Zinc-induced G2/M blockage is p53 and p21 dependent in normal human bronchial epithelial cells

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Wong SH, Shih RS, Schoene NW, Lei KY. Zinc-induced G2/M blockage is p53 and p21 dependent in normal human bronchial epithelial cells. Am J Physiol Cell Physiol 294: C1342–C1349, 2008.—The involvement of p53 and p21 signal pathway in the G2/M cell cycle progression of zinc-supplemented normal human bronchial epithelial (NHBE) cells was examined using the small interfering RNA (siRNA) approach. Cells were cultured for one passage in a cycle progression of zinc-supplemented normal human bronchial epithelial cells, which demonstrated that the p21 induction is p53 dependent. Furthermore, the normalization of p53 protein by siRNA treatment in ZS cells alleviated cell growth depression and G2/M blockage, which indicates that p53 is not required for the initial arrest of G2/M transition in HCT116 cells. In another case, expression of a truncated form of p53 that formed inactive tetraters with endogenous wild-type p53 shortened the G2 delay in IMR-90 fibroblasts.

Involvement of p53 in G2/M transition in response to DNA damage-induced stress has been elucidated by two major approaches. The first approach was to investigate the effect of eliminating p53 on the G2/M checkpoint. When p53 protein was inactivated by the human papilloma virus E6 protein, the number of IMR-90 normal fibroblasts entering mitosis after exposure to ionizing radiation was much higher, which suggested that p53 was required for the G2 arrest. In another case, expression of a truncated form of p53 that formed inactive tetraters with endogenous wild-type p53 shortened the G2 delay stimulated by exposure to ionizing radiation, which was explained by inactivation of p53 protein bound by large T antigen. The p53 null cell line, human colorectal tumor cell line HCT116, more clearly revealed the role of p53 played in the G2 checkpoint. After exposure of HCT116 to ionizing radiation, although the cells initially arrested at G2/M, the arrest was not stable and cells eventually entered mitosis. This indicates that p53 is not required for the initial arrest of HCT116 cells in G2 but is essential for the long-term maintenance of the arrest. The second approach was to examine the effect of overexpressing p53 on the G2/M transition. Agarwal et al. (1) have used a tetracycline-inducible system in p53-null human fibroblasts to demonstrate the role of p53 in G2 arrest. They first synchronized all p53-null human fibroblast populations to the beginning of S phase with mimosine. Thereafter, mimosine was removed and p53 was induced with tetracycline. They found that up to 60% of cells became arrested at G2/M and the level of phosphorylated histone H1b, which is normally highest during mitosis, was very low in these arrested cells (1, 38). These results indicate that p53 is involved in G2 arrest and that the arrest is not due to an aborted attempt at mitosis, followed by arrest in G1.

Successful G2/M transition is governed by cyclin-dependent kinase Cdc2 (31). Cdc2 binds to Cyclin B to form a complex that is activated by Cdc25 at the onset of mitosis (11). The mechanism by which p53 regulates the G2/M transition is proposed to mediate its immediate downstream transcriptional target p21, which is a Cdc2 inhibitor (42). The involvement of p21 in G2 checkpoint has been widely documented. Induction of p21 by a tetracycline-regulated system caused a number of different cell lines, including human Hela cervical carcinoma cells, Saos-2 and U2OS osteosarcoma cells, RKO colorectal carcinoma cells, H1299 lung carcinoma cells, and Rat

THE ACCURACY of genomic DNA replication is ensured by two major checkpoint controls at G1-S and G2/M transition during cell division. Therefore, dysregulation of these checkpoint controls would cause genomic instability, which has been implicated in carcinogenesis (15). The tumor suppressor gene p53 has been found to be mutated in a large fraction of human cancers and is a major gatekeeper of cell cycle division (26). p53 protects mammals from carcinogenesis by inducing apoptosis, DNA repair, and cell cycle arrest in response to a variety of stresses (1, 25, 26). In response to ionizing or ultraviolet radiation or other chemical stresses, p53 has been found to mediate cell cycle arrest mainly at G1-S transition through the immediate downstream target p21/waf1 (13, 22, 43). However, circumstantial evidence has emerged to support that p53 is also involved in the regulation of G2/M transition through the modulation of p21 expression.

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fibroblast cells, to arrest at G2 (4, 29, 30). Similar to HCT116 colorectal tumor cells lacking p53, the cells lacking p21 also did not arrest stably in G2 after exposure to ionizing radiation (6). This failure to arrest was associated with levels of Cdc2 kinase activity that is higher than those observed in cells with p21. Additional evidence linking p21 to the G2/M transition comes from its restored abundance when the cells enter G2 (12).

Metal ions are vital for many biological processes, such as transcription, respiration, and growth. However, excess accumulation of essential metals such as zinc, copper, cadmium, and mercury can be detrimental. Although, zinc is not redox active under physiological concentrations and is less toxic than most metal ions, at high concentration, zinc toxicity is associated with reduced iron absorption, impaired immune function, and neuronal death (9, 24). In our previous study, we found that G2/M arrest coupled with depressed growth were observed in normal human bronchial epithelial (NHBE) cells cultured in zinc-supplement media (35). Signal cascade of Gadd45 and p21 was measured by 10.220.33.6 on May 2, 2017 http://ajpcell.physiology.org/ Downloaded from

### MATERIALS AND METHODS

**Cell culture.** NHBE cells were purchased from Cambrex Bio Science (Walkersville, MD). Cells were cultured with bronchial epithelial cells growth medium (BEGM) supplemented with 0.5 μg/ml epinephrine, 10 μg/ml transferrin, 5 μg/ml insulin, 0.1 ng/ml retinoic acid, 52 μg/ml bovine pituitary extract, 0.5 μg/ml hydrocortisone, 0.5 pg/ml of a rabbit anti-p21 antibody conjugated with horseradish peroxidase (Santa Cruz Biotechnology) and exposed to film. The optical densities of the protein bands were quantified by the Alpha Innotech Imaging System (San Leandro, CA).

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NHBE cells were cultured in ZD, ZN, ZA, and ZS for 6 days. The cell number was determined by trypan blue dye exclusion. Cell morphology was evaluated by using a phase-contrast microscope (Olympus, Tokyo, Japan).

**Cellular zinc and DNA determination.** After reaching 80% confluence, cells were harvested by trypsinizing with trypsin-EDTA for 5 min in a 37°C incubator. Cell suspensions were then centrifuged at 500 g for 5 min at room temperature. Cell pellets were washed with phosphate-buffered saline (PBS) buffer, centrifuged again, and then resuspended into 1.5 ml PBS and sonicated for two 30-s intervals. Thereafter, an aliquot of the sonicated cell suspension was used to measure cellular zinc content by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA). Zinc standard solutions (Fisher, Pittsburgh, PA) ranging from 0.05 to 1.0 ppm were used to generate a linear standard curve. The zinc content of the cells was determined based on these zinc reference solutions. In addition, the certificated zinc solutions were compared with bovine Liver Standard Reference (Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. From the same sample, a small aliquot of the sonicated cell suspension was used to measure cellular DNA content using diphenylamine (41). Data were expressed as cellular zinc per microgram of DNA because of the linear relationship between cellular DNA and cell number we previously established (41).

**Rnase protection assay.** Total cellular RNA was isolated from HepG2 cells by using the RNeasy Mini Kit (Qiagen, Valencia, CA), according to manufacturer’s instructions. The integrity of the RNA was verified by RNA gel electrophoresis. The mRNA abundance of p21 was measured by the nonradioactive RNase Protection Assay System (BD Biosciences, San Diego, CA), according to manufacturer’s instructions. The human GAPDH probe was used as internal reference for normalization. Biotin-labeled riboprobes were synthesized using non-Rad In Vitro Transcription kit with T7 RNA polymerase (BD Biosciences) according to manufacturer’s instructions.

**Nuclear and cytoplasmic extract preparation.** The NE-PER Nuclear and Cytoplasmic Extraction Reagents and the Halt Protease Inhibitor Cocktail Kits (Pierce Biotechnology, Rockford, IL) were used for nuclear and cytoplasmic extracts preparation according to the manufacturer’s instructions, which were based on the method of Smirnova et al. (36). Nuclear and cytoplasmic extracts were then stored in aliquots at −80°C. Protein concentrations were determined by using the BCA Protein Assay Reagent kit (Pierce). Contaminations of nuclear extracts by cytoplasmic proteins or contamination of cytoplasmic extracts by nuclear proteins, detected by Western blot analysis of heat shock protein 90 (Hsp90) or POU family homeodomain protein (Oct-1), respectively, were routinely found to be <5% in our lab.

**Western blot analysis.** Forty micrograms of nuclear and cytoplasmic protein were resolved on a 10% SDS-polyacrylamide gel electrophoresis and transferred to Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) by using a minitransfer system (Bio-Rad, Hercules, CA). Membranes were blocked with 5% nonfat dry milk in PBS-T (10 mM phosphate buffer, pH 7.3, 137 mM NaCl, 2.7 mM KCl, and 0.1% Tween 20) for 1 h at room temperature before incubation with 1 μg/ml of a rabbit anti-p21 polyclonal antibody (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA), in PBS-T containing 5% nonfat milk at 4°C overnight. Membrane was then washed three times with PBS-T and blotted with a secondary antibody conjugated with horseradish peroxidase (Santa Cruz) at room temperature for 1 h, followed by three washes in PBS-T. The protein was visualized by using the Western Blot Lumiloid Reagent (Santa Cruz Biotechnology) and exposed to film. The optical densities of the protein bands were quantified by the Alpha Innotech Imaging System (San Leandro, CA).

**p21 promoter activity.** The influence of zinc status on the human p21 gene promoter activity was studied by transient transfection of a p21-promoter-luciferase gene into NHBE cells. This approach was used to provide further evidence that the transcription process is
depressed by a reduction in p21 gene promoter activity in zinc-deficient NHBE cells.

Preparation of luciferase construct. The p21 promoter construct was a kind gift provided by Dr. Lieberman (44), which consisted of a 2,337-bp fragment of the human p21 promoter (base pairs −2258 to −4594) (14), containing two p53-binding consensus sites, and with HindIII and XhoI sites added on the ends. It was isolated and inserted into the plasmid pG53-basic (Promega, Madison, WI) to generate construct pG53-p21-Luci plasmid. The pG53-p21-Luci plasmid was transfected into Escherichia coli DH5α competent cells (Invitrogen) by standard protocol for mass production. The plasmid was prepared by using Wizard PurePrep Plasmid DNA purification system from Promega.

Transient transfection and luciferase assay. NHBE cells were transfected by using Tfx-20 reagent according to the protocol provided by the manufacturer (Promega). Cells in the ZN bronchial epithelial cell growth medium were seeded at a density of 2 × 10⁵ cells/well in 24-well plates and then cultured for 4 days in their respective treatment media (ZD, ZN, ZA, and ZS) as described in Cell culture (33). Just before transfection, the medium will be removed. Transfections were performed in triplicate with 500 ng of the plasmid DNA containing the wild-type p21 promoter luciferase reporter construct and 10 ng of an internal control plasmid pRL-SV40 (Promega).

One hour after transfection, the transfection medium was changed to their respective media and cultured for 2 more days. Luciferase activity was measured in the Luminometer TD-20/20 (Turner Designs, Sunnyvale, CA) by using the Dual-Luciferase reporter Kit according to recommendations by the manufacturer (Promega). Changes in firefly luciferase activity was calculated and plotted after normalization with changes in renilla luciferase activity in the same sample.

Cell cycle analysis. DNA contents of cells were assayed by fluorescence-activated cell sorting (FACS). NHBE cells were cultured in ZD, ZN, ZA, and ZS media for one passage, trypsinized, washed in PBS (Ca²⁺, Mg²⁺ free), and fixed in 70% cold ethanol. Cells were stored at 4°C. For staining, cells were collected by centrifugation, and pellets were suspended in 1.0 ml propidium iodide staining solution (50 mg/ml propidium iodide, 100 U/ml RNase in PBS) and incubated at room temperature for 1 h. Staining was quantitated with a FACS-Calibur cytometer (Becton Dickinson, San Jose, CA). The data files were analyzed by using the CELLQuestPro software program (Becton Dickinson). Cell cycle distribution percentages of stained nuclei were calculated by using Modfit LT software (Verity Software House, Topsham, ME). The calibration standard LinearFlow green and the DNA QC Particle kit, for verification of instrument performance, were purchased from Molecular Probes (Eugene, OR) and Becton Dickinson, respectively.

siRNA transfection. Small interfering RNA (siRNA) duplex against p21 (sc-29427) and p53 (SC-29435) were purchased from Santa Cruz Biotechnology. A control siRNA specific targeting to the Luciferase DNA sequence was used as a negative control. For cell transfection with siRNA, NHBE cells were cultured on culture dishes at 2 million cells/well in 24-well dishes and incubated for 1 day in their respective media and cultured for 2 more days. Luciferase activity was measured in the Dual-Luciferase reporter Kit (Promega). Statistical analysis. Each experiment was repeated at least three times with each experiment yielding essentially identical results. Data were expressed as means ± SE. Statistical comparisons were carried out by one-way analysis of variance (ANOVA). Means were examined by the Least Significant Difference post hoc analysis (SPSS, Chicago, IL). P < 0.05 was considered statistically significant.

RESULTS

Zinc supplementation reduces cell growth of NHBE cells. In vitro content of cellular zinc in ZD cells, as measured by flame atomic absorption spectrophotometry, was 70% lower than that in ZN control cells (Fig. 1A). Moreover, cellular zinc levels in ZA and ZS cells were 118% and 435%, respectively, higher than ZN cells. Growth as measured by cell number was significantly depressed in ZD, ZA, and ZS cells to 85%, 90%, and 70%, respectively, of ZN cells (Fig. 1B).

Zinc supplementation suppresses G2/M progression. Cell cycle analysis of three independent experiments demonstrated that the proportion of cells in G2/M progression was the following: 11.9 ± 0.4% for ZD; 12.9 ± 0.3% for ZN; 15.9 ± 0.6% for ZA; and 19.8 ± 0.2% for ZS cells. A significant delay in G2/M cell cycle progression was observed in ZS NHBE cells (19.8%) compared with that in ZN cells (12.9%). The
finding confirmed our previous report of G2/M blockage in ZS NHBE cells (35). The G2/M suppression was smaller in magnitude in ZA cells, with 15.9% of cells in G2/M. In contrast, no change was observed in ZD cells, with 11.9% cells in G2/M, compared with ZN cells (Fig. 2). This result indicates that the impaired G2/M progression in zinc-supplemented NHBE cells is responsible for depressed cell growth.

Zinc supplementation upregulates p21 protein and mRNA levels as well as p21 promoter activity. p21 mRNA levels in the ZA and ZS cells were drastically upregulated to almost 200% of ZN cells (Fig. 2). Consistent with the pattern of p21 mRNA expression, nuclear p21 protein levels in ZA and ZS cells were similarly upregulated (Fig. 3). In contrast, no significant differences were detected in nuclear p21 protein levels as well as p21 mRNA abundance between the ZD and ZN cells (Figs. 2 and 3). Transient transfection of a p21-promoter-luciferase reporter gene into NHBE cells indicated that the p21 promoter activity of ZA and ZS cells was significantly induced to 150% and 200% of ZN cells, respectively. In contrast, no difference was observed between ZD and ZN cells (Fig. 4).

Transfection of the p21 siRNA, at 200 pmol/plate, normalizes the induced p21 protein level in ZS cells to that of ZN control cells. By transfecting graded amounts of p21 siRNA (600, 400, and 200 pmol/plate), the amount of p21 siRNA at 200 pmol per transfection was able to abrogate the elevated p21 protein level in ZS cells and normalize it to that of ZN control cells (Fig. 5). In addition, ZN cells transfected with 200 pmol of p21 siRNA showed a near 20% decrease in p21 protein level than ZN control cells. The same batch of cells with normalized p21 protein level in ZS cells were used for G2/M cell cycle analysis to determine whether G2/M arrest was altered or not.

Normalization of p21 protein level in ZS cells with p21 siRNA alleviates the G2/M blockage and restores depressed cell growth. After the induced protein level of p21 in ZS cells was knocked down to a similar level as in ZN cells with p21 siRNA, the cells were subjected to cell cycle analysis to examine the association of p21 induction with G2/M arrest in

Fig. 2. High intracellular zinc status induces p21 mRNA level. Total RNA was isolated from cells by using RNeasy Kit (Qiagen, Valencia, CA). The abundance of p21 mRNA was measured by using a nonradioactive RPA kit (BD Biosciences, San Diego, CA) according to the manufacturer’s instructions. The RPA probe set, which included p21 and GAPDH probe, was from the same manufacturer. The optical densities of the protected p21 bands were quantified by the Alpha Innotech imaging system (San Leandro, CA) and then normalized to that of GAPDH. Values shown in bar graphs represent means ± SE from 3 separate experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicate no significant difference.

Fig. 3. High intracellular zinc status induces p21 protein level. Nuclear proteins (40 μg) prepared from NHBE cells were separated on SDS-PAGE, Western blotted, and probed with antibody p21 (C-19). The nuclear protein p21 band was normalized to the corresponding housekeeping gene Oct-1 and expressed as a percentage of ZN cells. Values shown in bar graphs represent means ± SE from 3 separate experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicate no significant difference.

Fig. 4. High intracellular zinc status enhances p21 promoter activity. NHBE cells were cultured in media with respective zinc concentration (ZD = 0.4 μM; ZN = 4 μM; ZA = 16 μM; and ZS = 32 μM zinc supplemented to the basal medium) for 4 days. Thereafter, cells were transiently transfected with 500 ng of luciferase reporter construct (PGL3-p21) containing the p21 promoter, together with 10 ng pRL-SV40 as internal control. PGL3 basal vector without carrying any promoter was used as the vector control. After transfection, the cells were cultured in corresponding media for 2 more days. Cells extracts were then assayed by Dual-Luciferase reporter system and signals were measured by a Luminometer. Values represent means ± SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicate no significant difference.
was the following: 12.9 demonstrated that the proportion of cells in G2/M progression ZS cells. Cell cycle analysis of three independent experiments NHBE cells were cultured in media with respective zinc concentration (ZN abolishes the induced p21 protein level in zinc-supplemented NHBE cells. NHBE cells were cultured in media with respective zinc concentration (ZN = 4 µM and ZS = 32 µM) for 4 days. Thereafter, cells were transiently transfected with either p21 siRNA at concentration of 200 pmol/plate or Luc siRNA as a negative control. After transfection, the cells were cultured in corresponding media for 2 more days. Total cell numbers were counted using hemacytometer and were expressed as percentage of ZN control. Values represent means ± SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicates no significant difference.

Fig. 5. Transfection of p21 small interfering RNA (siRNA), at 200 pmol/plate, abolishes the induced p21 protein level in zinc-supplemented NHBE cells. NHBE cells were cultured in media with respective zinc concentration (ZN = 4 µM and ZS = 32 µM) for 4 days. Thereafter, cells were transiently transfected with graded amounts of p21 siRNA (600, 400, and 200 pmol/plate). After transfection, the cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to protein expression analysis by Western blot. Values represent means ± SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicates no significant difference.

ZS cells. Cell cycle analysis of three independent experiments demonstrated that the proportion of cells in G2/M progression was the following: 12.9 ± 0.3% for ZN; 12.8 ± 0.5% for ZN + LUC; 11.0 ± 0.1% for ZN + si-p21; 20.8 ± 0.9% for ZS; 19.8 ± 0.4% for ZS + si-LUC; and 12.0 ± 0.3% for ZS + si-p21 cells. Thus the normalization of p21 protein level in ZS cells alleviated the G2/M blockage from 20% and normalized it back to 12%, which was similar to the percentage of ZN control cells in G2/M phase. As for ZN cells transfected with same amount of p21 siRNA, the percentage of cells in G2/M was slightly reduced. The cell growth as measured by cell numbers per plate indicated that, in ZS+si-p21 cells, the normalization of p21 protein level by using p21 siRNA restored the originally depressed growth of ZS cells back to the same level of ZN control cells (Fig. 6).

Upregulation of p21 in ZS cells is dependent on p53 expression. p53 was widely reported as an immediate upstream regulator of p21 for its role played in G1/S cell cycle regulation (13). In addition, p53 has also been widely reported to be the major regulator of p21 for another role in G2/M cell cycle checkpoint (reviewed in Ref. 38). To investigate whether in ZS cells, the upregulation of p21 and its involvement in G2/M arrest were modulated by p53, the gene knockdown approach with p53 siRNA was applied. First, p53 protein level was found to be induced to more than twofolds higher in ZS cells than that in ZN cells (Fig. 7). By transfecting graded amounts of p53 siRNA (600, 400, and 200 pmol/plate), the amount of p53 siRNA at 200 pmole per transfection was found to be able to abrogate the amount of elevated p53 protein level in ZS cells and normalize it to that of ZN control cells. The same batch of cells with normalized p53 protein level in ZS cells were used for p21 protein level measurement. Interestingly, in ZS-si-p53 cells with normalized p53 level, the originally elevated p21 protein level was found to be abolished and reduced to the level almost identical to that of ZN control cells (Fig. 8). In addition, ZN cells transfected with the same amount of p53 siRNA showed a near 40% decrease in p21 protein level than ZN

Fig. 6. Cell growth depression in zinc-supplemented NHBE cells is abolished after abrogation of induced p21 protein level. NHBE cells were cultured in media with respective zinc concentration (ZN = 4 µM and ZS = 32 µM) for 4 days. Thereafter, cells were transiently transfected with either p21 siRNA at concentration of 200 pmol/plate or Luc siRNA as a negative control. After transfection, the cells were cultured in corresponding media for 2 more days. Total cell numbers were counted using hemacytometer and were expressed as percentage of ZN control. Values represent means ± SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicates no significant difference.

Fig. 7. Transfection of p53 siRNA, at 200 pmol/plate, abolishes the induced p53 protein level in zinc-supplemented NHBE cells. NHBE cells were cultured in media with respective zinc concentration (ZN = 4 µM and ZS = 32 µM) for 4 days. Thereafter, cells were transiently transfected with graded amounts of p53 siRNA (600, 400, and 200 pmol/plate). After transfection, the cells were cultured in corresponding media for 2 more days. Total proteins were then extracted and subjected to protein expression analysis by Western blot analysis. Values represent means ± SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicates no significant difference.
control cells. The ZS cells, with normalized level of p53 protein, were then used for cell growth determination.

Normalization of p53 protein level in ZS cells with p53 siRNA alleviates the G2/M blockage and restores depressed cell growth. p53 is a well-known tumor suppressor gene. In response to various stresses, upregulation of p53 induces immediate downstream target p21, which arrests cell cycles at different phases for DNA repair (13, 22, 43). To establish that G2/M blockage is caused by elevated p53 expression, ZS-siRNA alleviates the G2/M blockage and restores depressed cell growth. Therefore, the enhancement of cellular zinc status depressed G2 to M phase progression, which resulted in cell growth reduction. Moreover, Gadd45 induction in ZS NHBE cells was found to be partially responsible for the depressed G2/M progression. In view of available evidence indicating that p21, which is a major cell regulator at G1 and is also involved in G2/M regulation (12), we have designed the present study to examine whether p21 and p53 expression are affected and whether they are also involved in the G2/M arrest in zinc-supplemented NHBE cells.

In the present report, we have presented multiple lines of evidence for the first time to substantiate that the transcriptional process and expression of p21 are upregulated in zinc-supplemented NHBE cells in primary culture. The observed induction of almost twofold higher nuclear p21 protein and p21 mRNA in ZA and ZS cells than control ZN cells (Figs. 2 and 3) indicates that the transcriptional process is enhanced in ZS cells. The p21 promoter activity was measured to address the question whether the induced expression of mRNA and protein were caused by an upregulation of promoter activity. A similar pattern of induction of p21 promoter activity, as in p21 mRNA and protein levels, was observed in ZA and especially ZS cells compared with ZN cells (Fig. 4). These findings indicate that an enhanced promoter activity, instead of another mechanism such as mRNA stability, may be mainly responsible for the upregulation of p21 gene and protein expression.

Available data indicate that p21 is involved in G2/M transition. For example, in human fibroblasts, p21 mRNA abundance shows bimodal periodicity with peaks in G1 and G2/M (27) and that nuclear p21 protein reaccumulates at the onset of mitosis (12). In addition, inducible p21 expression was demonstrated to be responsible for cell cycle arrest at both G1 and G2 (7). Moreover, p21 was found to induce G2 arrest in human

DISCUSSION

In a previous study (35), we have reported that in ZS NHBE cells, the enhancement of cellular zinc status depressed G2 to M phase progression, which resulted in cell growth reduction. Moreover, Gadd45 induction in ZS NHBE cells was found to be partially responsible for the depressed G2/M progression. In view of available evidence indicating that p21, which is a major cell regulator at G1 and is also involved in G2/M regulation (12), we have designed the present study to examine whether p21 and p53 expression are affected and whether they are also involved in the G2/M arrest in zinc-supplemented NHBE cells.

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Fig. 8. Transfection of p53 siRNA abrogates the elevated p21 level in zinc-supplemented NHBE cells with normalized p53 protein level. NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μM and ZS = 32 μM) for 4 days. Thereafter, cells were transiently transfected with p53 siRNA at concentration of 200 pmol/plate. After transfection, the cells were cultured in corresponding media for 2 more days. Total proteins were extracted and subjected to p21 protein expression analysis by Western blot analysis. Values represent means ± SE from 3 experiments. Data were analyzed by one-way ANOVA. Different letters indicate significantly different means (P < 0.05); whereas means with the same letter indicates no significant difference.

Fig. 9. Cell growth depression in zinc-supplemented NHBE cells is abolished after abrogation of induced p53 protein level. NHBE cells were cultured in media with respective zinc concentration (ZN = 4 μM and ZS = 32 μM) for 4 days. Thereafter, cells were transiently transfected with either p53 siRNA at concentration of 200 pmol/plate or Luc siRNA as a negative control. After transfection, the cells were cultured in corresponding media for 2 more days. Total cell numbers were counted using hemacytometer and was expressed as percentage of ZN control cells. The ZS cells, with normalized level of p53 protein, were then used for cell growth determination.
fibroblasts cells (37). Thus we have designed the present study to investigate whether the enhanced expression of p21 in ZS cells was associated with the G2/M arrest. A gene knockdown approach was employed to examine whether G2/M blockage would be alleviated after abrogation of the enhanced p21 expression in ZS cells. By transfecting graded amounts of p21 siRNA, the transfection of siRNA at 200 pmol/plate was found to abolish the elevated level of p21 protein in ZS cells to that of ZN cells (Fig. 5). The ZS-si-p21 cells with normalized level of p21 protein were subjected to cell cycle analysis. The result indicated that, after the normalization of p21 protein level in ZS cells, the original G2/M blockage was alleviated to the same level in ZN control cells. Whereas for ZN cells transfected with same amount p21 siRNA, G2/M cell cycle transition was slightly reduced compared with ZN control cells. Furthermore, the alleviation of G2/M blockage in ZS cells with normalized p21 protein level was also accompanied by a normalized cell growth. The original growth repression in ZS cells was abolished and cell growth was restored to a similar level as in ZN controls cells (Fig. 6). Thus the elevation of p21 expression in zinc-supplemented NHBE cells is largely, if not entirely, responsible for the observed G2/M blockage as well as growth repression induced by high zinc status in NHBE cells. In fact, three mechanisms have been proposed for how upregulation of p21 participates in inhibiting Cdc2 activity to cause G2 arrest. First, p21 directly binds to cdc2/cyclin B1 complexes and inhibits Cdc2 activity (5). p21 was found in cyclin B1 immunoprecipitates in cells overexpressing p21 (29, 38). However, in some studies, it was not found in cyclin B1 immunoprecipitates (4, 12). It was suggested that binding to Cdc2/cyclin B1 may be only observed when there are very high levels of p21 (39). A second mechanism for inhibiting Cdc2 has been suggested by a report showing that p21 may inhibit Cdk2, which is an activator of Cdc2 and p21, and resulted in the loss of Cdc2 activity (20). A third mechanism of Cdc2 inhibition by p21 was suggested by the report of Smits et al. (37), which showed that overexpression of p21 reduced the phosphorylation of Cdc2 on threonine-161 and the Cdc2 activity. By adding recombinant CAK protein, which phosphorylated Cdc2 on threonine 161, the immunoprecipitated Cdc2 activity was restored (37).

We then investigated whether the elevation of p21 expression in ZS cells was dependent on p53. Although p53-independent regulation of p21 expression has been widely reported, p53 is still well recognized as a major immediate upstream regulator of p21 expression (13). In particularly, in the modulation of G2/M progression, p53 has been proposed to employ p21 in the regulation of Cdc2/cyclin B complex activity, which is a cell-cycle regulator of G2 to M transition (reviewed in Ref. 39). In ZS cells, p53 was found to be induced to almost twofold higher than ZN control cells (Fig. 7). The magnitude of elevation of p53 is similar to that of p21 protein. Therefore, a similar gene knockdown approach used to examine whether p21 protein induction would be dependent on p53 expression. By testing graded amounts of p53 siRNA, the transfection of 200 pmole of siRNA per plate was found to abolish the elevated level of p53 protein in ZS cells and normalized it to that of ZN cells (Fig. 8). The ZS-p53-siRNA cells, with normalized level of p53 protein, were subjected to p21 protein measurement as well as cell growth determination. Our results indicated that in ZS-siRNA cells, after the normalization of p53 protein level, the original twofolds-elevated p21 protein was abolished and the protein level was normalized to a level as in ZN control cells (Fig. 8). The ZN cells transfected with same amount of p53 siRNA showed 50% less p21 protein than that of ZN control cells, indicating the efficacy of p53 knockdown by using p53 siRNA. The ZS-si-p53 cells with normalized level of p53 protein were then subjected to cell cycle analysis to examine whether G2/M arrest would be alleviated. The result indicated that, after the normalization of p53 protein level in ZS cells, the original G2/M blockage was alleviated to the same level in ZN control cells. However, for ZN cells transfected with same amount p53 siRNA, the G2/M cell cycle transition was only slightly reduced compared with ZN control cells. Furthermore, with the normalization of p53 protein in ZS-Si-p53 cells, original growth repression was abolished and cell growth was restored back to 20% higher than ZN control cells (Fig. 9). This result is consistent with previous contention that p53 functions as a negative growth regulator. In addition, ZN cells transfected with the same amount of p53 siRNA showed an almost 40% higher cell growth than ZN control cells. Thus high intracellular zinc content appeared to induce p53, which in turn led to the elevation of p21 gene expression, blockage of G2/M progression, and suppression of cell proliferation.

Several studies have indicated that high cellular zinc may exert adverse effect on human cell growth (3, 17). Moreover, studies also reported that treatment with high level of zinc inhibited cancer cells proliferation by mediating G2/M arrest (23, 28). In one report, human primary liver cells cultured in 100 μM zinc were found to exhibit DNA fragmentation (32). However, the present report is the first to show that 32 μM of zinc concentration, which represents the high end of plasma zinc attainable by oral supplementation, can cause G2/M arrest and cell growth repression in primary cell line. In our NHBE study, the cytotoxic stress induced by zinc supplementation may be one of the stress factors that elevates p53 expression, which then suppresses G2/M progression through the upregulation of cyclin-dependent kinase inhibitor p21. In fact, preliminary results in our laboratory indicated that the amount of endogenous reactive oxygen species (ROS) generated in ZS NHBE cells was higher than that in ZN cells (Shih et al., personal communication). ROS have been implicated in DNA damage by genotoxic agents such as ultraviolet and ionizing radiation or doxorubicin. Subsequent accumulation of ROS-damaged DNA is a critical event during carcinogenesis and aging (2). DNA damage triggers a variety of signaling pathways that lead either to apoptosis or to DNA damage repair that is coupled with cell cycle regulation. One function of such pathways is controlled by the tumor suppressor protein p53. The principal function of p53 is to promote survival or apoptosis for cells exposed to agents that cause DNA damage, such as hypoxia, ultraviolet radiation, ROS, or mutagens (10, 18, 19, 34). The p53 protein is involved in complex cellular responses to DNA damage. These responses involve DNA editing and repair followed either by normal cell division (33) or by apoptosis (21). Cells damaged by ultraviolet radiation and oxidative stress often remain in the G1/G2-phase of the cell cycle for long periods (16). Therefore, the oxidative stress induced in ZS cells may trigger the upregulation of p53 signal transduction pathway that causes the G2/M arrest in our cell system.
ZINC-INDUCED G2/M ARREST IS p53 DEPENDENT

In summary, the present data suggest that the upregulation in p53 expression in zinc-supplemented NHBE cells resulted in an elevation of p21 expression, which contributed to the G2/M blockage. Comprehensive studies will have to be designed to firmly establish that a possible induction of oxidative stress in zinc-supplemented NHBE cells may be the trigger of p53 signal transduction pathway responsible for the G2/M blockage.

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