

# Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase

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**Abstract** Oxidized low density lipoprotein (OxLDL) is one of the most important risk factors of cardiovascular disease. Here, we study the impact of OxLDL on endothelial progenitor cells (EPCs) and determine whether OxLDL affects EPCs by an inhibitory effect on endothelial nitric oxide synthase (eNOS). It was found that OxLDL decreased EPC survival and impaired its adhesive, migratory, and tube-formation capacities in a dose-dependent manner. However, all of the detrimental effects of OxLDL were attenuated by pretreatment of EPCs with lectin-like oxidized low density lipoprotein receptor (LOX-1) monoclonal antibody or L-arginine. Western blot analysis revealed that OxLDL dose-dependently decreased Akt phosphorylation and eNOS protein expression and increased LOX-1 protein expression. Furthermore, OxLDL caused a decrease in eNOS mRNA expression and an increase in LOX-1 mRNA expression. These data indicate that OxLDL inhibits EPC survival and impairs its function, and this action is attributable to an inhibitory effect on eNOS.—Ma, F. X., B. Zhou, Z. Chen, Q. Ren, S. H. Lu, T. Sawamura, and Z. C. Han. Oxidized low density lipoprotein impairs endothelial progenitor cells by regulation of endothelial nitric oxide synthase. *J. Lipid Res.* 2006. 47: 1227–1237.

**Supplementary key words** angiogenesis • stem cell • apoptosis • Akt

Postnatal neovascularization is a crucial compensatory response in chronically ischemic diseases. Increasing evidence indicates that postnatal neovascularization involves the incorporation of endothelial progenitor cells (EPCs) (1, 2). Transplantation of EPCs into patients can induce blood flow recovery in ischemic limbs and improve left ventricular function after myocardial infarction (3, 4). Therefore, this beneficial property of EPCs is appealing for cell-based therapeutic neovascularization in ischemic

tissue. However, in some diseases, such as coronary artery disease and diabetes, the number of circulating EPCs is decreased and their function is impaired (5–7). Therefore, further understanding of the regulation of EPCs and the mechanism of their regulation may provide new insights into therapeutic neovascularization. However, the exact mechanism leading to a deficiency of EPCs remains unknown. The level of oxidized low density lipoprotein (OxLDL) increases in patients with coronary artery disease or diabetes and serves as an independent predictor for future cardiac events in these patients (8, 9). Therefore, OxLDL may be one of the factors that influence the growth and bioactivity of EPCs. Wang and colleagues (10) recently investigated the effects of OxLDL on the number and activity of EPCs, but the underlying mechanism of action still remains largely unknown.

Recent studies have demonstrated that statins and vascular endothelial growth factor (VEGF) rapidly activate serine/threonine kinase Akt in EPCs and induce the mobilization of bone marrow-derived EPCs (11, 12). Akt serves as a multifunctional regulator of cell biology (13). Phosphorylated Akt becomes available to phosphorylate its downstream substrates, such as endothelial nitric oxide synthase (eNOS), leading to eNOS activation and nitric oxide (NO) release (14). Furthermore, it has been suggested that eNOS is indispensable for the mobilization of

Abbreviations: Dil Ac-LDL, 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (Dil)-labeled acetylated low density lipoprotein; eNOS, endothelial nitric oxide synthase; EPC, endothelial progenitor cell; HUVEC, human umbilical vein endothelial cell; L-NAME, N-nitro-L-arginine methyl ester; LOX-1, lectin-like oxidized low density lipoprotein receptor; LOX-1 MAb, lectin-like oxidized low density lipoprotein receptor monoclonal antibody; MNC, mononuclear cell; NO, nitric oxide; OxLDL, oxidized low density lipoprotein; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling; UEA-1, *Ulex europaeus* agglutinin-1; VEGF, vascular endothelial growth factor.

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stem/progenitor cells from bone marrow and serves as a significant regulator of hemangioblast activity (15, 16). Thus, we hypothesize that the Akt/eNOS pathway is involved in the effects of OxLDL on EPC biology.

OxLDL, through its receptors, exerts its effects on the vessels (17). The classical scavenger receptors are absent or present in very small amounts in endothelial cells (18). In 1997, a lectin-like oxidized low density lipoprotein receptor (LOX-1) was identified in endothelial cells (19). Since then, LOX-1 has been shown to mediate many biological effects of OxLDL in endothelial cells (20). The inhibition of LOX-1 can abrogate the downregulation of phosphorylated Akt and eNOS protein expression (21, 22). LOX-1 is expressed in EPCs and related to OxLDL-induced EPC senescence (23). It is of interest to know whether LOX-1 is involved in the effects of OxLDL on the survival and function of EPCs.

## METHODS

### Preparation and oxidation of LDL

Blood samples for LDL preparation were taken from healthy volunteers after 12 h of fasting. Written informed consent was obtained from all volunteers in accord with the Institutional Ethics Committee. LDL was separated by density gradient ultracentrifugation as we described previously (24). The purity of LDL was assessed by agarose gel electrophoresis, and the protein content was determined by the modified Lowry method. LDL was dialyzed for 24 h against 0.01 mol/l PBS, pH 7.4, at 4°C to remove EDTA and oxidized by exposure to CuSO<sub>4</sub>. EDTA was then added and LDL was dialyzed against PBS to stop oxidation. To assess the extent of LDL oxidation, the thiobarbituric acid-reactive substance and electrophoretic mobility on agarose gels were determined. The level of thiobarbituric acid-reactive substance was  $2.23 \pm 1.56$  and  $24.37 \pm 8.14$  nmol/mg protein in native LDL and OxLDL, respectively. Compared with the native LDL, the OxLDL showed increased electrophoretic mobility on agarose gels of  $1.8 \pm 0.4$  times. OxLDL was sterilized by passing it through a 0.22  $\mu$ m filter.

### Cell isolation and culture

Human umbilical cord blood samples were collected in sterile blood packs containing anticoagulant. Written informed consent was obtained from all mothers in accord with the Institutional Ethics Committee. Mononuclear cells (MNCs) were isolated by Histopaque-density gradient centrifugation within 4 h of sample collection. CD34<sup>+</sup> cells were isolated from MNCs with the CD34 Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. The viability and purity of CD34<sup>+</sup> cells were evaluated by trypan blue exclusion and flow cytometry. CD34<sup>+</sup> cells were depleted of adherent cells by incubation in fibronectin (Invitrogen)-coated culture flasks for 24 h. This process was repeated three times, with the purpose of removing CD34<sup>+</sup> mature endothelial cells, which shed off the vessel wall and adhered to the culture flasks. The nonadherent CD34<sup>+</sup> cells were reseeded in fibronectin-coated culture flasks and cultured in endothelial cell growth medium-2 (Clonetics) supplemented with 50 ng/ml VEGF (Pepro Tech), 100 ng/ml stem cell growth factor (Pepro Tech), and 20 ng/ml interleukin (kindly provided by Kirin Pharmaceuticals). After 7–10 days of culture, nonadherent cells were removed by washing with PBS, and adherent cells were continuously cultured in endothelial cell growth medium-2. Human umbilical vein endothelial cells (HUVECs) were isolated by in-

cubating umbilical vein with 0.25% trypsin for 15 min at 37°C and were cultured in M199 with 20% FBS. HUVECs in passages 1 and 2 were used.

### EPC characterization

EPCs and HUVECs were subjected to flow cytometric analysis to examine the expression of CD31, VE-cadherin, vWF, CD34, CD133, CD106, CD45, and CD14. Cells were incubated for 30 min at 4°C with monoclonal mouse anti-human antibodies against VE-cadherin and vWF (BD PharMingen). Cells were prepenetrated with 0.1% Triton X-100 for 15 min at 37°C before being labeled with vWF antibody. Then, cells were incubated with FITC-conjugated anti-mouse IgG. For additional analysis, cells were incubated for 30 min at 4°C with phycoerythrin-conjugated anti-human CD133 (Miltenyi Biotec) and FITC-conjugated anti-human CD34, CD31, CD106, and CD14 antibodies (BD PharMingen). Isotype-matched antibodies served as controls. Cells were then fixed in 1% polyformaldehyde and quantitatively analyzed by flow cytometry.

Binding of FITC-labeled *Ulex europaeus* agglutinin 1 (FITC-UEA-1), a lectin specific for human endothelium, and uptake of 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (Dil)-labeled acetylated low density lipoprotein (Dil Ac-LDL) were measured to determine EPCs. EPCs were incubated with 10  $\mu$ g/ml Dil Ac-LDL (Molecular Probes) at 37°C for 4 h and fixed with 2% paraformaldehyde for 10 min. After washing, EPCs were incubated with 10  $\mu$ g/ml FITC-UEA-1 (Sigma) at 4°C for 30 min. The incorporation of Dil Ac-LDL and binding of FITC-UEA-1 were detected with a confocal microscope (Leica Microsystems GmbH).

To reconfirm EPCs, attached cells were assessed for the uptake of Dil Ac-LDL and immunostaining with CD31 or vWF (Dako Corp.) by confocal microscopy. 4',6-Diamino-phenylindole were used to dye the nuclei of adherent cells.

### Treatment of EPCs

EPCs were treated without or with different concentrations of OxLDL (10, 25, and 50  $\mu$ g/ml) for 48 h. Among these EPCs, two groups of EPCs were pretreated with 10  $\mu$ g/ml lectin-like oxidized low density lipoprotein receptor monoclonal antibody (LOX-1 MAb; JTX92), a specific blocking antibody to LOX-1, or 100  $\mu$ mol/l L-arginine (Sigma), a precursor of NO, before exposure to 50  $\mu$ g/ml OxLDL. To confirm the role of Akt and eNOS in EPC biology, one group of EPCs was treated with 100  $\mu$ mol/l N-nitro-L-arginine methyl ester (L-NAME; Sigma), an inhibitor of eNOS. Another group of EPCs was treated with 1  $\mu$ mol/l triciribine, a specific inhibitor of the Akt signaling pathway.

### Apoptosis assay

The proapoptotic potential of OxLDL on EPCs was assessed by annexin V/propidium iodide binding assay according to the manufacturer's instructions. To exclude dead cells, only annexin V-positive and propidium iodide-negative cells were counted.

For in situ detection of apoptotic cells, EPCs were fixed with 2% paraformaldehyde. Then, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was applied according to the manufacturer's instructions (Roche Molecular Biochemicals). EPCs were counterstained with 4',6-diamino-phenylindole (Sigma). TUNEL-positive cells were examined in a blinded manner, and the percentage was evaluated.

### Adhesion assay

EPCs were labeled with Dil Ac-LDL as described above. After detachment and centrifugation, EPCs were resuspended in adhesion buffer (0.5% BSA in EBM-2), and identical numbers of

cells were replated onto fibronectin-coated 24-well culture plates, incubated for 30 min at 37°C, and then washed three times carefully with adhesion buffer to remove nonadherent cells. The adherent cells were counted by fluorescence microscopy at 200× magnification by independent blinded investigators. Six independent fields were assessed for each well, and the average number of adherent cells per 200× field was determined.

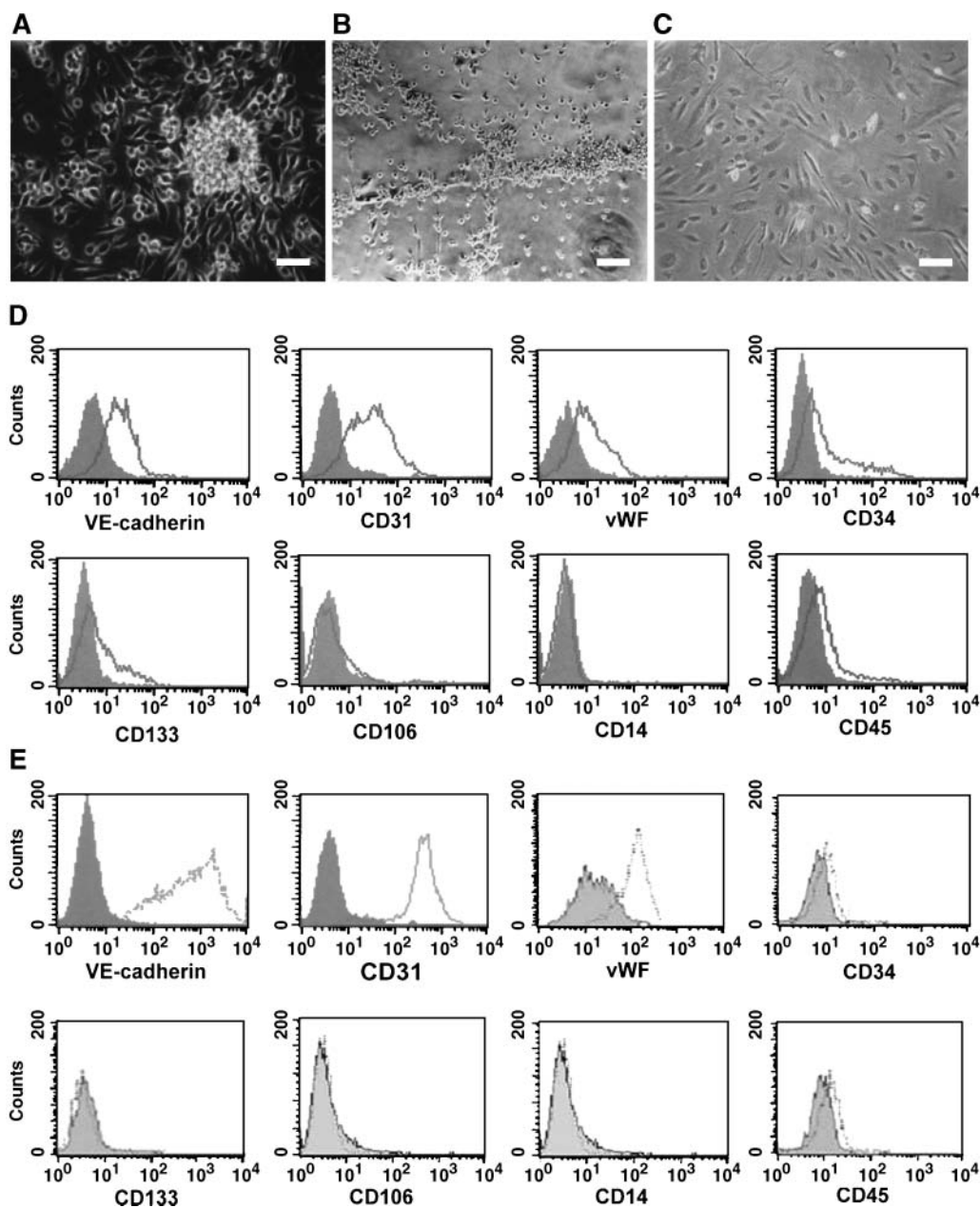
### Migration assay

The migration of EPCs was performed in a Transwell Chamber (Corning Costar). Briefly, EPCs were gently detached, harvested, and resuspended in chemotaxis buffer (EBM-2, 0.5% BSA). One hundred microliters of chemotaxis buffer containing  $1 \times$

$10^5$  cells was added to the upper compartment, and 600  $\mu$ l of chemotaxis buffer with 100 ng/ml VEGF was added to the lower compartment. After incubation at 37°C for 4 h, the filters were removed and the cells in the lower compartment were counted using flow cytometry, with appropriate gating, for 20 s at a high flow rate. The migratory rate was expressed as the percentage of input cells migrating into the lower chamber (average events for 20 s/average input cell events for 20 s  $\times$  100%). All groups of experiments were performed in triplicate.

### Tube-formation assay

An ECM gel (Sigma) was thawed at 4°C overnight and placed on a 96-well culture plate at 37°C for 1 h to allow solidification.



**Fig. 1.** Characteristics of endothelial progenitor cell (EPCs) developed from cord blood CD34<sup>+</sup> cells. A: Cell clusters and spindle-shaped cells sprouted from the edges of cell clusters. B: Cord-like structures formed by adherent cells. C: Adherent cells reached confluence with cobblestone morphology in 2 weeks. Bars = 10  $\mu$ m. D, E: Flow cytometric analysis of cell surface markers on EPCs (D) and human umbilical vein endothelial cells (E).

EPCs treated without or with OxLDL were harvested and replated (10,000 cells/well) on the top of the solidified ECM gel in EBM-2 medium supplemented with 0.5% BSA and VEGF (100 ng/ml). Cells were incubated at 37°C for 12 h. Tube formation was defined as a structure exhibiting a length four times its width. The networks of tubes were photographed from six randomly chosen fields with a microscope. The total length of the tube structures in each photograph was measured using Adobe Photoshop software (25).

### Determination of NO generation

NO is unstable, but it has the stable end products nitrite and nitrate. Hence, the best index of NO generation is the sum of nitrite and nitrate. The culture medium of EPCs was harvested and stored at -80°C until assay. The level of nitrite/nitrate was measured as described previously (26). Briefly, nitrate was converted to nitrite with nitrate reductase, and total nitrite was measured with the Griess reagent. The absorbance was determined at 540 nm with a spectrophotometer.

### Semiquantitative RT-PCR

Total RNA was extracted from cultured EPCs using the RNA extraction kit (Qiagen). To generate cDNA, the DNase-treated RNA (100 ng) was reverse-transcribed with oligo(dT) and Moloney murine leukemia virus reverse transcriptase at 37°C for 1 h for PCR amplification to estimate the expression of LOX-1 and eNOS. Two specific primers were used to identify and amplify LOX-1 (sense primer, 5'-TTACTCTCCATGGTGGTGCC-3'; antisense primer, 5'-AGCTTCTTCTGCTTGTTGCC-3'). The PCR product was 188 bp. For PCR, 32 cycles were used at 94°C for 40 s, 55°C for 1 min, and 72°C for 1 min. Two specific primers were used to identify and amplify eNOS (sense primer, 5'-ATGAAGCACCTGGAGAATGAG-3'; antisense primer, 5'-TCGGAGCCATACAGGATTG-3'). The PCR product was 299 bp. For PCR, 30 cycles were used at 94°C for 40 s, 50°C for 1 min, and 72°C for 1 min. The RT-PCR-amplified samples were visualized on 1% agarose gels using ethidium bromide. Each mRNA band was normalized with  $\beta$ -actin mRNA as an internal

reference. The band of interest was analyzed by scan analysis software and expressed as the ratio to the  $\beta$ -actin mRNA band.

### Western blot analysis

EPCs lysates were separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Millipore). The membranes were blocked by incubation in Tris-buffered saline (10 mmol/l Tris, pH 7.5, and 100 mmol/l NaCl) containing 0.1% (v/v) Tween 20 and 5% (v/v) nonfat dry milk for 2 h, followed by a 2 h incubation at room temperature with rabbit polyclonal antiphospho-Akt-Ser473 antibody (Cell Signaling Technology), mouse monoclonal anti-eNOS antibodies (BD Transduction Laboratories), or goat anti-LOX-1 polyclonal antibody (Biovision). The membranes were washed extensively in Tris-buffered saline containing 0.1% (v/v) Tween 20 before incubation for 1 h with a horseradish peroxidase-conjugated secondary antibody (Amersham Life Sciences). The membranes were then washed and developed using ECL substrate (Amersham Life Sciences). The membranes were reprobbed with an anti-actin antibody (Santa Cruz Biotechnology) and quantified by densitometry.

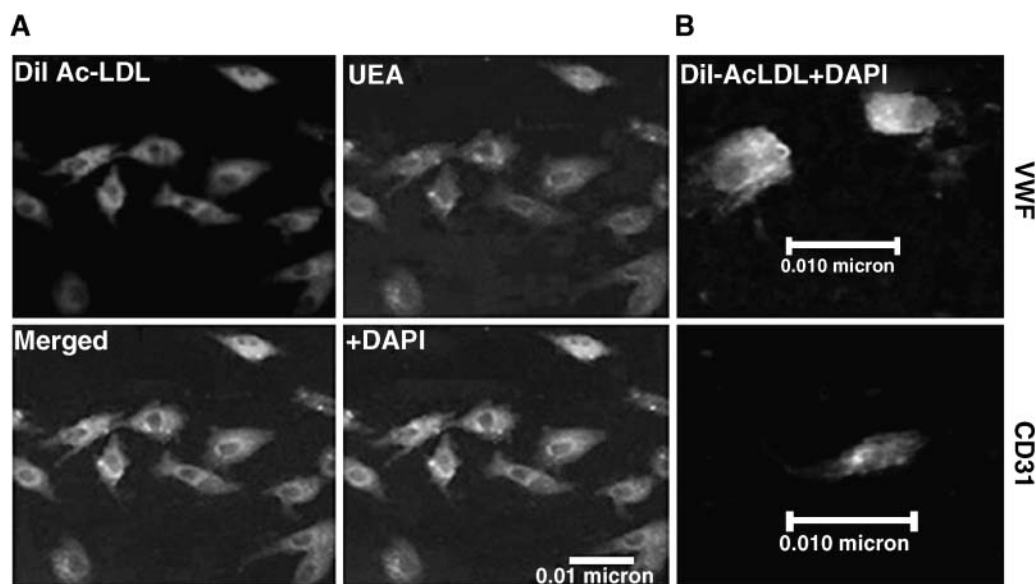
### Statistical analysis

All data are expressed as means  $\pm$  SD. Differences between group means were determined by one-way ANOVA followed by a Newman-Keuls test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Characteristics of EPCs developed from cord blood CD34<sup>+</sup> cells

After two rounds of immunomagnetic sorting, the purity of enriched CD34<sup>+</sup> cells was >90%. When CD34<sup>+</sup> cells were cultured on fibronectin, a fraction of cells adhered to fibronectin, whereas others still suspended in culture medium. A number of cell clusters appeared, and nu-



**Fig. 2.** 1,1'-dioctadecyl-3,3,3'-tetramethylindocarbocyanine perchlorate (Dil)-labeled acetylated low density lipoprotein (Dil Ac-LDL) uptake and FITC-*Ulex europaeus* agglutinin (UEA) binding of EPCs were determined by confocal microscopy. A: Double positive cells were identified as differentiating EPCs. B: Immunohistological staining of anti-CD31 or vWF and uptake of Dil Ac-LDL reconfirmed the characteristics of adherent cells. DAPI, 4',6-diamino-phenylindole.

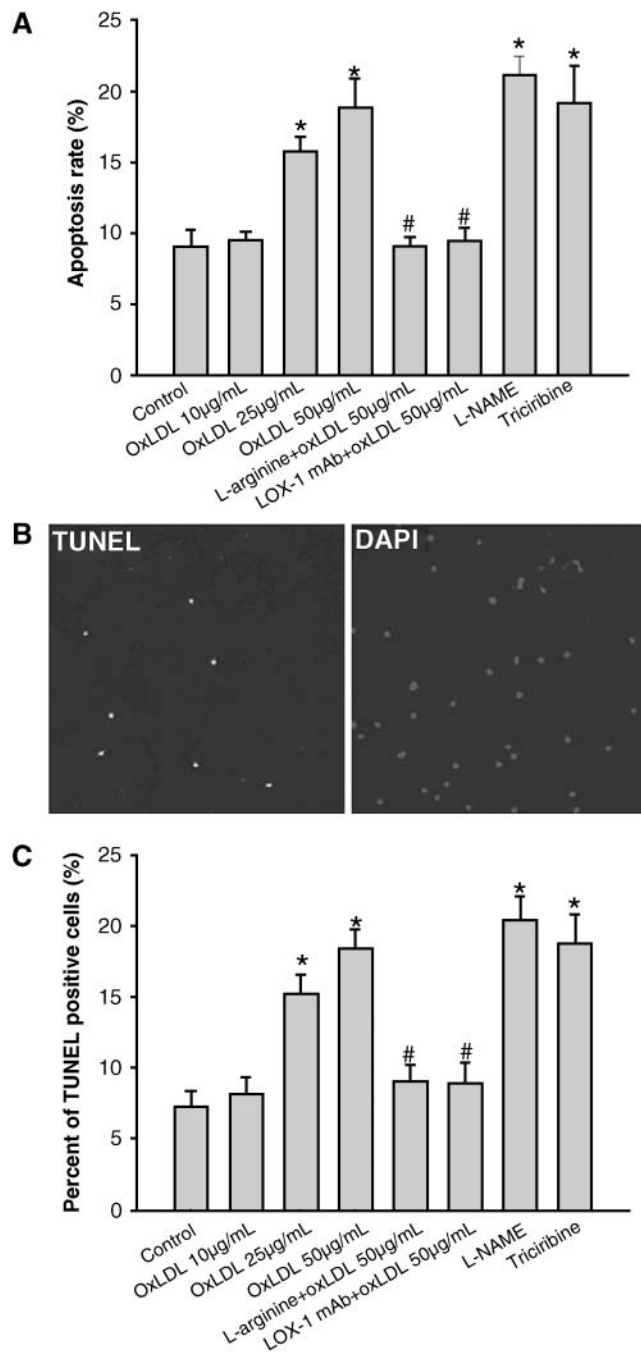
merous spindle-shaped cells sprouted from the edges of the cell clusters after 4–7 days in culture. Cell clusters composed of round cells at the center and spindle-shaped cells at the periphery might resemble the structure of blood islands (Fig. 1A). Cord-like structures, commonly formed by endothelial cells, were observed after 10 days in culture (Fig. 1B). After 2 weeks of culture, spindle-shaped adherent cells reached confluence (Fig. 1C). At this time, flow cytometric analysis disclosed that EPCs expressed endothelial markers, including CD31, vWF, VE-cadherin, and CD34 but not CD106, CD45, and CD14. Stem cell marker CD133 was still expressed (Fig. 1D). However, HUVECs only express the marker of endothelial cells (Fig. 1E).

After 4 h of culture with Dil Ac-LDL and 30 min of incubation with FITC-UEA-1, EPCs took up Dil Ac-LDL and bound FITC-UEA-1 (Fig. 2A), indicating that they expressed scavenger receptor for Ac-LDL and ligand for UEA-1. Figure 2B shows magnified confocal images ( $\times 1,000$ ) of a representative EPC demonstrating the uptake of Dil Ac-LDL and immunostaining with anti-CD31 or vWF antibody.

### Induction of EPC apoptosis by OxLDL

Figure 3A depicts the dose-dependent effect of OxLDL on EPC apoptosis. When EPCs were exposed to 10  $\mu\text{g/ml}$  OxLDL, no significant apoptosis was detected compared with the control (10  $\mu\text{g/ml}$  OxLDL,  $9.49 \pm 0.59\%$ , vs. control,  $9.02 \pm 1.23\%$ ;  $P > 0.05$ ). Incubation with OxLDL at concentrations of 25  $\mu\text{g/ml}$  or higher resulted in a dose-dependent increase of EPC apoptosis (25  $\mu\text{g/ml}$  OxLDL,  $15.78 \pm 1.01\%$ , 50  $\mu\text{g/ml}$  OxLDL,  $18.82 \pm 2.04\%$  vs. control;  $P < 0.05$ ). Pretreatment of cells with L-arginine or LOX-1 MAb markedly decreased the number of apoptotic cells in response to OxLDL (L-arginine + 50  $\mu\text{g/ml}$  OxLDL,  $9.07 \pm 0.63\%$ , LOX-1 MAb + 50  $\mu\text{g/ml}$  OxLDL,  $9.41 \pm 0.94\%$  vs. 50  $\mu\text{g/ml}$  OxLDL;  $P < 0.05$ ). However, pretreatment of cells with nonspecific IgG had no effect (data not shown). When NO production was inhibited by L-NAME, EPC apoptosis was induced significantly (L-NAME,  $21.12 \pm 1.32\%$  vs. control;  $P < 0.05$ ). These results suggest that EPC survival depends on NO production and that the detrimental effect of OxLDL may be mediated in part by interfering with NO generation. Moreover, when the Akt pathway was inhibited by triciribine, EPC apoptosis was increased significantly (triciribine,  $19.12 \pm 2.64\%$  vs. control;  $P < 0.05$ ).

Similar results were observed in the in situ TUNEL assay, in which TUNEL-positive cells were labeled with FITC and total adherent cells were dyed blue. A dose-dependent increase of EPC in situ apoptosis was evident when the concentration of OxLDL was 25  $\mu\text{g/ml}$  or greater (25  $\mu\text{g/ml}$  OxLDL,  $15.23 \pm 1.31\%$ , 50  $\mu\text{g/ml}$  OxLDL,  $18.41 \pm 1.36\%$ , vs. control,  $7.30 \pm 1.01\%$ ;  $P < 0.05$ ). The percentage of apoptotic cells in response to OxLDL was reduced when pretreated with L-arginine or LOX-1 MAb (L-arginine + 50  $\mu\text{g/ml}$  OxLDL,  $9.02 \pm 1.18\%$ , LOX-1 MAb + 50  $\mu\text{g/ml}$  OxLDL,  $8.87 \pm 1.54\%$ , vs. 50  $\mu\text{g/ml}$  OxLDL;  $P < 0.05$ ). Treatment with L-NAME or triciribine resulted in similar proapoptotic effects as treatment with OxLDL (L-NAME,  $20.43 \pm 1.68\%$ , triciribine,  $18.78 \pm 2.07\%$  vs. control;  $P < 0.05$ ) (Fig. 3B, C).



**Fig. 3.** Effects of oxidized low density lipoprotein (OxLDL) on the apoptosis of EPCs. A, C. Treatment of EPCs with OxLDL induced EPC apoptosis. Pretreatment of EPCs with L-arginine or lectin-like oxidized low density lipoprotein receptor monoclonal antibody (LOX-1 MAb) inhibited OxLDL-induced EPC apoptosis. N-Nitro-L-arginine methyl ester (L-NAME) or triciribine also induced EPC apoptosis. Data shown are means  $\pm$  SD;  $n = 6$ . \*  $P < 0.05$  versus control; #  $P < 0.05$  versus 50  $\mu\text{g/ml}$  OxLDL. B: FITC-labeled cells were terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) positive, and 4',6-diamino-phenylindole (DAPI) dyed nuclei.

### OxLDL attenuates EPC adhesion

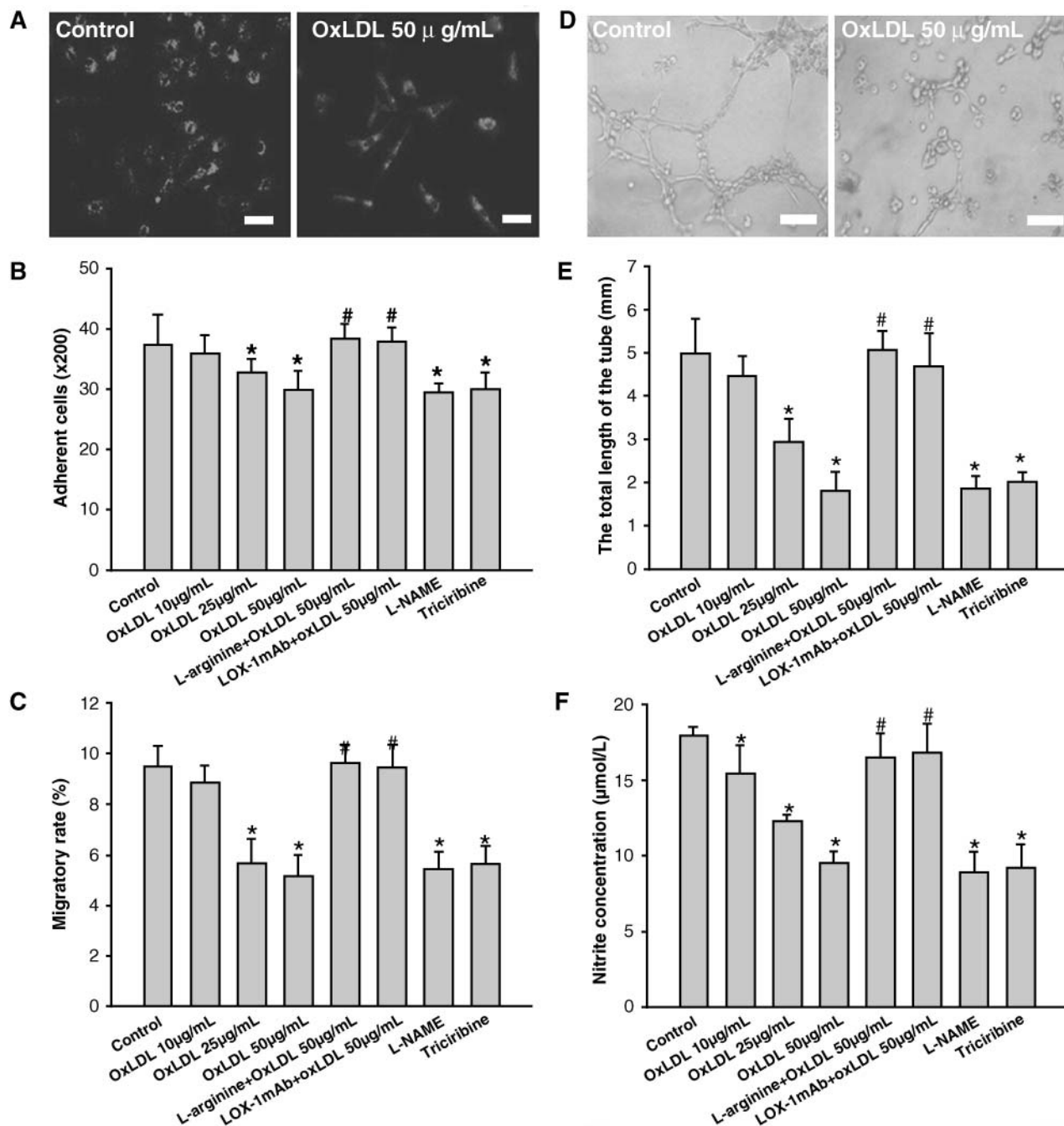
Adhesion of EPCs to the extracellular matrix seems important for the formation of new blood vessels. However, EPCs treated with OxLDL showed a dose-dependent de-

crease of adhesion to fibronectin (25  $\mu\text{g}/\text{ml}$  OxLDL,  $33 \pm 2$ , 50  $\mu\text{g}/\text{ml}$  OxLDL,  $30 \pm 3$ , vs. control,  $37 \pm 5$ ;  $P < 0.05$ ). Pretreatment of cells with L-arginine or LOX-1 MAb markedly increased the number of adhesive cells (L-arginine + 50  $\mu\text{g}/\text{ml}$  OxLDL,  $38 \pm 2$ , LOX-1 MAb + 50  $\mu\text{g}/\text{ml}$  OxLDL,  $38 \pm 2$ , vs. 50  $\mu\text{g}/\text{ml}$  OxLDL;  $P < 0.05$ ). L-NAME or triciribine, like OxLDL, decreased the num-

ber of adhesive cells (L-NAME,  $30 \pm 1$ , triciribine,  $30 \pm 3$  vs. control;  $P < 0.05$ ) (Fig. 4A, B).

#### OxLDL inhibits VEGF-induced EPC migration

To assess the effect of OxLDL on EPC migratory activity, we analyzed the migratory activity of EPCs in response to



**Fig. 4.** Effects of OxLDL on EPC function and nitric oxide (NO) generation. A, D: Representative images of the adhesion (A) and tube formation (D) of EPCs treated with medium alone or 50  $\mu\text{g}/\text{ml}$  OxLDL. B, C, E: Treatment of EPCs with OxLDL, L-NAME, or triciribine decreased the adhesive (B), migratory (C), and tube-formation (E) abilities of EPCs. Pretreatment of EPCs with L-arginine or LOX-1 MAb restored OxLDL-impaired EPC function. F: Treatment of EPCs with OxLDL decreased NO generation, which was restored by pretreatment with L-arginine or LOX-1 MAb. Treatment of EPCs with L-NAME or triciribine also decreased NO generation. Data shown are means  $\pm$  SD;  $n = 6$ . \*  $P < 0.05$  versus control; #  $P < 0.05$  versus 50  $\mu\text{g}/\text{ml}$  OxLDL.

VEGF. Figure 4C shows a significantly reduced migratory rate of EPCs after treatment with OxLDL (25  $\mu\text{g/ml}$  OxLDL,  $5.65 \pm 0.99\%$ , 50  $\mu\text{g/ml}$  OxLDL,  $5.15 \pm 0.82\%$ , vs. control,  $9.51 \pm 0.82\%$ ;  $P < 0.05$ ). Pretreatment of cells with L-arginine or LOX-1 MAb markedly inhibited the reduction of migratory rate in response to OxLDL (L-arginine + 50  $\mu\text{g/ml}$  OxLDL,  $9.63 \pm 0.40\%$ , LOX-1 MAb + 50  $\mu\text{g/ml}$  OxLDL,  $9.48 \pm 0.73\%$  vs. 50  $\mu\text{g/ml}$  OxLDL;  $P < 0.05$ ). L-NAME or triciribine also reduced the migratory rate of EPCs (L-NAME,  $5.45 \pm 0.67\%$ , triciribine,  $5.61 \pm 0.73\%$  vs. control;  $P < 0.05$ ).

### OxLDL impairs tube formation from EPCs

EPCs incubated on Matrigel for 24 h formed an extensive and enclosed network of tubes. Treatment with OxLDL (25 and 50  $\mu\text{g/ml}$ ) impaired the ability of EPCs to form tube structures. The total length of the tube structures in each photograph was decreased (25  $\mu\text{g/ml}$  OxLDL,  $2.93 \pm 0.54$  mm, 50  $\mu\text{g/ml}$  OxLDL,  $1.78 \pm 0.46$  mm, vs. control,  $4.98 \pm 0.61$  mm;  $P < 0.05$ ). Furthermore, the tube structure was severely disrupted, resulting in an incomplete and sparse tube network. Pretreatment with L-arginine or LOX-1 MAb significantly restored the ability of EPCs to form tubes (L-arginine + 50  $\mu\text{g/ml}$  OxLDL,  $5.06 \pm 0.46$  mm, LOX-1 MAb + 50  $\mu\text{g/ml}$  OxLDL,  $4.69 \pm 0.75$  mm vs. 50  $\mu\text{g/ml}$  OxLDL;  $P < 0.05$ ). L-NAME or

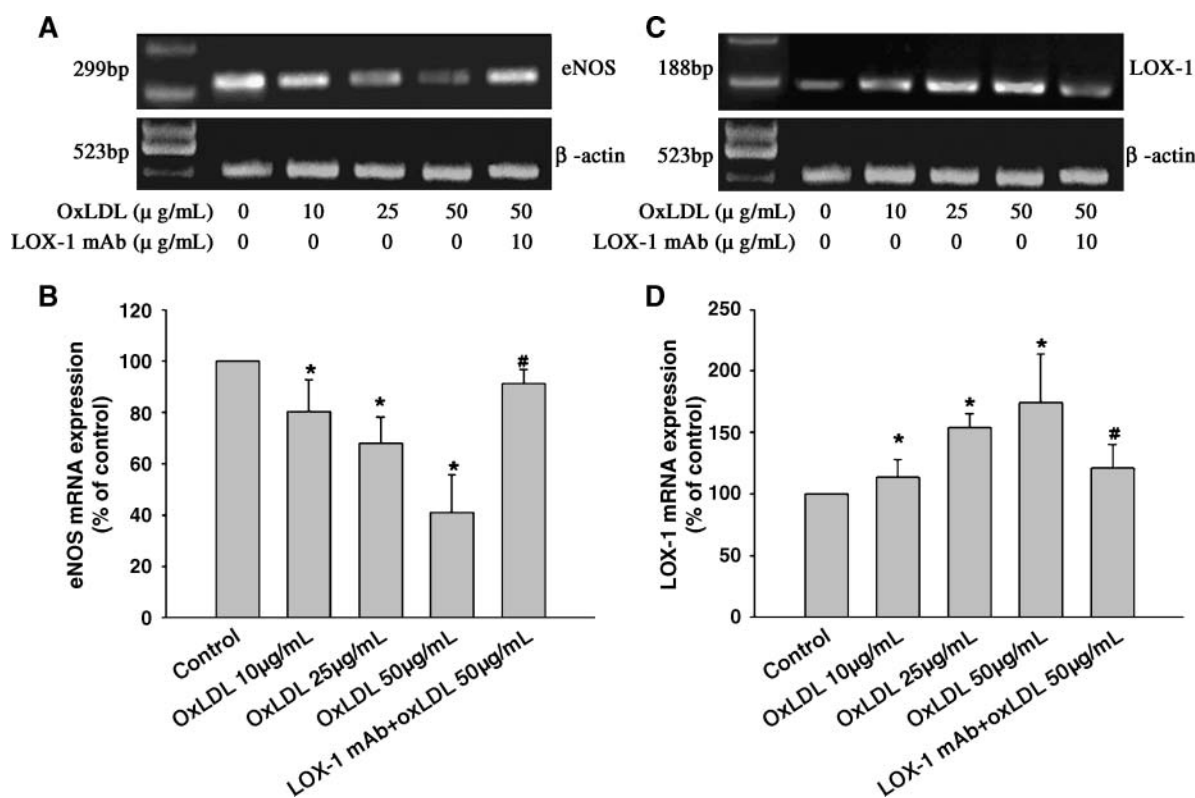
triciribine also impaired EPC tube formation (L-NAME,  $1.82 \pm 0.32$  mm, triciribine,  $2.00 \pm 0.24$  mm vs. control;  $P < 0.05$ ) (Fig. 4D, E).

### OxLDL decreases NO generation

A decrease of NO generation was observed at every OxLDL concentration assayed (nitrite concentrations in 10  $\mu\text{g/ml}$  OxLDL,  $15.44 \pm 1.89$   $\mu\text{mol/l}$ , in 25  $\mu\text{g/ml}$  OxLDL,  $12.32 \pm 0.43$   $\mu\text{mol/l}$ , and in 50  $\mu\text{g/ml}$  OxLDL,  $9.54 \pm 0.77$   $\mu\text{mol/l}$ , vs. control,  $17.96 \pm 0.58$   $\mu\text{mol/l}$ ;  $P < 0.05$ ). This inhibitory effect of OxLDL was abrogated by L-arginine or LOX-1 MAb (L-arginine + 50  $\mu\text{g/ml}$  OxLDL,  $16.46 \pm 1.62$   $\mu\text{mol/l}$ , LOX-1 MAb + 50  $\mu\text{g/ml}$  OxLDL,  $16.85 \pm 1.92$   $\mu\text{mol/l}$  vs. 50  $\mu\text{g/ml}$  OxLDL;  $P < 0.05$ ). Treatment with L-NAME or triciribine also significantly decreased NO generation (L-NAME,  $8.94 \pm 1.33$   $\mu\text{mol/l}$ , triciribine,  $9.18 \pm 1.60$   $\mu\text{mol/l}$  vs. control;  $P < 0.05$ ). (Fig. 4F).

### Regulation of eNOS and LOX-1 mRNA expression by OxLDL

We set eNOS and LOX-1 mRNA expression in controls at 100%. Figure 5A, B depicts a significant decrease in eNOS mRNA expression of EPCs after incubation with OxLDL (eNOS mRNA expression in 10  $\mu\text{g/ml}$  OxLDL,  $80 \pm 12\%$ , in 25  $\mu\text{g/ml}$  OxLDL,  $67 \pm 10\%$ , and in 50  $\mu\text{g/ml}$  OxLDL,  $41 \pm 14\%$ ). Pretreatment with LOX-1 MAb



**Fig. 5.** Effects of OxLDL on endothelial nitric oxide synthase (eNOS) and LOX-1 mRNA expression. Treatment of EPCs with OxLDL decreased eNOS mRNA expression and increased LOX-1 mRNA expression. Pretreatment of EPCs with LOX-1 MAb inhibited OxLDL-induced downregulation of eNOS mRNA expression and upregulation of LOX-1 mRNA expression. A, C: Representative images from agarose gel electrophoresis. B, D: Summary of data for eNOS and LOX-1 mRNA expression. Data are means  $\pm$  SD;  $n = 6$ . \*  $P < 0.05$  versus control; #  $P < 0.05$  versus 50  $\mu\text{g/ml}$  OxLDL.

partially restored eNOS mRNA expression (eNOS mRNA expression in LOX-1 MAb + 50  $\mu$ g/ml OxLDL,  $91 \pm 6\%$ ).

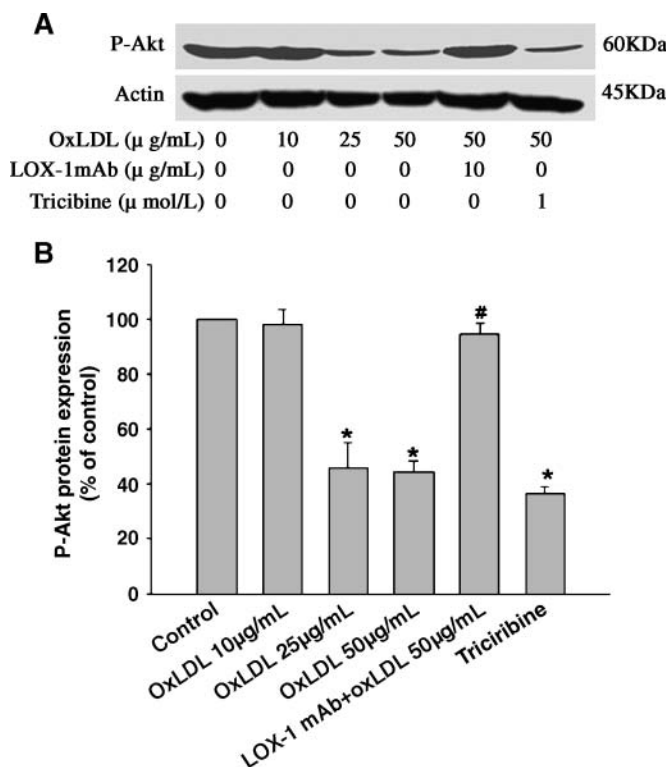
Incubation of EPCs with OxLDL for 48 h increased LOX-1 mRNA expression (LOX-1 mRNA expression in 10  $\mu$ g/ml OxLDL,  $114 \pm 15\%$ , in 25  $\mu$ g/ml OxLDL,  $154 \pm 11\%$ , and in 50  $\mu$ g/ml OxLDL,  $174 \pm 39\%$ ). The upregulation of LOX-1 by OxLDL was blocked by pretreatment of EPCs with LOX-1 MAb (LOX-1 mRNA expression in LOX-1 MAb + 50  $\mu$ g/ml OxLDL,  $121 \pm 19\%$ ) (Fig. 5C, D).

### Regulation of Akt phosphorylation by OxLDL

To test our hypothesis that OxLDL influences eNOS activity through the Akt/eNOS pathway, immunoblotting was performed with a specific phosphorylated Akt antibody at the serine 473 phosphorylation site. OxLDL induced a dose-dependent decrease of Akt phosphorylation, with a  $56 \pm 4\%$  reduction at 50  $\mu$ g/ml. Triciribine also decreased Akt phosphorylation, with a  $64 \pm 3\%$  reduction. However, pretreatment with LOX-1 MAb inhibited the OxLDL-induced decrease of Akt phosphorylation (Fig. 6A, B).

### Regulation of eNOS and LOX-1 protein expression by OxLDL

To determine whether OxLDL influences eNOS protein expression, eNOS protein expression was assessed by



**Fig. 6.** Effects of OxLDL on Akt phosphorylation. A: Representative blots showing Akt phosphorylation from three independent experiments. B: OxLDL dose-dependently decreased Akt phosphorylation, but pretreatment with LOX-1 MAb inhibited the decrease of Akt phosphorylation. Data are means  $\pm$  SD;  $n = 3$ . \*  $P < 0.05$  versus control; #  $P < 0.05$  versus 50  $\mu$ g/ml OxLDL.

immunoblotting. Incubation of EPCs with OxLDL dose-dependently reduced eNOS protein expression, with a  $58 \pm 7\%$  reduction at 50  $\mu$ g/ml. Pretreatment with LOX-1 MAb partially restored the downregulation of eNOS protein expression by OxLDL, with a reduction of only  $7 \pm 4\%$  (Fig. 7A, B).

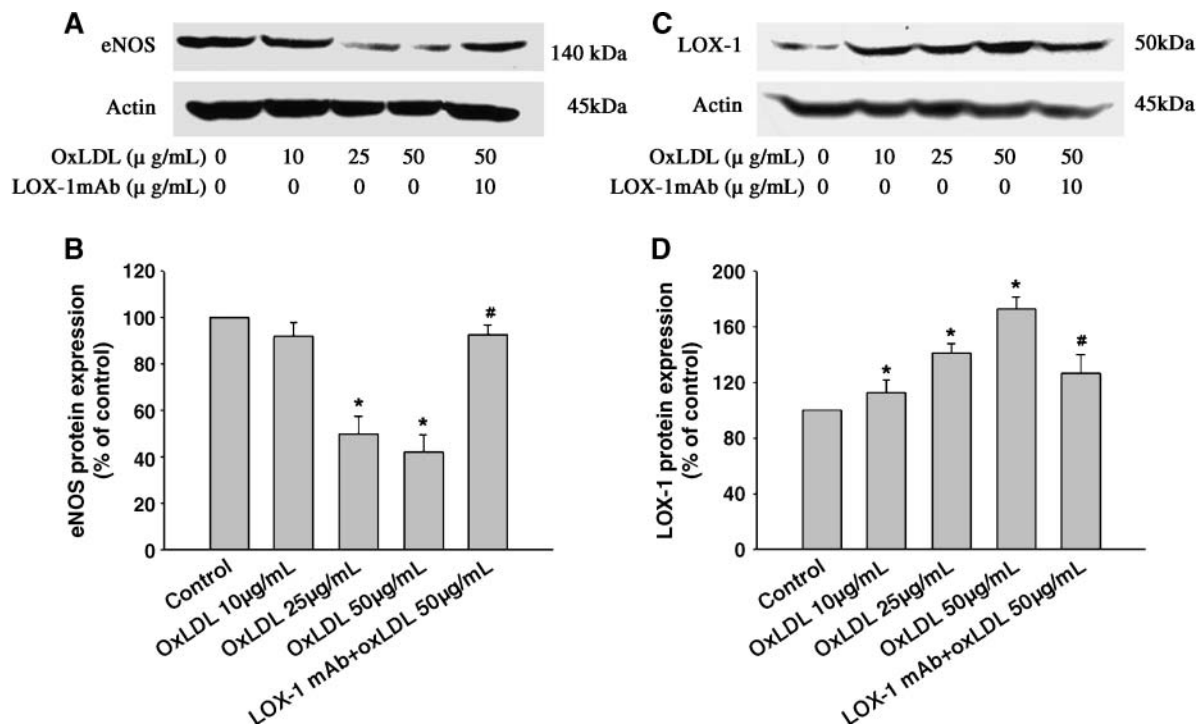
Incubation of EPCs with OxLDL dose-dependently increased LOX-1 protein expression, with a  $73 \pm 8\%$  increase at 50  $\mu$ g/ml. Pretreatment with LOX-1 MAb partially restored the upregulation of LOX-1 protein expression by OxLDL, with an increase of  $27 \pm 13\%$  (Fig. 7C, D).

## DISCUSSION

Recent studies suggest that circulating EPCs serve as a biological marker for vascular function (27) and that reduced numbers of circulating EPCs predict future cardiovascular events (28). In this study, we found that treatment of EPCs with OxLDL resulted in significant apoptosis by annexin V/propidium iodide binding assay and in situ TUNEL assay. Because the level of OxLDL increases in patients with coronary artery disease or diabetes (8, 9), our finding may explain why the number of circulating EPCs is decreased in these patients. Not only does OxLDL inhibit the differentiation of stem cell to EPCs, it also induces the apoptosis of differentiated EPCs. Imanishi et al. (29) reported that OxLDL at concentrations of  $\leq 10$   $\mu$ g/ml inhibited EPC differentiation but did not induce a significant apoptosis of EPCs, which was in accordance with our results. Only at a dose of  $>10$   $\mu$ g/ml did OxLDL significantly induce EPC apoptosis. However, pretreatment of EPCs with L-arginine, a precursor of NO, markedly decreased the number of apoptotic cells in response to OxLDL. Furthermore, when NO production was inhibited by L-NAME, a known inhibitor of eNOS, EPC apoptosis was increased significantly. These results suggest that EPC survival depends on NO production and that the pro-apoptotic effect of OxLDL may be mediated in part by interfering with NO generation.

NO is a significant regulator of neovascularization. Mice deficient in eNOS display impaired ischemia-induced angiogenesis and reduced EPC mobilization (15, 30). Estrogen was discovered to increase EPC number, which was attributable to its ability to induce NO (31). Our study provided new evidence for this point and demonstrated that in the presence of either OxLDL or the eNOS antagonist L-NAME, the adhesive, migratory, and tube-formation activities of EPCs were reduced. However, this reduction could be abrogated with L-arginine coculture. We further showed that OxLDL dose-dependently decreased Akt phosphorylation and eNOS protein as well as mRNA expression in EPCs. Phosphorylated Akt is available to phosphorylate eNOS, leading to eNOS activation (14). So the level of Akt phosphorylation partially reflects eNOS activity (32). This study showed that triciribine, a specific inhibitor of Akt, inhibited Akt phosphorylation and the function of EPCs. These data suggest that OxLDL not only inhibited eNOS activity through the Akt/eNOS pathway but also down-





**Fig. 7.** Effects of OxLDL on eNOS and LOX-1 protein expression. A, C: Representative blots showing eNOS and LOX-1 protein expression from three independent experiments. B, D: OxLDL dose-dependently decreased eNOS protein expression and increased LOX-1 protein expression, but pretreatment with LOX-1 MAb inhibited the downregulation of eNOS protein expression and the upregulation of LOX-1 protein expression. Data are means  $\pm$  SD;  $n = 3$ . \*  $P < 0.05$  versus control; #  $P < 0.05$  versus 50  $\mu$ g/ml OxLDL.

regulated eNOS expression, leading to a significant decrease in NO generation, which may promote EPC apoptosis and overall poor function.

In agreement with our study, a recent study demonstrated that in the presence of L-NAME, the tube formation of EPCs was reduced and that this reduction was abrogated with diethylenetriamine-NO, a donor of NO, coculture (33). Another study further demonstrated that asymmetric dimethylarginine, an endogenous inhibitor of eNOS, inhibited the mobilization, differentiation, and function of EPCs (34). However, one article showed that the capacity of circulating EPCs to migrate was not affected by eNOS inhibition with L-NAME (35). It is difficult to explain these discordant findings, because the culture conditions, including culture duration, culture medium, and concentrations of FBS and growth factors, were distinct and the characteristics of EPCs strongly depended on the culture conditions (36, 37). Moreover, in our study, CD34<sup>+</sup> cells were used as the source of EPCs, which have been suggested to have higher NO-producing capacity than MNC-derived EPCs (38).

We also revealed that the reduction of eNOS expression and activity by OxLDL was mediated by LOX-1. It has been shown that OxLDL exerts its biological effects via the activation of its receptors. LOX-1, found predominantly on endothelial cells, has a different biochemical structure from other receptors. Several investigators have demonstrated that LOX-1 and eNOS can regulate each other. For example, the inhibition of LOX-1 upregulates eNOS expression, and eNOS deficiency leads to the upregulation

of LOX-1 expression (21, 39). Almost all previous studies about LOX-1 were performed with mature endothelial cells. Imanishi et al. (23) showed that LOX-1 was expressed by EPCs and that its mRNA expression was upregulated by OxLDL in a concentration-dependent manner. We demonstrated that the protein expression was also upregulated by OxLDL in a concentration-dependent manner. Furthermore, the detrimental effects of OxLDL on EPC survival and activity, the downregulation of eNOS expression and activity, and the upregulation of LOX-1 expression were all inhibited by pretreatment with specific blocking antibody to LOX-1. Hence, the detrimental effects of OxLDL on EPCs and eNOS were mediated by LOX-1.

Wang and colleagues (10) have described the effects of OxLDL on EPCs derived from MNCs but not from purified CD34<sup>+</sup> cells; in addition, they did not investigate the mechanism underlying the effects of OxLDL on EPCs, which was the emphasis of our study. In our study, CD34<sup>+</sup> cells were used as the source of EPCs, rather than MNCs. This is because MNC-derived EPCs expressed monocyte/macrophage markers but only a few expressed the endothelium-specific marker CD144 or the stem/progenitor marker CD133 (40). However, CD34<sup>+</sup> cell-derived EPCs displayed endothelial features while keeping progenitor markers.

In summary, OxLDL inhibits EPC survival and function, which occurs in part by attenuating EPC eNOS expression and activity. Accumulating evidence indicates that the impairment of EPCs may contribute to the progression of atherosclerosis (41). Therefore, our study may bring new

evidence for the noxious effects of OxLDL in the pathogenesis of atherosclerosis. Moreover, this may be of clinical relevance for patients with cardiovascular disease and those who undergo neovascularization procedures. Gene and cytokine therapy with VEGF in such patients may not produce a maximal angiogenic response because eNOS, the downstream signaling of VEGF, is inhibited. Therefore, it is conceivable that supplemental therapy with downstream targets of VEGF, such as NO, may be more effective at inducing neovascularization. Because the adverse effects of OxLDL could be restored by LOX-1 MAb, the antagonist of LOX-1 may be a promising agent to promote neovascularization in patients with cardiovascular disease. ■

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## REFERENCES

- Asahara, T., H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Wagner, and J. M. Isner. 1999. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ. Res.* **85**: 221–228.
- Murayama, T., O. M. Tepper, M. Silver, H. Ma, D. W. Losordo, J. M. Isner, T. Asahara, and C. Kalka. 2002. Determination of bone marrow-derived endothelial progenitor cell significance in angiogenic growth factor-induced neovascularization in vivo. *Exp. Hematol.* **30**: 967–972.
- Yang, C., Z. H. Zhang, Z. J. Li, R. C. Yang, G. Q. Qian, and Z. C. Han. 2004. Enhancement of neovascularization with cord blood CD133<sup>+</sup> cell-derived endothelial progenitor cell transplantation. *Thromb. Haemost.* **91**: 1202–1212.
- Ott, I., U. Keller, M. Knoedler, K. S. Gotze, K. Doss, P. Fischer, K. Urbauer, G. Debus, N. von Bubnoff, M. Rudelius, et al. 2005. Endothelial-like cells expanded from CD34<sup>+</sup> blood cells improve left ventricular function after experimental myocardial infarction. *FASEB J.* **19**: 992–994.
- Vasa, M., S. Fichtlscherer, A. Aicher, K. Adler, C. Urbich, H. Martin, A. M. Zeiher, and S. Dimmeler. 2001. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ. Res.* **89**: E1–E7.
- Loomans, C. J., E. J. de Koning, F. J. Staal, M. B. Rookmaaker, C. Verseyden, H. C. de Boer, M. C. Verhaar, B. Braam, T. J. Rabelink, and A. J. van Zonneveld. 2004. Endothelial progenitor cell dysfunction: a novel concept in the pathogenesis of vascular complications of type 1 diabetes. *Diabetes.* **53**: 195–199.
- Tepper, O. M., R. D. Galiano, J. M. Capla, C. Kalka, P. J. Gagne, G. R. Jacobowitz, J. P. Levine, and G. C. Gurtner. 2002. Human endothelial progenitor cells from type II diabetics exhibit impaired proliferation, adhesion, and incorporation into vascular structures. *Circulation.* **106**: 2781–2786.
- Shimada, K., H. Mokuno, E. Matsunaga, T. Miyazaki, K. Sumiyoshi, K. Miyauchi, and H. Daida. 2004. Circulating oxidized low-density lipoprotein is an independent predictor for cardiac event in patients with coronary artery disease. *Atherosclerosis.* **174**: 843–847.
- Shimada, K., H. Mokuno, E. Matsunaga, T. Miyazaki, K. Sumiyoshi, A. Kume, K. Miyauchi, and H. Daida. 2004. Predictive value of circulating oxidized LDL for cardiac events in type 2 diabetic patients with coronary artery disease. *Diabetes Care.* **27**: 843–844.
- Wang, X., J. Chen, Q. Tao, J. Zhu, and Y. Shang. 2004. Effects of ox-LDL on number and activity of circulating endothelial progenitor cells. *Drug Chem. Toxicol.* **27**: 243–255.
- Asahara, T., T. Takahashi, H. Masuda, C. Kalka, D. Chen, H. Iwaguro, Y. Inai, M. Silver, and J. M. Isner. 1999. VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells. *EMBO J.* **18**: 3964–3972.
- Levadot, J., S. Murasawa, Y. Kureishi, S. Uchida, H. Masuda, A. Kawamoto, K. Walsh, J. M. Isner, and T. Asahara. 2001. HMG-CoA reductase inhibitor mobilizes bone marrow-derived endothelial progenitor cells. *J. Clin. Invest.* **108**: 399–405.
- Ma, F. X., and Z. C. Han. 2005. Akt signaling and its role in postnatal neovascularization. *Histol. Histopathol.* **20**: 275–281.
- Fulton, D., J. P. Gratton, T. J. McCabe, J. Fontana, Y. Fujio, K. Walsh, T. F. Franke, A. Papapetropoulos, and W. C. Sessa. 1999. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature.* **399**: 597–601.
- Aicher, A., C. Heeschen, C. Mildner-Rihm, C. Urbich, C. Ihling, K. Technau-Ihling, A. M. Zeiher, and S. Dimmeler. 2003. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat. Med.* **9**: 1370–1376.
- Guthrie, S. M., L. M. Curtis, R. N. Mames, G. G. Simon, M. B. Grant, and E. W. Scott. 2005. The nitric oxide pathway modulates hemangioblast activity of adult hematopoietic stem cells. *Blood.* **105**: 1916–1922.
- Steinbrecher, U. P. 1999. Receptors for oxidized low density lipoprotein. *Biochim. Biophys. Acta.* **1436**: 279–298.
- Bickel, P. E., and M. Freeman. 1992. Rabbit aortic smooth muscle cells express inducible macrophage scavenger receptor messenger RNA that is absent from endothelial cells. *J. Clin. Invest.* **90**: 1450–1457.
- Sawamura, T., N. Kume, T. Aoyama, H. Moriwaki, H. Hoshikawa, Y. Aiba, T. Tanaka, S. Miwa, Y. Katsura, T. Kita, et al. 1997. A novel endothelial receptor for oxidized low density lipoprotein. *Nature.* **386**: 73–77.
- Oka, K., T. Sawamura, K. Kikuta, S. Itokawa, N. Kume, T. Kita, and T. Masaki. 1998. Lectin-like oxidized low-density lipoprotein receptor 1 mediates protein kinases implicated in signaling downstream of phagocytosis of aged/apoptotic cells in endothelial cells. *Proc. Natl. Acad. Sci. USA.* **95**: 9535–9540.
- Mehta, J. L., D. Y. Li, H. J. Chen, J. Joseph, and F. Romeo. 2001. Inhibition of LOX-1 by statins may relate to upregulation of eNOS. *Biochem. Biophys. Res. Commun.* **289**: 857–861.
- Li, D. Y., H. J. Chen, and J. L. Mehta. 2001. Statins inhibit oxidized-LDL-mediated LOX-1 expression, uptake of oxidized-LDL and reduction in PKB phosphorylation. *Cardiovasc. Res.* **52**: 130–135.
- Imanishi, T., T. Hano, T. Sawamura, and I. Nishio. 2004. Oxidized low-density lipoprotein induces endothelial progenitor cell senescence, leading to cellular dysfunction. *Clin. Exp. Pharmacol. Physiol.* **31**: 407–413.
- Ma, F. X., L. Y. Liu, and X. M. Xiong. 2003. Protective effects of lovastatin on vascular endothelium injured by low density lipoprotein. *Acta Pharmacol. Sin.* **24**: 1027–1032.
- Soeda, S., T. Kozako, K. Iwata, and H. Shimeno. 2000. Oversulfated fucoidan inhibits the basic fibroblast growth factor-induced tube formation by human umbilical vein endothelial cells: its possible mechanism of action. *Biochim. Biophys. Acta.* **1497**: 127–134.
- Nims, R. W., J. C. Cook, M. C. Krishna, D. Christodoulou, C. M. Poore, A. M. Miles, M. B. Grisham, and D. A. Wink. 1996. Colorimetric assays for nitric oxide and nitrogen oxide species formed from nitric oxide stock solutions and donor compounds. *Methods Enzymol.* **268**: 93–105.
- Hill, J. M., G. Zalos, J. P. Halcox, W. H. Schenke, M. A. Waclawiw, A. A. Quyyumi, and T. Finkel. 2003. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N. Engl. J. Med.* **348**: 593–600.
- Schmidt-Lucke, C., L. Rossig, S. Fichtlscherer, M. Vasa, M. Britten, U. Kamper, S. Dimmeler, and A. M. Zeiher. 2005. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation.* **111**: 2981–2987.
- Imanishi, T., T. Hano, Y. Matsuo, and I. Nishio. 2003. Oxidized low-density lipoprotein inhibits vascular endothelial growth factor-induced endothelial progenitor cell differentiation. *Clin. Exp. Pharmacol. Physiol.* **30**: 665–670.
- Murohara, T., T. Asahara, M. Silver, C. Bauters, H. Masuda, C. Kalka, M. Kearney, D. Chen, J. F. Symes, M. C. Fishman, et al. 1998. Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J. Clin. Invest.* **101**: 2567–2578.
- Strehlow, K., N. Werner, J. Berweiler, A. Link, U. Dirnagl, J. Priller, K. Laufs, L. Ghaeni, M. Milosevic, M. Bohm, et al. 2003. Estrogen increases bone marrow-derived endothelial progenitor cell production and diminishes neointima formation. *Circulation.* **107**: 3059–3065.
- Dimmeler, S., I. Fleming, B. Fisslthaler, C. Hermann, R. Busse, and

- A. M. Zeiher. 1999. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature*. **399**: 601–605.
33. Verma, S., M. A. Kuliszewski, S. H. Li, P. E. Szmitko, L. Zucco, C. H. Wang, M. V. Badiwala, D. A. Mickle, R. D. Weisel, P. W. Fedak, et al. 2004. C-reactive protein attenuates endothelial progenitor cell survival, differentiation, and function: further evidence of a mechanistic link between C-reactive protein and cardiovascular disease. *Circulation*. **109**: 2058–2067.
34. Thum, T., D. Tsikas, S. Stein, M. Schultheiss, M. Eigenthaler, S. D. Anker, P. A. Poole-Wilson, G. Ertl, and J. Bauersachs. 2005. Suppression of endothelial progenitor cells in human coronary artery disease by the endogenous nitric oxide synthase inhibitor asymmetric dimethylarginine. *J. Am. Coll. Cardiol.* **46**: 1693–1701.
35. Hoetzer, G. L., H. M. Irmiger, R. S. Keith, K. M. Westbrook, and C. A. Desouza. 2005. Endothelial nitric oxide synthase inhibition does not alter endothelial progenitor cell colony forming capacity or migratory activity. *J. Cardiovasc. Pharmacol.* **46**: 387–389.
36. Gulati, R., D. Jevremovic, T. E. Peterson, S. Chatterjee, V. Shah, R. D. Vile, and R. D. Simari. 2003. Diverse origin and function of cells with endothelial phenotype obtained from adult human blood. *Circ. Res.* **93**: 1023–1025.
37. Stefanoa, R. D., T. Santoni, M. C. Barsottia, C. Armania, B. Chifentia, C. Guidac, R. Vanacorec, M. T. Loccid, M. Marianib, A. Balbarinib, et al. 2002. Different growth conditions for peripheral blood endothelial progenitors. *Cardiovasc. Radiat. Med.* **3**: 172–175.
38. Bompais, H., J. Chagraoui, X. Canron, M. Crisan, X. H. Liu, A. Anjo, C. T. Le Port, M. Leboeuf, P. Charbord, A. Bikfalvi, et al. 2004. Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells. *Blood*. **103**: 2577–2584.
39. Smirnova, I. V., T. Sawamura, and M. S. Goligorsky. 2004. Upregulation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in endothelial cells by nitric oxide deficiency. *Am. J. Physiol. Renal Physiol.* **287**: F25–F32.
40. Rehman, J., J. Li, C. M. Orschell, and K. L. March. 2003. Peripheral blood “endothelial progenitor cells” are derived from monocyte/macrophages and secrete angiogenic growth factors. *Circulation*. **107**: 1164–1169.
41. Rauscher, F. M., P. J. Goldschmidt-Clermont, B. H. Davis, T. Wang, D. Gregg, P. Ramaswami, A. M. Pippen, B. H. Annex, C. Dong, and D. A. Taylor. 2003. Aging, progenitor cell exhaustion, and atherosclerosis. *Circulation*. **108**: 457–463.