EFFECTS OF WORTMANNIN ON THE CYTOSKELETAL STRUCTURE OF HeLa CELLS

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ABSTRACT

Wortmannin (WM), a fungal metabolite that is a selective inhibitor of phosphatidylinositol 3-kinase (PI3 kinase) at 10^{-9} M and of myosin light chain kinase (MLCK) at 10^{-6} M, was applied extracellularly to HeLa cells. WM caused marked shape changes of HeLa cells at 10^{-6} M but not at 10^{-9} M. Within 20 min after the addition of 10^{-4} M WM, almost all of the cells started to round out and lost their attachment to the substratum. The rounded-out cells showed blebbing at the surface, and stress fibers and microtubules in the round cells, prominent in the attached control cells, were not evident. The results suggest that WM changes the cytoskeletal structures in HeLa cells by inhibition of MLCK activity rather than PI3 kinase.

In animal cells, cell division is considered to take place through the interaction between actin and myosin II in the contractile ring (1, 7) at the position specified by the mitotic spindle (4). However, important questions remain as to how the interactions between actin and myosin II are spatially and temporally regulated.

One of the well-characterized mechanisms for the regulation of actin-myosin interactions is through phosphorylation of the 20-kD regulatory myosin light chain of myosin II (MRLC) by the Ca^{2+} /calmodulin-dependent enzyme, myosin light chain kinase (MLCK), in both smooth muscle and non-muscle cells (8, 9). This enzyme catalyzes the specific phosphorylation of MRLC on serine 19, which in turn stimulates the actin-activated Mg^{2+} -ATPase of myosin II, the assembly of myosin filaments under physiological conditions, and the ability of myosin to generate force. Thus, it has been generally agreed that the phosphorylation of

MRLC by MLCK may control the organization and contractile activity of myosin in smooth muscle and non-muscle cells. In the present study, we investigate whether this agreement is true using a MLCK inhibitor, wortmannin, *in vivo*. Wortmannin (WM) has been found as an antifungal antibiotic and can inhibit phosphatidylinositol 3-kinase activity at a concentration of 10^{-9} M (3), and the smooth muscle MLCK selectively at 10^{-6} M (2). Another inhibitor for MLCK, ML-9, whose activity has been reported (5, 6), also inhibits other protein kinases, including A-kinase or C-kinase.

The objective of the present study was to examine the effects of WM on cultured HeLa cells. Since the structure of these cells changed dramatically on addition of WM at 10^{-6} M but not at 10^{-9} M, the data suggest important roles of MLCK in changes associated with the cytoskeleton of non-muscle cells.

MATERIALS AND METHODS

Materials

Wortmannin was dissolved in dimethylsulfoxide (DMSO) at 4.7 mM and stored at -80°C until use.

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Fig. 1 Changes in the cytoskeletal structure of HeLa cells by wortmannin (WM). Cells were treated with (b and d) or without (a and c) WM at a concentration of 4.7×10^{-5} M. Rhodamine-phalloidin was used to localize F-actin (a and b). The mitotic cells (c) and rounded-out cells (d) were stained with anti-tubulin monoclonal antibody for indirect immunofluorescence microscopy.

Rhodamine-conjugated phalloidin was purchased from Molecular Probes. A mouse monoclonal antibody against chick brain alpha-tubulin was purchased from Amersham. FITC-conjugated goat anti-mouse IgG (Tago, Inc.) was used at a dilution of 1:20.

Cell Culture

HeLa cells (RCB0007; just like ATCC CCL2, HeLa) were obtained from Riken Cell Bank (Tsukuba, Japan) and were grown in DMEM medium (Nissui Pharmaceuticals) supplemented with 10% fetal calf serum and streptomycin in plastic tissue culture dishes containing circle glass microscope coverslips.

Calculation of Rounded-Out Cells

The ratio of round cells was calculated as rounded-

out cells/(normal cells + rounded-out cells). More than 200 cells were observed in each sample.

Indirect Immunofluorescence

The cells were prepared for indirect immunofluorescence by fixation for 15 min with acetone at -20°C . After washing in phosphate-buffered saline (PBS), 10–20 μl of anti-tubulin antibody (1:400 diluted) were applied directly to the coverslip. Coverslips were incubated for 60 min in a moist chamber and then washed for 15 min with several changes of PBS. The cells were then stained with 20 μl of FITC-conjugated goat anti-mouse IgG containing 1:30 diluted rhodamine phalloidin in a manner identical to that in the first staining. After several 10-min washes in PBS, coverslips were mounted onto a glass slide with a drop of 10% glycerol containing DABCO. Alternatively, cells were fixed with absolute methanol (-20°C)

for 15 min, rinsed with PBS and stained with anti-tubulin monoclonal antibody.

Stained cells were examined with a fluorescent microscope (Carl Zeiss) equipped with a $\times 40$ or a $\times 10$ lens. Photographs were taken with Fuji film, ISO400.

RESULTS AND DISCUSSION

WM caused marked morphological changes of HeLa cells. After addition of WM, many cells began to be rounded out and finally detached from culture dishes (Fig. 1b). Microfilaments staining was bright at the cell periphery including blebbing and stress fibers in the cytoplasm had disappeared in the rounded-out cells, when examined by using rhodamine-conjugated phalloidin as a probe for F-actin (Fig. 1b). In the absence of WM, cells were generally well spread and the long narrow pseudopods appeared to overlap extensively with those of neighboring cells (Fig. 1a). However, about 10% of cells are round (Fig. 1a). These spherical cells could be normal mitotic cells.

To obtain evidence for the difference between rounded-out and mitotic cells, we stained both cells using anti-tubulin monoclonal antibody. In the rounded-out cells, no regular mitotic spindle was observed (Fig. 1d). In contrast, in the normal dividing cells, mitotic spindles were observed in metaphase or telophase cells (Fig. 1c), indicating that the rounded-out cells were entirely different from the mitotic cells. These observations strongly suggested that shape changes of HeLa cells resulted from effects of WM on the cytoskeleton, such as microfilaments and/or microtubules.

Next, we examined the effects of varying concentrations of WM on the susceptibility of HeLa cells. Until 5 h after the addition of WM, the cells were apparently affected by WM at concentrations higher than 10^{-6} M but not at concentrations lower than 10^{-8} M (Fig. 2A). It is well known that WM selectively inhibits phosphatidylinositol 3-kinase activity at 10^{-9} M (2) and myosin light chain kinase activity at concentrations higher than 3×10^{-6} M (3). Therefore, it is highly probable that WM inhibited myosin light chain kinase activity in HeLa cells.

The effect of DMSO on HeLa cells was examined, since stock solutions of WM were made in DMSO. When less than 2% (v/v) DMSO was added into the cell culture, the ratio of round cells remained at a low level (Fig. 2B). The ratio of round cells was, however, rapidly increased after

Fig. 2 (A) Induction of rounded-out cells by WM. Cells were incubated with various concentrations of WM for 1, 3, 5 and 24 h. It is noteworthy that the ratio of round cells was significantly decreased 1 day after incubation with WM at any concentrations. (B) Effects of DMSO on HeLa cells. HeLa cells were treated with various concentrations of DMSO for 1 to 6 h.

the addition of 4% (v/v) DMSO (Fig. 2B). The results suggest that DMSO lower than 2% (v/v) exerts no effect on HeLa cells. In this experiment, WM dissolved in DMSO lower than 2% (v/v) was used.

The ratio of rounded-out cells was increased in a dose-dependent manner in the concentration range from 10^{-6} to 10^{-4} M WM (Fig. 2A). However, it is noteworthy that the ratio of rounded-out cells was decreased when cells were exposed to WM at concentrations lower than 10^{-5} M for a longer period. Furthermore, at 1 day after the addition of WM, cells being once rounded out became flat. These results suggest that the effect of WM on HeLa cells was canceled by longer incubation. Since it has been reported that WM inhibits irreversibly MLCK activity *in vitro* (3), MLCK might be newly synthesized to compensate the activity of MLCK inactivated by WM *in vivo*.

Our observation using WM provides evidence that MLCK may play an important role in the regulation of cytoskeletal structures in HeLa cells.

This research was supported in part by grants for Scientific Research from the Ministry of Education, Science and Culture of Japan and from the Fugaku Trust for Medicinal Research (to H. H.) and from Research Fellowships of Japan Society for the Promotion of Science for Young Scientists (to M. M.-H.).

Received 5 June 1997; and accepted 20 June 1997

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