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Differential modulation of zinc-stimulated p21$^{Cip/WAF1}$ and cyclin D1 induction by inhibition of PI3 kinase in HT-29 colorectal cancer cells

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Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular signal regulated kinase; FBS, fetal bovine serum; PBS, phosphate-buffered saline; PD98059, 2-(2'-amino-3'-methoxyphenyl) oxanaphthalen-4-one; BrdU, bromodeoxyuridine; DAPI, 4',6'-diamidine-2-phenylindole dihydrochloride; PI3K, phosphoinositide 3-kinase.

Abstract

Activation of the extra cellular signal regulated kinase (ERK) pathway is involved in both proliferation and growth arrest of cells depending on intensity and duration of stimuli. In this study, we have elucidated differential regulation of the zinc-stimulated p21$^{Cip/WAF1}$ and cyclin D1 activation by inhibition of phosphoinositide 3-kinase (PI3K). In HT-29 colorectal cancer cells, the ERK activities were increased by zinc, which was accompanied by the induction of p21$^{Cip/WAF1}$ and cyclin D1. However, in the HT-29 cells pre-treated with PI3K inhibitor, LY294002, zinc induced further the p21$^{Cip/WAF1}$ induction whereas abrogated cyclin D1 induction. In addition, the induction of p21$^{Cip/WAF1}$ expression completely inhibited the incorporation of bromodeoxyuridine (BrdU) into the nucleus, indicating that p21$^{Cip/WAF1}$ is an important indicator for ERK-dependent growth arrest. These studies suggest presence of an inter-related regulatory mechanism of cell proliferation by ERK and PI3K pathways.

Keywords: MAP kinase, ERK, Zinc, cyclin D1, p21$^{Cip/WAF1}$, cell growth regulation, PI3 kinase

Introduction

The mitogen-activated protein kinase (MAPK) pathway (also known as extra cellular signal regulated kinase, ERK, pathway) is an important signal network for the proliferation and differentiation of cells (Blenis, 1993; Cobb and Goldsmith, 1995; Gutkind, 1998; Kang *et al*., 1999; Yeo *et al*., 2000). The role of activation of the MAPK pathway in the proliferation of cells has been well elucidated (Blenis, 1993; Pages *et al*., 1993; Kerkhoff and Rapp, 1998). However, a role of the MAPK pathway in growth inhibition has also been reported (Hirakawa and Ruley, 1988; Lloyd *et al*., 1997; Pumiglia and Decker, 1997; Hu *et al*., 1999). The conflicting results were partly explained by the MAPK pathway playing dual roles of progression and inhibition of the cell cycle regulators depending on the signaling intensity or duration (Pumiglia *et al*., 1997; Sewing *et al*., 1997; Woods *et al*., 1997). Moderate activation of the MAPK pathway has induced the proliferation of cells, but strong activation of the MAPK pathway has resulted in growth arrest of cells (Qiu and Green, 1991; Sewing *et al*., 1997; Woods *et al*., 1997; also reviewed in Kerkhoff and Rapp, 1998). p21$^{Cip/WAF1}$ is one of the CDK inhibitory proteins which is induced by both p53 dependent and independent mechanisms (Sherr and Roberts, 1995; Liu *et al*., 1996), and induction of p21$^{Cip/WAF1}$ is an important indicator for MAPK dependent growth arrest of cells (Sewing *et al*., 1997; Woods *et al*., 1997; Hu *et al*., 1999). The p53 independent induction of p21$^{Cip/WAF1}$ was acquired by high level of Raf-1 activation involving subsequent activation of MEK and ERK (reviewed in Kerkhoff and Rapp, 1998). A positive cell cycle regulator, cyclin D1, is also induced by activation of ERK pathway (Sewing *et al*., 1997; Woods *et al*., 1997). However, a physiological role of the concomitant induction of cyclin D1 together with p21$^{Cip/WAF1}$ is not illustrated. Recent studies identified several signaling molecules, which concomitantly activate ERK pathway and p21$^{Cip/WAF1}$ (Bottazzi *et al*., 1996; Liu *et al*., 1996; Archer *et al*., 1998; Liang *et al*., 1999). Zinc is involved in anti-proliferation of prostate cancer cells through the induction of p21$^{Cip/WAF1}$ (Liang *et al*., 1999). The ERK activities were also increased by zinc treatment in bronchial epithelial or Swiss3T3 fibroblast cells without known physiological function (Hansson *et al*., 1996; Samet *et al*., 1998). In addition, zinc also increased PI3K signaling pathway (Kim *et al*., 2000).

In this study, we identified induction of both cyclin D1 and p21$^{Cip/WAF1}$ cell cycle regulators by treatment of extra cellular zinc in HT-29 human colorectal cancer cells. Induction of the cyclin D1 and p21$^{Cip/WAF1}$ is dependent upon the activation of ERK pathway, and differentially

modulated by inhibition of PI3K activity. The activation of $p21^{Cip/WAF1}$ related with the potent inhibition of colorectal cancer cells. These results suggest the presence of a mechanism for an efficient regulatory mechanism of colorectal cancer cell proliferation by inter-related ERK and PI3K signaling pathways.

Materials and Methods

Cell culture

The HT-29 human colorectal cell line (Shiff *et al*., 1995; obtained from American Type Culture Collection) was maintained in McCoy's 5A medium supplemented with 10% (vol/vol) FBS, 100 units/ml of penicillin and 250 ng/ml of streptomycin in 5% CO_2 at 37°C. Experiments were performed on cells at 70% confluence. To observe zinc effects, the cells were serum-starved by growing in the medium containing 1% FBS for 16 to 20 h and treated with $ZnCl_2$ at a concentration of 100 μM. When required, the MEK-specific inhibitor, PD98059, or PI3K inhibitor, LY 294002, was added to different concentrations at 30 min prior to $ZnCl_2$ treatment.

Western Blot Analysis

For preparation of proteins, attached cells were rinsed with ice-cold PBS, harvested and then Laemmli sample buffer (Laemmli, 1970) was added to the samples. The samples were boiled and then subjected into 8-10% SDS polyacrylamide gel (acrylamide: bis-acrylamide at a ratio of 29:1), and the blots were prepared on a Protran membrane (Schleicher & Schuell GmbH, Dassel, Germany). Activation of endogenous ERKs was analyzed by using phospho-specific anti-ERK antibody (New England Biolabs Inc., Beverly, MA, USA). The level of ERKs was analyzed by using anti-ERK antibody (Stratagene, La Jolla, CA, USA). The levels of cyclin D1 and $p21^{Cip/WAF1}$ proteins were determined by using anti-cyclin D1 and -$p21^{Cip/WAF1}$ antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), respectively. Blots were probed with HRP-conjugated secondary antibody (Transduction Laboratories), and proteins were visualized using enhanced chemiluminescence (ECL) (Genepia, Seoul, Korea).

Immunocytochemistry and BrdU incorporation

For immunocytochemistry, HT-29 cells were plated onto cover-slips at a density of 2×10^5 cells/coverslip into 6-well plates, grown in McCoy's 5A and treated with $ZnCl_2$ to 100 μM. If required, 10 μM PD98059 or 50 μM LY294002 was added 30 min before zinc treatment. Twenty μM of BrdU was treated for 4 h before performing the immunocytochemistry. After 9 h, the 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 each. Cells were then treated with blocking solution (PBS containing 1% BSA, 0.1% gelatin, and 5% goat serum) for 30 min, the cover slips were further incubated with anti-rabbit-p21 antibody for 2 h, and washed 5 times with PBS containing 1% BSA and 0.1% gelatin for 5 min. They were then further incubated with goat anti-rabbit-Rhodamin Red™-X-conjugated secondary antibody at a dilution of 1:100 for 1 h, and washed 5 times with PBS for 5 min.

For the BrdU incorporation study, the cells were then fixed in 3.7% formaldehyde at room temperature for 10 min and rinsed once with PBS for 5 min 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 monoclonal antibody at a dilution of 1:20 for 2 h and washed 5 times with PBS containing 1% BSA and 0.1% gelatin for 5 min. The goat anti-mouse-Cy™2-conjugated secondary antibody at a dilution of 1:100 was incubated for 1 h and the cells washed 5 times with PBS for 5 min. Each experiment was performed at least three times. DAPI was then treated at a final concentration of 1 μg/μl in PBS for 10 min, and the cells extensively washed 5 times with PBS and 3 times with distilled water. The samples were mounted for photography, which was conducted using a Radiance 2000/MP, multi-photon imaging system (Zeiss, Germany).

Results and Discussion

The MAPK signaling pathway is an important signaling route involving cell proliferation (Blenis, 1993). Recent studies, however, also reported a role of the MAPK pathway in growth arrest of the cells. Zinc is an essential trace element in body and numerous studies have been reported involvement of dietary zinc in the proliferation as well as growth arrest of cells. Recent studies identified zinc-induced activation of ERK MAPKs in several types of cells (Hansson, 1996; Wu *et al*., 1999) and enhanced expression of $p21^{Cip/WAF1}$ cell cycle regulator in human prostatic carcinoma cells (Liang *et al*., 1999). However, the interrelationship between biochemical changes and cell growth related with those changes has not been clarified.

To better understand a role of the MAPK pathway in the regulation of $p21^{Cip/WAF1}$, we monitored ERK activation and $p21^{Cip/WAF1}$ induction in HT-29 colorectal cancer cell line (Shiff *et al*., 1995). The ERKs were strongly activated at 9 h after treatment with 100 mM $ZnCl_2$ in serum starved medium containing 1% FBS, and that concomitantly induced $p21^{Cip/WAF1}$, and cyclin D1 cell cycle regulators (Figure 1). Induction of the positive and negative cell cycle regulators, cyclin D1 and $p21^{Cip/WAF1}$,

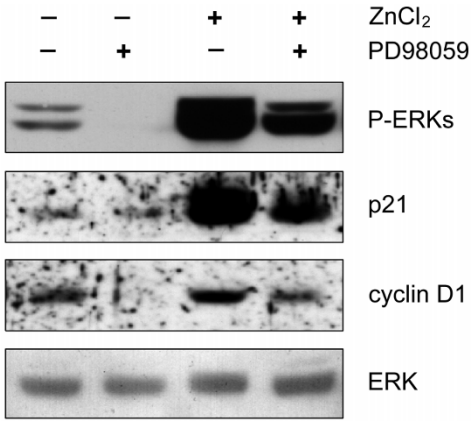

Figure 1. Zinc-dependent induction of p21$^{Cip/WAF1}$ and cyclin D1 inhibited by PD98059. HT-29 colorectal cells were grown in McCoy's 5A medium, and serum starved for 16 h in the medium containing 1% FBS before treatment with 100 μM $ZnCl_2$. When required, 10 μM of PD98059 was also treated for 30 min before zinc treatment. The cells were harvested at 9 h after zinc treatment for assay. ERK activities and protein levels of ERK, cyclin D1, and p21$^{Cip/WAF1}$ were measured by Western blot analysis using anti-phospho-ERK, ERK, cyclin D1, and p21$^{Cip/WAF1}$ antibodies, respectively.

by the MAPK pathway was also observed by other studies in NIH3T3 cells (Sewing *et al*., 1997; Woods *et al*., 1997). Activation of ERKs as well as the p21$^{Cip/WAF1}$ and cyclin D1 were significantly blocked by pre-treatment of an MEK inhibitor, PD98059 (Alessi *et al*., 1995) (Figure 1). These results suggest that the p21$^{Cip/WAF1}$ and cyclin D1 induction by zinc may be resulted form the activation of the ERK pathway. It is known that extra-cellular zinc also activates PI3K pathway (Kim *et al*., 2000) that related with ERK pathway. The PI3K pathway which is important for growth regulation and apoptosis often cross-talks with ERK pathway (Rommel *et al*., 1999; Zimmermann and Moelling, 1999), and expression of target genes by those signaling pathways could affect simultaneously by common signaling molecule (Kerkhoff and Rapp, 1998). However, relationship between these pathways in the regulation of target gene expression was not investigated. Here, we investigated the role of PI3K pathway in the ERK-dependent induction of p21$^{Cip/WAF1}$ and cyclin D1. We treated HT-29 colorectal cancer cells with a PI3K inhibitor, LY294002, prior to the zinc treatment, and also measured subsequent effects. As shown in Figure 2, the increase of p21$^{Cip/WAF1}$ protein by zinc treatment was further up-regulated by pre-treatment of LY294002 in HT29 cells. On the other hand, induction of cyclin D1 by zinc was lowered by pre-treatment of LY294002 (Figure 2A). The differential effects of LY294002 in the induction of p21$^{Cip/WAF1}$ and cyclin D1 were more convincingly demonstrated by using different dosages of LY294002. Although p21$^{Cip/WAF1}$ protein levels were dose-dependently increased by LY294002, cyclin D1 levels were oppositely decreased by increasing concentrations of LY294002 in the cells stimulated with extracellular zinc (Figure 2B).

The zinc-dependent induction and up-regulation of p21$^{Cip/WAF1}$ were also confirmed by immunocytochemistry. In a resting status, cells did not significantly induced p21$^{Cip/WAF1}$. When treated with 100 μM $ZnCl_2$, 8-9% of cells showed the induction and nuclear localization of p21$^{Cip/WAF1}$ (activation) (Figure 3A; representative cases are shown in panel B). The up-regulation of zinc-stimulated p21$^{Cip/WAF1}$ induction by Western blot analysis was also confirmed by immunocytochemistry. The percentage of cells with activated p21$^{Cip/WAF1}$ by zinc treatment was increased from 8-9% to 18% by pre-treatment of LY294002. It is currently unknown how zinc-dependent inductions of p21$^{Cip/WAF1}$ and cyclin D1 are differentially modulated by PI3K inhibitor, LY294002. The ERK activity was not significantly affected by pre-treatment of LY294002 whereas the zinc-induced p21$^{Cip/WAF1}$ induction was increased by LY294002 pre-treatment

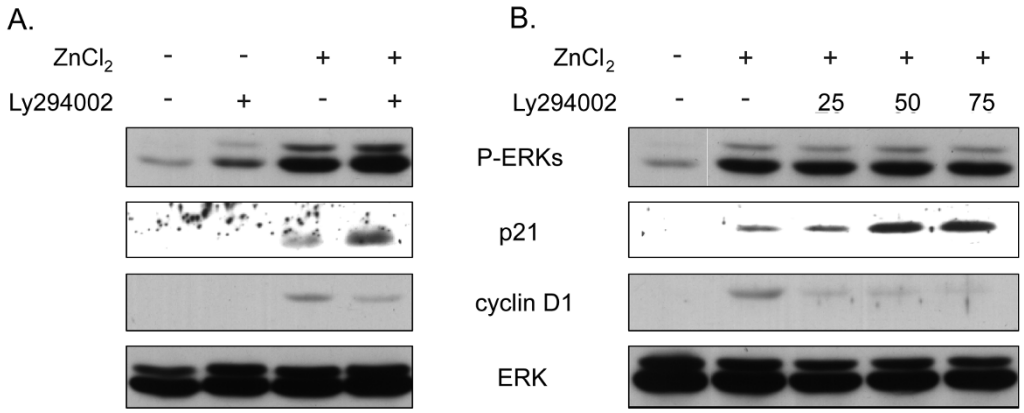

Figure 2. Differential induction of p21$^{Cip/WAF1}$ and cyclin D1 cell cycle regulators by LY294002 in zinc stimulated HT-29 colorectal cancer cells. The HT-29 cells were grown as shown in Figure 1 except that 25 μM (**A**) or different concentrations (25-75 μM) (**B**) of LY294002 were added before zinc treatment in cases required. The cells were harvested at 9 h after zinc treatment. The activities of ERKs and levels of ERK, p21$^{Cip/WAF1}$, and cyclin D1 were measured by Western blot analysis.

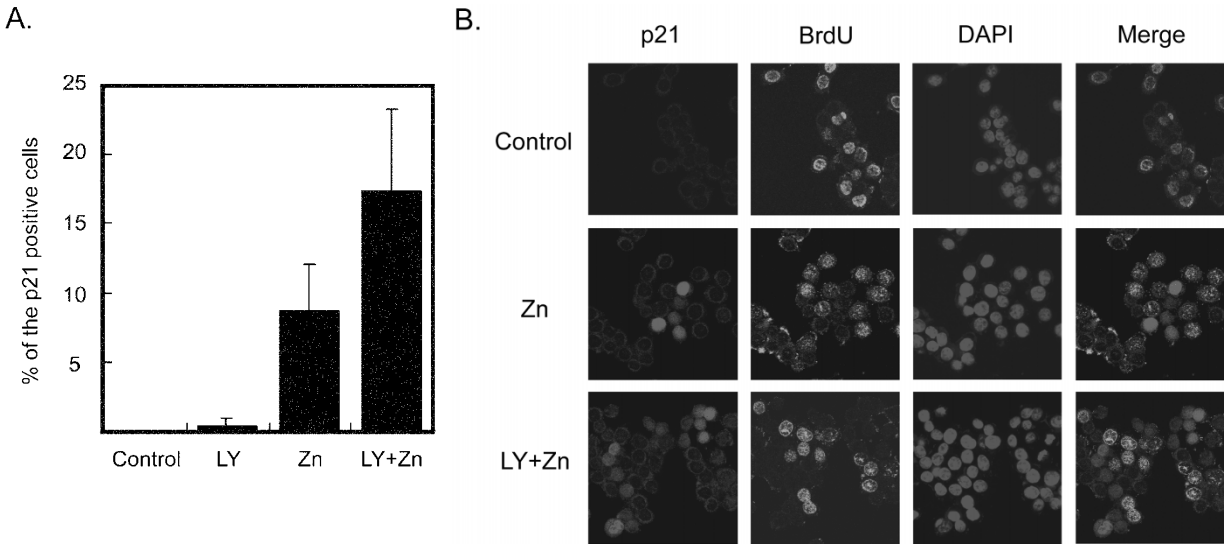

Figure 3. Differential activation of $p21^{Cip/WAF1}$ in HT-29 cells and inhibition of BrdU incorporation by the $p21^{Cip/WAF1}$. (**A**) The cells showing induction and nuclear localization $p21^{Cip/WAF1}$ (red colored) were recorded as $p21^{Cip/WAF1}$ positive cells. (**B**) Representative data of immunocytochemistry. No cells with activated $p21^{Cip/WAF1}$ incorporated BrdU in its nucleus. HT29 cells were grown in McCoy's 5A medium containing 1% FBS. The cells were then treated or not treated with 100 μM of $ZnCl_2$ for 9 h before immunocytochemistry. In the required cases, LY294002 (50 μM) was also treated for 30 min prior to zinc treatment. The cells were labeled with 20 μM of BrdU for last 4 h before assay. The expression and localization of $p21^{Cip/WAF1}$ was revealed by immunocytochemistry using the anti-$p21^{Cip/WAF1}$ antibody together with Rhodamin-conjugated goat anti-rabbit IgG. The BrdU incorporated into nuclei was detected as a green color by using anti-BrdU antibody followed by Cy2-conjugated goat anti-mouse IgG. Cell nuclei were stained with DAPI. Representative cells showing BrdU incorporation by $p21^{Cip/WAF1}$ induction are visualized in the merged panel.

(Figure 2A). Therefore, the up-regulation of $p21^{Cip/WAF1}$ by LY294002 may occur independent of ERK activation. Treatment of cells with LY294002 without zinc barely caused induction of $p21^{Cip/WAF1}$ (Figure 2A and 3A). Thus, the up-regulation of $p21^{Cip/WAF1}$ by LY294002 only occurs under the zinc-stimulating condition.

The fact that no BrdU uptake in the $p21^{Cip/WAF1}$ induced cells (representative cases are shown in Figure 3B) suggests that the induction of $p21^{Cip/WAF1}$ cell cycle regulator is an important indicator for anti-proliferation of cells by G1 cell cycle arrest. Zinc induced activation of $p21^{Cip/WAF1}$ may directly related with anti-proliferation of HT29 colorectal cancer cells. The differential effects of LY294002 in the zinc mediated induction of $p21^{Cip/WAF1}$ or cyclin-D1 suggest that roles of the PI3K pathway in the zinc-stimulated induction of $p21^{Cip/WAF1}$ and cyclin D1 may be different. The differential regulation of $21^{Cip/WAF1}$ and cyclin D1 by LY294002 in zinc-stimulated cells may be related with the cooperative role of ERK and PI3K pathways for efficient regulation of cell growth by differential regulation target gene expression.

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