Toronto, ON, Canada) (1 mg·kg$^{-1}$·day$^{-1}$ sc) was administered to treated mice in vivo. In in vitro experiments, cells were treated with apocynin (300 μM).

TGF-β Inhibition

LY364947 (Sigma, 1 mg·kg$^{-1}$·day$^{-1}$ ip) was administered to treated groups in vivo. In in vitro experiments, cells were treated with LY364947 (2 μM).

Euk-134 Treatment

For treatment in vivo with the superoxide dismutase (SOD) and catalase mimetic Euk-134, mice were given 10 mg/kg Euk-134 (Cayman Chemical, Ann Arbor, MI) in normal saline once daily intraperitoneally.

Statistical Analysis

Values are shown as means ± SE unless otherwise described, or medians (25-75th percentile). The results were analyzed by the Mann-Whitney test for comparison between any two groups, and by non-parametric equivalents of ANOVA for multiple comparisons. GraphPad Prism software (Version 6.03; GraphPad Software, San Diego, CA) was used to analyze the data. The level of statistical significance was set at $P < 0.05$.

RESULTS

Transient Decrease of the Total Number of PVECs Was Associated with Increased Apoptosis

Mice challenged with intraperitoneal LPS showed a decrease of body weight until day 2 (Fig. 1A) and development of protein-rich pulmonary edema until day 2 (Fig. 1, B and C).

Intraperitoneally injected LPS induced a marked influx of leukocytes (CD31+/CD45+ cells) including neutrophils (Gr-1+/CD11b+/CD31+/CD45+ cells) into the lungs (Fig. 1, D–F). The influx of inflammatory cells was confirmed by HE staining (Fig. 1F).

The total number of PVECs (CD31+/CD45− cells) decreased significantly by day 2 (median; 30.2% decrease) but recovered by day 7 (Fig. 1, D and G). Hoechst 34580 staining revealed an increase in apoptotic PVECs with fragmented DNA from day 1 to day 2 and a decrease thereafter (Fig. 1, H and I).

Reactive Oxygen Species in PVECs Transiently Increased Antecedent to Apoptosis

Oxidative stress plays a significant role in the development of acute lung injury (10). Therefore, we determined the amount of intracellular ROS in PVECs by flow cytometry (FCM) analysis using DCFDA and CellRox Deep Red, both of which are well-known quantitative markers of cellular oxidative stress (11). Following LPS challenge, both DCFDA and CellRox Deep Red geometric mean fluorescence intensity significantly increased within 12 h (1.9-fold) and then gradually decreased from day 2 until day 7 (Fig. 1, J and K). ROS production preceded the increase in PVEC apoptosis, which began to increase on day 1.

Pulmonary Vascular EndMT Increased During PVECs Injury In Vivo

Recent reports have indicated that LPS induces a fibrotic phenotype in HUVECs (15, 16). Thus we evaluated whether EndMT occurred in PVECs after LPS challenge in vivo.

We defined the PVECs that underwent EndMT (EndMT-PVECs) as those showing double labeling with antibodies against CD31 and α-SMA, or with antibodies against CD31 and S100A4 (15–17). Changes in the number of EndMT-PVECs after LPS challenge are shown in Fig. 2A. Representative panels of FCM analyses are shown in Fig. 2B. There was a significant increase in the number of PVECs expressing α-SMA (α-SMA-PVECs) and S100A4 (S100A4+PVECs) on day 2 (Fig. 2, A and B), while the total number of PVECs significantly decreased (Fig. 2G). We isolated endothelial cells (CD31+/CD45− cells) using magneeto-beads and performed immunocytochemistry (ICC) experiments. As shown in Fig. 2C, a significant increase of EndMT-PVECs (CD31+/α-SMA− cells and CD31+/S100A4− cells) was detected on day 2 after LPS challenge. Quantitative RT-PCR analyses of the isolated PVECs showed that LPS-PVECs expressed less endothelial cell markers such as Pecam1, Cdh5, and Vwf on day 1 (Fig. 2D), more mesenchymal cell markers such as Coll1a1, Coll1a2, S100a4, Vim, and Myh11 on day 2 (Fig. 2D), and more
transcriptional factors related to EndMT such as Snai1, Snai2, Twist1, Twist2, and Zeb1 on day 2 (Fig. 2D).

Characterization of EndMT-PVECs

**Proliferation of EndMT-PVECs.** To examine the proliferative characteristics of EndMT-PVECs, we performed BrdU incorporation experiments. FCM analyses revealed significantly higher percentages of BrdU-positive α-SMA⁺PVECs compared with α-SMA⁺PVECs on day 2 of LPS challenge (Fig. 3A). Immunofluorescent histological analyses also revealed the presence of α-SMA⁺ cells on day 2 of LPS-induced injury in capillary vessels where smooth muscle cells are usually absent (Fig. 3B). These cells coexpressed CD31, indicating they corresponded to EndMT-PVECs (Fig. 3B). In addition, most EndMT-PVECs expressed Ki67, a marker of cell proliferation (Fig. 3B). Subsequently, the number of pro-
The Majority of EndMT-PVECs Originated from Tissue-Resident Cells

To determine the origin of EndMT-PVECs observed after LPS challenge, we used mice reconstituted with GFP-expressing (GFP⁺) BM cells to distinguish BM-derived from non-BM derived (GFP⁻) cells (Fig. 4). More than 12 wk after BM reconstitution, the percentage of GFP⁺ cells in peripheral blood cells, lung interstitial leukocytes, and PVECs of the recipients (Fig. 4, A–C) was 96.8 ± 1.6 (n = 30), 85.5 ± 1.8 (n = 3), and 0.83 ± 0.92% (n = 3), respectively. After LPS administration, the percentage of GFP⁺ in PVECs transiently increased on day 2 (day 0 vs. day 2: 1.08 ± 0.32 vs. 5.72 ± 0.43% vs. 1.46 ± 0.16%; Fig. 4D). Representative panels of FCM analyses are shown in Fig. 4E. However, the percentage of GFP⁺ in α-SMA⁺ PVECs did not increase significantly. The percentage on days 0, 2, and 7 of LPS challenge (Fig. 4F) was 0.83 ± 0.92, 1.25 ± 1.18, and 0.91 ± 1.13%, respectively, indicating that tissue-resident GFP⁺ PVECs continued to significantly predominate during the follow up term after LPS challenge. These results indicated that most of the new EndMT-PVECs originated not from BM derived cells, but from tissue-resident endothelial cells. Representative panels of FCM analyses are shown in Fig. 4G.

Fig. 2. LPS induction of pulmonary vascular endothelial-to-mesenchymal transition (EndMT). A: FCM analyses revealed the percentage of α-SMA⁺ PVECs and S100A4⁺ PVECs was significantly increased on day 2 after LPS challenge (*P < 0.05, n = 5). Values are means ± SE. B: representative FCM panels with α-SMA⁺-gated and S100A4⁺-gated PVECs are shown. C: isolated PVECs were immunocytochemically stained before and 2 days after LPS challenge. Time course examination by confocal microscopy showed few EndMT-PVECs defined as CD31⁻/α-SMA⁺ or CD31⁻/S100A4⁺ before LPS challenge whereas they were noticeable on day 2. Scale bars, 20 μm. D: gene expression of mesenchymal-specific markers increased after LPS challenge, whereas gene expression of endothelial specific markers decreased after LPS challenge. Moreover, expression of transcription factors related to EndMT was significantly increased after LPS challenge (*P < 0.05, no. of mice from which PVECs were isolated = 3). Values are means ± SE.
Fig. 3. Characterization of EndMT-PVECs. A: FCM analyses revealed higher increases in the percentage of bromodeoxyuridine (BrdU)-positive cells among α-SMA+ PVECs compared with α-SMA- PVECs, on day 2 after LPS challenge (*P < 0.05, n = 5). Values are means ± SE. B: immunohistochemistry also revealed the presence of CD31+ (green)/α-SMA- (blue) cells in capillaries on day 2 after LPS challenge (arrows). Most EndMT-PVECs expressed Ki67 (red) (arrowheads). Yellow scale bars, 100 μm. White scale bars, 20 μm. C: histological quantification revealed that the number of Ki67+ cells among α-SMA+ PVECs was higher than that in α-SMA- PVECs (*P < 0.05; n = 5). Values are means ± SE. D: α-SMA+ PVECs showed higher expression of EPC markers such as CD133 and c-kit (*P < 0.05; n = 5). Values are means ± SE. E: representative FCM panels of Prom1/CD133-positive cells, c-kit-positive cells, and CD34-positive cells in α-SMA+/α-SMA- PVECs are shown. F: immunohistochemistry revealed higher expression of CD133 in α-SMA+ PVECs on day 2 after LPS challenge. α-SMA, blue; CD133, red; α-SMA+/CD133+, purple (arrowheads). Yellow scale bar, 100 μm. White scale bar, 20 μm. G: immunohistochemistry revealed higher expression of c-kit in α-SMA+ PVECs on day 2 after LPS challenge. α-SMA, blue; c-kit, red; α-SMA+/c-kit+, purple (arrowheads). Yellow scale bar, 100 μm. White scale bar, 20 μm.
LPS-Induced EndMT via NAD(P)H Oxidase-Dependent ROS Production

TGF-β is a key inducer of EndMT, although little is known about other signaling events that trigger EndMT. Since LPS-induced EndMT-PVECs was associated with an increase of ROS production, we tested whether LPS-induced EndMT was mediated by oxidative stress. As excessive production of ROS by NAD(P)H oxidase is responsible for tissue injury associated with respiratory inflammatory diseases/injuries including ARDS (30), we injected apocynin intraperitoneally or DPI subcutaneously to C57BL/6 mice to block NAD(P)H oxidase activation. In addition, to confirm the importance of other source of ROS in EndMT, we tested Euk-134, which mimics the catalytic function of SOD coupled with catalase. We also used LY364947, which is a selective inhibitor of TGF-β receptor type I (TGF-βRI), to evaluate the role of TGF-β and ROS in EndMT.

Inhibition of TGF-β signaling attenuated ROS production and EndMT in PVECs. Intracellular ROS measured ex vivo in PVECs isolated by FCM analysis using DCFDA and CellRox Deep Red significantly decreased in LPS-PVECs treated with LY364947, a TGF-β inhibitor (Fig. 5, A and B). In addition, LY364947 significantly attenuated pulmonary vascular EndMT, which was confirmed by a significant decrease in CD31+/α-SMA+ cells and CD31+/S100A4+ cells (Fig. 5, C and D). We also evaluated EndMT-associated gene expression in PVECs. This revealed that LY364947 attenuated EndMT at the level of gene expression (Fig. 5E). These results indicated that inhibition of the TGF-β pathway attenuates production of both ROS and EndMT in PVECs.

Inhibition of NAD(P)H oxidase attenuated EndMT and expression of Tgfb1 and Tgfb2 transcripts in PVECs. As expected, the expression of ROS was significantly decreased in LPS-PVECs treated with apocynin, DPI, or Euk-134 (Fig. 5, A and B). Apocynin and DPI significantly attenuated pulmonary vascular EndMT, which was confirmed by a significant decrease in CD31+/α-SMA+ cells and CD31+/S100A4+ cells (Fig. 5, C and D). Interestingly, NAD(P)H oxidase inhibitors attenuated Tgfb1 and Tgfb2 gene expression, implying an interaction between ROS and the TGF-β pathway (Fig. 5E). In addition, NAD(P)H oxidase inhibition attenuated EndMT as assessed by transcription of EndMT-related genes (Fig. 5E). Euk-134 also slightly attenuated EndMT, although not statistically significant (Fig. 5, C and D). These results indicated that LPS-induced EndMT is closely affected by NAD(P)H oxidase-dependent ROS production.

Fig. 4. Most of EndMT-PVECs originated from non-bone marrow (BM)-derived cells. BM chimera mice reconstituted with GFP-expressing BM cells were analyzed by FCM to detect the origin of lung cells. Representative FCM panels of GFP in peripheral blood cells (A), lung interstitial leukocytes (B), and PVECs (C) in control lungs are shown. GFP+ cells were predominant among α-SMA+ PVECs before and after LPS challenge. D: GFP+ PVECs transiently increased on day 2 after LPS challenge and decreased thereafter (*P < 0.05, n = 3). Values are means ± SE. E: representative panels of time course FCM analyses related to D. F: GFP+ PVECs before and after LPS challenge (comparison between GFP+ and GFP- cells, n = 3) N.S., not significant. Values are means ± SE. G: representative panels of time course FCM analyses related to Fig. 4F. GFP+ cells among α-SMA+ PVECs were gated and the percentages are displayed.
Fig. 5. Changes in α-SMA or S100A4 expression during LPS-induced EndMT depended on NAD(P)H oxidase activity. A and B: LY364947 (LY), apocynin (Apo), diphenyleneiodonium sulfate (DPI) and Euk-134 decreased dichlorofluorescein-sensitive cellular ROS (A) and CellRox Red-sensitive cellular ROS (B) in PVECs 2 days after LPS challenge (*P < 0.05 vs. vehicle-treated mice, #P < 0.05 vs. LPS-treated mice; n = 5). Values are means ± SE. C and D: LY364947, apocynin, and DPI decreased the percentage of α-SMA⁺PVECs (C) and S100A4⁺PVECs (D) 2 days after LPS challenge (*P < 0.05 vs. vehicle-treated mice, #P < 0.05 vs. LPS-treated mice; n = 5). Euk-134 also slightly decreased them, although not statistically significantly. Values are means ± SE. E: PVECs a day after LPS challenge with LY364947, apocynin, or DPI failed to decrease the expression of endothelial markers, as well as to increase the expression of mesenchymal markers. In PVECs isolated 2 days after LPS challenge, NAD(P)H oxidase inhibitors and LY364947 decreased the expression of Tgfb1 and Tgfb2 (*P < 0.05; no. of mice from which PVECs were isolated = 3). Values are means ± SE.
LPS Induced EndMT of HMVEC-Ls in the Absence of Immune Cells

Clinical and experimental studies have provided circumstantial evidence of neutrophil-mediated inflammation in acute lung injury (48), but still acute lung injury does develop in patients with profound neutropenia. Thus we next tested whether LPS induced pulmonary vascular EndMT in the absence of immune cells in vitro.

LPS exposure induced morphological change to a spindle-shaped phenotype (Fig. 6A). ICC analyses revealed that LPS treatment increased α-SMA\(^{-}\)HMVEC-Ls (Fig. 6B). These findings imply that EndMT was reproducible in vitro. FCM analyses also revealed that LPS treatment induced an increase of α-SMA expression onto HMVEC-Ls (Fig. 6C). FCM analyses using DCFDA revealed that LPS exposure increased ROS generation in HMVEC-Ls prior to the increase of EndMT (Fig. 6D).

Expression of ROS was significantly decreased in LPS-treated HMVEC-Ls treated with inhibitors of NAD(P)H oxidase (apocynin) and TGF-βRI (LY364947) (Fig. 6D). EndMT was suppressed by NAD(P)H oxidase inhibitor or the TGF-βRI inhibitor, which was confirmed by FCM (Fig. 6E) and quantitative RT-PCR (Fig. 6F).

These results indicated that LPS provokes EndMT of HMVEC-Ls even in the absence of immune cells, mediated by simple ROS generation.

Long-Term Exposure to LPS Induced a Higher EndMT Response In Vitro

As shown in Fig. 6D, ROS generation in HMVEC-L reached its maximum level at 2 h after the start of LPS exposure and then gradually decreased. Since we could not evaluate the effect of long-term exposure to LPS in vivo due to high mortality, we tested it using HMVEC-Ls. To maintain concentration, LPS-containing media were replaced every 48 h.

As shown in Fig. 7, A and B, the percentage of α-SMA\(^{-}\)HMVEC-Ls reached ~50% at 96 h and reached a plateau thereafter. When LPS was removed from media at 96 h, the percentage of α-SMA\(^{-}\)HMVEC-Ls significantly decreased at 144 h (not removal vs. removal: 46.6 ± 2.41 vs. 10.3 ± 4.95%; P < 0.05). This indicated that most of the phenotypic change was reversible, or so-called “partial EndMT,” whereas the rest of the irreversible phenotypic change was “complete EndMT” (49).

EndMT-HMVEC-Ls Showed Several Characteristics of Progenitor Cells

Since our results in vivo showed EndMT-PVECs were highly capable of proliferation and expressed markers of putative progenitor cells such as Prom1/CD133 and c-kit when the total number of PVECs decreased in lung injury, we speculated that most of EndMT-PVECs behaved as progenitor-like cells in a process critical to lung injury repair. To confirm this, we conducted the following experiments using HMVEC-Ls that exhibited a higher EndMT response after long-term (144 h) exposure to LPS.

Gene expression of progenitor cells and proliferation marker in EndMT-HMVEC-Ls. We first examined whether LPS-treated HMVEC-Ls, ~50% of which coexpressed α-SMA, showed enhanced expression of putative progenitor genes: PROM1/CD133, KIT, and CD34. Among these genes, PROM1/CD133 and KIT were significantly increased in EndMT-HMVEC-Ls (Fig. 7C).

Given that proliferative ability is a crucial factor of progenitor cells, we next examined the gene expression of MKI67, a marker of proliferation by quantitative PCR analyses of EndMT-HMVEC-Ls. As shown in Fig. 7D, a significant increase of MKI67 was observed in EndMT-HMVEC-Ls (5.4-fold), which supported the result of BrdU-incorporation assay in vivo.

Expression of drug resistance genes in EndMT-HMVEC-Ls. Because several ATP-binding cassette (ABC) transporter genes, which are associated with the efflux of noxious reagents from the cells, have been used as functional markers of stem/progenitor cells (27, 47), we examined the expression of three ABC transporter genes. Figure 7E shows that the expression of ABCB1 significantly increased in EndMT-HMVEC-Ls (2.1-fold), suggesting the survival potential of EndMT-HMVEC-Ls in acute lung injury.

ALDH activity of EndMT-HMVEC-Ls. Aldehyde dehydrogenase (ALDH) activity has been reported to increase in stem/progenitor cells (25, 29). We analyzed two genes related to ALDH activity (25). The expression of ALDH1B1 significantly increased in EndMT-HMVEC-Ls (2.7-fold) (Fig. 7F).

Vasculogenic capacity of EndMT-HMVEC-Ls. Increased vasculogenesis is one of the fundamental properties of progenitor cells (2, 18, 46); therefore we performed tube formation assays on Matrigel-coated chamber slides using HMVEC-Ls subjected or not to LPS challenge (Fig. 7G). LPS-treated HMVEC-Ls, ~50% of which coexpressed α-SMA (EndMT-HMVEC-Ls), began to form tubelike networks from day 1 and this was expanded until day 7. In contrast, HMVEC-Ls that were not subjected to LPS challenge exhibited very short tubular structure on day 7.

DISCUSSION

In this study, we first demonstrated that pulmonary vascular EndMT occurred in a murine model of endotoxin-induced lung injury. Although LPS induced apoptosis in pulmonary endothelial cells in vivo, surviving endothelial cells underwent mesenchymal transition from the early phase of acute lung injury. We also determined that ROS, a major cause of acute lung injury, directly induced EndMT in pulmonary endothelial cells, which was significantly blocked by NAD(P)H oxidase. Importantly, LPS-induced EndMT-PVECs expressed progenitor cell markers and showed increased cell proliferation ability and angiogenesis.

Acute lung injury is characterized by substantial oxidative stress in the lung, which leads to cellular injury and disease (10). Our experiment in vivo showed that the increase of intracellular ROS in PVECs was followed by an increase of EndMT-PVECs. This suggested that ROS has a driving potential for EndMT and we proved this by administering two different antioxidants that block NAD(P)H oxidase, a major source of superoxide in vascular endothelial cells (14, 21). As far as we know, this is the first study demonstrating that oxidative stress mediates LPS-induced pulmonary vascular EndMT in vivo. Interestingly, TGF-β might increase ROS production and ROS might activate/induce TGF-β in LPS-
induced acute lung injury. Although we did not show the synergism between the antioxidants and the TGF-βRI inhibitor in vascular EndMT in this study, the two likely interact. The endotoxemia generated by LPS induces a burst of other pro-inflammatory cytokines such as tumor necrosis factor-α and interleukin-6. These pro-inflammatory cytokines also might be involved in EndMT in acute lung injury (33, 37).

Endotoxin-induced vascular injury of the lung occurs mainly at the peripheral capillary level, where interstitial leaked fluid blocks the diffusion capacity, leading to progressive respiratory failure (13, 24). In the acute phase of acute lung injury, EndMT could contribute to the angiogenic repair of injured PVECs. Indeed, EndMT-PVECs were increased in capillary vessels on day 2 after LPS challenge, showed high proliferative potential, and expressed EPC markers such as CD133 and c-kit. Recently, it has become evident that some forms of cells, recruited from the BM and from some other tissues, circulate in the peripheral blood and have the ability to be embedded in the injured endothelium and differentiate into mature cells with endothelial characteristics. These cells were called EPCs, and they seemed to be an important mechanism for maintenance and repair of the endothelium (7, 8, 26, 42). Our experiments in vitro also showed that EndMT cell-rich HMVEC-Ls had higher vasculogenic capacity, expression of drug resistance genes, and ALDH activity, which strongly suggested that EndMT-PVECs include functional EPCs.

In previous studies we have shown that pulmonary microvascular endothelial cells were enriched with progenitor cells that had vasculogenic capacity (27, 38). LPS-induced EndMT-PVECs occurred at the capillary level where tissue-resident EPCs are also located (27, 38). Although it has been reported that the expression of mesenchymal progenitor cell markers is upregulated during the process of EndMT, our findings suggested the possibility that some survived pulmonary endothelial cells underwent EndMT to dedifferentiate toward endothelial progenitor-like cells, contributing thereby to the replacement of the injured endothelium. The endothelium itself is believed to have a relatively weak capacity for self-repair, because it is built from mostly terminally differentiated cells with low proliferative capacity (1). However, our study suggests that mature endothelial cells surrounding the injured locus in the endothelium can replicate in situ and replace lost and damaged cells via EndMT.

In addition, we investigated the origin of LPS-induced EndMT-PVECs in vivo. To do so, we used BM-chimeric mice to distinguish tissue-resident from BM-derived circulating cells. Setting time as 3 mo between BM transplantation and LPS challenge enabled us to establish ~95% chimerism of peripheral blood cells (27), avoiding underestimation due to insufficient BM reconstitution; lung interstitial leukocytes were efficiently replaced by BM-derived cells, whereas ~98% of EndMT-PVECs were non-BM-derived cells before and after LPS challenge. These results indicated that the majority of EndMT-PVECs originated from tissue-resident PVECs, not from the BM. To our knowledge, this study is the first to describe the origin of EndMT-PVECs. Accumulating evidence has demonstrated that BM-derived endothelial progenitor cells are involved in tissue repair (6, 7). However, the sources of these cells are matter of debate, i.e., from BM or from the tissue-resident cells, especially in lung injury. We previously reported that tissue-resident endothelial cells had progenitor-like capacity and were involved in tissue repair in the murine models of acute lung injury and pulmonary hypertension (27, 38). In the present study, EndMT-PVECs that could be involved in endothelial repair were derived from lung-resident cells. We focused on a mouse model in which injury could be repaired to better support our underlying hypothesis; it is not a severe lung injury model. With higher doses of LPS (15 μg/kg), we had significant mortality. Although there is a possibility that BM-derived EndMT-PVECs could increase with more significant lung injury, in our study with moderate injury, BM-derived EndMT-PVECs did not significantly increase while BM-derived PVECs transiently increased at the same time in the phase of acute lung injury. However, it is possible that these phenomena are dependent on the severity of injury. Taking them together, we have to cast a spotlight on the cell transformation not only of BM-derived cells but also of tissue-resident cells.

Several limitations should be mentioned in the present study. First, we did not clearly establish the role of LPS-induced EndMT in any pathological process, such as post-ARDS pulmonary fibrosis. Mice treated with LPS recovered within a week and lung inflammation regressed without any fibrotic change in this model. Thus our findings demonstrated so-called partial EndMT, a process in which cells retain reversibility to their original phenotype when stimulation ceases (49). Although partial EndMT is apparently associated with the development of acute lung injury in each phase, endothelial cell lineage tracing transgenic mice would be necessary for demonstrating “complete EndMT” (pulmonary fibrosis), using a post-ARDS pulmonary fibrosis model (49). Although our murine model exhibited normal repair by day 7 after LPS challenge, intraperitoneal injection of LPS for 3 consecutive days is reported to induce pulmonary fibrosis (23). In such a model of continuous LPS stimulation, EndMT might be involved not only in pulmonary fibrosis but also in pathological angiogenesis as is observed in tumor progression (54). Indeed, LPS were
reported to induce lung metastasis of breast cancer (31). Thus how EndMT changes according to dose or duration of LPS or with other changes to the mouse model will be an issue to be investigated in the future.

Second, more precise assessment of gene expression associated with EndMT-PVECs is needed. In this study, we couldn’t isolate live EndMT-PVECs because we used intracellular antigens (α-SMA and S100A4) as EndMT markers. Therefore further research for detecting cell-surface antigens as EndMT markers will be needed to elucidate the characteristics of EndMT-PVECs.

Third, a more rigorous evaluation of NAD(P)H oxidase isoform and/or another sources of ROS responsible for EndMT would be useful. These studies may provide novel insight into ROS as potential therapeutic targets for treatment of acute lung injury.

In conclusion, our data provide the first evidence in vivo that endotoxin directly induces pulmonary vascular EndMT from
the early phase of acute lung injury by ROS activation. The EndMT-PVECs share progenitor cell-like characteristics, which may contribute to repair after pulmonary vascular injury. Our findings are useful for further studies to elucidate the role of EndMT in acute lung injury, which would lead to new approaches and treatments.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


