COMMUNICATIONS TO THE EDITOR

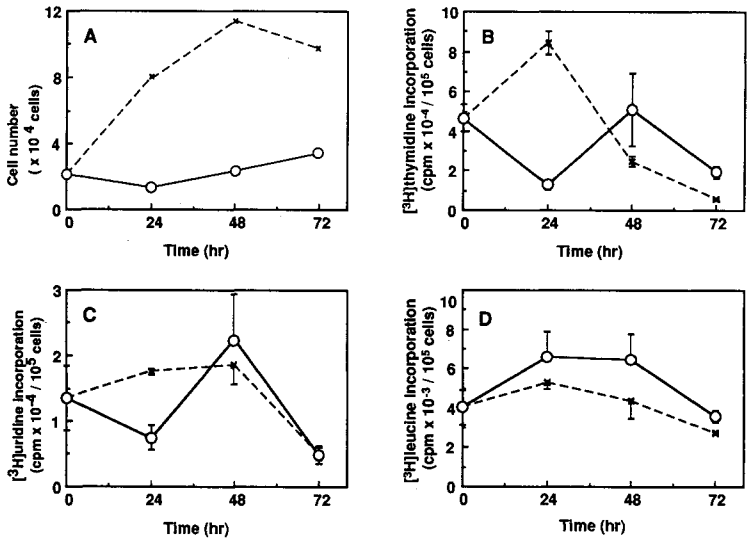
The Neuritogenesis Inducer Lactacystin Arrests Cell Cycle at Both G0/G1 and G2 Phases in Neuro 2a Cells

Sir:

Lactacystin was isolated as a neuritogenesis inducer from cultured broth of a soil actinomycete[1,2]. It induces the formation of bipolar projections at both sides of the cell body 1 day after treatment and a marked morphological change characterized by a neurite-like structure at 3 or 4 days in Neuro 2a mouse neuroblastoma cells[1]. Because the compound inhibited the proliferation of the cells but did not affect macromolecular synthesis within 2 hours after treatment, the effects on cell cycle and related enzymes were examined in Neuro 2a cells. Recently, FENTEANY *et al.*[3] reported that lactacystin inhibits progression of M phase of Neuro 2a cells and MG-63 osteosarcoma cells beyond the G1 phase of the cell cycle. We report here that lactacystin arrests the cell cycle at both G0/G1 and G2 phases in Neuro 2a cells.

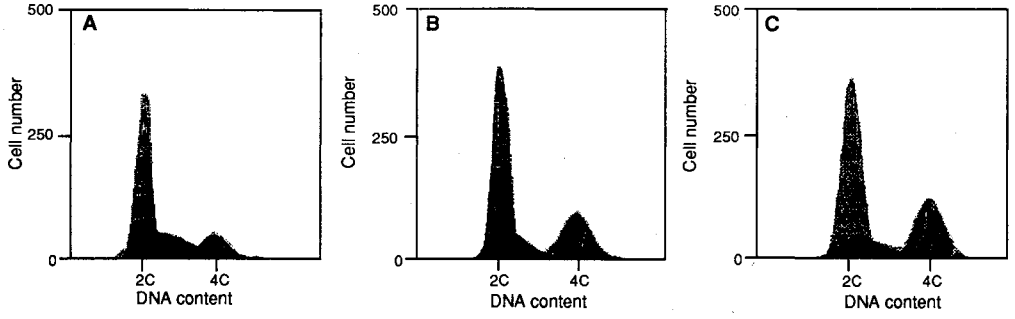
As reported previously, when neuritogenesis is induced in the presence of lactacystin in Neuro 2a cells, cell proliferation is inhibited[1]. So, the effect of lactacystin on the synthesis of macromolecules in the cells was examined. Neuro 2a cells were purchased from Dainippon Pharmaceutical (Osaka, Japan). They were grown at 37°C in EAGLE's minimum essential medium with HANK's salt (MEM-H) containing 10% fetal bovine serum (FBS) and 1% non-essential amino acids. Cells were subcultured twice weekly. The viability of cells was determined by trypan blue exclusion. Synthesis of DNA, RNA and protein was assayed by the incorporation of [*methyl*-^{3}H]thymidine (185 GBq/m mol, Amersham), [5-^{3}H]uridine (999 GBq/m mol, Amersham), [4,5-^{3}H]leucine (2.04 TBq/m mol, Amersham), respectively, into the macromolecular fraction. Each radioisotope was added at 37 kBq/ml. After incubation of culture (0.5 ml) with an isotope for 1 hour, the culture medium was removed. Cells were washed with 2 ml of cold phosphate buffered saline (PBS) and then solubilized with 0.1 N NaOH containing 0.1% sodium dodecyl sulfate. Three volumes of 13.3% trichloroacetic acid (TCA) were added to the solution and placed in an ice bath for 1 hour. TCA insoluble materials were collected on a glass filter (GF/C, Whatman) and then washed three times with 2 ml of 5% TCA and twice with 2 ml of ethanol. After the filter was dried, its radioactivity was counted by using a liquid scintillation counter. Lactacystin did not inhibit the synthesis of DNA, RNA or protein within 2 hours in an experiment of incorporation of their precursors into the acid-insoluble fraction (data not shown). In a longer term experiment, however, lactacystin significantly affected macromolecular synthesis. In a control culture of Neuro 2a cells, DNA synthesis reached a maximum at 24 hours and then decreased rapidly, RNA synthesis somewhat increased until 72 hours and then decreased abruptly, and protein synthesis somewhat increased until 24 hours and then decreased gradually. On the other hand, in

Fig. 1. Effect of lactacystin on cell growth (A) and synthesis of DNA (B), RNA (C) and protein (D) in Neuro 2a cells.



Cells were cultured in MEM-H containing 10% FBS. After 24 hours, the culture medium was replaced with fresh one containing 4% FBS with (○) or without (×), 1.3 μM lactacystin. At the indicated time after the medium replacement, [^{3}H]thymidine, [^{3}H]uridine or [^{3}H]leucine was added to the culture (0.5 ml), and then incubated at 37°C. Radioactivities of acid insoluble fractions were determined 1 hour thereafter and expressed as the mean ± SD of the duplicate cultures. The experiments were performed three times.

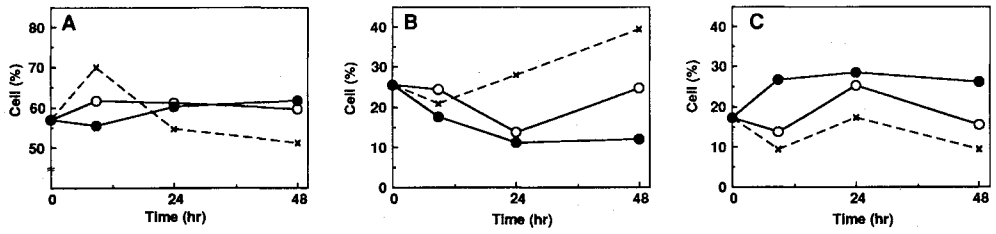
Fig. 2. Effect of lactacystin on distribution of DNA contents in Neuro 2a cells.



Cells were cultured in MEM-H without serum. After 24 hours, the serum-free medium was replaced with fresh one containing 4% FBS without (A) or with 1.3 μM (B) or 3.9 μM (C) lactacystin. After additional 24-hours incubation, cells were collected and their isolated nuclei were analyzed by a flow cytometer as described in the text. The experiments was done three times.

Fig. 3. Effect of lactacystin on each cell phase.

×, control; ○, lactacystin, 1.3 μM; ●, lactacystin, 3.9 μM. The data were expressed as the means of triplicate cultures.



Cells were collected at the indicated time after lactacystin addition and then analyzed as shown in the text. A, G_0/G_1 phase; B, S phase; C, G_2 phase.

the presence of lactacystin (1.3 μM), DNA synthesis was inhibited to one sixth at 24 hours, and RNA synthesis was decreased to about a half, while protein synthesis was rather enhanced (Fig. 1). The above results suggest that lactacystin is not a direct inhibitor of macromolecular synthesis and it may be a cell cycle inhibitor, and that protein synthesis is necessary for neuritogenesis in the presence of lactacystin.

The effect of lactacystin on distribution of DNA content in Neuro 2a cells was investigated by using a flow cytometer. Neuro 2a cells were plated at a density of 8×10^5 cells per ml per 100-mm dish and incubated for 24 hours in MEM-H without serum. The culture medium was changed to fresh MEM-H containing 4% FBS and then lactacystin (1.3 μM) was added to the culture. After incubation for 9, 24 or 48 hours, cells were washed with PBS, detached with trypsin/EDTA solution, and harvested by centrifugation for 3 minutes at $2,300 \times g$. The harvested cells were processed for a flow cytometer as follows. Cells were fixed with 70% ethanol on ice for 1 hour, and then treated with 0.25 mg/ml RNase (Sigma) at 37°C for 1 hour and with 50 μg/ml propidium iodide (Sigma) at 4°C for 30 minutes. DNA histograms were obtained by using a flow cytometer (Epics Erite, Coulter) equipped with an argon-ion laser at 488 nm. Figures 2 and 3 show the effect of lactacystin on the cell cycle. In the control cells, cells with 2C DNA (G0/G1 phase) and 4C DNA (G2/M phase) and cells between 2C and 4C (S phase) were observed in similar rates at both 0 and 24 hours after binding the assay. These data indicate that although we tried to synchronize the culture by incubation in a serum-free medium, the synchronizing on this method was not complete (G0/G1, 56.5% ; S, 25.5%; G2/M, 17.0%). The proportion of G0/G1 phase cells increased and reached 70% at 9 hours and then decreased (55% at 24 hours).The number of S phase cells begun to increase after 9 hours and continued until 48 hours. In the cells treated with lactacystin, the number of S phase cells decreased after 24 hours, while G0/G1 and G2/M phase cells were increased (Figs. 2 and 3). Such increase at 3.9 μM lactacystin was larger than that at 1.3 μM (Fig. 2 (B and C) and Fig. 3 (A and C)). At 9 hours, in spite of decreasing the number of G0/G1 phase cells, S phase was not so much affected and G2/M phase slightly increased, suggesting that the arrest at G2/M phase by lactacystin precedes that at G0/G1 phase.

Recently, FENTEANY *et al.*[3] reported that lactacystin inhibits progression of M-phase synchronized Neuro 2a cells beyond the G1 phase of the cell cycle. However, we found that lactacystin inhibits the progression of cell cycle not only beyond G1 phase but also beyond G2/M phase. The difference seems due to the cells used. That

is, FENTEANY *et al.* used M-phase synchronized cells incubated with lactacystin for 20 hours, whereas we used nonsychronized ones. Therefore, they could not observe inhibition of progression beyond G2 phase, but we could find the arrest at G2/M phase in the presence of lactacystin. This arrest is considered to be at the G2 phase because the M-phase synchronized cells can progress to G0/G1 phase in the presence of lactacystin as reported by FENTEANY *et al.*[3]

Because it was found from the above results that lactacystin arrests the cell cycle at G0/G1 and G2 phase in Neuro 2a cells, we examined its effect on enzymes, cdc2 kinase, casein kinase II, mitogen-activated protein (MAP) kinase and histone deacetylase[4,5], which are known as target enzymes of cell cycle inhibitors arresting at both G1 and G2 phases. Assays for cdc2 kinase, casein kinase II and MAP kinase were performed as reported by KITAGAWA *et al.*[4] Histone deacetylase was assayed as described by YOSHIDA *et al.*[5] Lactacystin did not affect these enzymes even at 100 μM, but weakly inhibited histone deacetylase (30% inhibition at 26 μM). As compared with the concentrations of lactacystin (1.3 μM) causing neuritogenesis and arrest of cell cycle, the 50% inhibitory concentration ($>26\,\mu$M) for histone deacetylase is very high. The above data suggest that lactacystin has a target molecule other than the above enzymes tested. Defining the target molecule for lactacystin and clarifying the relationship between neuritogenesis and arrest of cell cycle are of great interest in elucidation of regulation mechanism involved in neuronal differentiation.

Acknowledgments

We thank Dr. M. YOSHIDA and Prof. T. BEPPU of the University of Tokyo for assay for histone deacetylase and Dr. M. KITAGAWA of Banyu Tsukuba Research Institute for assay for cdc2 kinase, casein kinase II and MAP kinase. This work was supported in part by Grant-in-Aid for Scientific Research (C) from The Ministry of Education, Science and Culture of Japan.

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(Received December 5, 1995)

References

1) ŌMURA, S.; T. FUJIMOTO, K. OTOGURO, K. MATSUZAKI, R. MORIGUCHI, H. TANAKA & Y. SASAKI: Lactacystin, a novel microbial metabolite, induces neuritogenesis of neuroblastoma cells. J. Antibiotics 44: 113~116, 1991
2) ŌMURA, S.; K. MATSUZAKI, T. FUJIMOTO, K. KOSUGE, T. FURUYA, S. FUJITA & A. NAKAGAWA: Structure of lactacystin, a new microbial metabolite which induces differentiation of neuroblastoma cells. J. Antibiotics 44: 117~118, 1991
3) FENTEANY, G.; R. F. STANDAERT, G. A. REICHARD, E. J. COREY & S. L. SCHREIBER: A β-lactone related to lactacystin induces neurite outgrowth in a neuroblastoma cell line and inhibits cell cycle progression in an osteosarcoma cell line. Proc. Natl. Acad. Sci. USA 91: 3358~3362, 1994
4) KITAGAWA, M.; T. OKABE, H. OGINO, M. MATSUMOTO, I. SUZUKI-TAKAHASHI, T. KOKUBO, H. HIGASHI, S. SAITO, Y. TAYA, H. YASUDA, Y. OHBA, S. NISHIMURA, N. TANAKA & A. OKUYAMA: Butyrolactone I, a selective inhibitor of cdc2 and cdc2 kinase. Oncogene 8: 2425~2432, 1993
5) YOSHIDA, M.; M. KIJIMA, M. AKITA & T. BEPPU: Reversible arrest of proliferation of rat 3Y1 fibroblasts in both the G1 and G2 phases by Trichostatin. J. Biol. Chem. 265: 17174~17179, 1990