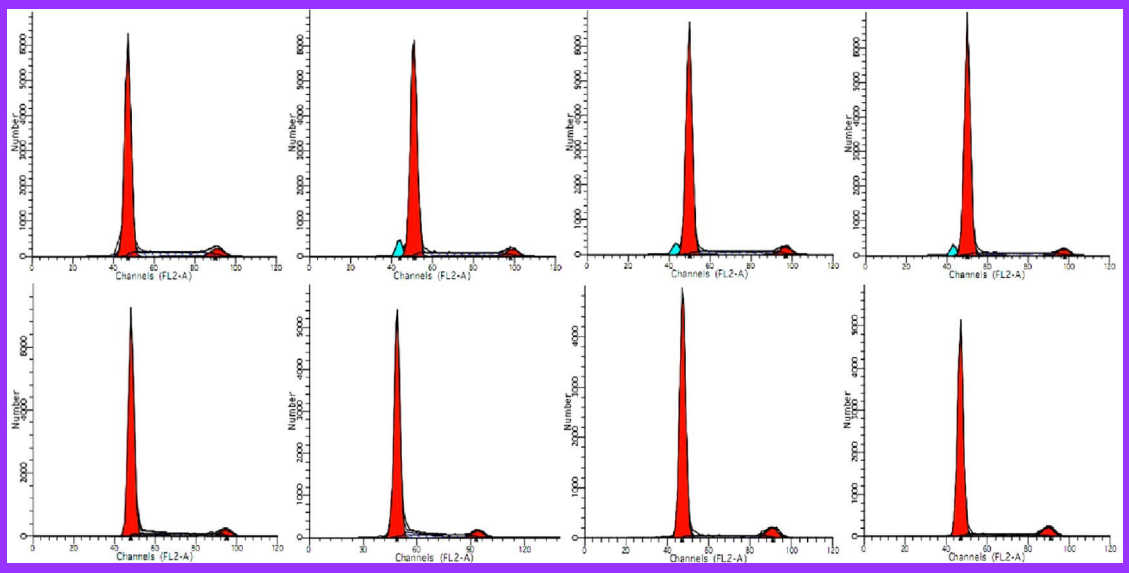

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Lovastatin induces G1-phase synchronization of HEC-1-A cells and JEC cells

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Abstract

Our study investigated lovastatin-induced G_1 phase synchronization of HEC-1-A cells and JEC cells and the cell cycle progression after desynchronization of these cells. HEC-1-A cells and JEC cells were treated with by lovastatin and the proportion of cells in G_1 phase was detected. Both cell types were incubated with lovastatin and the synchronization was determined. The cell cycle progression of both cell types was investigated. The proportion of Both cell types in G1 phase was 87.9 ± 0.7% and 85.0 ± 0.8%, respectively. At 20 and 16 hours after G1-phase desynchronization, the proportion of cells in S phase reached a maximal level and that in G1 phase was at the minimal level for HEC-1-A cells and JEC cells, respectively. Thus, at 20 and 16 hours after desynchronization, the proportion of cells in S phase was the highest and that in G1 phase the lowest for HEC-1-A cells and JEC cells, respectively.

Introduction

Tumor has been regarded as a group of diseases related to impairment of cell cycle (Sherr, 1996; Evan and Vousden, 2001; Go et al., 2012) and the pathogenesis of tumor attributes to the uncontrolled cell cycle. Under this condition, the cells are autonomous and uncontrolled and can unlimitedly proliferate. The cell cycle of tumor cells has been a hot topic in studies on molecular biology of tumors. In the studies on cell cycle, cell phase synchronization is usually required to collect cells in the same phase. According to the specific phase in synchronization, synchronization is classified as G0/G1 phase synchronization, G1 phase synchronization, G2 phase synchronization, M phase synchronization and S phase synchronization. In addition, there are some methods that have been developed for synchronization. For example, for G0/G1 phase synchronization, serum starvation or amino acid starvation; for G1 phase synchronization, mimosine and lovastatin are used; for S phase synchronization, treatment with thymidine for arrest is usually performed.

To date, few studies have conducted for the synchronization of endometrial cancer cells. The present study employed endometrial cancer cell lines (HEC-1-A cells and JEC cells) to investigate the efficacy of lovastatin in G1-phase synchronization of both cell lines and to explore the optimal duration of lovastatin treatment and optimal lovastatin concentration. Subsequently, the cell cycle progression was investigated after G1-phase desynchronization. Our findings may provide evidence for the role of cell cycle regulation in the occurrence and development of endometrial cancer.

Materials and Methods

Materials

Human endometrial cancer cell lines (HEC-1-A cells and JEC cells) were preserved in our lab. DMEM low glucose, fetal bovine serum (FBS) (GIBCO), trypsin

(Genom Biotech, Co., Ltd), lovastatin, mevalonic acid (MVA), propidium iodide (PI), Ribonuclease A (RNase A) (Sigma, USA), CCK-8 (Dojindo, Japan) and FACS caliber flow cytometer (Becton Dickinson) were used in the present study.

Cell culture

Human endometrial cancer HEC-1-A cells and JEC cells were adherent cells and seeded in DMEM low-glucose containing 10% FBS followed by incubation at 37°C in an atmosphere with 5% CO_2.

Detection of doubling time by CCK-8 assay

The HEC-1-A cells and JEC cells were digested, centrifuged, suspended and counted. Then, these cells were re-suspended in DMEM low-glucose containing 10% FBS and then seeded into a 96-well plate (3,000 cells/well). These cells were divided into 6 groups and 5 wells were included in each group. Incubation was performed at 37°C in an atmosphere with 5% CO2. CCK-8 was added at 24 hours (0 hour) and 40 hours (16 hours) after incubation and thereafter every 2~4 hours followed by incubation at 37°C in an atmosphere with 5% CO_2 for 2 hours. Optical density (OD) was measured on a microplate at the detection wavelength of 490 nm and reference wavelength of 630 nm. The time to doubling of OD was used as the doubling time of cell proliferation.

Induction of G1-phase synchronization by lovastatin treatment

The induction of G1-phase synchronization was performed according to previously described with modification (Javanmoghadam-Kamrani and Keyomarsi, 2008). In brief, the HEC-1-A cells and JEC cells were digested, centrifuged, re-suspended and counted. After re-suspending in DMEM low-glucose containing 10% FBS, cells were seeded into a 6-well plate (5 × 105 cells/well). Cells were divided into 4 group and 3 wells included in each group. Incubation was done at 37°C in an atmosphere with 5% CO_2 for 24 hours. Then, cells in different groups were treated with lovastatin at 10, 20, 30, and 40 µmol/L, respectively for a doubling time. Then, these cells were collected and subjected to flow cytometry. The concentration at which the proportion of cells in G1 phase was the highest was used for further experiments and regarded as the respective optimal concentration. Then, HEC-1-A cells and JEC cells were seeded into 6-well plate, independently, and three wells included in each group. These cells were treated with lovastatin at the optimal concentration for 0.5~2-fold of doubling time. Cells were harvested every 4 hours and subjected to flow cytometry. The time when the proportion of cells in G1 phase was the highest was regarded as the optimal duration for lovastatin treatment.

G1-phase desynchronization

HEC-1-A cells and JEC cells were seeded into 6-well plate and 3 wells included in each group. These cells were treated with lovastatin at optimal concentration for optimal duration and the supernatant containing lovastatin was removed. These cells were washed in PBS thrice and then treated with MVA at a concentration of 100-fold of the optimal lovastatin concentration followed by incubation at 37°C in an atmosphere with 5%CO_2 for more than one doubling time. Cells were harvested every 4 hours followed by flow cytometry.

Detection of cell cycle by flow cytometry

Cells were digested with 0.25% trypsin and harvested into centrifuge tube followed by centrifugation at 1,000 rpm/min for 4 min. The supernatant was removed and cells were washed in PBS by centrifugation at 1,000 rpm/min for 4 min. The supernatant was removed and cells were re-suspended in 1 mL of 70% alcohol overnight. Following centrifugation at 1,000 rpm/min for 5 min, the supernatant was removed and cells were washed in PBS once. After centrifugation at 1,000 rpm/min for 5 min, the supernatant was removed and cells were re-suspended in 50 µL of 0.5% RNase A and 450 µL of PI solution followed by incubation in dark at 2~8° C for 30 min. Then, flow cytometry was performed.

Statistical analysis

Data were expressed as mean (± SD) and statistical analysis was performed with SPSS version 13.0 statistics package. Comparisons of means were done with t-test between two groups and with one way analysis of variance among different groups. A value of $p<0.05$ was considered statistically significant.

Results

CCK-8 assay revealed, at 0, 16, 20, 22, 24 and 28 hours, the OD was 0.3447, 0.5568, 0.6188, 0.6303, 0.6782 and 0.7262, respectively, for HEC-1-A cells and 0.3778, 0.5829, 0.6670, 0.6970, 0.7423 and 0.7691, respectively, for JEC cells. At 24 hours, the OD of HEC-1-A cells and JEC cells was increased by about 100% and thus 24 hours was defined as the doubling time.

HEC-1-A cells were divided into 4 groups and treated with lovastatin at 10, 20, 30 and 40 µmol/L, respectively. After treatment for 24 hours, the proportion of cells in G1 phase was (76.7 ± 0.5)%, (79.7 ± 0.9)%, (80.4 ± 1.6)%, and (84.6 ± 1.3)%, respectively (Figure 1). Statistical analysis showed the proportion of cells in G1 phase after treatment with 40 µmol/L lovastatin for 24 hours was dramatically increased when compared with other treatments ($p<0.05$). This suggests the optimal concentration of G1-synchronization of HEC-1-A cells was 40 µmol/L.

JEC cells were divided into 4 groups and treated with lovastatin at 10, 20, 30 and 40 µmol/L, respectively.

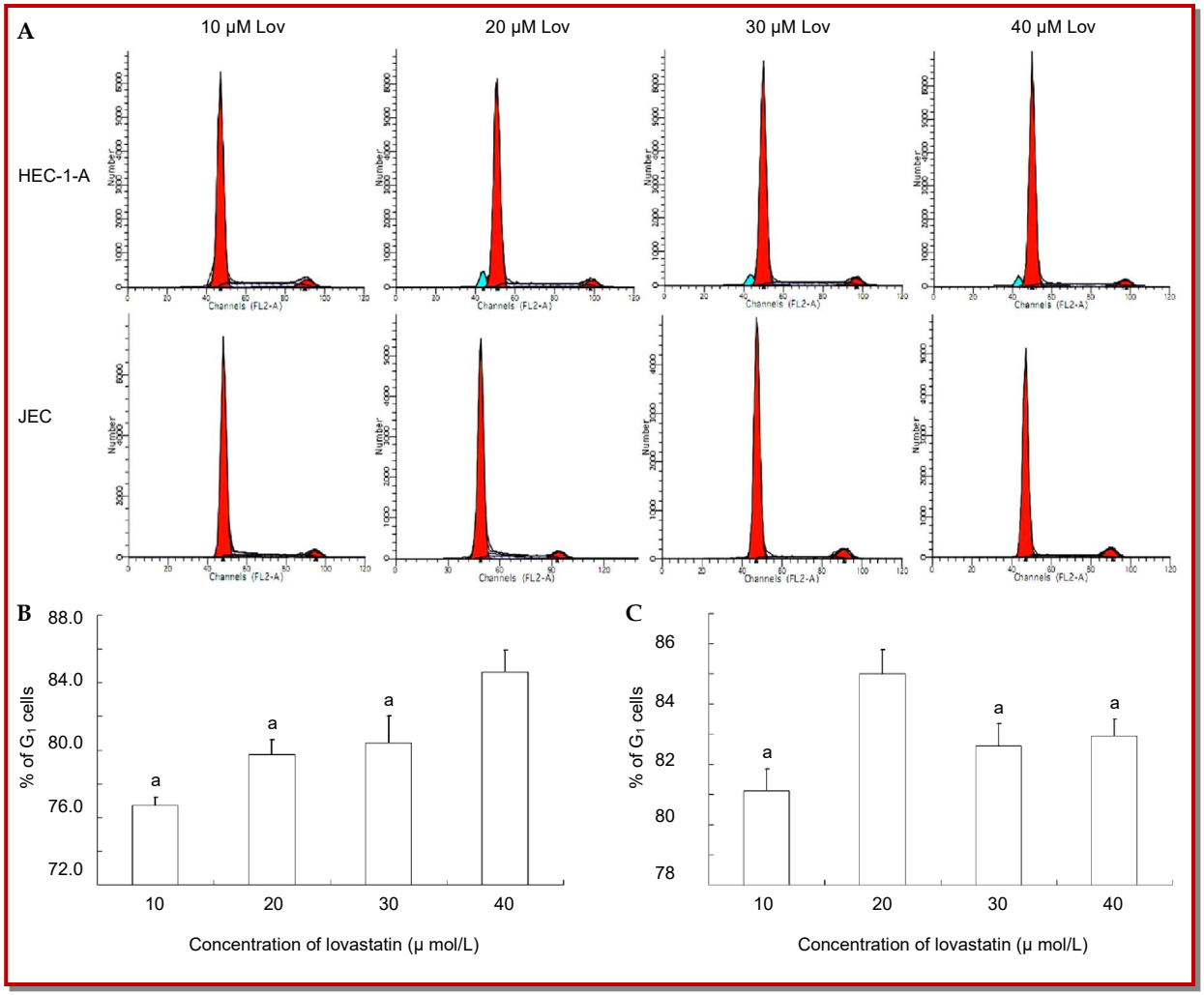

Figure 1: Optimal lovastatin concentration for G1-synchronization. (A) Flow cytometry of HEC-1-A cells and JEC cells following treatment with lovastatin at different concentrations for 24 hours. (B) The ratio of HEC-1-A cells in G1 phase induced by lovastatin for 24 hours at different concentrations. [a]p<0.05 vs 40 μmol/L. (C) The ratio of JEC cells in G1 phase induced by lovastatin for 24 hours at different concentrations. [a]p<0.05 vs 20 μmol/L

After treatment for 24 hours, the proportion of cells in G1 phase was (81.1 ± 0.7)%, (85.0 ± 0.8)%, (82.6 ± 0.7)%, and (82.9 ± 0.6)%, respectively (Figure 1). Statistical analysis showed the proportion of cells in G1 phase after treatment with 20 μmol/L lovastatin for 24 hours was dramatically increased when compared with other treat -ments ($p<0.05$). This suggests the optimal concentration of G1-synchronization of JEC cells was 20 μmol/L.

HEC-1-A cells were divided into 10 groups and then treated with 40 μmol/L lovastatin for 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours, and the proportion of cells in G1 phase was (77.8 ± 1.8)%, (81.1 ± 0.5)%, (83.6 ± 0.9)%, (84.6 ± 1.3)%, (87.9 ± 0.7)%, (81.4 ± 1.1)%, (72.6 ± 0.8)%, (69.3 ± 0.9)%, (61.3 ± 0.6)% and (56.9 ± 0.5)%, respectively (Figure 2). Statistical analysis showed treatment with 40 μmol/L lovastatin for 28 hours could maximize the proportion of HEC-1-A cells in G1 phase ($p<0.05$). This suggests the optimal concentration of lovastatin and duration of lovastatin treatment was 40 μmol/L and 28 hours for G1-phase synchronization of HEC-1-A cells.

JEC cells were divided into 10 groups and then treated with 20 μmol/L lovastatin for 12, 16, 20, 24, 28, 32, 36, 40, 44 and 48 hours, and the proportion of cells in G1 phase was (70.4 ± 1.2)%, (75.5 ± 1.1)%, (82.5 ± 1.1)%, (85.0 ± 0.8)%, (83.0 ± 0.4)%, (80.7 ± 0.5)%, (67.6 ± 0.6)%, (55.6 ± 0.5)%, (47.8 ± 1.8)% and (50.4 ± 2.1)%, respectively (Figure 2). Statistical analysis showed treatment with 20 μmol/L lovastatin for 24 hours could maximize the proportion of JEC cells in G1 phase ($p<0.05$). This suggests the optimal concentration of lovastatin and duration of lovastatin treatment was 20 μmol/L and 24 hours for G1-phase synchronization of JEC cells.

HEC-1-A cells were divided into 8 groups and treated with 40 μmol/L lovastatin for 28 hours for synchro-

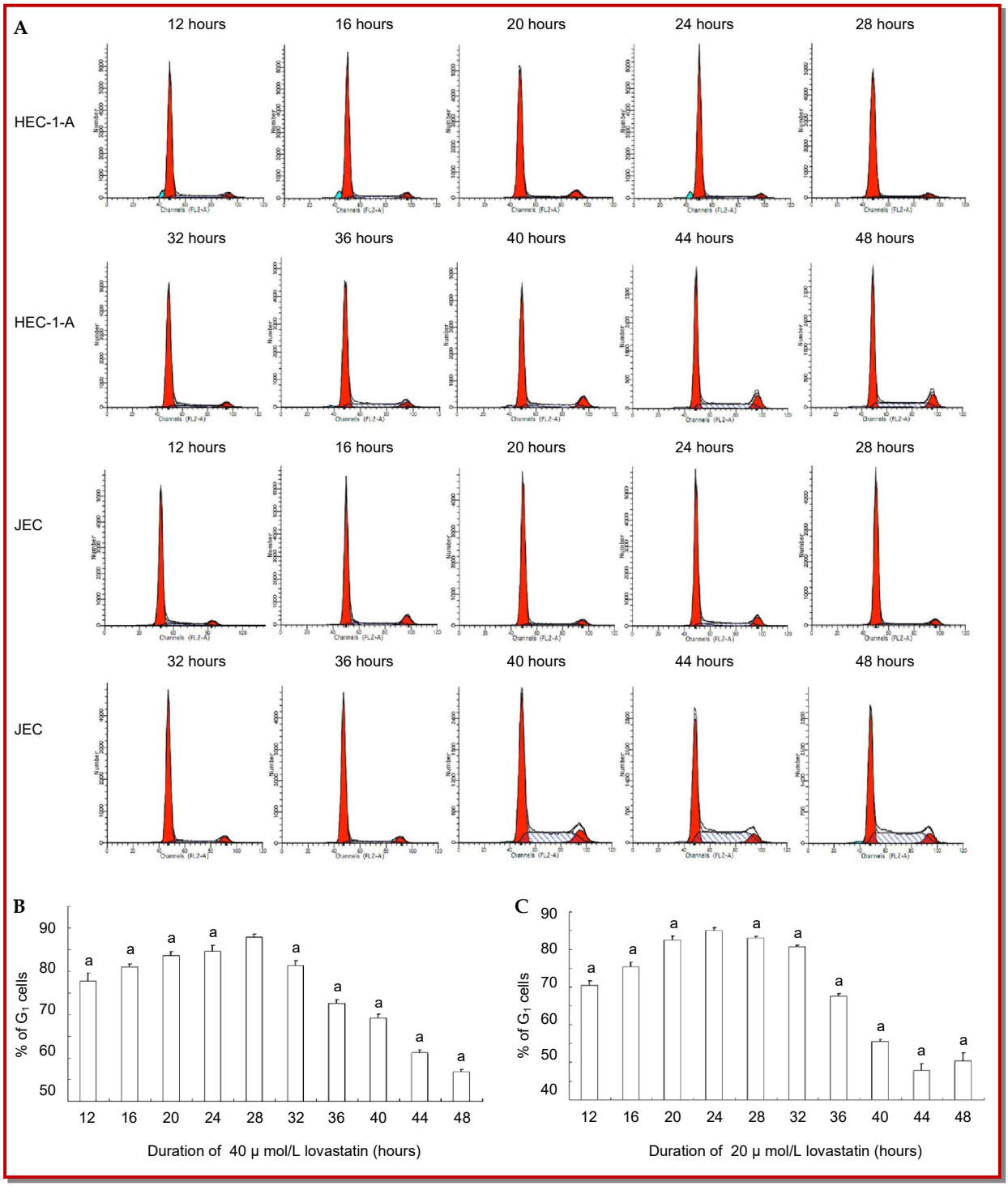

Figure 2: Optimal duration of Lovastatin treatment for G1-synchronization. (A) Flow cytometry of HEC-1-A cells following treatment with 40 μmol/L lovastatin and JEC cells following treatment with 20 μmol/L lovastatin for different durations. (B) The ratio of HEC-1-A cells in G1 phase induced by 40 μmol/L lovastatin for different durations. [a]p<0.05 vs 28 hours (C) The ratio of JEC cells in G1 phase induced by 20 μmol/L lovastatin for different durations. [a]p<0.05 vs 24 hours

nization and then with 4 mmol/L MVA for desynchronization. Results showed the proportion of cells in G1 phase reached a minimal level (58.4 ± 0.5)% but that in S phase was at a maximal level (33.6 ± 0.6)% at 20 hours after desynchronization, which were markedly different from those in the remaining groups (4, 8, 12, 16, 24, 28 and 32 hours) (p<0.05) (Table I and Figure 3).

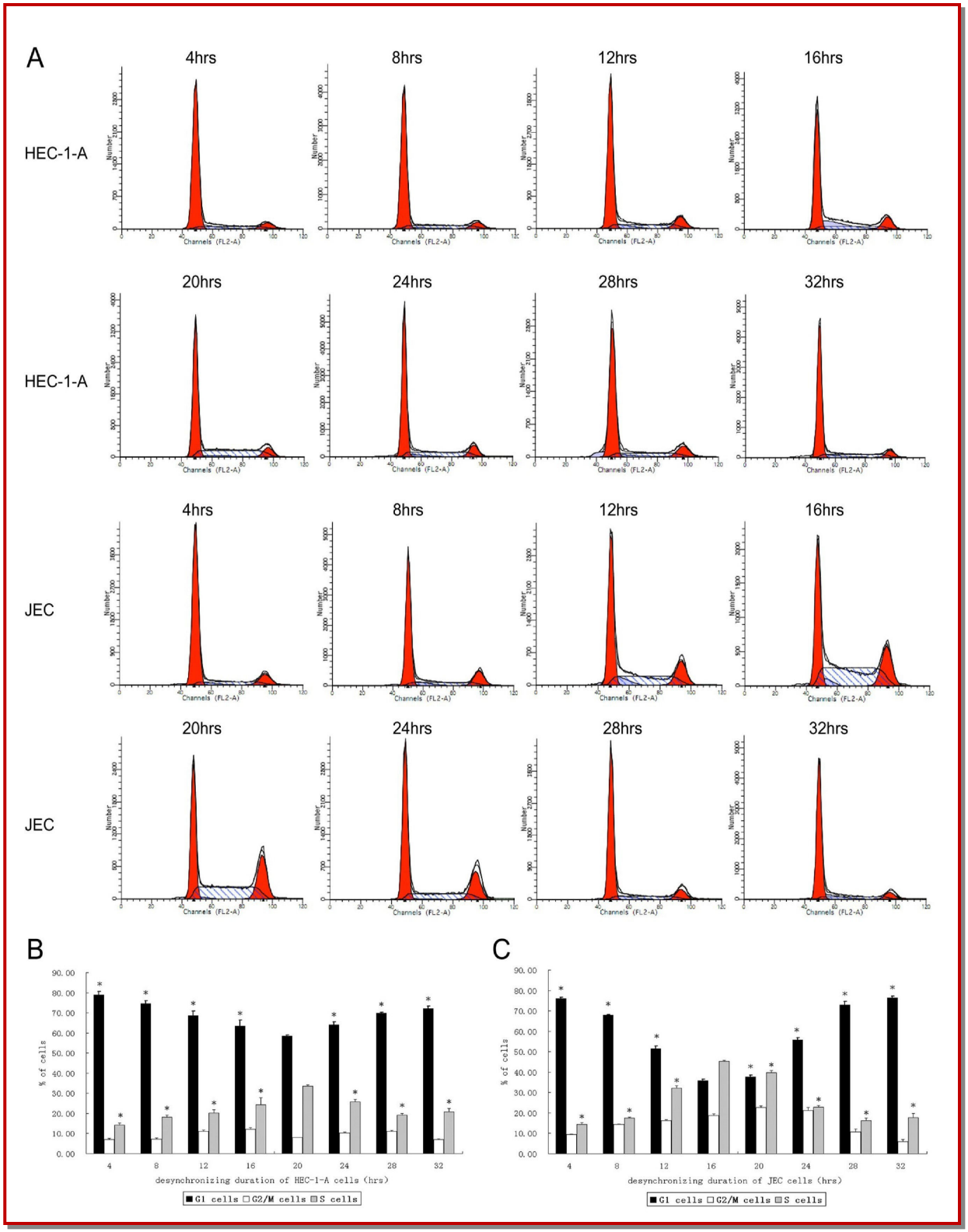

Figure 3: Cell cycle progression after G1-phase desynchronization. (A) Flow cytometric analysis of HEC-1-A cells and JEC cells desynchronized for different durations after induced by optimal lovastatin concentration and duration. (B) Cell cycle progress of HEC-1-A cells desynchronized for different durations after induced by 40 µmol/L lovastatin for 28 hours. *p<0.05 vs 20 hours (C) Cell cycle progress of JEC cells desynchronized for different durations after induced by 20 µmol/L lovastatin for 24 hours. *p<0.05 vs 16 hours

Table I

Cell cycle progress after desynchronization of HEC -1-A cells

Group (hours)	G_1 (%)	S (%)	G_2/M (%)
4	78.9 ± 1.8	14.2 ± 1.2	7.0 ± 0.7
8	74.5 ± 1.4	18.2 ± 0.9	7.3 ± 0.5
12	68.6 ± 2.2	20.2 ± 1.7	11.1 ± 0.6
16	63.5 ± 2.9	24.3 ± 3.5	12.6 ± 0.7
20	58.4 ± 0.5	33.6 ± 0.6	8.0 ± 0.1
24	64.0 ± 1.6	25.7 ± 1.3	10.4 ± 0.3
28	69.7 ± 0.5	19.1 ± 0.9	11.2 ± 0.5
32	72.2 ± 1.1	20.9 ± 1.5	6.9 ± 0.5

Data are (mean ± SD)

Table II

Cell cycle progress after desynchronization of JEC cells (mean ± SD)

Group (hours)	G_1 (%)	S (%)	G_2/M (%)
4	76.1 ± 0.6	14.5 ± 0.8	9.5 ± 0.2
8	68.0 ± 0.3	17.6 ± 0.4	14.5 ± 0.2
12	51.5 ± 1.3	32.2 ± 1.1	16.3 ± 0.4
16	35.9 ± 0.4	45.3 ± 0.5	18.8 ± 0.9
20	37.6 ± 1.0	39.7 ± 1.0	22.7 ± 0.9
24	55.9 ± 0.9	22.9 ± 0.7	21.2 ± 1.4
28	72.8 ± 1.7	16.4 ± 1.1	10.8 ± 1.4
32	76.3 ± 1.0	17.7 ± 2.1	5.9 ± 1.2

Data are (mean ± SD)

JEC cells were divided into 8 groups and treated with 20 μmol/L lovastatin for 24 hours for synchronization and then with 2 mmol/L MVA for desynchronization. Results showed the proportion of cells in G1 phase reached a minimal level (35.9 ± 0.4)% but that in S phase was at a maximal level (45.3 ± 0.5)% at 16 hours after desynchronization, which were markedly different from those in the remaining groups (4, 8, 12, 16, 24, 28 and 32 hours) ($p<0.05$) (Table II and Figure 3).

Discussion

Lovastatin can inhibit the HMG-CoA reductase resulting in deficiency of mevalonate which is a precursor of cholesterol and also essential for the prenylation of substrates of p21 and ras (Wejde et al., 1993). Lovastatin can arrest suspended and adherent cells of a lot of types in the G1 phase and has following advantages (Keyomarsi et al., 1991; Javanmoghadam-Kamrani and Keyomarsi, 2008): 1) lovastatin-induced synchronization is not toxic to cells and reversed by mevalonate; 2) in the lovastatin-induced synchronization, the nutritional substances and growth factors are sufficient, which may not cause disorder of cell metabolism; 3) lovastatin-induced synchronization can be used to achieve a large number of cells in G1 phase; 4) the efficiency of lovastatin-induced synchronization is unrelated to the type of cells, the cell density and the culture medium; 5) synchronization can be applied repeatedly. Wu et al. applied 5 μmol/L lovastatin to treat CHO cells for 32 hours and the proportion of cells in G1 phase was 90% (Wu and Gilbert, 2000). In addition, lovastatin at 10 and 20 μmol/L was applied to treat M6 cells and MCF-1 cells for 24 and 33 hours, respectively, and 78% and 77~96% of cells were identified to be in the G1 phase (Gupta et al., 2003; Shibata et al., 2003). Treatment of Pr14 cells and Pc-3M cells with 10 μmol/L lovastatin for 24 hours and 24~48 hours can achieve 82% and 68~90% of cells in G1 phase, respectively (Park et al., 2001; Shibata et al., 2003). Treatment of T-24 cells with 30 μmol/L lovastatin may achieve 80% of cells in G1 phase and about 70~90% of Calu-1 cells were identified to be in the G1 phase following treatment with 30 μmol/L lovastatin (Jakobisiak et al., 1991; Vogt et al., 1997). In addition, the G1-phase synchronization of HL-60 cells, T47D cells, HELA cells and U2-OS cells was also favorable following lovastatin treatment (Kishi et al., 2001; Reimers et al., 2001; Jurchott et al., 2003; Audic and Hartley, 2004).

In the present study, G1-phase synchronization of HEC-1-A cells and JEC cells was performed by lovastatin treatment. Firstly, the optimal concentration of lovastatin for G1-phase synchronization was determined. Cells were treated with lovastatin at 10, 20, 30 and 40 μmol/L, independently for one doubling time (24 hours). Results showed the optimal concentration of lovastatin was 40 μmol/L for HEC-1-A cells and 20 μmol/L for JEC cells under which the proportion of cells in G1 phase was (84.6 ± 1.3)% and (85.0 ± 0.8)%, respectively. Lovastatin at this concentration was applied for further experiment. Then, the optimal time for lovastatin treatment in the G1-phase synchronization was further studied. HEC-1-A cells and JEC cells were treated with lovastatin at 40 and 20 μmol/L, respectively, for 0.5~2-fold of doubling time and G1-phase synchronization was measured every 4 hours. Results showed the optimal duration for lovastatin treatment in the G1-phase synchronization was 28 hours for HEC-1-A cells and 24 hours for JEC cells under which the proportion of cells in G1 phase was (87.9 ± 0.7)% and (85.0 ± 0.8)%, respectively. Both synchronizations were favorable. Thus, we speculate that the optimal lovastatin concentration and duration of lovastatin treatment for G1-phase synchronization were 40 μmol/L and 28 hours for HEC-1-A cells and 20 μmol/L and 24 hours for JEC cells. Following desynchronization of these cells, the cell cycle progression was further investigated. Results showed the proportion of HEC-1-A cells in S phase was markedly increased (33.6 ± 0.6)% and that in G1 phase was the

highest (58.4 ± 0.5)% at 20 hours after desynchronization. In addition, the proportion of JEC cells in S phase was markedly increased (45.3 ± 0.5)% and that in G1 phase was the highest (35.9 ± 0.4)% at 16 hours after desynchronization. Both desynchronizations were favorable. These, these conditions for synchronization with lovastatin and subsequent desynchronization can be used in related studies.

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