Expression of the p53 Tumor Suppressor Gene Is Up-Regulated by Depletion of Intracellular Zinc in HepG2 Cells[1,2]

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ABSTRACT Expression and activation of the p53 tumor suppressor protein are modulated by various cellular stimuli. The objective of this work was to examine the influence of zinc depletion on the expression of p53 in HepG2 cells. Two different low Zn (ZD) media, Zn-free Opti-MEM and a ZD medium containing Chelex-100 treated serum, were used to deplete cellular zinc over one passage. Cellular zinc levels of ZD cells were significantly lower than in their controls in both the Opti-MEM and Chelex studies. p53 mRNA abundance was 187% higher in ZD Opti-MEM cells and >100% higher in ZD Chelex cells compared with their respective controls. To examine whether the effects were specific to zinc depletion, a third, zinc-replenished group (ZDA) was included in the Opti-MEM study in which cells were cultured in ZD media for nearly one passage before a change was made to zinc-adequate (ZA) medium for the last 24 h. Zinc levels in the ZDA cells were significantly higher than in ZD cells, and p53 mRNA abundance was normalized to control levels. Nuclear p53 protein levels were >100% higher in the ZD Opti-MEM cells than in ZA cells. Interestingly, the ZDA Opti-MEM cells had significantly lower levels of nuclear p53 protein than both the ZA and ZD cells. These data suggest that expression of p53, a critical component in the maintenance of genomic stability, may be affected by reductions in cellular zinc. J. Nutr. 130: 1688–1694, 2000.

KEY WORDS: • *p53* • *zinc deficiency* • *HepG2 cells* • *tumor suppressor gene.*

The tumor suppressor gene product, p53 protein, is one of the most well-studied molecules in recent cancer research because more than half of all known human malignancies involve p53 mutations (Greenblatt et al. 1994, Hollstein et al. 1991). It has been called the "guardian of the genome" (Lane 1992) because of its ability to cause cell cycle arrest in response to certain types of DNA damage, thereby allowing DNA repair to occur before cell cycle progression (Kastan et al. 1991). Furthermore, after DNA damage in some cell types, p53 can trigger the genetically altered cells to be eliminated by inducing apoptosis (Lowe et al. 1993). In addition to its DNA-damage response, p53 is also involved in the response by abnormal or stress conditions such as hypoxia (Graeber et al. 1994), oxidative stress (Yin et al. 1998), the presence of genotoxic chemicals (Sun et al. 1995) and depletion of ribonucleotides (Linke at al. 1996). Therefore, normal p53 expression and function are crucial to prevent the propagation of genetically damaged cells and to prevent proliferation of cells under stress conditions.

p53 is a 393-amino acid transcription factor that binds its consensus DNA sequence through high affinity interactions to control the transcription of several sets of genes. Some of the genes regulated by p53 include regulators of apoptosis such as Bax-1 and also inhibitors of cell cycle progression such as $p21^{waf\text{-}1}$ and GADD45. p53 can also transactivate genes involved in the metabolism of reactive oxygen species (Yin et al. 1998). In addition to its transactivation properties, p53 can repress a number of positive regulators of cell growth or survival, such as c-fos, c-jun and bcl-2 [reviewed in Donehower and Bradley (1993)].

The earliest report to show that suboptimal zinc and apoptosis may be linked was published in 1977 by Elmes who found significantly increased numbers of apoptotic bodies in the small intestine of zinc-deficient rats (Elmes 1977). Subsequently, several investigators have used zinc-depleted culture medium or zinc chelators to show that low cellular zinc induced apoptosis in several different cell lines [reviewed in Fraker and Telford (1996)]. These data led to speculation that a chelatable or depletable pool of intracellular zinc may influence the propensity of cells to undergo apoptosis; however, the mechanism(s) responsible for the induction of apoptosis remains unclear.

Because of the prevalence of marginal zinc deficiency in certain subpopulations of the United States (Sandstead 1995), these studies were designed to investigate how compromised cellular zinc status affects the expression of the p53 tumor suppressor gene. We utilized two different methods to deplete cellular zinc in HepG2 cells. Compared with the alternative of exposing cells directly to a chelator, we believe that culturing

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cells in a low zinc medium more closely resembles physiologic conditions. We considered the findings of numerous other investigators as well as extensive efforts in our own laboratory (Wu et al. 1998) in establishing these models. An essential criterion for a successful model is that zinc-dependent processes such as growth must not be impaired. The systems that we chose for these studies depleted cellular zinc levels without affecting cell morphology or growth. Therefore, our system may reflect a state of marginal zinc deficiency.

MATERIALS AND METHODS

Cell culture and treatment. The human hepatoblastoma cell line, HepG2, was purchased from the American Type Culture Collection (Rockville, MD). Cell culture reagents were purchased from Life Technologies (Grand Island, NY). Cells were maintained in Dulbecco's modified Eagle medium (DMEM)[4] containing 10% fetal bovine serum (FBS) and antibiotics (100,000 U/L penicillin and 100 mg/L streptomycin). Medium was replaced every 2 d; ~6.5 d 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.

Two different methods were utilized to deplete cellular zinc levels for these studies. For the first method, cells were cultured in the specially formulated medium Opti-MEM (Life Technologies). This medium contains supplemented growth factors and was designed for experiments in which serum-free or serum-reduced conditions may be desirable such as DNA transfection experiments. We obtained a customized Zn-free Opti-MEM formulation from Life Technologies and have used this method to deplete HepG2 cellular zinc in these as well as previous studies. Appropriate growth conditions for this medium have been determined and described previously (Wu et al. 1999). Briefly, HepG2 cells were cultured in Opti-MEM with 1% FBS, and various levels of zinc (0–16 μmol/L) were added. Cellular zinc responded in a dose-dependent manner and no morphological differences were apparent among the cells cultured in different levels of zinc. Therefore, our zinc-depleted (ZD) cells were cultured in Opti-MEM plus 1% FBS. This medium contained 0.4 μmol/L zinc. For the zinc-adequate group (ZA), $ZnSO_4$ was added to the ZD medium to 4.0 μmol/L, the level in normal HepG2 medium with 10% FBS. In the third group (ZDA), ZD cells were replenished for the last 24 h with the ZA medium to examine the specificity of the zinc effects.

The second strategy for zinc depletion utilized a divalent ion–chelating resin with a high affinity for zinc. Chelex 100 resin (Bio-Rad, Richmond, CA) was used to deplete zinc from FBS before the FBS was combined with DMEM. The resin was mixed with FBS at a ratio of 1:4 and shaken for 2 h at 4°C as described previously (Flynn and Yen 1981, Messer et al. 1982). Chelex resin was separated from FBS by centrifugation followed by filtration through a 0.4-μm filter for sterilization and removal of residual Chelex resin. The amount of zinc in chelexed sera was not above the background level (1.0 μmol/L) of detection by flame atomic absorption spectrophotometry. Therefore, DMEM with 10% chelexed FBS was termed the zinc-free basal treatment medium. However, for cells cultured in the zinc-free basal medium, growth was slowed slightly by the low level of zinc compared with cells grown in regular medium. Studies in our laboratory were performed with increasing amounts of media zinc to establish the optimal growth conditions for HepG2 cells (Wu et al. 1999). From these studies, it was determined that the zinc-free basal medium supplemented with 0.4 μmol/L $ZnSO_4$ was suitable to deplete cellular zinc without affecting overall growth; thus it was used as the Zn-depleted medium (ZD). The Zn-adequate (ZA4) medium contained 4.0 μmol/L $ZnSO_4$ added to the zinc-free basal medium, and the medium of the Zn-supplemented group (ZA16) contained 16 μmol/L $ZnSO_4$ added to the zinc-free basal medium. The ZA medium was used as a comparison to standard culture medium, and ZA16 was used as a representative of human plasma Zn levels. Cells were grown for 6.5 d (one passage) in their respective treatment media and then harvested.

Determination of cellular DNA and zinc levels. Cells and media were collected from each tissue culture plate and centrifuged at 500 × *g* for 5 min at 4°C. Cell pellets were then washed two times with PBS, resuspended in 1.5 mL PBS and sonicated. An aliquot of the sonicant was used to measure cellular zinc by flame atomic absorption spectrophotometry (Hitachi, San Jose, CA) as previously described (Wu et al. 1999). The zinc concentration of the cell samples was determined on the basis of standard curves 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 sonicant was also used to measure cellular DNA content by the method of Williams et al. (1986). Cellular zinc was expressed per DNA because a linear relationship between cellular DNA and cell numbers was previously established.

HepG2 nuclear extracts. Nuclear extracts were prepared as previously described by Schreiber et al. (1989) with slight modifications. Cells were harvested with trypsin-EDTA, and 1 mL ice-cold Tris-buffered saline (TBS) was added to each flask to collect the cells. Cells were centrifuged at 1500 × *g* for 5 min at 4°C. Pelleted cells were then washed with 10 mL ice-cold TBS, shaken vigorously for 30 s and spun as described above followed by an additional wash. TBS was removed and the cell pellet was resuspended in 5 mL ice-cold buffer A [10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT)] with freshly added DTT and protease inhibitors [0.5 mmol/L phenylmethylsulfonylfluoride (PMSF), 0.5 mg/L leupeptin, 1 mg/L pepstatin A, 1 mmol/L benzamidine-HCl] and mixed gently by pipetting. Cells were allowed to swell by incubating on ice for 15 min; then 312 μL of 10% NP-40 solution was added for each 5 mL of buffer A, and tubes were vortexed vigorously for 20 s. Samples were transferred to 1.5-mL microfuge tubes and spun at 16,000 × *g* for 50 s at 4°C. The supernatant fraction composed of cytoplasm and RNA was discarded, and the nuclear pellet was resuspended in ice-cold buffer C (20 mmol/L HEPES pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, 1 mmol/L PMSF, 0.5 mg/L leupeptin, 1 mg/L pepstatin A, 1 mmol/L benzamidine-HCl) by vigorously shaking at 4°C for 15 min on a shaking platform. Nuclear extract was centrifuged at 16,000 × *g* for 15 min at 4°C and the supernatant fraction was frozen in aliquots at −80°C. Protein concentrations were determined by the method of Lowry (1951).

Western blot analysis. Nuclear extract (20 μg) was combined with an equal volume of sample loading buffer (20% glycerol, 10% 2-mercaptoethanol, 5% SDS, 200 mmol/L Tris-HCl, pH 6.7, 0.01% bromophenol blue), boiled for 3 min and then subjected to 7.5% SDS-polyacrylamide electrophoresis. After electrophoresis, gels were equilibrated briefly in transfer buffer (20% methanol, 192 mmol/L glycine, 25 mmol/L Tris-aminomethane, 0.05% SDS) before transfer onto nitrocellulose membranes. Transfer was performed at 30 V overnight at 4°C. Equal loading of samples was verified by staining a duplicate gel with Coomassie Brilliant Blue R-250 and scanning with a laser densitometer to compare optical density units between lanes. After blocking [5% (wt/v) nonfat dried milk, 10 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 0.05% Tween 20] for at least 1 h, blots were washed twice in TBST (10 mmol/L Tris-HCl pH 8.0, 150 mmol/L NaCl, 0.05% Tween 20) for 10 min each. Blots were then incubated with mouse anti-p53 antibody (Clone PAb421; Oncogene Research Products, Cambridge, MA) diluted to 1 mg/L in TBST 0.5% milk at room temperature for 1 h, followed by two 10 min washes in TBST. Incubation with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Santa Cruz Biotechnology, Santa Cruz, CA), diluted to 0.1 mg/L in TBST, was for 30 min followed by a 10 min wash in TBST and two 10 min washes in TBS (no Tween 20).

[4] Abbreviations used: DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; FBS, fetal bovine serum; MT, metallothionein; NF, nuclear factor; PMSF, phenylmethylsulfonylflouride; RPA, ribonuclease protection assays; RT-PCR, reverse transcriptase-polymerase chain reaction; TBS,Tris-buffered saline; ZA, zinc-adequate; ZD, zinc-deficient; ZA4, zinc-adequate media containing 4.0 μmol/L Zn; ZA16, zinc-adequate media containing 16 μmol/L Zn; ZDA, zinc-replenished.

Autoradiography was performed utilizing enhanced chemiluminescence according to the manufacturer's instructions (Amersham, Arlington Heights, IL). p53 bands were verified by running p53-GST fusion protein (Oncogene Research Products) or A431 cell nuclear extract (Santa Cruz Biotechnology) on a lane in each gel. Blots were also stained with Amido black and photographed to document equivalent protein loading. Laser densitometry (Molecular Dynamics, Sunnyvale, CA) was used to quantify p53 bands after establishing linearity curves.

RNase protection assays. Total cellular RNA was isolated from cells using a RNaqueous kit (Ambion, Austin, TX) according to the manufacturer's instructions, and the integrity of each sample was checked by electrophoresis. The abundance of p53, metallothionein (MT)-II, β-actin and cyclophilin mRNA was measured by RNase Protection Assays (RPA). The p53 human probe antisense template was purchased from Ambion. The template consisted of a 300-bp cDNA fragment of the human p53 tumor suppressor gene, spanning exons 2, 3 and 4. The human cyclophilin probe (Ambion), which protects a fragment 103 nt in length, was used as an internal reference for normalization in the Chelex experiments. For the Chelex study, we also measured mRNA abundance of the zinc-responsive MT-II gene as an assessment of cellular zinc status. The human MT-II antisense template was prepared by reverse transcriptase-polymerase chain reaction (RT-PCR), as previously described (Sullivan and Cousins 1997, Wu et al. 1998). A pair of human MT primers, MT5 and MT3, corresponding to the 5′ and 3′ regions of the human MT-II cDNA (Sullivan and Cousins 1997) were synthesized. RT-PCR products were cloned into pGEM-T PCR cloning vector (Promega, Madison, WI). Plasmid DNA was isolated from a correct clone, which contained a MT-II cDNA fragment in the antisense orientation with respect to the T7 promoter. A pair of primers (puc/M13F and Rev-T), which correspond to upstream/downstream vector sequences, were used to prepare the final cDNA template for MT-II RPA probe synthesis in a PCR reaction. The resulting fragment was 354 nt in length and the PCR product was used to synthesize labeled probe. The RNA probe transcribed from the T7 promoter was 288 nt in length, which contained 201 nt of human MT-II antisense sequence. β-Actin riboprobe was synthesized from pTRI-β-actin template (Ambion) and was used as the internal control for the Opti-MEM experiments. All RNA probes for p53, MT-II, β-actin, cyclophilin and RNA century size markers (Ambion) were synthesized using the MAXI-script in vitro transcription system kit (Ambion) with T7 RNA polymerase. These probes were labeled at predetermined specific activities by changing the ratio of α-P^{32}-UTP to cold UTP in the labeling reaction to provide roughly comparable band intensities in the final RPA gel.

RPA were done using the RPA-II Kit (Ambion). HepG2 RNA (40 μg) was combined with a molar excess of labeled RNA probe. The RNA and labeled antisense probes were coprecipitated with ammonium acetate and ethanol and then resuspended in hybridization buffer at 43.5°C for 10 h. RNase cocktail was then added and samples were incubated at 37°C for 30 min. The RNase digestion was then stopped by adding inactivation/precipitation buffer and samples were precipitated at −80°C. Protected fragments were separated by PAGE on 6% acrylamide, 8 mol/L urea gels. Controls for the digestion step were included in which yeast RNA replaced HepG2 RNA. No protected bands appeared in these lanes, indicating that the digestions were complete. Conversely, controls were also included in which reactions were prepared as the other samples but were not digested with RNase. Without digestion, only full-length probes were observed. RPA gels were dried and exposed to film. Band intensities of protected signals were quantified by a laser densitometer (Molecular Dynamics). The relative mRNA abundance in each sample was expressed as the arbitrary units of the p53 or MT-II band per arbitrary unit of cyclophilin or β-actin in the same RPA reaction.

Statistics. Values are means ± SEM. Differences were considered significant at $P < 0.05$. The data were analyzed using one-way ANOVA and Duncan's new multiple range test (Jaccard and Becker 1990).

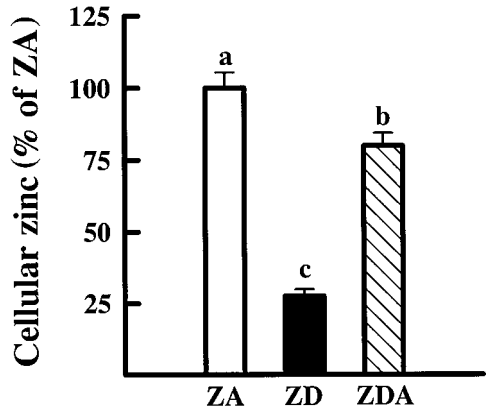

FIGURE 1 Relative cellular zinc levels in zinc-adequate (ZA), zinc-depleted (ZD) and zinc-replenished (ZDA) HepG2 cells. Cells were cultured for one passage in Opti-MEM media with or without zinc added as a supplement to ZD medium. Zinc-replenished (ZDA) cells were ZD cells cultured in ZA medium for the last 24 h. Cellular zinc was measured by atomic absorption spectrophotometry and DNA by the diphenylamine method. Cellular zinc levels were expressed as a percentage of ZA. Cellular zinc concentration in ZA cells was 1143 ± 61 pg Zn/μg DNA. Values are means ± SEM, $n = 4$. Different letters indicate significantly different means, $P < 0.05$.

RESULTS

Zn-deficient medium depleted cellular zinc. HepG2 cells were cultured for nearly one passage in two types of zinc-reduced media. Culture in both types of zinc-deficient media resulted in significant reductions in cellular zinc. Cellular zinc levels were expressed per cellular DNA to correct for any differences in cell numbers between plates. Growth was not affected by the medium zinc concentration because no significant differences in DNA were observed among treatment groups (data not shown). Cellular zinc was reduced to 27 or 64% in the Opti-MEM and Chelex studies, respectively, compared with their zinc-adequate controls (**Figs. 1**, **2**). In the Opti-MEM experiments, culturing ZD cells for the last 24 h in

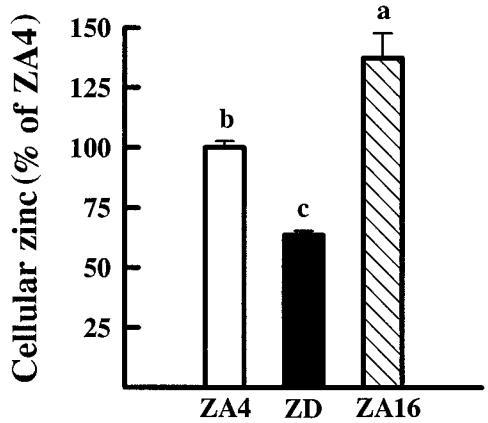

FIGURE 2 Relative cellular zinc levels in zinc-adequate (ZA4), zinc-depleted (ZD) and zinc-supplemented (ZA16) HepG2 cells. Cells were cultured for one passage in media prepared containing Chelex-treated serum with zinc added as a supplement. Cellular zinc was measured by atomic absorption spectrophotometry and DNA by the diphenylamine method. Cellular zinc levels were expressed as a percentage of ZA4. Cellular zinc concentration in ZA4 cells was 1570 ± 39 pg Zn/μg DNA. Values are means ± SEM, $n = 6$ for ZA4 and ZD, and $n = 5$ for ZA16. Different letters indicate significantly different means, $P < 0.05$.

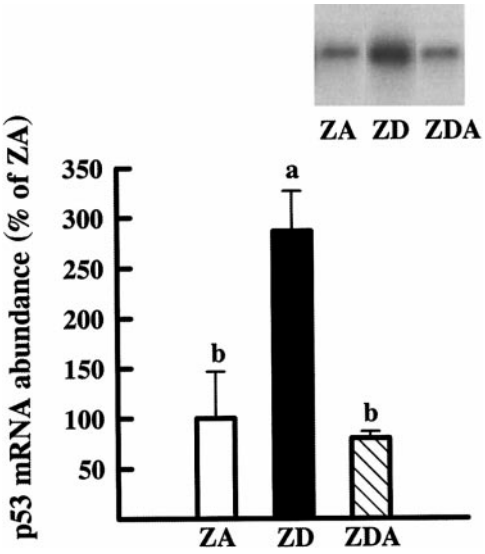

FIGURE 3 Relative p53 mRNA abundance in zinc-adequate (ZA), zinc-depleted (ZD) and zinc-replenished (ZDA) HepG2 cells as determined by RNase protection assays. Cells were cultured for one passage in various Opti-MEM media with or without zinc added as a supplement to ZD medium. ZDA cells were ZD cells cultured in ZA medium for the last 24 h. RNase protection products were separated on 6% acrylamide, 8 mol/L urea gels and quantitated by laser densitometry. β-Actin was used as a reference and values were expressed as a percentage of ZA. Representative samples of the p53 protected fragment from each treatment group are shown in the inset. Values are means ± SEM, $n = 3$. Different letters indicate significantly different means, $P < 0.05$.

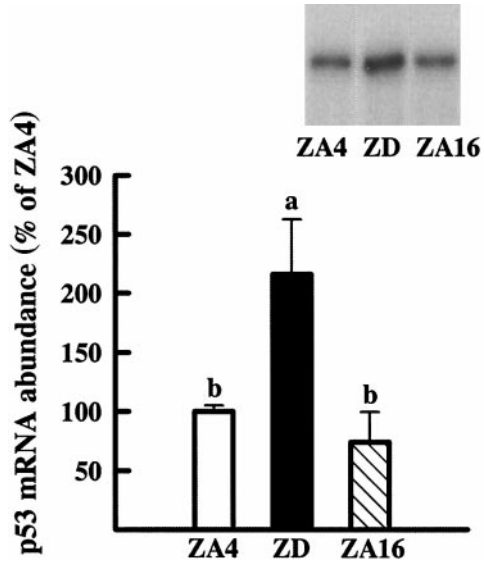

FIGURE 4 Relative p53 mRNA abundance in zinc-adequate (ZA4), zinc-depleted (ZD) and zinc-supplemented (ZA16) HepG2 cells as determined by RNase protection assays. Cells were cultured for one passage in various media containing Chelex-treated serum and with zinc added as a supplement. RNase protection products were separated on 6% acrylamide, 8 mol/L urea gels and quantitated by laser densitometry. Cyclophilin was used as a reference and values were expressed as a percentage of ZA. Representative samples of the p53 protected fragment from each treatment group are shown in the inset. Values are means ± SEM, $n = 3$. Different letters indicate significantly different means, $P < 0.05$.

ZA medium (ZDA cells) resulted in a significant increase in cellular zinc to a level ~80% of the ZA cells (Fig. 1). For the Chelex study, cells cultured in medium containing 16 μmol/L Zn (ZA16) had significantly higher cellular zinc than both ZA4 and ZD cells (Fig. 2). Because Chelex can reportedly bind copper under certain conditions, we also analyzed cellular copper level but found no differences among groups (data not shown).

Effects of treatment on p53 and MT-II mRNA abundance. p53 mRNA abundance was higher in the ZD cells in both studies. The p53 mRNA abundance in the ZD cells of the Opti-MEM study was almost twofold higher than the level found in ZA cells (**Fig. 3**). Similarly, ZD cells in the Chelex study exhibited >100% higher levels of p53 mRNA compared with their ZA4 controls (**Fig. 4**). Zinc replenishment of ZD cells (ZDA cells) in the Opti-MEM study resulted in p53 mRNA levels not different from those of ZA controls (Fig. 3). Nevertheless, the p53 mRNA abundance was significantly different between the ZA16 and ZD cells (Fig. 4). The mRNA abundance of the zinc-responsive MT-II is also shown for the Chelex study. MT-II mRNA levels were drastically lower in the ZD cells than those found in ZA4 cells (**Fig. 5**). In addition, MT-II was also sensitive to increasing levels of cellular zinc because MT-II mRNA was >100% higher in ZA16 cells than in ZA4 cells (Fig. 5).

Nuclear p53 protein was higher in Opti-MEM ZD cells. Western blot analysis of nuclear extracts was used to quantitate nuclear p53 protein levels in each of the studies. In the Opti-MEM study, nuclear p53 protein in ZD cells was significantly higher than in both ZA and ZDA cells (**Fig. 6**). One-day zinc replenishment resulted in reducing p53 to levels lower than both ZA and ZD cells (Fig. 6). However, no significant differences were detected in nuclear p53 protein

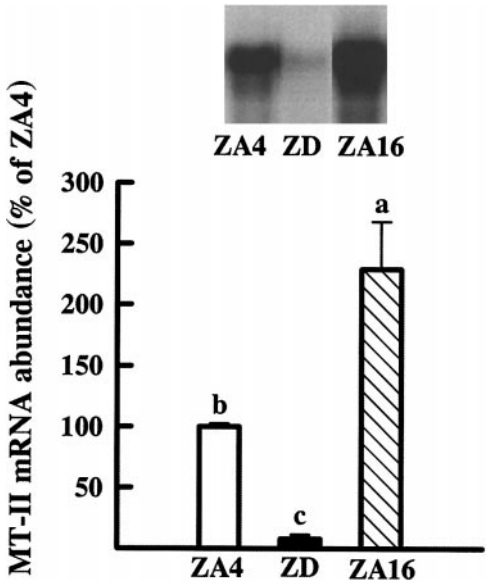

FIGURE 5 Relative metallothionein (MT)-II mRNA abundance in zinc-adequate (ZA4), zinc-depleted (ZD) and zinc-supplemented (ZA16) HepG2 cells as determined by RNase protection assays. Cells were cultured for one passage in various media containing Chelex-treated serum with zinc added as a supplement. RNase protection products were separated on 6% acrylamide, 8 mol/L urea gels and quantitated by laser densitometry. Cyclophilin was used as a reference and values were expressed as a percentage of ZA. Representative samples of the MT-II protected fragment from each treatment group are shown in the inset. Values are means ± SEM, $n = 3$. Different letters indicate significantly different means, $P < 0.05$.

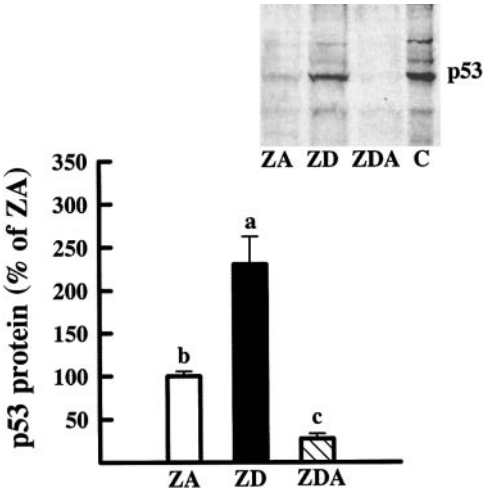

FIGURE 6 Relative p53 protein levels in zinc-adequate (ZA), zinc-depleted (ZD) and zinc-replenished (ZDA) HepG2 cells as determined by Western blot analysis. Cells were cultured for one passage in Opti-MEM medium with or without zinc added as a supplement to ZD medium. ZDA cells were ZD cells cultured in ZA medium for the last 24 h. Nuclear protein extracts were separated on 7.5% polyacrylamide-SDS gels, transferred onto nitrocellulose membranes and incubated with anti-p53 antibody. Autoradiography was performed using enhanced chemiluminescence and quantitated by laser densitometry. Values were expressed as a percentage of ZA. Representative samples from each treatment group are shown in the inset. C, control using nuclear extract from A431 cells. Values are means ± SEM, $n = 3$. Different letters indicate significantly different means, $P < 0.05$.

among the treatment groups in the Chelex study (ZA, 100 ± 16%; ZD, 95 ± 10%; ZA16, 107 ± 19%).

DISCUSSION

In these studies, we used two different methods to deplete cellular zinc from HepG2 cells. Both strategies significantly reduced cellular zinc within one passage. The first method utilized a special medium from Life Technologies, Opti-MEM, that was originally developed by adding growth factors so that the requirement of serum could be reduced significantly. Similar to the present studies, Opti-MEM has also been used previously as a means to deplete cellular zinc from BHK (Palmiter 1994) and HepG2 cells (Wu et al. 1999). Previous work in our laboratory (Wu et al. 1999) demonstrated that HepG2 cells could be cultured in Zn-free Opti-MEM with 1% FBS for one passage without altering growth or morphology. Therefore, the combination of Zn-free Opti-MEM and 1% FBS constituted our ZD medium. For the ZA treatment group, zinc in the form of $ZnSO_4$ was added so that the level of zinc in ZA medium was equal to that found in Opti-MEM with 10% FBS. Therefore, the only difference between ZA and ZD media was zinc concentration. Because Opti-MEM had been used successfully to examine zinc-regulated expression of metallothionein (Palmiter 1994) and apolipoprotein A-I (Wu et al. 1999), we chose to use Opti-MEM to examine the response of p53 to alterations in cellular zinc.

For the second study, we utilized Chelex 100, a divalent ion–chelating resin that has been used extensively in biological research for numerous applications (Flynn and Yen 1981, Messer et al. 1982, Prasad et al. 1996). To avoid exposing cells directly to the chelating resin, FBS was incubated with Chelex and then removed before FBS was used for the cell culture medium. This provided an effective yet noninvasive technique to control zinc concentrations in medium because serum is the major source of zinc in most culture media. A separate experiment demonstrated that HepG2 cells cultured in ZA4 treatment medium (medium containing Chelexed FBS and $ZnSO_4$ added as a supplement) for three consecutive passages did not appear to be morphologically distinct from control cells cultured in normal medium (data not shown). Even though Chelex has a high affinity for zinc, it has also been reported to sequester other divalent metals under certain conditions (Prasad et al. 1996). Because of the divalent properties of copper and the interrelationships of copper and zinc, we also measured intracellular copper levels in each group. No significant differences in cellular copper existed (data not shown). Although we acknowledge that other divalent ions besides zinc may be affected by Chelex, levels of the ions would be the same for all groups. Therefore, we believe our findings are due to differences in cellular zinc status. As an additional marker of cellular zinc status, we also measured MT-II mRNA abundance in the Chelex study. The regulation of the MT genes by zinc has been well documented and is mediated by metal responsive elements located within their promoters (Hamer 1986). Indeed, we found that MT-II mRNA abundance was related to cellular zinc levels because ZA4 MT-II mRNA was dramatically higher than ZD cells and ZA16 MT-II mRNA abundance was significantly higher than ZA4.

HepG2 cells have been used extensively as a model with which to study p53 because of several key attributes that make them distinct from many other hepatic cell lines. Of seven hepatic cell lines recently examined, the hepatoblastoma-derived HepG2 cells were the only cell line in which the p53 gene and its expression appeared to be unaltered (Bressac et al. 1990). Other reports have indeed confirmed that HepG2 cells express a wild-type p53 protein (Hsu et al. 1993) that can be activated to elicit normal p53 function (Muller et al. l977). Because of these characteristics and our ability to deplete cellular zinc from HepG2 cells, we believe this model could provide important information about how a nutrient such as zinc may affect the expression of p53.

In the current studies, reductions in cellular zinc were associated with an increase in cellular p53 mRNA abundance regardless of the method used to deplete cellular zinc. Zinc depletion in the Opti-MEM study (reduced to 27% of controls) was slightly more drastic than in the Chelex study (64% of controls). It is possible that the efficiency of zinc depletion between the two studies is different because of differences in the serum concentrations used; however, we have not determined the mechanism. Of interest was that the p53 mRNA was higher relative to controls (nearly 200%) in the Opti-MEM study compared with the Chelex study (>100%). Replenishing zinc levels in ZD cells in the Opti-MEM study reduced p53 mRNA to control levels even though zinc was replenished to only 80% of controls. For the Chelex study, culturing cells in ZA16 media did not significantly affect p53 mRNA abundance even though MT-II mRNA was elevated by the increase in zinc. Relative differences in cellular zinc from controls were comparable for the ZA16 and ZD cells (137 and 64%, respectively) in the Chelex study; however, the regulation of p53 mRNA may be more sensitive to decreases in cellular zinc than to increases in cellular zinc. Because zinc is relatively nontoxic, it would seem unlikely that p53 expression would be affected by moderate increases in cellular zinc. This is supported by the findings that moderately high and very high levels of zinc are required to modulate apoptosis (Fraker and Telford 1996). However, because p53 is a stress-response factor, one or more types of cellular stress (e.g., oxidative

stress, DNA damage) may have been imposed by the level of depletion in our studies and up-regulated p53 expression.

Although data from both of our studies showed that p53 mRNA was higher in zinc-depleted cells, the mechanism responsible for these changes remains to be determined. Several investigators have reported that zinc deficiency is capable of inducing cellular oxidative stress, depending on the severity of the deficiency and the model or cell line used [reviewed by Bray and Bettger (1990)]. One factor that we suspect may be involved in the observed changes for p53 expression is the well-described nuclear factor (NF)-κB. Because NF-κB is sensitive to cellular reactive oxygen species [reviewed by Schreck et al. (1992)], and has been shown to bind to and regulate p53 promoter activity (Wu and Lozano 1994), it would seem to be a likely candidate as a regulatory factor in these conditions. Electrophoretic mobility shift assays and transfection experiments using p53 promoter sequences must be performed under these zinc-deficient conditions to identify regulatory factors and elements affected by zinc status.

Nuclear p53 protein levels were also higher in the ZD cells of the Opti-MEM study. It is interesting that the increase in nuclear p53 protein did not affect cell growth. As postulated in a recent review by Meplan et al. (1999), several aspects of p53 may be affected by a deficiency in cellular zinc. The authors point out that p53 is sensitive to oxidative stress because of key cysteine residues present in both the zinc coordination motif as well as the DNA-binding domain. Studies using metal chelators as well as oxidizing agents have shown that zinc chelation (Verhaegh et al. 1998) and oxidation (Hainaut and Milner 1993) cause p53 to adopt a "mutant-like" form with decreased DNA-binding activity. It should also be noted that p53 also exhibits functions other than suppressing growth, including the regulation of genes involved in the oxidative stress response as well as other cytotoxic stresses. The finding that there was no significant difference in p53 protein between ZA and ZD cells of the Chelex experiments was interesting and somewhat surprising in light of the Opti-MEM data. Perhaps these findings were related to a less drastic zinc depletion in the Chelex ZD cells (64% of controls) compared with the ZD cells in the Opti-MEM study (27% of controls). Indeed, the increase in p53 mRNA was less dramatic in Chelex ZD cells compared with Opti-MEM ZD cells, and this smaller change in mRNA may be responsible at least in part for the lack of change in the p53 protein level. A distinct post-transcriptional regulatory mechanism, one that is less sensitive to zinc deprivation, may also be involved in controlling nuclear p53 protein levels.

Cellular zinc is thought to exist either in stable, fixed pools or in more dynamic, labile pools. Many researchers believe that these labile pools regulate important cellular processes, including gene expression, and are influenced by zinc supplementation or deprivation. In a recent study, cultured cells were exposed to a membrane-permeable chelator with a high affinity for zinc; p53 conformation was altered and DNA-binding activity was decreased (Verhaegh et al. 1998). At this point, however, it is difficult to determine whether these labile pools of zinc may be affected differently depending on whether a cell-permeable chelator or a strategy such as the one utilized for these studies is used to deplete cellular zinc. Obviously, integrating findings from studies using membrane-permeable chelators with our present findings is difficult, and each strategy has distinct advantages. However, we believe our strategy of depleting zinc in the media or sera, the extracellular environment of these cells, better reflects suboptimal plasma zinc levels, a common characteristic of dietary zinc deficiency. To the best of our knowledge, this is the first reported use of a nonchelator strategy (which did not involve the culture of cells with chelators) to examine how depletion of cellular zinc affects p53 mRNA and protein levels.

In these studies, we examined the effects of zinc depletion on p53 mRNA abundance and nuclear p53 protein levels. Using the human hepatoblastoma HepG2 cell line, which expresses moderate levels of wild-type p53, we showed that zinc depletion increases p53 mRNA abundance. Zinc depletion in the Opti-MEM study showed a concomitant increase in nuclear p53 protein levels. Furthermore, zinc repletion of zinc-deficient cells resulted in a return of p53 mRNA abundance to control levels and lower levels of p53 protein than that of controls in the Opti-MEM study. On the basis of these results, we propose that the expression of p53 may be responsive to cellular zinc status under certain conditions. One potential implication for this study is that zinc deficiency may reduce the ability of p53 to protect cells from carcinogenic compounds or conditions such as radiation. On the basis of these studies, it appears that zinc depletion alters normal p53 expression; work from other laboratories indicates that zinc is crucial to maintain wild-type p53 conformation and DNA-binding activity. Therefore, compromised cellular zinc status may possibly enhance the susceptibility of an organism to cancer by attenuating the tumor-suppressive activity of p53.

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