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Zinc Replenishment Reverses Overexpression of the Proinflammatory Mediator S100A8 and Esophageal Preneoplasia in the Rat

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Abstract

Background & Aims—Zinc-deficiency is implicated in the pathogenesis of human esophageal cancer. In the rat esophagus, it induces cell proliferation, modulates genetic expression, and enhances carcinogenesis. Zinc-replenishment reverses proliferation and inhibits carcinogenesis. The zinc-deficient rat model allows the identification of biological differences affected by zinc during early esophageal carcinogenesis.

Methods—We evaluated gene expression profiles of esophageal epithelia from zinc-deficient and replenished rats versus sufficient rats using Affymetrix Rat Genome GeneChip. We characterized the role of the top-upregulated gene S100A8 in esophageal hyperplasia/reversal and in chemically-induced esophageal carcinogenesis in zinc-modulated animals by immunohistochemistry and real-time quantitative polymerase chain reaction.

Results—The hyperplastic deficient esophagus has a distinct expression signature with the proinflammation-gene S100A8 and S100A9 upregulated 57- and 5-fold. “Response to external stimulus” comprising S100A8 was the only significantly overrepresented biological pathway among the upregulated genes. Zinc-replenishment rapidly restored to control levels the expression of S100A8/A9 and 27 other genes and reversed the hyperplastic phenotype. With its receptor RAGE, co-localization and overexpression of S100A8 protein occurred in the deficient esophagus that overexpressed NF-κB p65 and COX-2 protein. Zinc-replenishment but not by a COX-2 inhibitor reduced the overexpression of these 4 proteins. Additionally, esophageal S100A8/A9 mRNA levels were directly associated with the diverse tumorigenic outcome in zinc-deficient and zinc-replenished rats.

Conclusions—*In vivo* zinc regulates S100A8 expression and modulates the link between S100A8-RAGE interaction and downstream NF-κB/COX-2 signaling. The finding that zinc regulates an

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inflammatory pathway in esophageal carcinogenesis may lead to prevention and therapy for this cancer.

Introduction

Esophageal cancer, including esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAC), is the sixth most common cancer in the world.¹ ESCC is the predominant histological subtype. Because of an absence of early symptoms, patients with ESCC are frequently diagnosed at an advanced stage of the disease and, consequently have a poor prognosis. Despite advances in treatment protocols that combine surgery with radiotherapy and chemotherapy, the overall 5-year survival rate remains at ~10% for ESCC worldwide. In order to better prevent and treat this deadly cancer, it is critical to understand its biology and to discover novel early biomarkers for chemopreventive and therapeutic regimens.

Epidemiologic studies have implicated dietary zinc-deficiency (ZD) in the etiology of ESCC.^{2,3} Abnet et al.⁴ provided the strongest evidence of an association between dietary ZD and esophageal cancer in humans by establishing an inverse relationship between zinc concentration in biopsy samples from a high ESCC incidence area (Linxian, China) and subsequent risk of developing cancer.

We have developed a ZD rat tumor model that reproduces this feature of human ESCC. By causing unregulated cell proliferation and extensive genetic changes in the squamous epithelium, dietary ZD creates a protumorigenic microenvironment in the rat esophagus and tongue. As a result, ZD rats are exquisitely sensitive to *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal^{5,6} and 4-nitroquinoline 1-oxide (NQO)-induced lingual carcinogenesis.⁷ Zinc replenishment (ZR) rapidly re-establishes a normal phenotype by reversing cell proliferation, restoring normal gene expression, stimulating apoptosis and, thereby, inhibiting carcinogenesis.⁷⁻⁹

DNA microarray analysis provides a powerful tool to understand how zinc might modify genetic events in esophageal carcinogenesis. Using Bioarray chips with ~8,000 genes we previously showed that 33 genes were differentially expressed in hyperplastic ZD *versus* control zinc-sufficient (ZS) esophagus, including upregulation of the zinc-sensitive gene metallothionein-1.¹⁰ Although this pattern of gene expression is different from those reported for small intestine and liver in which ZD does not cause proliferative lesions,^{11,12} the mechanism by which ZD induces esophageal hyperplasia is not known. We hypothesized that global transcriptome profiling using an expanded genome array (Affymetrix) that analyzes >30,000 transcripts and variants from ~28,000 rat genes would identify key biologic differences affected by nutritional ZD during early esophageal carcinogenesis. Here, we evaluated gene expression profiles of esophageal epithelia from zinc-modulated rats and focused on the role of the proinflammation gene S100A8 (S100 calcium binding protein A8) in early esophageal carcinogenesis and its reversal.

Materials and methods

Animals

Weanling male Sprague-Dawley rats were from Taconic Laboratory (Germantown, NY). Custom-formulated ZD and ZS diets (Harlan Teklad, Madison, WI) were identical except for the zinc content.⁵

Experimental design

This study was approved by The Ohio State University Institutional Animal Care and Use Committee. Male weanling rats were fed a ZD diet *ad libitum* (n=24) or pair-fed a control ZS diet (n = 12). After 6 weeks ZD rats evidenced increased cell proliferation in the esophagus, as assessed by increased expression of proliferating cell nuclear antigen PCNA.⁵ Zinc gluconate (1.0 mg elemental zinc) in saline was then administered intragastrically to 12 ZD rats, which were immediately switched to the ZS diet to form the ZR group. At 48 hours after replenishment, all animals (ZD, ZR, and ZS; n = 12/group) were killed. Whole esophagi (n = 4/group) were fixed in buffered formalin and embedded in paraffin. Esophageal epithelia prepared by using a blade (under microscopic guidance) to remove the submucosal and muscularis layers were snap-frozen in liquid nitrogen and stored at -80°C for RNA (n = 4/group) and protein (n = 4/group) preparation^{6, 7, 10}.

RNA preparation

Total RNA was prepared using TRIZOL reagent (Invitrogen, Carlsbad, CA). All RNA samples displayed intact ribosomal RNA 18S and 28S bands as determined by the Agilent 2100 Bioanalyzer.

Gene expression profiling

Microarray analysis was conducted on individual RNA samples from ZS, ZD, and ZR rats (n = 4/group) using Rat Genome 230 2.0 GeneChip (Affymetrix, Santa Clara, CA), containing more than 31,000 probe sets that analyze >30,000 transcripts and variants from ~28,000 rat genes. Five micrograms of total RNA was reverse transcribed into cDNA followed by *in vitro* transcription and labeling to produce biotin-labeled cRNA (IVT Labeling Kit). The labeled cRNA was hybridized to the array, followed staining with streptavidin-phycoerythrin. The distribution of fluorescent material on the oligonucleotide probes sets was obtained using GeneArray Scanner.

Expression data analysis

The Significance Analysis of Microarrays software (SAM, version 3.0) was used to identify differentially expressed mRNAs following normalization of array data by the BRBArrayTools software (Biometric Research Branch, National Cancer Institute). Based on the False Discovery Rate, the cutoff for significance was set so that the estimated q-value was between zero and 5%.¹³ The data were submitted to Array Express (Accession number: ETABM-504).

Pathway analysis of expression data

The Gene Ontology database was used to categorize genes into functional groups. The Expression Analysis Systematic Explorer (EASE) software that uses Fisher's exact test statistics (EASE score) for pathway analysis was employed to determine the probability of observing the number of genes within a list of interest *versus* the number of gene in each category on the array.¹⁴

Real-time quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

To confirm the differential expression for selected genes, qRT-PCR was performed on the same RNA samples used for array analyses, using pre-designed probes, GAPDH as the normalizer, and the comparative C_t method. The analysis used the ABI Prism 7900HT Sequence detection system (Applied Biosystems, Foster City, CA).

Immunoblotting analysis

Immunoblotting analysis was performed on all dietary groups (n=4/group) as described.⁷ Membranes were probed with goat polyclonal against S100A8, goat polyclonal against PTGES, and mouse monoclonal against SERPINB3 (Santa Cruz Biotechnology, Santa Cruz, CA), chicken IgY against DNMT3A (Abcam, Cambridge, MA), mouse monoclonal against PPP2R1a (Upstate), or mouse monoclonal against CSRP3 (Genway, San Diego, CA). GAPDH (Calbiochem, San Diego, CA) was used as a loading control. Band intensities were evaluated by GS800 calibrated densitometer (Bio-Rad, Hercules, CA).

S100A8 and RAGE immunohistochemistry (IHC)

Following deparaffinization and antigen retrieval, esophageal sections were incubated with mouse anti-S100A8 monoclonal antiserum (BMA Biomedicals, Augst, Switzerland) or mouse anti-RAGE monoclonal antiserum (R&D Systems, Minneapolis, MN) followed by incubation with appropriate biotinylated secondary antibodies and streptavidin horseradish peroxidase.⁷ Protein was localized by incubation with 3-amino-9-ethylcarbazole substrate-chromogen (AEC) (Dako Corporation, Carpinteria, CA).

The percentage of cells positive for S100A8 was determined as follows: 0 = 0%-5%, 1 = 6%-25%, 2 = 26%-50%, 3 = 51%-75%, and 4 = 76%-100%. The intensity of S100A8 staining was graded as follows: 0 = none, 1 = weak, 2 = moderate, and 3 = intense. An immunoreactive score was calculated by multiplying the grade of percentage of positive cells by the grade of intensity of staining.

Esophageal S100A8/A9 mRNA expression during carcinogenesis

S100A8/A9 mRNA expression was determined by qRT-PCR in esophagi from 6 ZD and 6 ZS rats at 0 hour (before NMBA dosing) and from zinc-modulated rats at 5 and 15 weeks after NMBA treatment in a tumorigenesis experiment. Here, 120 ZD and 60 ZS rats were given their respective diets for 6 weeks to establish increased esophageal proliferation in ZD rats (0 hour). All rats were then administered an intragastric dose of NMBA (2 mg/kg body weight). Immediately thereafter, 60 ZD rats were switched to a ZS diet to form the ZR group while the remaining ZD and ZS rats continued on their diets. Intragastric NMBA was administered again at week 6 and 12. At 0 hour and at various times after the first dose, 8 rats per group were killed for esophageal tissue isolation. All remaining rats were killed at 15 weeks for tumor incidence analysis.

Serum zinc measurement

Serum zinc analysis was by atomic absorption spectrometry.⁵

Statistical Analysis

Data on serum zinc and mRNA levels, tumor multiplicity were analyzed by one-way analysis of variance (ANOVA). Tumor incidence differences were by Fisher's exact test. Statistical tests were two-sided and were considered statistically significant at $P < .05$.

Results

Zinc replenishment reverses the hyperplastic esophageal phenotype

The effect of zinc treatments on esophageal histopathology and serum zinc levels was first determined. After 6 weeks of experimental diets, ZD rat esophageal epithelia were highly hyperplastic, as compared with control ZS mucosa (typically 2 to 3 cells thick). At 48 hours after intragastric zinc, ZR mucosa had thinned to a thickness of 3 to 5 cells (Figure 1D). Serum zinc levels ($\mu\text{g}/100 \text{ ml}$) in ZD rats were one-third those in control ZS rats (41 ± 8 versus 112

± 12 ; $P < .001$). Forty-eight hours after ZR, serum zinc contents in ZR rats rose from a ZD level of 41 ± 8 to 188 ± 36 ($P < .001$).

A distinct gene expression signature in ZD esophagus

A hierarchical clustering analysis of 30,000 transcripts showed that the gene expression patterns between ZD and ZS esophageal epithelia were distinct (Figure 1A). Using a cutoff point of q value $< 30\%$ and ≥ 2 -fold difference, 2305 dysregulated probe sets were identified in the hyperplastic ZD *versus* ZS esophagus (1060 up and 1245 downregulated; Supplementary Table 1). The present data included 8 (5 up: CA2, SCD1, ENO1, CNNB, and Lypla2; and 3 down: FETUB, STS, and GPLD1) of the 33 genes identified in the previous microarray analysis by the Rat 1 Bioarray chip.¹⁰

By filtering the data using a cutoff of q value $< 5.0\%$ and ≥ 4 -fold differences in expression levels, a group of 103 differentially expressed genes were identified in ZD esophagus (61 up and 42 down, Table 1), thereby revealing a distinct and novel molecular signature as compared with ZS esophagus (Figure 1B). Three of these genes (2 up- CA2 and SCD1, and 1 downregulated FETUB) were previously reported in ZD esophagus,¹⁰ the remaining 100 genes represent new biomarkers associated with esophageal hyperplasia.

The most upregulated genes were S100A8 (up 57-fold), SERPINB3, IFIT2, TMPRSS11D, IFIT3, and SECTM1 with fold-changes ranging from 57- to 13-fold; and the most downregulated genes were P2RX2, SLC15A1, GOS2, TRDN, KBTBD10, and CABP1 with fold-changes ranging from -12.5 to -7.7-fold (Figure 1C, scatter plot). At 48 hours after ZR, the altered expression of 14 genes, including P2RX2, SLC15A1, KLK6, PRSS35, SFTPC, DMNT3A, GDF15, CFTR, AREG, IFIT3, SCGB1A1, PTGES, GADD45A, and SERPINB3 returned to control levels of ZD/ZS ~ 1.0 (Figure 2A). The abnormal expression levels of another 15 genes, including the top-upregulated S100A8 (Figure 2A) and S100A9, RHOV, HPGD, OCM, IVL, FETUB, FXD4, IFIT2, PLAT, GOS2, MYL2, ACTC1, IGSF4A, CPT1A (data not shown) were reverted to a fold-change level of ZD/ZS ~ 2.0 . Importantly, these data show that 29 of 103 genes (28%) are highly regulated by zinc nutrition and the altered and corrected expression is associated with induction and reversal of esophageal hyperplasia.

Identification of overrepresented pathways

EASE pathway analysis program¹⁴ was used to identify biological pathways overrepresented among these 103 genes (Table 2). Among the upregulated genes, "response to external stimulus" pathway comprising S100A8 and 6 genes (CDKN1A, GJA1, MX2, PPP2R1A, SECTM1, and SERPINB3) was the only significantly overrepresented pathway (EASE score = .02), a novel result that supports S100A8 as a relevant marker in ZD-induced esophageal hyperplasia. Among the downregulated genes, EASE uncovered an intriguing correlation (EASE score $< .001$) between the genes associated with muscle contraction/development pathway (10 genes: ATP2A1, CKM, CRYAB, MYBPC1, MYH8, MYL3, MYH6, SMPX, TNNT3, and TRDN) and organogenesis pathway (7 genes: CRYAB, MYBPC1, MYH6, MYH8, MYL3, PCP4, and TNNT3) and esophageal hyperplasia. This finding is consistent with a microarray analysis of mouse skin carcinogenesis in which EASE indicated a correlation between muscle-associated genes and skin differentiation.¹⁵

Validation of expression data

The real-time qRT-PCR data verified the expression changes on the arrays, although with larger or smaller magnitudes (Figure 2B). As an example, qRT-PCR data for S100A8 in ZD and ZR esophagi were increased 77- and 12-fold above ZS, as compared with the array results (57- and 2.4-fold increase). qRT-PCR data for S100A9 (not shown in Figure 2B) in ZD esophagus was 3.1-fold above ZS as compared with the array data of 5-fold.

The protein products of the upregulated genes S100A8, SERPINB3, DNMT3A, PTGES, and PPP2R1A were 14.7-, 15.5-, 3.5, 4.2, and 2-fold higher in ZD than ZS esophagi, respectively. The protein product of the downregulated gene CSRP3 was 0.23-fold of ZS controls (Figure 2 C-D). ZR significantly reduced the abnormal protein expression of S100A8, SERPINB3, and PTGES, a result indicating that the zinc-modulating effects observed at the transcript level were reflected at the protein level for these genes.

In vivo link between S100A8-RAGE interaction and NF-κB p65 - COX-2 protein expression

We focused our study on the top-upregulated gene S100A8 (up 57-fold) because of its role in inflammation and cancer. Interaction of S100A8/S100A9 ligands with their receptors triggers intracellular signaling cascades, including NF-κB signaling.¹⁶ S100A9 (up 5-fold) was not selected to complement IHC studies for S100A8, because an appropriate antibody was not available.

We determined whether a link occurs between S100A8 overexpression and downstream NF-κB signaling in the earliest stage of esophageal carcinogenesis. S100A8 and its putative receptor RAGE (receptor for advanced glycation end products) protein expression were analyzed by IHC in near serial sections from archived esophageal tissues that had documented co-overexpression and co-reduction of COX-2 (cyclooxygenase-2) protein⁷ and the transcription factor NF-κB p65⁸ under identical ZD and ZR conditions; NF-κB controls the expression of the inducible enzyme COX-2. IHC was performed on esophageal tissue samples from 11 ZD, 10 ZR, and 6 control ZS rats.

ZS esophageal epithelia showed sporadic and weak cytoplasmic staining of S100A8 (Figure 3Q). In contrast, the hyperplastic ZD esophageal epithelia displayed frequent and intense cytoplasmic staining with notable spatial patterns. As examples, intense S100A8 staining was observed in many cell layers throughout the entire esophageal section (Figure 3A and E [higher magnification]) and in bands that alternated with zones of no expression (Figure 3I). As early as 8 hours after zinc treatment, S100A8 expression in the still proliferative ZR esophagus showed a substantial reduction in staining intensity and number of cells stained (Figure 4A3 *versus* 3A). By 48 hours S100A8 expression was weak and diffuse (Figure 3M) in the thinned ZR esophageal epithelia. The semi-quantitative mean immunoreactive scores for ZS, ZD, and ZR esophageal epithelia were 1.3 ± 0.4 , 7.8 ± 0.6 , and 3.4 ± 0.7 , respectively, translating to a ~6-fold increase in ZD *versus* ZS esophagi and a 2.7-fold increase in ZR *versus* ZS esophagi. These data are consistent with those of immunoblot analysis in which the 14.7-fold increase of S100A8 expression in ZD esophagi was reduced to 2.5-fold that of ZS esophagi following ZR (Figure 2C and D).

RAGE the putative receptor for S100A8 displayed a spatial pattern similar to S100A8. Parallel to S100A8 (Figure 3Q), RAGE protein expression was very low in ZS esophageal epithelia (Figure 3R). Under ZD conditions, RAGE was co-overexpressed with S100A8 in near serial esophageal tissue sections (Figure 3B, F, and J *versus* A, E, and I). Upon ZR, RAGE expression was concomitantly co-reduced with S100A8 (8 hours after ZR, Figure 4A4 and A3; 48 hours after ZR, Figure 3N and M). These data showed that co-overexpression of S100A8 and RAGE protein occurs during early esophageal carcinogenesis and is reduced upon ZR. Thus, *in vivo* zinc regulates RAGE protein expression in the same manner as S100A8.

Importantly, our IHC data revealed that the spatial and temporal localization of S100A8 and RAGE protein expression mirrored that of NF-κB p65 and COX-2.^{7, 8} For the purpose of comparison, our published IHC data on NF-κB p65 and COX-2 expression are presented (NF-κB p65, Figure 3C, G, K, O, and S; COX-2, Figure 3D, H, L, P, and T). In normal ZS esophagus, all 4 proteins showed very low levels of expression (Figure 3, Q-T). Under ZD conditions all 4 proteins were overexpressed. They exhibited a strikingly similar spatial pattern in

hyperplastic esophageal epithelia (Figure 3, A-D, E-H, and I-L). Within hours after ZR, the expression of all 4 proteins was concomitantly reduced (Figure 3, M-P). These results demonstrate an *in vivo* link between S100A8-RAGE co-overexpression and downstream NF- κ B-COX-2 signaling that is modulated by zinc nutrition.

Effect of COX-2 inhibitor on S100A8/RAGE protein expression in ZD esophagus

The ability was determined of the COX-2 inhibitor celecoxib to inhibit S100A8/RAGE overexpression events upstream of COX-2. IHC was performed on archived esophageal samples from ZD rats that were treated with an oral dose of celecoxib.⁷ Within hours ZR reduced S100A8 and RAGE overexpression in the still hyperplastic esophagus (Figure 4A3 and A4), celecoxib had little effect in curbing S100A8 or RAGE overexpression in hyperplastic ZD esophagus (Figure 4A1 and A2). The result provides an explanation for the inefficacy of celecoxib in UADT cancer prevention in ZD animals.⁸

S100A8/A9 mRNA expression levels correlate with esophageal tumor outcome

The influence of dietary zinc on esophageal S100A8/A9 mRNA expression was further defined by qRT-PCR analysis in a tumorigenesis study (Figure 4B). Consistent with the array data (Table 1), hyperplastic ZD esophagi had significantly higher S100A8 ($P<.001$) and S100A9 ($P<.01$) mRNA levels than ZS esophagi. Importantly, S100A8/A9 mRNA overexpression in ZD esophagi was sustained during esophageal carcinogenesis (5 and 15 weeks after NMBA dosing) and was associated with a high tumorigenic outcome in ZD versus ZS rats (tumor incidence, 100% *versus* 16.6%, $P=7.8\times 10^{-6}$; multiplicity, 11 ± 3.8 *versus* 0.5 ± 0.3 , $P<.001$). Replenishing zinc led to reduced S100A8/A9 mRNA levels at both time points and a low tumorigenic outcome in ZR versus non-replenished ZD rats (incidence, 100% *versus* 28.9%, $P=5.0\times 10^{-5}$; multiplicity, 11 ± 3.8 *versus* 0.6 ± 0.4 , $P<.001$). Thus, esophageal S100A8/A9 mRNA levels were regulated by zinc and were directly associated with the risk of developing tumors in zinc-modulated rats.

S100A8 protein expression in human and mouse ESCC

Finally, we investigated whether expression of the marker S100A8 identified in the hyperplastic ZD rat esophageal mucosa was overexpressed in human and mouse esophageal ESCC. IHC was performed on 16 human ESCC and 5 mouse ESCC from NQO-treated ZD:p53 +/- mice.¹⁷

In human ESCC, cytoplasmic S100A8 immunostaining was observed in all 16 samples with varying degree of intensities. As examples: 5 showed moderate staining (+2; Figure 5C), 8 had strong staining in tumor tissues (+3; Figure 5A and B), and 3 had weak staining (+1; data not shown). Additionally, strong S100A8 immunostaining was detected in tumor stroma (Figure 5A-C; arrows). Adjacent normal esophageal epithelium showed very weak staining of S100A8 (Figure 5D).

ESCC from NQO-treated, zinc-deficient p53 +/- mice showed strong cytoplasmic immunostaining of S100A8 protein in invasive tumor areas (Figure 5E and F). By contrast, carcinoma free control zinc-sufficient p53 +/- esophagus displayed weak staining (data not shown).

Discussion

The present expression profiling analysis shows that the hyperplastic ZD rat esophagus has a distinct and novel molecular signature (Figure 1B). Importantly, the proinflammation gene S100A8 (up 57-fold, q value = .0%) and its heterodimer S100A9 (up 5-fold, q value = .4%) and 29 other genes were highly zinc-responsive. Upon ZR their altered expression rapidly

reverted to control levels, accompanied by the reversal of the hyperplastic phenotype. S100A8 together with 6 other genes are members of the only significantly overrepresented “response to external stimulus” biological pathway (EASE score = .02, Table 2) among the upregulated genes. This result suggests that S100A8 is a relevant marker belonging to a causal pathway that drives esophageal cell proliferation rather than simply an epiphenomenon of this process or of nutritional zinc deficit.

S100A8 and S100A9 encode the S100 family member of calcium binding proteins that also bind zinc. Originally discovered as immunogenic protein expressed and secreted by neutrophils, S100A8/A9 have emerged as important mediators in inflammation. As a result, they may play a key role in inflammation-associated cancers.¹⁶ Indeed, S100A8/A9 are overexpressed in a variety of human cancers,¹⁶ including skin SCC¹⁸ and Barrett's esophagus, a precancerous condition of EAC.¹⁹ In ESCC conflicting cDNA microarray data have been published. S100A8 downregulation was reported in a Chinese population,²⁰ whereas upregulation was noted in an Indian population.²¹

Here in a tumorigenesis experiment (Figure 4B), we provide evidence to show that a ZD diet induces and maintains S100A8/A9 mRNA overexpression before and during NMBA-induced esophageal carcinogenesis. Switching to a ZS diet during carcinogenesis leads to substantially reduced S100A8/A9 mRNA levels in ZR animals. Importantly, the diverse tumorigenic outcome in ZD and ZR animals are directly associated with their elevated and reduced S100A8/A9 mRNA levels during carcinogenesis. The data establish for the first time that zinc regulates S100A8/A9 mRNA expression in vivo in esophageal epithelial cells, thereby influencing the risk for developing cancer.

RAGE is the putative receptor of these two proteins. *In vitro* engagement of RAGE by S100A8/A9 activates NF-κB signaling in various cells and tumor cells.^{22, 23} In vivo in a multistage mouse skin carcinogenesis model, Gebhardt et al.²⁴ provided direct genetic evidence that S100A8/A9 binds to RAGE, and RAGE signaling mediates sustained skin inflammation and promotes tumor development. Blockade of RAGE suppresses tumor growth and metastasis.^{24, 25} Here we showed that RAGE was co-overexpressed with S100A8 in the hyperplastic ZD esophageal epithelia that evidenced overexpression of NF-κB p65 and COX-2 proteins. ZR reduced the overexpression of all four proteins, thereby suppressing RAGE/S100A8 signaling, and reversed esophageal hyperplasia (Figure 3). These data demonstrate in vivo that zinc regulates the proinflammatory mediator S100A8 and modulates its interaction with RAGE and the downstream NF-κB-COX-2 signaling pathway, providing the first evidence for an inflammation-modulating role of zinc in early esophageal carcinogenesis and its reversal. Consistent with this finding, NF-κB is recognized as a link between inflammation and cancer development/progression. The IκB kinase/NF-κB activation pathway is a target for cancer prevention.²⁶ In vivo zinc supplementation in humans led to downregulation of inflammatory cytokines and inhibition of induced NF-κB activation.²⁷ In vitro zinc supplementation also inhibited NF-κB activation and suppressed the tumorigenic potential of human prostate cancer cells.²⁸ Our data show that the protumorigenic and antitumorigenic effects of zinc may well operate through activation and suppression of S100A8-RAGE interaction and downstream NF-κB signaling.

Other classes of genes common among the upregulated genes include those associated with immune responses, apoptosis, cell proliferation and regulation of transcription (Table 1). Many of these genes are zinc-responsive and their roles in esophageal carcinogenesis are unclear. As examples, the highly upregulated gene SERPINB3 is overexpressed in several human cancers, including head and neck SCC.²⁹ The CDKN1A gene that encodes the cyclin-dependent kinase inhibitor p21 has divergent roles in tumorigenesis. CDKN1A overexpression correlates with chromosomal instability and is an adverse prognostic predictor for patients with ESCC.³⁰

Another upregulated gene, PTGES, the eicosanoid pathway enzyme that converts PGH₂ to PGE₂, is elevated in various types of cancer, and is often associated with increased expression of COX-2.³¹ Importantly, a similar association between PTGES and COX-2 overexpression is found in the ZD esophagus.

The finding here that the de novo methyltransferase gene DMNT3A is zinc-responsive suggests that DNA methylation³² may be a mechanism for transcriptional gene silencing of candidate genes in ZD esophagus. Thus, the downregulated gene G0S2 is epigenetically silenced through promoter hypermethylation in primary head and neck SCC development.³³ Loss of the structural protein UPK1B expression in bladder carcinoma is attributed to methylation of a CpG island within the UPK1B promoter³⁴ and hypermethylation of TPM1 gene is a mechanism for TPM1 silencing in breast cancer cell lines.³⁵ Together, this study has identified relevant genetic changes, induced by zinc-deficiency, that may act to produce the protumorigenic microenvironment in the ZD esophagus that favors carcinogenesis.

The role of zinc-deficiency as a cause of disease and as a determinant factor in the progression of disease is gaining attention.³⁶ This concept is based on the many biological functions of zinc. Zinc is an essential nutrient. Zinc plays an important role in the immune system. There are more than 300 zinc-containing enzymes. Zinc ions are key structural components of nearly 2000 proteins that are mainly nuclear transcription factors, which contribute to control of cell proliferation, differentiation, and apoptosis through regulation of gene expression.³⁷ It is now established that zinc deficiency induces oxidative stress and activates/inhibits oxidant-sensitive transcription factors that can affect cell function, proliferation and survival, thereby predisposing to disease.³⁸ Interestingly, S100A8 was strongly induced in mouse keratinocytes in response to UV-induced oxidative stress.³⁹ It is not known, however, whether oxidative stress induced by zinc deficiency would lead to S100A8 induction in the ZD esophagus. Given the recent interest in the idea of an association of inflammation and the genesis and perpetuation of cancer,^{40, 41} the finding here that zinc regulates a key inflammation pathway in esophageal carcinogenesis provides a new understanding of the role of zinc in esophageal cancer initiation, progression, and prevention.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

S100A8, S100 calcium binding protein A8
 RAGE, receptor for advanced glycation end products
 COX-2, cyclooxygenase-2
 UADT, upper aerodigestive tract cancer
 ZD, zinc- deficiency
 ZR, zinc-replenishment
 ZS, zinc-sufficiency
 NF-κB, nuclear factor-κB
 SCC, squamous cell carcinoma
 PCNA, proliferating cell nuclear antigen
 EASE, Expression Analysis Systematic Explorer

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Figure 1.

Expression profiling of zinc-modulated rat esophageal mucosa. (A) Dendrogram illustrating clustering of 30,000 transcripts in ZD, ZR, and ZS esophagi. (B) 103 genes in ZD versus ZS esophagi (fold-change ≥ 4). (C) Scatterplot of ZD versus ZS esophagi. A-C, red and green denote up- and down-regulated genes; $n = 4$ /group. (D) Hematoxylin and eosin-stained sections of normal ZS, hyperplastic ZD, and restored ZR esophagus; scale bar = 25 μ m.

Figure 3.

Spatial and temporal localization of S100A8 and RAGE protein (AEC, red) in near serial sections of archived esophageal tissues from zinc-modulated rats that overexpressed NF- κ B p65 and COX-2 (3,3'-diaminobenzidine tetrahydrochloride, brown). S100A8 (A, E, and I) and RAGE (B, F, and J) were intensely and abundantly coexpressed in ZD esophagus, and occurred in similar spatial patterns as those of NF- κ B p65 (C, G, and K: reproduced from *Int J Cancer*, 2008; 122:978-989) and COX-2 (D, H, and L: reproduced from *J Natl Cancer Inst* 2005;97:40-50). 100A8 (M) and RAGE (N) expression was greatly reduced at 48 hours after ZR, as was NF- κ B p65 (O) and COX-2 (P). ZS esophagi showed sporadic and weak expression (arrows) of S100A8 (Q) and RAGE (R) and weak or absent staining of NF- κ B p65 (S) and COX-2 (T). Scale bars: 100 μ m (A-D and Q-T); 50 μ m (I-P); 25 μ m (E-H, bracketed area in A-D).

Figure 4.

(A) Effects of celecoxib and zinc on S100A8 and RAGE protein expression. S100A8 and RAGE protein (AEC, red) immunostaining was intense in hyperplastic ZD esophagi at 8 hours after celecoxib treatment (A1, A2) but sporadic/weak after zinc treatment (A3, A4). Scale bars: 50 μ m. (B) Correlation of S100A8/A9 mRNA expression levels (normalized to GAPDH; values are mean $\pm$ SEM; n = 6-8/group) with esophageal tumorigenic outcome in NMBA-treated zinc-modulated rats. S100A8 (ZD versus ZS: 0 hour, 5 and 15 wk; ZD versus ZR: 5 and 15 wk, $P < .001$). S100A9, (ZD versus ZS: 0 hour, 5 and 15 wk; ZD versus ZR: 5 and 15 wk, $P < .01$). Inset: Tumor incidence: ZD [100%] versus ZS [16.6%], $P = 7.8 \times 10^{-6}$; ZD [100%] versus ZR

[28.9%], $P = 5.0 \times 10^{-5}$. Multiplicity: ZD [11 ± 3.8] *versus* ZS [0.5 ± 0.3] and ZD [11 ± 3.8] *versus* ZR [0.6 ± 0.4], $P < .001$.

Figure 5.

S100A8 protein expression in human (A-D) and mouse (E-F) ESCC. Panels A-C show moderate to intense S100A8 immunostaining (AEC, red) in tumor tissues and stroma (arrows), and D shows very weak staining in adjacent near-normal esophageal mucosa in an ESCC section. Panels E-F are ESCC from NQO-treated zinc-deficient p53^{+/-} mice. Intense (E) and moderately strong (F) cytoplasmic S100A8 staining occurred in invasive tumor areas and stroma (arrows). Scale bars: 25 μ m (A-D, F) and 50 μ m (E).

Table 1
Gene expression signature in precancerous zinc-deficient rat esophagus

Affymetrix id	Gene Symbol	Gene Title	Fold-Change ZD versus ZS	Score	q-value (%)	Function
Sixty-one up-regulated genes associated with dietary ZD						
1368494_at	S100A8	S100 calcium binding protein A8 (calgranulin A)	57	8.7	0	chemotaxis
1384973_at	SERPINB3	serpins protease inhibitor B3	56	8.0	0	serine-type endopeptidase inhibitor activity
1379568_at	IFIT2	interferon-induced protein with tetratricopeptide repeats 2	16	8.6	0	binding
1387620_a_at	TMPRSS11D	transmembrane protease, serine 11d	16	8.9	0	proteolysis
1376908_at	IFIT3	interferon-induced protein with tetratricopeptide repeats 3	15	3.9	0.6	binding
1376976_at	SECTM1	secreted and transmembrane 1B	13	7.9	0	immune response
1379747_at	PRSS35	Protease, serine, 35	12	8.9	0	proteolysis
1367800_at	PLAT	plasminogen activator, tissue	10	5.7	0.1	protein modification process
1367733_at	CA2	carbonic anhydrase 2	9.5	7.4	0	kidney development
1369202_at	MX2	myxovirus (influenza virus) resistance 2	8.7	3.0	2.0	defense response
1386889_at	SCD2	stearoyl-Coenzyme A desaturase 2	8.4	3.6	0.9	lipid metabolic process
1369871_at	AREG	amphiregulin	8.3	5.4	0.2	epidermal growth factor receptor signaling pathway
1367566_at	SCGB1A1	secretoglobulin, family 1A, member 1 (uteroglobulin)	8.2	4.9	0.3	signal transduction
1371100_at	ES2	esterase 2	7.8	2.9	2.4	carboxylesterase activity
1370355_at	SCD1	stearoyl-Coenzyme A desaturase 1	7.6	6.8	0	lipid metabolic process
1384960_at	CFTR	cystic fibrosis transmembrane conductance regulator homolog	7.4	8.6	0	transport
1392678_a_at	DNMT3A	DNA methyltransferase 3A	7.2	3.1	2.0	DNA methylation
1369640_at	GJA1	gap junction membrane channel protein alpha 1	6.7	4.4	0.5	in utero embryonic development
1387391_at	CDKN1A	cyclin-dependent kinase inhibitor 1A	6.6	5.3	0.2	regulation of progression through cell cycle
1380083_at	KLK10	kallikrein 10	6.5	4.8	0.3	proteolysis
1381024_at	PPP2R1A	protein phosphatase 2 (formerly 2A)	6.4	5.3	0.2	protein phosphatase type 2A activity
1395886_at	ACTR3	ARP3 actin-related protein 3 homolog	6.3	3.4	1.2	structural constituent of cytoskeleton

Affymetrix id	Gene Symbol	Gene Title	Fold-Change ZD versus ZS	Score	q-value (%)	Function
1391481_at	CAST	Calpastatin	6.2	6.0	0.1	myoblast fusion
1380533_at	APP	amyloid beta (A4) precursor protein	6.2	3.0	2.0	G2 phase of mitotic cell cycle
1369248_a_at	BIRC4	baculoviral IAP repeat-containing 4	6.2	6.5	0	apoptosis
1368025_at	DDIT4	DNA-damage-inducible transcript 4	6.2	9.4	0	response to hypoxia
1368325_at	EGF	epidermal growth factor	6.1	5.7	0.1	activation of MAPKK activity
1397675_at	WBSR1	Williams-Beuren syndrome chromosome region 1 homolog	5.8	4.7	0.4	translation
1370153_at	GDF15	growth differentiation factor 15	5.6	3.5	1.1	signal transduction
1384784_at	CLCA2	chloride channel calcium activated 2	5.5	4.2	0.5	translation
1369846_at	IVL	involucrin	5.3	3.5	1.1	keratinocyte differentiation
1398143_at	PRKX	protein kinase, X-linked	5.2	3.7	0.7	protein amino acid phosphorylation
1370627_at	RHOV	ras homolog gene family, member V	5.1	6.6	0	signal transduction
1387982_at	TLR4	toll-like receptor 4	5.1	3.8	0.7	inflammatory response
1368213_at	POR	P450 (cytochrome) oxidoreductase	5.0	7.0	0	electron transport
1369517_at	PSCD1	pleckstrin homology, Sec7 and coiled-coil domains 1	5.0	4.9	0.3	Golgi to secretory vesicle transport
1387125_at	S100A9	S100 calcium binding protein A9 (calgranulin B)	5.0	4.5	0.4	inflammatory response
1397674_at	EIF3S8	eukaryotic translation initiation factor 3, subunit 8, 110kDa	4.9	4.0	0.6	translational initiation
1368489_at	FOSL1	fos-like antigen 1	4.9	6.3	0.1	regulation of transcription, DNA-dependent
1369568_at	STX6	syntaxin 6	4.9	12	0	transport
1370255_at	SFTPC	surfactant associated protein C	4.8	9.0	0	respiratory gaseous exchange
1370792_at	MAPRE1	microtubule-associated protein, RP/EB family, member 1	4.7	3.4	1.2	cell cycle
1387605_at	CASP12	caspase 12	4.6	4.0	0.6	proteolysis
1371108_a_at	ATP1A1	ATPase, Na ⁺ /K ⁺ transporting, alpha 1 polypeptide	4.6	3.5	1.1	ATP catabolic process
1369406_at	ASAH2	N-acylsphingosine amidohydrolase 2	4.6	3.1	1.7	lipid metabolic process
1387631_at	HPGD	hydroxyprostaglandin dehydrogenase 15 (NAD)	4.4	3.1	1.7	lipid metabolic process
1371618_s_at	TUBB3	tubulin, beta 3	4.3	5.5	0.2	microtubule-based process

Affymetrix id	Gene Symbol	Gene Title	Fold-Change ZD versus ZS	Score	q-value (%)	Function
1387788_at	JUNB	Jun-B oncogene	4.3	9.8	0	regulation of progression through cell cycle
1370174_at	MYD116	myeloid differentiation primary response gene 116	4.3	5.5	0.2	myeloid differentiation primary response gene 116
1369587_at	VAPB	vesicle-associated membrane protein, associated protein B and C	4.3	5.6	0.1	protein complex assembly
1370123_a_at	CTTN	cortactin	4.3	5.5	0.2	receptor-mediated endocytosis
1396262_at	PBEF1	pre-B-cell colony enhancing factor 1	4.3	4.5	0.4	cell cycle
1368024_at	QSCN6	quiescin Q6	4.2	7.2	0	regulation of progression through cell cycle
1370477_at	OCM	oncomodulin	4.2	3.5	1.1	calcium ion binding
1389123_at	CCL	chemokine (C-C motif) ligand 6	4.2	2.6	3.4	apoptosis
1384328_at	TOM1	target of myb1 homolog (chicken)	4.2	6.0	0.1	intracellular protein transport
1368947_at	GADD45A	growth arrest and DNA-damage-inducible 45 alpha	4.2	3.9	0.6	regulation of progression through cell cycle
1387353_at	AKT2	thymoma viral proto-oncogene 2	4.2	7.2	0	protein amino acid phosphorylation
1395614_at	U2AF2	U2 small nuclear ribonucleoprotein auxiliary factor (U2AF) 2	4.1	2.8	2.4	nuclear mRNA splicing, via spliceosome
1387870_at	ZFP36	zinc finger protein 36	4.1	6.7	0	mRNA catabolic process
1368014_at	PTGES	prostaglandin H synthase	4.1	4.2	0.5	prostaglandin biosynthetic process
Forty-two down-regulated genes associated with dietary ZD						
1387578_a_at	P2RX2	purinergic receptor P2X, ligand-gated ion channel, 2	0.08	-6.3	0.1	transport
1369381_a_at	SLC15A1	solute carrier family 15 (oligopeptide transporter), member 1	0.09	-3.8	0.5	transport
1388395_at	G0S2	G0/G1 switch gene 2	0.11	-3.4	0.7	integral to membrane
1370198_at	TRDN	triadin	0.12	-2.4	2.8	calcium ion homeostasis
1368252_at	KBTBD10	kelch repeat and BTB (POZ) domain containing 10	0.13	-2.8	1.7	cell motility
1372536_at	CABC1	chaperone, ABC1 activity of bc1 complex like	0.13	-4.2	0.4	protein folding
1398243_at	CSRP3	cysteine and glycine-rich protein 3	0.13	-2.1	4.6	blood vessel remodeling
1371247_at	TNNT3	troponin T3, skeletal, fast	0.14	-2.9	1.7	muscle contraction
1383875_at	UPK1B	uroplakin 1B	0.14	-3.5	0.7	epithelial cell differentiation

Affymetrix id	Gene Symbol	Gene Title	Fold-Change ZD versus ZS	Score	q-value (%)	Function
1387181_at	MYF6	myogenic factor 6	0.14	-2.6	2.4	somitogenesis
1374248_at	MYBPC1	myosin binding protein C, slow type	0.14	-2.9	1.5	muscle contraction
1367572_at	MYL3	myosin, light polypeptide 3	0.14	-2.9	1.5	muscle contraction
1371053_at	MYH8	myosin, heavy polypeptide 8, skeletal muscle, perinatal	0.14	-3.8	0.6	striated muscle contraction
1374391_at	SLN	sarcolipin	0.14	-3.2	1.1	transport
1370165_at	SMPX	small muscle protein, X-linked	0.15	-2.7	2.0	striated muscle contraction
1368108_at	ATP2A1	ATPase, Ca++ transporting, cardiac muscle, fast twitch 1	0.16	-2.7	2.0	ATP catabolic process
1368145_at	PCP4	Purkinje cell protein 4	0.16	-4.9	0.3	central nervous system development
1370026_at	CRYAB	crystallin, alpha B	0.16	-3.6	0.6	glucose metabolic process
1372639_at	TRIM54	tripartite motif-containing 54	0.16	-3.8	0.6	negative regulation of microtubule
1370982_at	PYGM	muscle glycogen phosphorylase	0.16	-3.4	0.7	response to hypoxia
1391429_at	HFE2	hemochromatosis type 2 (juvenile) homolog (human)	0.16	-2.9	1.5	GPI anchor binding
1367782_at	COX6A2	cytochrome c oxidase, subunit VIa, polypeptide 2	0.17	-2.9	1.5	electron transport
1378430_at	MOXD1	monooxygenase, DBH-like 1	0.17	-8.2	0	catecholamine metabolic process
1367626_at	CKM	creatine kinase, muscle	0.17	-2.3	4.0	phosphocreatine metabolic process
1387082_at	FETUB	fetuin beta	0.17	-5.9	0.1	cysteine protease inhibitor activity
1390355_at	RYR1	ryanodine receptor 1, skeletal muscle	0.17	-3.7	0.6	transport
1387122_at	PLAGL1	pleiomorphic adenoma gene-like 1	0.17	-8.3	0	induction of apoptosis
1367962_at	ACTN3	actinin alpha 3	0.18	-4.2	0.4	muscle contraction
1372383_at	GPSM1	G-protein signalling modulator 1	0.18	-6.2	0.1	signal transduction
1372190_at	AQP4	Aquaporin 4	0.18	-4.8	0.3	transport
1367964_at	TNNI2	troponin I type 2 (skeletal, fast)	0.18	-2.8	1.7	hydrogen transport
1368724_a_at	TPM1	tropomyosin 1, alpha	0.18	-3.1	1.2	cell motility
1386907_at	ENO3	enolase 3, beta	0.18	-2.8	1.7	glycolysis
1387092_at	FXYD4	FXYD domain-containing ion transport regulator 4	0.19	-2.6	2.4	transport
1377499_a_at	HRC	histidine rich calcium binding protein	0.19	-3.8	0.6	structural molecule activity

Affymetrix id	Gene Symbol	Gene Title	Fold-Change ZD versus ZS	Score	q-value (%)	Function
1369067_at	NR4A3	nuclear receptor subfamily 4, group A, member 3	0.20	-2.3	4.0	mesoderm formation
1367951_at	PGAM2	phosphoglycerate mutase 2	0.20	-2.4	2.8	glycolysis
1370033_at	MLC3	myosin, light polypeptide 1	0.21	-3.1	1.2	cardiac muscle contraction
1378698_at	MYH13	myosin, heavy polypeptide 8, skeletal muscle, perinatal	0.21	-2.3	4.0	striated muscle contraction
1368966_at	MYBPH	myosin binding protein H	0.21	-2.2	4.6	striated muscle contraction
1367739_at	COX8H	Cytochrome c oxidase subunit VIII-H (heart/muscle)	0.22	-2.5	2.8	electron transport
1390822_at	CDH16	cadherin 16	0.22	-2.5	2.8	tRNA aminoacylation for protein translation

A group of 103 differentially expressed genes were identified, using a cuipoint of q value <5% and fold-change ≥4.0. Score is the T-statistic value; q-value is the lowest False Discovery Rate at which the gene is considered significant. ZD = zinc-deficient; ZS = zinc-sufficient.

Table 2

EASE analysis of functional pathways among the 103 differentially expressed genes in zinc-deficient rat esophagus

Functional pathway	EASE score	Gene Symbol
Upregulated with zinc-deficiency		
response to external stimulus	0.02 [*]	CDKN1A; GJA1; MX2; PPP2R1A; S100A8; SECTM1; SERPINB3
immune response	0.07	CDKN1A; MX2; S100A8; SECTM1; SERPINB3
apoptosis	0.08	APP; BIRC4; CDKN1A; PPP2R1A
tyrosine phosphorylation	0.09	EGF; PPP2R1A
response to chemical substance	0.12	PPP2R1A; S100A8
cell cycle	0.12	CDKN1A; EGF; PPP2R1A
response to abiotic stimulus	0.2	GJA1; PPP2R1A; S100A8
cell proliferation	0.41	AREG; CDKN1A; EGF; PPP2R1A
protein modification	0.54	EGF; PLAT; PPP2R1A
protein metabolism	0.59	CA2; EGF; PLAT; PPP2R1A; SERPINB3
cell growth and/or maintenance	0.63	APP; AREG; BIRC4; CA2; CDKN1A; EGF; GJA1; PPP2R1A
cell motility	0.67	ACTR3; GJA1
cell adhesion	0.69	APP; PPP2R1A
protein catabolism	0.7	PLAT; SERPINB3
regulation of transcription	0.71	DNMT3A; PPP2R1A
cell organization and biogenesis	0.73	BIRC4; EGF
nucleotide and nucleic acid metabolism	0.73	DNMT3A; EGF; PPP2R1A
cell communication	0.81	APP; AREG; EGF; GJA1; PPP2R1A; SCGB1A1
development	0.81	DNMT3A; GJA1; PPP2R1A; SCGB1A1
macromolecule biosynthesis	0.84	CA2; PPP2R1A
cell-cell signaling	0.85	AREG; GJA1
signal transduction	0.92	APP; EGF; PPP2R1A; SCGB1A1
transport	0.94	APP; CA2; GJA1
Downregulated with zinc-deficiency		
muscle contraction/development	1.0E-10 [*]	ATP2A1; CKM; MYF6; CRYAB; MYBPC1; MYH8; MYL3; SMPX; TNNT3; TRDN
organogenesis	5.3E-03 [*]	CRYAB; MYBPC1; MYF6; MYH8; MYL3; PCP4; TNNT3
energy pathways	1.4E-01	CKM; COX6A2; PYGM
cytoskeleton organization and biogenesis	4.9E-01	MYH8; MYL3
neurogenesis	7.8E-01	CRYAB; PCP4
ion transport	8.4E-01	ATP2A1; P2RX2
transport	8.5E-01	ATP2A1; P2RX2; SLC15A1; TRDN
cell growth and/or maintenance	9.4E-01	ATP2A1; MYH8; MYL3; P2RX2; SLC15A1; TRDN

EASE = Expression Analysis Systematic Explorer

A group of 103 differentially expressed genes (61 up and 42 down) were identified, using a cutoff point of $q < 5\%$ and fold-change ≥ 4.0 (zinc-deficiency versus zinc-sufficiency).

* Significant at EASE score < 0.05