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INHIBITION OF SERUM-INDUCED M-PHASE PROGRESSION BY A TYROSINE KINASE INHIBITOR, ERBSTATIN

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A tyrosine kinase inhibitor, erbstatin, increased the number of M-phase cells in exponentially growing NRK, NIH3T3, and RSV-NRK cells. In synchronized NRK cells prepared by serum starvation or hydroxyurea treatment, erbstatin also increased the number of M-phase cells in the presence of serum when added before M-phase. Erbstatin did not inhibit serum-induced DNA synthesis at all. The accumulated M-phase cells later divided even in the presence of erbstatin, indicating that the effect of erbstatin is reversible. An inactive analogue, 5'-*O*-methylerbstatin, did not increase the number of mitotic figures. Flow cytometric analysis indicated that erbstatin reversibly accumulated 4n DNA containing cells when added to G_1/S-synchronized NRK cells. Increased cellular H1 kinase activity at M-phase is decreased after mitosis, and this decrease was inhibited by erbstatin. Thus tyrosine kinase is suggested to be involved in the progression of the M-phase.

Protein kinases are considered to be involved in the regulatory mechanisms governing the cell cycle. Recent studies have shown that the $p34^{cdc2}$/cyclin B complex is an essential regulator of cell division in eukaryotes (1). Mammalian $p34^{cdc2}$ is a cell cycle-regulated serine/threonine protein kinase maximally activated at the G_2/M transition (2). The activity of $p34^{cdc2}$ is regulated by tyrosine kinase (e.g., the *wee*1 gene product) and tyrosine phosphatase (e.g., *cdc25* gene product) (3-6). And it is also regulated by association of $p34^{cdc2}$ with cyclin B (7). The cdc2 protein is a catalytic subunit of maturation-promoting factor, having histone H1 kinase activity (8-11). The high degree of conservation between human and fission yeast *Schizosaccharomyces pombe* $p34^{cdc2}$ proteins (12) suggests a central role for $p34^{cdc2}$ in mitotic progression. Mitogen-activated protein (MAP) kinase is also known to be activated in M-phase (13,14), and its activity is regulated by phosphorylation of its serine/threonine and tyrosine residues (15).

Erbstatin was isolated from *Streptomyces* as an inhibitor of EGF receptor tyrosine kinase (16). It inhibits EGF-induced cytoskeletal change and DNA synthesis reversibly

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in EGF receptor-overexpressing NIH3T3 cells (17). Erbstatin also inhibits Src-induced cytoskeletal changes (18) and DNA synthesis (19) in murine fibroblasts.

In the course of our cell-cycle studies with tyrosine kinase inhibitors we found that erbstatin markedly increased the mitotic index of various cell lines when added before M-phase.

MATERIALS AND METHODS

Materials

NRK-49F cells (ATCC CRL-1570) were obtained through Flow Laboratories, Inc. Calf serum (CS) was purchased from Gibco. Erbstatin was isolated from *Streptomyces* as described before (16). 5'-*O*-methylerbstatin was synthesized in our laboratory. [^{3}H]-thymidine (47 μCi/mmol) was obtained from Amersham.

Cell culture

NRK cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 5% CS at 37°C in a 5% CO_2/95% air atmosphere.

Cell synchronization

NRK cells were synchronized at the G_0 phase by serum starvation. Exponentially growing cells ($2x10^4$) were seeded in 24-well plates and cultured for 2 days in medium containing 5% CS, followed by a 3-days' incubation in medium containing 0.2% CS. Synchronization of cells at the G_1/S boundary was then achieved by a further 20-hr incubation in medium containing 5% CS and hydroxyurea (190 μg/ml).

Measurement of mitotic index

Mitotic index was determined by counting the number of rounded cells in two fields under a phase-contrast microscope. Mitotic indices were calculated as the percentage of rounded cells based on the counting of at least 500 total cells for each datum point.

DNA synthesis

Cells were incubated in medium containing 1 μCi/ml [^{3}H]-thymidine for 1 hr. After having been washed with CS-free medium, the cells were incubated with cold 10% trichloroacetic acid (TCA) at 4°C for 10 min. The insoluble fraction of the cells was washed with 10% TCA and dissolved in 0.5N NaOH at 37°C for 1 hr, and an aliquot was counted for radioactivity in a liquid scintillation counter.

Assay of histone H1 kinase

Cells ($1x10^6$) synchronized at G_0 or G_1/S phase were harvested and suspended in a hypotonic buffer (10 mM Tris-HCl [pH 7.4], 3 mM $MgCl_2$, 1 mM PMSF) for 10 min. Nonidet P-40 was then added to a final concentration of 0.1%, and the cell suspension was subsequently vortexed and centrifuged. The pellet was washed twice with the hypotonic buffer, extracted for 30 min with 0.4M NaCl, and centrifuged at 105,000 x g for 1 hr. The supernatant (10 μl) was diluted with 30 μl of 20 mM Tris-HCl (pH 7.4) containing 4.5 mM β-mercaptoethanol, 1 mM EGTA and 10 mM $MgCl_2$. Then, 20 μg/ml of H1 histone and 2 μCi of [γ-^{32}P]ATP were added, and the mixture was incubated for 20 min at 30°C. The reaction was terminated by addition of the same volume of sample buffer (42 mM Tris-HCl [pH 6.3], 10% glycerol, 2.3% SDS, 5% β-mercaptoethanol, 0.002% bromophenol blue), and the mixture was boiled for 10 min. The samples were electrophoresed on a 12.5% polyacrylamide gel and autoradiographed.

RESULTS

Addition of 5% calf serum to quiescent NRK cells induced cell rounding after 24 hrs, although there was none at 16 hrs, as shown in Fig. 1A and B. These rounded cells

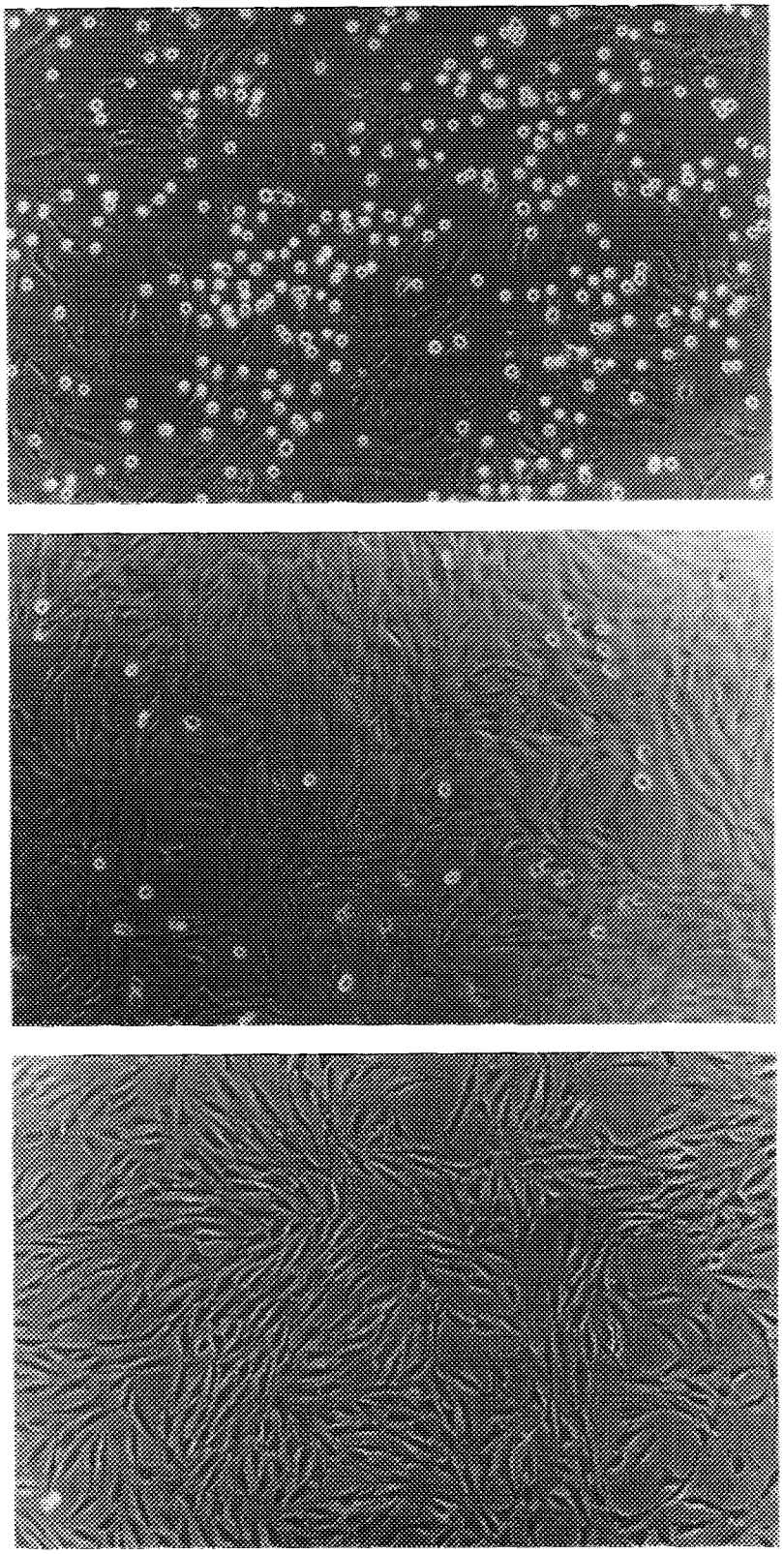

Fig. 1. Accumulation of mitotic figures caused by erbstatin in NRK cells. Quiescent NRK cells were incubated in 5% CS for 16 hrs (A); then, they were cultured for a further 8 hrs without additive (B) or with 4 μg/ml of erbstatin (C).

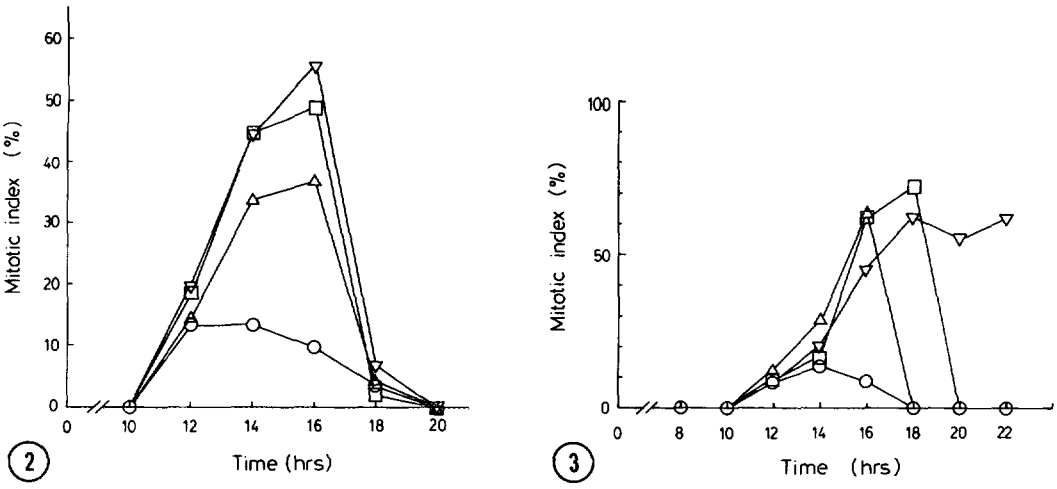

Fig. 2. Increase in mitotic cells caused by erbstatin in G1/S-synchronized NRK cell cultures. After the removal of hydroxyurea the cells were further incubated in 5% CS without additive (○) or with 1 μg/ml (△), 2 μg/ml (□), or 4 μg/ml (▽) of erbstatin. Values are means of duplicate samples.

Fig. 3. Accumulation of mitotic cells due to erbstatin added at different times to G1/S-synchronized NRK cells. After the removal of hydroxyurea, the cells were incubated further with 5% CS in the absence (○) or presence of erbstatin (4 μg/ml) added at 4 hrs (△), 8 hrs (□), or 12 hrs (▽). Values are means of duplicate samples.

were confirmed to be in metaphase with chromosome aggregation by Giemsa staining. When 4 μg/ml of erbstatin was added at 16 hrs, the number of mitotic cells seen at 24 hrs greatly increased (Fig. 1C). The drug had a similar effect on NIH3T3 and RSV-NRK cells as well (data not shown). On the other hand, addition of erbstatin at 0 hr did not increase mitosis after 24 hrs. This would be because erbstatin was degraded before 16 hrs (20) rather than because the S-phase was inhibited. Erbstatin at 4 μg/ml did not inhibit serum-induced DNA synthesis at all.

Next, NRK cells were synchronized at the G1/S boundary by hydroxyurea. After the release from the G1/S boundary, mitosis was observed at 12-16 hrs, as shown in Fig. 2. Erbstatin at 1-4 μg/ml increased the mitotic index when it was added 6 hrs after the removal of hydroxyurea. However, the effect of erbstatin was reversible, and mitotic figures disappeared by 18 hrs from the removal of hydroxyurea. When erbstatin was added later, at a time approaching the start of M-phase, the effect of erbstatin was enhanced, as shown in Fig. 3. Mitotic figures did not disappear even after 22 hrs with erbstatin added at 12 hrs. According to the inhibition of mitosis, increase of cell number delayed by addition of erbstatin (data not shown). Again, the later the addition, the more prominent was the effect. As shown in Fig. 4, erbstatin clearly accumulated 4n DNA containing cells in the presence of serum when added to the G1/S-synchronized NRK cells. The effect of erbstatin was again reversible, since after 24 hrs these 4n cells all moved to the 2n cells.

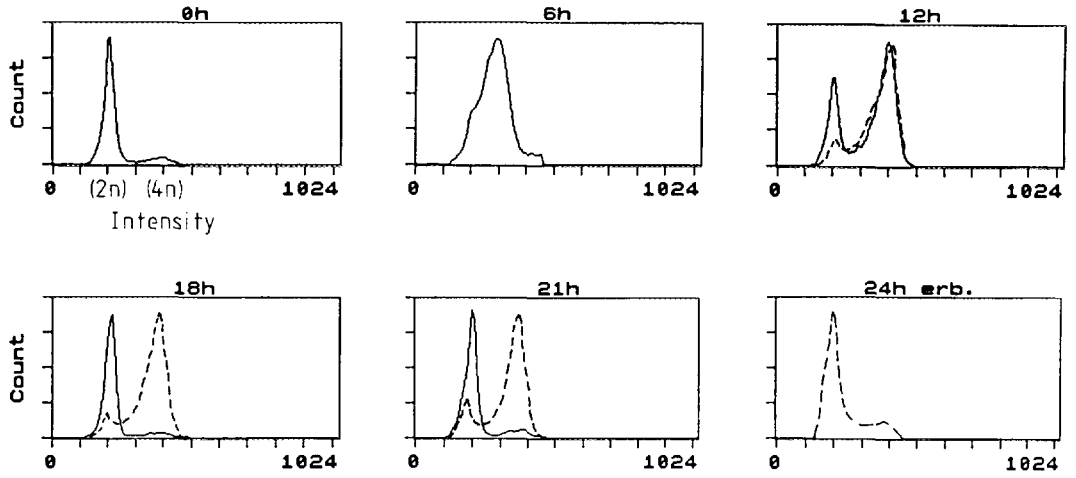

Fig. 4. Effect of erbstatin on distribution of DNA content in G_1/S-synchronized NRK cells. After the removal of hydroxyurea, the cells were incubated further with 5% CS in the absence (solid line) or the presence (dotted line) of erbstatin (6 μg/ml) added at 6 hrs. At the indicated times, the cells were collected and their DNA content of isolated nuclei were analyzed by flow cytometry.

Cycloheximide blocks the cell cycle at G_2 in mammalian cell cultures by inhibiting protein synthesis (21). The increase in mitosis effected by erbstatin was inhibited by cycloheximide in exponentially growing NRK cells (data not shown).

Histone H1 kinase activity is known to increase transiently during the M-phase (2). Erbstatin at 4 μg/ml did not inhibit the increase in this activity, but it clearly inhibited the decrease in histone H1 kinase activity of the cell lysate, as shown in Fig. 5.

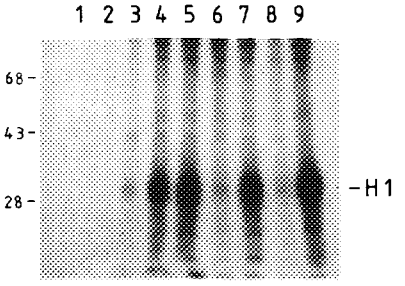

Fig. 5. Time course of cellular histone H1 kinase activity in synchronized NRK cells. The NRK cells were synchronized by serum starvation (G_0, Lanes 1 and 2) or by hydroxyurea (G_1/S, Lanes 3-9). In the former case calf serum (5%) was added, and incubation was carried out for 0 hr (1) or 10 hrs (2). In the latter case, after the removal of hydroxyurea the cells were cultured for 6 hrs without additive (3), for 11 hrs without additive (4) or with 4 μg/ml of erbstatin (5), for 13 hrs without additive (6) or with erbstatin (7), or for 15 hrs without additive (8) or with erbstatin (9).

DISCUSSION

We found in this study that erbstatin transiently inhibited M-phase progression, as evidenced by the accumulation of cells in mitosis (rounded cells), thereby delaying proliferation. We previously reported that erbstatin inhibited EGF-induced DNA synthesis. It did not, however, inhibit serum-induced DNA synthesis, indicating that there would be tyrosine kinase-independent pathway for induction of DNA synthesis in the presence of serum. But erbstatin clearly increased the mitotic index in exponentially growing NRK, NIH3T3, and RSV-NRK cells in the presence of calf serum. An inactive analogue, 5'-*O*-methylerbstatin, did not accumulate metaphase cells, even at a higher concentration (20 μg/ml) (data not shown).

The increase in mitotic index elicited by erbstatin was blocked by cycloheximide, a G_2 blocker, so the transition point of erbstatin may be in the M-phase including cytokinesis. The activity of c-Src tyrosine kinase was reported to be activated during M-phase (22). Since the decrease in cellular histone H1 phosphorylation was blocked by erbstatin during the M-phase, the kinase activity including that of cdc2 kinase may be controlled by tyrosine kinase. We are currently studying the precise role of tyrosine kinase in mitosis both biochemically and morphologically by electron microscopy.

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