Simvastatin inhibits cell growth and induces apoptosis and G0/G1 cell cycle arrest in hepatic cancer cells

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Abstract. Hepatocellular carcinoma (HCC) is one of major health concerns worldwide and one of leading causes of cancer death after lung and gastric cancers. Simvastatin is a cholesterol-lowering drug which inhibits 3-hydroxy-3-methylglutarylcoenzyme CoA (HMG-CoA) reductase. Simvastatin exhibits numerous pleiotropic effects including anti-cancer activity. Yet, the anticancer effects in HCC remain poorly characterized. Therefore, in this study, we investigated the effects of simvastatin on tumor cell growth, apoptosis and cell cycle. HepG2 and Huh7 cell lines were treated with simvastatin (32 and 64 μM) for different time periods. Tumor cell growth was assessed using MTT assay. Apoptosis and cell cycle analysis were also evaluated. Analysis of cell cycle proteins involved in simvastatin-induced manipulation was performed by Western blot and quantitative RT-PCR analyses. Simvastatin induced a reduction of tumor cell growth. In both cell lines, simvastatin induced apoptosis and impaired cell cycle progression as depicted by the greater rates of G0/G1-phase cells than the rates of S-phase cells. Protein expression levels of cell cycle regulating proteins CDK1, CDK2, CDK4, cyclin D1, cyclin E, p19 and p27 were markedly altered by simvastatin. Moreover, *CDC2, CCND1* and *CDCN2D* mRNA expressions were also altered by drug treatment. Collectively, these results suggest that simvastatin induces apoptosis in tumor cells and its anti-proliferative activity was accompanied by inhibition of cyclin-dependent kinases and cyclins, whereas CDK inhibitors p19 and p27 were enhanced. These results may provide novel insights into simvastatin tumor-suppressive action.

Introduction

Hepatocellular carcinoma (HCC), one of most common malignancies and causes of cancer death worldwide, is

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characterized by a poor prognosis and recurrence within a short time (1-5). HCC is frequently derived from chronic liver diseases, such as alcoholic liver disease or viral hepatitis, with increasing incidence in Europe, the United States and in certain areas in Asia (6,7). Radical therapies, such as resection, liver transplantation or percutaneous ablation are applicable only for early or small HCC. At an advanced stage, in selected patients transarterial chemoembolization (TACE) is possible, but systemic chemotherapy often remains the only alternative therapy for the majority of patients, despite its modest anti-tumor capacity against HCC (8-11). Therefore, there is a need for alternative strategies focusing on HCC.

Simvastatin, one of the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors, is currently widely used safe and well tolerated therapeutic agent for treating hypercholesterolemia, atherosclerosis, coronary heart disease and stroke (12). Emerging research suggests that simvastatin exerts despite of its effective cholesterol lowering role, antiproliferative, proapoptotic and anti-invasive effects in cancer cells (13,14). In several human malignancies, including breast, colon, prostate cancer and melanoma, simvastatin had selective anticancer effects, which were attributed to cell cycle arrest in the G1 and G2/M phases, thereby inhibiting cell proliferation as well as apoptotic and necrotic cell death induction (14-18). However, the efficacy and the molecular mechanism of simvastatin on HCC progression have yet to be clarified.

Given the need for alternative therapeutic strategies, these findings in parallel with limited knowledge about simvastatin's influence on HCC prompted us to evaluate its potential therapeutic options for HCC. The objective of this study was to determine i) whether simvastatin exerts anticancer effects in HepG2 and Huh7 and ii) the mechanism by which simvastatin exerts these effects. We evaluated reduced tumor cell growth and enhanced tumor cell death of both, apoptotic and necrotic cells after simvastatin treatment. Cell cycle analyses by Western blot and qRT-PCT revealed simvastatin-induced cell cycle arrest in G0/G1 and the cell cycle regulating proteins involved in these modifications.

Materials and methods

Cell cultures. Human HCC cell lines HepG2 and Huh7 were purchased from Cell Line Services (Heidelberg, Germany). Tumor cells were maintained at 37°C under 5% CO_2 in

RPMI-1640 medium (Seromed, Berlin, Germany) supplemented with 10% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin (Gibco, Karlsruhe, Germany) and 20 mM HEPES buffer (Sigma, Steinheim, Germany).

Drug treatment. Simvastatin was obtained from Calbiochem (Darmstadt, Germany) and activated prior to the experiments by alkaline hydrolysis of the lactone moiety according to the manufacturer's protocol. Tumor cells were treated 24, 48 and 72 h with simvastatin (32 and 64 μM) or with vehicle (ctrl) with fresh changes of culture medium and simvastatin after 48 h.

Tumor cell growth. Cell proliferation kit I (3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide, MTT; Roche Diagnostics, Penzberg, Germany) was used to evaluate MTT-reducing activity of the cellular mitochondria. HepG2 and Huh7 cells (100 μl, 1x10^4/ml) were seeded onto 96-well tissue culture plates and incubated with simvastatin or vehicle as described above to monitor time- and dose-response. Twenty-four hours before the evaluation of each time-point, MTT (0.5 mg/ml) was added to each well, and the cells were incubated for 4 h at 37˚C. Thereafter, cells were lysed in a solubilization solution containing 10% sodium dodecyl sulphate (SDS) in 0.01 M hydrogen chloride (HCl) and incubated overnight at 37˚C in 5% CO_2. The following day the absorbance of each well was measured with a multimode microplate reader (Infinite M200; Tecan, Crailsheim, Germany) at 550 nm. Each experiment was performed in triplicate. After substracting the background absorbance, results were expressed as mean cell number.

Measurement of apoptosis. After treatment with simvastatin or vehicle (ctrl), HepG2 and Huh7 cells were incubated with propidium iodide (PI) and Annexin V-conjugated fluorescein isothiocyanate (FITC) from an Annexin V-FITC Apoptosis detection kit 1 (Becton Dickinson) according to manufacturer's instructions. Annexin V-FITC/PI binding was evaluated by flow cytometry with a FACScan flow cytometer (excitation wavelength 488 nm, emission wavelength 530 nm; Becton Dickinson). The population of PI-negative/Annexin V-positive cells was related to early apoptosis, and that of PI-positive/Annexin V-positive cells was related to late apoptosis/secondary necrosis.

Cell cycle analysis. HepG2 and Huh7 cells were grown to 70% confluency and then treated with simvastatin or vehicle (ctrl). Cell cycle analyses were carried out after 48 h. Tumor cells were stained with propidium iodide using a Cycle Test Plus DNA reagent kit (Becton Dickinson) and then subjected to flow cytometry with a FACScan flow cytometer (Becton Dickinson). From each sample 10,000 cells were measured. Data acquisition was carried out using Cell-Quest software and cell cycle distribution calculated using ModFit software (Becton Dickinson). The number of gated cells in G1, G2/M or S-phase was presented as percentage (%). Each experiment was performed in triplicate.

Western blotting. Cell cycle regulating proteins were analyzed in Western blotting. Total cdk1, cdk2, cdk4, cyclin D1, cyclin E, p19 and p27 contents in HepG2 and Huh7 cells were evaluated by Western blot analysis using mouse anti-Cdk1 (clone 1), anti-Cdk2 (clone 55), anti-Cdk4 (clone 97), anti-human Cyclin D1 (clone DCS-6), anti-human Cyclin E (HE12), anti-p19 [Skp1] (clone 52) and anti-p27 [Kip1] (clone G173-524; all BD Biosciences, Heidelberg, Germany). Tumor cell lysates (50 μg protein) were separated by electrophoresis on 12% polyacrylamide SDS gels and transferred to nitrocellulose membranes (Amersham-Buchler, Braunschweig, Germany). Determination of ß-actin with anti-ß-actin antibody (clone AC-15; Sigma, Taufkirchen, Germany) served as loading control. Blots were blocked (10% non-fat dry milk in 1 mM Tris, 150 mM NaCl, pH 7.4) for 1 h, incubated 1 h at RT with primary antibody (diluted according to manufacturer's instructions in blocking buffer with 0.5% Tween-20 and 0.5% bovine serum albumin) and then incubated 1 h with horseradish peroxidase-conjugated secondary antibody (HRP-conjugated goat anti-mouse IgG, Upstate Biotehcnology, Lake Placid, NY, USA) diluted 1:5000 in blocking buffer with 0.5% Tween-20 and 0.5% bovine serum albumin at RT. Proteins were detected with ECL™ Western blot detection reagents (GE Healthcare, Munich, Germany) and visualized after exposure on X-ray film (Hyperfilm™ ECTM, Amersham, Freiburg, Germany). The film was digitized, and the integrated density of individual bands was determined using the software Multianalyst (Bio-Rad, Munich, Germany). By densitometric measurements using the same software the amount of protein expression was normalized to ß-actin.

Ribonucleic acid (RNA) isolation, quantitative reverse-transcription-polymerase chain reaction (RT-PCR). Total RNA was isolated using the RNeasy-system (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The RNA was stored immediately at -80˚C. Quality and amount of the RNA were determined photometrically using the NanoDrop ND-1000 device (NanoDrop Technologies, Wilmington, DE, USA).

RNA was subsequently used for qRT-PCR. In brief, 100 ng of total RNA was reversely transcribed using the Affinity script QPCR-cDNA synthesis kit (Stratagene, La Jolla, CA, USA) following the manufacturer's instructions. To determine the mRNA expression of Cdk1 (*CDC2*), Cyclin D1 (*CCND1*) and p19 (*CDKN2D*), qRT-PCR was carried out on a Stratagene MX3005p QPCR system (Stratagene) using gene-specific primers for human CDC2 (NM 001786, UniGene no. Hs.334562, Cat no. PPH00116B), human CCND1 (NM 053056, UniGene no. Hs.523852, Cat no. PPH00128E) and human CDKN2D (NM 001800, UniGene no. Hs.435051, Cat no. PPH00210B) purchased from SABiosciences (SuperArray, Frederick, MD, USA). As reference gene, the expression of *GAPDH* with human GAPDH (NM 002046, UniGene no. Hs.592355, Cat no. PPH00150E; SABiosciences) was measured. Sequences of these primers are not available. PCR reaction was set up with 1X RT2 SYBR Green/Rox qPCR Master mix (SABiosciences) in a 25 μl volume according to manufacturer's instructions. A two-step amplification protocol consisting of initial denaturation at 95˚C for 10 min followed by 40 cycles with 15 sec denaturation at 95˚C and 60 sec annealing/extension at 60˚C
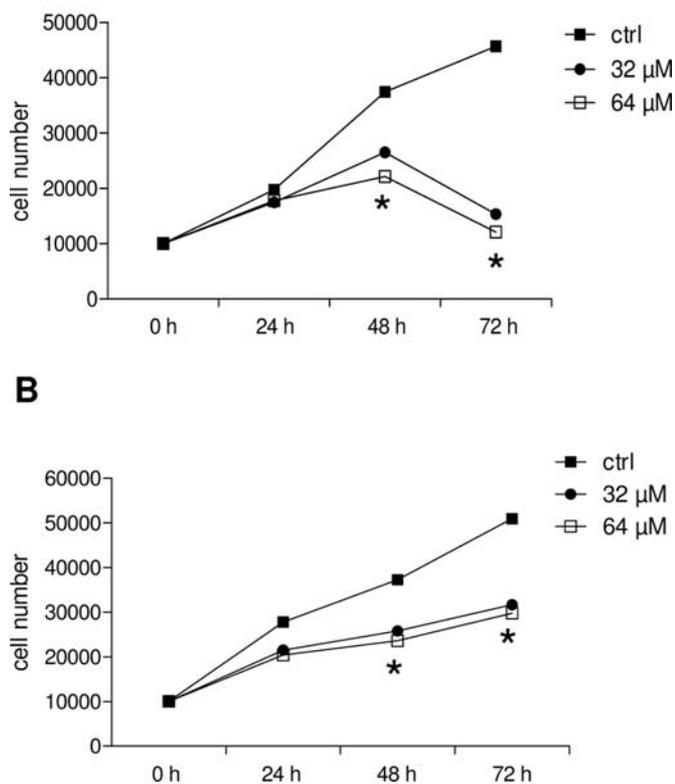
Figure 1. Effects of simvastatin on the cell growth. HepG2 and Huh7 cells were treated with simvastatin (32 and 64 μM) for 24, 48 and 72 h or vehicle (ctrl). Then, cells were placed on a 96-well culture plate and cell proliferation was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay. The assays were carried out in triplicate and data are given as mean ± SEM. Representative figure from one out of three experiments is shown (*$p < 0.05$ vs. ctrl).
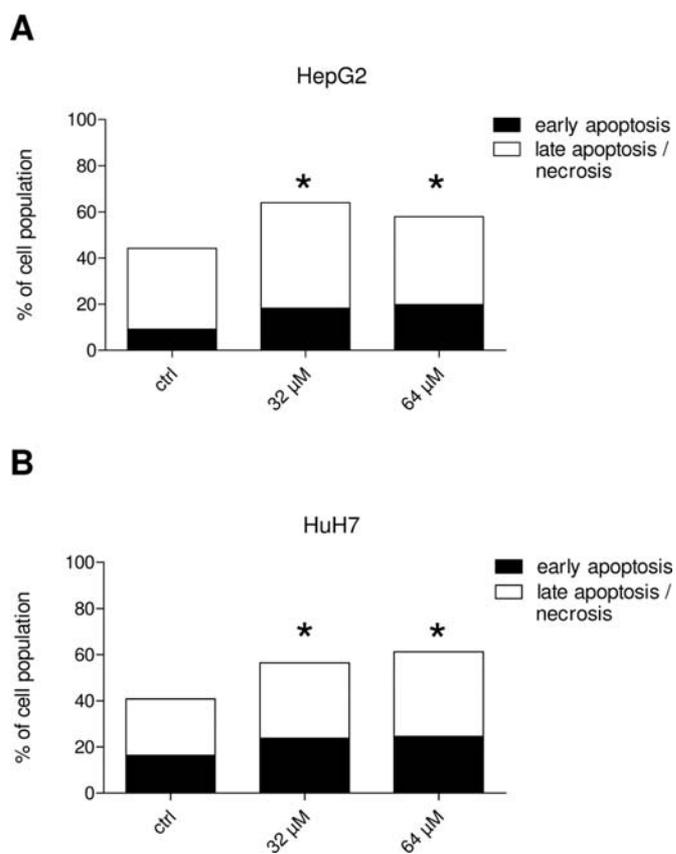

Figure 2. Simvastatin induces cell death by apoptosis in HepG2 and Huh7 cells. The mechanism of cell death in HepG2 and Huh7 cells was determined 48 h after 32 or 64 μM simvastatin exposure by staining cells with Annexin V-FITC and propidium iodide (PI). PI-negative/Annexin V-positive cells were related to early apoptosis, and PI-positive/Annexin V-positive cells were related to late apoptosis/secondary necrosis. The percentage of early apoptotic and late apoptotic/necrotic cells was averaged from three independent experiments (*$p < 0.05$ vs. ctrl).

was chosen. A melting-curve analysis was applied to control the specificity of amplification products. The amount of target mRNA in each sample was normalized to the amount of *GAPDH* mRNA. We conducted three replicates for the arrays and had three replicates of the individual genes in the PCR. The relative mRNA expression of target genes after normalization to *GAPDH* is presented.

Statistical analysis. All experiments were performed 3-6 times. Differences between groups were determined by Wilcoxon-Mann-Whitney U-test. $p < 0.05$ was considered significant. Data are given as mean ± standard error of the mean (SEM).

Results

Simvastatin inhibits tumor cell growth. Tumor cell growth analysis revealed rapid cell growth of both cell lines HepG2 and Huh7. Fig. 1 demonstrates that both doses of simvastatin (32 and 64 μM) had a significant inhibitory effect on tumor cell growth as compared to controls (ctrl, $p < 0.05$). This effect was time-dependent, since a simvastatin pre-treatment for 48 h or 72 h significantly reduced cell growth as compared to 24 h pre-incubation with simvastatin ($p < 0.05$).

Induction of apoptosis in HepG2 and Huh7 cells by simvastatin. To study the effect of simvastatin on cell survival HepG2 and Huh7 cells were treated with 32 or 64 μM for 48 h. Fig. 2 shows cell death rates of treated versus vehicle-treated cells (ctrl). In HepG2 cells, early apoptosis markedly increased by simvastatin treatment from 9.2% in non-treated ctrl cells to 18.2% (32 μM simvastatin) and 19.8% (64 μM), respectively ($p < 0.05$). Late apoptosis/necrosis rates increased from 35.0% in ctrl cells to 56.9% (32 μM simvastatin) and 48.0% (64 μM), respectively ($p < 0.05$). In Huh7 cells, early apoptosis rates increased from 16.3% in non-treated ctrl cells, to 23.8% and 24.5% in simvastatin pre-treated cells (32 μM and 64 μM, respectively). Late apoptosis/necrosis rates increased markedly from 24.7% in ctrl cells to 32.8% (32 μM simvastatin) and 36.8% (64 μM), respectively ($p < 0.05$).

Simvastatin-induced cell cycle arrest in G1. To elucidate the mechanism of simvastatin-induced tumor cell growth inhibition, we examined the effect of simvastatin on cell cycle distribution by flow cytometry. As shown in Fig. 3, concomitant with the inhibitory effect on tumor cell growth, simvastatin treatment induced a strong G1 phase arrest after 48 h treatment. Cell populations in the G0/G1, S and G2/M
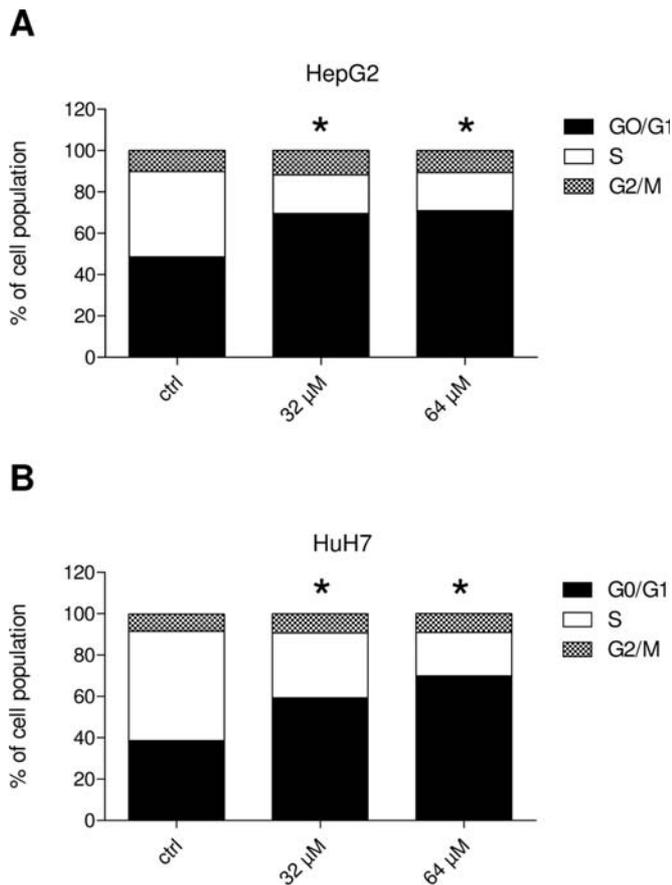
A

HepG2

B

HuH7

Figure 3. Simvastatin effects on cell cycle of HepG2 and Huh7. Cells were treated with simvastatin (32 or 64 μM) or vehicle (ctrl). Distribution of cell cycle fractions (G0/G1, S and G2/M phase) was determined using propidium iodide staining as described in Materials and methods. Percentages of populations in each cell cycle phase were averaged from three independent experiments (G0/G1 cell cycle arrest = *p<0.05 vs. ctrl).
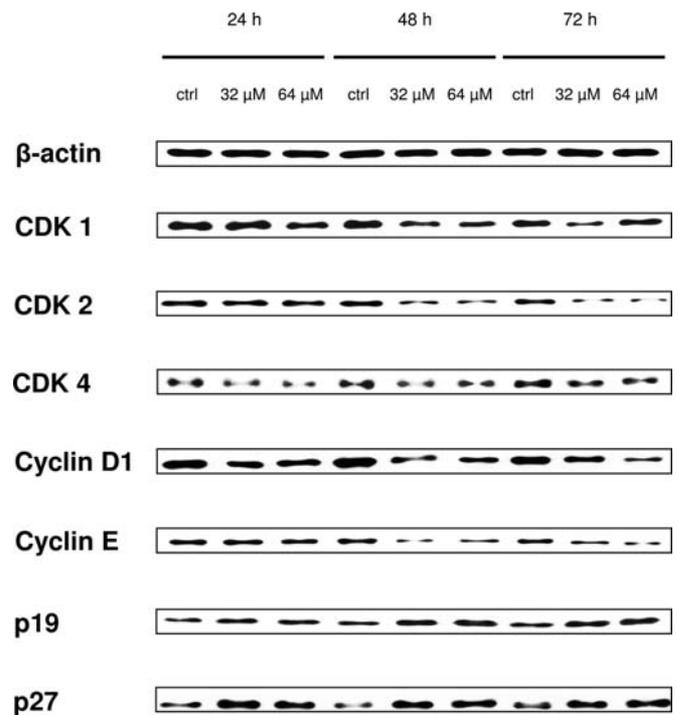

Figure 4. Effect of simvastatin on expressions of cell cycle regulating proteins, CDK1, CDK2, CDK4, cyclins D1 and E as well as p19 and p27 in Western blotting. HepG2 and Huh7 cells were treated with simvastatin (32 and 64 μM) for 24, 48 and 72 h or vehicle (ctrl). Cell lysates were analyzed by specific antibodies as denoted in Materials and methods. ß-actin served as internal loading control. Representative gel from 3 experiments in HepG2 cells is shown.

phases were 44, 45.8 and 10.2%, respectively, in ctrl HepG2 cells, and 38.6, 53.1 and 8.3%, respectively, in Huh7 ctrl cells. After 48 h incubation with 32 μM or 64 μM simvastatin, the S phase population was largely reduced to 17.8% and 18.1% in HepG2 and 30.4% and 20.1% in Huh7 cells, respectively (p<0.01 vs. ctrl). After 48 h incubation with 32 μM or 64 μM simvastatin this reduction in the S phase was accompanied by a concomitant increase in the G0/G1 phase cell populations to 70% and 70.9% in HepG2 and 59.6% and 70% in Huh7 cells, respectively (p<0.01 vs. ctrl).

As another index for G1 phase arrest, we measured the expression levels of cyclin-dependent kinases CDK1, CDK2, CDK4, of cyclins D1 and E, and of cyclin-dependent kinase inhibitors p19 and p27, because the cyclin D/E-CDK2/4 complexes are the primary regulators of the G1/S phase reconstruction points. As representatively demonstrated in Fig. 4, HepG2 cells exposed to simvastatin treatment for 48 or 72 h exhibited downregulation of CDK1, CDK2, CDK4 and cyclins D1 and E as compared to ctrl tumor cells. Relative expression of cyclin-dependent kinase inhibitors p19 and p27 enhanced with simvastatin treatment for 24, 48 and 72 h as compared to ctrl tumor cells. These findings coincide with the simvastatin-induced cell cycle arrest in the G0/G1 phase.

Differential gene expression in simvastatin treated tumor cells. To analyze target genes responsive to the effects of simvastatin in HCC, we examined changes in *CDC2* (CDK1), *CCND1* (cyclin D1) and *CDKN2D* (p19) gene expression in HepG2 and Huh7 cells after treatment with simvastatin. The qRT-PCR showed evident expression of *CDC2* and *CCND1* in non-treated control cells, which was significantly reduced after simvastatin treatment for 24, 48 or 72 h (Fig. 5, representative for HepG2). Simvastatin up-regulated gene expression of the cyclin-dependent kinase inhibitor p19 as compared to ctrl (Fig. 5).

Discussion

The present study showed that simvastatin effectively suppressed tumor cell growth and survival of tumor HepG2 and Huh7 cells. These effects were associated with reduced expression of cyclin-dependent kinases (CDKs) and cyclins, whereas CDK inhibitors p19 and p27 were enhanced, suggesting that simvastatin's cell cycle-suppressive action is involved in its anti-cancer properties.

In cancer cells, apoptosis is deregulated and resistance to apoptosis has been correlated with increased metastatic potential in animal models (19,20). Many naturally occurring as well as synthetic drugs comprise apoptosis in their anti-cancer activity (21). Thus, it is likely that simvastatin acting through apoptotic activation may counteract metastasis, recurrence and progression of HCC.
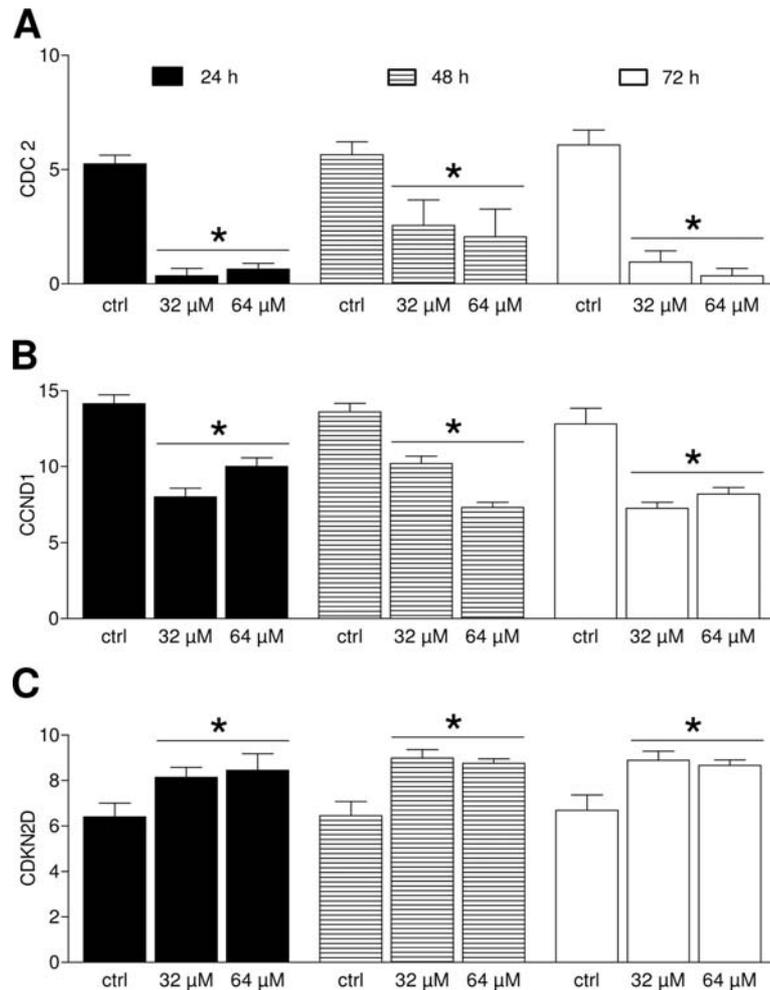
Figure 5. Effects of simvastatin on gene expressions of *CDC2* (CDK1), *CCND1* (cyclin D1) and *CDKN2D* (p19). Quantitative real-time (qRT)-PCR analyses of the three cell cycle regulators 24, 48 and 72 h after treatment of HepG2 cells with 32 or 64 μM simvastatin or vehicle (ctrl) is shown. The relative mRNA expressions of target genes after normalization to GAPDH as described in Materials and methods are represented. Data are given as mean ± SEM. Representative figure from one out of three similar experiments is shown ([*]p<0.05 vs. ctrl).

Statins are known to alter several cellular mechanisms resulting in apoptosis, reduced tumor cell growth as well as angiogenesis and impaired metastatic process (22). Although simvastatin has been demonstrated to exert *in vitro* and *in vivo* anti-tumor effects on various cancer types including human myeloma cells, breast cancer, leukemia cells, prostate cancer and malignant glioblastoma cells by inhibiting tumor cell growth and inducing apoptosis, the anti-tumor effects of simvastatin on hepatocellular carcinoma have not yet been investigated in depth (23-27). The present *in vitro* model reveals that simvastatin is effective in inhibiting the cell growth and also induces apoptosis in human carcinoma cell lines HepG2 and Huh7. Interestingly, the HMG-CoA reductase inhibitors fluvastatin and pravastatin inhited proliferation and induced apoptosis in hepatocellular carcinoma cells but also demonstrated anti-cancer effects in rat HCC *in vivo* models by inhibiting cancer progression and improving survival (28-30). As statins are commonly used drugs for treatment of hypercholesterolemia, the cancer risk and recurrence among statin users versus non-users is gaining increasing interest. Recent clinical data showed that pravastatin improved survival of patients with advanced HCC compared with non-users (31,32). Combined therapy of leucovorin with

simvastatin demonstrated beneficial effects in metastatic colorectal cancer patients (33). Therefore, a multidrug therapy including simvastatin could be useful in HCC therapy.

Eukaryotic cell cycle progression is regulated by CDKs, and cyclin-dependent kinase inhibitors whose activity is highly controlled and coordinated by their association with cyclins (34). Cyclin-dependent kinase inhibitors bind with active CDK-cyclin complexes and thereby exert tumor-suppressive role that downregulates the cell cycle progression (35,36). Analyses of human cancers revealed that cell cycle regulators are frequently mutated in most common malignancies (37,38). Therefore, control of the cell cycle progression in malignant cells may deliver an effective strategy for the control of tumor cell growth. In the current study, simvastatin induced a G0/G1 arrest of the cell cycle progression in HepG2 and Huh7 cell lines, indicating an additional mechanism by which simvastatin may impact the proliferation of HCC cells. In several human malignancies including breast, colon, prostate cancer and melanoma, simvastatin had anti-cancer effects, which were indeed attributed to cell cycle arrest in the G1 and G2/M phases (14-18). Nevertheless, the efficacy and the molecular mechanism of simvastatin on HCC progression have yet to be clarified.

To analyze the underlying biological mechanisms in simvastatin-induced HCC cell cycle arrest in G0/G1, protein and gene expressions of CDKs, cyclins and CDK-inhibitors, p19 and p27 were evaluated. Simvastatin-induced reduction of the cell population in the S phase and G0/G1 cell cycle arrest were associated with a marked reduction in the protein levels of CDK1, CDK2, CDK4, cyclins D1 and E. Moreover, gene expressions of CDK1 and cyclin D1 were also reduced markedly after simvastatin treatment. Furthermore, we suggest that simvastatin-induced G1 arrest in cell cycle progression in HepG2 and Huh7 cells is thought to be mediated via an upregulation of p19 and p27 proteins, which was accompanied by enhanced p19 gene expression. In line with these results, another group has shown that enhancement in p19 or p27 in other cancer types by statins induced a cell cycle arrest (39,40). Recently, induction of p19 in Ataxia Telangiectasia Mutated-knockout mice resulted in cell cycle arrest, apoptosis and abrogated hepatocarcinogenesis in a long-term *in vivo* model (41). Simvastatin has the potential to enhance p27 and induce cell cycle arrest (18,42). However, its role in HCC has not been described previously within this context. Our study demonstrated that simvastatin-induced cell cycle arrest in G0/G1 by down-regulating CDKs and cyclins and enhancing p19 and p27 (Figs. 3-5). Based on our results and reports of others, we conclude that HCC development might be caused by altered expression of cell cycle regulators. However, simvastatin-induced cell cycle arrest was accompanied by apoptosis, necrosis and reduced cell proliferation.

In conclusion, CDK1, cyclin D1 and p19 play a decisive role in the carcinogenesis of HCC, since their up-regulation by simvastatin may be associated with reduced tumor cell growth and cell death. Hence, this study demonstrates the anti-carcinogenic and protective role of simvastatin in our HCC model and suggests that simvastatin may be a promising candidate for therapeutic intervention.

Acknowledgements

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