Both Wortmannin and Simvastatin Inhibit the Adipogenesis in 3T3-L1 Cells During the Late Phase of Differentiation

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ABSTRACT—As we reported previously, both wortmannin and lovastatin inhibit the differentiation in 3T3-L1 cells when these drugs were applied during the insulin-induced cell differentiation. In the present study, 3T3-L1 cells were treated with wortmannin and simvastatin after the completion of the insulin-stimulation, and differentiation was found to be significantly decreased by these drugs. This suggests intracellular signaling pathways play roles in the differentiation of 3T3-L1 cells even after the completion of the insulin-stimulation and also suggests that not only the early phase (day 0 to day 2) but also the late phase (day 2 to day 4) of differentiation is important for the differentiation of the cells.

Keywords: Simvastatin, Wortmannin, Adipocytic differentiation

As we reported previously (1), the adipocytic differentiation of 3T3-L1 cells is inhibited by wortmannin, when it was added to the culture medium not only during the induction of differentiation by insulin, an essential growth factor for 3T3-L1 cells (2), but also after the removal of insulin. In the process of differentiation, 3T3-L1 cells showed various morphological changes that continued even after the removal of insulin (3). This suggested to us the possibility that differentiation might be affected when cells are treated with the drug after the removal of insulin. To examine this possibility was the purpose of this study.

3T3-L1 cells (Japanese Cancer Research Resources Bank, Tokyo) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS) (JRH Bioscience, Tokyo). Differentiation was induced two days after reaching confluence, and the day of inducing differentiation was designated as day 0. To induce adipocytic differentiation, the cells were cultured for 48 hr in DMEM containing 10% FBS, 1 mM isobutylmethylxanthine (Wako Pure Chemicals Industry, Osaka), 1 μM dexamethasone (Wako) and 100 nM (in the case of simvastatin treatment) or 1.7 μM (in the case of wortmannin treatment) of insulin (Wako). At 48 hr after day 0, the medium was changed to DMEM containing only 10% FBS every 48 hr. Control cells were not induced to differentiate and maintained in DMEM containing 10% FBS alone from day 0 to day 2. Simvastatin (gift from Banyu Pharmaceutical Co., Ltd., Tokyo) or wortmannin (Wako) was added to the medium at scheduled times and concentrations. On day 8, the extent of differentiation was evaluated by measuring the activity of glucose 3-phosphate dehydrogenase (GPDH) as follows: Sonicated extract of cells on day 8 (4) was added to the reaction buffer containing 100 mM triethanolamine-HCl (pH 7.5), 2.5 mM EDTA, 0.1 mM 2-mercaptoethanol and 0.12 mM NADH (Wako); and then the reaction was started by adding 0.2 mM dihydroxyacetone phosphate (Sigma Chemicals Co., Ltd., St. Louis, MO, USA). Changes in absorbance at 340 nm were followed at 25℃ by a spectrophotometer (UV2000; Shimadzu, Kyoto). The results were expressed as mU per mg protein, where 1 mU is equivalent to the oxidation of 1 nmol NADH/min. Protein concentration was measured with a BCA protein assay kit (Pierce, Rockford, IL, USA) using BSA as the standard.

The effect of 1 μM of wortmannin on adipocytic differentiation was first examined. This concentration had previously been confirmed to clearly inhibit the adipogenesis (1). Cells were pretreated with 1 μM wortmannin for 30 min and further treated for up to 96 hr after the onset of differentiation. As shown in Fig. 1E, 1 μM wortmannin inhibited the adipogenesis of 3T3-L1 cells.

Since 3T3-L1 cells showed various morphologies with a major change on day 2 (3), the process of adipogenesis of the cells was divided into two phases, an early phase from day 0 to day 2 and a late phase from day 2 to day 4. To examine the effect of wortmannin during the early phase,

3T3-L1 cells were pretreated with 1 μM wortmannin for 30 min and further treated with wortmannin up to 48 hr through the onset of differentiation (early phase). As shown in Fig. 1C, the differentiation of 3T3-L1 cells during the early phase was inhibited by about 50% by 1 μM wortmannin. Then, the cells were treated with 1 μM wortmannin from day 2 to day 4 (late phase). As shown in Fig. 1D, the differentiation was halved again. These results indicate that not only the early phase but also the late phase is important for 3T3-L1 cells to show adipocytic differentiation.

We have previously shown that lovastatin, an inhibitor of 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase, inhibited the adipogenesis of 3T3-L1 cells in a dose-dependent manner, and lovastatin at a concentration of 15 μM reduced the GPDH activity by 52% (5). Although no data were presented in our previous report (5), we also observed that 3T3-L1 cells detached in the presence of higher concentrations of lovastatin. Since the lovastatin treatment of the cells was continued until day 10 where the extent of differentiation was measured in our previous study (5), the effect of lovastatin on differentiation during the late phase was involved. In the present study, therefore, we examined if simvastatin, another HMG-CoA reductase inhibitor, could reproduce the effect of lovastatin during the late phase. If both simvastatin and lovastatin showed similar effects, such an activity may be suggested to be common for HMG-CoA reductase inhibitors and would support our previous observation with lovastatin. To know the general effect of simvastatin on 3T3-L1 cell differentiation, we first treated the cells with simvastatin during the early phase. The cells took a spherical shape in the presence of 10 μM simvastatin and became detached from culture dishes at 48 hr after the onset of differentiation (Fig. 2C). This change of cell morphology was observed as early as 6 hr after starting the induction of differentiation and was not seen at lower simvastatin concentrations. Next, we treated cells with simvastatin during the late phase and examined its effect on adipogenesis. However, simvastatin had to be added 12 hr before the removal of insulin because HMG-CoA reductase inhibitors generally need a longer time to exert their effect (6) than wortmannin, which shows its effect as early as 30 min after addition (7). Under this condition, simvastatin inhibited the adipocytic differentiation of 3T3-L1 cells as shown in Fig. 3, indicating that simvastatin reproduced the effect of lovastatin on the adipocytic differentiation during the late phase. On the other hand, the morphological change that was observed when the cells were treated with 10 μM simvastatin during the early phase was not seen at the same concentration of the simvastatin added at the late phase. These results support the suggestion that the late phase is important for the adipogenesis of 3T3-L1 cells.

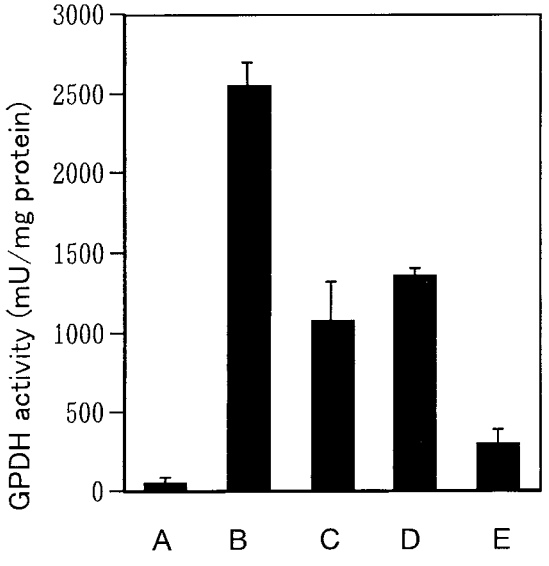

Fig. 1. Wortmannin during both the early and late phases inhibited differentiation. Differentiation was induced and the extent of differentiation was evaluated by measuring the GPDH activity on day 8 as described. Differentiation was A) not induced, B) induced without wortmannin, C) induced in the presence of 1 μM wortmannin from 30 min before the onset of differentiation to 48 hr after the onset of differentiation (early phase), D) induced in the presence of 1 μM wortmannin from day 2 to day 4 (late phase), and E) induced with wortmannin from 30 min before the onset of differentiation to day 6. Data are means±S.E.M. (n=3).

How do wortmannin and simvastatin inhibit the differentiation of 3T3-L1 cells during late phase? By the late phase, mitogen-activated protein (MAP) kinase is thought to have already been activated. In a study on cardiac myocyte hypertrophy, the activation and gene expression of MAP kinase have been observed when Raf-1 kinase was being activated, but the morphological changes associated with cardiac hypertrophy were not induced (8). These findings suggest some factors other than the members of the Ras-Raf1-MAP kinase cascade are needed to induce morphological changes. Some of the small GTP-binding proteins, Rac1, RhoA and Cdc42, which play a role in cytoskeletal rearrangement, are known to regulate the activity of the JNK signaling cascade instead of the Ras-Raf-ERK (extracellular-signal-regulated kinase) (or MAP kinase) cascade (9–11). Wortmannin is known to inhibit phosphatidylinositol (PI) 3-kinase with an IC_{50} value of less than 10 nM. In the present study, we used 1 μM wortmannin, and this concentration of wortmannin has been shown to inhibit some factors other than PI 3-kinase, such as PI 4-kinase and myosin light chain kinase. In the Discussion of our previous report (1), we have speculated that impairment of the adipogenesis of 3T3-L1 cells by 1 μM wortmannin may

result from the inhibition of PI 3-kinase. In fact, 1 μM wortmannin has been used to inhibit the PI 3-kinase activity (12, 13). Involvement of PI 3-kinase in the formation of cytoskeletal fragments has also been suggested (14), and PI 3-kinase is said to be functionally related to some of small GTP-binding proteins. Thus, we hypothesized that the wortmannin applied during the late phase may have inhibited PI 3-kinase and this led to the inhibition of small GTP-binding proteins to affect cytoskeletal components. As a result, the adipocytic differentiation of 3T3-L1 cells was impaired.

The differentiation was inhibited also by simvastatin during the late phase, reproducing the effect of lovastatin

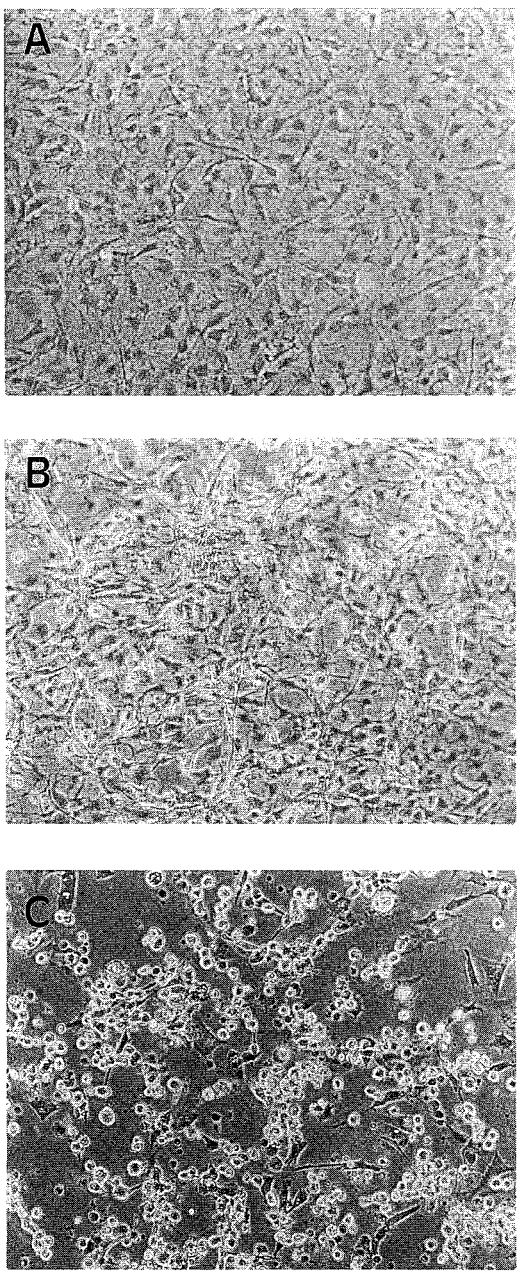

Fig. 2. Simvastatin during the early phase caused rounding-up of cell morphology. Differentiation was induced as described and microphotographs (×400) of culture dishes were obtained 48 hr after the onset of differentiation. Differentiation was A) not induced, B) induced without simvastatin, and C) induced in the presence of 10 μM simvastatin from day 0 to 48 hr after the onset of differentiation. Data represent four separate experiments.

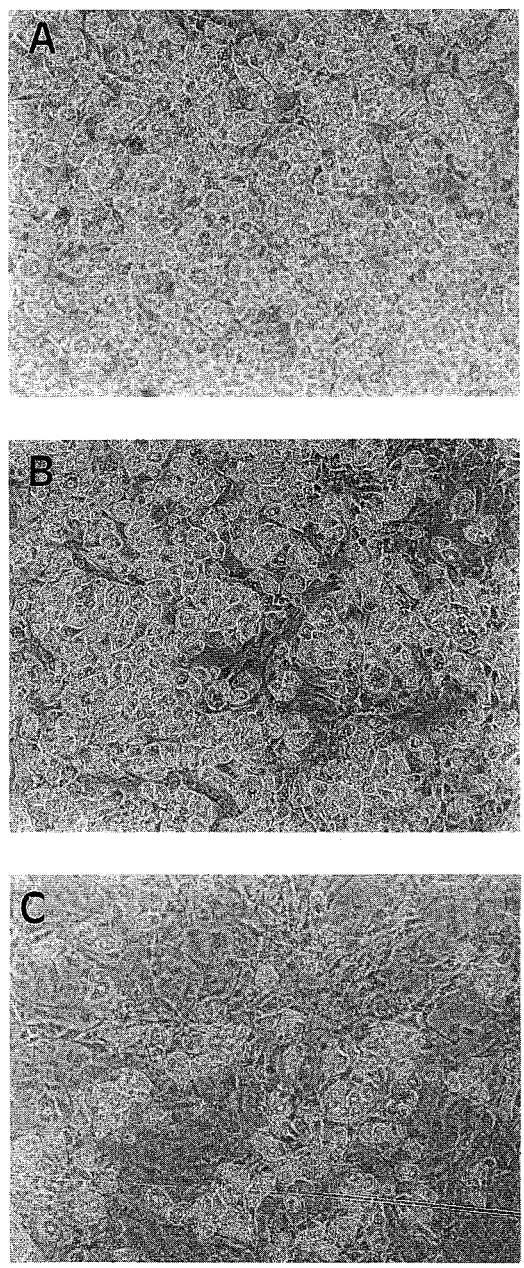

Fig. 3. Simvastatin during the late phase inhibited differentiation. Differentiation was induced as described. Cells were treated with A) 0.1, B) 1 and C) 10 μM of simvastatin from 36 hr to 96 hr after the onset of differentiation. On day 8, microphotographs (×400) of culture dishes were obtained. Data represent four separate experiments.

observed previously. Lovastatin has been found to inhibit the cell growth in a ras-independent way, although impairment of the isoprenoid synthesis turned out to be the essential mechanism (15). There is a report that showed the inhibition of PI 3-kinase by lovastatin (6). We also reported that simvastatin inhibited the PI 3-kinase activity in a dose-dependent manner, producing nearly complete inhibition at 10 μM (16). Thus, it is inferable that, like wortmannin, simvastatin applied during the late phase affected some small GTP-binding proteins through the inhibition of the PI 3-kinase activity, resulting in the morphological changes that occurred during the late phase.

The rounding-up of 3T3-L1 cells and their detachment from culture dishes appeared to suggest the cytotoxicity of 10 μM simvastatin. However, simvastatin at this concentration during the late phase did not have such a morphological effect and the cells remained adherent to the culture dishes. In addition, when differentiation was not induced, the cells did not detach in the presence of 10 μM simvastatin. These results suggest that rounding-up of cells during the early phase was not always due to cytotoxicity. Although it is inferable that simvastatin might affect the cells via different mechanisms in the early and late phases, and wortmannin and simvastatin might affect differentiation via a pathway other than the Ras-Raf1-MAP kinase cascade, these possibilities remained for further studies.

The purpose of the present study was to examine whether the differentiation of 3T3-L1 cells is affected even when wortmannin or simvastatin was added to culture media during the late phase of adipocytic differentiation. In fact, both wortmannin and simvastatin applied during the late phase were confirmed to reduce the adipogenesis of 3T3-L1 cells. This suggest that not only the early phase (from day 0 to day 2) but also the late phase (from day 2 to day 4) is important for the adipocytic differentiation of 3T3-L1 cells. Further studies are certainly needed and actually in progress to explore the intracellular signal transduction mediating the actions of wortmannin and HMG-CoA reductase inhibitors including simvastatin and lovastatin.

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