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The ERK pathway involves positive and negative regulations of HT-29 colorectal cancer cell growth by extracellular zinc

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Park, Ki-Sook, Nam-Gu Lee, Ki-Hoo Lee, Jeong Taeg Seo, and Kang-Yell Choi. The ERK pathway involves positive and negative regulations of HT-29 colorectal cancer cell growth by extracellular zinc. *Am J Physiol Gastrointest Liver Physiol* 285: G1181–G1188, 2003. First published June 19, 2003; 10.1152/ajpgi.00047.2003.—Dietary zinc is an important trace element in the body and is related to both cell proliferation and growth arrest. A recent study found that extracellular zinc-sensing receptors trigger intracellular signal transduction in HT-29 human colorectal cancer cells. However, the signaling mechanism causing this growth regulation by extracellular zinc is not clearly understood. At 10- and 100-μM levels of $ZnCl_2$ treatment, HT-29 cell growth and proliferation increased and decreased, respectively, in a minimally serum-starved medium (MSSM). A lack of significant increase in intracellular zinc levels after zinc treatment suggested that this differential growth regulation of HT-29 cells by extracellular zinc is acquired by receptor-mediated signal transduction. Moreover, this zinc-induced growth regulation was differentially affected by PD-98059, suggesting the involvement of the ERK pathway. Transient ERK activation and subsequent cyclin D1 induction were observed on adding 10 μM $ZnCl_2$ in MSSM in the presence of cell proliferation. On the other hand, prolonged ERK activity was observed with a subsequent increase of cyclin D1 and $p21^{Cip/WAF1}$ on adding 100 μM $ZnCl_2$ in MSSM, and this was associated with nonproliferation. Moreover, this ERK activation and cyclin D1 and $p21^{Cip/WAF1}$ induction were abolished by PD-98059 pretreatment. The differential regulations of cell growth, ERK activities, and cyclin D1 and $p21^{Cip/WAF1}$ inductions were also observed in serum-enriched medium containing higher zinc concentrations. Therefore, differential cell cycle regulator induction occurs by a common ERK pathway in the differential growth regulation of HT-29 cells by extracellular zinc.

signal transduction; extracellular signal-regulated kinase; $p21^{Cip/WAF1}$

ZINC IS INVOLVED IN THE STIMULATION of cell growth, and a zinc deficiency inhibits growth in animals, causes proliferation in cultured cells, and accelerates established tumor growth (23, 38). However, it has also been reported that zinc has an antitumor effect that is associated with growth inhibition (2, 9, 19, 39). The levels of zinc in tumorous and normal tissues differ (8, 21, 37), and cell growth may be affected by zinc dosage (8, 29). The epithelium of the colonic epithelial mucosa undergoes continuous renewal, and the growth and tumorigenesis of colorectal cells are highly sensitive to zinc levels (5, 24, 25). However, the mechanism of the differential growth regulation of cells by zinc remains unknown.

Extracellular zinc is reported to activate signaling pathways related to cell growth regulation, such as MAPK and phosphoinositol 3-kinase pathways (17, 20, 28, 32), and the chelation of intracellular zinc does not change the activation status of MAPK by extracellular zinc (14). Moreover, cellular growth regulation by zinc is not likely to be mediated by direct interaction with intracellular metalloproteins, because symptoms of zinc deficiency precede changes in the intracellular zinc content (6, 20). Recently, Hershfinkel et al. (15) reported the presence of an extracellular zinc-sensing receptor (ZnR) in HT-29 colorectal cancer cells and suggested its involvement in extracellular receptor-mediated signaling pathway(s) for growth regulation.

In the present study, we investigated the mechanism of the differential regulation of HT-29 colorectal cancer cell growth at nontoxic micromolar concentrations of extracellular zinc. HT-29 colorectal cancer cell growth was found to be positively and negatively regulated by different extracellular zinc concentrations, which resulted from differential ERK activation. Moreover, cyclin D1 and $p21^{Cip/WAF1}$ cell cycle regulators were also differentially induced, and this was also found to depend on ERK activation and to be related to the differential regulation of cell growth. No significant accumulation of intracellular zinc by the application of micromolar concentrations of extracellular zinc suggests that the effects of zinc are mediated by receptor-mediated signal transduction rather than being due to complex effects associated with intracellular zinc accumulation (18, 34).

METHODS

Cell culture. HT-29 cells were maintained in McCoy's 5A medium supplemented with 10% FBS, 100 U/ml penicillin,

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and 250 ng/ml streptomycin in 5% CO_2 at 37°C. Unless otherwise stated, the experiments were performed on cells at 70% confluence. To observe the effects of zinc, the cells were grown in McCoy's 5A containing either 1 or 10% FBS for 16–20 h before zinc treatment. In required cases, 1, 5, or 10 μM of the MEK inhibitor PD-98059 (Calbiochem, La Jolla, CA) was given 30 min before $ZnCl_2$ addition.

Cell counting and thymidine incorporation. HT-29 colorectal cells were grown in minimally serum-starved medium (MSSM) containing 1% FBS for 16 h. The cell culture medium was replaced with fresh 1 or 10% FBS medium containing different concentrations of $ZnCl_2$ (0–500 μM). The attached cell numbers were counted by mounting 10 μl of the cell mixture (1:1 mixture of 0.4% trypan blue and HT-29 cell suspension) onto a Tiefe Depth Profondeur 0.0025-mm^2 cell-counting plate (Superior) and observing plated cells under a microscope.

For thymidine incorporation, the HT-29 cells were plated in 12-well culture dishes at 1.3×10^5 cells/well and then grown in MSSM for 16–20 h. The medium was then replaced with fresh MSSM containing different $ZnCl_2$ concentrations (0, 10, or 100 μM), and the cells were further incubated for 40 h. When required, PD-98059 was also added 30 min before $ZnCl_2$ treatment. Finally, the incubations were continued in the presence of [^{3}H]thymidine (0.5 μCi/well) for 8 h. After cells were washed twice with 5% trichloroacetic acid, they were solubilized by the addition of 0.2 N NaOH, and DNA-associated ^{3}H activity was counted by using a liquid scintillation counter.

Western blot analysis. Cell extracts were prepared from harvested cells as previously described (28). Samples of 10–50 μg protein from the whole cell extracts were separated by SDS-PAGE, and the blots were prepared on a Protran membrane. The following antibodies were used as probes: for ERK, phosphospecific anti-ERK (New England Biolabs, Beverly, MA) and anti-ERK (Stratagene, La Jolla, CA) antibodies; for cyclin D1 and $p21^{Cip/WAF1}$, anti-cyclin D1 and -$p21^{Cip/WAF1}$ antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). The blots were probed with horseradish peroxidase-conjugated secondary antibodies (Transduction Laboratories, San Diego, CA), and the proteins were visualized by enhanced chemiluminescence.

Measurement of intracellular zinc. The intracellular zinc concentration ($[Zn^{2+}]_i$) in HT-29 cells was measured by using a modification of a method described previously (34). HT-29 cells were plated onto coverslips at a density of 3×10^5 cells/coverslip into six-well plates, and the cells were grown overnight in McCoy's 5A medium containing 1 or 10% FBS and treated with $ZnCl_2$ at 1, 100, or 500 μM. Cells were then loaded with the fluorescent indicator mag-fura-2 at 37°C in a 5% CO_2 incubator by including 3 μM of the acetoxymethyl ester of mag-fura-2 (mag-fura-2 AM; Molecular Probes, Eugene, OR) for 20 min in a HCO_3^--buffered solution containing (in mM): 110 NaCl, 4.5 KCl, 1 NaH_2PO_4, 1 $MgSO_4$, 1.5 $CaCl_2$, 5 HEPES-Na, 5 HEPES-free acid, 25 $NaHCO_3$, and 10 D-glucose (pH 7.4). Cells were then rinsed twice and incubated in the HCO_3^--buffered solution for at least 20 min before use.

$[Zn^{2+}]_i$ was measured on the stage of an inverted microscope (Nikon, Tokyo, Japan) by spectrofluorometry (Photon Technology International, New Brunswick, NJ) while cells were superfused at a constant perfusion rate of 2 ml/min with HCO_3^--buffered solution equilibrated with 95% O_2-5% CO_2 to maintain a pH of 7.4. All experiments were performed at 37°C. The excitation wavelength alternated between 340 and 380 nm, and emission fluorescence was recorded at 510 nm. The values of $[Zn^{2+}]_i$ were calculated by using a modification of the equation described by Grynkiewicz et al. (12): $[Zn^{2+}]_i = K_d \cdot Sf2/Sb2[(R - R_{min})/(R_{max} - R)]$, in which K_d of mag-fura-2 for Zn^{2+} was assumed to be 20 nM (36), R is the observed 340/380 fluorescence ratio, R_{min} is the 340/380 fluorescence ratio value determined in mag-fura-2-loaded HT-29 cells exposed to 100 μM TPEN, R_{max} is the 340/380 fluorescence ratio value [obtained by adding 20 μM 1-hydroxypyridine-2-thione (pyrithione; Sigma, St. Louis, MO), a Zn^{2+} ionophore, in the presence of 1 mM Zn^{2+}], Sf2 is the fluorescence intensity at 380 nm at R_{min}, and Sb2 is the fluorescence intensity at 380 nm at R_{max}.

Statistical analysis. The statistical significances of differences between the positive- and negative-regulated groups were determined by using a one-way ANOVA and Bonferroni's test. A P value <0.05 was considered statistically significant. The results are expressed as means ± SD.

RESULTS

HT-29 cell growth and proliferation were positively or negatively regulated by 5–100 μM of $ZnCl_2$ in MSSM. The number of cells was measured after the extracellular zinc treatments to determine the role of zinc in regulating the growth of HT-29 cells. The numbers of HT-29 cells increased up to 72 h after a $ZnCl_2$ treatment at <10 μM in MSSM containing 1% FBS (Fig. 1*A*). On the other hand, the number of cells decreased after increasing the $ZnCl_2$ concentration to 80–100 μM (Fig. 1*A*). Unlike the cells grown in MSSM, the number of cells increased as a result of the treatments in the range of 5–100 μM $ZnCl_2$ in a medium containing 10% FBS (Fig. 1*B*). The lack of growth inhibition by 100 μM $ZnCl_2$ in the medium containing 10% FBS suggests that $ZnCl_2$ at this concentration is not toxic to HT-29 cells. Because 10 and 100 μM $ZnCl_2$ resulted in the most significant effects in terms of the positive and negative regulation of HT-29 cell growth in MSSM, we used these two zinc concentrations in further studies involving MSSM.

The level of DNA synthesis was measured by determining the extent of [^{3}H]thymidine incorporation and was found to increase in cells by more than twofold on adding 10 μM $ZnCl_2$ to MSSM (Fig. 1*C*). The amount of thymidine incorporated by 10 μM $ZnCl_2$ was only slightly lower than that level acquired by growing cells in medium containing 10% FBS without zinc treatment (Fig. 1*C*). However, the thymidine incorporation level was reduced by adding 100 μM $ZnCl_2$ to cells grown in MSSM.

Positive and negative regulation of zinc-treated HT-29 cell growth by PD-98059 in MSSM. The ERK pathway is involved in cell cycle arrest (7, 35, 40) and is well known to be a function of cell cycle progression (4, 7). The ERKs were previously found to be strongly activated in a prolonged manner by 100 μM $ZnCl_2$ treatments in MSSM, and this activation was related to the antiproliferation of colorectal cancer cells, including HT-29 cells (28). However, ERK activities were also found to be transiently and weakly activated by extracellular zinc treatment (14, 33). This growth regulation was not further investigated with respect to differential ERK regulation. In this study, we investigated the growth and proliferation induced by the

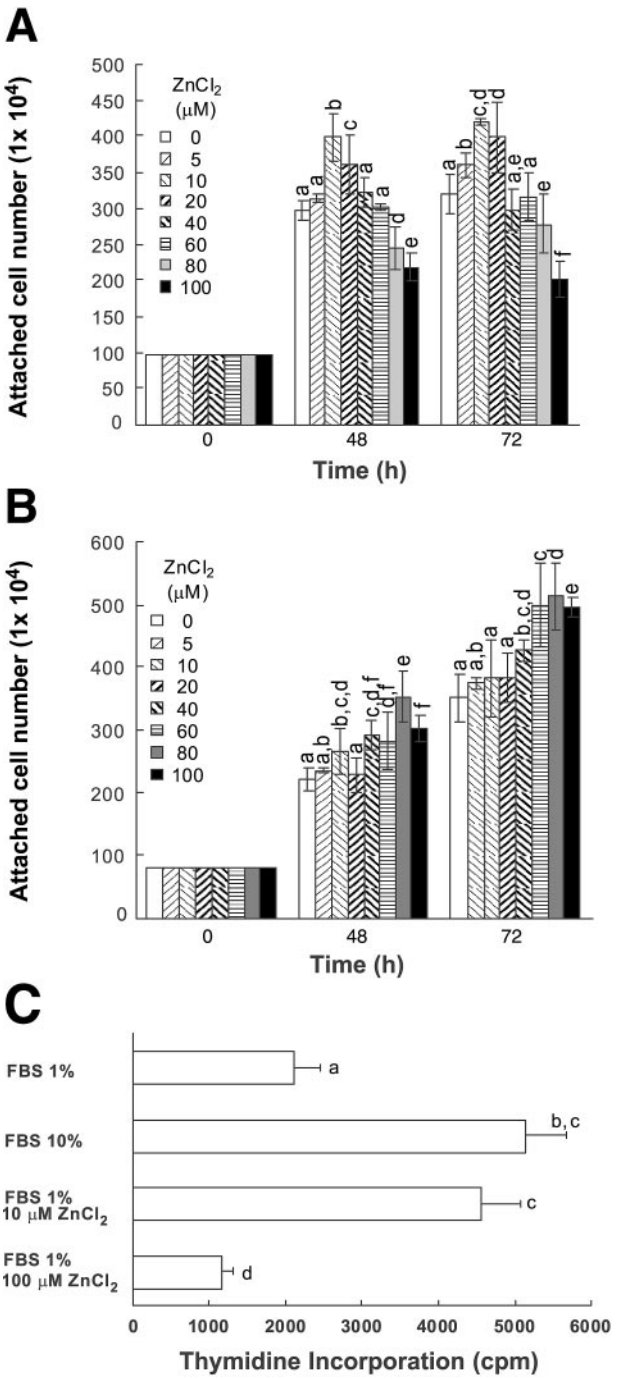

Fig. 1. Effects of different concentrations of extracellular zinc on HT-29 cell growth. Positive and negative regulation of cell growth and proliferation were affected by extracellular zinc treatment. The HT-29 colorectal cells were minimal serum starved (the cells were grown in McCoy's 5A media containing 1% FBS, called MSSM) for 16 h, and the medium was replaced with MSSM (*A*) or 10% FBS medium (*B*) with different concentrations of $ZnCl_2$ (0, 5, 10, 20, 40, 60, 80, and 100 μM). The numbers of attached cells were counted at 0, 48, and 72 h after zinc treatments. *C*: HT-29 cells were cultured as shown in *A* in MSSM, and [^{3}H]thymidine levels were measured 48 h after replacing medium with media containing different concentrations of zinc as described in METHODS. Values are means ± SD. Means with different lowercase letters are significantly different at $P < 0.05$.

different zinc concentrations and their relationship with the ERK activation profiles.

Cell numbers, which had been previously increased by 10 μM $ZnCl_2$ in MSSM, were dose-dependently decreased by treatment with the MEK inhibitor PD-98059 (Fig. 2*A*), suggesting that ERK activation affects the proliferation of HT-29 cells by 10 μM $ZnCl_2$ in MSSM. Surprisingly, cell numbers reduced by 100 μM $ZnCl_2$ treatment in MSSM were increased by subsequently treating the cells with 1 μM PD-98059 (Fig. 2*B*). These results suggest that the ERK activation by 100 μM $ZnCl_2$ in MSSM, which caused growth inhibition (28), was blocked by 1 μM PD-98059. A further increase in PD-98059 to 5 and 10 μM in the cells treated with 100 μM $ZnCl_2$ resulted in a further reduction in the number of cells (Fig. 2*B*). These results suggest that the ERK pathway may be involved in both the positive and negative regulation of cell growth and further suggest that the zinc-induced growth regulation of HT-29 cells may be highly sensitive to the level of ERK activation. The upregulation of cell numbers by PD-98059 in 100 μM $ZnCl_2$-treated cells in MSSM confirms that 100 μM $ZnCl_2$ is not a toxic concentration to HT-29 cells. The thymidine incorporation level, which was increased by adding 10 μM $ZnCl_2$, was also dose-dependently inhibited by pretreating the cells with PD-98059 in MSSM (Fig. 2*C*). However, the thymidine incorporation level was also increased by the 1 μM PD-98059 cotreatment in the cells treated with 100 μM $ZnCl_2$ and was reduced by cotreatment with 10 μM PD-98059 (Fig. 2*C*) compared with 1 μM PD-98059 cotreatment. Therefore, cell numbers in this system were found to be highly correlated with proliferation status. The differential modulations of cell growth and proliferation by PD-98059 suggest that the ERK pathway participates in both the proliferation and the antiproliferation of cellular growth.

The cyclin D1 and p21$^{Cip/WAF1}$ are differentially induced by 10 and 100 μM $ZnCl_2$ in MSSM and are dependent on ERK activations. The ERK pathway is involved in the progression and inhibition of the G_1-to-S phase progression by the differential activations of Raf-1 → MEK → ERK cascade kinases, which is followed by the differential induction of cell cycle regulators such as cyclin D1 and p21$^{Cip/WAF1}$ (35, 40).

In this study, we monitored the inductions of cyclin D1 and p21$^{Cip/WAF1}$ at 10 min and 9 h after treating HT-29 cells with 10 and 100 μM $ZnCl_2$ in MSSM, which induced transient and prolonged ERK activations, respectively (28). Cyclin D1 was induced 9 h after treatment with 10 or 100 μM $ZnCl_2$, and this was higher in cells treated with the higher concentration of $ZnCl_2$ (Fig. 3). On the other hand, the p21$^{Cip/WAF1}$ was significantly induced only in cells treated with 100 μM $ZnCl_2$ (Fig. 3). The cyclin D1 and p21$^{Cip/WAF1}$ inductions by 100 μM $ZnCl_2$ were reduced by subsequently treating cells with PD-98059 (Fig. 4), which suggests that both cyclin D1 and p21$^{Cip/WAF1}$ inductions are dependent on ERK activations.

The effects of zinc on ERK activation, as well as cyclin D1 and p21$^{Cip/WAF1}$ induction, resulted in different

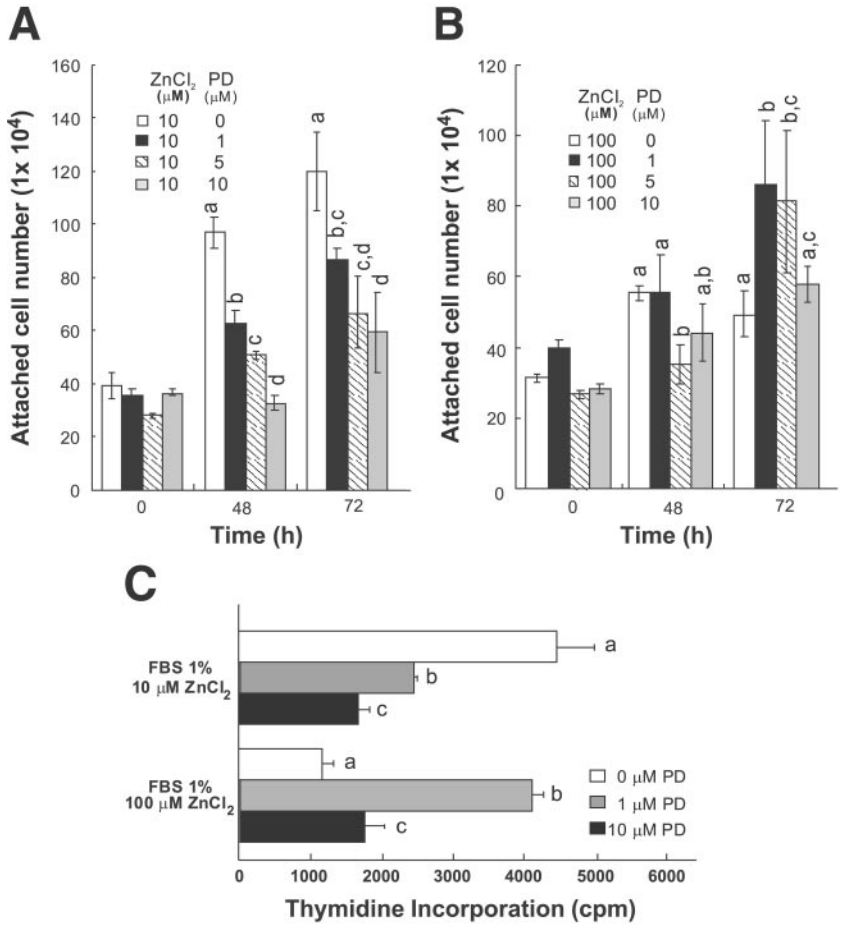

Fig. 2. Effects of PD-98059 on the zinc-induced positive and negative regulations of cell growth and proliferation. Essentially, these experiments are the same as those in Fig. 1*A*. In the required cases, cells were incubated with different PD-98059 (PD) concentrations (0, 1, 5, or 10 μM) for 30 min before treatment with 10 μM $ZnCl_2$ (*A*) or 100 μM $ZnCl_2$ (*B*). *C*: [^{3}H]thymidine was measured 48 h after treatment with zinc. In the required cases, the cells were incubated with 1 or 10 μM PD-98059 for 30 min before zinc treatment. Values are means ± SD. Means with different lowercase letters are significantly different at $P < 0.05$.

sensitivities to the zinc concentrations in an extracellular serum concentration-dependent manner. In contrast to the cells grown in MSSM, we did not observe inhibition of cell growth by 100 μM $ZnCl_2$ in 10% FBS medium (Fig. 1*B*). To further characterize the role of ERK activation in zinc-induced growth regulation, we measured the effects of 100 μM $ZnCl_2$ on ERK activation and the subsequent cyclin D1 and $p21^{Cip/WAF1}$ inductions in media containing 10% FBS. We did not observe prolonged ERK activation by 100 μM $ZnCl_2$ in this medium; only transient ERK activation was observed 10 min after the zinc treatment (Fig. 5). Furthermore, we observed the weak induction of cyclin D1 9 h after treatment with 100 μM $ZnCl_2$ in medium containing 10% FBS, without significant $p21^{Cip/WAF1}$ induction. These observations of the effects of zinc, including prolonged ERK activation and $p21^{Cip/WAF1}$ induction in MSSM, may have been caused by an inhibitory factor(s) present in the serum or because the effect of zinc may have been attenuated by signaling homeostasis of the cells grown in 10% FBS medium. Both the transient and prolonged ERK activations, detected within 10 min and 9 h of zinc treatment, respectively, were redetected in cells grown in 10% FBS medium containing 500 μM $ZnCl_2$ (Fig. 5). In addition, cyclin D1 and $p21^{Cip/WAF1}$ inductions were also observed in cells grown in 10% FBS medium con-

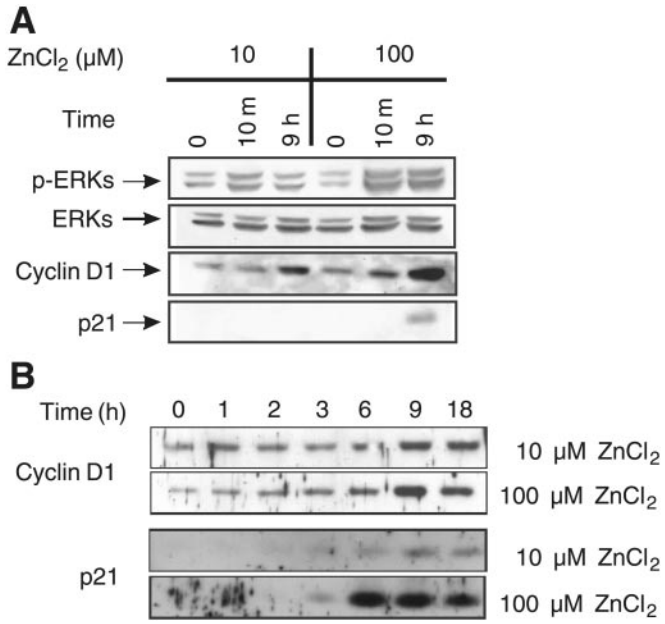

Fig. 3. ERK activations and inductions of cyclin D1 and $p21^{Cip/WAF1}$ by 10 and 100 μM $ZnCl_2$. *A*: serum-starved HT-29 cells grown in MSSM were treated with 10 or 100 μM $ZnCl_2$. The cells were then harvested at different time points (0 min, 10 min, and 9 h), and Western blot analysis was performed using phospho-ERK (p-ERK), ERK, cyclin D1, or $p21^{Cip/WAF1}$ (p21) antibodies. *B*: same experiment as in *A* except that samples were harvested over a greater period of time to detect cyclin D1 or $p21^{Cip/WAF1}$.

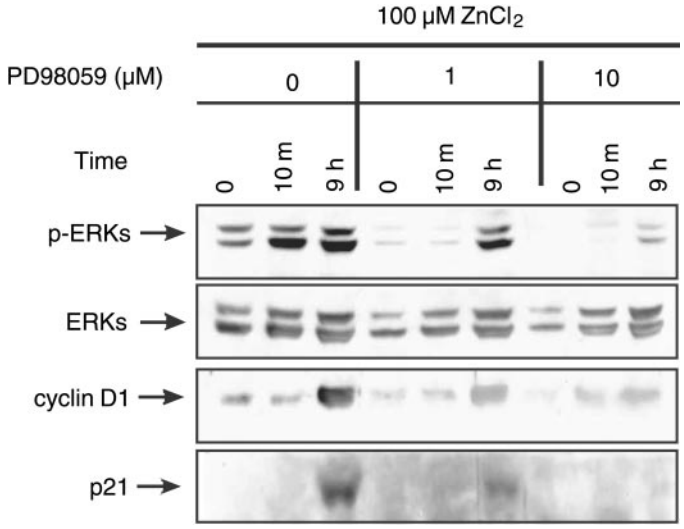

Fig. 4. Effects of PD-98059 on cyclin D1 and $p21^{Cip/WAF1}$ induction. Serum-starved HT-29 cells grown in MSSM were treated with 100 μM $ZnCl_2$. Cells were incubated with 1 or 10 μM PD-98059 for 1 h before zinc treatment. Cells were then harvested, and Western blot analysis was performed as described in Fig. 3*A*.

taining 500 μM $ZnCl_2$. The patterns of ERK activations and subsequent cyclin D1 and $p21^{Cip/WAF1}$ inductions by 100 and 500 μM $ZnCl_2$ in 10% FBS medium were similar to the patterns observed in cells grown in MSSM containing 10 and 100 μM $ZnCl_2$, respectively (Fig. 3*A*).

Differential growth regulations by zinc in 10% FBS medium were associated with prolonged ERK activation and subsequent $p21^{Cip/WAF1}$ induction. To measure the differential effects of zinc in more detail, HT-29 cells were treated with a range of $ZnCl_2$ concentrations (0, 100, 200, 300, and 500 μM) in 10% FBS medium, and the effects of different zinc concentrations on cell growth were measured. As observed in MSSM, the positive and negative regulations of cell growth were also observed by the higher zinc concentrations in the 10% FBS medium. The number of HT-29 cells was increased by 100 μM $ZnCl_2$ (Fig. 6*A*). However, the cell numbers were unaffected by 200 and 300 μM $ZnCl_2$ and were reduced as a result of the 300 and 500 μM $ZnCl_2$ treatments (Fig. 6*A*). $p21^{Cip/WAF1}$ protein levels were found to be increased in 10% FBS medium by only 200–500 $ZnCl_2$, and this occurred concomitantly with prolonged ERK activation (Fig. 6*B*). On the other hand, cyclin D1 protein induction was weak at 100 μM $ZnCl_2$ and at higher zinc concentrations (200–500 μM $ZnCl_2$) in 10% FBS medium. Both cyclin D1 and $p21^{Cip/WAF1}$ inductions by zinc in 10% FBS medium were inhibited by pretreatment with 10 μM PD-98059 (Fig. 6*B*).

Extracellular zinc was not significantly taken up into HT-29 cells. To identify uptake level of extracellular zinc in HT-29 colorectal cancer cells, we measured the

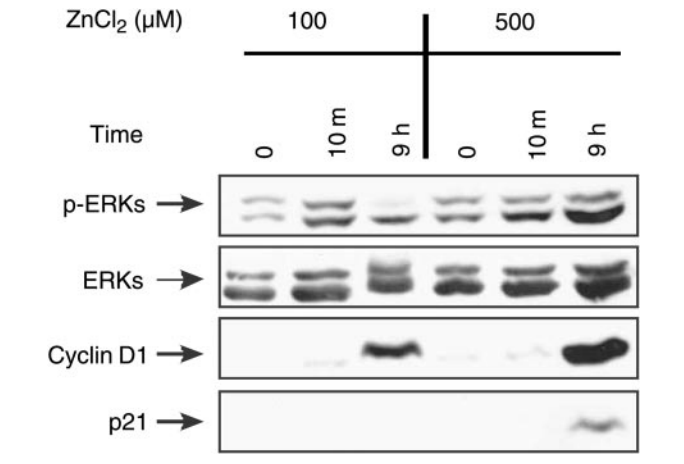

Fig. 5. Prolonged ERK activations and cyclin D1 and $p21^{Cip/WAF1}$ induction were not observed in 10% FBS medium but were observed again at a higher extracellular zinc level. Experiments shown are essentially the same as in Fig. 3*A* except for the higher $ZnCl_2$ concentrations (100 or 500 μM) and that 10% FBS medium was used.

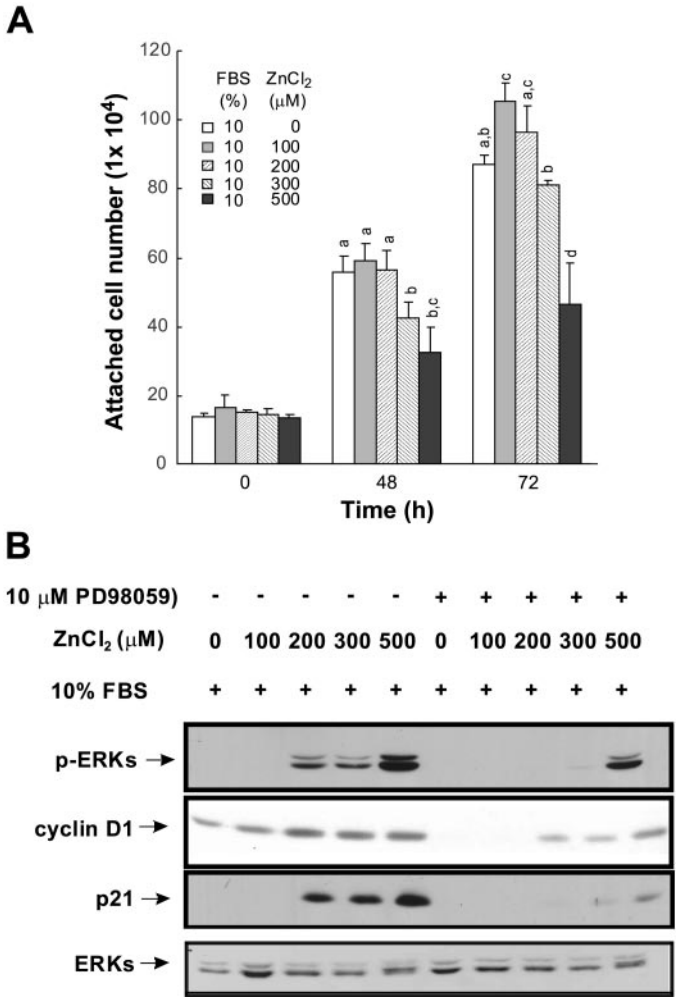

Fig. 6. Positive and negative regulations of cell growth and the differential activation of ERK and induction of $p21^{Cip/WAF1}$ in 10% FBS medium by $ZnCl_2$ at concentrations > 100 μM $ZnCl_2$. *A*: experiments are as in Fig. 1*B* except that higher $ZnCl_2$ concentrations (0, 100, 200, 300, and 500 μM) were used. Values are means ± SD of 3 independent experiments. Means with different lowercase letters are significantly different at $P < 0.05$. *B*: experiments are essentially the same as in Fig. 5, except that a broader range of $ZnCl_2$ concentrations (0, 100, 200, 300, or 500 μM) was used. When required, 10 μM PD-98059 was added 1 h before zinc treatment. Cells were harvested at 9 h after treatment with zinc for Western blot analysis.

intracellular zinc levels after adding 10 or 100 μM $ZnCl_2$ to cells grown in MSSM and after adding 500 μM $ZnCl_2$ to cells grown in 10% FBS medium. Intracellular zinc levels were not found to be significantly increased by treating cells grown in MSSM with 10 and 100 μM $ZnCl_2$ or in cells grown in 10% FBS containing 500 μM $ZnCl_2$ (Fig. 7). However, the intracellular zinc levels started to increase soon after adding the Zn^{2+} ionophore pyrithione to cells previously treated with 100 or 500 μM $ZnCl_2$. Therefore, the high levels of zinc applied to the culture medium were not significantly taken up by HT-29 colorectal cancer cells regardless of the serum levels, and ERK activation may have been effected via an extracellular zinc sensor such as ZnR (15) rather than via the activation of intracellular zinc-sensing protein(s).

DISCUSSION

Zinc is a trace element in the human body and is known to participate like a growth factor in cellular proliferation (13, 27, 30). However, zinc has also been reported to have a growth-inhibitory role (3, 9, 19). The physiological roles and the mechanisms of zinc participation in the regulation of cell growth are not yet fully understood. The intestinal mucosa undergoes continuous renewal and needs zinc constantly (39). In addition, zinc was suggested to be a primary determinant of risk in human colorectal cancer (5, 24, 25, 37), and low zinc intake may increase the incidence of colorectal cancer (22). Therefore, it is possible that zinc levels in the extracellular plasma environment may affect the growth of colorectal tumors (1, 18).

We recently identified growth arrest in colorectal cancer cells grown in MSSM containing 100 μM $ZnCl_2$ and found that this may have been mediated by the activation of $p21^{Cip/WAF1}$ via ERK pathway activation (28). In the current study, we also observed the growth stimulation of HT-29 colorectal cancer cells in MSSM containing 10 μM $ZnCl_2$ and in MSSM with 10% FBS containing 100 μM $ZnCl_2$. Therefore, extracellular zinc is involved in both the positive and negative regulation of colorectal cancer cell growth, and the direction of this regulation is dependent on the levels of extracellular zinc. The pH was slightly changed during the culture period and was not significantly affected by zinc or FBS concentrations. Therefore, the differential cell growth induced by zinc may not have been caused by nonspecific effects such as pH change.

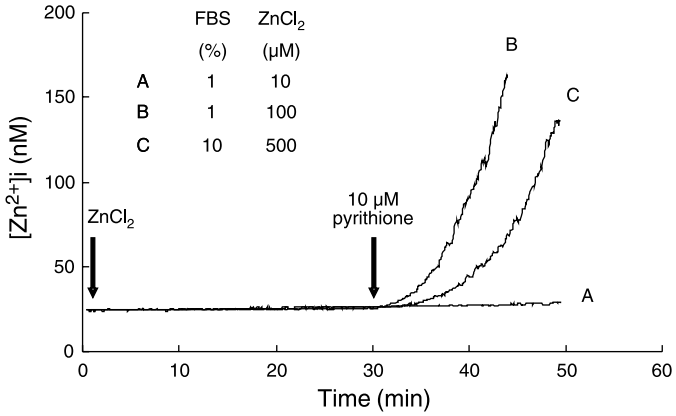

Fig. 7. Profile of intracellular zinc accumulations caused by extracellular zinc treatment in HT-29 cells and the effect of 1-hydroxypyridine-2-thione (pyrithione). Cells were grown on coverslips MSSM or 10% FBS medium and were treated with $ZnCl_2$ at 1, 100, or 500 μM. Intracellular zinc concentration ($[Zn^{2+}]_i$) was monitored vs. time. Changes in $[Zn^{2+}]_i$ were measured by using a ratiometric fluorescence-recording technique (33). After 30 min, 10 μM pyrithione was added and zinc uptake was monitored.

To identify an intracellular signaling mechanism for the differential regulation of cell growth by extracellular zinc, we used HT-29 colorectal cancer cells, which retain putative ZnR (15). These observations of the positive and negative regulations of HT-29 cell growth and proliferation by 10 and 100 μM $ZnCl_2$ in MSSM are surprising and suggest that the extracellular zinc-mediated signal transduction in colorectal cancer cells is highly dependent on the extracellular zinc level. Moreover, in MSSM, this cell growth switching point lies in the range of 10–20 μM $ZnCl_2$. The positive and negative regulations of HT-29 cell growth by 10 and 100 μM $ZnCl_2$ were further modulated by subsequent treatment with PD-98059, suggesting the involvement of the ERK pathway. The growth inhibition of HT-29 cells by 100 μM $ZnCl_2$ in MSSM was abrogated by 1 μM PD-98059, which suggests that at 100 μM $ZnCl_2$ is not toxic to HT-29 cells. Moreover, the ERK pathway is known to be involved in both cellular proliferation and growth arrest (35, 40). In those studies, only cyclin D1 was weakly induced by the moderate activation of the ERK pathway, and this induced G_1-to-S phase cell cycle progression. However, both cyclin D1 and $p21^{Cip/WAF1}$ were substantially induced by strong ERK pathway activation, and this blocked G_1-to-S phase progression (40).

The patterns of cyclin D1 and $p21^{Cip/WAF1}$ inductions by 10 and 100 μM $ZnCl_2$ in MSSM were similar to those reported in a study of the transient and sustained activation of the ERK pathway (40). The inhibition of cyclin D1 and $p21^{Cip/WAF1}$ induction by PD-98059 suggest that these inductions occur via ERK activation. Furthermore, the regulation of cell growth and proliferation by different zinc treatments was highly correlated with the G_1-to-S phase progression observed for different levels of ERK pathway activation (16, 40). Therefore, we suggest that the ERK pathway is involved in the positive and negative regulation of cell growth and proliferation by extracellular zinc and that this regulation is achieved via the differential inductions of cell cycle regulators, such as cyclin D1 and $p21^{Cip/WAF1}$. It is not known why cyclin D1 was induced with $p21^{Cip/WAF1}$ in growth- and proliferation-inhibited cells. The effects of zinc on ERK activation and cyclin D1 and $p21^{Cip/WAF1}$ induction were found to be highly sensitive to serum levels and were not observed in cells grown in 10% FBS medium containing 10 and 100 μM $ZnCl_2$. However, the positive and negative regulation of cell growth were reobserved in 10% FBS medium containing 100 and 500 μM $ZnCl_2$. These results suggest that the lack of the differential zinc

effects in cells treated with 10 and 100 μM $ZnCl_2$ in 10% FBS medium may be due to an intracellular homeostasis, which reduces zinc sensitivity.

Correlation between $p21^{Cip/WAF1}$ induction and prolonged ERK activation and the inhibition of $p21^{Cip/WAF1}$ induction by PD-98059 confirmed that $p21^{Cip/WAF1}$ induction occurs after prolonged ERK activation. The serum-modulated effects of zinc on cyclin D1 and $p21^{Cip/WAF1}$ induction were found to correlate well with cellular growth. For example, cellular growth was not inhibited but slightly enhanced by 100 μM $ZnCl_2$ in 10% FBS medium, and under this condition cyclin D1 was induced and $p21^{Cip/WAF1}$ was not. On the other hand, the level of cell growth was reduced by treatment with 300–500 μM $ZnCl_2$, where the $p21^{Cip/WAF1}$ levels were most increased.

The zinc concentrations (100–500 μM of $ZnCl_2$) used in the present study to activate ERKs and differential cell growth in media containing 10% FBS were higher than the zinc levels in plasma and in the luminal surface of enterocytes, where relatively high zinc concentration was observed (11). However, we found that intracellular zinc levels were not increased by treatment with 100–500 μM $ZnCl_2$. These results suggest that the zinc-mediated differential growth regulation of HT-29 colorectal cancer cells may be achieved via extracellular receptor-mediated ERK pathway activation and the subsequent induction of cell cycle regulators such as $p21^{Cip/WAF1}$.

Our identification of positive and negative growth regulation of colorectal cancer cells by extracellular zinc and other epidemiological studies related to zinc (9, 10, 26, 37) suggest that an adequate intake and the right dosage of zinc is important to maintain good health and prevent disease.

DISCLOSURES

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