Temporal Gene Expression Analysis of Human Coronary Artery Endothelial Cells Treated With Simvastatin

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Increasing evidence indicates that the beneficial “pleiotropic” effects of statins on clinical events involve nonlipid mechanisms including the modification of blood vessel endothelial cell function. However, the involved molecular events and pathways are not completely understood. In the present study, Affymetrix microarrays were used to monitor the temporal gene expression of human coronary artery endothelial cells (HCAEC) treated with simvastatin (Sim) to gain insight into statins’ direct effects on the endothelial function. We isolated and labeled mRNA from HCAEC treated with Sim for 0, 3, 6, 12, 24, and 48 h and hybridized these samples to Affymetrix GeneChip HG-U95Av2 to analyze the temporal gene expression profile. Out of 12,625 genes present on the HG-U95Av2 GeneChip, expression of 5,432 genes was detected. There were 1,475 of 5,432 genes that displayed the differential expression compared to baseline (0 h). Fifty-four genes were upregulated ($\leq$ twofold) while 61 genes were downregulated ($\geq$ twofold) at 24–48 h after the Sim treatment. Many new target genes and pathways modulated by Sim were uncovered. This study indicates that many aspects of the pleiotropic effect of Sim on the endothelial cell function can be mediated by transcriptional control. Physiological function of 22% of 115 differentially expressed genes in Sim-treated HCAEC are currently unknown. These newly identified genes could be useful for new mechanistic study and new therapeutic modalities. Expressions of 13 out of 18 genes ($>70\%$) in the cell cycle/proliferation control process were significantly inhibited by the Sim treatment. CDC25B and ITGB4 gene expressions were validated by RT-PCR and Western blotting. Sim’s inhibitory effect of on HCAEC growth was confirmed by the measurement of $[^3\text{H}]$ thymidine incorporation into the DNA synthesis. Further in-depth analysis of this effect may shed light on molecular mechanisms of Sim’s beneficial inhibition of neointima formation in the atherosclerotic artery stenosis.

Key words: Simvastatin; Coronary artery endothelial cells; Microarray; Gene expression

INTRODUCTION

Several large primary and secondary intervention trials have established that statins are the powerful class of hypolipemic drugs in reducing coronary mor-

bidity and mortality (9,10,13). Statins include simvastatin, lovastatin, pravastatin, fluvastatin, cerivastatin, atorvastatin, and others. Their well-recognized mechanism of action is that statins competitively inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-

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CoA) reductase, which catalyzes the rate-limiting step in cholesterol biosynthesis (7). The resultant reduction in intracellular cholesterol synthesis triggers increased expression of cell membrane LDL receptor, which clears LDL and LDL precursors from the circulation. The study by Istvan and Deisenhofer (16) revealed the physical and chemical mechanism of the stain inhibition. The bulky, hydrophobic compounds of statins occupy the HMG-binding pocket of HMG-CoA reductase and part of its binding surface for CoA, thus blocking the access of its substrate HMG-CoA. Growing evidences indicate that the beneficial effects of statins on clinical events may involve non-lipid mechanisms, which modify endothelial function, smooth muscle cells, monocyte-macrophage, vasomotor function, inflammatory responses, and plaque stability (3,18,21). The detailed cellular and molecular mechanisms for these beneficial effects of statins unrelated to lipid lowering are not completely understood.

Improvement of endothelial function may represent a significantly important beneficial "pleiotropic" effect of statins unrelated to their lipid-lowering effects. Hypercholesterolemia impairs endothelial function, and endothelial dysfunction is one of the earliest manifestations of atherosclerosis, occurring even in the absence of angiographic evidence of disease (17, 33). An important characteristic of endothelial dysfunction is the impaired synthesis, release, and activity of endothelium-derived NO. Acute plasma LDL apheresis improves endothelium-dependent vasodilatation, suggesting that statins could restore endothelial function, in part, by lowering serum cholesterol levels. However, studies indicates that statins restored endothelial function before significant reduction in serum cholesterol levels (34), suggesting that there may be additional effects on endothelial function beyond that of cholesterol reduction.

Laufs and Liao (19) reported that the effects of statins on Ras and Rho isoprenylation were reversed in the presence of farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), respectively, whereas the effects of statins on eNOS expression were reversed only with GGPP and not with FPP or LDL cholesterol. These findings are consistent with a non-cholesterol-lowering effect of statins. A recent study has shown that statins can suppress the inflammatory response independent of HMG-CoA reductase inhibition by binding directly to a novel regulatory site of the $\beta 2$ integrin, leukocyte function antigen-1, which serves as a major counterreceptor for intercellular adhesion molecule-1 on leukocytes (35).

One important mechanism of statins' beneficial effects, which is unrelated to their lipid lowering, is that statins regulate endothelial cell NO production.

Statins increase NO bioavailability either by upregulating endothelial NO synthase (eNOS) or by decreasing oxidative stress. Statins upregulate eNOS expression by prolonging eNOS mRNA half-life, not eNOS gene transcription. Endothelial NO has been shown to inhibit several components of the atherogenic process. For example, endothelium-derived NO mediates vascular relaxation and inhibits platelet aggregation, vascular smooth muscle proliferation, and endothelium-leukocyte interactions. Inactivation of NO by superoxide anion ($O_2^{\bullet -}$) limits the bioavailability of NO and leads to nitrate tolerance, vasoconstriction, and hypertension. Furthermore, statins have been shown to restore eNOS activity in the presence of hypoxia and oxidized LDL, conditions that lead to endothelial dysfunction.

Statins also increase the expression of tissue-type plasminogen activator and inhibit the expression of endothelin-1, a potent vasoconstrictor and mitogen. Therefore, statins exert many favorable effects on the endothelium and attenuate endothelial dysfunction in the presence of atherosclerotic risk factors. Brouet et al. (6) showed that statins promoted the tyrosine phosphorylation of hsp90 and the direct interaction of hsp90 with Akt, which further potentiated the NO-dependent angiogenic processes. Their study provides new mechanistic insights into the NO-mediated angiogenic effects of statins, and it also underscores the potential of these drugs and other modulators of hsp90 and caveolin abundance to promote neovascularization in disease states associated or not with atherosclerosis. Mulder et al. (22) reported that pravastatin reduces clinical and angiographic restenosis 2 years after PCTA (regress trial). Although the list of cellular effects of statins on the vascular wall continues to grow, it remains to be determined what the molecular mechanism is and which, if any, of these effects accounts for the clinical benefits of statin therapy in cardiovascular disease.

In the present study, Affymetrix microarrays were used to monitor the temporal gene expression of human coronary artery endothelial cells (HCAEC) treated with simvastatin (Sim) to gain insight into an important role in mediating the direct effects of statins on the endothelial function. We isolated and labeled mRNA from HCAEC treated with Sim from 0, 3, 6, 12, 24, and 48 h and hybridized these samples to Affymetrix GeneChip HG-U95Av2. From these data, temporal gene expression profile and pathways affected by Sim were analyzed. Selected target genes in the control of cell growth were validated by alternative approaches such as RT-PCR and Western blotting. The inhibitory effect of Sim on HCAEC growth was confirmed by the measurement of [3H]thymidine incorporation into the DNA synthesis.

MATERIALS AND METHODS

Cell Culture

Human coronary artery endothelial cells (HCAEC, Cat. No. CC 2585) were obtained from Cambrex Bio Science Walkersville, Inc. (Walkersville, MD) and maintained in Microvascular Endothelial Cell Medium-2 (EGMTM-2 MV). Cells were incubated at 37°C in 5% CO₂ and 95% air. They were used at passages 6.

Simvastatin Treatment

HCAEC were grown to 90–95% confluency in T75 flasks. Cells in 12 different flasks, duplicate in each time point, were then incubated in the presence of Sim (5 µM) for 0, 3, 6, 12, 24, and 48 h, respectively. At the end of each time point, supernatant from each flask was aspirated and 2 ml TRIzol® solution (Invitrogen, Carlsbad, CA) was added to cover the cell floors and stored at a –80°C freezer until use.

Microarray Methods

The Affymetrix GeneChip System was used as a gene expression profiling platform in this study as described previously (37). Briefly, total HCAEC RNA of each time point was extracted using TRIzol® reagent (Cat. No. 15596-026, Invitrogen) with additional purification using the RNeasy MiniElute Cleanup Kit (Cat. No. 74204, Qiagen, Inc., Valencia, CA). Total RNA (5 µg) from each sample was converted into double-stranded cDNA using SuperScript Choice system (Cat. No. 11917-010, Invitrogen). Each double-stranded cDNA was subsequently used as a template to make biotin-labeled cRNA using the BioArray HighYield RNA Transcript Kit (Cat. No. 42655-10, Enzo Life Sci., Inc., Farmingdale, NY) and 15 µg of fragmented, biotin-labeled murine liver cRNA from each sample was hybridized to the Affymetrix GeneChip® HGU95A v2 at 45°C for 16 h. The HGU95A v2 Array allows the detection of 12,625 transcripts. The arrays were washed and stained in the Affymetrix GeneChip® Fluidics Station 450 using the supplier's reagents and scanned using Affymetrix GeneChip® Scanner 3000. Data acquisition and initial basic analysis of the GeneChip gene expression experiments were carried out using the Affymetrix GeneChip® Operating Software (GCOS, ver1.0). Initial global normalization to target intensity of 150 was applied to each chip. Those chips that failed to pass quality control standards as specified in Affymetrix Guideline for Assessing Sample and Array Quality were excluded for further analysis. The array data were subjected to three progressive filtering steps. First, only those genes with the present call

($p < 0.05$) were allowed to further the comparison analysis. Second, those genes with change calls (increase or decrease, $p < 0.05$) after comparison analysis to 0 h time point were further filtered. Third, those genes in 24-h time point with $\geq$ twofold or $\leq$ twofold changes relative to 0 h time point were subjected to the Netaffex software (Affymetrix, Santa Clara, CA) and K-mean clustering analysis using MV4 program (27).

Semiquantitative Multiplex RT-PCR Analysis

Reverse transcription was performed at 42°C for 50 min in a final volume of 20 µl, containing 1 µg total RNA, 11 pmol random hexamer, 500 µM each dNTP, 20 U RNasin® Ribonuclease inhibitor (Cat. No. N2111, Promega, Madison, WI), 200 U Superscript II RNase H- reverse transcriptase (Cat. No. 18064-014, Life Technologies, Inc., Rockville, MD) and 4 µl 5 × first strand buffer, which came along with the reverse transcriptase.

PCR was carried out in 50 µl final volume containing 1 µl cDNA (1/20 of RT products), 0.5 µM of each primer, 0.2 mM of each dATP, dTTP, dCTP, and dGTP, 50 mM KCl, 20 mM Tris-HCl, pH 8.0, 1.5 mM MgCl₂, 2.5 U of Taq DNA polymerase (Cat. No. N808-0160, Perkin-Elmer, Foster City, CA). The primers for genes of interest and control 18S rRNA were designed using Primer 3 software (Primer 3.cgi, V0.2b, Whitehead Institute/MIT Center for Genome Research) based on the published GenBank sequences. The primers were synthesized on an Applied Biosystems model 381A synthesizer in the DNA Synthesis and Analysis Core Facility, Johns Hopkins University School of Medicine.

Thermal cycling was done in a Perkin-Elmer Gene Amp PCR System 9600 Thermal Cycler with an initial 2-min denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, and a final extension of 5 min at 72°C.

One fifth of the PCR product was separated on 2% agarose gel electrophoresis for 1.5–2 h at constant voltage of 110 V. The gels were stained by 0.5 µg/ml of ethidium bromide. The image was captured on the Alpha Imager (Alpha InfoTech Corp., San Leandro, CA).

Western Blotting

Western blot analysis was performed as described previously (38). Briefly, after washing with PBS, cells were lysed with 200 µl of cell lysis buffer containing 10 mM Tris (pH 7.4), 1% Triton X-100, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, 0.2 mM EGTA, 0.2 mM vanadate, 0.2 mM PMSF, and 0.5%

protease inhibitor cocktail. Total cell lysates were cleared by centrifugation, boiled with the same amount of 3× SDS sample buffer for 5 min. Total proteins of EC lysates were quantified using the BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL). The same amount (10 µg) of total protein from each sample was then subjected to 10% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred to PVDF membranes by electrotransfer. The blots were subsequently blocked with 5% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 (PBS-T) at room temperature for 1 h, and then incubated at 4°C overnight with primary antibodies of a rabbit anti-human integrin β 4 polyclonal antibody (Cat. No. sc-9090, Santa Cruz Biotechnology, Santa Cruz, CA) in 1:2000 dilution or a rabbit anti-human CDC25B polyclonal antibody (Cat. No. sc-5619, Santa Cruz Biotechnology) in 1:500 dilution. After washing three times for 10 min with PBS-T, the membrane was incubated with horse radish peroxides-linked secondary antibodies of a mouse anti-rabbit IgG (Sigma, St. Louis, MO) at room temperature for 1 h. The blots were then visualized with the ECL Western blot detection system. The band image was acquired using the Alpha Imager (Alpha InfoTech Corp.) and analyzed by the AlphaEase™ Stand Alone Software.

Determination of [³H]Thymidine Incorporation

HCAEC were seeded in 96-well plates (5,000 cells/well). After attachment overnight, cells were treated with Sim (5 µM) for various times. [³H]Thymidine (55.5 kBq/ml) was added for the final 12 h. The dishes were rinsed twice with ice-cold PBS before 10% trichloroacetic acid was added for 20 min. After dishes were washed once with ice-cold 10% trichloroacetic acid, cells were lysed with 500 µl 0.2 mol/L NaOH/1% SDS, and the lysates were neutralized with 0.2 mol/L HCl. The incorporated radioactivity was determined by liquid scintillation counting.

RESULTS

Global Analysis of Gene Expression Profile in HCAEC Treated With Sim for 0, 3, 6, 12, 24, and 48 h

Overall effect of Sim on the gene expression in HCAEC is summarized in Table 1. Out of 12,625 genes present on the HGU95A GeneChip, expression of 5,432 genes was detected as present calls based on the gene expression at the 24-h time point. There were 1,475 of the 5,432 genes that displayed differen-

tial expression compared to baseline (0 h). Six hundred and one genes increased expression and 874 genes decreased gene expression. Among them, 54 genes were upregulated ($\geq$ twofold) while 61 genes were downregulated ($\leq$ twofold) at 24–48 h after the Sim treatment.

To examine which major biological processes are affected by the Sim treatment in HCAEC, 115 differentially expressed genes (based on those genes at the 24-h time point $\geq$ twofold or $\leq$ twofold relative to those 0 h) were classified based on the current available GO database (www.GO.org) using the Netaffex software (Affymetrix). As presented in Supplementary Table 1 (Ygeneexpression, Supplementary Table 1, available from: <http://web.missouri.edu/~yes>), the top of the list is 25 genes whose biological process is unknown, accounting for nearly 25%. The known biological processes affected by Sim in HCAEC are as follows in the order of percentage rank: cell cycle/proliferation control (18 genes, 16%) (Table 2); metabolism (14 genes, 12%) and posttranslational modification (14 genes, 12%); cell adhesion (9 genes, 8%); transcriptional regulation (8 genes, 7%); inflammatory response (7 genes, 6%); signal transduction (6 genes, 5%); proteolysis (5 genes, 4%) and cytoskeletal organization (5 genes, 4%); apoptosis (3 genes, 3%); and transport (1 genes, 1%).

Further Pathway Analysis of the Differentially Expressed Genes in Inflammatory Response/Cell Adhesion Processes and Cell Cycle/Proliferation Control

To further characterize the differentially expressed gene profile after the Sim treatment in HCAEC, we subjected those differential expressed genes to the K-mean clustering procedure, relevant network analysis with the focus on genes involved in inflammatory response/cell adhesion processes and cell cycle/proliferation control.

The 115 differentially expressed genes were divided into 10 different groups by the K-mean clustering procedure (Fig. 1). The complete lists of genes of each cluster are provided in Supplementary Table 2 (Ygeneexpression, Supplementary Table 2, available from: <http://web.missouri.edu/~yes>). It is of interest that 6 out of 7 genes in Cluster 9 [interleukin 32, interleukin 8, selectin E, chemokine (C-X3-C motif) ligand 1, endothelial cell-specific molecule 1, chemokine (C-X-C motif) ligand 1] are known genes involved in inflammation. Atherosclerosis is an inflammatory disease (1,25). This experiment demonstrated that indeed the inflammatory/cell adhesion process is one of major targets by Sim treatment. This alterna-

TABLE 1
SUMMARY OF GENE EXPRESSION FILE IN HCAEC TREATED
WITH SIM BASED ON 24-h TIME POINT

	Total Probe Sets	Present Calls	Change Calls	Increase Expression	Decrease Expression	Increase Expression ≥Twofold	Decrease Expression ≤Twofold
<i>N</i>	12,625	5,432	1,475	601	874	54	61
%	100	43*	27†	12†	16†	0.99†	1.12†

*Based on 12,625 as 100%.

†Based on 5,432 as 100%.

tive analysis lend further support that the inhibition of the inflammatory/cell adhesion process is one of major molecular mechanisms by which Sim exerts its antiatherogenesis.

Furthermore, 11 of 18 genes classified in biological process of cell cycle/proliferation control in Supplementary Table 1 are linked together as revealed by the relevant network analysis (Fig. 2). These alternative analyses strengthen the conclusion derived from the GO annotation that the cell cycle/proliferation control is one of major biological processes affected by the sim treatment.

Validation of Microarray Experiments by Semiquantitative RT-PCR and Western Blotting

To validate microarray experiment by alternative approaches, we employed semiquantitative RT-PCR and Western blotting to determine the level of inte-

grin β B4 and CDC25B. Both RT-PCR and Western blotting on the analysis of integrin β B4 expression at its mRNA (Fig. 3a) and protein level (Fig. 3b) at the 24-h time point confirm the corresponding microarray result: Sim increases the expression of integrin β 4. A representative gel pattern in a time-dependent increase in CDC25B mRNA levels by RT-PCR and CDC25B protein levels by Western blotting is demonstrated in Figure 4a and Figure 4b, respectively. Housekeeping genes RPS18 and β -actin showed no change in their expression as internal controls. These results corroborated the microarray findings.

Inhibition of [3 H]Thymidine Incorporation Into HCAEC by the Sim Treatment

Because the analyses by several computer programs indicate that the cell cycle/proliferation control is one of major biological processes affected by the

TABLE 2
DIFFERENTIALLY EXPRESSED GENES IN CELL CYCLE/PROLIFERATION CONTROL
(≥TWOFOLD OR ≤TWOFOLD) TREATED WITH SIMVASTATIN FOR 0–48 h

Gene Title	Gene Symbol	Gene Accession	3 h	6 h	12 h	24 h	48 h
Histone deacetylase 5	HDAC5	AF039241	1	1.15	1.41	2.07	2.3
Kinetochore associated 2	KNTC2	AF017790	1.07	1.07	-1.08	-2	-4
MAD2 mitotic arrest deficient-like 1 (yeast)	MAD2L1	AJ000186	-1.41	-1.32	-2	-2.63	-5.26
Exostoses (multiple) 1	EXT1	S79639	-1.08	-1.19	-1.56	-2	-2.44
Ataxia telangiectasia mutated	ATM	U26455	-1.19	-1.11	-1.23	-2.3	-1.37
Non-metastatic cells 1, protein (NM23A)	NME1	X17620	-1.28	-1.08	-1.75	-3.45	-4
ZW10 interactor	ZWINT	AF067656	-1.11	-1.11	-1.37	-2.13	-5
Proliferating cell nuclear antigen	PCNA	M15796	-1.11	-1.08	-1.37	-2.08	-2.56
Cyclin B1	CCNB1	M25753	-1.03	-1.08	-1.15	-2.56	-6.25
Cell division cycle 25B	CDC25B	S78187	-1.11	-1.07	2	-2.38	-2.46
Dual specificity phosphatase 4	DUSP4	U48807	-1.23	-1.07	-1.74	-2.38	-2.83
AXL receptor tyrosine kinase	AXL	M76125	-1	-1.19	-1.32	-2.56	-5.26
Endothelial cell-specific molecule 1	ESM1	X89426	-1.11	-1.23	-2.44	-3.7	-3.13
Cysteine-rich, angiogenic inducer, 61	CYR61	Y11307	-1.41	-1.23	-2.08	-3.85	-3.45
Insulin-like growth factor binding protein 6	IGFBP6	M62402	-1.23	-1.07	-1.8	-2.14	-1.15
CDC28 protein kinase regulatory subunit 2	CKS2	X54942	-1.15	-1.11	-1.37	-2.08	-2.56
DIRAS family, GTP-binding RAS-like 3	DIRAS3	U96750	1.04	-1.08	-1.85	-2.56	-2.44
Cyclin-dependent kinase inhibitor 3	CDKN3	L25876	-1.41	-1.37	-1.41	-2.08	-4.35

Figure 1. K-mean clustering of temporal gene expression profile of 115 genes in HCAEC treated with simvastatin. K-mean clustering analysis was performed using MV4 program. One hundred and fifteen genes were clustered into 10 different clusters based on Euclidean distance matrix with 50 maximum iterations. The x-axis indicates time points (3, 6, 12, 24, 48 h) after Sim treatment. The y-axis indicates fold changes relative to baseline (0 h) as 1. The value in each time point represents the mean value of two samples. Red color represents gene upregulation and green color gene downregulation. The variation in color intensity corresponds to the difference in the gene expression level. The corresponding gene names and their temporal expression levels of each cluster are presented in Supplemental Table 1 (Yegeneexpression, Supplementary Table 1, available from: <http://web.missouri.edu/~yes>).

Sim treatment and point to the inhibitory effect on the cell growth by the Sim treatment, we determined whether Sim could inhibit [3 H]thymidine incorporation into HCAEC. As demonstrated in Figure 5, the Sim treatment significantly inhibited [3 H]thymidine incorporation into HCAEC by 25% at the 24-h time point and 75% at the 48-h time point. This evidence supports that indeed the Sim treatment inhibited the growth of HCAEC.

DISCUSSION

Temporal gene expression profiling of HCAEC treated with Sim using the Affymetrix oligonucleotide array revealed that expressions of many genes were either induced or suppressed by the treatment of Sim, a cholesterol-lowering drug with an ever-

growing list of pleiotropic effects unrelated to its direct lipid-lowering function. This study not only confirms previous reports of some known genes affected by statins but also find a number of new targets of statins, which are not documented in the published literature.

Examples of known genes affected by statin treatment include increased expression in nitric oxide synthase 3 (NOS3) and decreased expression in Rho family GTPase 3 (RND3). They both demonstrated time-dependent changes after the Sim treatment. Increase in NOS3 expression could promote the production of nitric oxide, which is an endothelial vasodilation factor (36). Rho is a key signal transducer in the inflammatory response (39). Decreased expression in Rho may in part contribute to the inhibition of inflammatory response by the Sim treatment.

Figure 2. Relevance network of differentially expressed cell cycle/proliferation control genes in HCAEC after Sim treatment. Relevance networks were constructed from the list of 18 cell cycle/proliferation control genes in Table 2. The network containing the 11 genes is presented. Each gene is represented by an oval with the corresponding gene symbol. Genes connected by red lines show positive relationship; green lines show negative relationship. The corresponding full gene names for each gene symbol are CDC28 protein kinase regulatory subunit 2 (CKS2), proliferating cell nuclear antigen (PCNA), non-metastatic cells 1, protein (NME1), histone deacetylase 5 (HDAC5), kinetochore associated 2 (KNTC2), dual specificity phosphatase 4 (DUSP4), exostoses 1 (EXT1), MAD2 mitotic arrest deficient-like 1 (MAD2L1), cyclin B1 (CCNB1), cyclin-dependent kinase inhibitor 3 (CDKN3), and ZW10 interactor (ZWINT). Their temporal expression levels can be found in Table 2.

Figure 3. Validation of ITGB4 expression by RT-PCR and Western blotting analyses. (a) RT-PCR analysis. Total RNAs (1 µg) of HCAEC from time point 0 and 24 h after Sim treatment were reverse transcribed. One tenth (2 µl) of the first-strand cDNA products (20 µl) was subjected to a semiquantitative duplex PCR amplification using CDC25B and RSP gene-specific primers. One fifth (10 µl) of the PCR products (50 µl) was separated on 2% agarose gel electrophoresis and visualized by 0.5 µg/ml ethidium bromide staining. A representative gel pattern of three separate experiments is presented. (b) Western blotting analysis. HCAEC were treated with Sim for 0 or 24 h. Total cell lysate protein (10 µg) of each sample was separated by 10% SDS-PAGE and immunodetected by Western blotting using the anti-human ITGB4 antibody. M.M., molecular marker

Figure 4. Validation of CDC25B expression by RT-PCR and Western blotting analyses. (a) RT-PCR analysis. Total RNAs (1 μ g) of HCAEC from each time point after Sim treatment were reverse transcribed. One tenth (2 μ l) of the first-strand cDNA products (20 μ l) was subjected to a semiquantitative duplex PCR amplification using CDC25B and RSP gene-specific primers. One fifth (10 μ l) of the PCR products (50 μ l) was separated on 2% agarose gel electrophoresis and visualized by 0.5 μ g/ml ethidium bromide staining. A representative gel pattern of three separate experiments is presented. (b) Western blotting analysis. HCAEC were treated with Sim for different time durations as indicated. Total cell lysate protein (10 μ g) of each sample was separated by 10% SDS-PAGE and immunodetected by Western blotting using a rabbit anti-human CDC25B(H-85) polyclonal antibody.

Many lines of evidence ranging from the in vitro cell culture experiments to epidemiologic studies and clinical investigations demonstrate that atherosclerosis is intrinsically an inflammatory disease (25). Adhesion of circulating leukocytes to the endothelium is one of the earliest steps in atherogenesis (20). The entry of inflammatory cells into the arterial wall depends on the interaction between adhesion/inflammatory molecules on the surface of endothelial cells and their counterligand on the leukocytes. It is still not completely understood how many genes are involved and what the molecular mechanisms are in this process.

It has been reported that this inflammatory/cell adhesion process is affected by the Sim treatment; however, present high-throughput gene expression profiling expands the repertoire of genes in the process affected by the Sim treatment. At least 6 out of 16 genes classified in inflammatory/cell adhesion process (KIAA0527 protein, neuroligin, pentraxin-related gene, chemokine C-X-C motif ligand 1, and interleukin 32) represents newly discovered targets by the high-throughput approach. Expression in 12 out of 16 genes (66%) in the process was inhibited by Sim,

Figure 5. [³H]Thymidine incorporation into DNA of HCAEC treated with Sim. The x-axis indicates time after Sim (5 μ M) treatment. The y-axis indicates the percentage of [³H]thymidine incorporation into DNA of HCAEC based on the 0-h time point as 100%.

indicating that the overall effect of Sim on the inflammatory/cell adhesion process is inhibitory.

PathwayAssist™ analysis (data not shown) indicates that IL-6 gene is the main hub in a network interconnected with 14 genes mostly involved in the inflammatory/cell adhesion process. IL-6 directly linked to 7 out of 14 genes in the network. Increased expression in the IL-6 gene was reported to be associated with the pathogenesis of atherosclerosis (2,31). It directly affects expression of three known inflammatory response genes: IL-8, CD44, and ILR1. IL-8 is known to be a risk factor to atherogenesis (26). Increased expression of CD44 was observed in the atherogenic plaque, correlated with the severity of atherosclerosis (8,28). ILR1 is a well-known inflammatory response gene (29). IL-8 is also directly linked to a newly identified cell adhesion/inflammatory gene, chemokine C-X-C motif ligand 1, which is induced on activated primary endothelial cells and it may constitute part of the molecular control of leukocyte traffic at the endothelium (5). In addition, IL-8 is also directly linked to caspase-8 and FADD-like apoptosis regulator, tuberose sclerosis 2, and CCAAT/enhancer binding protein δ .

Caspase-8 and FADD-like apoptosis regulator appear to be endogenous modulators of apoptosis sensitivity in mammalian cells, including the susceptibility of cardiac myocytes to apoptotic death following ischemia/ reperfusion injury (24). Tuberose sclerosis 2 is involved in the regulation of cell mobility and cell adhesion, whose dysfunction is associated with the pathobiology of tuberose sclerosis complex and lymphangioleiomyomatosis (12). CCAAT/enhancer binding protein δ is an important transcriptional activator in regulation of the genes involved in the immune and inflammatory responses (4). It remains to be elucidated how these linkages are relevant to the inflammatory response or other processes.

Over 1,000 genes were differentially expressed after the Sim treatment. There were 115 genes that were either upregulated by $\geq$ twofold or downregulated by $\leq$ twofold at the 24-h time point of the Sim treatment. Among them, 25 genes (23%) fall under the unknown category of biological processes (Yegene-expression, Supplementary Table 1, available from: <http://web.missouri.edu/~yes>). These will provide ample reagents for us to explore the molecular mechanisms of Sim's pleiotropic effects. One unexpected observation is that 18 genes (16%) in the cell cycle/proliferation control process, classified according to the current gene ontology annotation, top the list among the biological processes affected by Sim. A relevant network analysis demonstrates that 11 out of 18 genes in the cell cycle/proliferation control process links into two subnetworks.

Alternative approaches such as RT-PCR and Western blotting validated the microarray results for the selected genes (ITGB4 and CDC25B). Expressions in 13 out of 18 genes ($>70\%$) in the cell cycle/proliferation control process were significantly inhibited after the Sim treatment in a time-dependent manner, suggesting that the overall effect of Sim on the cell cycle/proliferation control process is inhibitory. Figure 5 demonstrates that the Sim treatment significantly inhibited [^3H]thymidine incorporation into HCAEC by 25% at the 24-h time point and 75% at the 48-h time point, supporting that Sim inhibited the growth of HCAEC. This result may provide new reagents for us to explore the molecular mechanisms underlying the inhibitory effect of Sim on the neointima formation. Schwartz et al. (30) showed that the severity of vessel injury strongly correlates with neointimal thickness and percent diameter stenosis. Although the implantation of stents can prevent vessels from the elastic recoil after percutaneous transluminal coronary angioplasty (PTCA) and appears to limit adverse remodeling, the problem of restenosis and consecutive need for reinterventions remain the major limitation of long-term success of PTCA (4,14,15,30). In a regress trial, Mulder et al. (22) found that pravastatin reduces 2-year clinical and angiographic restenosis. Herdeg et al. (15) reported that atorvastatin treatment reduced in-stent stenosis in both normal and hypercholesterolemic rabbits. Porter et al. (23) observed that simvastatin inhibited the neointima formation in human saphenous vein bypass grafts.

A key event in atherogenesis is the migration and proliferation of arterial smooth muscle cell in the arterial wall (23,25). The inhibitory effect of statins on smooth muscle cell proliferation has been recently shown in different models of proliferation cells such as cultured arterial myocytes and rapidly proliferating carotid and femoral intimal lesions in rabbits (11,32). However, molecular mechanisms underlying statins' inhibitory effect on the proliferation of different cell types in vessel wall are still largely unknown. Statins may attenuate the pathological stimulation or inhibition of endothelial cells by controlling those involved genes at the mRNA levels. This study could provide potential new leads of Sim's direct targets in the pathogenesis of atherosclerosis.

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