Nuclear Accumulations of p53 and Mdm2 Are Accompanied by Reductions in c-Abl and p300 in Zinc-Depleted Human Hepatoblastoma Cells

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The influence of zinc status on the expression of proteins known to be involved in the stability of p53, the human tumor suppressor gene product, was examined in hepatoblastoma (HepG2) cells. Cells were cultured in zinc-deficient (ZD0.2, ZD0.4), zinc normal (ZN), zinc adequate (ZA), or zinc-supplemented (ZS) medium, which contained 0.2, 0.4, 4, 16, or 32 μM zinc, respectively. Nuclear p53 levels were almost 100% and 40% higher in ZD0.2 and ZD0.4 cells, respectively, than in ZN cells. Mdm2 protein, which mediates p53 degradation, was 174% and 148% higher in the nucleus of ZD0.2 and ZD0.4 cells, respectively, than in ZN cells. In addition, the observed reductions of nuclear c-Abl in ZD0.2 and ZD0.4 cells to 50% and 60% of ZN cells, respectively, may be a cellular response attempting to normalize nuclear p53 accumulation because nuclear c-Abl is known to down-regulate ubiquitination and nuclear export of p53. Moreover, no changes in total cellular p53, Mdm2, and c-Abl or nuclear Mdmx were observed among the treatment groups. Furthermore, in ZD0.2 and ZD0.4 cells, the reduction in total and nuclear p300, which is known to complex with CREB-binding protein and Mdm2 in the nucleus for the generation of degradable polyubiquitinated form of p53, may have depressed the degradation pathway for p53 and Mdm2, and contributed to the nuclear accumulation of these proteins in ZD cells. Exp Biol Med 231:611–618, 2006

Key words: p53; Mdm2; p300; c-Abl; zinc deficiency

In human cancer the tumor suppressor gene, *p53*, is one of the most frequently mutated genes. p53 is a transcription factor that regulates many downstream genes by the activation or repression of target genes. Many cellular functions, including the induction of cell cycle arrest or apoptosis, are mediated by p53 in response to DNA damage, genetic recombination, DNA repair, and the preservation of genetic stability (1).

The human p53 protein, consisting of 393 amino acids, can be divided into several structural and functional domains, many of which contribute to the ability of p53 to function as a transcription factor (2). Activation of p53 induces or inhibits the expression of numerous *BAX* genes including $p21^{WAF\text{-}1/CIP\text{-}1}$, *GADD45*, and *IGFBP3* (3). These genes are known to mediate arrest of mammalian cells at one of two major cell cycle checkpoints, in G1 near the border of S phase, or in G2 before mitosis; or to induce other responses, including apoptosis, a programmed cell death. Arrest of cell cycle progression is believed to provide time for the repair of DNA damage, and recent evidence suggests that p53 may modulate DNA repair processes (4). p53 has been shown to induce cell cycle arrest at the G1 phase (5).

In normal unstressed cells, p53 is maintained at very low levels through constant degradation, which is mediated primarily by murine double minute 2 (Mdm2) (2), the product of a p53 inducible gene (6). Mdm2 and p53 are linked through an autoregulatory negative feedback loop to keep the p53 level low. In response to stress, p53 has to escape the degradation-promoting actions of Mdm2 for p53 to accumulate. Mdm2 encodes an E3 ubiquitin ligase activity (7), which promotes p53 degradation through an ubiquitin-dependent pathway on cytoplasmic and nuclear proteosomes (8–10). In the nucleus, the Mdm2-mediated p53 ubiquitination occurs in a complex with p300/CREB-binding protein (CBP) transcriptional coactivator proteins, which serve as a scaffolding (11). Most nuclear Mdm2 is bound to p300/CBP. Although Mdm2 catalyzes p53

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monoubiquitination (12), the product is not a substrate for proteasome degradation. In contrast, the complex of Mdm2 with p300, which harbors an E4 ubiquitin ligase activity, is needed to mediate the final p53 polyubiquitination (13). Thus, the E3 and the E4-ubiquitin ligase activities of Mdm2 and p300/CBP are needed to produce the degradable polyubiquitinated form of p53.

Mdmx, with a strong homology to Mdm2 within the zinc and RING finger domains, is capable of inhibiting Mdm2-mediated p53 degradation and enhancing subsequent accumulation of p53. The interaction of these proteins inhibits the degradation of Mdm2 as well as Mdm2-mediated turnover of p53 (14). The mechanism by which Mdmx modulates Mdm2 and p53 stability remains unclear. Mdmx may inhibit Mdm2 degradation by blocking a site on Mdm2, which is important for the E3 ligase to function (15). Alternatively, Mdmx may inhibit nucleocytoplasmic shuttling of Mdm2.

The c-Abl, with protein-tyrosine kinase activity, can shuttle between the cytoplasm and nucleus. Cytoplasmic c-Abl is activated by oxidative stress and is closely involved in mediating growth and survival signals. In response to genotoxic agents, nuclear c-Abl is phosphorylated and activated by ATM protein kinase (16, 17), and plays an important role in mediating apoptosis (18). Moreover, nuclear c-Abl is known to block ubiquitination and nuclear export of p53 (19) and to enhance phosphorylation of Mdm2, which contributes to apoptosis by blocking the ability of Mdm2 to downregulate p53. Goldberg *et al.* (20) recently demonstrated that the Mdm2 Tyr394 is a key site for c-Abl–dependent phosphorylation. The use of a mutant at this site revealed that the c-Abl–dependent phosphorylation of this site depresses Mdm2-mediated degradation of p53 and downregulation of p53-dependent transactivation, and decreases the ability of Mdm2 to inhibit p53-mediated apoptosis.

Previous studies in our laboratory have demonstrated increases in nuclear p53 level in zinc-deficient human normal bronchial epithelial (21) and aortic endothelial (22) cells in primary culture as well as in the human hepatoblastoma HepG2 cell line (23). The present study was designed to examine the influence of zinc status on the expression of Mdm2, Mdmx, c-Abl, and p300 proteins, which are known to modulate the p53 cellular level in HepG2 cells.

Materials and Methods

Cell Culture and Treatment. The human hepatoblastoma cell line, HepG2, was purchased from the American Type Culture Collection (Manassas, VA) and was used to mimic the human hepatic responses to different zinc statuses. Cell culture reagents were purchased from Life Technologies, Inc. (Grand Island, NY). Cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS), and antibiotics (100 U/ml penicillin and 100 μg/l streptomycin). Medium was replaced every 2 days and approximately 6.5 days of culture constituted one passage. At the end of Passage 80, nearly confluent cells were subcultured at a ratio of 1:8 for the initiation of experimental treatments. Chelex 100 resin (Bio-Rad, Richmond, CA), a divalent ion-chelating resin, was used to deplete zinc from FBS before the FBS was combined with DMEM. The resin was first neutralized to physiologic pH with 0.25 *M* HEPES pH 7.4, and then mixed with FBS at a ratio of 1:4 and shaken for 2 hrs at 4°C as described previously (23). Chelex resin was separated from FBS by centrifugation followed by filtration through a 0.2-μm filter for sterilization and removal of residual Chelex resin. Zinc was added to the media in the form of $ZnSO_4$ so that the only difference between these media was the zinc concentration. Cells cultured in the zinc-free basal media had slower growth compared with cells grown in the regular medium. Studies in our laboratory were performed with increasing amounts of medium zinc in order to establish the optimal growth conditions for HepG2 cells (24). From these studies the zinc-free basal medium supplemented with 0.4 μ*M* $ZnSO_4$ was determined to be suitable for the depletion of cellular zinc without affecting overall growth. The DMEM with 10% Chelexed FBS containing 0.2 and 0.4 μmol of zinc per liter were termed the zinc-deficient (i.e., ZD0.2 and ZD0.4) media, respectively. For the other treatment groups, the zinc-normal (ZN), zinc-adequate (ZA), and zinc-supplemented (ZS) media contained 4, 16, and 32 μ*M* $ZnSO_4$, respectively, added to the zinc-free basal medium. The ZN medium was used as a comparison to standard culture medium, and ZA was used as a representative of human plasma zinc levels. The ZS medium contained 32 μ*M* zinc and was used to represent the high end of plasma zinc level attainable by oral zinc supplementation. After the HepG2 cells were subcultured, they were maintained overnight in the ZN medium before being changed to their respective media. Cells were grown for one passage in their respective treatment media (ZD0.2, ZD0.4, ZN, ZA, or ZS medium), or for the time period as indicated, and then harvested.

Determination of Cellular DNA and Zinc Levels. Cells and media were collected from each tissue culture plate and then centrifuged at 500 *g* for 5 mins at 4°C. Cell pellets were then washed twice with phosphate-buffered saline (PBS), resuspended in 1.5 ml PBS, and sonicated. An aliquot of the sonicated cells was used to measure cellular zinc content by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA) as previously described (24). The zinc concentration of the cells was determined on the basis of standard curves of 0.05–1.0 ppm, generated with certified zinc reference solutions (Fisher Scientific, Fair Lawn, NJ). In addition, the certified zinc solutions were compared with Bovine Liver Standard Reference (U.S. Department of Commerce, National Institute of Standards, Gaithersburg, MD). Appropriate blanks were employed for all measurements. An aliquot of

the sonicated cells was also used to measure cellular DNA content according to the method described by Williams *et al.* (25). Cellular zinc was expressed per microgram of DNA because a linear relationship between cellular DNA and cell number was previously established (25).

HepG2 Nuclear Extracts. Nuclear extracts were prepared according to the method described by Schreiber *et al.* (26) with slight modifications. Cells were washed with ice-cold Tris-buffered saline (TBS), scraped off the dish, and collected by centrifugation at 1500 *g* for 5 mins at 4°C. Cell pellets were resuspended in five times the original packed cell volume of ice-cold buffer A (10 m*M* HEPES pH 7.9, 10 m*M* KCl, 0.1 m*M* EDTA, 0.1 m*M* EGTA, 1 m*M* dithiothreitol [DTT]) with freshly added DTT and protease inhibitors (0.5 m*M* phenylmethylsulfonyl fluoride [PMSF], 0.5 μg/l leupeptin, 1 μg/l pepstatin A, 1 m*M* benzamidine-HCl) and mixed gently by pipetting. Cells were allowed to swell by incubating on ice for 15 mins, 10% NP-40 solution was added to attain a final concentration of 0.5%, and the mixture was vigorously mixed for 20 secs. Samples were transferred to 1.5-ml microfuge tubes and centrifuged at 13,000 *g* for 50 secs at 4°C. The supernatant fraction composed of cytoplasm and RNA was kept at −80°C and the nuclear pellet was resuspended in ice-cold buffer C (20 m*M* HEPES pH 7.9, 0.4 *M* NaCl, 1 m*M* EDTA, 1 m*M* DDT, 1 m*M* PMSF, 0.5 μg/l leupeptin, 1 μg/l pepstatin A, 1 m*M* benzamidine-HCl) by vigorously shaking at 4°C for 15 mins on a shaking platform. Nuclear extract was centrifuged at 13,000 *g* for 15 mins at 4°C and the supernatant fraction was frozen in aliquots at −80°C. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad). Contamination of nuclear extracts by cytoplasmic proteins, as detected by Western blot analysis of Hsp90, was routinely found to be less than 5% in our laboratory.

Western Blot Analysis. The nuclear extract of HepG2 cells was prepared as described above. For whole cell lysate, cells were washed twice with ice-cold PBS, and lysed in a buffer containing 20 m*M* Tris-HCl pH 7.5, 150 m*M* NaCl, 1 m*M* EDTA, 1 m*M* EGTA, 1% Triton X-100, 2.5 m*M* sodium pyrophosphate, 1 m*M* α-glycerolphosphate, 1 m*M* Na_3VO_4, 1 μg/ml leupeptin, and 1 m*M* PMSF. Protein concentrations were determined using the Bradford protein assay kit (Bio-Rad). Forty micrograms of proteins (nuclear or total protein) were combined with an equal volume of sample loading buffer (20% glycerol, 10% 2-mercaptoethanol, 5% sodium dodecyl sulfate [SDS], 200 m*M* Tris-HCl pH 6.7, and 0.01% bromophenol blue), boiled for 4 mins, and then subjected to 10% SDS-polyacrylamide gel electrophoresis (PAGE). Following electrophoresis, gels were briefly equilibrated in transfer buffer before transfer onto nitrocellulose membranes. Transfer was performed at 30 V overnight at 4°C. After blocking with 5% dried nonfat milk in TBS-T (20 m*M* Tris-HCl pH 7.4, 8 g/l NaCl, 0.1% Tween-20) for at least 1 hr, blots were then incubated with appropriate antibodies diluted to 1 μg/ml in TBS-T with 5.0% nonfat dried milk at 4°C overnight, followed by four 10-minute washes with TBS-T. Blots were incubated with horseradish peroxidase-conjugated appropriate IgG (Santa Cruz Biotechnology, Santa Cruz, CA), diluted to 0.5 μg/ml in TBS-T with 5% nonfat dried milk for 1 hr followed by four 10-min washes with TBS-T. Protein was visualized by using Super West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) by following the manufacturer's instructions. Prestained SDS-PAGE standard was used as molecular weight marker (Bio-Rad). Then the membrane was incubated with a stripping buffer (100 m*M* 2-mercaptoethanol, 2% SDS, and 62.5 m*M* Tris-HCl pH 6.7) at 52°C for 1 hr and then detected with antihistone H1 (Santa Cruz Biotechnology), to control for equal loading. Protein bands were quantified by using laser densitometry (Bio-Rad).

Antibodies. p53 (DO-1) mouse monoclonal antibody, Mdm2 (C-18) rabbit polyclonal antibody, Mdmx (D-19) goat polyclonal antibody, histone H1 (N-19) mouse monoclonal antibody, c-Abl (C-19) rabbit polyclonal antibody, p300 (N-15) rabbit polyclonal antibody, β-actin (I-19) goat polyclonal antibody, and normal mouse antibody were purchased from Santa Cruz Biotechnology. Secondary antibodies including goat anti-mouse IgG1, goat anti-rabbit IgG1, and bovine anti-goat IgG1, were also purchased from Santa Cruz Biotechnology.

Statistical Analysis. The data were analyzed with one-way analysis of variance by using SAS 8.1 Windows software (SAS Institute, Cary, NC). The means were further analyzed by the least significant differences method. Values were expressed as means ± SEM and differences were considered significant at a level of $P < 0.05$.

Results

Zinc-Deficient Medium Depleted Cellular Zinc. HepG2 cells were cultured for one passage in the ZD and ZS media. Growth as measured by DNA content per plate was depressed in ZD0.2 to 78.2% of ZN cells (Fig. 1A). In addition, no differences in DNA contents were observed among the ZD0.4, ZN, and ZA cells. However, DNA content in ZD0.4 cells was lower than that of ZS cells. Cellular zinc levels were expressed per cellular DNA to correct for any differences in cell numbers between plates. In ZD0.2 and ZD0.4 cells, cellular zinc concentration was reduced to 33% and 44%, respectively, of ZN controls (Fig. 1B). Moreover, cellular zinc levels in ZA and ZS were 84% and 127% higher than those in ZN cells, respectively. Furthermore, the cellular zinc level in ZS cells was higher than in ZA cells.

Zinc Depletion Increased Nuclear but Not Total Cellular p53 Protein Levels. Nuclear p53 protein levels in ZD0.2 and ZD0.4 cells were 96% and 43% higher than they were in ZN control cells, respectively (Fig. 2A). Moreover, no significant difference in the nuclear p53 protein was detected among ZN, ZA, and ZS cells. In contrast, total cellular p53 protein was not different among all treatment groups (Fig. 2B).

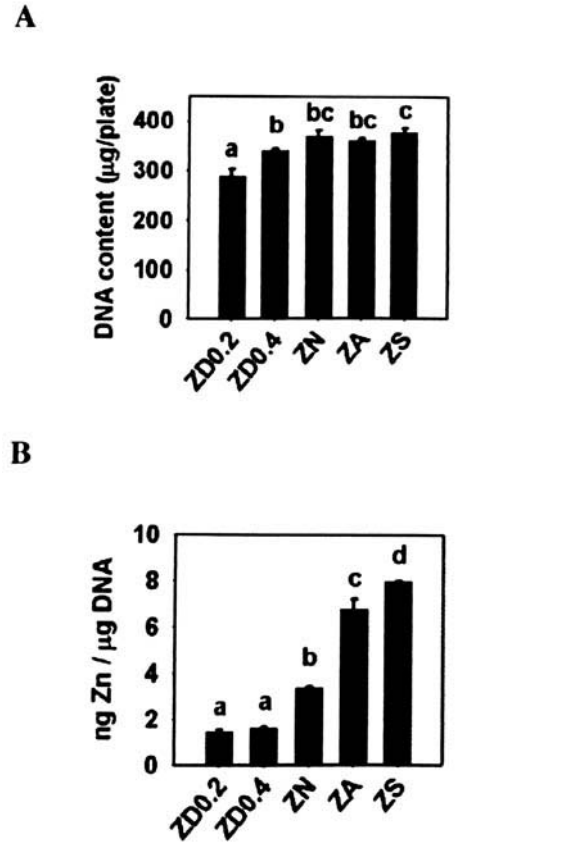

Figure 1. Zinc-deficient medium depleted cellular zinc. (A) DNA content, in micrograms per plate: ZD0.2, 0.2 µ*M* zinc; ZD0.4, 0.4 µ*M* zinc; ZN, 4.0 µ*M* zinc; ZA, 16.0 µ*M* zinc; and ZS, 32.0 µ*M* zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. DNA was measured by the diphenylamine method. Values are mean ± SEM from three experiments. Different letters indicate significantly different means, $P < 0.05$. (B) Cellular zinc, in nanograms of zinc per micrograms of DNA: ZD0.2, 0.2 µ*M* zinc; ZD0.4, 0.4 µ*M* zinc; ZN, 4.0 µ*M* zinc; ZA, 16.0 µ*M* zinc; and ZS, 32.0 µ*M* zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. Cellular zinc was measured by atomic absorption spectrophotometry. Values are mean ± SEM from three experiments. Different letters indicate significantly different means, $P < 0.05$.

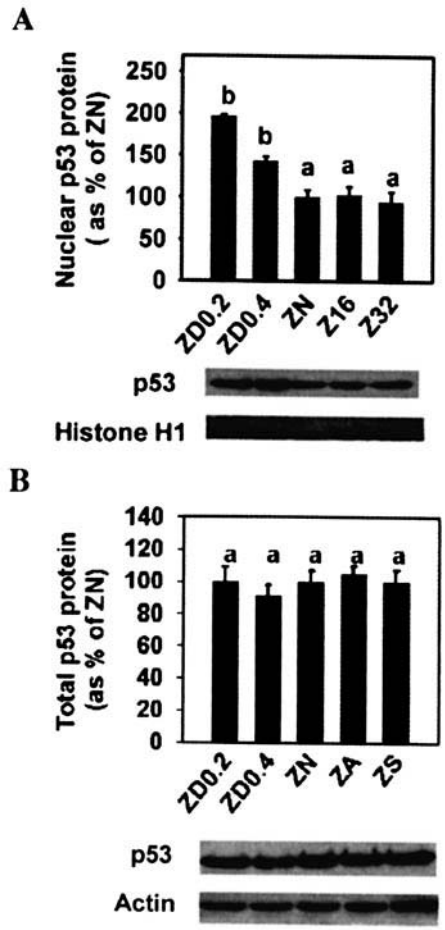

Figure 2. Zinc depletion increased nuclear but not total cellular p53 protein levels. Relative nuclear p53 (A) and total cellular p53 (B) protein levels in ZD0.2, 0.2 µ*M* zinc; ZD0.4, 0.4 µ*M* zinc; ZN, 4.0 µ*M* zinc; ZA, 16.0 µ*M* zinc; and ZS, 32.0 µ*M* zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. p53 protein levels in nuclear or total cellular protein extracts were measured by Western blot analysis using p53 antibody. To control equal loading, the nuclear protein or the total cellular protein blot was reprobed with histone H1 or β-actin antibody. Data are expressed as a percentage of ZN controls. Values shown in the bar graph represent mean ± SEM from three separate experiments. Different letters indicate significantly different means, $P < 0.05$.

Zinc Depletion Increased Nuclear but Not Total Cellular Mdm2 Levels. Nuclear Mdm2 protein was markedly increased in the ZD groups (ZD0.2, 270.6% ± 24.1%; ZD0.4, 242% ± 50%) compared with that of the ZN control group (100% ± 15.4%) (Fig. 3A). However, nuclear Mdm2 levels were not different among ZN, ZA, and ZS cells. In addition, total cellular Mdm2 was not different among all treatment groups.

Nuclear and Total Cellular Mdmx Levels Were Not Altered by Zinc Treatments. No difference in nuclear Mdmx protein was detected among the treatment groups (Fig. 4A). Total Mdmx protein levels were lower in ZD0.2 and ZD0.4 cells than in ZN cells (Fig. 4B). In addition, no difference in total Mdmx protein was detected among ZN, ZA, and ZS cells.

Zinc Depletion Depressed Nuclear c-Abl but Did Not Affect Total c-Abl Protein Levels. That c-Abl protects p53 by neutralizing the inhibitory effects of Mdm2 in response to stress encouraged us to examine the influence of zinc status on the expression of c-Abl protein. Our results indicate that in response to zinc deficiency, the nuclear c-Abl protein levels of ZD0.2 and ZD0.4 cells were significantly depressed to 49% and 64%, respectively, of ZN cells (Fig. 5A). Moreover, no difference was detected among ZN, ZA, and ZS cells. In contrast, no difference in total c-Abl protein was detected among all treatment groups (Fig. 5B).

Nuclear and Total Cellular p300 Protein Levels Were Depressed in Zinc-Deficient Cells. Surprising and important findings of marked reductions in both nuclear

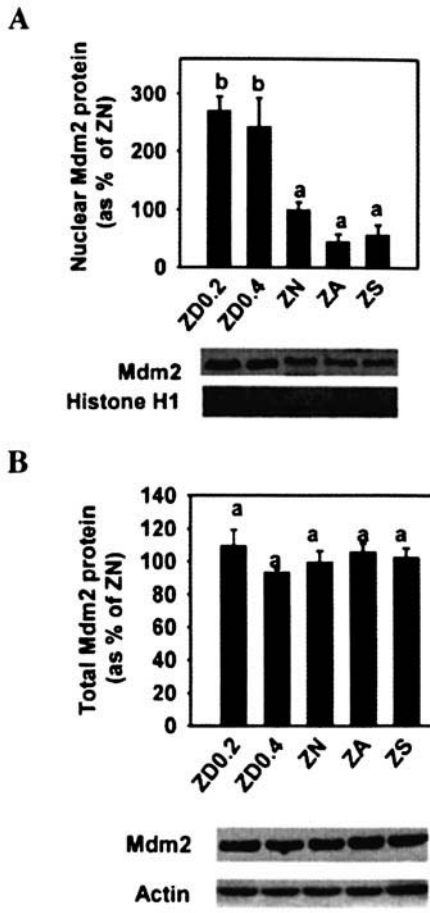

Figure 3. Zinc depletion increased nuclear but not total cellular Mdm2 levels. Relative nuclear Mdm2 (A) and total cellular Mdm2 (B) protein levels in ZD0.2, 0.2 μM zinc; ZD0.4, 0.4 μM zinc; ZN, 4.0 μM zinc; ZA, 16.0 μM zinc; and ZS, 32.0 μM zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. Mdm2 protein levels in nuclear or total cellular protein extracts were measured by Western blot analysis using Mdm2 antibody. To control equal loading, the nuclear protein or the total cellular protein blot was reprobed with histone H1 or β-actin antibody. Data are expressed as a percentage of ZN controls. Values shown in the bar graph represent mean ± SEM from three separate experiments. Different letters indicate significantly different means, $P < 0.05$.

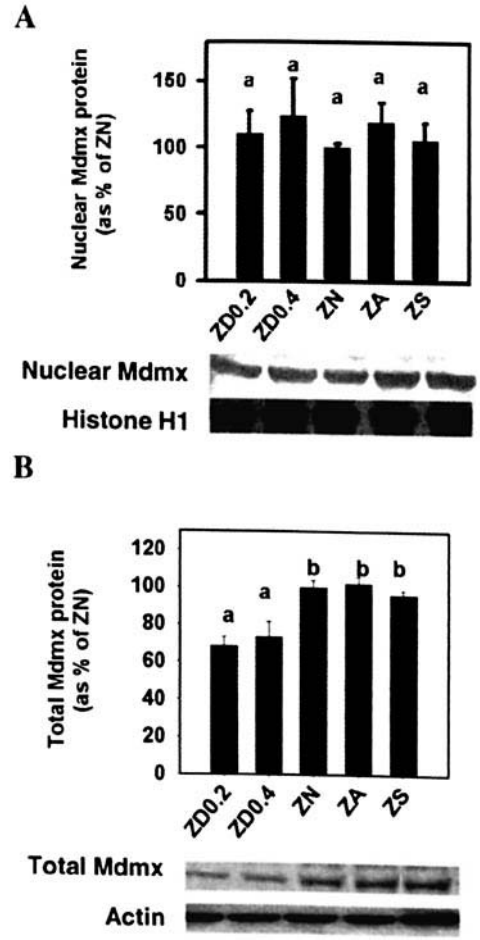

Figure 4. Nuclear and total cellular Mdmx levels were not altered by zinc status. Relative nuclear Mdmx (A) and total cellular Mdmx (B) protein levels in ZD0.2, 0.2 μM zinc; ZD0.4, 0.4 μM zinc; ZN, 4.0 μM zinc; ZA, 16.0 μM zinc; and ZS, 32.0 μM zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. Mdmx protein levels in nuclear or total cellular protein extracts were measured by Western blot analysis using Mdmx antibody. To control equal loading, the nuclear protein or the total cellular protein blot was reprobed with histone H1 or β-actin antibody. Data are expressed as a percentage of ZN controls. Values shown in the bar graph represent mean ± SEM from three separate experiments. Different letters indicate significantly different means, $P < 0.05$.

and total p300 protein levels were detected in ZD cells. Nuclear p300 levels in ZD0.2 and ZD0.4 cells were significantly reduced to 19.6% ± 3.8% and 58.8 ± 0.6%, respectively, of that in ZN cells (100% ± 3.6%) (Fig. 6A). Nuclear p300 level was also significantly lower in ZD0.2 cells compared with that of ZD0.4 cells. Moreover, no difference was detected among ZN, ZA, and ZS cells. Similarly, total cellular p300 levels in ZD0.2 and ZD0.4 cells were significantly reduced to 52.9% ± 4% and 60.3% ± 3.8%, respectively, of that of ZN cells (100% ± 3.6%) (Fig. 6B). In addition, no significant difference in total p300 level was observed in ZD0.2 cells compared with that of ZD0.4 cells. Moreover, no difference in total cellular p300 protein level was detected among the ZN, ZA, and ZS cells.

Discussion

This study was performed to establish the influence of zinc status on the expression of Mdm2, Mdmx, c-Abl, and p300 proteins, which are known to modulate p53 cellular level, in HepG2 cells. In the present study, the ZD0.4 cells did not exhibit a decrease in cell growth as measured by DNA content per plate (Fig. 1A). Data derived from ZD0.4 cells without growth reduction may be relevant to marginal zinc-deficient states found in certain subpopulations of the United States. Reaves *et al.* (23) and Fanzo *et al.* (21) have also found that zinc depletion with 0.4 μM zinc culture medium did not alter cell growth in zinc-depleted HepG2 cells and normal human bronchial epithelial cells, respectively. Thus, zinc depletion with 0.4 μM zinc culture medium may not hinder

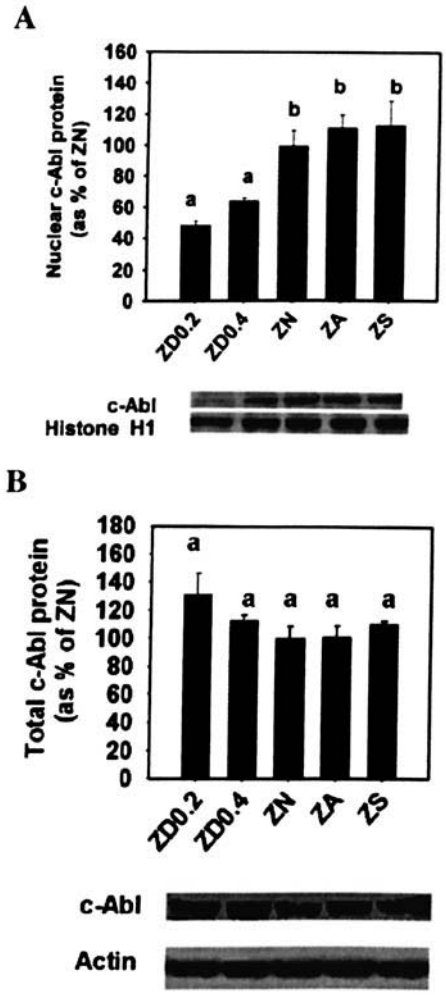

Figure 5. Zinc depletion depressed nuclear but not total cellular c-Abl levels. Relative nuclear c-Abl (A) and total cellular c-Abl (B) protein levels in ZD0.2, 0.2 μM zinc; ZD0.4, 0.4 μM zinc; ZN, 4.0 μM zinc; ZA, 16.0 μM zinc; and ZS, 32.0 μM zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. c-Abl protein levels in nuclear or total cellular protein extracts were measured by Western blot analysis using c-Abl antibody. To control equal loading, the nuclear protein or the total cellular protein blot was reprobed with histone H1 or β-actin antibody. Data are expressed as a percentage of ZN controls. Values shown in the bar graph represent mean ± SEM from three separate experiments. Different letters indicate significantly different means, $P < 0.05$.

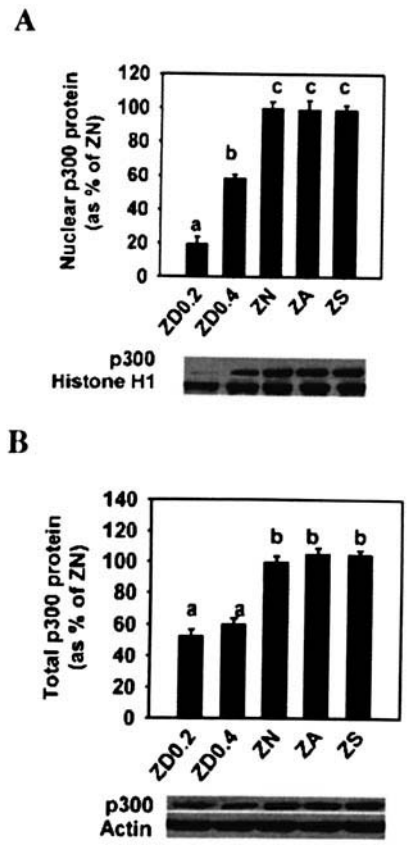

Figure 6. Zinc depletion depressed nuclear and total cellular p300 levels. Relative nuclear p300 (A) and total cellular p300 (B) protein levels in ZD0.2, 0.2 μM zinc; ZD0.4, 0.4 μM zinc; ZN, 4.0 μM zinc; ZA, 16.0 μM zinc; and ZS, 32.0 μM zinc, HepG2 cells. Cells were cultured for one passage in media containing Chelex-treated serum with zinc added as a supplement. p300 protein levels in nuclear or total cellular protein extracts were measured by Western blot analysis using p300 antibody. To control equal loading, the nuclear protein or the total cellular protein blot was reprobed with histone H1 or β-actin antibody. Data are expressed as a percentage of ZN controls. Values shown in the bar graph represent mean ± SEM from three separate experiments. Different letters indicate significantly different means, $P < 0.05$.

cell cycle progression. However, DNA content per plate was depressed in ZD0.2 cells to 78.2% of ZN cells (Fig. 1A). This reduction in cell growth was previously established in our laboratory to be due to an increased percentage of ZD HepG2 cells in the G1 phase, and a markedly decreased proportion of cells in S phase (27). This finding suggests that zinc is critical for the normal progression of HepG2 cells from G1 to S phase. Similarly, Chesters *et al.* (28) reported a blockage of G1/S phase transition in zinc-deficient rodent fibroblasts. However, the mechanism by which zinc depletion depresses the G1-to-S phase transition remains unclear. Recently, Nakatani *et al.* (29) found that zinc depletion by N,N′,N′-tetrakis (2-pyridylmethyl)-ethylenediamine [TPEN], a membrane-permeable chelator, induced apoptosis in hepatocytes, whereas zinc depletion by diethylenetriamine pentaacetic acid (DTPA), a membrane-impermeable chelator, did not induce apoptosis. As a membrane-impermeable chelator, DTPA cannot enter the cells to deplete tightly bound cellular zinc and may cause a less severe zinc-deficient state that is not capable of inducing apoptosis, compared with TPEN. Similarly, our system of culturing cells in a zinc-depleted medium may not deplete cellular zinc to such a severe extent that it results in apoptosis. Nevertheless, in the present study, the zinc-depleted cells had a much lower concentration of zinc than ZN cells. Moreover, the intracellular zinc level in each treatment group was significantly distinct from each other, indicating that HepG2 cells had taken up zinc depending on the zinc concentration of the medium.

The expression of p53 is under firm negative and positive control. In addition, the activity and stability of p53 protein are tightly regulated. Under the normal condition, the p53 protein is very labile and is an inactive protein. The

degradation of p53 requires the binding of Mdm2 to p53 (8, 9). Mdm2, a principle regulator of p53 degradation by proteasome, functions as an E3 ubiquitin ligase, which catalyzes the addition of single ubiquitin to a cluster of six COOH-terminal lysines of p53 (12, 30). Although total cellular p53 and Mdm2 protein levels were not affected by zinc depletion, our data indicate that zinc deficiency increases nuclear p53 and Mdm2 protein levels (Figs. 2A and 3A). Reaves *et al.* (23) and Fanzo *et al.* (21) have also found that p53 mRNA and nuclear p53 protein levels were increased in zinc-depleted HepG2 cells and normal human bronchial epithelial cells, respectively. In the present study, we report for the first time a marked increase in nuclear Mdm2 in ZD0.2 cells compared with that of ZN cells. The magnitude of this increase in nuclear Mdm2 was, interestingly, almost 2-fold that of the extent of elevation in nuclear p53 in ZD0.2 cells. Thus, the accumulation of p53 in the nucleus of ZD cells is not due to a lack of Mdm2 expression but is accompanied by a much larger magnitude of increase in nuclear Mdm2 accumulation. These findings are consistent with the proposed model in which cellular p53 undergoes fast nuclear import and export, as well as degradation by the proteasome-dependent pathway mediated by Mdm2 and p300. Three mechanisms may be responsible for the observed nuclear accumulation of p53 and Mdm2 in ZD cells. First, p53 and Mdm2 nuclear export may be depressed by zinc deficiency. Second, the nuclear p53 polyubiquitin and proteasome degradation pathway may not be fully functional in zinc deficiency due to a reduction in p300. Third, p53 and Mdm2 nuclear import may be enhanced by zinc deficiency. However, this is unlikely because total and cytoplasmic levels[1] appeared not to be affected by zinc deficiency.

Mdmx is known to stabilize p53 protein by binding directly to Mdm2, and to interfere with Mdm2-dependent p53 nuclear export (31, 32). The zinc finger and RING finger domain of Mdm2 have been established to be responsible for the binding of Mdm2 to Mdmx (33). Moreover, the RING finger of Mdmx may be involved in the hetero-oligomerization with the RING finger of Mdm2, leading to the inhibition of the ubiquitin ligase activity of Mdm2. In addition, Mdmx is known not to target p53 for proteasome-mediated degradation, which results in elevated levels of p53 and Mdm2 (34). The importance of the RING finger domain in ubiqitination is supported by the fact that many RING finger proteins have been observed to be associated with ubiqitin, or to target specific proteasome-dependent degradation, or both (7). Our data demonstrated that nuclear Mdmx protein levels were not affected in all treatment groups, which indicated that Mdmx distribution in the nucleus was maintained even in zinc-depleted cells. Thus, Mdmx may not be involved in the nuclear accumulation of p53 and Mdm2 in ZD cells.

In response to zinc deficiency, nuclear c-Abl was found to be decreased (Fig. 4A), whereas total cellular c-Abl protein level was not affected. These findings suggest that the importation of c-Abl into the nucleus may be impaired or the exportation of the c-Abl out of the nucleus may be enhanced by zinc deficiency. The cytoplasmic form of c-Abl has been reported to be activated by oxidative stress, whereas the nuclear form of c-Abl is activated in response to genotoxic stress (35). In addition, in response to stress, c-Abl protects p53 by neutralizing the effect of Mdm2 on p53 degradation. In the present study, nuclear c-Abl protein levels of ZD0.2 and ZD0.4 cells were depressed to 49% and 64%, respectively, of ZN cells. These data suggest that c-Abl may not contribute to the accumulation of p53 and Mdm2 in zinc-deficient cells. In addition, the depressed nuclear c-Abl may be a feedback cellular response that attempts to adjust to or to normalize the nuclear buildup of p53 and Mdm2.

Most importantly, we have shown for the first time that nuclear and total p300 levels were markedly reduced in response to zinc deficiency (Fig. 6A and B). The depressed level of p300 in ZD cells may severely impair the p300/Mdm2 interaction, which is essential for p53 degradation. In unstressed cells, the interaction of p300 with Mdm2 is an important element in the Mdm2-dependent p53 turnover (11). Degradation of p53 by the proteasome requires the ubiquitin ligase of Mdm2, which catalyzed the addition of single ubiquitin in p53 (12, 30), but does not polyubiquitinate p53 *in vitro*. Polyubiquitinated forms of p53 are considered to be a signal for proteasome degradation (36). A recent study (13) reported that after monoubiquitination by Mdm2, the polyubiquitination of p53 could be performed by an ubiquitin ligase of p300. In view of the importance of p300 in the generation of the degradable form of p53 for the proteosome-mediated degradation pathway, the depressed p300 may well be the limiting factor responsible for the observed nuclear accumulation of p53 and Mdm2 in ZD cells. In order to establish that the depressed p300 is responsible, we plan to transfect recombinant p300 protein (Active Motif, Carlsbad, CA) into zinc-deficient cells using a commercially available protein transfection system (Chariot; Active Motif). This approach may provide us with data to support the hypothesis that a depressed p300 expression is responsible for the nuclear accumulation of p53 and Mdm2 in ZD cells.

Finally, the changes in p53-related proteins were observed solely in zinc-deficient cells. Although compared with ZN treatment, the ZS medium contained an eight times higher zinc concentration, resulting in a 2-fold higher level of cellular zinc, the p53-related proteins appeared not to be affected. One possible explanation is that liver cells may be capable of storing and sequestering a high level of zinc at cellular sites to prevent any effect on the expression of these proteins. Future studies should be designed to examine the ability of different cell types to handle higher zinc status and

[1] Lei, personal communication, 2005.

the expression of genes involved in zinc storage and metabolism.

In summary, the present data indicate that the nuclear accumulation of p53 in zinc deficiency is not due to a lack of Mdm2 expression. Moreover, our data seem to support the hypothesis that the markedly elevated level of nuclear Mdm2 may be unable to maintain optimal nuclear p53 export and degradation due to the marked reduction in p300. To understand the responsible mechanism(s), future studies will have to be designed to examine the influence of zinc status on nuclear import and export of p53 and Mdm2, as well as on the Mdm2/p300-mediated polyubiquitination and the proteasome degradation pathway of p53.

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