Prolonged ERK Activation by Extracellular Zinc Inhibits the Proliferation of Colorectal Cancer Cells by Activating $p21^{Cip/WAF1}$

Thesis by
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Brain Korea 21 Project for Medical Sciences

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Directed by Professor Yong Ho Ahn

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Abstract

Prolonged ERK Activation by Extracellular Zinc Inhibits the Proliferation of Colorectal Cancer Cells by Activating $p21^{Cip/WAF1}$

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(Directed by Professor Yong Ho Ahn)

Numerous studies have been reported on the involvement of dietary zinc, which is an important trace element in the body in the proliferation of cells related with cancer. However, the role of zinc in arresting cell growth related to an anti-tumor effect has also been reported. In this study, a mechanism for growth arrest of colorectal cancer cell by extracellular zinc was identified. Zinc activated ERKs in two different phases in HT29 human colorectal cells, the initial weak activation followed by strong and prolonged activation after several hours. The prolonged activation of ERKs by extracellular zinc was acquired through the Raf-1→MEK→ERK module by a positive regulatory loop, and induced Elk-1-dependent *trans*-reporter gene expression. The cell growth and [^{3}H] thymidine incorporation were inhibited by zinc, and that related with p53 independent induction and nuclear localization of the $p21^{Cip/WAF1}$. The inhibition of the prolonged ERK

activation by pre-treatment of the cells with PD98059 or transfection of catalytically inactive MEK resulted in the inhibition of induction and nuclear localization of the $p21^{Cip/WAF1}$. Therefore, the growth arrest of the cells by extracellular zinc was related with the prolonged ERK activation dependent induction of the $p21^{Cip/WAF1}$.

Key Words : Zinc, ERK, $p21^{Cip/WAF1}$

Prolonged ERK Activation by Extracellular Zinc Inhibits the Proliferation of Colorectal Cancer Cells by Activating $p21^{Cip/WAF1}$

<Directed by Professor **Yong Ho Ahn**>

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I. Introduction

Zinc has been known to be an important trace element in the body and an optimal availability of zinc is essential for normal growth and proliferation of cells. The effect of zinc on cell growth varies, depending upon the level of zinc and types of cells.[1] However, the mechanisms of cell growth regulation by zinc are unclear. In a recent study, it has been reported that zinc can inhibit prostatic carcinoma cell growth by the induction of the cyclin-dependent kinase inhibitor, $p21^{Cip/WAF1}$ (Ref 2). Zinc can also induce the activation of the extra-cellular signal regulated kinase (ERK) pathway in

Swiss 3T3 fibroblasts and bronchial epithelial cells.[3,4] However, the mechanisms and physiological roles involved in $p21^{Cip/WAF1}$ induction and ERK activation by zinc, including the regulation cell growth, have not been clearly elucidated.

$p21^{Cip/WAF1}$ inhibits cell cycle progression through its interaction with cyclin-dependent kinase complexes, which are required for various cell cycle transitions, to induce growth arrest or differentiation of cells.[5,6] An important role of $p21^{Cip/WAF1}$ in the protection of cells against apoptosis has also been proposed in the colorectal carcinoma cell line.[7] Recent studies have demonstrated that $p21^{Cip/WAF1}$ expression is induced by both p53 and ERK pathway dependent mechanism.[8]

ERK is involved in proliferative signaling triggered by a diverse group of exracellular stimuli, which generally are coupled with Ras activation.[9-12] ERK functions in many different cell types and mediates various cellular responses.[13-15] There are accumulating evidences suggesting that the duration and intensity of ERK activity can significantly influence on the biological responses.[9-14] Whereas the mild and transient activation of ERK followed by low Raf-1 activation causes cell-cycle progression, the strong and prolonged activation of ERK followed by high Raf-1 activation causes cell-cycle arrest.[16-19] The differential regulation of ERK activity was observed during human colorectal tumorigenesis and high levels of ERK activity were observed in normal colorectal epithelial tissue, as compared with the paired tumor tissue.[20] These results suggest the high ERK activity may be involved in the growth regulation of colorectal cells.

In this study, a mechanism for the negative regulation of human colorectal cancer cell growth by extracellular zinc and its relationship with ERK pathway activation were identified. Here, the inducibility and nuclear localization of a cell-cycle regulator $p21^{Cip/WAF1}$, which is dependent upon the activation of the ERK pathway, were identified as important indicators of the negative growth behavior of the cells by zinc.

II. Materials and Methods

1. Materials

HT29 (ATCC HTB-38), DLD-1 (ATCC CCL-221), and HCT116 (ATCC CCL-247) human colorectal cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). McCoy's 5A and RPMI1640 media, fetal bovine serum (FBS), antibiotics and Lipofectamin plus reagent were purchased from Life Technologies, Inc. (Grand Island, NY, USA). The Tri Reagent used for RNA isolation was purchased from Molecular Research Center (Cincinnati, OH, USA). Phospho-ERK, phospho-MEK antibodies, and the ERK kinase assay kit were obtained from Cell Signaling Biotechnology (Beverly, MA, USA), and ERK antibody was from Stratagene (La Jolla, CA, USA). The $p21^{Cip/WAF1}$ antibody was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA), and Raf-1 antibody, horseradish peroxidase (HRP)-conjugated goat anti-rabbit and anti-mouse IgG antibodies were from Transduction Laboratories (Lexington, KY, USA). The Protran nitrocellulose membrane and nylon membrane were purchased from Schleicher and Schuell Co. (Dassel, Germany), and the enhanced chemiluminescence (ECL) system from Genepia (Seoul, Korea). The hyper-sensitive X-ray films and Radiprime II random prime labeling system were from Amersham Inc. (Buckinghamshire, UK), Elk-1-dependent

pathdetect plasmids (pFR-Luc and pFA2-Elk1) were from Stratagene (La Jolla, CA, USA), the Luciferase assay kit and U0126 were from Promega Co. (Madison, WI, USA), PD98059 was from Calbiochem (La Jolla, CA, USA), the protein assay solution was from Bio-Rad Laboratories (Hercules, CA), 4',6'-diamidine-2'-phenylindole dihydrochloride (DAPI) was from Boehringer Mannheim (Mannheim, Germany), the goat anti-mouse-Cy™2- or goat anti-rabbit Rhodamin Red ™-X-conjugated secondary antibodies were purchased from Jackson Immuno Research Laboratories Inc. (West Grove, PA, USA), and finally the the anti-BrdU monoclonal antibody was purchased from DAKO Co. (Carpinteria, CA, USA).

2. Cell Culture

HT29[21] and HCT116[22] colorectal cell lines were maintained in McCoy's 5A medium supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin and 100 μg/ml of streptomycin in 5% CO_2 at 37°C. The DLD-1 cells[22] were cultured with RPMI1640 medium using the same conditions and supplements. Experiments were performed on the cells at 70% confluence, unless otherwise stated. To observe the effects of zinc, the cells were grown in a media (McCoy's 5A or RPMI1640) containing 1% FBS for 16 to 20 hr and then treated with 100 μM of $ZnCl_2$. Thirty minutes prior to the $ZnCl_2$ treatment, the MEK inhibitor, PD98059 or U0126, was

added to 1-50 μM in the required cases.

3. Cell Transfection

HT29, HCT116 and DLD-1 cells were plated into 6-well plates at 4×10^5 cells per well using McCoy's 5A or RPMI1640 media supplemented with 10% FBS, 100 units/ml of penicillin and 100 μg/ml of streptomycin. After allowing 24 hr for growth, cells were transfected with plasmids using Lipofectamine plus reagent according to the manufacturer's instructions.

4. Cell Counting and Thymidine Incorporation

HT29 cells were seeded into 12-well plates at 0.8×10^5 cells per well. After growing for 24 hr in McCoy's 5A, the cells were placed in the same medium containing 1% FBS for 16-20 hr. Then 100 μM of $ZnCl_2$ was added into the medium. When required, PD98059 was also added 30 min before the $ZnCl_2$ treatment. Cells were incubated for the indicated times at 37°C. The attached cells were washed with phosphate buffered saline (PBS), and harvested using trypsin (0.05% trypsin, 0.5 mM EDTA in PBS), and finally the cell numbers determined. The cell numbers were

counted by mounting 10 µl of the cell mixture (1:1 mixture of 0.4% trypan blue and HT29 cells suspension) onto a Tiefe Depth Profondeur 0.0025 mm^2 cell counting plate (Superior Co., Germany) under an optical microscope. For thymidine incorporation, the HT29 cells were plated into 12-well plates with 0.8 x 10^5 cells per well. After growing overnight, cells were starved with fresh media for 16-20 hr. The medium was then replaced with same medium containing 100 µM of $ZnCl_2$ and the cells were further incubated for 48 hr. Finally, the incubations were continued in the presence of [^{3}H] thymidine (0.5 µCi/well) for 12 hr. After the incubation period, the medium was removed, and the cell monolayer was washed with ice-cold PBS and then detached by treatment with 1 ml of 0.05% trypsin-0.5 mM EDTA. Then, cells were centrifuged and washed three times with PBS. After addition of 3% perchloric acid, the acid-precipitable material was dissolved overnight in 1 N NaOH, 1% SDS and counted by liquid scintillation and the DNA-associated [^{3}H] activity was counted in a liquid scintillation counter.

5. Extract Preparation

The cells were rinsed twice with PBS and harvested by scraping the cells into 500 µl of ice-cold PBS. They were then centrifuged and resuspended in 300 µl of lysis buffer (70 mM β-glycerophosphate pH 7.2, 0.1 mM each meta- and ortho-vanadate, 2

mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 0.5% Triton X-100, 0.2 mM phenylmethylsulphonyl fluoride, and 5 μg/ml each of pepstatin A, chymostatin, leupeptin and peptin). After incubation on ice for 30 min, the lysate was sonicated for 20 sec on ice and unbroken cell debris removed by centrifugation at 23,000 g for 15 min. Samples were aliquoted immediately and stored at −80°C after protein quantification.

6. Western Blot Analysis and *in vitro* ERK Kinase Assay

Samples of protein (10-50 μg) from whole-cell extracts were separated by 8-10% sodium dodecyl sulfate (SDS) polyacrylamide gel (acrylamide: bis-acrylamide at a ratio of 29:1) and Western blot analysis was performed as described previously.[20] Activation of endogenous ERK and MEK was analyzed using phospho-specific anti-ERK and anti-MEK-1 antibodies, respectively. The expression of ERK was analyzed using anti-ERK antibody, and the expression and modification of Raf-1 were analyzed using anti-Raf-1 antibody, and levels of the $p21^{Cip/WAF1}$ proteins were determined using anti-$p21^{Cip/WAF1}$ antibody. Blots were probed with HRP-conjugated goat anti-rabbit and anti-mouse IgG secondary antibodies, and bands were visualized using ECL. The $p21^{Cip/WAF1}$ antibody detected $p21^{Cip/WAF1}$ protein as a single band when Western blots were performed. ERK kinase assay was performed *in vitro* using the ERK kinase assay

kit with GST-Elk-1 as a substrate as described by the manufacturer.

7. Elk-1 Dependent *trans*-Reporting System and Luciferase Assay

To determine the activation status of ERK pathway *in vivo*, we adapted Elk-1 dependent *trans*-reporting luciferase assay system, using a reporter vector (pFR-Luc) containing five tandom repeats of GAL4 binding elements and a basic promoter element (TATA box), followed by a coding sequence of firefly luciferase, and a fusion *trans*-activator plasmid (pFA2-Elk-1) expressing a *trans*-activator protein consistent with the yeast GAL4 binding domain and the activation domain of *trans*-activator Elk-1. HT29, HCT116 or DLD-1 cells were co-transfected with 0.5 μg of reporter plasmid pFR-Luc along with 25 ng of *trans*-activator pFA2-Elk-1 as described above. For normalization, the cells were co-transfected with plasmid containing the gene for β-galactosidase under the control of a CMV promoter. The transfected cells were transferred into the media for an additional 16 hr, and this was replaced with fresh media with or without 100 μM of $ZnCl_2$. The extracts were prepared 12 hr after treatment with the zinc. Luciferase activity was measured using the Luciferase assay kit, and normalized using the β–galactosidase level as an internal control. When required, 0.5 μg of pCMV-MEK-2A, pSV-Sport1-Raf-1-dn or the empty vector was co-transfected to express catalytically inactive MEK or dominant negative Raf-1,

respectively.

8. Northern Blot Analysis

Total RNAs were prepared by using Tri Reagent as recommended by the manufacturer. 20 μg of total RNA was resolved on a 1% denaturing agarose gel containing 6% formaldehyde, and the RNA was transferred onto a nylon membrane and UV-cross-linked. The membrane was pre-hybridized with hybridization buffer and then hybridized at 65°C for 2 hr with $p21^{Cip/WAF1}$ probes. The membrane was then washed four times for 15 min with 0.2X SSC, 0.1% SDS at 65°C. Membrane was placed under film and exposed for 4 hr at –80°C. As a control, the blot was stripped by boiling in 1% SDS and re-probed with ^{32}P-labeled cDNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ^{32}P-labeled probes were generated by the Rediprime II random prime labeling system using human $p21^{Cip/WAF1}$ (Ref 23) or GAPDH[24] cDNAs as a template, respectively.

9. Immunocytochemistry and BrdU Incorporation

For immunocytochemistry, HT29 cells were plated onto cover slips at a density of 2 x 10^5 cells/coverslip into 6-well plates. The cells were grown overnight and starved

with media containing 1% FBS for 16-20 hr and treated with 100 μM $ZnCl_2$ for 9 hr before being subjected to immunocytochemistry. In required cases, they were transfected with 0.5 μg of pCMV-MEK-2A. After transfection, the cells were starved with media containing 1% FBS in and treated with 100 μM of $ZnCl_2$ as described above. The HT29 cells were then washed twice with PBS, fixed in methanol/formaldehyde (99:1) mixture at –20°C for 15 min, permeabilized with PBS containing 0.2% Triton-X-100, and finally gently washed 5 times with PBS for 5 min. After treatment with blocking solution (PBS containing 1% BSA, 0.1% gelatin, and 5% goat serum), the cover slips were further incubated with primary antibody (anti-p21 or anti-MEK antibody at a 1:100 dilution) for 2 hr, and washed 5 times with PBS containing 1% BSA, and 0.1% gelatin. They were then further incubated with goat anti-mouse-Cy™2- or goat anti-rabbit-Rhodamin Red ™-X-conjugated secondary antibody at a 1:100 dilution for 1 hr, and washed 5 times with PBS.

For the BrdU incorporation study, HT29 cells were grown in McCoy's 5A media containing 20 μM of BrdU for 12 hr before immunocytochemistry. After performing immunocytochemistry, as described above, the cells were fixed in 3.7% formaldehyde for 10 min at room temperature and rinsed with PBS before being incubated for 30 min in 2 N HCl. They were then washed three times with PBS for 5 min. After blocking, the cells were incubated with anti-BrdU moncoclonal antibody at a 1:20 dilution for 2 hr and washed with PBS containing 1% BSA, 0.1% gelatin. The goat anti-mouse-Cy™2-conjugated secondary antibody at a 1:100 dilution was incubated for 1 hr and

washed the cells 5 times with PBS. Each experiment was performed at least three times. DAPI was then treated at a final concentration of 1μg/ml in PBS for 10 min, and the cells were extensively washed with PBS and mounted for photography by a Radiance 2000/MP, multi-photon imaging system (Bio-Rad, UK).

10. Statistics

One-way analysis of variance was performed using GraphPad Instat® (GraghPad software, San Diego, CA, USA). A value of $p<0.05$ was considered statistically significant**.**

III. Results

1. Effect of Extracellular Zinc on the Growth of Colorectal Cancer Cells

We investigated the growth regulatory effect of exracellular zinc on colorectal cancer cell line HT29. It had been known that the zinc levels in blood plasma were 10 - 20 μM.[25] However, zinc levels in the colorectal normal tissue and colorectal cancer cells are not clear. When HT29 cells were treated by higher zinc level (0 - 200 μM) than levels in blood plasma, the cell growth was inhibited (data not shown). In the other colorectal cancer cell lines, HCT116 and DLD-1, the cell growth was also inhibited zinc dosage-dependently (data not shown). When HT29 cells were treated with $ZnCl_2$ at 100 μM, cell numbers decreased step-wisely during a time course (Figure 1A). [^{3}H] thymidine incorporation, which represents cell proliferation status, was also lower in the cells treated with $ZnCl_2$ at 100 μM compared to the cells which were not treated with zinc (Figure 1B). Thus, 100 μM $ZnCl_2$ could inhibit colorectal cancer cells.

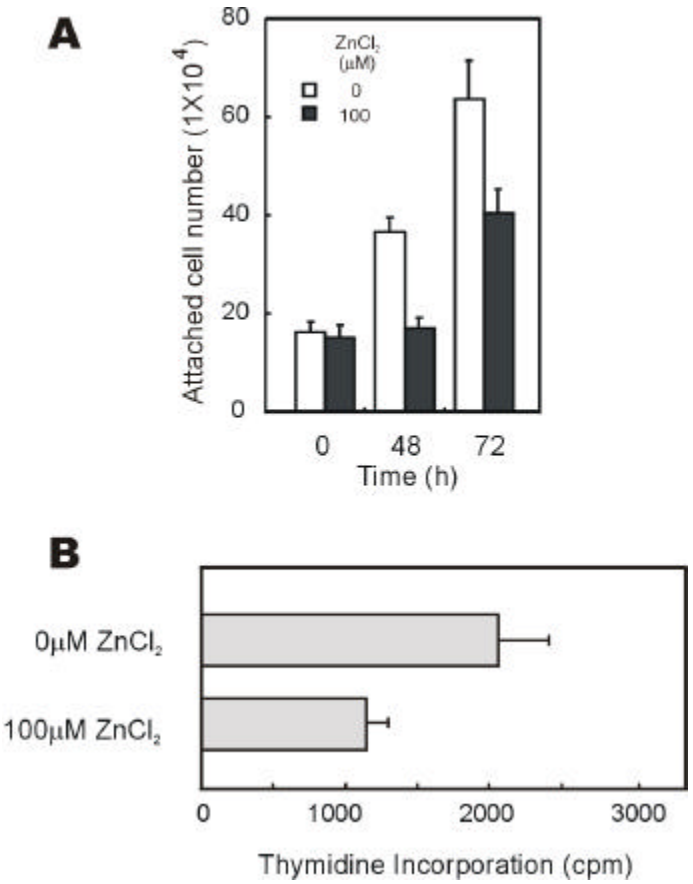

Figure 1. Cell growth and [^{3}H] thymidine incorporation were inhibited by exracellular zinc. (A) The HT29 cells were seeded at a density of 0.8 x 10^5 cells per well in 6-well plates. The cells were starved for 16 hr and either treated or not treated with $ZnCl_2$ at 100 μM. The numbers of attached cells were counted at 0, 48, 72 hr after zinc treatment, as described in Materials and Methods. Each data point represents the average value of three independent experiments. Error bars indicate the standard deviations of three independent experiments. (B) HT29 cells were seeded at a density of 0.8 x 10^5 cells per well into 6-well plates and starved for 16 hr. After treatment with $ZnCl_2$ at 100 μM the cells were further cultured for 48 hr before addition of [^{3}H] thymidine (0.5 μCi/well). The cells were further incubated for 12 hr, and the incorporation of [^{3}H] thymidine was determined as described in Materials and Methods. Results represent the average of three independent experiments. Error bars indicate the standard deviations of three independent experiments.

2. The Zinc-Induced Prolonged ERK Activation through the Raf-1-MEK-ERK Module

Recent studies have reported that extracellular zinc activates ERK within several cultured cell lines.[3,4] In these studies, it was tested whether extracellular zinc can activate ERK pathway in colorectal cancer cells. Phospho-ERK levels, which represent ERK activities, increased in two different phases in the HT29 colorectal cancer cells treated with $ZnCl_2$ at 100 μM (Figure 2A). Initially, the level of phospho-ERK increased within 5 min about 3-4 fold (Figure 2A) as previous reports have observed in other cell types.[3,4] However, the activities of ERKs were further strongly increased and prolonged after initial activation in cells treated with $ZnCl_2$ at 100 μM (Figure 2A). The strong activation of ERK, which peaked 6-to-9 hr after zinc treatment (see also Figure 5A), was also observed in other colorectal cancer cells like HCT116 and DLD-1 (Figure 2B). When the ERK activation was maximal, phospho-MEK protein levels also increased and the Raf-1 protein, further upstream, was mostly shifted to higher molecular weight form (Figure 3A). These results suggeste that the prolonged ERK activation could follow Raf-1 modification/MEK activation.

We used Elk-1 *trans*-reporting system to confirm ERK activation by zinc. Elk-1 *trans*-reporting system showed the ability of zinc to phosphorylate the nuclear transcription factor Elk-1 by activation of ERK pathway. The system employs a fusion construct that contains a GAL4 DNA binding domain of Elk-1 to induce expression of

a luciferase reporter that has five GAL4 binding elements. After ERK activation, the *trans*-activation domain of Elk-1 becomes phosphorylated and subsequently binds to and induces transcriptional activation of the luciferase reporter. Elk-1 dependent *trans*-reporter gene expression was found to be concomitantly increased in both HCT116 and DLD-1 cells (Figure 3C) as well as in the HT29 cells (Figure 3B) after treatment with $ZnCl_2$ at 100 μM.

To further characterize the zinc-induced prolonged activation of ERK, we pre-treated the MEK-specific inhibitor, PD98059,[26] and determined whether the drug could block the zinc-induced prolonged ERK activation. In the present study, the zinc-dependent increase of phospho-ERK level was significantly blocked by pre-treatment with 10 μM of PD98059 (Figure 4A). Interestingly, modification of the upstream component, Raf-1, was also observed by treating cells with PD98059 before stimulating them with $ZnCl_2$ at 100 μM (Figure 4A; under-shift of Raf-1 band from band "a" to band "b" in the cells pre-treated with PD98059). Elk-1 *trans*-reporter gene expression by zinc was also down-regulated in cells treated with 10 μM of PD98059 (Figure 4B). In addition, the activation of Elk-1 dependent *trans*-reporter gene expression by $ZnCl_2$ was also blocked by the transient transfection of dominant negative Raf-1 (dn-Raf-1) (Figure 4C). We also observed down-regulation of Elk-1 dependent *trans*-reporter gene expression, which was increased by $ZnCl_2$, by expressing dn-Raf-1 or catalytically inactive MEK (MEK-2A) in the other colorectal cancer cell, HCT116 (Figure 4D). Overall, the prolonged activation of ERK by zinc

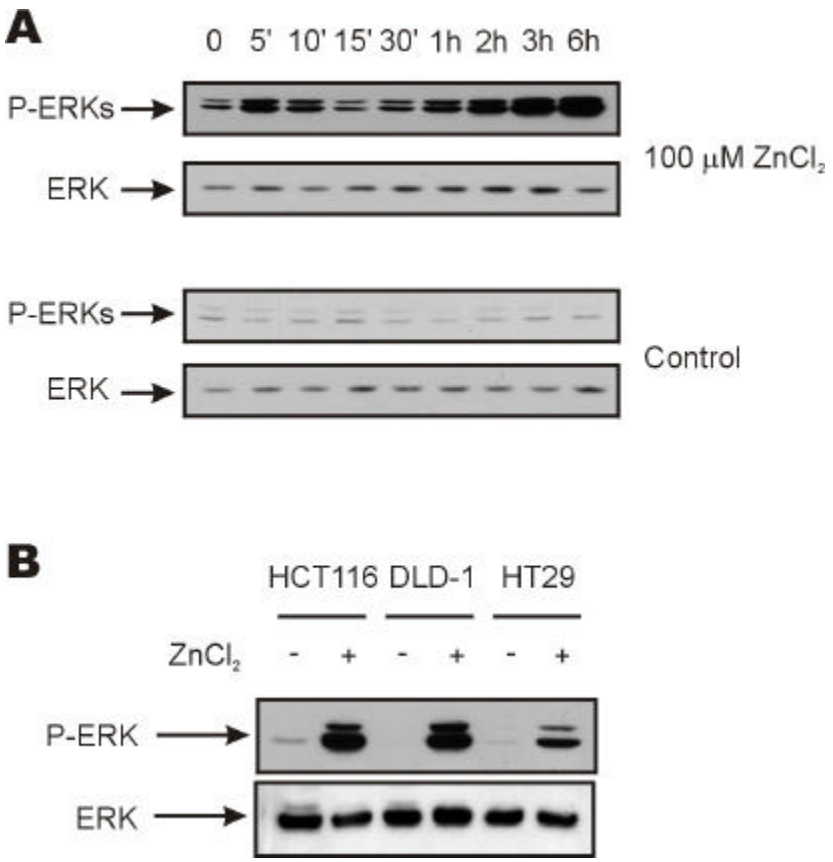

Figure 2. Extracellular zinc induced biphasic ERK activation. (A) The HT29 cells were starved for 16 hr. Cells were then either treated or not treated with $ZnCl_2$ at 100 μM before the cells were harvested at different time points for extract preparations. (B) The HCT116, DLD-1, and HT29 cells were grown as shown in Figure 2A and treated or not treated with $ZnCl_2$ at 100 μM for 9 hr before the preparation of cell extracts. Western blot analysis was performed using anti-phospho-ERK or anti-ERK antibody, respectively.

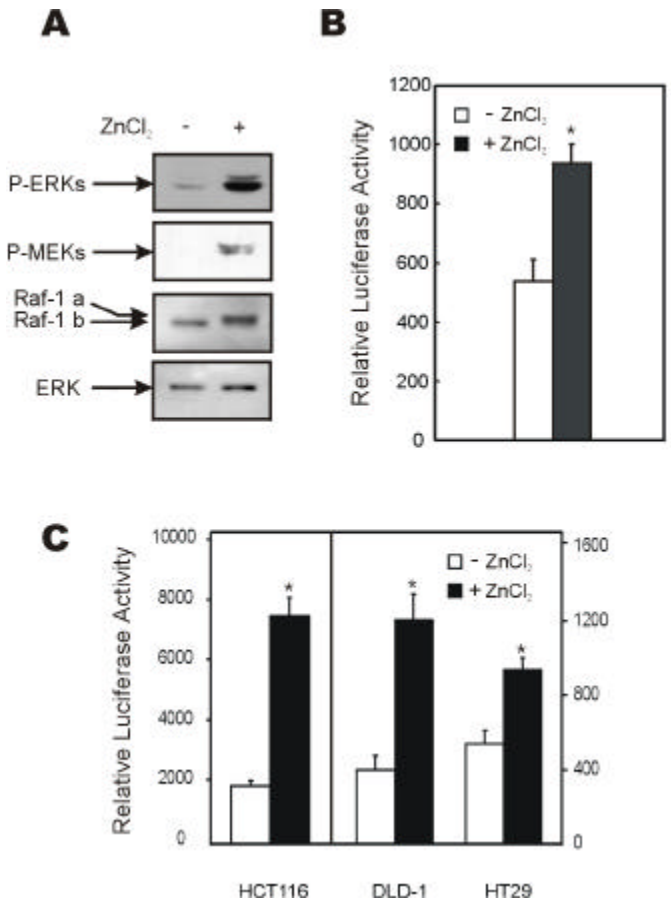

Figure 3. Extracellular zinc activated the ERK pathway. (A) Cells were harvested 9 hr after the 100 μM zinc treatment. Phospo-ERK, Raf-1 and ERK were detected by Western blot analysis. A shift in the Raf-1 protein band generated by zinc treatment was identified as Raf-1a, while the un-shifted band was marked as Raf-1b. (B) HT29 cells were co-transfected with 0.5 μg of Luciferase reporter plasmid pFR-Luc and 25 ng of *trans*-activator pFA2-Elk-1, as described in Materials and Methods. For normalization, the cells were co-transfected with 50ng of plasmid containing the gene for β-galactosidase under the control of a CMV promoter. Cells were incubated for an additional 16 hr. The medium was replaced with fresh media with or without 100 μM $ZnCl_2$ and extracts were made 12 hr after the zinc treatment. Luciferase activity was measured and normalized using the β–galactosidase level as an internal control. Each result represents the average value of three independent experiments. Error bars indicate standard deviations. (C) HCT116, DLD-1, HT29 cells were co-transfected with 0.5 μg of Luciferase reporter plasmid pFR-Luc and 25 ng of *trans*-activator pFA2-Elk-1, as Figure 3B. *, $p<0.002$ *vs* without 100 μM $ZnCl_2$.

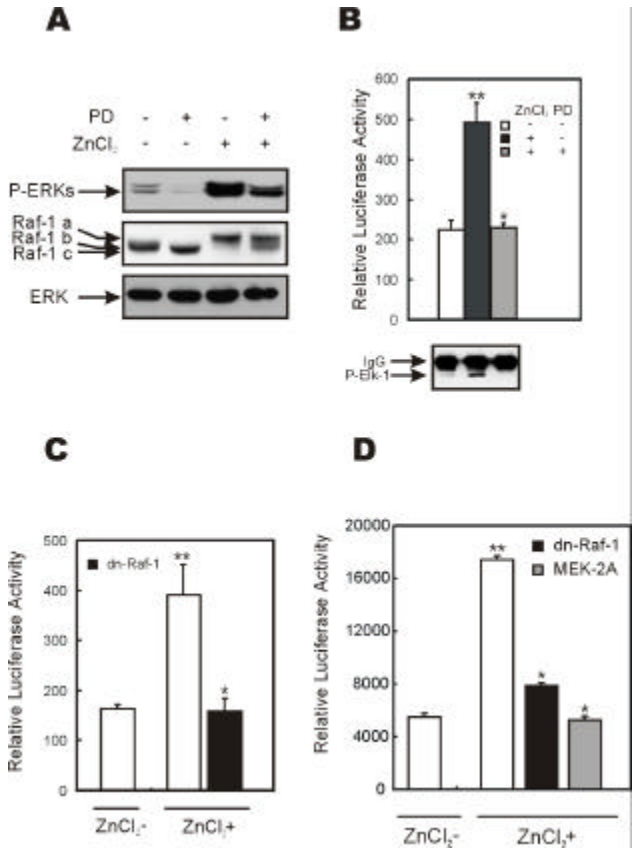

Figure 4. The zinc-induced ERK activation was down-regulated after inhibition of ERK module kinases. (A) HT29 cells were serum starved as described in Figure 3A, and treated with $ZnCl_2$ at 100 μM for 9 hr before harvesting for extract preparation. In required cases, cells were incubated with 10 μM of PD98059 for 30 min before zinc treatment. Western blot analysis was performed to visualize the phospho-ERK, Raf-1, and ERK protein bands. (B) HT29 cells were grown and transfected with pFR-Luc and pFA2-Elk-1 vectors as in Figure 3B, except that 10 μM of PD98059 was administered 30 min prior to treatment with $ZnCl_2$ at 100 μM, in the required cases. Elk-1 dependent *trans*-reporter gene expression was measured 12 hr after treatment with $ZnCl_2$ at 100 μM. The *in vitro* kinase activity of ERK was measured using purified GST-Elk-1 as a substrate as described in Materials and Methods. (C) After transfection with 0.5 μg of pFR-Luc and 25 ng of pFA2-Elk-1 plasmids, the HT29 cells were serum starved and treated with $ZnCl_2$ at 100 μM as described in Figure 3B. In selected cases, 0.5 μg of the empty vector or dominant negative Raf-1 (dn-Raf-1) was cotransfected together with the plasmids mentioned above. Elk-1 dependent *trans*-reporter gene expression was measured 12 hr after treatment with $ZnCl_2$ at 100 μM. (D) After transfection with 0.5 μg of pFR-Luc and 25 ng of pFA2-Elk-1 plasmids, the HCT116 cells were serum starved and treated with $ZnCl_2$ at 100 μM as described in Figure 4C. In selected cases, 0.5 μg of the empty vector, catalytically inactive MEK(MEK-2A) or dn-Raf-1 was cotransfected together with the plasmids mentioned above. Elk-1 dependent *trans*-reporter gene expression was measured 12 hr after treatment with $ZnCl_2$ at 100 μM. *, $p<0.02$ *vs* with $ZnCl_2$ and **, $p<0.03$ *vs* without $ZnCl_2$.

was acquired through the Raf-1-MEK-ERK module to activate expression of target gene(s) and it was presupposed that this prolonged ERK activation was involved in the mechanisms of colorectal cancer cell growth arrest by zinc.

3. Zinc Induced $p21^{Cip/WAF1}$ through ERK Activation

Recent studies have suggested that the induction of a negative cell cycle regulator, $p21^{Cip/WAF1}$, was related with cell growth arrest by the prolonged ERK activation.[18,19] Furthermore, the induction of $p21^{Cip/WAF1}$ is a good indicator of colorectal cancer cell growth arrest.[27,28] Therefore, we investigated the expression levels of $p21^{Cip/WAF1}$ in colorectal cancer cells treated with $ZnCl_2$ at 100 μM in order to identify a relationship between ERK activation and $p21^{Cip/WAF1}$ induction related to cell growth inhibition. Strong $p21^{Cip/WAF1}$ induction was also observed after treating $ZnCl_2$ at 100 μM, moreover, the profile of this induction was highly correlated with prolonged ERK activation (Figure 5A). In addition, patterns of the $p21^{Cip/WAF1}$ induction and ERK activation were also correlated well in the cells treated with different zinc concentrations (Figure 5B).

To identify a direct link between $p21^{Cip/WAF1}$ induction and ERK activation, we examined the effect of PD98059 upon the induction of $p21^{Cip/WAF1}$. The degree of ERK activity inhibition was dependent on the pre-treated PD98059 concentration. Phospho-ERK levels were significantly reduced by 10 μM of PD98059 and completely

abolished at 50 μM and the $p21^{Cip/WAF1}$ induction was dose-dependently reduced by PD98059 (Figure 5C). However, the $p21^{Cip/WAF1}$ band was not completely abolished at the higher PD98059 concentration above 10 μM. Alternatively, we also used U0126 to block ERK activation by MEK. Both the ERK activation and $p21^{Cip/WAF1}$ induction of the cells were similarly reduced by pre-treating the cells with U0126 (Figure 5D), but the $p21^{Cip/WAF1}$ band was also not completely abolished at the higher concentrations above 10 μM. Therefore, extracellular zinc increased the $p21^{Cip/WAF1}$ protein level by a mechanism, which was dependent on the ERK cascade, although some of the $p21^{Cip/WAF1}$ may have been induced by ERK independent mechanism(s). To know an induction mechanism of $p21^{Cip/WAF1}$ by zinc, we performed Northern blot analysis. We found the increase of $p21^{Cip/WAF1}$ mRNA by $ZnCl_2$ at 100 μM treatment and that was blocked by pre-treating cells with PD98059 (Figure 6A). These results suggested that the induction of $p21^{Cip/WAF1}$ could be caused by the increase of $p21^{Cip/WAF1}$ mRNA through the activation of ERK pathway. However, we did not know whether the increase of mRNA was caused by the transcriptional activation of the $p21^{Cip/WAF1}$ promoter or by the increase of $p21^{Cip/WAF1}$ mRNA stability.

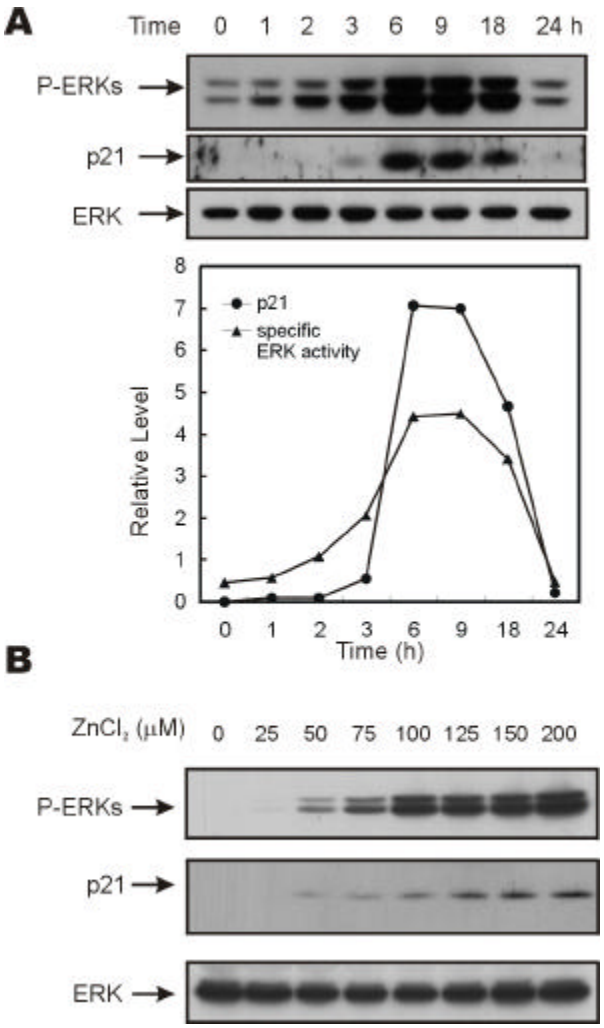

Figure 5. Induction of p21$^{Cip/WAF1}$ by zinc depended on ERK activation. (A) HT29 cells were serum starved and further grown in the identical media containing $ZnCl_2$ at 100 μM over the time course as described in Figure 2A. ERK activity and p21$^{Cip/WAF1}$ level were quantified by densitometric scanning of the protein bands as visualized by Western blot analysis. Specific ERK activities represent densitometric values of phospho-ERK divided by ERK. (B) HT29 cells were serum starved and further grown in the identical media containing different concentration of $ZnCl_2$ (0-200 μM). The cells were harvested 9 hr after treatment of $ZnCl_2$.

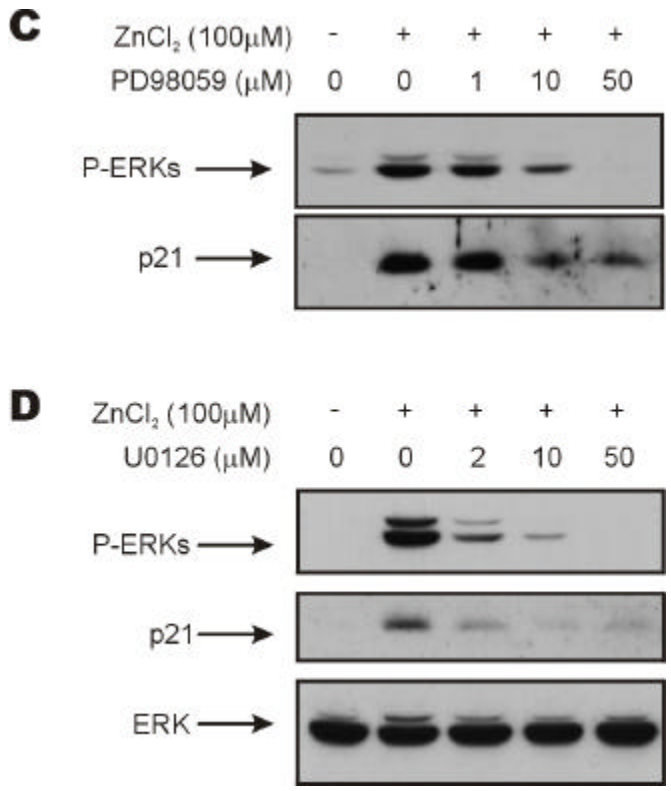

Figure 5. Induction of p21$^{Cip/WAF1}$ by zinc depended on ERK activation. (C) The cells were similarly grown in the media containing 1% FBS. In required cases, the cells which were treated with $ZnCl_2$ at 100 μM were pre-incubated with PD98059 (0-50 μM) for 30 min before zinc treatment. Phospho-ERK and p21$^{Cip/WAF1}$ protein bands were detected by Western blot analysis. (D) U0126 (0-50 μM) was treated for 30 min before zinc treatment instead of PD98059. Phospho-ERK and p21$^{Cip/WAF1}$ protein bands were detected by Western blot analysis.

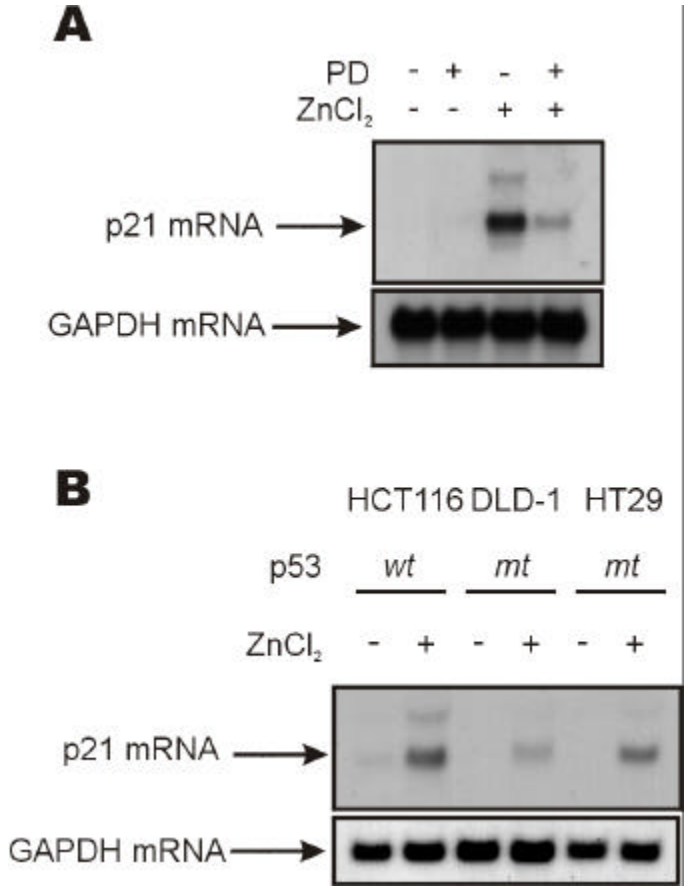

Figure 6. p21$^{Cip/WAF1}$ mRNA increased by an ERK-dependent mechanism. (A) HT29 cells were serum starved in media containing 1% FBS and the cells were treated with $ZnCl_2$ at 100 μM for 5 hr before harvesting for total RNA preparation. The cells were incubated with 10 μM of PD98059 for 30 min prior to zinc treatment in the required cases. Northern blot analyses were performed using ^{32}p-labeled p21$^{Cip/WAF1}$ or GAPDH cDNA as probes. (B) The HT29, HCT116 and DLD-1 cells were grown and serum starved as shown in Figure 6A except that RPMI1640 medium was used in case of the DLD-1 cells.

4. Nuclear Localization of $p21^{Cip/WAF1}$ by Zinc Depended on ERK Activation

The cell cycle inhibitory activity of $p21^{Cip/WAF1}$ is intimately related with its induction and nuclear localization, and these are directly related to cell growth.[29,30] Therefore, we investigated the localization of $p21^{Cip/WAF1}$ after treatment with $ZnCl_2$ at 100 μM to further characterize its role in cell growth regulation. In HT29 cells, the $p21^{Cip/WAF1}$ protein level was low and the protein was mainly localized to the perinuclear compartment and the cytosol (Figure 7A). However, the $p21^{Cip/WAF1}$ protein was both increased in level and localized to the nucleus in 100 μM $ZnCl_2$ treated cells. The induction and nuclear localization of $p21^{Cip/WAF1}$, caused by 100 μM of $ZnCl_2$, blocked by 10 μM PD98059 pre-treatment (Figure 7A). The percentage of cells induced and nuclear localized $p21^{Cip/WAF1}$ was increased by treatment of the cells with $ZnCl_2$ at 100 μM and that was inhibited by PD98059 pre-treatment (Figure 7C).

In addition, $p21^{Cip/WAF1}$ induction and especially its nuclear localization by 100 μM of $ZnCl_2$ were also significantly blocked in cells expressing catalytically inactive MEK mutant (MEK-2A) (Figure 7B). Therefore, it is demonstrated that the induction and nuclear localization of $p21^{Cip/WAF1}$ protein were dependent on ERK activation by zinc.

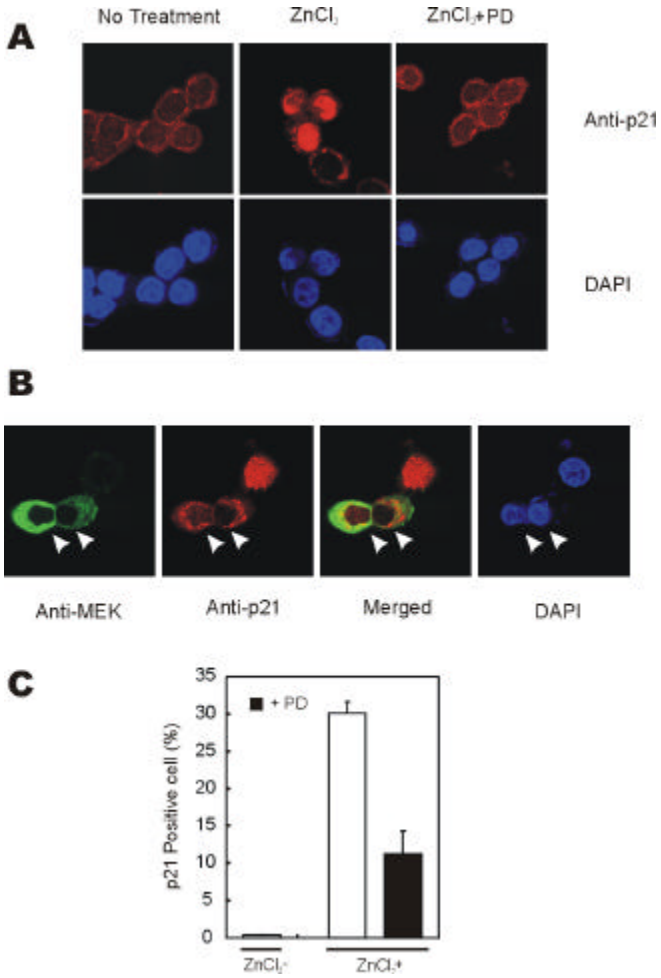

Figure 7. Nuclear localization of $p21^{Cip/WAF1}$ induced by zinc and inhibited by PD98059 or by expression of catalytically inactive MEK (MEK-2A). (A) HT29 cells were serum starved in media containing 1% FBS. The cells were then treated or not treated with $ZnCl_2$ at 100 μM for 9 hr before immunocytochemistry. In the required cases, they were incubated PD98059 (10 μM) for 30 min before treatment with zinc. (B) HT29 cells were grown and transfected with 0.5 μg of plasmid containing expressible catalytically inactive MEK (MEK-2A). The cells were treated with $ZnCl_2$ at 100 μM for 9 hr before immunocytochemistry. The expression and localization of $p21^{Cip/WAF1}$ and MEK-2A was revealed by immunocytochemistry using the anti- $p21^{Cip/WAF1}$ antibody together with Rhodamin-conjugated goat anti-rabbit IgG, or anti-MEK antibody together with Cy-2-conjugated goat anti-mouse IgG antibody; nuclei were stained by DAPI. The representative cells inhibiting induction and nuclear locaization of $p21^{Cip/WAF1}$ by expressing MEK-2A were marked by arrows. (C) The percentage of cells induced and nuclear localized $p21^{Cip/WAF1}$ was increased by treatment of the cells with $ZnCl_2$ at 100 μM and that inhibited by pre-treatment of PD98059.

5. The ERK Dependent Induction and Nuclear Localization of $p21^{Cip/WAF1}$ by Zinc Inhibited BrdU Incorporation

To obtain direct evidence upon the anti-proliferative effect of the induced and nuclear localized $p21^{Cip/WAF1}$ by zinc, we also monitored DNA synthesis at the single cell level by measuring BrdU incorporation after treatment of HT29 cells with $ZnCl_2$ at 100 μM. We checked whether BrdU incorporation was blocked in an individual cell containing nuclear localized $p21^{Cip/WAF1}$ which induced by treatment $ZnCl_2$ at 100 μM. In a resting status, around 60.8% of the cells incorporated BrdU. However, the percentage of cells to incorporate BrdU was decreased to 35.5% when cells were treated with zinc (Figure 8A; representative results are shown in Figure 8B). When we checked the BrdU incorporation at the single cell level, no cell induced and nuclear localized $p21^{Cip/WAF1}$ incorporated BrdU (representative cases are shown in Figure 8B). Therefore, ERK dependent induction and nuclear localization of the $p21^{Cip/WAF1}$ could directly be related with the inhibition of BrdU incorporation in HT29 colorectal cancer cells.

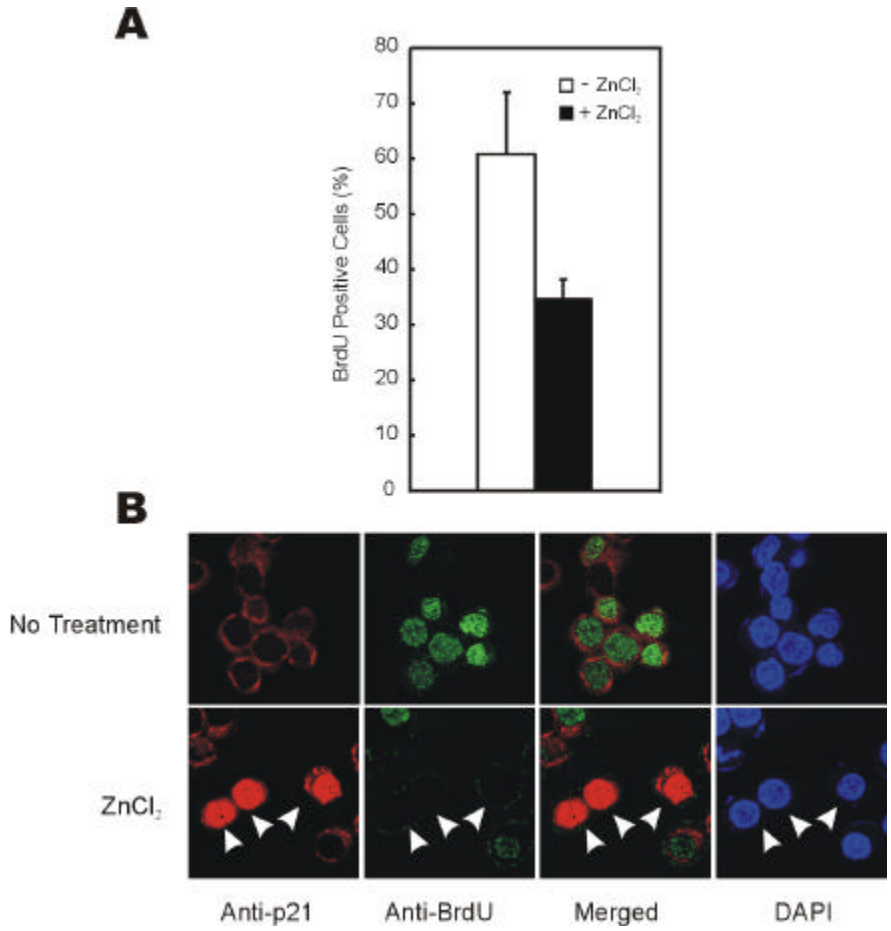

Figure 8. Inhibition of BrdU incorporation by zinc was directly related to the induction and nuclear localization of p21$^{Cip/WAF1}$. (A) The percentage of cells incorporating BrdU was lowered by $ZnCl_2$ at 100 μM treatment. (B) Zinc dependent induction and nuclear localization of p21$^{Cip/WAF1}$ inhibited BrdU incorporation. The representative 100 μM of $ZnCl_2$ treated cells blocked the BrdU incorporation by induction and nuclear localization of p21$^{Cip/WAF1}$ were marked by arrows. HT29 cells were serum starved with the media containing 1% FBS, then further grown in the media without or with $ZnCl_2$ at 100 μM for 20 hr. The cells were labeled with 20 μM of BrdU for last 12 hr before assay. p21$^{Cip/WAF1}$ was detected by anti-p21$^{Cip/WAF1}$ antibody together with Rhodamin-Red-conjugated goat anti-rabbit IgG, and BrdU incorporated nuclei were revealed with green color by immunocytochemistry by using anti-BrdU antibody followed by Cy2-conjugated goat anti-mouse IgG; cell nuclei were stained by DAPI.

IV. Discussion

Numerous studies have reported the roles of zinc in both the induction and prevention of cancer[31,32] and recent studies also have shown that zinc plays a crucial role in the regulation of cell proliferation.[1,2,33] The continuous renewal growth in colorectal epithelium and colorectal tumorigenesis have been suggested to be highly sensitive to zinc levels.[34-38] In this study, a role of extracellular zinc in the growth regulation of colorectal cancer cells was studied. Here, the ERK signaling pathway was identified as the pathway for zinc-induced growth arrest of the cells and this is related to the induction of the $p21^{Cip/WAF1}$ cell cycle regulator.

The treatment of cells with $ZnCl_2$ at 100 μM caused ERK activities to be transiently increased, which is in agreement with the previous observations in several other cell types.[3,4] However, we observed a further strong activation of ERKs followed by its initial activation in colorectal cancer cell line, HT29 (Figure 2A). The Zinc dependent prolonged ERK activation was also observed in several colorectal cancer cell lines.

Recent studies have shown the transient activation of ERK by zinc, however, the studies do not show the mechanism(s) of ERK activation.[3,4] A recent study suggested the involvement of EGF receptor in the zinc-stimulated activation of ERK. But it neither showed the necessity of Ras in the activation of ERK nor indicated the necessity of zinc accumulation in cells.[39] In this study, zinc-induced ERK activation was observed regardless of Ras genetic status. Through, Zinc may not need Ras in the

activation of ERK. Therefore, dominant negative *ras* was used to confirm the Ras dependence in the ERK activation. We unexpectedly observed the reduction of the induction of the Elk-1 *trans*-reporter gene by transfection of dominant negative *ras* construct in colorectal cancer cell line containing wild type Ras gene (data not shown). These results suggested involvement of Ras in the zinc-induced ERK activation. However, the Ras dependence is not clear because the activation of Raf-1 is acquired by highly complex Ras-dependent and independent mechanisms.[40] To illustrate the initial mechanism of the activation of ERK pathway by zinc, we checked whether zinc could accumulate in colorectal cancer cells when cells were treated with extracellular zinc. When HT29 cells were treated with 100 μM $ZnCl_2$ or even higher than zinc concentration of 100 μM, the intracellular zinc concentration did not increase (data not shown). Therefore, it was suggested that zinc-induced ERK activation in colorectal cancer cells was not occurred by the intracellular signaling system rather it might be acquired by a mechanism using extracellular receptors such as EGF or Ca^{2+} receptors.[39]

To know the mechanism for the strong and prolonged activation of ERKs by zinc, PD98059 was used. The zinc-dependent increase of phospho-ERK was significantly blocked by pre-treatment with PD98059 (Figure 4A). Interestingly, modification of the upstream component, Raf-1, was also observed by treating cells with PD98059 before stimulating them with $ZnCl_2$ at 100 μM (Figure 4A). Therefore, this result may suggest that the strong and prolonged activation of ERK induced by the 100 μM of

$ZnCl_2$ treatment may have been caused by a positive activation of the Raf-1→MEK→ERK loop after the transient activation of ERK.

The strong and prolonged activation of ERK by the treatment of 100 μM $ZnCl_2$ was related to cell growth inhibition and that may be caused by the activation of $p21^{Cip/WAF1}$. A substantial body of evidences supports that the activation of $p21^{Cip/WAF1}$ is the result of the zinc-induced ERK activation. First, the profiles of specific ERK activation and $p21^{Cip/WAF1}$ induction by zinc treatment are highly correlated (Figure 5A). Second, $p21^{Cip/WAF1}$ induction is inhibited by pretreatment with the MEK inhibitors, PD98059 and U0126 (Figure 5C and Figure 5D). Finally, both the induction and the nuclear localization of $p21^{Cip/WAF1}$ were blocked at the single cell level by either PD98059 pre-treatment or the transient expression of catalytically inactive MEK-2A (Figure 7A and Figure 7B). Therefore, zinc activated $p21^{Cip/WAF1}$ by ERK pathway dependent mechanism.

What was the mechanism(s) of the link between the induction of $p21^{Cip/WAF1}$ and the prolonged ERK activation? The $p21^{Cip/WAF1}$ was induced by two distinct mechanisms. One is the p53-dependent mechanism. The other is the p53-independent mechanism involving ERK activation.[8] To identify dependence of p53 in the zinc dependent induction of $p21^{Cip/WAF1}$, we used colorectal cancer cell lines containing different status of p53 gene. The zinc-dependent induction of $p21^{Cip/WAF1}$ mRNA was observed in all tested colorectal cancer cells regardless of p53 genetic status (Figure 6B) and these results indicated that induction of $p21^{Cip/WAF1}$ by zinc may be acquired by p53-

independent and ERK signaling-dependent mechanism at the level of transcriptional activation. According to recent studies, the ERK dependent regulation of $p21^{Cip/WAF1}$ mRNA stability was found to be as important as the transcriptional activation, which was also regulated by ERK activation.[41,42] In this study, it is not clear whether the increase of $p21^{Cip/WAF1}$ mRNA by zinc was obtained by the transcriptional activation or by the increase of mRNA stability.

Is the induction of $p21^{Cip/WAF1}$ dependent only on the zinc-induced ERK activation? In this study, levels of $p21^{Cip/WAF1}$ mRNA induced by zinc were variable and did not correlate directly with ERK activation levels in different colorectal cancer cell lines (compare Figure 2B and Figure 6B). These results suggest that unknown factor(s) may partly be involved in zinc dependent $p21^{Cip/WAF1}$ activation. The presence of an alternative route for the induction of $p21^{Cip/WAF1}$ by zinc was suggested by an experiment upon the effect of zinc after pre-treating with PD98059 or U0126 at a concentration higher than 10 μM. While 50 μM of PD98059 completely inhibited the ERK activities which were increased by $ZnCl_2$ at 100 μM, the $p21^{Cip/WAF1}$ level decreased but stopped at a certain level after pre-treatment of the cells with more than 10 μM of PD98059 or U0126. Therefore, the amount of $p21^{Cip/WAF1}$ induced by $ZnCl_2$ at 100 μM may be partly attributed to a route independent of ERK pathway. What's the other mechanism(s) to induce $p21^{Cip/WAF1}$ by zinc? In recent studies, it is suggested that PI3K and PKC are involved in the mechanism of $p21^{Cip/WAF1}$ induction.[42,43] To investigate the role of the other candidates, PI3K and PKC, in the induction of

$p21^{Cip/WAF1}$ by zinc, we used the PI3K pathway blocker, LY294002 and the PKC pathway blocker, GF109203X. We could not observe the decrease of the induced $p21^{Cip/WAF1}$ level by zinc when cells were pre-treated by these blockers (data not shown). Therefore, it was suggested that PI3K pathway or PKC pathway was not involved at least in the $p21^{Cip/WAF1}$ induction by zinc in colorectal cancer cells.

In this study, it was suggested that the ERK activation dependent induction and nuclear localization of $p21^{Cip/WAF1}$ inhibited the colorectal cancer cell growth. The importance of $p21^{Cip/WAF1}$ in the growth control of colorectal cancer cells, which depends on ERK signaling, is supported by the recent study which showed the promotion of DNA synthesis by prolonged activation of ERK pathway in $p21^{Cip/WAF1}$–null mice.[44] In addition, a cell cycle arrest caused by the induction of $p21^{Cip/WAF1}$ acquired by high intensity of Raf-1 signals was abolished in $p21^{Cip/WAF1}$-/- fibroblasts.[18] In the present study, a detailed mechanism for the induction and nuclear localization of $p21^{Cip/WAF1}$ by zinc followed by the regulation of cell growth were identified. Here, the prolonged activation of ERK signaling was found to play an important role in the $p21^{Cip/WAF1}$ induction by zinc and the negative regulation of colorectal cancer cell proliferation.

This study suggested that the maintenance of adequate zinc levels may be important for good health and that at the right dosage zinc usage has the potential to both prevent and inhibit tumorigenesis of colorectal cells.

V. Conclusion

1. Extracellular zinc inhibits colorectal cancer cell growth.

2. Zinc induces the strong and prolonged ERK activation through the Raf-1-MEK-ERK module kinases.

3. Zinc induces the negative cell cycle regulator, $p21^{Cip/WAF1}$ via the strong and prolonged ERK activation.

4. The induction of $p21^{Cip/WAF1}$ by zinc is acquired by both $p21^{Cip/WAF1}$ mRNA and $p21^{Cip/WAF1}$ protein levels.

5. The induced $p21^{Cip/WAF1}$ by zinc is localized in the nucleus of colorectal cancer cells and involved in the inhibition of colorectal cancer cell growth.

References

1. Fraker PJ, Telford WG. A reappraisal of the role of zinc in life and death decisions of cells. Proc Soc Exp Biol Med 1997;125:229-36.

2. Lian JY, Liu YY, Zou J, Franklin RB, Costello LC, Feng P. Inhibitory effect of zinc on human prostatic carcinoma cell growth. Prostate 1999;40:200-7.

3. Hansson A. Extracellular zinc ions induces mitogen-activated protein kinase activity and protein tyrosine phosphorylation in bombesin-sensitive swiss 3T3 fibroblasts. Arch Biochem Biophy 1996;328:233-8.

4. Samet JM, Graves, LM, Quay J, Dailey LA, Devlin RB, Ghio AJ, et al. Activation of MAPKs in human bronchial epithelial cells exposed to metals. Am J Physiol 1998;275:L551-L8.

5. Hunter T, Pines J. Cyclins and cancer. II: Cyclin D and CDK inhibitors come of age. Cell 1997;79:573-82.

6. Pardee AB. G1 events and regulation of cell proliferation. Science 1989;246:603-8.

7. Polyak K, Waldman T, He TC, Kinzler KW, Vogelstein B. Genetic determinants of p53-induced apoptosis and growth arrest. Genes Dev 1996;10:1945-52.

8. Liu Y, Martindale JL, Gorospe M, Holbrook NJ. Regulation of $p21^{WAF/CIP}$ expression through mitogen-activated protein kinase signaling pathway. Cancer Res 1996;56:31-5.

9. Davis RJ. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem 1993;268:14553-6.

10. Cobb MH, Goldsmith EJ. How MAP Kinases Are Regulated J Bio Chem 1995;270:14843-6.

11. Marshall CJ. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell 1995;80:179-85.

12. Robinson MJ, Cobb MH. Mitogen-activated protein kinase pathways. Curr Opin Cell Biol 1997;2:180-6.

13. Marshall CJ, Nigg EA. Oncogenes and cell proliferation. Cancer genes: lessons from genetics and biochemistry. Curr Opin Genet Dev 1998;8:11-3.

14. Mansour SJ, Matten WT, Herman AS, Candia JM, Rong S, Fukaskwa K, et al. Transformation of mammalian cells by constitutively active MAP kinase kinase. Science 1994;265:966-70.

15. Cowley S, Paterson H, Kemp P, Marshall CJ. Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. Cell 1994;77:841-52.

16. Kyriakis IM, App H, Zhang XF, Banerjee P, Brautigan DL, Rapp UR et al. Raf-1 activates MAP kinase-kinase. Nature 1992;358:417-21.

17. Marshall CY. Cell signalling. Raf gets it together. Nature 1996;383:127-8.

18. Woods D, Parry D, Cherwinski C, Bosch E, Lees E, McMahon M. Raf-

Induced proliferation or cell cycle arrest is determined by the level of Raf activity with arrest mediated by $p21^{Cip}$. Mol Cell Biol 1997;17: 5598-11.

19. Sewing A, Wiseman B, Lloyd AC, Land H. High-intensity Raf signal causes cell cycle arrest mediated by p21Cip1. Mol Cell Biol 1997;17:5588-97.

20. Park KS, Kim NG, Kim JJ, Kim H, Ahn YH, Choi KY. Differential regulation of MAP kinase cascade in human colorectal tumorigenesis. Br J Cancer 1999;81:1116-21.

21. Shiff SJ, Qiao L, Tsai LL, Rigas B. Sulindac sulfide, an aspirin-like compound, inhibits proliferation, causes cell cycle quiescence, and induces apoptosis in HT-29 colon adenocarcinoma cells. J Clin Invest 1995;96:491-3.

22. Shirasawa S, Furuse M, Yokoyama N, Sasazuki T. Altered growth of human colon cancer cell lines disrupted at activated Ki-ras. Science 1993;260:85-8.

23. El-Deiry W, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent M, Lin D, et al. WAF1, a potential mediator of p53 tumor suppression. Cell 1993;75:817-25.

24. Ercolani L, Florence B, Denaro M, Alexander M. Isolation and complete sequence of a functional human glyceraldehyde-3-phosphate dehydrogenase gene. J Biol Chem 1988;263:15335-41.

25. Costello LC, Franklin RB. Novel role of zinc in the regulation of prostate citrate metabolism and its implications in prostate cancer. Prostate 1998;35:285-96.

26. Pages G, Lenormand P, L'Allemain G, Chambard JC, Meloche S, Pouyssegur J. Mitogen-activated protein kinases p42mapk and p44mapk are required for fibroblast proliferation. Proc Natl Acad Sci U S A 1993;90:8319-23.

27. Archer SY, Meng S, Shei A, Hodin RA. p21(WAF1) is required for butyrate-mediated growth inhibition of human colon cancer cells. Proc Natl Acad Sci U S A 1998;95:6791-6.

28. Nakano K, Mizuno T, Sowa Y, Orita T, Yoshino T, Okuyama Y, et al. Butyrate activates the WAF1/Cip1 gene promoter through Sp1 sites in a p53-negative human colon cancer cell line. J Biol Chem 1997;272:22199-06.

29. Asada M, Yamada T, Ichijo H, Delia D, Miyazono K, Fukumuro K, et al. Apoptosis inhibitory activity of cytoplasmic p21(Cip1/WAF1) in monocytic differentiation. EMBO J 1999;18:1223-34.

30. Halevy O, Novitch BG, Spicer DB, Skapek SX, Rhee J, Hannon GJ, et al. Correlation of terminal cell cycle arrest of skeletal muscle with induction of p21 by MyoD. Science 1995; 267:1018-21.

31. Rojas E, Herrera LA, Poirier LA, Ostrosky-Wegman P. Are metals dietary carcinogens? Mutat Res 1999;443:157-81.

32. Vallee BL, Falchuk KH. The biochemical basis of zinc physiology. Physiol Rev 1993;73:79-118.

33. Seo SR, Chong SA, Lee SI, Sung JY, Ahn YS, Chung KC, et al. Zn^{2+}-induced ERK activation mediated by reactive oxygen species causes cell death in differentiated PC 12 cells. J Neuroch 2001;78:1-12.

34. Carter JW, Lancaster H, Hardman WE, Cameron IL. Zinc deprivation promotes progression of 1,2-dimethylhydrazine-induced colon tumors but reduces malignant invasion in mice. Nutr Cancer 1997; 27: 217-21.

35. Song MK, Heng MC, Rolandelli R, Ament ME, Heng MK. Possible link between zinc intake and colon cancer. J Natl Cancer Inst 1993;85:667-9.

36. Nelson RL. Dietary minerals and colon carcinogenesis. Anticancer Res 1987;7: 259-9.

37. Mulder TP, Verspaget HW, Janssens AR, de Bruin PA, Griffioen G, Lamers CB. Neoplasia-related changes of two copper (Cu)/zinc (Zn) proteins in the human colon. Free Radic Biol Med 1990;9:501-16.

38. Martin Mateo MC, Martin G. Influence of metallic carcinogenesis in lung and colorectal neoplasia. Metals in neoplastic processes. Clin Physiol Biochem 1988;6:321-6.

39. Wu W, Graves LM, Jaspers I, Devlin RB, Reed W, Samet JM. Activation of the EGF receptor signaling pathway in human air way epithelial cells exposed to metals. Am J Physiol 1999;277:L924-31.

40. Sharon LC, Khosravi-Far R, Rossman KL, Clark GJ, Der CJ. Increasing complexity of Ras signaling. Oncogene 1998;17:1395-431.

41. Park JS, Qiao L, Gilfor D, Yang YM., Hylemon PB, Benz C, et al. A role for both Ets and C/EBP transcription factors and mRNA stabilization in the MAPK-dependent increase in p21(Ci-1/WAF-1/mda6) protein levels in primary hepatocytes. Mol Biol Cell 2000;11:2915-32.

42. Park JW, Jang MA, Lee YH, Passaniti A, Kwon TG. P53-independent elevation p21 expression by PMA results from PKC-mediated mRNA stabilization. Bioch Biophy Res Com 2001;280:244-8.

43. Mitsuuchi Y, Johnson SW, Selvakumaran M, Williams SJ, Hamilton TC, Testa JR. The phosphatidylinositol 3-kinase/AKT signal transduction pathway plays a critical role in the expression of p21WAF1/CIP1/SDI1 induced by cisplatin and paclitaxel. Cancer Res 2000;60:5390-4.

44. Auer KL, Park JS, Seth P, Coffey RJ, Darlington G, Abo A, et al. Prolonged activation of the mitogen-activated protein kinase pathway promotes DNA synthesis in primary hepatocytes from $p21^{Cip-1/WAF1}$-null mice, but not in hepatocytes from $p16^{INK4a}$-null mice. Biochem J 1998;336:551-60.

국문 요약

Zinc 에 의한 ERK pathway 와 $p21^{Cip/WAF-1}$ 의 활성화가 대장암 세포주 성장 저해에 미치는 영향

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생체 내에서 zinc 가 세포 성장 촉진과 암 형성 촉진에 있어서 중요한 역할을 한다는 것은 수많은 연구에 의해 보고 되었다. 그러나 세포 성장 억제와 암 형성 억제에 있어서의 zinc 의 역할 또한 잘 밝혀져 왔다. 본 연구는 세포 외부에서 처리한 zinc 에 의한 대장암 세포주들의 세포 성장 저해 기전을 분자 수준에서 연구하였다. 100 μM 농도의 $ZnCl_2$ 를 HT29 대장암 세포주에 처리했을 때 ERK 단백질은 두 가지 다른 형태로 활성화 되었다. 먼저 15 분 이내의 짧은 시간대에 일시적인 ERK 활성화가 일어났으며 수시간 후에 강력하고 지속적인 ERK 활성화가 동반되었다. 세포 외부로부터 처리한 zinc 에 의한 지속적인 ERK 활성화는 Raf-1/MEK/ERK 모듈을 통해 유도되었으며 Elk-1 *trans*-reporter 유전자 발현을 현저하게 증가시켰다. 100 μM

농도의 $ZnCl_2$ 처리는 대장암 세포주의 세포 수 증가 억제와 세포 DNA 의 [^{3}H] thymidine 삽입을 현저하게 감소시켰으며 이러한 현상에서 p53 비의존적인 방법에 의한 $p21^{Cip/WAF-1}$ 의 핵으로의 국한된 발현 증가가 필수적인 요소로서 관찰되었다. PD98059 를 세포에 전 처리함에 의해 지속적인 ERK 활성화가 억제되었으며 이에 따라 $p21^{Cip/WAF-1}$ 의 핵으로의 국한된 발현 증가가 억제되었다. 그러므로 zinc 에 의한 세포 성장 억제는 강하고 지속적인 ERK 활성화에 따른 $p21^{Cip/WAF-1}$ 의 핵으로의 국한된 발현에 의존적임을 알 수 있다.

핵심되는 말 : Zinc, ERK, $p21^{Cip/WAF1}$