Modulation of Gene Expression in Precancerous Rat Esophagus by Dietary Zinc Deficit and Replenishment

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Abstract
Zinc deficiency in rats enhances esophageal cell proliferation, causes alteration in gene expression, and promotes esophageal carcinogenesis. Zinc replenishment rapidly induces apoptosis in the esophageal epithelium thereby reversing cell proliferation and carcinogenesis. To identify zinc-responsive genes responsible for these divergent effects, we did oligonucleotide array-based gene expression profiling analyses in the precancerous zinc-deficient esophagus and in zinc-replenished esophagi after treatment with intragastric zinc compared with zinc-sufficient esophagi. Thirty-three genes (21 up-regulated and 12 down-regulated) showed a ≥2-fold change in expression in the hyperplastic zinc-deficient versus zinc-sufficient esophageal epithelia. Expression of genes involved in cell division, survival, adhesion, and tumorigenesis were markedly changed. The zinc-sensitive gene metallothionein-1 (MT-1) was up-regulated 7-fold, the opposite of results for small intestine and liver under zinc-deficient conditions. Keratin 14 (KRT14), a biomarker in esophageal tumorigenesis, carbonic anhydrase II (CAII, a regulator of acid-base homeostasis), and cyclin B were up-regulated >4-fold. Immunochemistry showed that metallothionein and keratin 14 proteins were overexpressed in zinc-deficient esophagus, as well as in lingual and esophageal squamous cell carcinoma from carcinogen-treated rats, emphasizing their roles in carcinogenesis. Calponin 1 (CNN1, an actin cross-linking regulator) was down-regulated 0.2-fold. Within hours after oral zinc treatment, the abnormal expression of 29 of 33 genes returned to near zinc-sufficient levels, accompanied by reversal of the precancerous phenotype. Thus, we have identified new molecular markers in precancerous esophagus and showed their restoration by zinc replenishment, providing insights into the interaction between zinc and gene expression in esophageal cancer development and prevention. (Cancer Res 2005; 65(17): 7790-9)

Introduction
Esophageal cancer is an important cause of morbidity and mortality worldwide, with a 5-year survival of only ~10% (1). In 2003 in the United States, 13,900 new cases were diagnosed and 13,000 deaths were attributed to this malignancy (2). Whereas alcohol and tobacco consumption are major risk factors in industrialized countries, other factors, such as nutritional deficiencies, including that of zinc (3–5), and exposure to environmental carcinogens, including N-nitrosomethylbenzylamine (NMBA; ref. 6, 7), play an important role in the pathogenesis of esophageal squamous cell cancer (ESCC) in high incidence areas in northern China and Iran. We have developed zinc-deficient rat and mouse esophageal and oral cancer models (8–16) and found that zinc deficiency creates a precancerous condition in the tongue, esophagus, and forestomach by causing unrestrained cell proliferation and altering gene expression (9–11, 16). In particular, cyclooxygenase-2 (COX-2), an enzyme that is overexpressed in a variety of human premalignant and malignant lesions (17–19), is up-regulated by zinc deficiency (16). Thus, zinc deficiency accelerates carcinogenesis in the esophagus and forestomach caused by a single exposure to the carcinogen NMBA in rats (10), in p53-deficient mice (12), and in mice overexpressing cyclin D1 (13). In addition, zinc deficiency, by inducing cell proliferation in the entire upper aerodigestive tract, facilitates the development of tumors at multiple sites (tongue, esophagus, and forestomach) in rats exposed to the tongue carcinogen 4-nitroquinoline 1-oxide (NQO; ref. 16). On the contrary, zinc replenishment in zinc-deficient rats rapidly reduces COX-2 expression (16) and stimulates apoptosis by inducing Bax expression thereby inhibiting NMBA-induced esophageal carcinogenesis (14).

In a 2005 report, Abnet et al. (5) established a connection between zinc deficiency and ESCC in humans by using X-ray fluorescence spectroscopy to measure zinc, copper, iron, nickel, and sulfur in esophageal biopsy samples obtained from residents in a high ESCC incidence area in China. Subjects were matched on baseline histology and followed for 16 years. Ninety percent of subjects in the highest zinc quartile versus 65% of the subjects in the lowest quartile were alive and cancer free after 16 years. There were no associations with cancer risk for any of the other elements studied. These findings in humans are consistent with our conclusion from rodent model studies that zinc deficiency promotes esophageal cancer.

The rapid tumor initiation and reversal in zinc-deficient rat esophagus offers a unique opportunity to identify molecular markers in the precancerous esophagus that are responsible for the divergent tumorigenic potential caused by zinc deficit and replacement. To date, only a few studies have used DNA array and differential display techniques to identify genes in rodent tissues. In vivo studies that are sensitive to dietary zinc supply (20–23). Blanchard et al. (20) first reported that dietary zinc deficiency modulates expression of genes of rat intestine in vivo; 32 genes were modulated, representing genes that influence signaling pathways, growth, redox, and energy use. Differential display screening of the murine thymic transcriptome during dietary zinc deprivation and
supplementation identified only four zinc-modulated genes, including heat shock proteins (21). DNA array analysis in zinc-deficient rat liver identified 66 zinc-regulated genes, which participate in growth, signal transduction, and metabolism (22). In addition, liver from adult zinc-deficient rats showed severely repressed expression of MT-1 and MT-2 genes (23). Notably, zinc deficiency is not reported to cause a preneoplastic condition in any of the above organs.

Our earlier study identified differentially expressed genes in rat esophagus that are associated with the anticancer effect of zinc replenishment (24). Using cDNA microarray analysis, we found five up-regulated and three down-regulated transcripts in zinc-replenished rat esophageal mucosa ~27 hours after the zinc-deficient rats were dosed with intragastric zinc versus zinc-deficient esophagus. We identified two novel prouptogenetic genes ZD7 and ZD10 that were induced by zinc replenishment in rat esophageal epithelial cells displaying increased apoptosis. However, the specific pathway(s) or markers that might be aberrant in the esophagus when dietary zinc is in short supply have not been established. Recently, we showed that the hyperplastic esophagus and tongue of zinc-deficient rats overexpressed COX-2 protein and mRNA and zinc replenishment rapidly reduced COX-2 overexpression and reversed the hyperplastic phenotypes (16). To further investigate whether (i) the preneoplastic phenotype of zinc-deficient esophagus is brought about by global alterations in gene expression and (ii) the anticancer effect of zinc replenishment is due to its ability to restore gene expression to normal, we used oligonucleotide arrays to compare the expression of ~8,000 target genes in zinc-deficient versus control zinc-sufficient esophagus and in the zinc-replenished esophagus shortly after the zinc-deficient rats received an intragastric zinc dose.

Materials and Methods

Chemicals and animal diets. Custom-formulated, egg white–based, zinc-deficient, and zinc-sufficient diets were prepared by Teklad (Madison, WI). The two diets were identical except for the amount of zinc carbonate, which was 3 and 70 ppm for the zinc-deficient and zinc-sufficient diets, respectively (9).

Experimental design. The animal studies were approved by the Institutional Animal Care and Use Committee guidelines. The experimental design follows that described previously (16). Briefly, 24 male weaning Sprague-Dawley rats (Taconic Laboratory, Germantown, NY) were fed a zinc-deficient diet ad libitum and six control animals pair-fed a zinc-sufficient diet to match the reduced food consumption of zinc-deficient rats (9). After 5 weeks, zinc-deficient rats showed evidence of increased cell proliferation (as assessed by increased proliferating cell nuclear antigen and 5-bromo-2’-deoxyuridine labeling) in the esophagus (9, 10). Zinc gluconate (containing 1.2 mg elemental zinc) in 250-μL saline was then given intragastrically to 18 of the zinc-deficient rats, which were immediately switched to the zinc-sufficient diet, forming the zinc-replenished groups. At 8, 12, and 48 hours after zinc replenishment, six rats were killed after anesthesia with isoflurane (Abbott Lab., North Chicago, IL), forming the ZR8, ZR12, and ZR48 groups. Control zinc-sufficient and zinc-deficient rats (n = 6 per group) were treated intragastrically with saline lacking zinc and were killed after 8 hours. At sacrifice, whole esophagi were excised. Esophageal epithelium, prepared from the esophagus by using a blade to strip off the connective tissue layer, was snap-frozen in liquid nitrogen and stored at ~80°C until RNA preparation.

For Western blot analysis, we pooled esophageal epithelia from three to five rats of each group from another study of identical experimental design, comprising zinc-sufficient, zinc-deficient, ZR2, ZR4, ZR8, ZR12, ZR24, and ZR48 groups. Immunohistochemistry studies were done on esophageal sections from six rats of groups zinc-sufficient, zinc-deficient, ZR8, ZR12, and ZR48. Esophagi were fixed in phosphate-buffered formalin and embedded in paraffin. Serial cross-sections (4 μm) were stained with H&E or left unstained for immunohistochemical studies.

RNA isolation and oligonucleotide array analysis. Total RNAs were prepared separately from esophageal epithelia pooled from six zinc-sufficient, zinc-deficient, ZR8, ZR12, and ZR48 esophageal mucosae using TRIzol reagent (Invitrogen Corp., Carlsbad, CA), according to the manufacturer’s protocol. Samples were further purified using the Qiagen RNeasy Mini Kit (Qiagen, Valencia, CA). RNA concentrations were determined spectrophotometrically. Integrity of purified RNA samples was determined using agarose gel electrophoresis with ethidium bromide staining. Figure 1A shows that all RNA samples for oligonucleotide array analysis have distinct and clear 18S and 28S rRNA bands, indicating high-quality RNAs.

About 10 μg of each RNA sample was submitted to the Microarray Facility, Kimmel Cancer Center, Thomas Jefferson University, which provides sample labeling, chip hybridization, post-hybridization signal detection, chip scanning, and initial informatics data analysis. The CodeLink Uniset Rat 1 Bioarray chip (Amersham Biosciences, Piscataway, NJ) was used. This Bioarray contains single-stranded oligonucleotide probes from a broad range of rat genes derived from the National Center for Biotechnology Information database and clustered to generate consensus sequences. This Bioarray contains ~8,000 rat genes, one specific, prescreened 30-mer probe per gene, functionally validated; one set of housekeeping genes that may be used for baseline normalization; 68 bacterial control probes, 18 positive control probes to monitor cDNA and cRNA synthesis and array activity, and 50 negative control probes to determine threshold and assess background. In addition, each Bioarray contains probes for an independent assay of all genes in the set. A list of genes on the chip is available at the Amersham Biosciences web site.

Array data analysis. Array images and data were analyzed by GeneSpring 6.1 (Silicon Genetics, Redwood City, CA). Normalization was done as follows: values below 0.01 were set to 0.01. Each measurement was divided by the 50th percentile of all measurements in that sample. Each gene was divided by the median of its measurements in all samples. If the median of the raw values was below 10, then each measurement for that gene was divided by 10. Array elements in zinc-deficient esophagus exhibiting mean signal difference of ≥2-fold for duplicate comparisons with those in control zinc-sufficient esophagi were considered differentially expressed and associated with zinc deficiency. To evaluate whether zinc replenishment has an effect in reversing these changes in expression due to zinc deficiency, expression ratios among ZR8, ZR12, and ZR48 relative to zinc sufficiency were computed separately for these genes.

Confirmation of zinc modulation by real-time quantitative reverse transcription-PCR. Quantitative reverse transcription-PCR (RT-PCR) was done on the same RNA samples used for array analyses. Two micrograms of total RNA were reverse transcribed by Moloney murine leukemia virus reverse transcriptase (Stratagene Corp., La Jolla, CA) using oligo d(T) (Promega Corp., Madison, WI). Triplicate samples of 20, 50, or 100 ng of cDNA were amplified by PCR in the ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). Unlabeled PCR primer Taqman MGB probe sets (Applied Biosystems: “Assay-on-Demand” gene expression products) were obtained for metallothionein-1 (MT-1), adrenal secretory serine protease precursor, Enolases 1 α, neurturin (NTN), lingual lipase (RNLIIP), fetuin β, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to detect and quantify these sequences in RNA samples. The amplification reaction mixture (10 μL) contained cDNAs, primer-probe set, and Taqman Universal PCR Master Mix (Applied Biosystems). GAPDH was amplified separately as an endogenous control to normalize for variable amounts of cDNA in each sample. Dilutions (range, 200 ng to 10 pg) of cDNA samples prepared from total zinc-sufficient rat esophageal RNA were used to construct standard curves for candidate cDNAs and GAPDH amplifications (16). Expression results for all samples were normalized to GAPDH. Normalized expression results for zinc-deficient and zinc-replenished esophagi were calculated using zinc-sufficient esophagi as a
sufficient esophagi.

ZS in all experimental conditions ordered by their level of expression in zinc deficiency (ZS), ZD, zinc deficient. ZR8, ZR12, and ZR48 represent 8, 12, and 48 hours after zinc replenishment.

Confirmation of zinc modulation by Western blot analysis. Western blot analysis was used to confirm zinc modulation of high-mobility group protein 2 (HMGB2), carbonic anhydrase 2 (CAII), cyclin B (CCNB), and calponin 1 (CNN1). Briefly, proteins were extracted from esophageal epithelia pooled from three to five rats by homogenizing in buffer as previously described (16). Proteins (100 μg) were separated by 10% or 15% SDS-PAGE and electrophoretically transferred to nitrocellulose membranes. Blot analysis was used to confirm zinc modulation of high-mobility group protein 2 (HMGB2), rabbit polyclonal antisera against calponin 1 (CNN1). Immunodetection was done using the enhanced chemiluminescence method for Western blot detection (Pierce Biotechnology, Inc., Rockford, IL) followed by exposure to X-ray film. Band intensities were evaluated by the Image Station 440CF (Kodak, Rochester, NY). Jurkat cell lysate (BD Biosciences) and MCF-7 nuclear extract (Santa Cruz Biotechnology) were used as positive controls for CCNB and HMGB2, respectively.

Immunohistochemical localization of metallothionein and keratin 14. Immunohistochemistry was used to show spatial and temporal localization of metallothionein and keratin 14 (KRT14) in zinc-sufficient, zinc-deficient esophagi, and zinc-replenished esophagi. Following deparaffinization and rehydration in a graded alcohol series, esophageal sections were heated in citrate buffer (0.01 mol/L, pH 6.0) and nonspecific binding sites blocked with goat serum. Sections were incubated overnight at 37°C in a humidified chamber with mouse anti-KRT14 monoclonal antiserum (Clone LL002, Novocastra Laboratories, Newcastle upon Tyne, United Kingdom) at a 1:100 dilution, or mouse anti-metallothionein monoclonal antiserum (Clone E9, DakoCytomation, Carpinteria, CA) at a 1:50 dilution. Sections were then incubated with goat anti-mouse biotinylated secondary antibody (DakoCytomation) and streptavidin horseradish peroxidase (DakoCytomation). Staining of individual proteins was visualized by incubating sections with 3,3′-diaminobenzidine tetrahydrochloride and lightly counterstaining with hematoxylin.

Zinc determination. At sacrifice, blood was collected from the retro-orbital venous plexus of each animal after anesthesia with isoflurane; serum was prepared for zinc analysis by atomic spectrophotometry (9).

Results

Rat phenotypes. Weanling rats fed a zinc-deficient diet (3 ppm zinc) typically showed overt manifestation of zinc deficiency after 5 weeks, including retarded growth, loss of hair, and foci of alopecia, whereas pair-fed zinc-sufficient control rats showed only slowed growth (9). At sacrifice, zinc-sufficient, zinc-deficient, ZR8, and ZR12 rats had comparable body weights: 105 ± 13, 90 ± 11, 99 ± 8, 97 ± 13, respectively. However, ZR48 rats that received 2 days of zinc-sufficient diet after zinc treatment weighed significantly more than zinc-deficient rats (118 ± 12 versus 90 ± 11, P < 0.01).

We first determined the effect of an oral dose of zinc on serum zinc content (Fig. 2). Consistent with previous reports (16,24), serum zinc content (μg/100 mL ± SD) rose from a zinc-deficient level of 43 ± 14 to 727 ± 221 and 610 ± 255 at 8 and 12 hours after zinc replenishment, respectively. At 48 hours, the serum zinc level had decreased to 286 ± 55, approaching the level in zinc-sufficient rats. Although we did not determine the level of zinc in zinc-replenished esophagus, there is a correlation between zinc level in rat serum and esophagus.

Changes in esophageal gene expression due to zinc deficiency and restoration by zinc replenishment. To identify changes in esophageal gene expression that are responsive to chronic dietary zinc deficiency, we carried out gene expression profile analysis using oligonucleotide-based arrays (Uniset Rat 1 Bioarray, Amersham Biosciences). After array data processing, 33 genes exhibiting ≥2-fold differences in expression in precancerous zinc-deficient esophageal epithelium relative to control zinc-sufficient esophagus (Table 1). Among the 21 up-regulated genes, the apoptosis/proliferation/differentiation-related gene, MT-1, was unexpectedly up-regulated 7-fold in zinc-deficient esophagus relative to zinc-sufficient controls (Table 1), a finding opposite to published microarray and RT-PCR results for other tissues (liver, against carbonic anhydrase II (Abcam, Inc., Cambridge, MA; 1:10,000), goat polyclonal antiserum against HMGB2 (Santa Cruz Biotechnology, Santa Cruz, CA; 1:800 dilution), mouse monoclonal antiserum against calponin (Labvision, Fremont, CA; 1:500), or GAPDH as a loading and transfer control (Abcam Ltd, Cambridge, United Kingdom; 1:80,000 dilution). Immunodetection was done using the enhanced chemiluminescence method for Western blot detection (Pierce Biotechnology, Inc., Rockford, IL) followed by exposure to X-ray film. Band intensities were evaluated by the Image Station 440CF (Kodak, Rochester, NY). Jurkat cell lysate (BD Biosciences) and MCF-7 nuclear extract (Santa Cruz Biotechnology) were used as positive controls for CCNB and HMGB2, respectively.

A, quality of RNA samples submitted for oligonucleotide analyses. B, ordered list of 33 genes that are 2-fold up-regulated or down-regulated in all experimental conditions ordered by their level of expression in zinc sufficiency (ZS), ZD, zinc deficient. ZR8, ZR12, and ZR48 represent 8, 12, and 48 hours after zinc replenishment.
Figure 2. Serum zinc content (μg/100 ml) in zinc-deficient rats after oral zinc treatment. Zinc-sufficient control (ZS) and zinc-deficient (ZD). ZR8, ZR12, and ZR48 represent 8, 12, and 48 hours after zinc replenishment.

small intestine, pancreas, and kidney) from zinc-deficient rats, in which MT-1 was invariably down-regulated (20, 22, 23). Other up-regulated genes include tumor marker KRT14, G2-M-specific CCNB, acid-base balance molecule CAII, signaling intermediate molecules plasminogen activator inhibitor 2 type A (PAI2A), and aldoxe reductase-like protein, chromatin-associated transcription factor HMGB2, extracellular matrix protein Matrix Gla protein (MGP), cellular protein detoxification molecule, blemycin hydrolase, RNA binding molecule, nucleolar protein, and metabolism-related genes lysophospholipase and adrenal secretory serine protease precursor. The down-regulated genes include actin-cross linking regulator CNN1 (down 5-fold), signaling intermediate selenium binding protein 2 (SELENBP2), prosapoptotic gene tissue-type transglu-taminase (TG2M2), cell adhesion-related gene α-1 type 1 collagen, and metabolism-related genes RNLIP, cholesterol esterase, and steroid sulfatase.

Within 48 hours after zinc replenishment, the levels of gene expression of all 33 genes returned to near zinc-sufficient levels (Table 1; Fig. 1B). Among the 21 up-regulated genes, nine were expressed at zinc-sufficient levels (0.9- to 1.1-fold), eight were restored to 0.6- to 1.3-fold that of zinc-sufficient esophagi, and KRT14 and CAII were 1.8- and 1.6-fold above zinc sufficiency (ZR48 versus zinc sufficient). Only two genes, UV B radiation-activated genes and MT-1, still displayed a 3-fold increase above zinc-sufficient esophagi at 48 hours. In addition, MT-1, which is highly sensitive to dietary zinc status, showed a 26-fold increase at 8 hours that decreased to 11-fold at 12 hours and 3-fold by 48 hours. Among the 12 down-regulated genes, eight were restored to about the levels in zinc-sufficient esophagi by 48 hours (0.8- to 1.1-fold), CNN1 and NTN improved from <0.3-fold in the zinc-deficient state (zinc-deficient versus zinc-sufficient) to 0.6-fold (ZR48 versus zinc-sufficient), and fetuin β (FETUB) increased from 0.33-fold in the zinc-deficient state (zinc-deficient versus zinc-sufficient) to 1.7-fold at 48 hours (ZR48 versus zinc-sufficient). However, RNLIP remained at a pre-zinc-replenished low expression level of ~0.2 at 48 hours, after a transient surge in expression at 12 hours (1.9-fold, ZR12 versus zinc-sufficient).

Verification of changes in gene expression. We did quantitative RT-PCR for six selected genes from zinc-deficient, zinc-sufficient, and zinc-replenished esophagi to evaluate the reliability of the array results. Three up- (MT-1, adrenal secretory serine protease precursor, and Enolase 1 α) and three down-regulated genes (RNLIP, NTN, and FETUB) were chosen (Table 2). In general, the real-time data confirmed the expression changes on the arrays (Table 1), although in some cases with larger magnitudes. For example, quantitative RT-PCR data for MT-1 in zinc-deficient, ZR8, ZR12, and ZR48 esophagi, were 7-, 38-, 18-, and 2.8-fold increases above zinc-sufficient, respectively, compared with the array results of 7-, 26-, 12-, and 3-fold increases. Only in one case, real-time and array results were at odds: real-time PCR showed RNLIP improved from 0.06 (zinc-deficient versus zinc-sufficient) to ~0.6 (ZR12 versus zinc-sufficient and ZR48 versus zinc-sufficient) but on array, RNLIP increased from 0.2 (zinc-deficient versus zinc-sufficient) to 1.19 (ZR12 versus zinc-sufficient), but declined to 0.2 at 48 hours (ZR48 versus zinc-sufficient).

Immunoblot and immunohistochemistry analyses were used to determine if the zinc-elicited effects observed at the transcript level are reflected at the protein level. Based on commercial availability of antisera, three up-regulated (HMGB2, CAII, and CCNB) and one down-regulated gene (CNN1) were chosen for immunoblot analysis, and two up-regulated markers, metallothio-nein and KRT14, for immunohistochemistry. Consistent with array data (Table 1), the protein products of HMGB2, CAII, and CCNB were respectively, 8-, 9-, and 3-fold higher in zinc-deficient esophagi than zinc-sufficient controls; and a decline from the high zinc-deficient levels to near zinc-sufficient status was evident in all three within hours after zinc treatment (Fig. 3A-C and E-G). Also in agreement with array results (Table 1), the zinc-deficient CNN1 protein was 0.27-fold of CNN1 in zinc-sufficient controls, and a gradual increase was seen within hours after zinc replenishment (Fig. 3D and H).

Differential expression was confirmed for MT-1 and KRT14 by immunohistochemistry. In zinc-sufficient esophageal sections moderate nuclear staining of metallothionein was found in fewer than 5% of basal cells (Fig. 4A, inset). In contrast, the highly proliferative zinc-deficient esophagi displayed strong cytoplasmic as well as nuclear metallothionein staining in focal hyperplastic lesions (FHL) and >50% of basal and suprabasal cells throughout an entire esophageal section (Fig. 4B, inset). Within hours of zinc replenishment, metallothionein staining in the still proliferative esophagus of zinc-replenished rats showed a substantial increase in staining intensity and number of cells.
### Table 1. Regulation of gene expression in rat esophagus by dietary zinc deficiency and zinc replenishment

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<th>Function</th>
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**NOTE:** Red and green, respectively, >2-fold increase and decrease in gene expression in ZD versus ZS esophagi. Black, return to near ZS status in ZR48 esophagi 48 hours after an intragastric dose of zinc.

Abbreviations: ZD, zinc deficiency; ZR, zinc replenishment; ZS, zinc sufficiency.
Gene Expression Profiling of Precancerous Rat Esophagus

Discussion

This study shows that exposing rats to zinc-deficient conditions induced differential expression of 33 genes in the hyperplastic esophagus compared with control zinc-sufficient rats. Within 48 hours, zinc treatment reversed the precancerous phenotype and restored the aberrant expression of 29 of 33 (88%) genes to near zinc-sufficient status and reduced overexpression of UV B radiation-activated UV 103 gene and MT-1 from ~7- to ~3-fold that in control rats (Table 1). COX-2, a protein that is overexpressed in a variety of human cancers, including esophageal cancer (17–19), was not on the bioarray chip used here. COX-2 overexpression promotes cell proliferation and modulates apoptosis, cell adhesion, and angiogenesis thereby contributing to carcinogenesis (25). Our previous work showed that the hyperplastic esophagus and tongue of zinc-deficient rats expressed COX-2 protein and mRNA at 8- to 147-fold higher levels than control rats. Within hours, zinc replenishment reduced COX-2 overexpression and reversed the hyperplastic phenotypes (16). Thus, a select number of genes in the esophagus are induced or repressed during dietary zinc deficit but are restored during the rapid transition from zinc deficiency to sufficiency thereby reversing the precancerous environment in the zinc-deficient esophagus.

Among the overexpressed genes, we identified three, KRT14, MT-1, and CAII, with high levels of expression (>3.5-fold), which we believe could be potential predictors of esophageal cancer progression. KRT14 is a biomarker in human and rodent esophageal (Fig. 4P; refs. 12, 26–28) and lingual (Fig. 4R) carcinogenesis. Here we showed for the first time that KRT14 was highly expressed at both mRNA and protein levels in the precancerous esophagus, a phenotype induced solely by dietary zinc deficit. In addition, zinc treatment reversed the hyperplastic phenotype and restored KRT14 expression to near normal level to dietary zinc deficit. In addition, zinc treatment reversed the hyperplastic phenotype and restored KRT14 expression to near normal level to dietary zinc deficit. In addition, zinc treatment reversed the hyperplastic phenotype and restored KRT14 expression to near normal level to dietary zinc deficit. In addition, zinc treatment reversed the hyperplastic phenotype and restored KRT14 expression to near normal level to dietary zinc deficit. In addition, zinc treatment reversed the hyperplastic phenotype and restored KRT14 expression to near normal level 29 of 33 (88%) genes to near zinc-sufficient status and reduced overexpression of UV B radiation-activated UV 103 gene and MT-1 from ~7- to ~3-fold that in control rats (Table 1). COX-2, a protein that is overexpressed in a variety of human cancers, including esophageal cancer (17–19), was not on the bioarray chip used here. COX-2 overexpression promotes cell proliferation and modulates apoptosis, cell adhesion, and angiogenesis thereby contributing to carcinogenesis (25). Our previous work showed that the hyperplastic esophagus and tongue of zinc-deficient rats expressed COX-2 protein and mRNA at 8- to 147-fold higher levels than control rats. Within hours, zinc replenishment reduced COX-2 overexpression and reversed the hyperplastic phenotypes (16). Thus, a select number of genes in the esophagus are induced or repressed during dietary zinc deficit but are restored during the rapid transition from zinc deficiency to sufficiency thereby reversing the precancerous environment in the zinc-deficient esophagus.

Table 2. Real-time PCR verification for selected genes

<table>
<thead>
<tr>
<th>Group</th>
<th>GAPDH ng total esophageal RNA</th>
<th>Data expressed as a ratio to ZS group after normalization to GAPDH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MT-1</td>
<td>Enolase 1α</td>
</tr>
<tr>
<td>ZS</td>
<td>1.9 ± 0.09</td>
<td>1.0 (reference)</td>
</tr>
<tr>
<td>ZD</td>
<td>2.8 ± 1.03</td>
<td>7.0 ± 0.2</td>
</tr>
<tr>
<td>ZR8</td>
<td>3.5 ± 0.70</td>
<td>38 ± 0.3</td>
</tr>
<tr>
<td>ZR12</td>
<td>2.8 ± 0.97</td>
<td>18 ± 0.6</td>
</tr>
<tr>
<td>ZR48</td>
<td>2.3 ± 0.50</td>
<td>2.8 ± 0.03</td>
</tr>
</tbody>
</table>

NOTE: All results are presented as mean values from three to five esophagi.

Abbreviations: ZD, zinc deficiency; ZR, zinc replenishment; ZS, zinc sufficiency.

stained compared with zinc-deficient esophagus. For example, intense nuclear and cytoplasmic metallothionein staining covering many cell layers was evident in a proliferative ZR8 (Fig. 4C) and ZR12 esophageal section (Fig. 4D). However, 48 hours after zinc replenishment, the esophageal epithelium was mostly thinned (14, 16), and metallothionein expression was sporadic (Fig. 4E), with a staining pattern resembling that in control zinc-sufficient esophagi (Fig. 4A).

Cytoplasmic KRT14 staining in zinc-sufficient esophagus was moderate and restricted to basal cell layers (Fig. 4F, inset), but in the hyperplastic zinc-deficient esophagus, it was strong in many cell layers (Fig. 4K, inset), consistent with our previous reports (12, 16). Within hours after oral zinc administration, KRT14 staining was greatly reduced in intensity and extent, as shown in a ZR8 (Fig. 4L) and ZR12 esophagus (Fig. 4M). By 48 hours, the esophageal epithelium was mostly restored (14, 16), and KRT14 staining was weak and sporadic in basal and suprabasal cell layers (Fig. 4N).

Metallothionein and keratin 14 overexpression in lingual and esophageal squamous cell cancer from 4-nitroquinoline 1-oxide–treated zinc-deficient rats. We next investigated whether the expression of the two signature markers for precancerous esophagus, metallothionein and KRT14, correlated with prognosis in esophageal and lingual cancer, by doing immunohistochemistry on archival samples of esophageal and lingual SCC from zinc-deficient rats treated with the tongue carcinogen NQO (16). Esophageal and lingual SCC from zinc-deficient rats typically showed strong cytoplasmic and nuclear metallothionein staining (Fig. 4G, esophageal SCC; Fig. 4H, lingual SCC) and strong to intense cytoplasmic KRT14 staining in tumor areas (Fig. 4P, esophageal SCC; Fig. 4R, lingual SCC). By contrast, NQO-treated zinc-sufficient rats at end point showed moderate staining of metallothionein restricted to a few basal cells of the esophageal epithelia (Fig. 4F), but strong and extensive staining in basal cells and hyperplasia of lingual epithelia (Fig. 4H). Similarly, cytoplasmic staining of KRT14 was moderate in basal and outer cell layers of esophageal epithelia (Fig. 4O) but typically strong and extensive in basal cell layer and hyperplasia in lingual epithelia (Fig. 4Q).
basal cells (Fig. 4B versus A). These findings in zinc-deficient esophagus are the opposite of those reported for organs of absorption, storage, secretion, and excretion, such as small intestine, liver, pancreas, and kidney from growing and adult rats during dietary zinc deprivation. In these organs, depressed expression of metallothionein mRNA and protein is suggested as a marker for dietary zinc deficiency (20–23, 30–32). For example, expression of MT-1 mRNA in small intestine (20) and liver (23) of zinc-deficient rats was ~8- and ~20-fold lower than in control animals. Metallothionein staining was absent in zinc-deficient liver and weak and sporadic in zinc-deficient small intestine (31). Here we provide the first evidence that metallothionein overexpression is associated with hyperplasia in zinc-deficient esophagus, a result consistent with the known biological roles of metallothionein in cell proliferation, apoptosis, and differentiation (33).

In agreement with a previous report that MT-1 mRNA is short-lived and reaches maximum levels hours after zinc administration (34), we found that at 8 hours after intragastric zinc replenishment, esophageal MT-1 mRNA expression rose to ~26-fold and then decreased to ~12-fold at 12 hours and ~3-fold that in control rats by 48 hours (Tables 1 and 2). Similarly, metallothionein protein expression was enhanced in intensity and number of cells stained at 8 and 12 hours after zinc replenishment compared with zinc-deficient esophagus (Fig. 4C and D versus Fig. 4B) and decreased to

Figure 3. Confirmation of oligonucleotide array data by immunoblotting analysis for selected genes. Representative immunoblots. Each band represents a separate sample, pooled from three to five esophagi. A, HMGB2. B, CAIi. C, CCNB. D, CNN1. E-H, folds above zinc sufficiency (ZS) were calculated from the ratio of individual protein to GAPDH levels in esophagus. Positive controls for HMGB2 and CCNB were derived from MCF-7 nuclear extract (Santa Cruz Biotechnology) and Jurkat cell lysate (BD Biosciences), respectively. Zinc deficient (ZD). ZR2, ZR4, ZR8, ZR12, ZR24, and ZR48 represent 2, 4, 8, 12, 24, and 48 hours after zinc replenishment.
zinc-sufficient levels by 48 hours (Fig. 4E versus Fig. 4A). Notably, MT-1 alone among the 21 overexpressed genes shows a transient surge in expression after zinc administration (Table 1), consistent with its role in the regulation of zinc homeostasis (29).

There has been extensive interest in the role of metallothionein in carcinogenesis since an early report concerning its expression in human thyroid tumors (35). Metallothionein is overexpressed in a variety of human cancers, including SCC of the esophagus (36), tongue (37), and bladder (38). Metallothionein overexpression in ESCC is often positively correlated with the metastatic and proliferative activities of the cancer (36). In animal tumor models, overexpression of metallothionein has been shown in chemically induced rat and mouse bladder tumors (39, 40). Our results show that metallothionein overexpression occurs in precancerous zinc-deficient esophagus (Tables 1 and 2; Fig. 4B) and could be a good predictor of tumor progression, as illustrated by strong

**Figure 4.** Spatial and temporal localization of metallothionein and KRT14 protein in esophagus from zinc-modulated rats and in esophageal and lingual squamous cell carcinomas from NQO-treated zinc-deficient (ZD) rats. Representative immunohistochemically stained sections. A–I, metallothionein. J–R, KRT14. Top, metallothionein (A–I). Zinc-sufficient esophagus typically showed moderate nuclear metallothionein staining, restricted to a few cells in basal cell layer (A, inset). Conversely, zinc-deficient esophagus regularly displayed intense and abundant cytoplasmic and nuclear metallothionein staining, occurring in basal and suprabasal cell layers and FHLs (B, inset). 3,3′-diaminobenzidine tetrahydrochloride (dark brown). Within hours after zinc replenishment, an increase in intensity and extent of metallothionein overexpression was detected in ZR8 (C) and ZR12 (D) esophagi compared with ZD esophagus (B). By 48 hours, metallothionein staining was mostly weak and occurred only in a few cells in the nearly restored ZR48 epithelium (E). NQO-treated ZS rats typically showed sporadic but moderately strong nuclear metallothionein staining in basal cell layers of esophagus (F) and strong staining in basal cells and FHLs in tongue epithelium (H). In contrast, moderate to strong metallothionein staining was detected in basal cell layers (open arrows) and tumor areas (arrows) of NQO-treated zinc-deficient rats, exhibiting esophageal squamous cell carcinoma (G) and lingual squamous cell carcinoma (I). Bottom, KRT14 (J–R). KRT14 was weakly detected (3,3′-diaminobenzidine tetrahydrochloride, dark brown) in the cytoplasm of the basal cells of zinc-sufficient esophagus (J, inset) but strongly expressed in many cell layers of zinc-deficient esophagus, showing hyperplasia and FHLs (K, inset). Within hours after zinc replenishment, KRT14 expression was less in intensity and in the number of cells stained compared with zinc-deficient esophagus (K), ZR8 (L), and ZR12 (M) esophagi showed moderate basal and suprabasal staining, and ZR48 (N) very weak suprabasal and basal expression. KRT14 was strongly detected in ESCC (P) and lingual squamous cell carcinoma (R) from zinc-deficient rats exposed to NQO but moderately expressed in basal cell layers of esophagus (O) and tongue (Q) from similarly treated zinc-sufficient rats. ZR8, ZR12, and ZR48 represent zinc-replenished rats at 8, 12, and 48 hours after replenishment. Bar, 25 μm (A, B, D–G, and I) and 50 μm (C, H, and J–R).
Catalyze the reversible hydration of CO2 to form HCO3

Dietary zinc-deficient and esophageal hyperplasia participate in tumor growth (42).

Cells and a low acidic extracellular pH has been shown to enhance CAII in the zinc-deficient esophagus suggests that it may be involved in the regulation of pH homeostasis in these precancerous cells and a low acidic extracellular pH has been shown to enhance tumor growth (42).

The genes that showed decreased expression in association with dietary zinc-deficient and esophageal hyperplasia participate in a wide spectrum of functions, including stromal cell regulation, cell adhesion, cell death regulation, signal transduction, and metabolic regulation (Table 1). Two stromal or myoepithelial cell markers, α-1 type 1 collagen and CNN1 were expressed at 0.29- and 0.2-fold that in control rats and both increased to 0.6-fold after zinc treatment. CNN1 is a basic actin-binding protein of the calponin family and its major function is regulation of smooth muscle cell contraction. However, overexpression of CNN1 inhibits cell proliferation in cultured smooth muscle cells, fibroblasts, and tumor cells (47, 48). Interestingly, proteomic analyses of normal and malignant prostate tissue showed that calponin was lost during malignant transformation (49, 50). In addition, down-regulation of CNN3, an acidic member of the calponin family, was reported in human ESCC (28). At this point in our studies, it is not known if zinc modulation of calponin expression occurs in the precancerous epithelial or myoepithelial cells.

In summary, gene expression profiling showed that dietary zinc deficiency produces a unique profile in the precancerous esophagus, different from patterns reported for organs of storage or excretion, such as small intestine and liver (20–23), in which zinc deficiency does not cause preneoplasia. Zinc deficiency is implicated in the etiologies of human ESCC (3, 4). In particular, an association between zinc deficiency in humans and an increased risk of developing ESCC has recently been established (5). Here, in a zinc-deficient rat esophageal cancer model that mimics aspects of the human disease, we have successfully identified novel molecular markers KRT14, MT-1, CAII, and CNN1 in precancerous esophagus as candidates for early detection of ESCC in humans. Because these genes are responsive to zinc treatment they could also be used as markers in evaluation of chemoprevention strategies. More broadly, the data provide the beginnings of a molecular understanding of how zinc deficit promotes esophageal cancer progression.

Acknowledgments


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References


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