

# Electronegative LDL circulating in smokers impairs endothelial progenitor cell differentiation by inhibiting Akt phosphorylation via LOX-1

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**Abstract** Endothelial progenitor cells (EPCs), important for endothelial regeneration and vasculogenesis, are reduced by cigarette smoking. To elucidate the mechanisms, we examined the effects of electronegative LDL, circulating in chronic smokers, on EPC differentiation. Using ion-exchange chromatography, we purified smoker LDL into five subfractions, L1–L5. In matched, nonsmoking healthy subjects, L5, the most electronegative subfraction, was either absent or scanty. Sustained L5 treatment inhibited CD31 and KDR expression and EPC differentiation, whereas L1–L4 had no effect. L5 also inhibited telomerase activity to accelerate EPC senescence in correlation with reduced Akt phosphorylation. Transfection of day 3 EPCs with dominant negative Akt constructs inhibited CD31 and KDR expression, stalled EPC differentiation, and promoted early senescence. In contrast, transfection with constitutively active Akt rendered the EPCs resistant to L5, allowing normal maturation. L5 upregulated the lectin-like oxidized low density lipoprotein receptor 1 (LOX-1), and pretreatment of EPCs with TS20, a LOX-1-neutralizing antibody, blocked internalization of L5 by EPCs and prevented L5-mediated inhibition of EPC differentiation. Mixing L5 with L1 to physiological L5/L1 ratios did not attenuate L5's effects. **These findings suggest that cigarette smoking is associated with the formation of L5, which inhibits EPC differentiation by impairing Akt phosphorylation via the LOX-1 receptor.**—Tang, D., J. Lu, J. P. Walterscheid, H.-H. Chen, D. A. Engler, T. Sawamura, P.-Y. Chang, H. J. Safi, C.-Y. Yang, and C.-H. Chen. **Electronegative LDL circulating in smokers impairs endothelial progenitor cell differentiation by inhibiting Akt phosphorylation via LOX-1.** *J. Lipid Res.* 2008. 49: 33–47.

**Supplementary key words** electronegative low density lipoprotein • L5 • lectin-like oxidized low density lipoprotein receptor 1 • signal transduction

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Atherosclerosis is an inflammatory disease; one of its earliest manifestations is endothelial dysfunction. Replacement of neighboring endothelial cells (ECs) was long thought to be the chief or sole mechanism for replacing damaged ECs (1). However, increasing data now support bone marrow-derived endothelial progenitor cells (EPCs) as playing a key role in endothelial regeneration as well as in vasculogenesis (2–5). EPCs have been shown to be reduced in number and/or functional activity in the presence of traditional and emerging major risk factors, whether severally or as clusters (6). Reported relations include those with aging (7), subclinical and clinical atherothrombotic disease (8, 9), type 1 and type 2 diabetes mellitus (10, 11), hypercholesterolemia (12), smoking (6), C-reactive protein increase (13), and the metabolic syndrome (14).

The mechanism of tobacco's effects on EPCs is unknown. Michaud et al. (15), in an initial study, found the EC-specific markers vascular endothelial cadherin, vascular endothelial growth factor (VEGF) receptor 2 (VEGFR2; also known as KDR), and von Willebrand factor (vWF) to be reduced and the formation of reactive oxygen species to be increased in EPCs from smokers. The known effects of nicotine on postnatal vasculogenesis (16) may be mediated in part by the mobilization of EPCs (17). Whereas

Abbreviations: AcLDL, acetylated low density lipoprotein; Akt-CA, plncx-HA-myr-Akt (constitutively active Akt); Akt-DN, plncx-HA-myr-Akt179M (dominant negative Akt); DiI, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine; EC, endothelial cell; EGF, epidermal growth factor; EPC, endothelial progenitor cell; LOX-1, lectin-like oxidized low density lipoprotein receptor 1; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium; OxLDL, experimentally oxidized low density lipoprotein; SA- $\beta$ -gal, senescence-associated  $\beta$ -galactosidase; VEGF, vascular endothelial growth factor; vWF, von Willebrand factor.

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nicotine is cytotoxic at high concentrations, at low concentrations it increases EPC number and activity (18).

In the current study, we hypothesized that smoking's effects on EPCs are, at least in part, lipid-mediated. It is well established that smoking and dyslipidemia increase the risk for coronary artery and peripheral vascular disease synergistically. Cigarette smoke extracts such as acrolein and other gas-phase oxidants (19) increase superoxide anion (20) by stimulation of NADPH (19), which in turn inhibits reactive oxygen species function with a reduction of nitric oxide production and bioactivity (21) and results in impaired endothelium-dependent vasodilation (22) and direct cell death (23). Moreover, cigarette smoke is a source of free radicals that lead to oxidative stress and depletion of antioxidants, including vitamin C, carotene, and folate (24–26). The increased oxidants in smokers act directly to depress nitric oxide production by the endothelium, independent of any effect on mitochondrial respiration.

Besides directly damaging the endothelium, cigarette smoke can adversely modify lipoproteins and make them atherogenic through oxidation of their apolipoprotein (27) and lipid (28) components. By increasing cholesterol oxidation and lipid peroxidation, cigarette smoke extracts can reduce the affinity of human LDL to LDL receptors on hepatocytes (28). Cigarette smoke-modified LDL showed higher anodic electrophoretic mobility (28), implicating cigarette smoke in the generation of electronegative LDL through mechanisms yet to be elucidated. We previously showed that L5, a highly electronegative LDL, was present in nonsmoking hypercholesterolemic subjects (29). Here, we report on L5 in the plasma of chronic smokers without increased LDL-cholesterol. In addition to their role in the replacement of ECs in macrovessels, EPCs are now considered contributory to the building of microvessels and neovascularization in ischemic or damaged tissues (4, 5, 30). The focus of the current study is whether and how smoker L5 inhibits EPC differentiation at an early stage.

Akt, a family member of serine/threonine protein kinases, is closely linked to neovascularization through a variety of stimuli in ECs and EPCs, including the promotion of EC survival and migration and nitric oxide production (2, 31–33). Dominant negative Akt (Akt-DN) overexpression leads to functional interruption of EPC bioactivity (34). Recent studies showed that experimentally (copper) oxidized low density lipoprotein (OxLDL) inhibits VEGF-induced EPC differentiation through dephosphorylation of Akt and accelerates the onset of EPC senescence (35, 36). Here, we provide evidence derived from the naturally modified L5 to support the observations made with experimentally prepared OxLDL.

Since its cloning by Sawamura and colleagues (37), lectin-like oxidized low density lipoprotein receptor (LOX-1) has been well characterized in its role in transducing the signaling of copper-OxLDL. Previous studies showed that OxLDL can enhance LOX-1 expression in both EPCs (36, 38, 39) and human ECs (40, 41). In this study, we examined how smoker L5 may affect EPC differentiation through this receptor.

## Preparation of LDL

Total LDL (density = 1.019–1.063 g/ml) was separated from normal human plasma by ultracentrifugation (29). All reagents were purchased from Sigma-Aldrich Biotechnology. To minimize *in vitro* oxidation and microorganism contamination, the plasma was adjusted to contain 100  $\mu$ M EDTA, 0.056 U/ml aprotinin, 0.06% sodium azide, and 50  $\mu$ g/ml gentamycin. Protein concentrations were determined using the modified Lowry assay method.

## LDL subfractionation

Fasting blood samples were obtained from six smokers and six healthy, nonsmoking subjects (three males and three females, age 30–48 years, both groups). All smokers consumed one to two packs of cigarettes per day, and each had at least 1.5 years of continuous smoking history. Written approval was received by the Baylor College of Medicine Institutional Review Board and an informed consent was signed by all study subjects. All subjects were normocholesterolemic, with LDL cholesterol 9 120 mg/dl (means  $\pm$  SEM, 110  $\pm$  8 and 110  $\pm$  7 mg/dl;  $P > 0.01$ ). Plasma triglyceride was <180 mg/dl in all subjects, none of whom had a history of diabetes mellitus, hypertension, clinical manifestations of atherothrombotic disease, or any other systemic disorder.

The LDL fractions were separated using UNO Q12<sup>®</sup> ion-exchange columns (Bio-Rad Laboratories) and two Pharmacia P-500 HPLC pumps with a Pharmacia LCC-500 controller (42). The columns were preequilibrated with buffer A (0.02 M Tris HCl, pH 8.0, and 0.5 mM EDTA) in a 4°C cold room. Subfractions were eluted by the use of a multistep gradient of buffer B (1 M NaCl in buffer A). Samples equilibrated with buffer A were eluted with a linear gradient program at a flow rate of 2 ml/min. The effluent was monitored at 280 nm and protected from *ex vivo* oxidation with 5 mM EDTA. The LDL fractions were concentrated with Centriprep<sup>®</sup> filters (YM-30; Millipore Corp.) and sterilized by passing through 0.20  $\mu$ m filters. The isolated fractions were stored at 4°C while samples were being characterized.

## Isolation and cell culture of EPCs

Circulating mononuclear cells were isolated fresh from normolipidemic peripheral blood donations from nonsmokers, then cultured as described previously (2). In brief, mononuclear cells were separated from other components in the buffy coat by centrifugation using Histopaque<sup>®</sup>-1077 density-gradient tubes (Sigma-Aldrich Biotechnology). After three washings with PBS, 1  $\times$  10<sup>6</sup> purified mononuclear cells were seeded on 2% gelatin-coated six-well plates. Cells were cultured in EC basal medium 2 (EBM-2<sup>®</sup>; Clonetics) supplemented with the EGM-2-MV Bullet-Kit<sup>®</sup> (Clonetics), containing 5% FBS, 50 ng/ml VEGF (R&D Systems), 50 ng/ml human fibroblast growth factor 2 (R&D Systems), human epidermal growth factor (EGF), insulin-like growth factor 1, and ascorbic acid.

EPCs were defined by the expression of EC lineage markers, including vWF, KDR, and CD34 (35). Adherent cells at day 3 were rinsed with HBSS and immediately fixed with 4% paraformaldehyde for 10 min at 37°C. Direct and indirect immunostainings were performed with the use of EC-specific antibodies directed against vWF (GeneTex, Inc.), KDR, CD31, and CD34 (BD Pharmingen<sup>™</sup>, BD Biosciences). Detection of KDR required permeabilization by a 10 min incubation with 0.1% Triton<sup>®</sup> X-100 (Sigma-Aldrich Biotechnology). Staining of biotin-conjugated mouse anti-human KDR (Sigma-Aldrich Biotechnology) was visualized using streptavidin-FITC conjugate (BD Pharmingen<sup>™</sup>, BD Biosciences). The anti-CD31, anti-CD34, and anti-vWF anti-

bodies were linked directly to FITC-phycoerythrin (BD Pharmingen™, BD Biosciences). As a negative control, we used nonspecific antibodies of the same isotypes and species. After staining, cells were viewed with an inverted fluorescence microscope (Carl Zeiss MicroImaging) using standard FITC and phycoerythrin excitation and emission filter combinations. Additional confirmation was made by internalization of LDL or acetylated low density lipoprotein (AcLDL; Invitrogen) labeled, as described (35), with 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI). The medium was changed and adherent cells were washed with medium and incubated with 2.4 µg/ml DiI-labeled LDL or DiI-labeled AcLDL for 1 h before microscopy.

### RNA preparation and RT-PCR

RNA preparation with purification of total RNA was performed using the RNeasy® Mini Kit (Qiagen, Inc.), including a DNase step. In brief, cells were lysed in guanadine isothiocyanate buffer and RNA was purified according to the manufacturer's instructions. The purified RNA was suspended in diethyl pyrocarbonate-treated water. To generate cDNA, 1 µg of total RNA was treated with DNase I (Ambion, Inc.) to remove any contaminating genomic cDNA. The first strand of cDNA was synthesized using the SuperScript® First-Strand Synthesis System for RT-PCR (Invitrogen). The transcribed cDNA was then used for PCR amplification to estimate the expression of LOX-1. RT-PCR was conducted using the Qiagen two-step RT-PCR kit. Human LOX-1 primers were 5'-GAAACTGGAGGGACAGATCTC-3' (forward) and 5'-CGCATAAACAGCTCCTCGTTG-3' (reverse), and human GAPDH primers were 5'-ACCACAGTCCATGCCATCAC-3' (forward) and 5'-TCCACCACCCTGTTGCTGT-3' (reverse). PCR products were 523 and 484 bp. The thermal cycling conditions were 30 min at 50°C for reverse transcription, 15 min at 95°C for the initial PCR activation step, 30 s at 94°C for cycling denaturation, 30 s at 55°C for annealing, and 30 s at 72°C for extension, each for 30 cycles. The samples were run in a Bio-Rad Thermal Cycler.

### Plasmids and transfection

Constitutively active plncx-HA-myr-Akt (Akt-CA) and Akt-DN plasmids were provided by Dr. Marco Marcelli (43). Day 3 EPCs were transfected with Akt-DN and Akt-CA using Lipofectamine™ and PLUS® reagents (Life Technologies). The transfection mixture contained 0.5 µg of plasmid, 4 µl of PLUS®, and 150 µl of EBM® medium (Clonetics) and was incubated for 15 min before application. Cells were washed once with EBM and then incubated with the mixture and 1 ml of EBM for 3 h. Subsequently, 1 ml of EBM was added, and cells were incubated for a further 3 h before the medium was changed. Morphological changes of the cells were documented at 6 h after transfection, and cell differentiation was documented weekly during continuous exposure to 10 µg/ml L1 or L5 in culture medium. For the cell death and proliferation assays, cells were plated at  $1 \times 10^4$  cells/well onto 96-well plates to allow adherence to dish beds. After another 3 h, the cells were treated with L1 or L5 for 24 h. Before L1 or L5 treatment, serum-free EBM-2® was supplemented with 50 ng/ml VEGF. Whenever pharmacological inhibition of LOX-1 was indicated, we used 10 µg/ml TS20, a LOX-1-neutralizing antibody (developed by T. Sawamura).

### Cell death ELISA

Circulating monocytes were seeded at  $1 \times 10^6$  cells/well onto six-well plates in complete EBM-2® medium as described above. On day 3, after Akt-DN and Akt-CA transfection, cells were replated at  $1 \times 10^4$  cells/well onto 96-well plates and incubated with L5 at concentrations of 5, 10, 25, and 50 µg/ml for 24 h in

EBM-2® medium supplemented with 50 ng/ml VEGF. To further assess the effects of L5 under physiological conditions, additional experiments were performed using a mixture of L5 (1–50 µg/ml, protein concentration) and L1 (500 µg/ml, protein concentration), with a final L5/L1 ratio of 0.2–10%. A quantitative ELISA that detects DNA fragments was used in accordance with the manufacturer's instructions (Cell Death Detection ELISA Kit; Roche Diagnostics) (44). This ELISA detects mononucleosomal and oligonucleosomal DNA using the cytoplasmic fractions of cell lysates. In brief, anti-histone antibody was coated onto a microtiter plate. After a washing step, the wells were incubated with 200 µl of blocking buffer for 30 min and the wells were again washed and incubated with 100 µl of sample for 90 min at room temperature. After another washing step, the wells were incubated with 100 µl of anti-DNA peroxidase for an additional 90 min. The addition of substrate solution produces a color change after 15 min. The color change was compared with a blank well with added substrate. The absorbance was read at 450 nm on an automated ELISA plate reader.

### Proliferative activity assay

Mitogenic activity was assayed using a colorimetric MTS [for 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay kit (CellTiter 96 AQ®; Promega Corp.) (36). EPCs at day 3 after transfection were reseeded on 96-well plates ( $1 \times 10^4$  cells/well) in 0.1 ml of serum-free EBM-2® medium in the presence or absence of 50 ng/ml human VEGF. After 24 h in culture with increasing concentrations of L1 or L5, 20 µl of the combined MTS-phenazine methosulfate solution was added to each well and absorbance at 490 nm was detected with an ELISA plate reader.

### β-Galactosidase activity assay

Senescence-associated β-galactosidase (SA-β-gal) activity was measured as described (45). In brief, 24 h after L1 or L5 treatment, untransfected and Akt-CA/Akt-DN-transfected EPCs were washed with PBS and fixed for 5 min in 2% paraformaldehyde at room temperature. They were then washed again and incubated for 16 h at 37°C with fresh SA-β-gal stain solution: 1 mg/ml 5-bromo-4-chloro-3-indyl β-D-galactopyranoside, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate, and 0.02% Nonidet-40. Finally, the processed EPCs were counterstained with 4',6-diamino-phenylindole (0.2 µg/ml in 10 mM Tris-HCl, pH 7.0, 10 mM EDTA, and 100 mM NaCl) for 10 min to determine total cell numbers.

### Telomeric repeat amplification protocol assay

For quantitative analysis of telomerase activity, the *TeloTAGGG* PCR ELISA<sup>PLUS</sup> Kit® (Roche Applied Science) was used according to the manufacturer's instructions (46).

### Western blotting

EPCs were treated with increasing concentrations of L1 (10 or 500 µg/ml), L5 (1, 5, or 10 µg/ml), or L5 in various concentrations mixed with 500 µg/ml L1, with or without 10 µg/ml TS20 pretreatment, in the presence of 50 ng/ml VEGF. Cellular proteins were prepared and separated on SDS-PAGE gels, as described (29). Membranes were blocked by incubation in Tris-buffered saline (10 mM Tris, pH 7.5, and 100 mM NaCl) containing 0.1% (v/v) Tween-20 and 5% (v/v) nonfat dry milk for 2 h. Specific protein detection was accomplished with mouse polyclonal anti-KDR, anti-CD31 (BD Pharmingen™, BD Biosciences), rabbit polyclonal antiphospho-Akt, anti-Akt (Cell Signal-



ing Technology), or goat polyclonal anti-LOX-1 (GeneTex, Inc.) antibodies. The membranes were washed extensively in Tris-buffered saline containing 0.1% (v/v) Tween-20 before incubation for 1 h with a secondary anti-mouse or anti-rabbit antibody conjugated to horseradish peroxidase. Membranes were then washed and developed using ECL substrate (Amersham Pharmacia Biotech, now GE Healthcare).

### Statistical analysis

Data are expressed as means  $\pm$  SEM. Statistical analysis was performed by one-way ANOVA (least significant difference test) for multiple testing. Probability values were considered significant at  $P < 0.05$ .

## RESULTS

### Effects of L5 from smokers on EPC apoptosis and differentiation

L5 was present in all of the plasma LDL samples from smokers but in none from nonsmokers (Fig. 1A, B). Smokers' L5 was similar in apoptotic activity to that reported previously for L5 from hypercholesterolemic subjects (29). It induced apoptosis in day 3 EPCs at a concentration of 25 or 50  $\mu\text{g}/\text{ml}$ , as assessed by morphological changes of propidium iodide-stained nuclei. It is important to note that L5 at 10  $\mu\text{g}/\text{ml}$  or lower did not induce apoptosis. L1 had no apoptotic effect up to the highest concentration (50  $\mu\text{g}/\text{ml}$ ) examined (Fig. 1C, D).

To confirm that L5 remains unaltered in the milieu of other lipoproteins, we incubated L5 and the most abundant L1 in equal concentrations at 37°C for 24 h and then subjected the mixture to a secondary chromatography. As shown, two discrete spikes representing L1 and L5 reappeared in the second tracing at an interval identical to that of the original tracing (Fig. 1E, F). Of importance, in a mixture containing 500  $\mu\text{g}/\text{ml}$  L1, 25–50  $\mu\text{g}/\text{ml}$  L5 remained capable of inducing EPC apoptosis, whereas 1–10  $\mu\text{g}/\text{ml}$  L5 in the mixture did not (Fig. 1G, H). These findings strongly suggest that although L5 is only a small portion of total LDL, its chemical/physical and functional properties are not compromised by the presence of a large quantity of L1. For most of the subsequent experiments, 1–10  $\mu\text{g}/\text{ml}$  L5 (0.2–2% of the quantity of L1) was used to avoid apoptosis.

EPC colonies isolated from human blood buffy coats proliferated and smoothly differentiated into EC sheets, taking on a cobblestone-like appearance in the presence of VEGF after 28 days in culture (Fig. 2A). Initially, EPC colonies reorganized as a cell mass composed of a central cord of round cells with elongated, spindle-shaped cells sprouting at the periphery of the colony. By day 3, clusters of round cells that sat on top of spindle-shaped cells migrated from the base of the cell clusters. During the subsequent 2 weeks, the clusters of cells gradually disappeared and the spindle-shaped cells, displaying EC features, were left to adhere to the culture wells. By the 4th week, in the presence of EGF, insulin-like growth factor-1, fibroblast growth factor 2, and VEGF, endothelium-like cells proliferated and aggregated in the familiar cobble-

stone pattern. Similarly prepared cells have been reported to express KDR, CD31, CD34, and Tie-2 (angiopoietin 1 receptor) (47). As stated above, our cells were able to express KDR, CD34, and vWF and to internalize LDL and AcLDL (Fig. 2B).

Because L5 at concentrations  $> 10 \mu\text{g}/\text{ml}$  induces EPC apoptosis, we compared the effects of 10  $\mu\text{g}/\text{ml}$  total non-smoker LDL, smoker L1, and smoker L5 on EPC differentiation. As shown in Fig. 2A, neither nonsmoker LDL nor smoker L1 changed the course of EPC differentiation and maturation, compared with PBS treatment. In contrast, cells exposed to this subapoptotic concentration of L5 alone or in a mixture with 500  $\mu\text{g}/\text{ml}$  L1 dissociated without proliferation; furthermore, there was an absence of elongated, spindle-like ECs sprouting from the periphery of the colony. At day 28, there was no evidence of cobblestone-like EC aggregates, despite supplementation of 50 ng/ml VEGF.

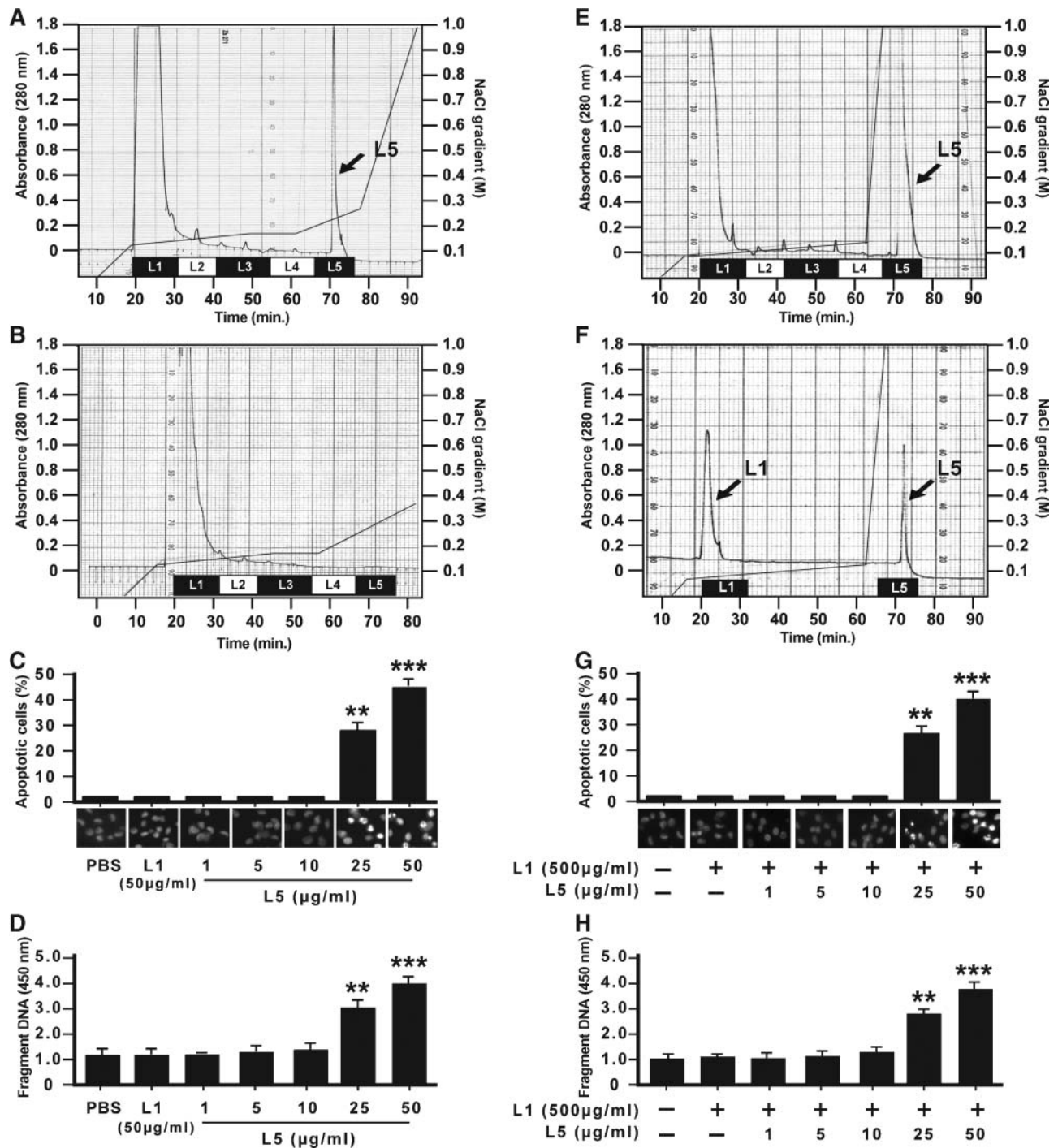
### Effects of L5 on Akt phosphorylation

Because Akt activation is important for EPC differentiation, we examined whether L5 at a low concentration could inhibit Akt phosphorylation at an early stage of differentiation. In day 3 EPCs, L5 alone or in a mixture with 500  $\mu\text{g}/\text{ml}$  L1 inhibited Akt phosphorylation in a concentration-dependent manner, without affecting total Akt content. The inhibitory effect was seen at a concentration as low as 5  $\mu\text{g}/\text{ml}$  (Fig. 3A, C). In comparison, L1 had no effect on Akt phosphorylation (Fig. 3B).

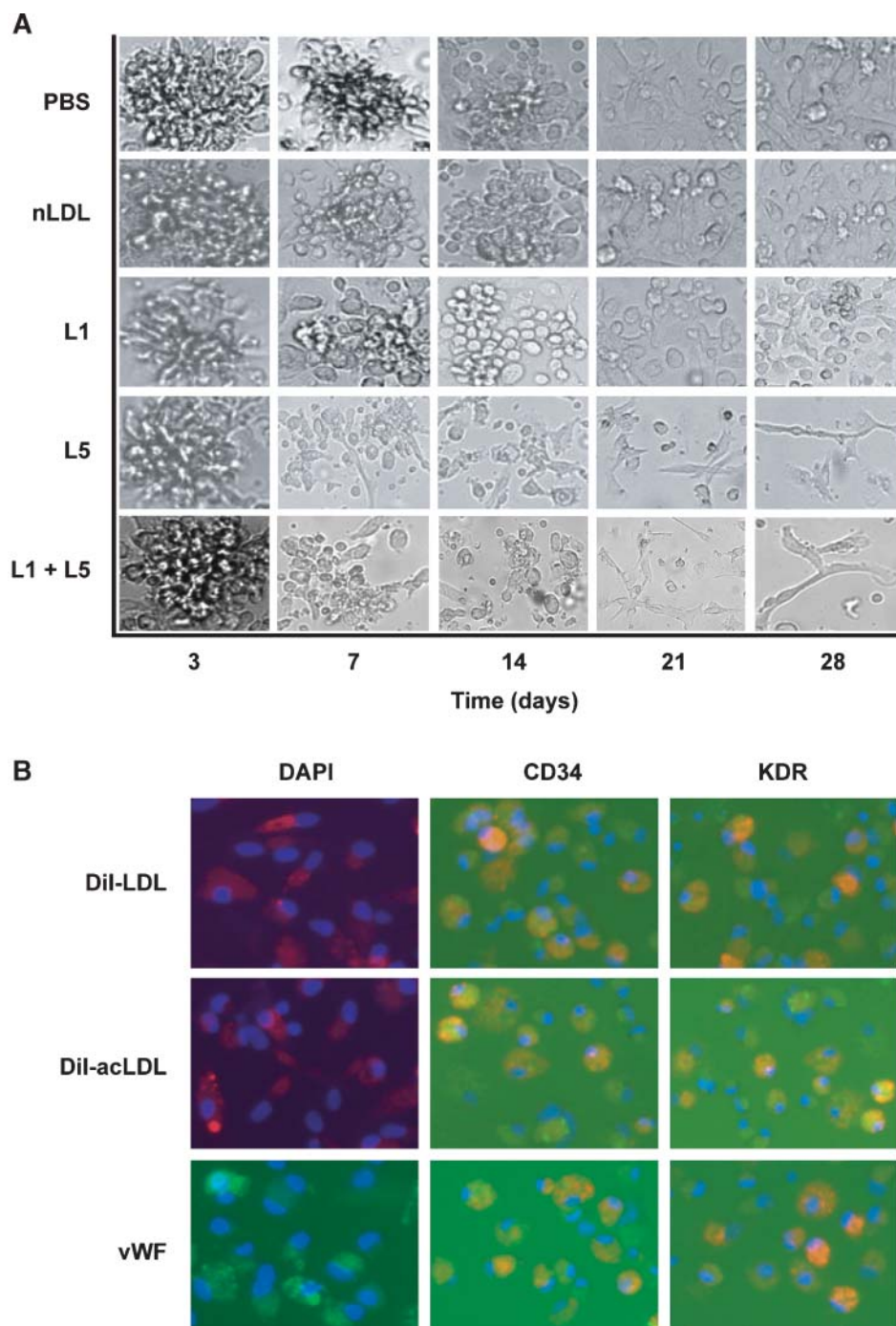
### Effects of Akt-DN and Akt-CA expression on EPC apoptosis and differentiation

To examine further the effect of Akt activation on EPC differentiation, we transfected day 3 EPCs with an Akt-DN or Akt-CA plasmid. Akt phosphorylation was reduced in Akt-DN-treated cells compared with untransfected EPCs, whereas it was enhanced in Akt-CA-treated cells (Fig. 4A). In the absence of L5, Akt-DN transfection resulted in spontaneous apoptosis in  $\sim 35\%$  of EPCs, as shown by propidium iodide nuclear staining, whereas Akt-CA cells appeared healthy (Fig. 4B).

To confirm that the L5-mediated inhibition of EPC differentiation was not a result of apoptosis, the extent of DNA fragmentation was measured by cell death ELISA. At concentrations  $> 10 \mu\text{g}/\text{ml}$ , DNA fragmentation increased in Akt-DN-transfected EPCs and decreased in Akt-CA-transfected EPCs, compared with native cells (Fig. 4C). The proapoptotic effect of Akt-DN and the antiapoptotic effect of Akt-CA are demonstrated in EPCs treated with L1, which did not affect DNA fragmentation, even at 50  $\mu\text{g}/\text{ml}$ . However, spontaneous apoptotic activity was slightly but nonsignificantly increased in Akt-DN-transfected EPCs. The proliferation rate of EPCs was determined to rule out the possibility that the inhibitory effect of L5 on VEGF-induced EPC differentiation was secondary to the induction of apoptosis. In this setting, neither L1 nor L5 at lower concentrations (5 and 10  $\mu\text{g}/\text{ml}$ ) increased the rate of EPC apoptosis. However, L5 inhibited proliferation in EPCs and Akt-DN-transfected EPCs (Fig. 4D), whereas L5 at 25 and 50  $\mu\text{g}/\text{ml}$  yielded an increase in EPC apoptosis

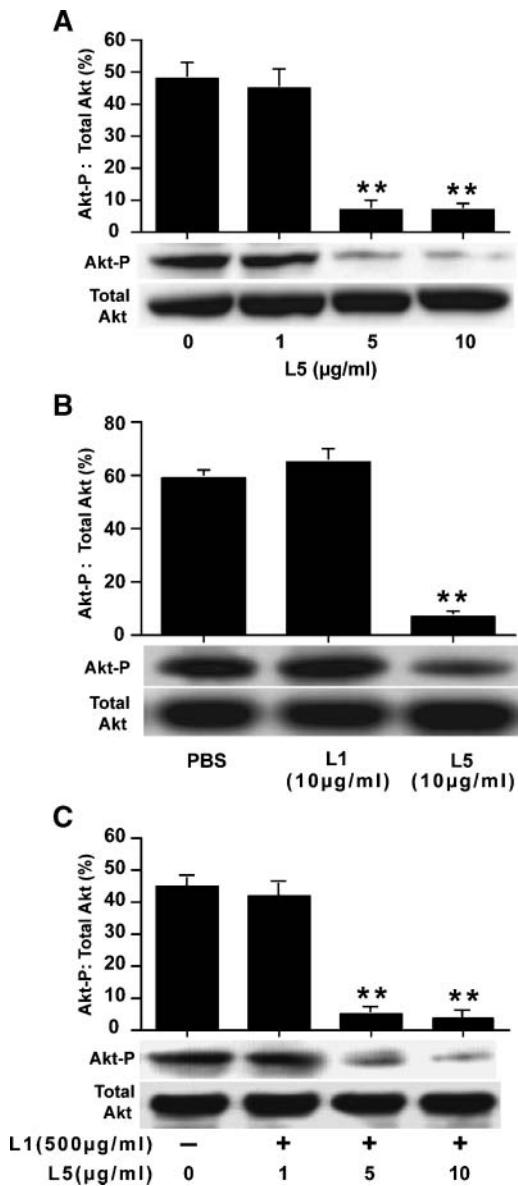


**Fig. 1.** L1–L5 distribution in smoker and nonsmoker LDL samples and concentration-dependent effects of smoker L5 on endothelial progenitor cell (EPC) apoptosis. **A:** Ion-exchange chromatogram of a typical LDL fractionation from a chronic smoking subject yields L1–L5. The arrow denotes the L5 peak. **B:** Chromatogram of LDL fractionation from a healthy, nonsmoking subject lacks L5. **C:** Evaluation of apoptosis in EPCs after exposure to 50  $\mu\text{g/ml}$  L1 (protein concentration) or increasing doses of L5 (protein concentration) in EBM-2<sup>®</sup> supplemented with 50 ng/ml vascular endothelial growth factor (VEGF). Cells entering apoptosis appear much brighter because of propidium iodide staining of condensed, fragmented DNA ( $\times 20$  magnification). **D:** Cell death ELISA measurement in EPCs after exposure to 50  $\mu\text{g/ml}$  L1 or increasing doses of L5 in EBM-2<sup>®</sup> supplemented with 50 ng/ml VEGF. **E:** Appearance of L1–L5 subfractions of another random smoker LDL sample. **F:** Typical example of "rechromatographic" separation of L1 and L5 after an equal concentration (mmol) mixture of the two subfractions retrieved from L1 and L5 of the subject shown in E. The mixture was incubated for 24 h at 37°C under 5% CO<sub>2</sub> before the second chromatography. **G:** Assay of EPC apoptosis after exposure to a mixture of 500  $\mu\text{g/ml}$  L1 and 1–50  $\mu\text{g/ml}$  L5 in EBM-2<sup>®</sup> supplemented with 50 ng/ml VEGF for 24 h. **H:** ELISA-assessed cell death in EPCs after exposure to a mixture of 500  $\mu\text{g/ml}$  L1 and 1–50  $\mu\text{g/ml}$  L5 for 24 h. \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$  versus PBS ( $n = 5$  for C, D) or no treatment with L1 or L5 ( $n = 5$  for G, H). Values are expressed as mean  $\pm$  SEM.



**Fig. 2.** Morphological and endothelial cell (EC) lineage marker changes in EPC colonies with or without sustained exposure to L5. **A:** Mononuclear cells were isolated from healthy human blood by density-gradient centrifugation and seeded on 2% gelatin-coated six-well plates in EBM-2<sup>®</sup> supplemented with VEGF and ascorbic acid. On day 3, when EPC colonies formed, the cells began to be exposed to nonsmoker total LDL, smoker L1, smoker L5 (10  $\mu\text{g}/\text{ml}$  each), or a mixture of smoker L1 (500  $\mu\text{g}/\text{ml}$ ) and L5 (10  $\mu\text{g}/\text{ml}$ ) in fresh medium with 50 ng/ml VEGF. The treatments were renewed every 3 days, and photomicrographs ( $\times 20$  magnification) were taken on days 7, 14, 21, and 28. **B:** Images ( $\times 20$  magnification) of single and double immunofluorescence stainings exhibiting EC lineage markers CD34 and KDR in healthy differentiating EPCs. Figures are representative of three independent experiments. AcLDL, acetylated low density lipoprotein; DAPI, 4',6-diamino-phenylindole; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine; vWF, von Willebrand factor.

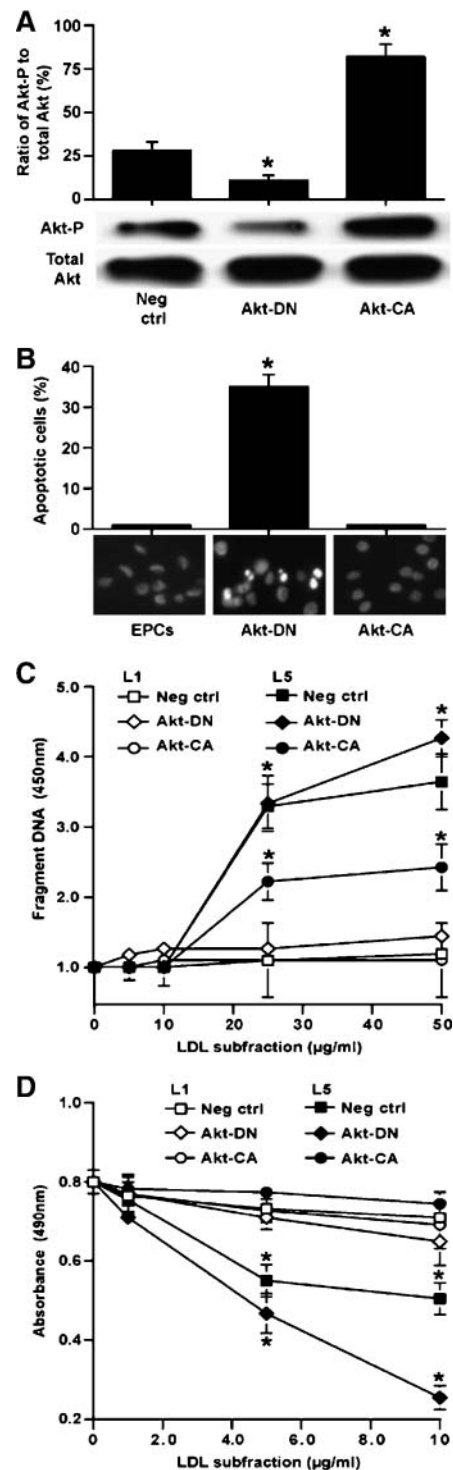




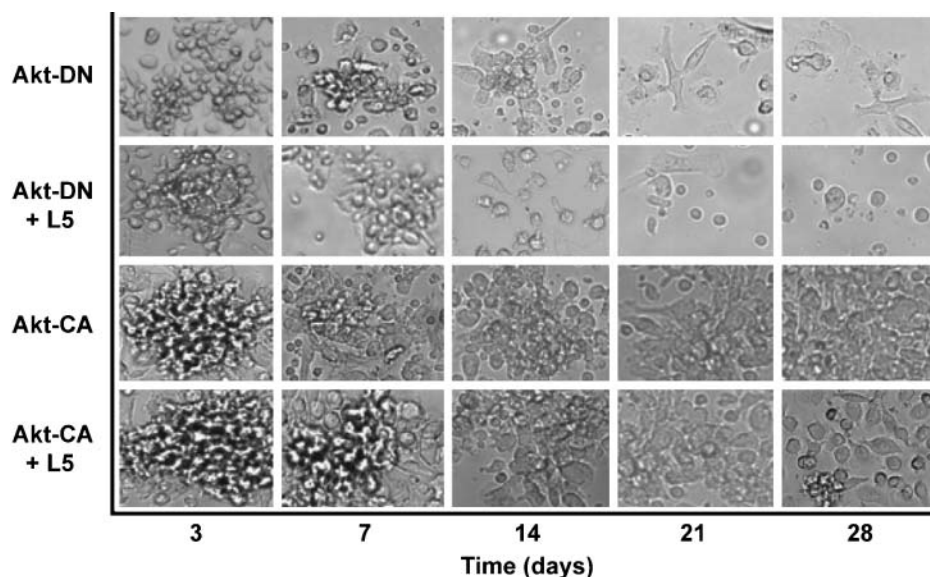
**Fig. 3.** Effects of L5 on phosphorylated Akt levels in early EPCs. **A:** EPCs at day 3 were treated with increasing concentrations of L5 for 1 h. Cells were lysed and applied to Western blots to determine the ratio of phosphorylated to total cellular Akt. **B:** EPCs treated with 10 µg/ml L1 or L5 were subjected to Western blot analysis and compared with respect to Akt phosphorylation ( $n = 5$ ). **C:** Akt phosphorylation in EPCs treated with 1–10 µg/ml L5 in the presence of 500 µg/ml L1. \*\*  $P < 0.01$  versus no treatment (**A, C**;  $n = 5$ ) or PBS (**B**). Values are expressed as mean  $\pm$  SEM.

(Fig. 4C). In addition, Akt-CA-transfected EPCs showed normal mitogenic activity according to MTS ELISA, even under L5 treatment at 5 and 10 µg/ml (see Fig. 6 below). These data suggest a significant inhibitory effect of L5 on VEGF-induced mitogenic activity of EPCs at day 3.

The effects of Akt-DN and Akt-CA on EPC differentiation were monitored for 28 days after transfection. As early as 6 h after Akt-DN transfection, the EPC colonies started to disintegrate and became fluffy in appearance. The cell number decreased progressively: by day 28, only a few cells remained and no cobblestone-like aggregations were



**Fig. 4.** Effects of Akt plasmid [dominant negative Akt (Akt-DN) or constitutively active Akt (Akt-CA)] transfection on Akt phosphorylation and L5-induced EPC apoptosis. **A:** Akt-DN or Akt-CA constructs were transfected into EPCs at day 3, as described in Methods. The status of Akt phosphorylation, without L5 treatment, was determined by immunoblotting. **B:** Fluorescent nuclear staining of EPCs transfected with Akt-DN or Akt-CA. Cells entering apoptosis appear much brighter as a result of propidium iodide staining of condensed, fragmented DNA ( $\times 20$  magnification). **C:** Results of cell death ELISA in EPCs transfected with Akt-DN or Akt-CA, followed by treatment with increasing doses of L1 or L5. **D:** Effects of L5 on mitogenic activity in EPCs transfected with Akt-DN or Akt-CA in a proliferation ELISA assay. \*  $P < 0.01$  versus EPCs (**A, B**) or versus the L1 negative control (**C, D**);  $n = 5$ . Values are expressed as mean  $\pm$  SEM.



**Fig. 5.** Morphological changes in Akt-transfected EPCs with or without sustained exposure to L5. Mononuclear cells were isolated from healthy human blood by density-gradient centrifugation and seeded on 2% gelatin-coated, six-well plates in EBM-2<sup>®</sup> supplemented with VEGF and ascorbic acid. EPC colonies formed at day 3 and were transfected with Akt-DN or Akt-CA; then, 10  $\mu\text{g}/\text{ml}$  L5 was added to fresh medium with 50 ng/ml VEGF. The medium was changed every 3 days, and photomicrographs were made at days 7, 14, 21, and 28 to document morphological changes ( $\times 20$  magnification).

seen. The addition of 10  $\mu\text{g}/\text{ml}$  L5 further accelerated EPC colony disassembly, and no mature EPCs were identified (**Fig. 5**). In contrast, EPCs transfected with Akt-CA appeared healthy and differentiated rapidly. By day 28, full-grown cobblestone-like cultures were formed. Akt-CA-transfected EPCs exposed to 10  $\mu\text{g}/\text{ml}$  L5 did not impair differentiation. By day 28, mature EPCs formed, although they were not as confluent as in Akt-CA-transfected cells without L5 exposure (**Fig. 5**).

#### Effects of Akt-CA and Akt-DN expression on VEGF-induced mitogenic activity in early EPCs

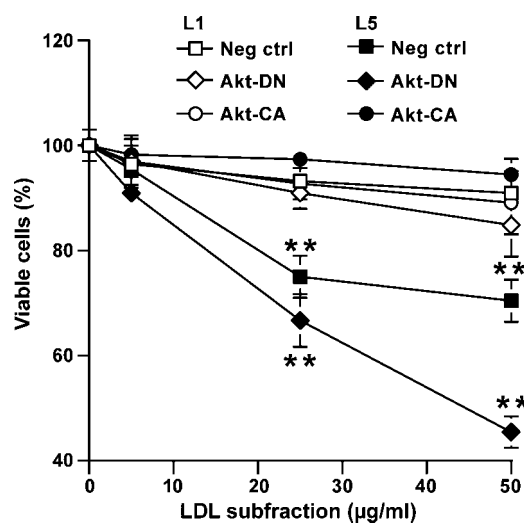
Cell proliferation plays an important role in EPC differentiation. To determine the importance of Akt activation in the mitogenic activity of early EPCs, an MTS assay was performed. Compared with untransfected EPCs, proliferation was suppressed by Akt-DN but supported by Akt-CA. L1 did not affect viability in untransfected EPCs or EPCs transfected with Akt-CA or Akt-DN plasmids (**Fig. 6**). In Akt-CA EPCs, L5 did not hinder VEGF-induced proliferation. However, it did inhibit proliferation in both the untransfected and the Akt-DN-transfected EPCs in a concentration-dependent manner.

#### Effects of L5 and Akt-DN or Akt-CA transfection on VEGF-induced expression of KDR and CD31 in early EPCs

KDR and CD31, both inducible by VEGF, are EC markers with important functions. KDR transduces VEGF signaling (48). CD31 (platelet-endothelial adhesion molecule 1) promotes adhesion between ECs (49). We examined how L5 inhibits EPC differentiation by affecting the expression of KDR and CD31. In untransfected EPCs, L5 almost completely suppressed the expression of both KDR and CD31 in

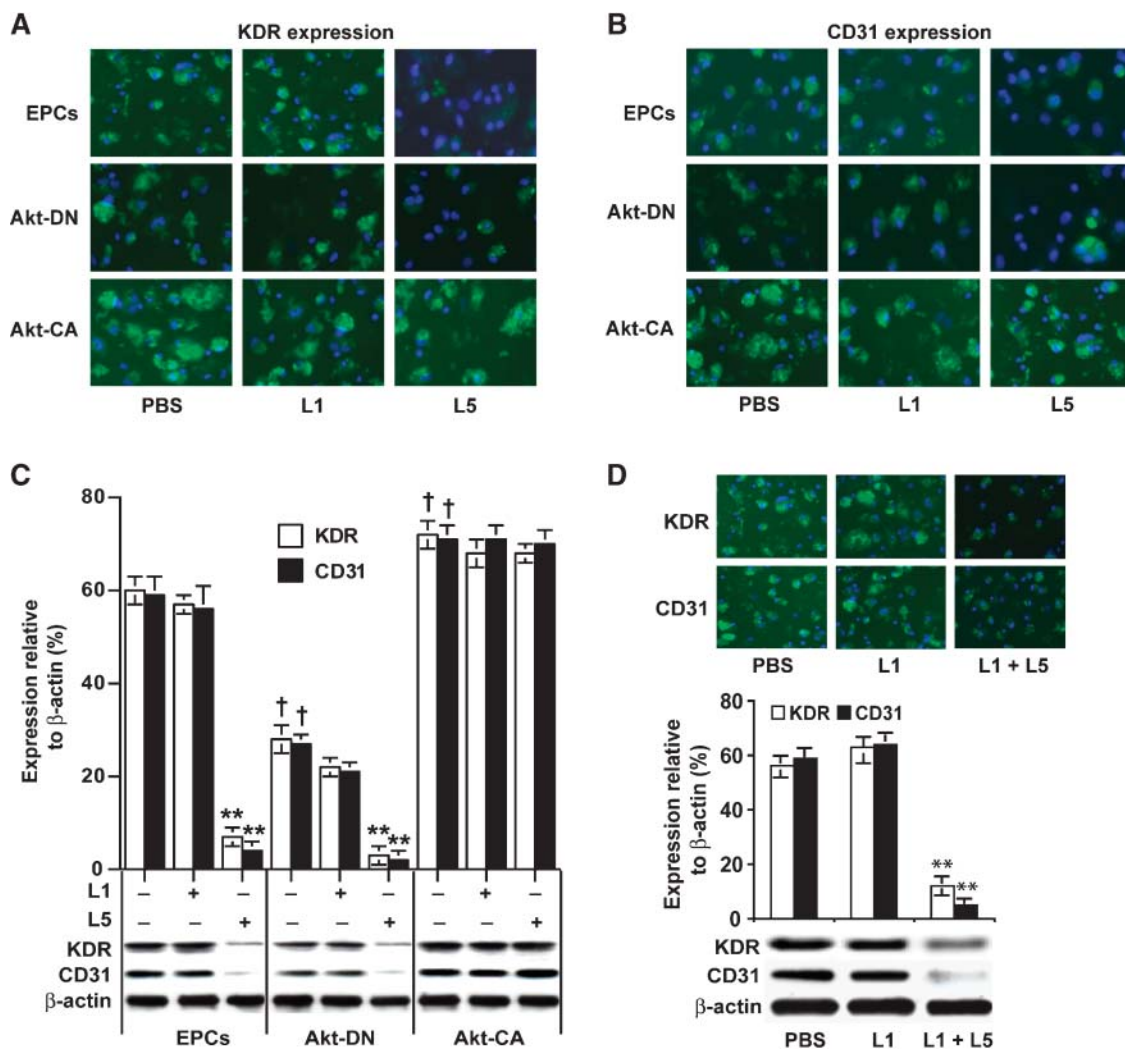
the setting of supplementation with 50 ng/ml VEGF, whereas L1 (10 and 500  $\mu\text{g}/\text{ml}$ ) had no effect compared with a PBS control (**Fig. 7A, B, D**). In a physiological mixture of L5 (10  $\mu\text{g}/\text{ml}$ ) and L1 (500  $\mu\text{g}/\text{ml}$ ), L5 was still able to inhibit KDR and CD31 expression, whereas 500  $\mu\text{g}/\text{ml}$  L1 alone did not (**Fig. 7D**).

In Akt-DN-transfected EPCs, immunofluorescence emission was reduced for both KDR and CD31, compared with untransfected EPCs. L1 did not exert additional effects, but L5 further suppressed the expression of both markers in Akt-DN EPCs (**Fig. 7A, B**). In contrast, the expressions of



**Fig. 6.** Viability of Akt-transfected EPCs treated with L1 or L5. EPCs were transfected with Akt-DN or Akt-CA and exposed to increasing concentrations of L1 or L5. \*\*  $P < 0.01$  versus L1 (negative control);  $n = 5$ . Values are expressed as mean  $\pm$  SEM.





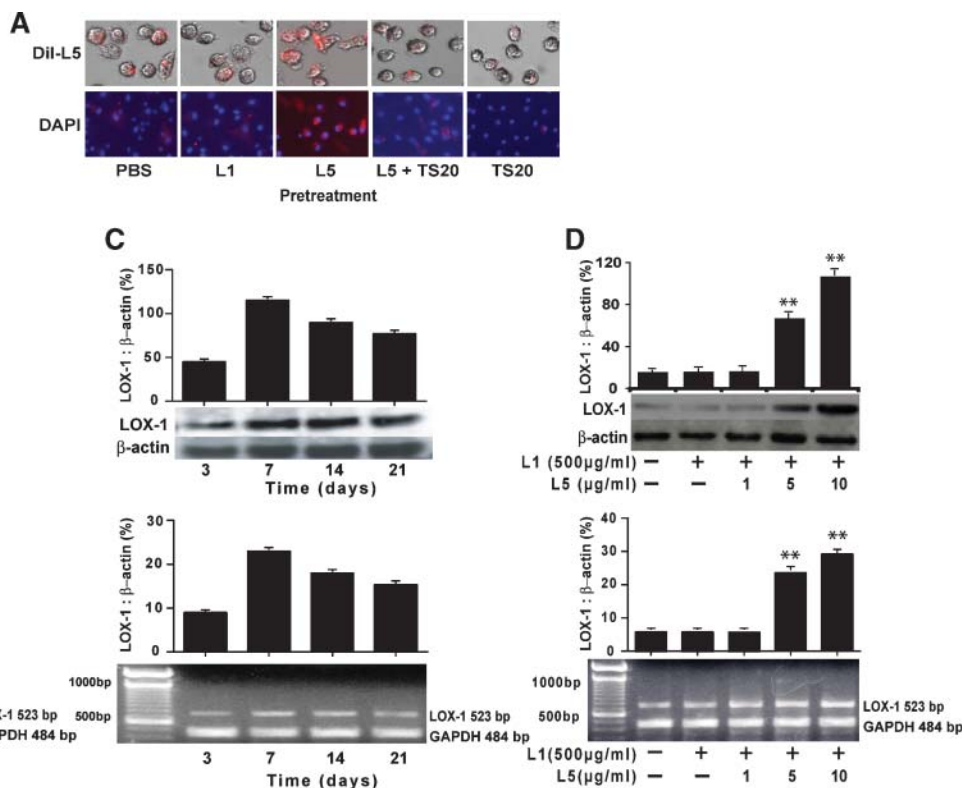
**Fig. 7.** Effects of L5 on EPC differentiation as measured by KDR and CD31 expression. EPCs transfected with Akt-DN or Akt-CA plasmids were treated with 10  $\mu$ g/ml L1 or L5 for 24 h in EBM-2<sup>®</sup> supplemented with 50 ng/ml VEGF. KDR and CD31 expression was detected by immunofluorescence antibody staining with respect to cellular counterstaining. A: KDR immunofluorescence in Akt-DN- and Akt-CA-transfected EPCs treated with 10  $\mu$ g/ml L1 or L5 ( $\times 20$  magnification). B: Representative photomicrographs of CD31 immunofluorescence in Akt-DN and Akt-CA EPCs treated with 10  $\mu$ g/ml L1 or L5 ( $\times 20$  magnification). C: Western blotting results for KDR and CD31 protein expression in Akt-DN- and Akt-CA-transfected EPCs exposed to 10  $\mu$ g/ml L1 or L5 for 24 h. \*\*  $P < 0.01$  versus no treatment within each cell line;  $\dagger P < 0.01$  versus no treatment in nontransfected EPCs ( $n = 5$ ). D: KDR and CD31 expression in EPCs treated with a mixture of 10  $\mu$ g/ml L5 and 500  $\mu$ g/ml L1 for 24 h as assessed by immunofluorescence antibody staining and Western blotting. \*\*  $P < 0.01$  versus PBS ( $n = 3$ ). Values are expressed as mean  $\pm$  SEM.

KDR and CD31 were sharply enhanced in Akt-CA EPCs; there was no additional effect with treatment by L1 or L5 (Fig. 7A, B). The immunofluorescence findings were confirmed by Western blot analyses. The baseline KDR and CD31 protein levels were decreased in Akt-DN but increased in Akt-CA EPCs. L1 had no effect, but L5 restricted expression in both the untransfected and Akt-DN EPCs. In contrast, L5 failed to reduce the augmentation of KDR and CD31 levels in Akt-CA EPCs (Fig. 7C). These findings indicate that the expressions of both KDR and CD31 are Akt-dependent and that the relations are impaired by L5.

#### Role of LOX-1 in L5 internalization and effect of L5 on LOX-1 expression

To visualize the binding of L5 to the LOX-1 receptor, we prepared DiI-labeled L5 and incubated it with day 3 EPCs.

At 10  $\mu$ g/ml, L5 but not L1 enhanced DiI-L5 binding to EPCs. Pretreatment with 10  $\mu$ g/ml TS20 abolished DiI-L5 binding (Fig. 8A). L1 did not enhance LOX-1 expression in day 3 EPCs, but L5 dose-dependently stimulated LOX-1 expression at both the transcriptional and translational levels (Fig. 8B). Expression of LOX-1 mRNA and of LOX-1 protein was observed in EPCs by day 3; both peaked by day 7 (Fig. 8C). This suggests that monocytes can commit to EPC differentiation as early as day 3 and that the pathway can be blocked by LOX-1 activation by L5. Moreover, LOX-1 internalization of L5 can also be enhanced by L5 in early-stage EPCs, as seen by the increased DiI-L5 staining in EPCs treated with L5. Because the effect was abolished by TS20, a LOX-1-specific neutralizing antibody, and TS20 by itself had no effects on DiI-L5 binding to EPCs, LOX-1 appears to be a critical mediator in the effects of L5 on EPC activity. In



**Fig. 8.** Role of lectin-like oxidized low density lipoprotein receptor 1 (LOX-1) in L5 internalization and effects of L5 on LOX-1 expression in differentiating EPCs. EPCs were pretreated with TS20 for 1 h after L1 and L5 treatment for 24 h. After medium was removed, cells were treated with DiI-L5 for 6 h. DiI-L5 internalization was confirmed by fluorescence microscopy after cells were washed three times. A: Typical fluorescence microscopy results for DiI-L5 internalization compared with DAPI nuclear staining in early-stage EPCs ( $\times 20$  magnification). Cells were pretreated with 10  $\mu\text{g/ml}$  L1, L5, or a combination of L5 with 10  $\mu\text{g/ml}$  TS20, followed by DiI-L5. B: Western blot results for LOX-1 mRNA and protein expression in day 3 EPCs exposed to increasing doses of L5. \*\*  $P < 0.01$  versus PBS ( $n = 5$ ). C: Time course of LOX-1 mRNA and protein expression during EPC differentiation after exposure to 10  $\mu\text{g/ml}$  L5. No significant differences were seen according to time ( $n = 5$ ). D: Concentration-dependent (1–10  $\mu\text{g/ml}$ ) effects of L5 on LOX-1 mRNA and protein expression in day 3 EPCs in a mixture with 500  $\mu\text{g/ml}$  L1. \*\*  $P < 0.01$  versus no treatment ( $n = 3$ ). Values are expressed as mean  $\pm$  SEM.

a physiological L5/L1 mixture, L5 was again able to augment LOX-1 expression at both the mRNA and protein levels in a concentration-dependent manner. In contrast, 500  $\mu\text{g/ml}$  L1 by itself had no effect (Fig. 8D).

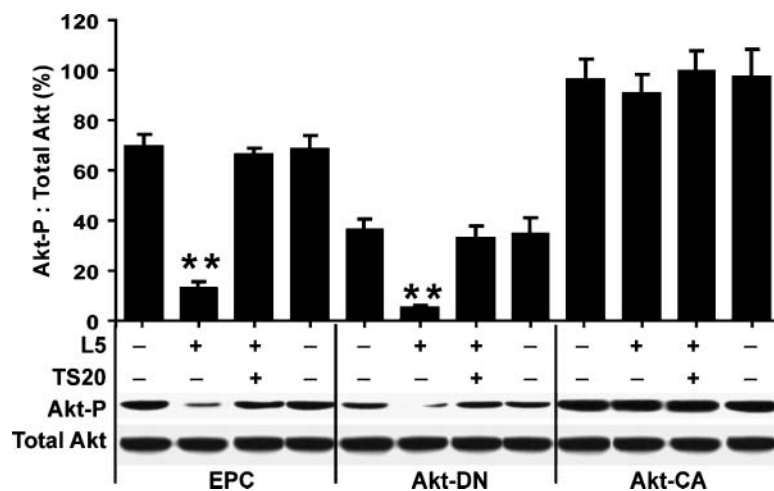
#### Akt pathway mediation of L5 inhibitory effects on EPCs

VEGF requires Akt to enhance EPC development, suggesting an essential role of Akt in regulating hematopoietic progenitor cell differentiation (2, 50). Experimental OxLDL has been shown to dose-dependently inhibit phosphorylated Akt in EPCs at day 7 under simultaneous stimulation with VEGF (35). In addition, OxLDL decreased EPC survival, adhesion, migration, and tube formation, effects that were attenuated by pretreatment with a LOX-1 monoclonal antibody (39). To test our hypothesis that L5-stimulated LOX-1 decreases phosphorylated Akt levels, we performed a series of experiments. Fresh mononuclear cells were cultured for 3 days in serum-free EBM-2<sup>®</sup> and stimulated by L5 in the presence of 50 ng/ml VEGF for 1 h. The cells were lysed and then electrophoretically separated by 10% SDS-PAGE, and immunoblotting was performed with a phosphospecific Akt antibody directed at the Ser<sup>473</sup> phosphorylation site. As shown in

Fig. 3, L5 produced a dose-dependent reduction of phosphorylated Akt in the presence of VEGF, whereas L1 at 10  $\mu\text{g/ml}$  did not inhibit phosphorylated Akt in EPCs (Fig. 3B). Pretreatment of EPCs at day 3 with 10  $\mu\text{g/ml}$  TS20, however, protected against L5-induced reductions of phosphorylated Akt (Fig. 9). Likewise, EPC transfection with various Akt constructs showed Akt-DN to decrease phosphorylated Akt, whereas Akt-CA-treated EPCs promoted phosphorylated Akt compared with control EPCs. Moreover, L5 at 10  $\mu\text{g/ml}$  inhibited phosphorylated Akt in control EPCs and in Akt-DN-treated but not Akt-CA-treated EPCs, whereas pretreatment with TS20 prevented L5's inhibitory effects (Fig. 9).

#### Effects of L5 on EPC senescence and telomerase activity

Some studies have shown that impairment of mitogenic activity in early EPCs by OxLDL can lead to accelerated EPC senescence (36). We used  $\beta$ -galactosidase to detect the acidification typical of the onset of cellular senescence. Extended cultivation of EPCs resulted in an increase of SA- $\beta$ -gal-positive cells; treatment with L5 accelerated senescence but L1 at those doses had no effect (Fig. 10A). By day 3, the proportion of SA- $\beta$ -gal-positive cells in EPCs treated by L5 at



**Fig. 9.** Akt phosphorylation in Akt-transfected EPCs treated with L5 or L5 and TS20. Akt-DN- and Akt-CA-transfected EPCs were exposed to L5 in the absence or presence of TS20. Akt phosphorylation levels were compared with total Akt levels by Western blot. \*\*  $P < 0.01$  versus negative controls without L5 or TS20 treatment, for L5 versus no treatment in EPCs or for L5 versus no treatment in Akt-DN constructs ( $n = 5$ ). Values are expressed as mean  $\pm$  SEM.

10  $\mu\text{g/ml}$  was markedly increased compared with control (Fig. 10B). As noted in Fig. 8, L5 upregulated the expression of LOX-1 in EPCs at day 3. To examine the interplay of L5 and LOX-1 in EPC senescence, we performed further experiments with TS20. The L5-induced increase in SA- $\beta$ -gal-positive cells was attenuated by 10  $\mu\text{g/ml}$  TS20 (Fig. 10B), suggesting that L5-induced EPC senescence is mediated through the LOX-1 receptor. In addition, SA- $\beta$ -gal staining showed accelerated senescence of Akt-DN-treated EPCs (Fig. 10A, B), and L5 treatment dramatically increased the effect, with early senescence prevented by TS20. Akt-CA-treated EPCs were strongly resistant to early senescence, maintaining their phenotype regardless of L5 or TS20 treatment. This suggests that L5-induced EPC senescence is mediated through the Akt signal transduction pathway.

Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the telomere length reduction induced by each cell division. It has been demonstrated that proatherosclerosis factors impair telomerase activity in mature ECs (50). Therefore, we measured telomerase activity using the *TeloTAGGG* PCR ELISA<sup>PLUS</sup> Kit<sup>®</sup>. Akt-DN EPCs showed decreased telomerase activity compared with the Akt-CA EPC control (Fig. 10C). This effect was exacerbated after treatment with L5. The observed decrease in telomerase activity was attenuated by pretreating cells with TS20, whereas TS20 by itself did not affect EPC senescence or telomerase activity (Fig. 10A–C). Parallel to earlier results, Akt-CA-treated EPC telomerase activity was unaffected by exposure to L5 or TS20.

In the L5/L1 mixture (10:500  $\mu\text{g/ml}$ ), L5 maintained its ability to accelerate senescence, as manifested by increased SA- $\beta$ -gal-positive cells, whereas L1 alone did not (Fig. 10D). Similarly, L5 concentration-dependently inhibited telomerase activity in the presence of L1, whereas L1 by itself did not (Fig. 10E).

## DISCUSSION

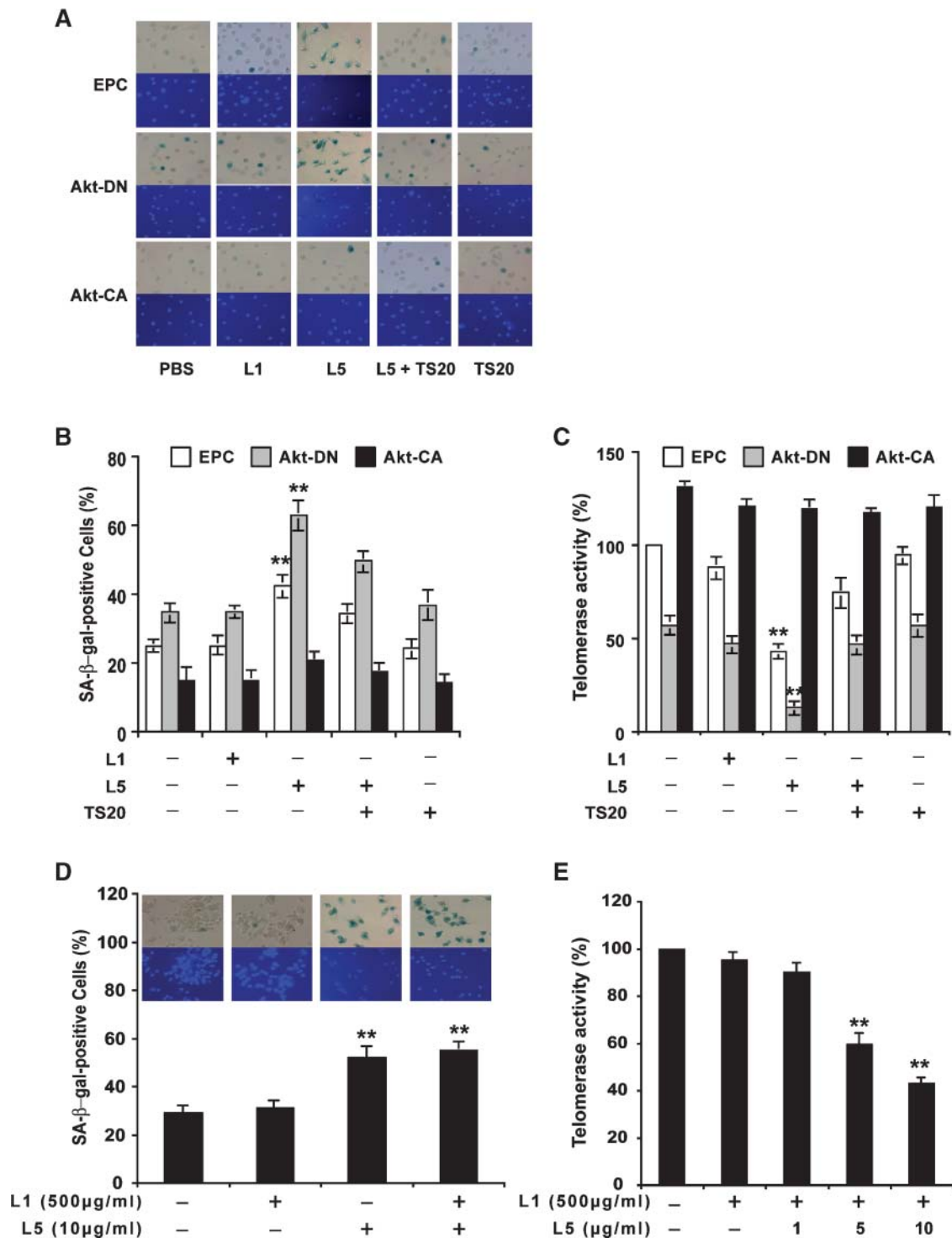
Our studies demonstrate that L5, a highly electronegative LDL subfraction present in the plasma of smoking

subjects, can inhibit the differentiation of EPCs derived from circulating monocytes. This effect was achieved by incubating monocytes with subapoptotic concentrations of L5, which suppressed typical EC markers that were otherwise expressed within 3 days of phenotypic transformation. Furthermore, L5 inhibited EPC telomerase activity, resulting in accelerated senescence. On the basis of our mechanistic studies, we propose that circulating L5 of cigarette smokers activates the LOX-1 receptor, which restricts Akt phosphorylation and other steps necessary to promote normal EPC maturation.

Bone marrow and peripheral blood of adults contain a special subtype of progenitor cells that are able to differentiate into mature ECs. These angiogenic EPCs are a heterogeneous population, derived chiefly from hematopoietic stem cells, and consist of cells at differing stages of maturation (51). Early functional angioblasts, located predominantly in the bone marrow, are characterized by three surface markers: CD133, CD34, and VEGFR2. After migrating to the systemic circulation, EPCs gradually lose their progenitor properties and start to express endothelial markers such as VE-cadherin, endothelial nitric oxide synthase, and vWF (51).

Isolated early peripheral blood-derived EPCs (4–7 days after isolation) represent a small subfraction of mononuclear cells that do not express EC markers. Within several weeks of culture, late-outgrowth cells start to express VE-cadherin and endothelial nitric oxide synthase and acquire a phenotype of mature ECs (4, 50, 52). Gradually, late-outgrowth EPCs functionally form monolayers with a typical cobblestone endothelial appearance (53). An additional feature of isolated EPCs is their ability to incorporate AcLDL and to bind an endothelium-specific lectin (4, 54). Such a smooth transition also may occur in vivo when more immature bone marrow-derived progenitor cells migrate to the systemic circulation. Studies have also shown that human peripheral blood contains pluripotent stem cells as a subset of circulating monocytes (55). These pluripotent monocytic cells can be induced by different culture conditions to acquire different phenotypes (55). Furthermore, it has been shown that monocytes coexpress endothelial markers and form cord-like





**Fig. 10.** Effects of L5 on EPC senescence and telomerase activity. EPCs transfected with Akt-DN or Akt-CA constructs were evaluated by  $\beta$ -galactosidase and telomerase assays. A: Representative photomicrographs ( $\times 20$  magnification) show senescence-associated  $\beta$ -galactosidase (SA- $\beta$ -gal)-positive cells (blue) in early EPCs treated with 10  $\mu$ g/ml L1 or L5 with or without 10  $\mu$ g/ml TS20 pretreatment. For comparison, cells were counterstained with DAPI. B: Percentages of SA- $\beta$ -gal-positive cells were determined after treatment with L1, L5, a combination of L5 and TS20, or TS20 alone. \*\*  $P < 0.01$  versus no treatment in nontransfected EPCs ( $n = 5$ ). C: Telomerase activity assay in early L5-treated EPCs and Akt-DN- or Akt-CA-transfected EPCs treated with 10  $\mu$ g/ml L1 or L5 with or without TS20 pretreatment. Telomerase activity was measured after 24 h of treatment. \*\*  $P < 0.01$  versus no treatment in nontransfected EPCs ( $n = 5$ ). D: Representative photomicrographs ( $\times 20$  magnification) and statistical analysis of SA- $\beta$ -gal-positive cells (blue) in nontreated EPCs and EPCs treated with 500  $\mu$ g/ml L1, 10  $\mu$ g/ml L5, or a mixture of L1 and L5. \*\*  $P < 0.01$  versus no treatment ( $n = 5$ ). E: Telomerase activity assay in early EPCs treated with 1–10  $\mu$ g/ml L5 in a mixture with 500  $\mu$ g/ml L1. \*\*  $P < 0.01$  versus no treatment ( $n = 5$ ). Values are expressed as mean  $\pm$  SEM.

structures *in vitro* under angiogenic conditions (56). Thus, ECs may be generated from both CD14<sup>+</sup> and CD14<sup>-</sup> cells. *Ex vivo* cultivation of young EPCs from CD14<sup>+</sup> or CD14<sup>-</sup> mononuclear cells improved neovascularization in a similar manner, whereas freshly purified CD14<sup>+</sup> or CD14<sup>-</sup> cells without previous culture did not (54). This close relation between monocytic and endothelial lineage cells suggests their possible common origin.

Here, we isolated human mononuclear cells from human blood and plated them on gelatin-coated plates with EC-specific medium supplemented with growth factor mixtures and ascorbic acid. After 3 days of cultivation, early EPC colonies were formed and showed characteristics of ECs, such as internalization of LDL/AcLDL and expression of vWF, VEGFR2/KDR, and CD34, indicating that ECs started differentiation as early as day 3 in culture with special growth factors. Overexpression of Akt-DN inhibited KDR and CD31 expression in EPCs and also blocked EPC proliferation and differentiation (cobblestone EPC formation) induced by VEGF, whereas overexpression of Akt-CA promoted this process, indicating that KDR and Akt signal transduction pathways are crucial elements in the process leading to EPC differentiation induced by VEGF.

It has been suggested that LDL modification may be more important than absolute LDL-cholesterol increase in contributing to atherosclerosis. Previously, we reported that L5, an electronegative LDL subfraction circulating in patients with hypercholesterolemia or type 2 diabetes, can induce marked EC apoptosis (29). Now we report that L5 was present in the LDL of smoking subjects with normal LDL-cholesterol concentrations. In healthy nonsmokers with normal lipid and glucose profiles, L5 is either absent or scanty. We provide evidence that L5 isolated from smoking subjects induces EPC apoptosis in a concentration-dependent manner, whereas sustained exposure to low-dose smoker L5 inhibits EPC proliferation and differentiation without inducing apoptosis. This finding is supported by the observed reduction of CD34- and KDR-positive EPC levels in chronic smokers (6). Our study proved that subapoptotic concentrations of L5 significantly inhibit VEGF-induced KDR and CD31 expression in differentiating EPCs. Resistance to these L5-mediated changes in Akt-CA-treated EPCs suggests that smoker L5 acts in part by decreasing levels of phosphorylated Akt. Indeed, our data demonstrated that L5 inhibits Akt phosphorylation in EPCs at day 3. This is compatible with the observations made with copper-OxLDL, which inhibits VEGF-induced EPC differentiation through dephosphorylation of the Akt kinase on Ser<sup>473</sup> in EPCs, as reported by Imanishi et al. (35). Akt is a serine-threonine protein kinase that is activated by a number of growth factors and cytokines in a phosphatidylinositol 3-kinase (PI3K)-dependent manner (57). Because VEGF induces EPC differentiation through the phosphatidylinositol 3-kinase-Akt pathway (2), L5 very likely inhibits VEGF-mediated effects by interfering with Akt signaling.

The inhibitory effect of Akt phosphorylation by L5 was abolished by neutralizing LOX-1 with its antibody TS20, indicating that most of L5's signals are transduced by

LOX-1. This is supported by the findings that baseline LOX-1 expression, noticeable in day 3 EPCs, was greatly enhanced by L5. Furthermore, internalization of DiI-L5 into EPCs was blocked by TS20, indicative of the mediation role of LOX-1.

Recent studies have demonstrated that atherosclerotic risk factors, including smoking, inversely correlate with the number of differentiated EPCs (6, 14). The proliferation of primary human EPCs is limited by the capacity to divide and the onset of senescence. Loss of telomerase activity has been suggested to constitute the molecular clock that triggers cellular senescence (58). Overexpression of human telomerase reverse transcriptase by adenovirus-mediated gene delivery could lead to a delay of senescence and a recovery/enhancement of the regenerative properties of EPCs (59). We provide evidence that L5 from smokers can accelerate the onset of EPC senescence, which leads to the impairment of proliferative capacity. This is in agreement with the findings by Breitschopf, Zeiher, and Dimmeler (50) that Akt-DN significantly reduces telomerase activity in human umbilical cord ECs. Our experiments showed that the L5-accelerated onset of senescence in EPCs is abolished by TS20. Furthermore, treatment with Akt-DN significantly reduces telomerase activity, whereas Akt-CA prevents the EPC senescence and diminishes telomerase activity induced by L5.

One of the most important observations in this study is that all of L5's adverse effects on differentiating EPCs cannot be compromised in a simulated physiological mixture with the harmless L1. In an L5/L1 ratio of 0.2–10%, L5 remained proapoptotic at higher concentrations (25–50 µg/ml, 5–10% of L1). At subapoptotic concentrations (1–10 µg/ml, 0.2–2% of L1), L5 continued to exert all inhibitory effects on EPCs to halt their differentiation and maturation.

In summary, monocyte differentiation into EPCs starts as early as day 3, as confirmed by the expression of the EPC markers CD31, KDR, and vWF. L5, a highly electronegative LDL found in the plasma of smoking subjects, prematurely terminates EPC differentiation by inhibiting Akt phosphorylation through the LOX-1 receptor signaling pathway. The clinical implications of these *in vitro* observations are strongly substantiated by the fact that mixing L5 with L1 in physiological ratios does not lessen any of its capacities in the absence of L1. Thus, our findings provide a new insight into the mechanism of smoking-related EPC damage, which may contribute to the impairment of endothelial regeneration and the compensatory neovascularization observed *in vivo* (60, 61). ■

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