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Different statins produce highly divergent changes in gene expression profiles of human hepatoma cells: a pilot study

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Statins are inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR), the key enzyme of the sterol biosynthesis pathway. Statin therapy is commonly regarded as well tolerated. However, serious adverse effects have also been reported, especially during high-dose statin therapy. The aim of our study was to investigate the effect of statins on gene expression profiles in human hepatoma HepG2 cells using Affymetrix Human Genome U133 Plus 2.0 arrays. Expression of 102, 857 and 1091 genes was changed substantially in HepG2 cells treated with simvastatin, fluvastatin and atorvastatin, respectively. Pathway and gene ontology analysis showed that many of the genes with changed expression levels were involved in a broad range of metabolic processes. The presented data clearly indicate substantial differences between the tested statins.

Keywords: gene expression, human hepatoma cells, microarrays, statins

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INTRODUCTION

An elevated level of plasma LDL-cholesterol is a widely recognized risk factor for coronary heart disease. Statins, by competitive inhibition of the HMG-CoA reductase (HMGR) reduce endogenous cholesterol production, increase the number of low-density lipoprotein (LDL) receptors and thereby lower the serum cholesterol level by as much as 50% (Mangravite *et al.*, 2006). The decrease in cholesterol levels is the primary cause of the therapeutic benefits of statins in cardiovascular disease. Apart from cardiovascular improvement, statins appear to exert other beneficial effects, such as reduction of the risk of dementia, Alzheimer disease, ischemic stroke, rheumatologic diseases (McCarey *et al.*, 2004), osteoporosis, and possibly breast cancer (Liao, 2002).

The overall clinical benefits associated with statin therapy, however, appear to be greater than what might be expected from changes in the lipid profile alone, suggesting that some beneficial effects of statins are independent of LDL cholesterol reduction (Liao, 2002). Lately, these non-lipid, or so-called pleiotropic effects of statins have been thoroughly investigated due to their potential impact on various aspects of cardiovascular disease. These include: improved endothelial function, atherosclerotic plaque stabilization, anti-inflammatory, immunomodulatory and antithrombotic effects, as well as induction of apoptosis and anti-proliferative properties (Buhaescu & Izzedine, 2007).

This complex characteristic of the HMG-CoA reductase inhibitors is further compounded by the disparities in pharmacokinetics, therapeutic efficacy and potency of particular compounds. Statins differ in terms of lipophilicity, which determines the rate and efficacy of their absorption, metabolism and excretion, as well as such properties as diffusion through the blood-brain barrier or circulation half-life. Comparative studies have reported significant differences in the reduction rate of LDL-cholesterol, triglycerides (Wierzbicki *et al.,* 1999; Olsson *et al.*, 2003) and phospholipid levels (Bergheanu *et al.*, 2008; Leszczynska *et al.*, 2009), as well as of paraoxonase-1 activity (Bergheanu *et al.*, 2007). Moreover, the risk of side effects is also different for each HMG-CoA reductase inhibitor (Wierzbicki *et al.*, 1999).

The HepG2 cell line is widely used as an *in vitro* model to evaluate potential effects of extrahepatic factors in the plasma of patients on hepatic metabolism (Pandak *et al.*, 1996; Van Greevenbroek *et al.*, 2002). Numerous studies investigating the effects of statin therapy used HepG2 cells as a model of human hepatocytes (Gerber *et al.*, 2004; Maeda *et al.*, 2010; Mullen *et al.*, 2010).

Here we present preliminary results of a comparative study of the effects of different HMGR inhibitors on gene expression profiles in human hepatoma HepG2 cells. The statins chosen for our research were of high clinical importance and both of natural (simvastatin) and synthetic (atorvastatin, fluvastatin) origin (Furberg, 1999). We used a high concentration of statins (100 μM) in order to facilitate identification of processes related to toxic effects as well as pleiotropic actions of statin therapy. Our data clearly indicate significant differences among the tested statins.

MATERIALS AND METHODS

Cell culture and treatment. Human hepatoma HepG2 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 100 IU/

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Abbreviations: ACAT2, acetoacetyl-CoA transferase; CVD, cardiovascular disease; HepG2, human hepatoma-derived cell line; HMGCS, HMG-CoA synthase; HMGR, 3-hydroxy-3-methylglutaryl-CoA reductase; LDL, low-density lipoprotein; LSS, lanosterol synthase; qRT-PCR, real time quantitative reverse transcription PCR; SQLE, squalene epoxidase.

ml penicillin and streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. When cells reached 70–75% confluency, the medium was replaced with fresh medium containing 100 μM atorvastatin, fluvastatin or simvastatin, or buffer alone as a control. The cells were harvested by trypsin treatment after 48 hours of statin exposure. The experiment was carried out in three replications.

Simvastatin was extracted from Zocor® tablets (MERCK & CO, INC, Whitehouse Station, NJ, USA) by dissolving in pH 7.0 buffer solution containing 0.5% sodium dodecyl sulfate (SDS) in 0.01 M sodium phosphate according to The United States Pharmacopoeia USP 26. Atorvastatin and fluvastatin were extracted as above from Atorvox® (Pliva Krakow, Zakłady Farmaceutyczne S.A.) and Lescol® (Novartis Pharma GmbH) tablets, respectively. Statins from the 10 mg/ml stock solutions were added to the media to a final concentration of 100 μM.

RNA isolation. Total RNA was isolated from cells using the RNeasy Mini Kit (QIAGEN, Germany) according to the manufacturer's recommendations and was eluted in RNase-free water. RNA samples were quantified by UV absorption (Nanodrop, LabTech International, UK). RNA quality was checked with the RNA 600 Nano Assay Kit using Bioanalyzer© in accordance with the manufacturer's procedures (Agilent, Santa Clara, CA, USA). Samples with an RNA integrity number of eight or above were considered suitable for use in microarrays.

cDNA microarrays. Total RNA (2 μg) was processed according to standard Affymetrix protocols (GeneChip One-Cycle Target Labelling and Control Reagents kit, Affymetrix, Santa Clara, CA, USA). Reverse transcription and second strand cDNA synthesis were followed by *in vitro* transcription, biotinylation and fragmentation. Samples were hybridized to the Human Genome U133 2.0 arrays for 16 h at 45 °C. Following hybridization, the probe arrays were washed and stained on a fluidics station and immediately scanned on an Affymetrix GCS 3000 GeneArray Scanner.

Data analysis of microarrays. Quality controls were performed using Microarray Suite 5.0 software provided by Affymetrix (www.affymetrix.com) according to the manufacturer's recommendations. Affymetrix raw gene array data were processed using the Partek Genomics Suite software (Partek Inc., St. Louis, MO, USA). Statistical significance of the results was estimated by variance analysis (ANOVA). Lists of differentially expressed genes were generated using a false discovery rate of FDR ≤ 0.01 and a fold change of ≥ 2 as significance criteria.

A bioinformatics approach was used to determine the biological context of the gene expression data generated by the microarray screen. Probes lacking gene names, designated as cDNA and those described as hypothetical were removed from the lists. Lists of genes showing significant differences in expression levels between experiments were submitted to Ingenuity Pathway Analysis (Ingenuity® Systems, www.ingenuity.com). Gene lists from each group were analyzed for functional categories. Genes were manually verified by searching the freely available databases KEGG and UniProt.

Determination of mRNA levels. qRT-PCR was used to confirm the effects of statins on expression of selected genes from the mevalonate pathway. Total RNA used for microarray analysis was also used to synthesize cDNA using the QuantiTect Reverse Transcription Kit (QIAGEN, Germany), according to the manufacturer's recommendations.

qPCR amplification was performed using a LightCycler 1.5 and LightCycler FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions. The relative expression software tool REST-MCS © — version 2 (Pfaffl, 2002) was used to estimate the relative changes in mRNA levels of the selected genes in the cultured cells treated with statins (compared with cultured cells treated with buffer). Data normalization was carried out against the transcript of the gene for TUBB (tubulin). The sequences of all primers and the qPCR amplification parameters are available upon request.

RESULTS AND DISCUSSION

Conversion of HMG-CoA to mevalonate is an early step in cholesterol biosynthesis. Mevalonate is also a

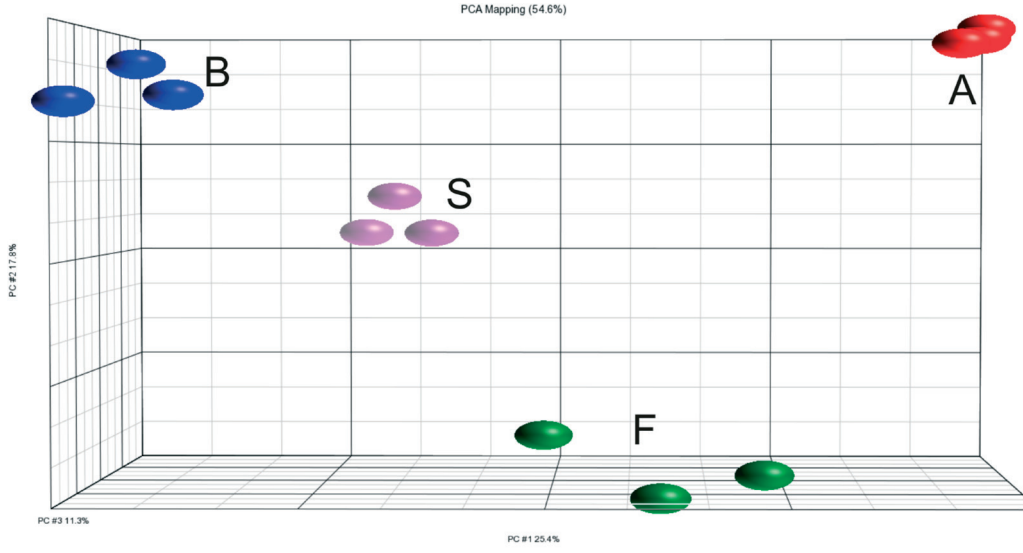

Figure 1. Principal component analysis of gene expression profiles in HepG2 cells treated with buffer (B), atorvastatin (A), fluvastatin (F) and simvastatin (S).
The intensity of whole human genome data was used.

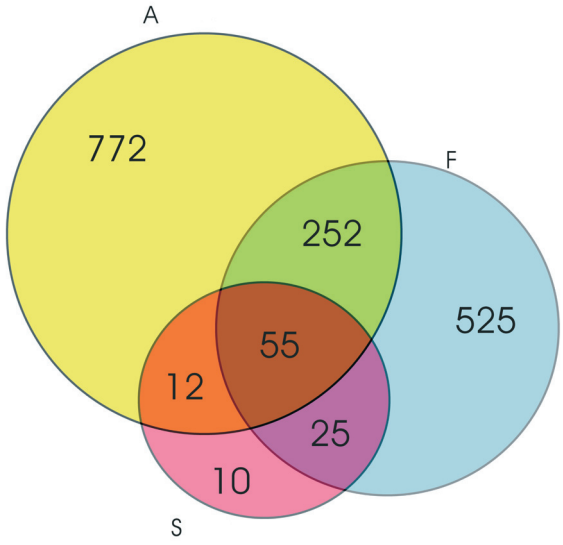

Figure 2. Venn diagram depicting changes in gene expression levels after atorvastatin (A), fluvastatin (F) or simvastatin (S) treatment.

precursor for biologically important nonsteroidal isoprenoids, for example: dolichol, ubiquinone, isopentenyl tRNA and prenylated proteins, which have an important role in the regulation of cellular processes. Therefore, it is postulated that the side effects of some statins may be due to the fact that statins suppress all post mevalonate biosynthesis steps including non-steroidal isoprenoids (Charlton-Meys & Durrington, 2007).

Numerous large-scale clinical trials have shown that statin therapy reduces the risk of cardiovascular events by 20–30% — an effect strongly related to the magnitude of LDL cholesterol (LDLC) reduction. Moreover, a well documented capacity for decreasing cardiovascular morbidity and mortality in both primary (Prosser *et al.*, 2000; Sever *et al.*, 2003) and secondary (Shepherd *et al.*, 2002; Heart Protection Study Collaborative Group, 2002) prevention settings has been demonstrated for statins. On the basis of these properties, statin treatment in conjunction with lifestyle changes is indicated as first-line therapy for the prevention of CVD in individuals who are considered at risk. Adoption of the current guidelines for plasma LDL reduction has led to the widespread and increasing use of statins, which are now the most often prescribed class of drugs worldwide (Mangravite *et al.*, 2006; Chopra *et al.*, 2007).

Statin therapy is commonly assumed to be well tolerated, but serious adverse effects have been reported. These include headaches (Ditschunheit *et al.*, 1991), transient global amnesia (Healy *et al.*, 2009) and myopathy possibly leading to rhabdomyolysis and death (Hodel, 2002). Recently, peripheral neuropathy has also been identified as a possible complication (Ahn, 2008).

To determine what changes in gene expression profiles occur in human hepatoma cells treated with atorvastatin, fluvastatin or simvastatin, we performed microarray analyses. Global transcriptomic profiles were analyzed by Principal Component Analysis (PCA plot) (Fig. 1) which demonstrated a clear separation between control and cells treated with particular statins.

The results obtained after filtering away unknown genes reveal considerable differences in gene expression among cells treated with the three statins. The number of genes whose expression changed upon treatment was

Table 1. Functional categories represented by genes altered by statin treatment.
Ingenuity Pathway Analysis software (Ingenuity Systems) was used to identify the number of genes significantly associated with molecular and cellular functions in the Ingenuity database. Functional categories with p values <0.01 are shown.

Category	A	F	S
Lipid Metabolism	123	16	20
Carbohydrate Metabolism	23	30	9
Nucleic Acid Metabolism	12	28	4
Vitamin and Mineral Metabolism	42	0	9
Small Molecule Biochemistry	144	37	25
Molecular Transport	59	11	16
Gene Expression	167	130	8
DNA Replication, Recombination, and Repair	77	138	12
Cell Cycle	132	135	23
Cell Death	272	205	38
Cell Morphology	126	42	15
Cell Signaling	42	23	8
Cell-To-Cell Signaling and Interaction	105	12	18
Cellular Assembly and Organization	66	127	24
Cellular Compromise	12	12	6
Cellular Development	208	121	25
Cellular Function and Maintenance	39	37	17
Cellular Growth and Proliferation	250	226	35
Cellular Movement	155	113	25
Other/Unknown	413	300	22
Total	1091	857	102

A, atorvastatin; F, fluvastatin; S, simvastatin. Note, total number of altered genes is smaller than the sum, as several genes have been classified into more than one category.

1091 for atorvastatin, 857 for fluvastatin, and 102 for simvastatin, corresponding to a total of 1651 differentially expressed genes. Among them, 466, 363 and 47 were upregulated after atorvastatin, fluvastatin and simvastatin treatment, respectively. To classify the 1651 genes whose expression change was unique or common in the comparison, a Venn diagram (Fig. 2) was used. Only 10 of the induced genes were specific to simvastatin treatment in comparison with 772 and 525 for atorvastatin and fluvastatin, respectively. As shown in Fig. 2, the expression of 55 genes was changed in response to all tested statins.

The affected genes were grouped into functional categories according to the Gene Ontology (GO) annotation. The numbers of altered genes according to their molecular or cellular function are shown in Table 1 for each of the statins tested using IPA software. The general tendency was that atorvastatin was the most effective (the highest number of genes changed) in contrast to simvastatin (the weakest impact on gene expression).

We observed that statin treatment (particularly atorvastatin and fluvastatin) induced changes in the expression of numerous genes. In our study a substantial proportion of genes modulated by three statins tested are involved in cell death, cell growth and proliferation as well as cell signaling. Similar results were obtained in an analysis of gene expression in C_2C_{12} myotubes following simvastatin application (Yu *et al.*, 2009). In that study, a

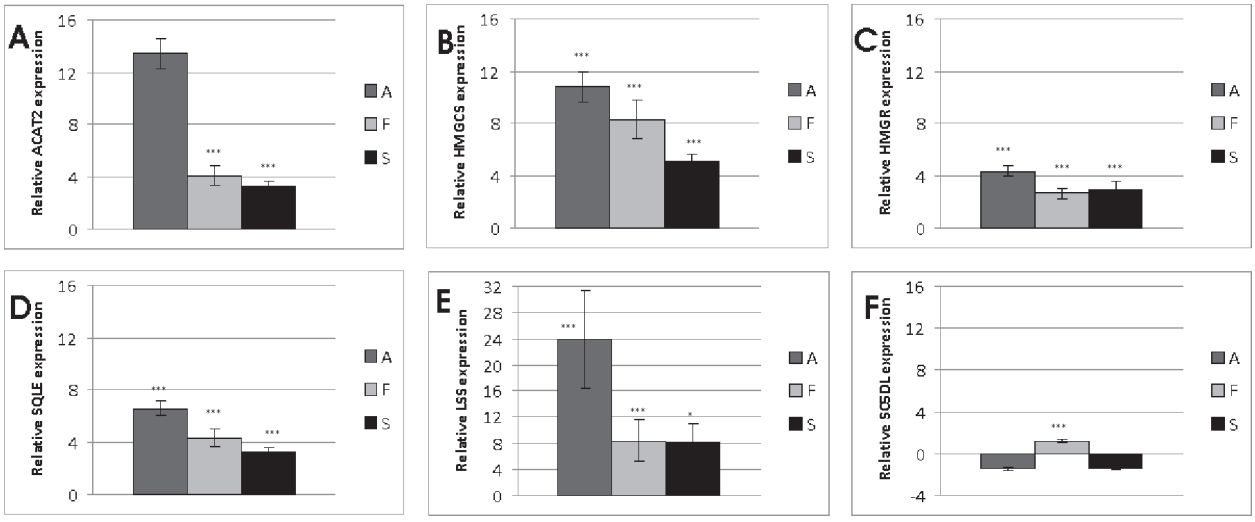

Figure 3. Quantitative real-time RT-PCR analysis of mevalonate pathway transcripts.
A, acetoacetyl-CoA thiolase, ACAT2; B, HMG-CoA synthase, HMGCS; C, HMG-CoA reductase, HMGCR; D, squalene epoxidase, SQLE; E, lanosterol synthase, LSS; F, sterol-c5-desaturase, SC5DL. *$p < 0.05$, ***$p < 0.001$.

high percentage of genes differentially expressed in response to statin treatment were associated with apoptosis, cell growth/maintenance and signal transduction.

Relative mRNA quantification of five genes from the mevalonate pathway (acetoacetyl-CoA transferase, ACAT2; HMG-CoA synthase, HMGCS; HMG-CoA reductase, HMGR; squalene epoxidase, SQLE, lanosterol synthase, LSS) revealed increased mRNA levels for all those genes in cultured cells treated with statins when compared to the cells treated with buffer (Fig. 3). Overall, the most prominent changes were found in cells cultured with atorvastatin, while the changes in mRNA expression induced by simvastatin were significantly lower. However, the expression of the sterol-c5-desaturase (SC5DL) gene did not change under statin treatment. The results of microarray analyses were generally in agreement with qRT-PCR data.

As statins are mainly prescribed to hypercholesterolemic patients to normalize the levels of serum lipoproteins, we have focused on the genes related to cholesterol biosynthesis. Both microarray and real-time PCR analysis revealed increased expression of genes involved in the mevalonate pathway. These results indicate that HMGR inhibitors influence the expression of practically all enzymes of the mevalonate pathway and this effect depends on the type of statin administered. Similar results were observed in human skeletal muscle-like cells (Morikawa *et al.*, 2005) — statins increased the expression of numerous cholesterol synthesis-related genes. This is in accordance with the results of Gerber *et al.* (2004), Hagemenas and Illingworth (1989) and ours (Leszczynska *et al.*, 2009) proving that inhibition of HMGR activity by statin treatment stimulates an adaptive response in the cell, including upregulation of genes involved in cholesterol biosynthesis, a compensatory increase in HMGR activity and partial reduction of sterol-lowering effect, all of which contribute to maintaining cholesterol homeostasis.

The results of our study demonstrate that the statins tested, although classified to the same therapeutic group, differ strikingly with regard to the range of genes affected as well as the potency of regulatory action on the levels of particular transcripts. Presumably, the widespread effects on genes involved in a broad range of cellular processes may play an important role in the pleiotropic actions of statins observed both *in vitro* and *in vivo*. Moreover, such diverse effects on gene transcription may be of special importance in clinical practice, as the statins are prescribed to patients of various health condition and different settings of cardiovascular diseases.

In conclusion, of the three statins tested, simvastatin was by far the least disruptive both in respect to the number of genes affected and the magnitude of their response. Genes involved in multiple cellular processes were affected by statins, which confirms that statins may cause pleiotropic effects. Further studies are needed to investigate the effects of high dose statin treatment.

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