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Zinc Supplementation Selectively Decreases Fetal Hepatocyte DNA Synthesis and Insulin-Like Growth Factor II Gene Expression in Primary Culture

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ABSTRACT. Zinc is important for normal cell growth and differentiation, DNA synthesis, and gene expression. IGF-II is a fetal growth and differentiation factor whose regulation is largely unknown. To assess the effect of zinc (Zn) on fetal hepatocyte IGF-II expression and DNA synthesis, primary cultures of ovine fetal hepatocytes were studied in serum-free medium containing 1 μmol/L Zn or supplemented to 10 or 50 μmol/L Zn. Fetal hepatocyte DNA synthesis, Zn and protein content, IGF-II mRNA, and IGF binding protein production were measured. Zn concentration in medium increased slightly in unsupplemented dishes, from 1 to 1.5 μmol/L; however, Zn concentration declined by 4 and 8 μmol/L over 24 h in culture medium supplemented to contain either 10 or 50 μmol/L Zn ($p < 0.05$). Zn content of cell pellets increased 155 and 204% after 24 h in supplemented cultures compared with unsupplemented controls, demonstrating uptake of Zn by the liver cells. Media Zn supplementation to 10 and 50 μmol/L decreased ^{3}H-thymidine incorporation of cells in culture by 11 and 13%, respectively, compared with 1 μmol/L Zn ($p = 0.001$). Addition of Zn caused a progressive 2- to 3-fold decline in the nuclear labeling index of fetal hepatocytes, whereas the labeling index of nonhepatocytes increased almost 2-fold at 50 μmol/L compared with 1 μmol/L Zn. Associated with decreased hepatocyte DNA synthesis, IGF-II mRNA abundance declined by almost 30%. IGF binding protein content of conditioned medium did not change with added Zn. Cellular DNA and protein contents did not vary after 24 h in culture with either 1, 10, or 50 μmol/L Zn, suggesting that Zn was not toxic to the cells. We conclude that Zn selectively decreases fetal hepatocyte proliferation in primary culture, whereas nonparenchymal cell growth is not inhibited. Some of this response may be caused by decreased expression of IGF-II, an autocrine growth factor for fetal hepatocytes. (*Pediatr Res* 35: 404–408, 1994)

Abbreviations

Zn, zinc
IGFBP, IGF binding protein

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Adequate Zn supply to the fetus is essential for normal growth (1, 2). However, the pathways in which Zn plays a role in fetal growth regulation are largely unknown. Nonetheless, the liver is thought to be a major site of Zn metabolism in the fetus, as it is in the newborn animal (1, 2). Zn is an important cofactor for DNA synthesis and gene transcription (2, 3), and Zn deficiency may affect growth in part through decreased DNA synthesis and RNA production.

The fetal liver plays a central role in many fetal metabolic pathways and is a source of circulating growth factors and regulatory proteins, such as IGF-II and IGFBP. IGF-II is a peptide hormone with structural homology to proinsulin whose importance in fetal growth regulation has recently been established (4). The IGFBP are a family of proteins that specifically bind IGF and regulate their biologic activity (5). Zn deficiency has been associated with decreases in a structurally and functionally related growth factor, IGF-I, in lambs and rats (6, 7). The effects of Zn on fetal IGF or IGFBP production have not been examined previously. To investigate the role of Zn in regulation of fetal liver cell IGF-II gene expression, IGF-BP biosynthesis, and DNA synthesis, we studied ovine fetal hepatocytes maintained in primary culture in medium containing minimal Zn (1 μmol/L), physiologic Zn (10 μmol/L), and excess Zn (50 μmol/L).

MATERIALS AND METHODS

Hepatocyte isolation and culture. Hepatocytes were isolated by *in situ* perfusion and collagenase digestion from late gestation Columbia-Rambouillet fetal sheep (131 ± 3 d gestation, term = 145 d) and cultured as described (8). All protocols were approved by the Animal Care and Use Committee of the University of Colorado Health Sciences Center. After hepatocyte isolation, cells were suspended in attachment medium (arginine-free α–minimum essential medium, with 1.1 mM glucose, 2 mM lactate, 0.4 mM ornithine, 10% FCS, 1×10^4 IU/L penicillin, 100 mg/L streptomycin, and 50 mg/L kanamycin) and plated at 8×10^6 cells/100-mm dish. Liver cells were allowed to adhere to dishes overnight in humidified air:CO_2 (19:1) at 37°C. The medium was then changed to serum-free α–minimum essential medium, with 0.2% BSA, and either no additional Zn or addition of $ZnSO_4$ stock solution (1000 g Zn/L) to achieve a final Zn concentration of 10 or 50 μmol/L. Unsupplemented medium contained 1 μmol/L Zn. The time of transfer to serum-free medium after cell attachment to culture dishes was considered time 0. Studies were performed after 24 h of culture in medium containing Zn concentrations as described.

Tritiated thymidine incorporation and DNA content. Isolated hepatocytes were plated in six-well dishes, adhered overnight as described above, and then transferred to serum-free medium

supplemented with 0.2% albumin and either 1, 10, or 50 μmol/L Zn. IGF-II (Lilly Research Laboratories, Indianapolis, IN) was added in some experiments to achieve a final concentration of 100 μg/L (100 ng/mL). Methyl-^{3}H-thymidine (~80 Ci/mmol, Amersham, Arlington Heights, IL) was added to the medium at a concentration of 1.5 mCi/L (1.5 μCi/mL), and cells were incubated for 24 h. Medium was removed at 24 h and cells were washed twice with ice-cold PBS before precipitation with 10% trichloroacetic acid. Triplicate wells were harvested for scintillation counting and DNA determination by the diphenylamine reaction (9). In some experiments, cells were fixed in 4% paraformaldehyde, then exposed *in situ* to photographic emulsion (NTB-3 Nuclear Track Emulsion, Eastman Kodak Co., Rochester, NY) for determination of nuclear labeling index by autoradiography. Counterstaining with hematoxylin and eosin (Sigma Chemical Co., St. Louis, MO) was performed to identify cell types morphologically. Cuboid cells with round nuclei in cords or sheets were considered hepatocytes; other cells with oblong or stellate shapes or isolated nuclei were considered nonhepatocytes. Nuclear labeling index for hepatocytes and nonhepatocytes was determined by two investigators (S.F.T., K.K.B.) unaware of treatment conditions. Interobserver agreement was greater than 98%.

RNA extraction and analysis. After removal of medium, cultured cells were harvested into a solution of 5 M guanidine thiocyanate, 25 mM sodium citrate, 0.5% sodium sarcosyl, and 100 mM β-mercaptoethanol, and total RNA was extracted according to the method of Chomczynski and Sacchi (10). RNA was quantified by UV absorbance at 260 and 280 nm and purity and integrity were assessed by agarose-gel electrophoresis with ethidium bromide staining. For slot blots, total cellular RNA was applied directly to nylon membranes with a slot-blot apparatus (Bio-Dot SF, Bio-Rad, Richmond, CA) after denaturation in buffer containing 50% formamide, 6% formaldehyde, and 20 mM 3-[N-morpholino]propanesulfonic acid and cross-linked by irradiation with UV light (UV-Stratalinker, Stratagene, La Jolla, CA). For Northern blots, RNA samples were electrophoresed in agarose/formaldehyde gels and transferred overnight to nylon membranes by standard methods (11).

IGF-II mRNA was identified using a full-length ovine IGF-II cDNA obtained from T. E. Adams (12). All cDNA were labeled by random primer extension (kit from Boehringer-Mannheim, Mannheim, Germany) with 5-(α^{32}P)-deoxycytidine triphosphate (Amersham) to a sp act of ~10^9 cpm/μg. Blots were washed under high-stringency conditions [0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0 (0.1× SSC), and 0.1% SDS at 65°C] (11). Blots were boiled and reprobed for 18S rRNA (probe kindly provided by V. Han, London, Ontario) to verify comparable loading. IGF-II was quantified by scanning densitometry of slot blots, with the IGF-II signal normalized to the 18S rRNA signal intensity before statistical analyses.

Western ligand blotting. Western ligand blot analyses of IGFBP were performed by a modification of standard methods (13, 14). In brief, samples of medium were denatured at 100°C in buffer containing 0.06 M Tris-HCl, 0.8% SDS, 4% glycerol, and 0.004% bromophenol blue for 5 min, then size fractionated on 8% SDS-polyacrylamide gels at 30 V for 20 h. Proteins were electroblotted onto nitrocellulose membranes overnight at 50 V, then blocked by washing in 0.15 M NaCl, 10 mM Tris-HCl buffers containing sequentially NaN_3, Nonidet P-40, BSA, and Tween 20 (Sigma Chemical Co.). The membranes were then incubated overnight with 200 000 to 400 000 cpm of ^{125}I-labeled IGF-II (~2000 Ci/mmol, Amersham), and binding proteins were detected by autoradiography. IGFBP were presumptively identified by molecular weight and by comparison with known fetal sheep IGFBP profiles (15).

Biochemical assays. After 24 h in culture, aliquots of media were saved and dishes were rinsed in succession with solutions containing 0.9% NaCl, 0.9% NaCl with 0.1% albumin and 1 mM EDTA, then 0.9% NaCl to remove residual medium. Cells were harvested by scraping and pelleted by centrifugation. All rinse solutions were aspirated from the cell pellet, then an aliquot was reserved for measurement of protein content by a modification of the Lowry method (16). Zinc content of the remaining cell pellet was measured by atomic absorption spectroscopy after ashing overnight in a low temperature asher (17). Zn in the medium was measured directly by atomic absorption spectroscopy with a Perkin-Elmer 2380 spectrophotometer (Perkin-Elmer Corp., Norwalk, CT) fitted with a single-slot burner head and a deuterium background correction lamp.

Statistical analyses. All assays were performed at least in duplicate, and the results from three to seven experiments were compared. Results are expressed as the mean ± SD. Data from Zn-supplemented dishes (10 and 50 μmol/L Zn) were compared with data from control, unsupplemented dishes (1 μmol/L Zn) by analysis of variance with a microcomputer statistical software package (SuperANOVA, Abacus Concepts, Berkeley, CA). Significance at $p < 0.05$ was determined by the Scheffe's S test for post hoc comparisons.

RESULTS

The concentration of Zn in unsupplemented medium (low Zn) was 1.0 ± 0.2 μmol/L ($n = 7$). After 24 h of culture of liver cells in serum-free medium, Zn concentration in unsupplemented medium increased to 1.5 ± 0.7 μmol/L ($p = 0.04$). In all cultures initially supplemented with Zn, the concentration of Zn in medium declined over 24 h from 10.8 ± 1.2 to 7.4 ± 2.3 μmol/L and 45.3 ± 9 to 37.3 ± 10.9 μmol/L (Fig. 1). Associated with the increase of Zn concentration in medium in low Zn cultures, cellular Zn and Zn/protein ratios in unsupplemented (low Zn) dishes declined by 55 ± 6 and 48 ± 6%, respectively, after 24 h ($n = 3$). Zn content of cell pellets was unchanged after 24 h in 10-μmol/L dishes and increased by 147 ± 12% in 50-μmol/L dishes, demonstrating uptake of Zn by the mixed liver cell population under these conditions. Therefore, after 24 h in culture in medium containing low Zn (1 μmol/L) or supplemented to physiologic (10 μmol/L) or high (50 μmol/L) Zn, the Zn content and Zn/protein ratios of cell pellets in supplemented cultures were approximately 150% and 200%, respectively, of values from low Zn dishes (Fig. 2). Protein content of cell pellets decreases by ~15% after 24 h of culture in serum-free medium but did not vary with Zn content of medium (Fig. 2, Table 1). Hepatocyte DNA content also did not differ after exposure to the varying Zn concentrations in medium (Table 1).

Supplementation of Zn in medium to 10 and 50 μmol/L decreased ^{3}H-thymidine incorporation of cells in culture by 11

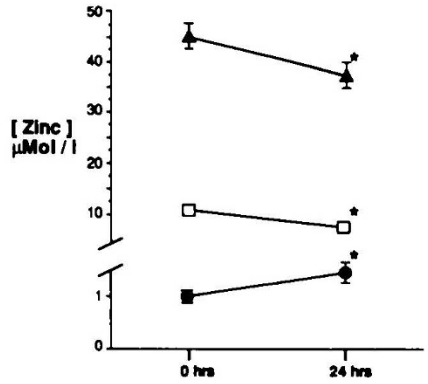

Fig. 1. Change in Zn concentration of medium conditioned by fetal liver cells over time in culture. Hepatocytes were transferred at 0 h to medium containing approximately 1, 10, or 50 μmol/L Zn, as described in Materials and Methods. Medium was removed after 24 h of incubation with hepatocytes in culture and assayed for Zn concentration as described. The results (mean ± SEM) of four experiments assayed in duplicate are shown; note change in scale. *, $p < 0.05$ compared with 0 h.

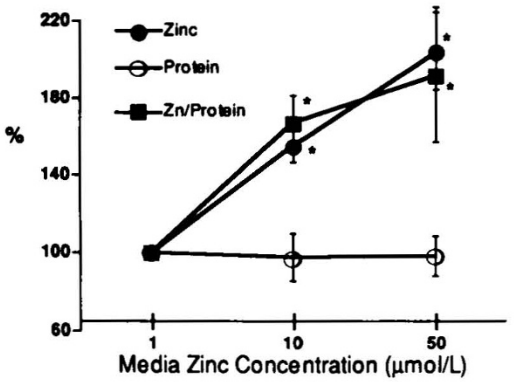

Fig. 2. Zn and protein contents and Zn/protein ratios of liver cell pellets after 24 h of culture in 1, 10, and 50 μmol/L Zn. Results are expressed as a percentage of the values obtained from cell pellets cultured in unsupplemented medium (1 μmol/L). The results of three experiments are shown. *, $p < 0.05$ compared with 1-μmol/L dishes.

Table 1. *Hepatocyte protein and DNA content after 24 h in culture with varying concentrations of Zn in medium*

Zn (μmol/L)	Protein (mg/dish, $n = 3$)*	DNA (μg/well, $n = 6$)*
1	324 ± 61	33 ± 4.6
10	331 ± 44	33.1 ± 5.1
50	356 ± 67	33.3 ± 4.8

* n = number of experiments.

± 1.4 and 13 ± 2.3%, respectively, compared with 1 μmol/L Zn ($n = 9$, $p = 0.001$). Because the decrease in ^{3}H-thymidine incorporation after Zn supplementation was variable and did not differ between the two higher Zn treatments, DNA synthesis was examined by autoradiography to determine a nuclear labeling index by cell type. These primary fetal liver cell cultures, assessed morphologically, contain predominantly hepatocytes, accounting for 85–95% of cells, as well as nonhepatocytes (biliary cells, monocytes, fibroblasts, *etc.*) (8). Addition of Zn caused a progressive 2- to 3-fold decrease in the nuclear labeling index of cells identified as fetal hepatocytes, whereas the labeling index of nonhepatocytes increased almost 2-fold at 50 μmol/L compared with 1 μmol/L Zn (Fig. 3). Photomicrographs demonstrating the differences in nuclear labeling of hepatocytes and nonhepatocytes in two separate experiments are shown in Figure 4. Panels *a* and *b* demonstrate appearance of nuclear labeling after culture in 1 μmol/L Zn and panels *c* and *d* after 24 h culture in 50 μmol/L Zn.

Associated with decreased hepatocyte DNA synthesis, IGF-II mRNA abundance assessed by scanning densitometry of slot blots (corrected to 18S rRNA) was decreased by 21 ± 11% in hepatocytes cultured in 10 μmol/L Zn and 31 ± 12% in hepatocytes cultured in 50 μmol/L Zn compared with hepatocytes maintained in 1 μmol/L Zn ($n = 4$). A representative Northern blot demonstrating this change in IGF-II mRNA from hepatocytes maintained in 1 and 50 μmol/L Zn for 24 h is shown in Figure 5. There are seven known IGF-II mRNA transcripts of varying size in the sheep (18), and culturing of fetal liver cells in the presence or absence of supplemental Zn did not appear to alter their relative abundance (Fig. 5). Addition of exogenous IGF-II (100 μg/L) to the culture medium increased DNA synthesis to 140% of unstimulated values; this increase was not affected by Zn supplementation.

We also examined the regulatory IGFBP produced by cultured cells over 24 h in three experiments. Medium conditioned by fetal liver cells in culture contained four species of IGFBP indicated by the arrows in Figure 6: IGFBP-3 migrating as a doublet, IGFBP-2, IGFBP-1, and IGFBP-4 (in descending order based on size). Increasing Zn content in medium did not alter the IGFBP amount or distribution in conditioned medium (Fig. 6).

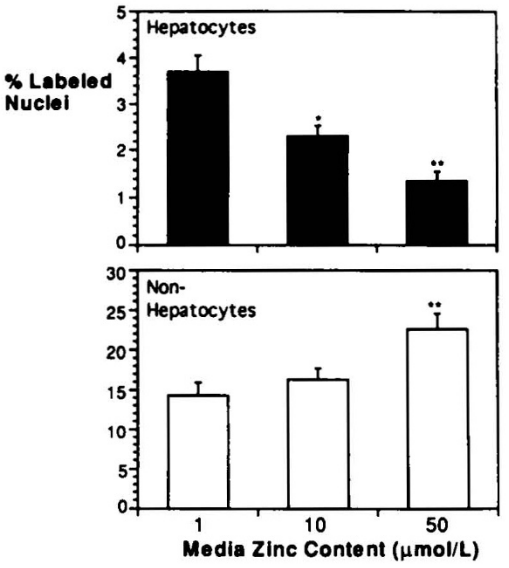

Fig. 3. Estimated nuclear labeling index of hepatocytes and nonhepatocytes after incubation with ^{3}H-thymidine in medium containing either 1, 10, or 50 μmol/L Zn as described in Materials and Methods. Cells were identified morphologically after staining in hematoxylin and eosin. *, $p < 0.05$ compared with 1 μmol/L Zn; **, $p < 0.05$ compared with 1 and 10 μmol/L Zn.

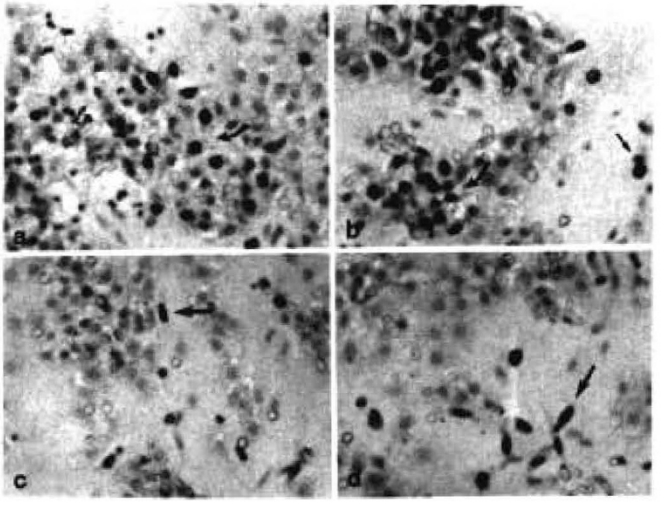

Fig. 4. Photomicrographs of liver cell cultures demonstrating nuclear labeling by ^{3}H-thymidine after culture in medium containing basal levels (1 μmol/L) of Zn (*a* and *b*) or supplemented to 50 μmol/L Zn (*c* and *d*). *Curved arrows* denote hepatocytes, *straight arrows* nonhepatocytes. Two separate experiments are shown in *a* and *c* compared with *b* and *d*. All panels are shown in magnification ×40; counterstain is hematoxylin and eosin.

DISCUSSION

These experiments demonstrate that Zn in physiologic concentrations selectively decreases fetal hepatocyte DNA synthesis, whereas nonhepatocyte DNA synthesis is increased. Decreased DNA synthesis is accompanied by a decline in IGF-II mRNA abundance, which is likely to result in decreased IGF-II synthesis and may in part mediate the response observed. We chose to study concentrations of Zn that reflected the basal concentration in culture medium as supplied by the manufacturer, a physiologic concentration comparable to that determined in plasma from

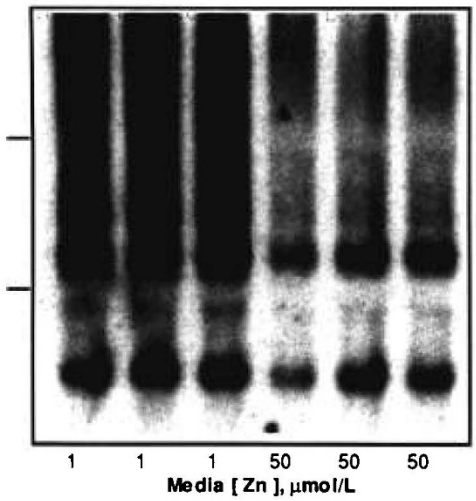

Fig. 5. A Northern blot of total RNA from fetal hepatocytes after 24 h of culture in medium with 1 or 50 μmol/L Zn, probed with a full-length IGF-II cDNA as described. There are seven IGF-II mRNA transcripts identified. Although 50 μmol/L Zn decrease total IGF-II mRNA abundance, the relative intensity of different transcripts is unchanged. Migration of 28S and 18S rRNA is indicated by the *dashes*.

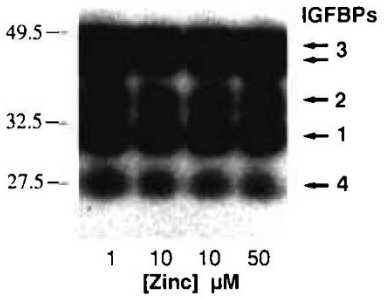

Fig. 6. A Western ligand blot of culture medium conditioned after incubation of fetal hepatocytes for 24 h in 1, 10, or 50 μmol/L Zn showing no effect of Zn on IGFBP production by fetal hepatocytes. The blot was probed with ^{125}I-labeled IGF-II to identify all IGFBP present, as indicated by the *arrows*. Note that IGFBP-3 migrates as a doublet. Molecular size markers are indicated on the left.

healthy lambs (~10 μmol/L) (6), and a supraphysiologic concentration (~50 μmol/L). Without Zn supplementation of serum-free medium, there is a decline in cultured fetal liver cell Zn content, and an increase in Zn concentration in the medium. Addition of Zn to the culture medium is associated with uptake of Zn by the fetal liver cells and a decline in Zn concentration in the medium. These responses are not associated with Zn toxicity to the cells: total DNA and protein content were not affected by Zn concentration in the medium, nor was production of a specific family of proteins, the IGFBP.

IGF-II is thought to mediate some of its growth-promoting effects via autocrine and paracrine mechanisms. There is abundant endogenous expression of IGF-II in cultured fetal hepatocytes and addition of exogenous IGF-II to primary cultures of fetal hepatocytes induces DNA synthesis (8). It is therefore possible that IGF-II is an autocrine growth factor for fetal hepatocytes. We hypothesize that Zn supplementation may act to decrease DNA synthesis in fetal hepatocytes in part through inhibiting expression of IGF-II, as seen by the decline in IGF-II mRNA abundance in response to increased Zn content in medium. Zn supplementation did not inhibit the proliferative response of fetal liver cells to exogenous IGF-II, suggesting that Zn was not directly interfering with the biologic actions of IGF-II.

Four IGFBP were identified in medium conditioned by cultured fetal liver cells. These have been presumptively identified as IGFBP-1, -2, -3, and -4, based on their molecular weights and reports of known fetal sheep plasma IGFBP profiles (15). The IGFBP are important physiologic modulators of IGF-II activity, and production of IGFBP by the liver is in part nutritionally regulated (5). Therefore, factors that alter IGF-II expression might be expected to alter hepatocyte IGFBP production. However, Zn supplementation did not change the content of IGFBP seen in medium conditioned by fetal liver cells, suggesting that Zn is not directly involved in regulation of IGFBP production by fetal hepatocytes.

Uptake of Zn by liver cells has been studied previously by using adult hepatocytes in primary culture (19–21). Our observations of loss of Zn into the culture medium by hepatocytes with culture in unsupplemented medium are similar to those of Guzelian *et al.* (21) but differ from the work of Cousin's group, which demonstrated no loss of Zn from cells after up to 48 h in culture (19, 20). The loss of Zn by fetal hepatocytes maintained in primary culture in low Zn may reflect differences in Zn kinetics (uptake and utilization) in a population of fetal hepatocytes that maintains a substantial level of DNA synthesis compared with quiescent, nonproliferating adult hepatocytes. In addition, adult hepatocytes are adhered in culture in the presence of insulin, which could alter cellular Zn metabolism. Uptake of Zn in cultures supplemented to 10 and 50 μmol/L was similar to that observed in previous reports (19, 20).

Effects of Zn on DNA synthesis in general have been studied by using models of Zn deficiency to demonstrate a decrease in DNA synthesis. Our finding of decreased DNA synthesis in hepatocytes in response to supplemental Zn contrasts with these widely reported observations. Nonetheless, dietary Zn deficiency has been associated with increased DNA synthesis in at least one other tissue, the buccal mucosa in rabbits (22). It is difficult to compare other *in vivo* studies using dietary restriction of Zn with these direct, *in vitro* observations of the effects of provision of adequate Zn to proliferating fetal hepatocytes. In our experiments, provision of Zn in physiologic concentrations augmented DNA synthesis in nonhepatocytes, more in keeping with previous whole-animal studies. Also, careful analysis of studies in mice in which Zn deprivation was begun during pregnancy and continued throughout gestation demonstrated larger liver size in Zn-deficient mice after birth than in Zn-sufficient, pair-fed controls (23). Other organs were not similarly affected (23). It is likely that Zn may have direct effects on control of cellular growth through actions on specific DNA or RNA polymerases and other enzymes (2, 3) as well as indirect effects that may be mediated through regulation of transcription of important growth factors, such as the IGF. Because dietary manipulation of Zn is associated with altered food intake and changes in metabolic pathways that also influence, for example, IGF production [*i.e.* those involving glucose and insulin (6)], the results of dietary studies are impossible to compare directly with the experiments reported here.

We conclude that provision of Zn selectively decreases fetal hepatocyte proliferation in primary culture, whereas nonparenchymal cell growth is augmented. We speculate that some of this response may be caused by decreased expression of IGF-II, an autocrine growth factor for fetal hepatocytes. Production of the family of regulatory IGFBP by the fetal liver is not modulated by Zn. These data suggest a complex role for Zn in fetal growth regulation, with different cell types capable of responding in diverse fashions to Zn supply.

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