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Up-regulation of skeletal muscle LIM protein 1 gene by 25-hydroxycholesterol may mediate morphological changes of rat aortic smooth muscle cells

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Abstract

Changes in the expression level of the skeletal muscle LIM protein 1 (SLIM1) in cultured A10 cells were monitored in response to 25-hydroxycholesterol (25-HC), an oxidized form of cholesterol present in the oxidized low-density lipoproteins. The level of SLIM1 mRNA was elevated in a time- and concentration-dependent manner by treatment of 25-HC. Expressions of smooth muscle (SM) α-actin and calponin-1 (CNN-1), early markers for SMC differentiation, were also increased by the 25-HC treatments. Expressions of all three genes (SLIM1, SM α-actin and CNN-1) were simultaneously elevated in the cells treated with 9-*cis* retinoic acid (RA). On the other hand, the SLIM1 expression induced by the 25-HC or 9-*cis* RA (as well as SM α-actin and CNN-1) was decreased by the treatment of 15d-PGJ2. Since the 25-HC, 9-*cis* RA and 15d-PGJ2 were ligands for the LXR, RXRα and PPARγ respectively, there might be a functional positive cross-talk between LXR and RXRα pathways and a negative cross-talk between PPARγ and LXR and/or RXRα pathways in the regulation of SLIM1 expression. The cells stably transfected with the expressional vector for SLIM1 also showed an elevation in the levels of SM α-actin and CNN-1. In addition, an over-production of SLIM1 in the cells resulted in a change in the cell-shape into a spindle-like form, which is identical to that observed after a prolonged treatment of the cells with cholesterol.

Keywords: SLIM1; 25-Hydroxycholesterol; Differentiation of SMC

Introduction

Phenotypic modulation of vascular smooth muscle cells (SMCs) from contractile to synthetic forms plays a pivotal role in the pathogenesis of vascular diseases including atherosclerosis (Owens et al., 2004). Since the oxidized low-density lipoproteins (oxLDL) has been known as a major component that gives rise to the atherogenesis, the role of constituents in oxLDL on the phenotypic transition of SMC was examined. The oxLDL are rich in oxysterols and taken up by the monocyte/macrophages or SMCs (Brown et al., 1996), which may eventually leads the cells to foam cells. The oxysterols, including 25-hydroxycholesterol (25-HC), are found in the foam cells in atherosclerotic plaques (Vine et al., 1998). The 25-HC has been shown to deliver its signal by binding to a nuclear receptor, liver X receptor (LXR) (Peet et al., 1998). The LXRs (LXRα and β), members of the nuclear receptor superfamily, have been shown to regulate the expression of target genes by binding to a specific response element in association with the retinoid X receptor α (RXRα), the receptor for 9-*cis* retinoic

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acid (9-*cis* RA) (Landis et al., 2002). In addition, it has been known that the RXR interacts with peroxisome proliferator-activated receptor γ (PPARγ) and plays a role in many biological processes including differentiation of adipocytes, regulation of lipid metabolism, atherogenesis, inflammation, diabetes, immune response and ageing (Kersten et al., 2000; Celi and Shuldiner, 2002).

In the previous study (Byun et al., 1998) conducted mainly by differential-display reverse transcription-PCR (DDRT-PCR), many genes were found that changed in their expression levels by the cholesterol-loading in the culture medium in the established aortic smooth muscle cells (A10). Among those genes, one showed a high homology to mouse skeletal muscle LIM protein 1 (SLIM1) gene in its nucleotide sequence containing four and a half LIM domains. The LIM domain has a double zinc finger motif in the consensus sequence ($CX_2CX_{17-19}HX_2C)X_2(CX_2CX_{16-20}CX_2$(H/D/C)) that mediates the protein–protein interactions of transcriptional factors, signaling proteins and cytoskeleton-associated proteins (Michelsen et al., 1993). The conserved cysteine and histidine residues form two zinc-binding pockets stabilizing the tertiary structure of the protein (Wadman et al., 1997). An increasing number of studies have demonstrated that the proteins with LIM domains participate in the regulation of the cellular development and differentiation of the skeletal muscle cells (Kosa et al., 1994; Freyd et al., 1990). The SLIM1 is expressed abundantly in the skeletal muscle cells. An alternatively spliced murine isoform of SLIM1, called KyoT2, has recently been identified as a binding partner of the DNA binding protein, RBP-Jκ (Taniguchi et al., 1998; Brown et al., 1999). It has been shown (Hwang et al., 2000; Loughna et al., 2000) that the SLIM1 is highly expressed in striated muscle. The increased level of SLIM1 expression is associated with both skeletal and cardiac muscle hypertrophy. In the progression of atherosclerosis, one of the major events is the onset of phenotypic alteration of vascular SMCs (Ross, 1995). During this process, vascular SMCs undergo a transition in phenotype from a differentiated to dedifferentiated state. Vascular SMCs thus gain two alternative abilities, proliferation and migration. Proliferated cells then migrate into the intima, causing intimal thickening. Finally, progression of atherosclerotic lesions in the intima is characterized by the accumulation of layers of dedifferentiated SMCs and lipid-laden macrophages. Therefore, phenotypic alteration of SMCs is one topic in current vascular biology. In the present study, we investigated the possibility that signals from 25-HC might be delivered to the SMCs, which results in the phenotypic alteration of the SMC, using cultured aortic smooth muscle cells.

Materials and methods

Cell culture

Media and materials for cell culture were purchased from Gibco-BRL (Rockville, MD, USA). Cholesterol, 25-hydroxycholesterol (25-HC), actinomycin D, brefeldin A, cycloheximide and 9-*cis* retinoic acid (9-*cis* RA) were purchased from Sigma (St. Louise, MO, USA), genistein and G418 were from Gibco-BRL and 15-deoxy-$\Delta^{12,14}$-prostagladin J2 (15d-PGJ2) was from Cayman Chemical (Ann Arbor, MI, USA). Rat aortic smooth muscle A10 cells (Kimes and Brandt, 1976; Zhang and Smith, 1996) were obtained from the American Type Culture Collection (ATCC; Manassas, VA. USA). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin (50 units/ml) and streptomycin (50 μg/ml) at 37 °C in a humidified atmosphere of 5% CO_2. The cells were fed every 2–3 days and sub-cultured once they reached 90–100% confluence. Cells were seeded with 1.0×10^6 cells followed by pre-incubation in serum-free media for 24 h and then treated with various factors for additional time periods. Sterols at concentrations 2–40 μg/ml were supplied in ethanol refer to previous report (Awad et al., 2001). Actinomycin D, brefeldin A, cycloheximide, genistein, 25-HC, 15d-PGJ2 and 9-*cis* RA were added to the culture media in a minimal volume (0.1%) of ethanol.

Isolation and oxidation of LDL

Whole blood was collected from normolipidemic subjects into tubes containing 1 mM EDTA and the plasma was separated by low-speed centrifugation (4000 rpm). Sodium azide (0.01%), aprotinin (5 μg/ml) and PMSF (1 mM) were added to the plasma. The LDL ($1.020<d<1.063$ g/ml) was isolated by the sequential flotation ultracentrifugation (Mills et al., 1984). Oxidized LDL was prepared as described previously (Steinbrecher, 1987). Before the oxidative modification, the LDL was dialyzed against PBS (without $Ca^{2+}$and Mg^{2+}, pH 7.4), filtered through a 0.22 μm Millipore membrane, and stored in PBS containing 1 mM EDTA at 4 °C. The concentrations of LDL protein were measured using BCA protein assay reagent (Pierce, Rockford, IL, USA).

RT-PCR and Northern blot analysis

Total RNA was prepared from cultured A10 cells by the acid guanidine thiocyanate–phenol–chloroform extraction method (Chomczynski and Sacchi, 1987). The RNA isolated from A10 cells was fractionated on a 0.9% agarose gel containing 2.2 M formaldehyde and transferred onto Hybond-N^+ membranes (Amersham pharmacia Biotech, Buckinghamshire, UK). Northern blot hybridization was performed as previously described (Sambrook and Russell, 2001). The cDNAs for probes used in Northern blot analysis were obtained by RT-PCR amplification, cloned into pT7blue® vector (Novagen, Madison, WI, USA) or pGEM®-T Easy vector (Promega, Madison, WI, USA), and their sequences were confirmed by DNA sequencing. To prepare the cDNAs for interested genes, total RNA isolated from A10 cells was reverse-transcribed using random hexamer primers and Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) and subsequently amplified by a PCR using the following primers; for SLIM1, 5′-CCGGATCCTCCAGCTATAAGGTGGG-3′ and 5′-GGAATTCGTTTTACAGGACAGGAGCC-3′ (fragment size: 1007 bp); for GAPDH, 5′-CGCCTGGTCACCAGGGC-3′ and 5′-GCCATGAGGTCCACCACCC-3′ (fragment size: 941 bp); for SM α-actin, 5′-CCATCGTGGGACGTCCCAG-3′ and 5′-CCTTGATGTCACGGACGATCTCAC-3′ (fragment size:

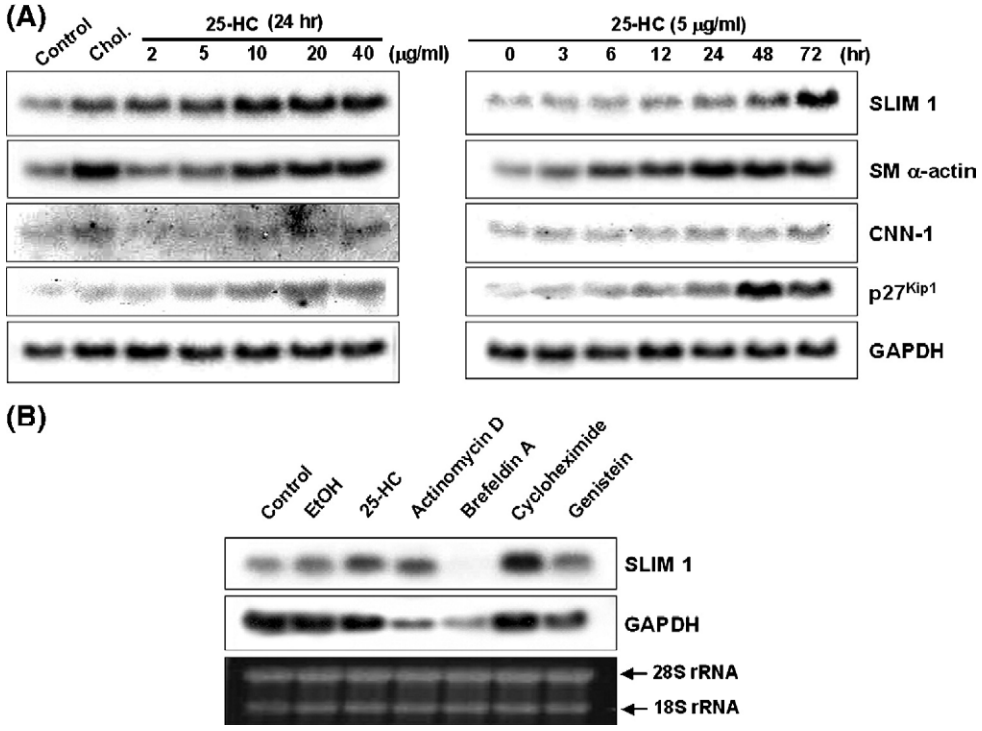

Fig. 1. Concentration- and time-dependence of 25-HC-induced SLIM1 expression in A10 cells. (A) Total RNA (15 μg) was isolated from A10 cells grown in the presence of 0, 2, 5, 10, 20 and 40 μg/ml of 25-HC for 24 h for concentration-dependence study, and in the presence of 25-HC (5 μg/ml) for 0, 3, 6, 12, 24, 48 and 72 h for the time-dependence study, followed by Northern blot analyses using [^{32}P]-labeled cDNAs for corresponding genes as probes. This figure represents one of four independent experiments. (B) The cells were pre-treated for 30 min with brefeldin A (0.5 μg/ml) and for 1 h with actinomycin D (5 μg/ml), cycloheximide (5 μg/ml) and genistein (15 μM), followed by incubation with 25-HC (10 μg/ml) for 12 h. Northern blot analyses were performed with total RNA isolated from the cells using [^{32}P]-labeled cDNAs for corresponding genes as probes. This figure represents one of three independent experiments.

543 bp); for CNN-1, 5′-GGCACCAGCTGGAGAACATAGG-3′ and 5′-GGCAGCCCATACACCGTCATG-3′ (fragment size: 547 bp); for p27^{Kip1} 5′-GCCTGGAGCGGATGGACG-3′ and 5′-GGCTTCTTGGGCGTCTGCTC-3′ (fragment size: 538 bp). The amplified cDNA fragments were purified and then labeled with [α-^{32}P]-dCTP using the Prime-a-Gene® Labeling system from Promega.

Production of recombinant SLIM1 and polyclonal antibody

Rat SLIM1 cDNA (GenBank Accession No. AF134773) was sub-cloned into the pET-14b vector (Novagen, Madison, WI, USA), followed by transformation into *E. coli* strain BL21(DE3) for production of recombinant SLIM1. The protein (His-tagged SLIM1) was massively produced by IPTG induction and purified with Ni-NTA-agarose (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. This protein was used as an antigen for the production of polyclonal antibody raised in New Zealand White rabbits.

Generation of SLIM1 over-expressing cells

Rat SLIM1 cDNA and antisense cDNA were generated by the PCR using the following primers: 5′-CCAAGCTTCCAGCTA-TAAGGTGGG-3′ and 5′-GCATCGAT ACAGGACAGGAGCC-3′ for SLIM1 cDNA; 5′-CCTCCATCGATAAGGTGGG CAC-3′ and 5′-GGAAGCTTTTTACAGGACAGGAGCC-3′ for anti-sense cDNA. These cDNAs were sub-cloned into the pLNCX (cytomegalovirus [CMV] promoter) vector (Clontech, Palo Alto, CA. USA) to generate the plasmids pLNCX-SLIM1^{sense} and pLNCX-SLIM1^{antisense}, respectively. Clones of A10 cells that over-express the SLIM1 or a control plasmid lacking the SLIM1 genes were generated by the transfecting the corresponding vector using lipofectamine (Life Technologies, Gaithersburg, MD) with the procedure recommended by manufacturer. The transfected cells were subsequently grown in the selection medium (complete medium containing 400 μg/ml of G418). Following 10–20 days in selection medium, colonies were picked and subsequently examined for the presence of SLIM1 proteins by immunocyto-chemistry. The cells were maintained in DMEM supplemented with 10% FBS and antibiotics.

Immunocytochemistry

After fixation with 4% paraformaldehyde, intrinsic peroxi-dase activity was inhibited by 3% H_2O_2 for 30 min and nonspecific binding was blocked with normal goat serum. The cells were then allowed to interact with the polyclonal antisera against SLIM1 (diluted 1:100) and CNN-1 (diluted 1:100, Santa Cruz, CA, USA) at 4 °C overnight. The cells were washed and incubated with the biotinylated secondary antibody (diluted 1:400) at 37 °C for 30 min followed by the incubation in avidin–biotin–peroxidase complex (Vector, Burlingame, CA, USA) for 30 min. Cells were then stained with 0.016% DAB

and 0.024% H_2O_2 in PBS at room temperature. The sections of samples were washed with distilled water at the end of every step. Finally, the cells were counterstained with hematoxylin.

Western blot analysis

Western blotting of cellular proteins (50 μg) separated by SDS-PAGE in a 12% gel and subsequently transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Inc., Hercules, CA. USA) was conducted as described previously. Membranes were probed with antibodies against the SLIM1, SM α-actin (R&D System, Inc., Minneapolis, MN. USA), β-actin (Santa Cruz, CA. USA) and horseradish peroxidase-conjugated secondary antibody (1:3000 diluted) was detected using the ECL chemiluminescent system (Amersham pharmacia Biotech.)

Results

25-Hydroxycholesterol induces SLIM1 gene expression

In this study, the response to the 25-HC on the SLIM1 expression was examined. Cellular RNAs from the established aortic smooth muscle cells (A10 cells) cultured in the presence of 25-HC were allowed to hybridize with the SLIM1 cDNA. A transcript (2.3 kb) corresponding to the SLIM1 gene was up-regulated in response to the 20 μg/ml of 25-HC (Fig. 1A), compared to those treated with free cholesterol. The up-regulation was time- and concentration-dependent. In order to determine whether the 25-HC affects on the SMC differentiation, the expressional patterns of smooth muscle (SM) α-actin and calpolin-1 (CNN-1), markers for differentiation of SMCs, were examined. The levels of SM α-actin and CNN-1 mRNAs were increased by 25-HC treatment. The expression of cyclin-dependent kinase inhibitor $p27^{Kip1}$, a critical regulator of cell-cycle progression (Braun-Dullaeus et al., 1998; Chellappan et al., 1998), was also increased in the same manner.

To determine whether the effects of 25-HC on the expression of the SLIM1 gene were dependent on RNA and protein syntheses de novo, the effects of actinomycin D, an inhibitor of DNA-dependent RNA synthesis (Frey et al., 1996) and cycloheximide, an inhibitor of protein synthesis (Im et al., 1987), were tested. The A10 cells were cultured in the media containing actinomycin D or cycloheximide for 1 h followed by an additional 12 h in the presence of 25-HC. The expression of SLIM1 gene in 25-HC-treated cells was further stimulated by those pre-treatments (Fig. 1B). Dependence of the SLIM1 expression to the protein translocation was also examined by culturing the cells in the media containing brefeldin A, which disrupts of the structure and function of the Golgi apparatus (Nakamura et al., 1995), prior to the addition of 25-HC. The stimulatory effects of the 25-HC were completely inhibited (Fig. 1B). To examine whether the stimulation of SLIM1 gene expression by 25-HC was mediated by the tyrosine kinase, the A10 cells were cultured in the presence of genistein, a tyrosine kinase inhibitor (Smirnov and Aaronson, 1995), prior to the treatment of 25-HC. The stimulation was mostly inhibited by the pre-treatment (Fig. 1B).

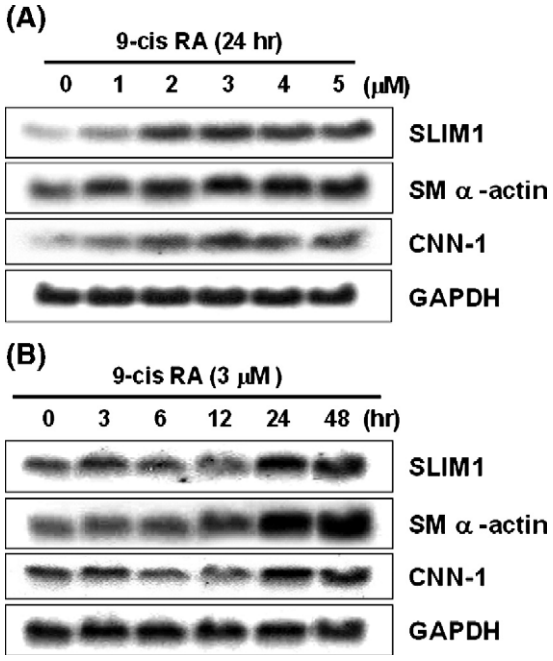

Fig. 2. Effects of 9-*cis* RA on the expression of SLIM1 and differentiation-related genes. Total RNA was isolated from the cells treated with 0, 1, 2, 3, 4 and 5 μM of 9-*cis* RA for 24 h for concentration-dependence study (A), and treated with 9-*cis* RA (3 μM) for 0, 3, 6, 12, 14 and 48 h for the time-dependence study (B), followed by Northern blot analyses using [^{32}P]-labeled corresponding cDNAs as probes. This figures represents one of three independent experiments.

Effects of RXRα and PPARγ ligands on the SLIM1 gene expression

Since the Liver X receptors (LXRs) and retinoid X receptor α (RXRα) were considered to be one of possible signaling molecules toward the SLIM1 in response to the 25-HC, the changes of expressional levels of SLIM1 and SMC marker genes were investigated in response to 9-*cis* RA. As shown in Fig. 2, the treatment of 9-*cis* RA in A10 cells resulted in an increase in the expressional levels of SLIM1, SM α-actin and CNN-1 mRNAs in a time- and concentration-dependent manners. The SLIM1 expression reached to a maximal level after 24 h of incubation and sustained for 48 h (Fig. 2B). Expressional patterns of SM α-actin and CNN-1 genes were similar to that of SLIM1. To demonstrate cross-talk among the LXR, RXRα and peroxisome proliferator-activated receptor γ (PPARγ) on the activation of SLIM1, Northern blot analyses were carried out with the total RNA from A10 cells treated with the corresponding ligands for those transcriptional factors. As shown in Fig. 3, the expressions of SLIM1, SM α-actin and CNN-1 were all elevated in the cells treated with 25-HC or 9-*cis* RA independently. There were strong elevations in the expression of all three genes when the cells were treated with 25-HC in combination with 9-*cis* RA. To determine effects of PPARγ ligand on the expression of SLIM1 and differentiation-relating genes, the cells were cultured in the media containing 15-deoxy-$\Delta^{12,14}$-prostagladin J2 (15d-PGJ2), an endogenous ligand for PPARγ. The expressions of SLIM1, SM α-actin and CNN-1 genes were all slightly declined by treatment of 15d-

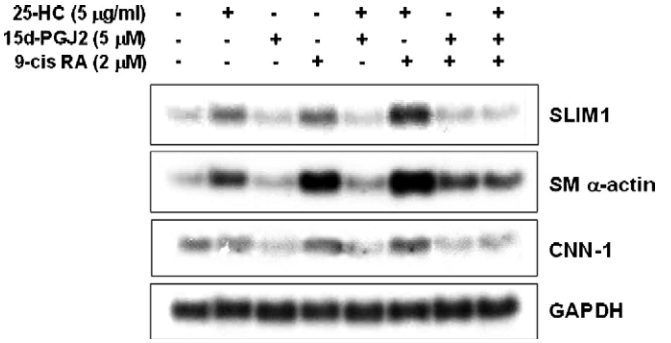

Fig. 3. Combinatorial effects of 15d-PGJ2, 9-*cis* RA and 25-HC on the expressions of SLIM1 and differentiation-related genes. The cells (A10) were treated with 15d-PGJ2 (5 μM), 9-*cis* RA (2 μM) and 25-HC (5 μg/ml) individually or in combination for 24 h. Total RNA was isolated from the cells followed by Northern blot analysis using [^{32}P]-labeled cDNAs for corresponding genes as probes. This figure represents one of four independent experiments.

PGJ2. Those expressional levels were reduced whenever 15d-PGJ2 was used as a partner. The expression remained at a basal level when the cells were treated with all three ligands.

Effects of over-expressed SLIM1 on the SMC differentiation

To elucidate the role of SLIM1 on the differentiation of SMCs, the expressional patterns of differentiation-related genes in response to the SLIM1 over-expression were monitored with the A10 cells transfected with plasmids which have previously constructed by inserting the full-length of SLIM1 cDNA into mammalian expression vector (pLNCX-SLIM1^{sense}). The levels of SM α-actin and CNN-1 mRNA were elevated in the pLNCX-SLIM1^{sense}-transfected (sense-transfected) cells and reduced in the pLNCX-SLIM1^{antisense}-transfected (antisense-transfected) cells (Fig. 4A). Additionally, the shape of sense-transfected cells was turned into a spindle-like form (arrowhead) (Fig. 4B-II), a characteristic phenotype of differentiated SMCs, indicating that the over-expression of SLIM1 might induce the cellular alteration. An increase in the expression of CNN-1 was clearly observed in the sense-transfected cells (Fig. 4B-g) when the cells were subjected to the immunocytochemistry performed with antibody against CNN-1. Expression of SM α-actin was also up-regulated in the sense-transfected cells, whereas it declined mostly in the antisense-transfected cells (Fig. 4C). In order to confirm that cholesterol or 25-HC induces the SMC differentiation, morphological changes was monitored in the cholesterol-treated A10 cells. As shown in Fig. 4D, the levels of SLIM1 mRNA were elevated in the cell treated with cholesterol for 2 and 14 days. Subsequently, shape of the cells treated with cholesterol for 14 days were turned into spindle-like form (arrowhead), suggesting that a prolonged treatment of cholesterol might give an influence to the induction of SMC differentiation. To determine the effects of SLIM1 over-expression on the lipid accumulation in the cell, cellular lipids were stained with Oil Red O after the cells were pre-treated with oxidized low-density lipoproteins (oxLDL). The neutral lipid inclusions in sense-transfected cells were substantially increased (Fig. 4B-h), compared to the controls (Fig. 4B-d and-l).

Discussion

It has been shown that the SLIM1 is highly expressed in the murine artery in response to a high-cholesterol diet (Byun et al., 1998). Oxidized form of cholesterol (such as 25-HC) existed in the oxLDL has been shown to alter morphology in cultured arterial smooth muscle and endothelial cells (Wohlfeil and Campbell, 1999). In the present study, the 25-HC increased mRNA level of SLIM1 in the cultured murine aortic SMCs, suggesting a possible role of SLIM1 in an action of 25-HC. The expression of SLIM1 gene in 25-HC-treated cells was stimulated by the pre-treatment of actinomycin D and cycloheximide, indicating that a de novo mRNA and protein syntheses do not affect on the elevation of SLIM1 expression by the 25-HC. This might implicate that the expression or the degradation SLIM1 mRNA might be suppressed by factors in the cell. The stimulation in the genistein-treated cells was mostly inhibited, suggesting that the stimulation of the SLIM1 expression by the 25-HC appears to be regulated through a tyrosine kinase.

Since it has been suggested that the SLIM1 may be involved in the differentiation of skeletal muscle cells (Kopan et al., 1994; Kuroda et al., 1999), the effects of 25-HC on the expression of SMC differentiation-related genes were also examined. Expressions of SM α-actin and CNN-1 were increased by the 25-HC treatments, suggesting that the 25-HC may play a role in the morphological change of SMCs. We also observed that an over-expression of SLIM1 leads to a change of the cell-shape, which is a characteristic of differentiated SMCs. It has been shown that over-expression of SLIM1 promotes integrin-dependent spreading, migration and differentiation of skeletal muscle myocyte (McGrath et al., 2003; Robinson et al., 2003). Additionally, the expression of SM α-actin and CNN-1 mRNA were elevated in the sense-transfected cells and reduced in antisense-transfected cells. It is well established that SMCs sequentially express specific contractile proteins during vascular development. Smooth muscle α-actin is an important determinant and considered to be an early marker for differentiated SMCs (Owens, 1995). The calponin has been shown to appear only after the cells make a commitment to be a SMC lineage in the artery or to be a multi-layered arterial wall (Duband et al., 1993). Thus, the SLIM1 appeared to function as not only a cytoskeleton reorganizer in the process of skeletal muscle cell differentiation, but also a transcriptional regulator in the process of SMC differentiation.

The 25-HC has been known as a natural ligand for the nuclear transcription factor, LXRs (Peet et al., 1998). The LXRs (LXRα and β), members of the nuclear receptor superfamily, have been shown to regulate the expression of target genes by binding to a specific response element in association with the RXRα, the receptor for 9-*cis* RA (Landis et al., 2002). In this study, experiments were performed to find a relationship among the 25-HC, SLIM1, LXRs and RXRα. The expressions of SLIM1, SM α-actin and CNN-1 genes were simultaneously elevated in the cells treated with 9-*cis* RA. Previous reports have demonstrated that retinoids inhibit cell-proliferation and induce a differentiation of cells in many systems (Chomienne et al.,

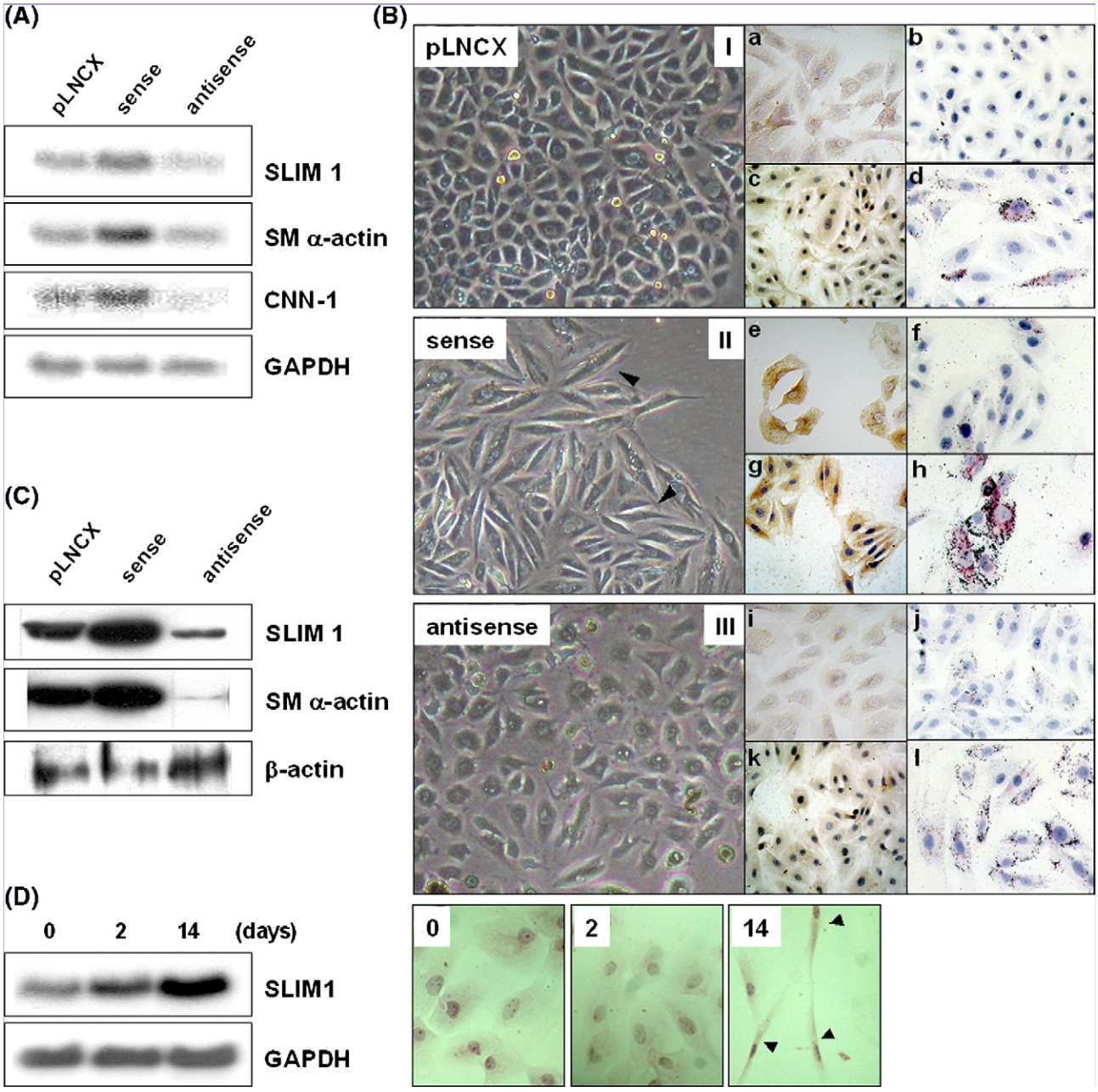

Fig. 4. Morphological changes in the SLIM1-over-expressed A10 cells. The cells (A10) were transfected with three kinds of plasmids (pLNCX, pLNCX-SLIM1^{sense} and pLNCX-SLIM1^{antisense}), which were previously constructed with full-length of SLIM1 cDNA and its antisense DNA inserted into a mammalian expression vector (pLNCX). Stably transfected cells were selected with G418 antibiotics. (A) Effects of over-expressed SLIM1 on the expression of differentiation-related genes were monitored by Northern blot analyses with total RNA isolated from the transfected cells. (B) Effects of SLIM1 over-expression on the long-term culture of A10 cells: stably transfected cells sub-cultured in DMEM for 15 days were subjected to the phase-contrast photomicrography (original magnification: 40). The cells over-expressing SLIM1 were cultured for 2 days on glass slides and stained with rabbit polyclonal antiserum against rat SLIM1 (a, e, i) and goat polyclonal antibody against human CNN-1 (c, g, k). Avidin–biotin–horseradish peroxidase system (Vector, Burlingame, CA, USA) was used for the visualization. The cells were counter-stained with the hematoxylin. Effects of oxLDL on lipid accumulation in the SLIM1-overexpressed A10 cells were monitored by Oil Red O staining. Stably transfected cells were incubated with PBS (b, f, j) or oxLDL (50 μg/ml, d, h, l) for 24 h. Cell-preparations were fixed with paraformaldehyde (4%), stained with Oil Red O, and then counterstained with hematoxylin (original magnification: ×200). pLNCX, pLNCX-transfected cells (I); sense, pLNCX-SLIM1^{sense}-transfected cells (II); antisense, pLNCX-SLIM1^{antisense}-transfected cells (III). (C) Cell lysates (50 μg) from stably transfected cells were fractionated by SDS-PAGE and blotted with anti-SLIM1 antiserum, anti-SM α-actin monoclonal antibody and anti-β-actin polyclonal antibody. (D) The cells were treated with cholesterol (20 μg/ml) for 0, 2 and 14 days in DMEM supplemented with 10% FBS. Total RNA was isolated from the cells followed by Northern blot analysis using [^{32}P]-labeled SLIM1 and GAPDH cDNA as probes. The cells fixed with paraformaldehyde were stained with hematoxylin and eosin Y.

1990; Gudas et al., 1994; Axel et al., 2001). It has also been shown that all-*trans* retinoic acid promotes differentiation of the SMCs through the expressional regulation of multiple genes essential for entry into the cell cycle and for the subsequent progression to G1 phase (Miano et al., 1996). In addition, it has been known that the RXR interacts with PPARγ and plays a role

in many biological processes including differentiation of adipocytes, regulation of lipid metabolism, atherogenesis, inflammation, diabetes, immune response and ageing (Kersten et al., 2000; Celi and Shuldiner, 2002; Na and Surh, 2003). In this study, the SLIM1 expression induced by the 25-HC or 9-*cis* RA (as well as SM α-actin and CNN-1) was decreased by the treatment of 15d-PGJ2. These data suggested a functional positive cross-talk between LXR and RXRα pathways and a negative cross-talk between PPARγ and LXR and/or RXRα pathways in the regulations of SLIM1, SM α-actin and CNN-1 expressions. To determine the effects of SLIM1 over-expression on the lipid accumulation in the cell, cellular lipids were stained after the cells were pre-treated with oxLDL, the neutral lipid inclusions in sense-transfected cells were substantially increased. Previous reports (Argmann et al., 2004) have demonstrated that spindle-shaped SMCs preferentially accumulate cholesteryl esters and triglycerides.

This study suggested a possibility that the 25-HC might function in the alteration of aortic smooth muscle cells via SLIM1. The expression of SLIM1 was regulated by LXR: RXRα and RXRα:PPARγ pathways which may in turn regulate the differentiation of SMCs.

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