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Zinc supplementation suppresses 4-nitroquinoline 1-oxide-induced rat oral carcinogenesis

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Dietary zinc (Zn) deficiency is implicated in the pathogenesis of human oral–esophageal cancers. In rats, Zn deficiency causes increased cell proliferation and cyclooxygenase-2 (COX-2) overexpression and enhances oral carcinogenesis by 4-nitroquinoline 1-oxide (NQO). Zn replenishment reverses all these effects. We questioned whether Zn has antitumor efficacy in a Zn-sufficient animal by investigating in Zn-sufficient rats (i) the efficacy of Zn supplementation on the progression of tongue squamous cell carcinogenesis induced by drinking water exposure to high (20–30 p.p.m.) and low (10 p.p.m.) doses of NQO and (ii) the modulating effects of Zn supplementation on biomarker expression in tongue lesions by immunohistochemistry. In rats exposed to high doses of NQO, Zn supplementation significantly reduced the incidence of papillomas from 100 to 64.7% ($P = 0.018$) and invasive carcinomas from 93.8 to 52.9% ($P = 0.017$). In rats exposed to low doses of NQO, where only minimally invasive carcinomas developed, Zn supplementation significantly reduced tumor multiplicity, incidence of tumors (1–2 mm), hyperplasia, dysplasia, papillomas and progression to carcinoma. Immunohistochemical analysis of carcinomas showed that Zn supplementation caused a shift to a less proliferative/aggressive cancer phenotype by reducing cell proliferation, stimulating apoptosis and decreasing expression of the key tumor markers cyclin D1, p53 and COX-2. Additionally, Zn supplementation significantly reduced cell proliferation in non-lesional tongue squamous epithelia, thereby suppressing tumor development. Together, the results demonstrate that Zn supplementation has chemopreventive efficacy against oral carcinogenesis in nutritionally complete animals. Our data suggest that Zn supplementation may be efficacious in the chemoprevention of human oral cancer.

Introduction

Oral cancer, most commonly in tongue, is a major cause of cancer deaths worldwide (1). In 2009, >36 000 cases of oral cancer were diagnosed in the USA, and 8000 deaths were attributed to this malignancy (American Cancer Society, 2009). Patients with oral cancer have a high mortality rate because they frequently develop second primary tumors in the esophagus owing to field cancerization effects (2,3). Risk factors for oral and esophageal cancers are chronic alcohol consumption, tobacco use and human papillomavirus infection (4). The incidence of oral cancer is increasing worldwide, particularly in young adults without documented risk factors (4). Epidemiological and clinical studies have implicated dietary zinc (Zn) deficiency in the pathogenesis of oral and esophageal squamous cell carcinoma (SCC) (5–9).

Zn is required for the activity of many enzymes, for proper immune function and for the conformation of many transcription factors that control cell proliferation, apoptosis and signaling pathways (10,11). Zn is known to undergo rapid ligand exchange reactions and is used as an information carrier in signal transduction pathways (12). Accordingly, Zn deficiency predisposes to disease by adversely affecting the immune system, by increasing oxidative stress and by increasing the generation of inflammatory cytokines (13).

We have developed *in vivo* cancer models that reproduce the Zn-deficient feature of human oral–esophageal cancers. In the rat, a Zn-deficient diet creates a precancerous condition in the upper digestive tract, including tongue, esophagus and forestomach (an expanded lower esophagus), by inducing proliferation (14) and gene expression changes, including overexpression of *cyclooxygenase-2* (*Cox-2*) and the proinflammation-genes *S100 calcium binding protein a8* (*S100a8*) and *a9* (*S100a9*) (14,15). As a result, Zn-deficient rats rapidly develop esophageal tumors after a single exposure to the environmental carcinogen *N*-nitrosomethylbenzylamine (16) and concurrent tongue, esophageal and forestomach tumors with exposure to the tongue carcinogen 4-nitroquinoline 1-oxide (NQO) (14). Zn replenishment inhibits tumorigenesis by inducing apoptosis and reversing cellular proliferation (16,17). Recently, we reported that dietary Zn replenishment reverses overexpression of S100A8 in Zn-deficient rat esophagus and modulates the link between S100A8 and its receptor for advanced glycation end products (RAGE) and downstream nuclear factor-kappaB/COX-2 signaling, thereby attenuating inflammation and reverses esophageal preneoplasia. Since chronic inflammation is a hallmark of cancer (18), our finding provides evidence that Zn has an inflammation-modulating role in esophageal cancer initiation/reversal (15).

Our previous studies have been focused on the mechanisms by which Zn deficiency and replenishment influence carcinogenesis (16,17). Since most cancers acquire similar hallmarks during their development albeit through different mechanistic routes (18,19), we hypothesized that Zn should exert similar effects on the signal pathways important in tumor suppression, independent of the nutritional Zn status of the animal. We tested this hypothesis by determining the ability of Zn supplementation to inhibit NQO-induced tongue carcinogenesis in Zn-sufficient animals and by examining its modulating effects on cell proliferation, apoptosis and expression of the tumor markers cyclin D1, p53 and COX-2 in lingual tumor lesions. Cell proliferation and COX-2 overexpression are highly correlated with oral cancers induced by NQO in nutritionally complete rats (20,21).

Abbreviations: COX-2, cyclooxygenase-2; IHC, immunohistochemistry; ISOL, *in situ* oligo ligation; NQO, 4-nitroquinoline 1-oxide; PCNA, proliferating cell nuclear antigen; SCC, squamous cell carcinoma; Zn, zinc.

Materials and methods

Animal, diet, carcinogen and Zn gluconate

Male Sprague–Dawley rats (5 weeks of age) were obtained from Taconic Laboratory (Germantown, NY) and were acclimatized for a week before experiment. In order to eliminate biological variation in long-term tumorigenesis studies introduced by non-purified protein ingredients in rodent lab diets and city water, we used a custom synthesized, egg-white based Zn-sufficient diet that contains 65 p.p.m. Zn as Zn carbonate (Harlan Teklad, Madison, WI) and deionized water as drinking water (16,17). The Zn content in the diet was regularly determined by atomic absorption spectroscopy in our laboratory.

NQO was from Wako Chemicals USA (Richmond, VA). Drinking water containing NQO was freshly prepared every week in deionized water and was administered to the rats in light-shielded water bottles. Zn gluconate was from Life Extension (Ft Lauderdale, FL), and drinking water containing Zn gluconate was freshly prepared every week in deionized water.

Experimental design

The animal studies were approved by The Ohio State University Institutional Animal Care and Use Committee and conducted under National Institutes of

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Health guidelines. The experimental design is depicted in Figure 1A. Experiment A (high NQO doses): 55 male Sprague–Dawley rats (6-week-old) were fed *ad libitum* a Zn-sufficient diet throughout the study period and exposed to deionized water containing high doses of NQO (20 p.p.m. for 4 weeks followed by 30 p.p.m. for 4 weeks). After 8 weeks, five rats were killed to determine the number of lingual lesions. The remaining animals were taken off NQO and immediately administered deionized water supplemented with 0, 5 and 20 p.p.m. Zn (as Zn gluconate) to form three groups, namely, control ($n = 16$), low Zn ($n = 17$) and high Zn (n = 17), respectively. At 13 weeks of Zn intervention, the animals were killed for tumor incidence analysis. Experiment B (low NQO doses): 56 rats were similarly fed and exposed to low doses of NQO (10 p.p.m. for 8 weeks). After 8 weeks, five rats were killed. The remaining animals were taken off NQO and immediately administered deionized water supplemented with 0 and 20 p.p.m. Zn (as Zn gluconate) to form the control ($n = 25$) and high Zn ($n = 26$) groups, respectively. At 10 weeks of Zn intervention, the animals were killed for tumor incidence analysis.

Zn gluconate is a widely used, over-the-counter dietary supplement and is very soluble in water, whereas dietary Zn carbonate is not soluble (10 versus 0.001%). We estimated that the daily Zn intake by a 470 g rat (average body weight at study termination) from the low Zn and high Zn-supplemented water was ∼0.17 and 0.68 mg, respectively, based on a water intake of ∼35 ml/day. These Zn doses translate to a human supplementation of ∼25 and 100 mg Zn/day for a 70 kg individual, a dose on the same order as those used for Zn supplement in commercial preparations. The animals were monitored daily for signs of ill health and weighed weekly. No animals became moribund and early removal from the study was not necessary for any animal subjects.

Tumor analysis

At killing, tongues were excised. Lesions >0.5 mm in diameter were mapped and counted. The tongue was longitudinally bisected and both halves were fixed in buffered formalin and embedded in paraffin. Five micrometer sections were cut and stained with hematoxylin and eosin for histopathology or left unstained for immunohistochemical studies.

Cell proliferation by proliferating cell nuclear antigen immunohistochemistry

Tongue sections were deparaffinized and rehydrated in a graded alcohol series and then incubated with mouse anti-proliferating cell nuclear antigen (PCNA) monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at a 1:300 dilution overnight at 37°C in a humidified chamber, followed by incubation with biotinylated goat anti-mouse antibodies (DakoCytomation, Carpinteria, CA) and then streptavidin–horseradish peroxidase (DakoCytomation). PCNA staining was visualized by incubation with the chromogen 3-amino-9-ethylcarbazole (DakoCytomation). Cells whose nuclei showed strong staining for a red reaction product were defined as positive for PCNA, i.e. as being in S phase. The PCNA-labeling index (%) was calculated by dividing the number of darkly stained PCNA-labeled nuclei by the total number of cells (∼1000 cells) counted.

Apoptosis detection

Apoptosis was detected in lingual sections by the *in situ* oligo ligation (ISOL) assay, using an ApopTag ISOL assay kit (Millipore, Billerica, MA) (14). This assay relies on the selective binding of biotin-labeled hairpin oligonucleotide probes to the types of genomic DNA ends that are characteristic of the double-strand breaks in apoptotic cells. Briefly, the sections were deparaffinized, rehydrated in a graded alcohol series and incubated with proteinase K. Endogenous peroxidase was inhibited with hydrogen peroxide. The slides were then incubated with T4 DNA ligase at 16–20°C overnight as to catalyze blunt-end ligation of biotinylated oligo B with fragmented double-strand DNA. Slides were then incubated with streptavidin–peroxidase conjugate, and oligo B binding (i.e. DNA fragmentation) was detected by staining with 3,3′-diaminobenzidine tetrahydrochloride (Santa Cruz), with methyl green as a counterstain. Sections from rat mammary gland, in which apoptosis is extensive, served as a positive control. Negative controls omitted T4 DNA ligase enzyme.

Immunohistochemical analysis of cyclin D1, p53 and COX-2 protein expression

Immunohistochemistry (IHC) on tissue sections was preformed as described (14). Sections were incubated with respective primary antisera: rabbit anti-cyclin D1 rabbit monoclonal at 1:20 (Cell Signaling, Danvers, MA), rabbit anti-COX-2 polyclonal at 1:50 dilution (Caymen Chemical, Ann Harbor, MI), rabbit anti-p53 polyclonal, which detects both mutated and wild-type proteins, at 1:100 dilution (Leica Microsystems, Bannockburn, IL). Expression of proteins was localized by incubation with 3-amino-9-ethylcarbazole or 3,3′-diaminobenzidine tetrahydrochloride.

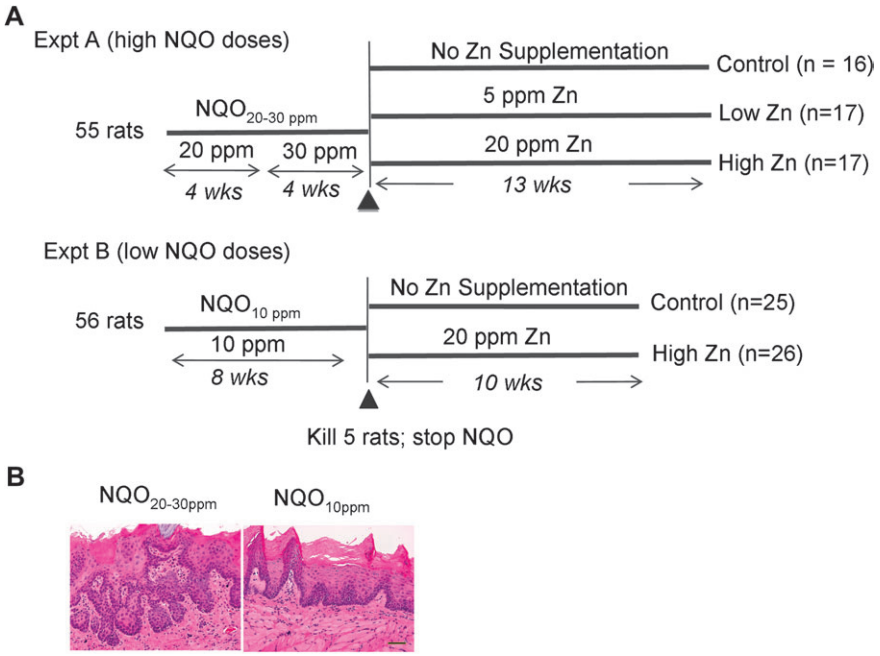

Fig. 1. Treatment protocol and histopathology of tongue at 8 weeks of NQO exposure. (**A**) Treatment protocol. Experiment A (high NQO doses): 55 male rats (6-week-old) were fed a Zn-sufficient diet (65 p.p.m. Zn as Zn carbonate) and received drinking water exposure to high doses of NQO for 8 weeks (20 p.p.m. for 4 weeks followed by 30 p.p.m. for 4 weeks). At 8 weeks, five rats were killed for the determination of the status of lingual lesions. The remaining rats were taken off NQO and given deionized water with 0, 5 or 20 p.p.m. Zn (as Zn gluconate) to form the control (16 rats), low Zn (17 rats) and high Zn (17 rats) groups. Experiment B (low NQO doses): 56 Zn-sufficient rats (6-week-old) received drinking water exposure to low doses of NQO (10 p.p.m.) for 8 weeks. At 8 weeks, five rats were killed; the remaining rats were taken off NQO and given deionized water with 0 and 20 p.p.m. Zn (as Zn gluconate) to form the control (25 rats) and high Zn (26 rats) groups. All animals were fed the Zn-sufficient diet throughout the study period. The animals were killed at 13 (Experiment A) and 10 weeks (Experiment B) of Zn intervention. (**B**) Histopathology of tongues at 8 weeks of NQO exposure (scale bar, 100 μm).

Immunoreactive scores were calculated by multiplying the percentage of positive cells by the grade of staining intensity (16). The percentage of positive cells was evaluated as follows: 0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–75% and 4 = 76–100%. The intensity of immunostaining was graded as follows: 0 = none, 1 = weak, 2 = moderate and 3 = intense.

Serum Zn measurement

At killing, blood was collected from the retro–orbital venous plexus of each rat after anesthesia with isoflurane (GE Healthcare, Waukesha, WI). Serum Zn analysis was by atomic absorption spectrometry, using AAnalyst 400 (Perkin Elmer, Waltham, MA).

Statistical analyses

Analysis of variance was used to analyze tumor multiplicity, serum Zn, immunoreactive scores, apoptosis and cell proliferation-labeling indices among the control, low Zn supplemented and high-Zn-supplemented animal groups. Differences among the three groups were assessed using the Tukey range test *post hoc t*-tests for multiple comparisons. When there were only two treatment groups (Experiment B), a standard *t*-test was used to analyze the data. Tumor, hyperplasia, dysplasia, papillomas and carcinoma incidence rates were assessed by Fisher's exact test. Statistical tests were two sided and were considered significant at $P < 0.05$.

Results

Serum Zn levels and general observations

That the animals received their assigned drinking water during Zn intervention was confirmed by measurement of serum Zn levels at study terminations (Tables I and II). Both high and low doses of Zn led to significantly higher serum Zn levels than controls without supplementation (Table I). Although serum Zn level was greater in high Zn than low Zn group, the difference was not statistically significant. No significant differences in body weights were present among Zn supplemented and control groups throughout the experiment (data not shown) and at study terminations (Tables I and II), indicating that Zn supplementation was well tolerated.

Zn supplementation suppresses NQO-induced tongue carcinogenesis

NQO is a synthetic DNA adduct-forming agent that has been widely used to induce oral cancer in rodents (22). In the rat, NQO induces ulcerated exophytic and endophytic oral lesions that progress from hyperplasia to dysplasia and carcinoma *in situ*, and finally to SCC, in a manner similar to human oral cancers (23). The NQO rat oral cancer model has been widely used to test efficacies of chemopreventive agents, including COX-2 inhibitors (21,24,25).

Table I. Effect of Zn supplementation on tongue carcinogenesis in rats exposed to high doses of NQO (20–30 p.p.m.) for 8 weeks[a]

Group (No. of rats at study conclusion)	Body weight (g)	Serum Zn levels (μg/100 ml)	Gross examination			Histologic examination			
			Tumor incidence (%)			Preneoplastic lesions incidence (%)		Neoplastic lesions incidence (%)	
			Total tumors	Large tumors (2–10 mm)	Tumor multiplicity	Hyperplasia	Dysplasia	Papilloma	Invasive SCC
Control (16)	477 ± 60	143 ± 16	16/16 (100)	15/16 (93.8)	17.9 ± 4.4	16/16 (100)	16/16 (100)	16/16 (100)	15/16 (93.8)
Low Zn (17)	478 ± 60	164 ± 15[b]	17/17 (100)	11/17 (64.7)	13.5 ± 4.8	17/17 (100)	11/17 (64.7[e])	11/17 (64.7[e])	10/17 (58.8[g])
High Zn (17)	489 ± 74	175 ± 16[c]	17/17 (100)	9/17 (52.9[d])	9.9 ± 4.0[c]	17/17 (100)	10/17 (58.8[f])	11/17 (64.7[e])	9/17 (52.9[d])

[a]Beginning at age 6 weeks, male rats were fed a Zn-sufficient diet and received drinking water exposure to high doses of NQO for 8 weeks (20 p.p.m. for 4 weeks followed by 30 p.p.m. for 4 weeks). The rats were taken off NQO and immediately and given drinking water supplemented with 0, 5 and 20 p.p.m. Zn, forming the control, low Zn and high Zn groups, respectively. The study was terminated 13 weeks after NQO cessation. Body weight, serum Zn levels and tumor multiplicity are presented as mean ± SD and differences among the three groups were assessed using the Tukey range test *post hoc t*-tests for multiple comparisons. Tumor and lesions incidence (number of rats with lesions/total number of rats) was compared by Fisher's exact test. Excluding animals with coalesced lesions, tumor multiplicity (number of discrete tumors/tongue) was analyzed in 11 controls, 12 low Zn and 15 high Zn-supplemented rats). All statistical tests were two sided.
[b]Compared with control: $P < 0.01$
[c]$P < 0.001$
[d]$P = 0.017$
[e]$P = 0.018$
[f]$P = 0.007$
[g]$P = 0.039$

Table II. Effect of Zn supplementation on tongue carcinogenesis in rats exposed to low doses (10 p.p.m.) of NQO for 8 weeks[a]

Group (No. of rats at study conclusion)	Body weight (g)	Serum Zn levels (μg/100 ml)	Gross examination			Histologic examination			
			Tumor incidence (%)			Preneoplastic lesions incidence (%)		Neoplastic lesions incidence (%)	
			Total tumors	Tumors (1–2 mm)	Tumor multiplicity	Hyperplasia	Dysplasia	Papilloma	Minimally invasive SCC
Control (25)	493 ± 39	144 ± 6.7	25/25 (100)	9/25 (36)	8.6 ± 3.4	22/25 (84)	12/25 (48)	15/25 (60)	9/25 (36)
High Zn (26)	495 ± 34	176 ± 9.8[b]	26/26 (100)	2/26 (7.7[c])	5.2 ± 2.3[b]	13/26 (50[d])	5/26 (19[e])	7/26 (26.9[f])	3/26 (11.5[g])

[a]Beginning at age 6 weeks, male rats were fed a Zn-sufficient diet and received drinking water exposure to NQO (10 p.p.m.) for 8 weeks. The animals were taken off NQO and immediately given drinking water supplemented with 0 and 20 p.p.m. Zn to form control and high Zn group, respectively. The study was terminated 10 weeks after NQO cessation. Body weight, serum Zn levels and tumor multiplicity (number of tumors/tongue) are presented as mean ± SD and analyzed by unpaired *t*-test. Tumor and lesions incidence (number of rats with lesions/total number of rats) was analyzed by Fisher's exact test. All statistical tests were two sided.
[b]Compared with control: $P < 0.001$
[c]$P = 0.019$
[d]$P = 0.006$
[e]$P = 0.040$
[f]$P = 0.025$
[g]$P = 0.052$

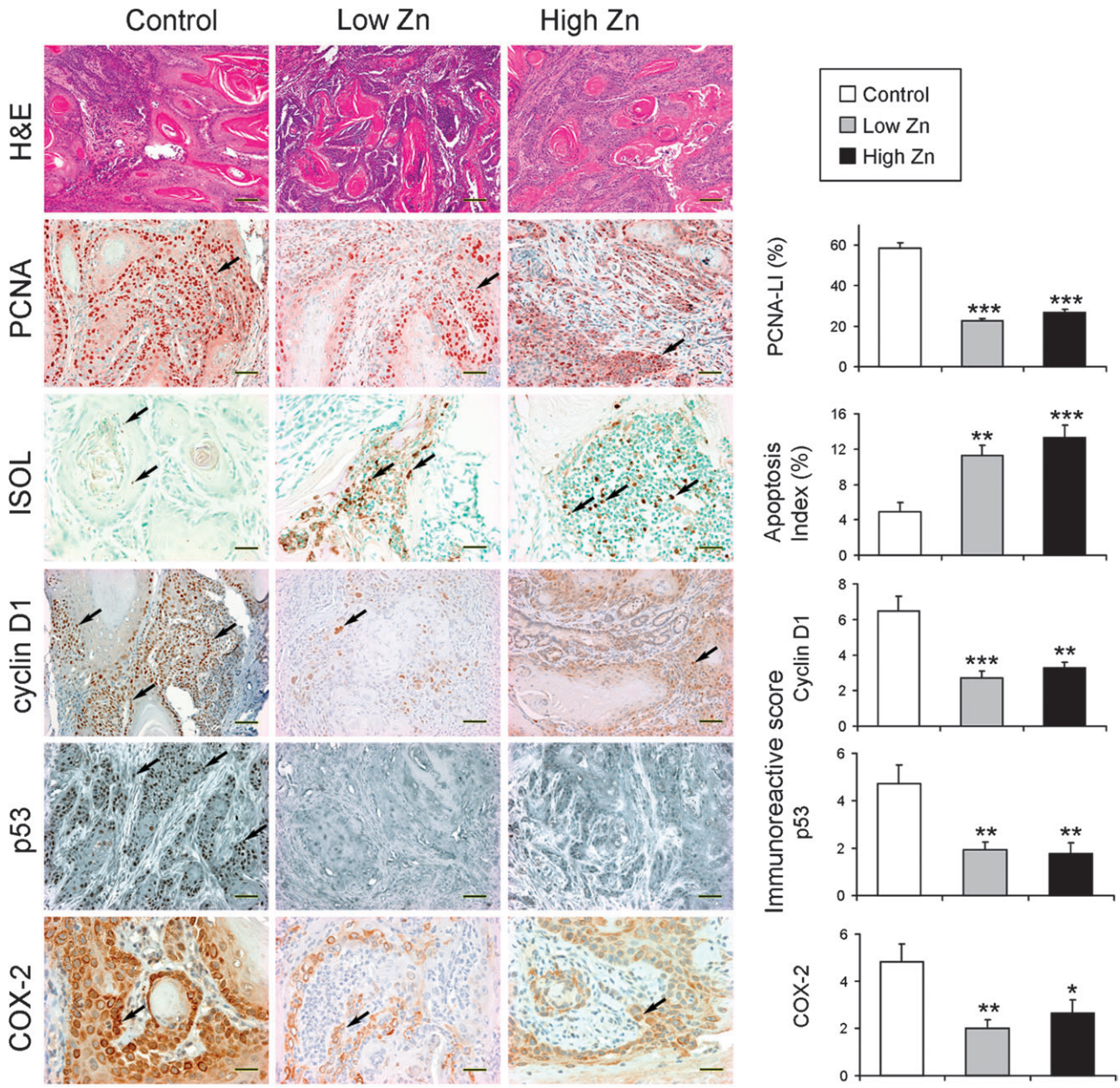

Fig. 2. Analysis of invasive tongue SCCs from rats exposed to high doses of NQO (20–30 p.p.m.). IHC was performed on SCC sections from 15 non-supplemented controls, 8 low- and 8 high-Zn-supplemented rats. Representative sections were presented [red, 3-amino-9-ethylcarbazole, (AEC)]; brown, 3,3′-diaminobenzidine tetrahydrochloride (DAB), DAB; black, DAB with cobalt chloride). Scale bars, 100 μm (hematoxylin and eosin-stained), 50 μm (PCNA, cyclin D1 and p53), 25 μm (ISOL and COX-2). Arrows show area of high immunostaining of these markers. The cell proliferation-labeling index (PCNA-LI) and apoptosis index (assessed by ISOL assay) and immunoscores (cyclin D1, p53 and COX-2) per ∼800–1000 tumor cells are quantified at right. Bar charts represent mean ± standard deviation. Compared with control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$

The chemopreventive effect of Zn supplementation against malignant oral tumor progression and tumor development was determined by exposing rats to drinking water containing high (20–30 p.p.m.) and low (10 p.p.m.) doses of NQO for 8 weeks, a regimen that produced a high and low carcinoma outcome, respectively (17,21,26). First, we determined lingual tumor incidence/multiplicity before the start of Zn intervention. At 8 weeks, rats exposed to high NQO doses had greater lingual tumor incidence/multiplicity than those exposed to low NQO doses [incidence, 100% (5 of 5) versus 20% (1 of 5); multiplicity, 3.4 ± 1.1 versus 0.6 ± 0.6]. Additionally, high doses of NQO (but not low doses) led to lingual dysplasia [60% (5 of 5) versus 0% (0 of 5)] and both doses resulted in lingual hyperplasia [100% (5 of 5) versus 60% (3 of 5)] (Figure 1B), thereby setting the stage for rapid tumor progression to malignancy in the former group.

In the first chemoprevention study (Table I), at termination high doses of NQO (20–30 p.p.m.) induced numerous exophytic and endophytic lingual tumors in all three animals groups, regardless of Zn supplementation. The tumors were found mainly on the posterior dorsum of the tongue and some tumors had coalesced into a single mass. Five of 16 control rats, 5 of 17 low Zn supplemented and 2 of 17 high Zn-supplemented rats had such coalesced tumors. Both doses of Zn resulted in a reduced incidence of large tumors (2–10 mm, including discrete and coalesced lesions) compared with control rats, with statistical significance achieved for the high Zn dose (52.9 versus 93.8%, $P = 0.017$). For tumor multiplicity (number of discrete

tumors/tongue), rats with coalesced tumors were excluded. Both doses of Zn resulted in a decreased tumor multiplicity compared with control rats, with statistical significance achieved for the high Zn dose.

Histopathologic examination (Table 1) showed that both doses of Zn significantly reduced the incidence of dysplasia and papilloma from 100% in controls to ∼60% and the incidence of invasive SCC from 93.8 to 58.9% in low Zn group ($P = 0.017$) and 52.9% in high Zn group ($P = 0.039$). Only 5 p.p.m. Zn (as Zn gluconate) in the drinking water >65 p.p.m. Zn (as Zn carbonate) in the diet exhibits chemopreventive efficacy, probably because the organic form of Zn that is highly soluble in water has greater bioavailability than the inorganic form. No dose–response relationship was evident between the dosage of Zn used and the incidence of SCC (Table I), perhaps because Zn supplementation above an optimal level does not provide increased benefit.

In the second study (Table II), low doses of NQO (10 p.p.m.) induced small discrete tumors (<2 mm) that occurred mainly in posterior dorsum of the tongue. Zn supplementation with high Zn doses resulted in significant reductions of tumor multiplicity and incidence of tumors (size 1–2 mm). Histopathologic examination showed that Zn treatment led to significant reductions in the incidence of hyperplasia, dysplasia, papillomas and minimally invasive SCCs (Table II). Taken together, these data demonstrate that in Zn-sufficient rats, Zn supplementation had efficacy in suppressing oral tumor progression to malignancy as well as tumor development.

Cell proliferation, apoptosis and immunohistochemical analysis of tumor marker expression

To investigate how Zn supplementation suppresses lingual tumor progression in rats exposed to high doses of NQO (20–30 p.p.m.), we analyzed invasive tongue SCCs from rats that received Zn supplementation versus those that did not. We analyzed a total of 15 SCCs from controls and 8 SCCs from each of the groups supplemented with low or high doses of Zn. Because uncontrolled cell proliferation and evasion of apoptosis are two hallmarks of cancer (19,27), we examined the effect of Zn supplementation on cancer cell proliferation by PCNA (an endogenous cell proliferation marker)-IHC (28) and apoptosis occurrence by ISOL assay. Additionally, we evaluated the expression of three tumor markers: cyclin D1, p53 and COX-2 in these carcinomas. Tumor cells have typically acquired damage to cell cycle gene *CCND1* that encodes cyclin D1 protein (27) and cyclin D1 overexpression is a common genetic alteration in human oral–esophageal cancer (29,30). The *TP53* tumor suppressor gene is mutated in ∼50% of all human cancers, including oral–esophageal cancers (31). COX-2 that catalyzes the formation of prostaglandins is overexpressed in a variety of human premalignant and malignant lesions, including esophageal and tongue SCC (32,33).

Histopathologic analysis shows that tongue carcinomas from all three animal groups were well-differentiated SCCs (Figure 2, hematoxylin and eosin). IHC analysis shows that control SCCs had high proliferative activity with abundant PCNA-positive nuclei in tumor areas but isolated occurrence of ISOL-positive apoptotic nuclei. Concurrently, these carcinomas displayed prominent accumulation of intensely stained cyclin D1-positive and p53-positive nuclei, as well as strong cytoplasmic and perinuclear immunostaining of COX-2 protein. In sharp contrast, both doses of Zn resulted in a less proliferative SCC with fewer PCNA-positive nuclei, and reduced expression of the same tumor markers. Importantly, the Zn-supplemented SCCs evidenced numerous ISOL-positive nuclei in tumor areas, documenting the induction of apoptosis by Zn. Quantitatively, Zn-supplemented SCCs showed significantly reduced PCNA-labeling index but increased apoptotic index than control SCCs. At the same time, these SCCs showed significantly reduced immunoscores of cyclin D1, p53 and COX-2 protein than controls (Figure 2, bar graphs). No significant differences were observed among immunoscores of these markers for carcinomas of high and low Zn-supplemented rodents.

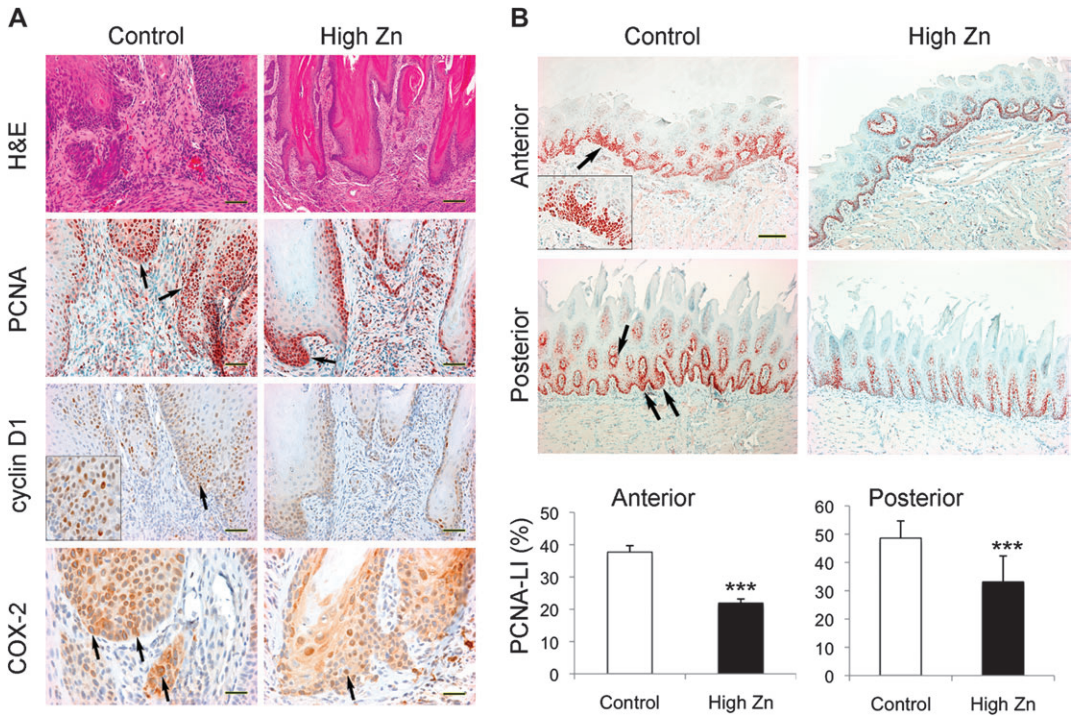

Fig. 3. Analysis of minimally invasive tongue SCCs and non-lesional tongue epithelia from rats exposed to low doses of NQO (10 p.p.m.). (**A**) IHC was performed on minimally invasive carcinoma sections from six non-supplemented controls and three high Zn-supplemented rats. Representative sections were presented. (**B**) Cell proliferation in non-lesional anterior and posterior tongue from control and high Zn-supplemented rats ($n = 15$ per rat group). Representative sections were presented [red, 3-amino-9-ethylcarbazole, (AEC); brown, 3,3′-diaminobenzidine tetrahydrochloride (DAB)]. Scale bars, 100 μm (hematoxylin and eosin-stained), 50 μm (PCNA, cyclin D1 and p53), 25 μm (COX-2). Arrows show areas of high immunostaining for these markers. Bar charts represent mean ± standard deviation. Compared with control: $***P < 0.001$.

Additionally, we investigated how Zn supplementation suppresses oral tumor development and progression to SCCs in rats exposed to low doses of NQO (Table II). Although minimally invasive SCCs from control rats displayed abundant PCNA-positive nucleic and overexpression of cyclin D1 and COX-2 protein, Zn supplementation led to a less proliferative tumor with fewer PCNA-positive nuclei and reduced expression of these same tumor markers (Figure 3A). In non-lesional squamous epithelia from these animals (n = 15 per rat group), Zn supplementation resulted in reduced cell proliferation in both anterior and posterior tongue. Although hyperplastic lingual epithelia from controls had abundant PCNA-positive nuclei in many cell layers and in preneoplastic focal hyperplastic lesions, non-proliferative Zn-supplemented epithelia showed PCNA-positive nuclei mainly in basal cell layers (Figure 3B). Quantitatively, the PCNA-labeling index (%) was significantly lower in epithelia from Zn-supplemented rats than controls (anterior tongue: 21.9 ± 2.6 versus 37.7 ± 3.6; posterior tongue: 33.1 ± 9.2 versus 48.6 ± 6.1, $P < 0.001$). Taken together, the data demonstrate that Zn supplementation suppresses oral tumor development and progression by restricting cell proliferation, stimulating apoptosis and reducing expression of key tumor biomarkers.

Discussion

Dietary Zn deficiency is implicated in the pathogenesis of human oral–esophageal cancers (5–9). Our previous studies showed that in Zn-deficient rodents, Zn replenishment reverses increased cell proliferation, stimulates apoptosis, corrects abnormal gene expression, attenuates inflammation and inhibits carcinogenesis (15–17,34). The goal of the present study was to determine whether in Zn-sufficient animals, Zn supplementation has similar antitumor activity against oral carcinogenesis by NQO, studied at exposure levels that elicit high and low incidence of SCCs. In rats exposed to high doses of NQO (20–30 p.p.m.), our data demonstrated that Zn supplementation significantly reduced tongue tumor size and multiplicity as well as incidence of papillomas and SCCs (Table I). Supplementation with high doses of Zn appeared to have better efficacy than with low doses, but the difference is not statistically significant (Table I). The lack of a clear dose–response relationship for the dose levels of Zn used should be further explored, with larger animal cohorts, to establish an optimal dose. In animals exposed to low doses of NQO (10 p.p.m.), Zn supplementation significantly reduced tumor size, tumor multiplicity and the incidence of hyperplasia, dysplasia, papillomas and minimally invasive SCCs (Table II). This finding demonstrating that Zn has tumor suppressor activity against NQO-induced oral carcinogenesis in Zn sufficient as in Zn-deficient animals (17) is supported by our recent studies in wild-type and tumor suppressor-deficient mice, which showed that Zn supplementation significantly reduced forestomach/lower esophagus tumor burdens after multiple carcinogen exposures (35).

IHC analysis of lingual SCCs from control rats exposed to high doses of NQO showed high PCNA proliferative activity and prominent accumulation of p53 protein (Figure 2). The elevation of p53 protein levels in tongue carcinomas from NQO-treated rats is well documented (36). Additionally, consistent with NQO-induced tongue-cancers of rats (21,25,37), these carcinomas from control rats displayed strong expression of cyclin D1 and COX-2 protein and low apoptotic index (Figure 2). This finding is reminiscent of human head and neck squamous cell cancer, which is a highly inflammatory, proliferative and aggressive cancer with strong expression of the same tumor markers (29,32,33,38,39). Importantly, supplementation with both doses of Zn-suppressed malignancy in these invasive SCCs by inducing a shift to a less proliferative/aggressive cancer phenotype. Compared with control SCCs, carcinomas from rats supplemented with both doses of Zn had significant reductions in PCNA-labeling index and expression of p53, cyclin D1 and COX-2 protein, but an increase in apoptotic index (Figure 2). Similarly, in minimally invasive SCCs from rats exposed to low NQO doses, Zn supplementation brought about a less proliferative/aggressive tumor phenotype by the same mechanism (Figure 3A). These data provide an evidence that under conditions of Zn sufficiency (Figures 2 and 3) and deficiency (15,17,40,41), Zn suppresses carcinogenesis by restricting cell proliferation, stimulating apoptosis and attenuating expression of inflammation-related cancer markers COX-2 and p53. Of particular interest, supplemental Zn was reported to reactivate wild-type p53 and stimulate apoptosis by enhancing MI-219 (MDM2 inhibitor) activity in colon and breast cancer cells (42). Thus, Zn supplementation in the present study may have inhibited NQO-induced oral carcinogenesis by a similar mechanism (42).

Safety is an important consideration for chemopreventive agents. Zn is an essential nutrient. The Recommended Dietary Allowance for Zn is ∼12–15 mg. The only adverse effect of oral Zn supplementation in amounts >50 mg Zn/day is copper deficiency (43), which can be readily remedied by supplementation with low doses of copper (2 mg/day). In this regard, an oral dose of 45 mg Zn/day (as Zn gluconate) for 6 months was shown to have a protective effect in atherosclerosis owing to the anti-inflammatory and antioxidant function of Zn (44). In a study to prevent blindness in patients with age-related macular degeneration, 80 mg Zn/day (as Zn oxide) (with copper supplementation) was used for 10 years with no evident side effects (45). In the present study, the efficacious doses of Zn (5 and 20 p.p.m. Zn as Zn gluconate) used to suppress NQO-induced tongue carcinogenesis, when translated to human use are ∼25 and 100 mg/day, should, therefore, be considered safe.

Owing to the many biological functions of Zn and the tumorigenic effects of Zn deficiency in rodent oral–esophageal cancer models (15,17,40,41), the role of Zn deficiency in cancer development/progression is gaining attention. At the same time, the role of Zn as a tumor suppressor agent in cancer prevention has been explored in several animal tumor models, including prostate and colon cancer (46,47). Recent studies reported that Zn supplementation improves clinical outcomes in patients receiving radiotherapy for head and neck squamous cell cancer (48) as well as concomitant chemotherapy and radiotherapy for advanced nasopharyngeal carcinoma (49). The present finding that Zn reduces cell proliferation, stimulates apoptosis, attenuates expression of key tumor markers and suppresses oral carcinogenesis in Zn-sufficient rats suggests that Zn may be efficacious in the chemoprevention of human oral cancer.

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