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Prevention of upper aerodigestive tract cancer in zinc-deficient rodents: Inefficacy of genetic or pharmacological disruption of COX-2

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Abstract

Zinc deficiency in humans is associated with an increased risk of upper aerodigestive tract (UADT) cancer. In rodents, zinc deficiency predisposes to carcinogenesis by causing proliferation and alterations in gene expression. We examined whether in zinc-deficient rodents, targeted disruption of the cyclooxygenase (COX)-2 pathway by the COX-2 selective inhibitor celecoxib or by genetic deletion prevent UADT carcinogenesis. Tongue cancer prevention studies were conducted in zinc-deficient rats previously exposed to a tongue carcinogen by celecoxib treatment with or without zinc replenishment, or by zinc replenishment alone. The ability of genetic *COX-2* deletion to protect against chemically-induced for-estomach tumorigenesis was examined in mice on zinc-deficient versus zinc-sufficient diet. The expression of 3 predictive bio-markers COX-2, nuclear factor (NF)-κ B p65 and leukotriene A_4 hydrolase (LTA_4H) was examined by immunohistochemistry. In zinc-deficient rats, celecoxib without zinc replenishment reduced lingual tumor multiplicity but not progression to malignancy. Celecoxib with zinc replenishment or zinc replenishment alone significantly lowered lingual squamous cell carcinoma incidence, as well as tumor multiplicity. Celecoxib alone reduced overexpression of the 3 biomarkers in tumors slightly, compared with intervention with zinc replenishment. Instead of being protected, zinc-deficient COX-2 null mice developed significantly greater tumor multiplicity and forestomach carcinoma incidence than wild-type controls. Additionally, zinc-deficient COX-2−/− forestomachs displayed strong LTA_4H immunostaining, indicating activation of an alter-native pathway under zinc deficiency when the COX-2 pathway is blocked. Thus, targeting only the COX-2 pathway in zinc-deficient animals did not prevent UADT carcinogenesis. Our data suggest zinc supplementation should be more thoroughly explored in human prevention clinical trials for UADT cancer.

Keywords

zinc deficiency; upper aerodigestive tract cancer; chemoprevention; COX-2; COX-2 null mice

Upper aerodigestive tract (UADT) cancer, including esophageal and oral cancer, is an important cause of morbidity and mortality worldwide.[1] With a 5-year survival of ~10%,

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esophageal cancers are deadly. The prognosis of oral cancer, the major site being the tongue, is equally dismal, with an increasing incidence worldwide, particularly in young adults.[2] Patients with oral cancer have a high mortality rate, because of field cancerization effects that result in second primary tumors, particularly in the esophagus.[3,4] Thus, new chemopreventive and therapeutic approaches are much needed to prevent and treat these deadly cancers.

While chronic alcohol consumption and tobacco use are the major risk factors for UADT cancer, epidemiologic and clinical studies have implicated dietary zinc deficiency (ZD) in the etiology of esophageal squamous cell carcinoma (SCC) and head and neck SCC.[5–9] In 2005, Abnet *et al*.[10] provided the strongest evidence of an association between ZD and esophageal cancer in humans, by establishing an inverse relationship between zinc concentration in biopsy samples from a high esophageal SCC incidence area and subsequent risk of developing cancer.

We have developed *in vivo* cancer models that reproduce features of human UADT cancer. In rodents, ZD creates a precancerous condition in the UADT, including esophagus, forestomach (considered a dilation of the lower esophagus) and tongue by causing unrestrained cell proliferation[11–13] and extensive changes in gene expression, including upregulated expression of genes that are relevant in UADT cancer *cyclooxygenase-2* (*COX-2*), *MT-1* and *cytokeratin 14 (KRT14)*.[14–19] Rats on a ZD diet are exquisitely sensitive to *N*-nitrosomethylbenzylamine (NMBA)-induced esophageal[20,21] and 4-nitroquinoline 1-oxide (NQO)-induced lingual carcinogenesis.[13] Zinc replenishment (ZR) rapidly reverses cell proliferation, stimulates apoptosis, corrects abnormal gene expression and inhibits esophageal tumorigenesis.[13,22] Additionally, *p53*-deficient mice or *cyclin D1* overexpressing transgenic mice on a zinc-deficient diet showed rapid development and progression of esophageal/forestomach tumors by NMBA,[16,23] as well as lingual/esophageal tumors by NQO.[24] Since UADT cancer patients are often zinc-deficient, our well-characterized ZD rodent cancer models offer opportunities to explore the biologic role of zinc in UADT cancer development and prevention.

Targeted molecular intervention and therapies have been explored in attempts to prevent or cure cancer. The rationale for targeting the COX-2 pathway for cancer prevention is supported by numerous preclinical and human studies, culminating in use of celecoxib, a selective COX-2 inhibitor with Food and Drug Administration approval for cancer prevention in patients with familial adenomatous polyposis.[25] COX-2 selective inhibitors are actively being tested in clinical trials for the prevention of several cancers, including colorectal, esophageal adenocarcinoma and head and neck SCC.[26–28]

COX-2, which catalyzes the formation of prostaglandins from arachidonic acid, is induced quickly by factors implicated in carcinogenesis, including growth factors, inflammatory stimuli, oncogenes and tumor promoters.[29] The finding that deletion of the *COX-2* gene in *Apc* knockout mice greatly reduces intestinal polyp formation provides genetic evidence that COX-2 plays a key role in tumorigenesis.[30] Additionally, *COX-2* knockout mice developed fewer chemically-induced skin tumors than wild-type mice,[31] and COX-2 selective inhibitors reduced tumorigenesis in a variety of tissues,[32] including rat tongue in nutritionally complete animals.[33,34]

We previously showed that an intragastric dose of the COX-2 inhibitor celecoxib or indomethacin is less effective than zinc replenishment in reversing the multiple genetic abnormalities in the esophagus caused by ZD.[13] To investigate the role of the COX-2 pathway in our UADT cancer models that incorporates dietary zinc deficiency, we examined the ability of the inhibitor celecoxib without ZR, with ZR, or the ability of ZR alone to prevent lingual carcinogenesis in ZD rats previously exposed to the tongue carcinogen NQO. We investigated whether genetic deletion of *COX-2* would provide protection against NMBA-induced

forestomach tumorigenesis in mice on ZD *versus* ZS diet. Additionally, we examined whether under ZD conditions disruption of *COX-2* might shift arachidonic acid to the 5-lipoxygenase (5-LOX) path-way. Activation of 5-LOX pathways leads to the production of the potent inflammatory mediator leukotriene B_4 (LTB_4), which has protumorigenic activities and is leukotriene A_4 hydrolase (LTA_4H)-dependent.[35]

Material and methods

Chemicals and diets

NQO was from Wako Chemicals USA (Richmond, VA), NMBA from Ash Stevens (Detroit, MI) and celecoxib from LKT Laboratories (St. Paul, MN). Custom-formulated ZD and ZS diets (Harlan Teklad, Madison, WI) were identical except for the amount of zinc, which was 1.5 and 3 ppm for ZD diets for mice and rats and 70 ppm for ZS diet.[16,22] Celecoxib at 100 and 500 ppm was mixed with rat diets. To ensure the animals were fed the assigned food, the diets were shape and color-coded.

Lingual cancer chemoprevention in ZD rats

The animal studies were approved by the Thomas Jefferson University Institutional Animal Care and Use Committee. Two-hundred sixty-nine weanling male Sprague-Dawley rats (Taconic Laboratory, Germantown, NY) were housed 6 to a stainless steel cage and divided into 2 dietary groups (Fig. 1*a*). One group ($n = 229$) was fed a ZD diet *ad libitum*, the other ($n = 40$) pair-fed a ZS diet to match the reduced food consumption of ZD animals. After 5 weeks, increased cellular proliferation was established in the tongue of ZD rats. Both groups were then given deionized water containing 10 ppm NQO for 9 weeks to establish lingual lesions in ZD rats.[13] To determine the number of lingual lesions before the start of chemodietary intervention, 19 ZD and 12 ZS rats were killed at 9 weeks. The remaining animals were taken off NQO administration. Twenty-eight ZS rats were untreated and continued on ZS diet. Two-hundred ten ZD rats were earmarked and randomly divided into 6 groups. Three groups remained on ZD diets with 0, 100 or 500 ppm celecoxib (Cxb), forming control ZD (48 rats), ZD/Cxb100 (24 rats) and ZD/Cxb500 (23 rats) groups. Three other groups were zinc replenished by switching to ZS diets with 0, 100 or 500 ppm Cxb, thus forming ZR (49 rats), ZR +Cxb100 (22 rats) and ZR +Cxb500 (23 rats) groups.

The intake of celecoxib by a ZD rat from the 500 ppm diet was about 5 mg/10 g/day, translating to an intake of ~680 mg for a 70-kg human subject consuming about 1,360 g (3 lb) of this rat diet, a dose in the same order as those used for the prevention of human sporadic colorectal adenomas and Barrett's esophagus.[28,36] Similarly, the intake of zinc by a 70-kg human from the ZD rat diet (3-ppm zinc) will be about 4 mg/day, translating to ~36 and 50% of the recommended daily allowance (RDA) for men and women (11 and 8 mg). Thus, both celecoxib and zinc levels used in the diet are highly relevant to the human situation.

In order to evaluate early biologic changes in tongue epithelia brought about by the various treatments, 5 ZR, 8 ZD/Cxb500 and 8 ZR + Cxb500 animals were killed 52 hr after intervention, as compared with 19 ZD rats killed after 9 weeks of NQO (Fig. 1*a*). The remaining rats were killed at 15 weeks of intervention for endpoint tumor incidence analysis. No animals became moribund and had to be killed, as the animal protocol would have demanded.

Forestomach carcinogenesis in zinc-deficient COX-2−/− mice

Breeding of heterozygous B6;129S7-*Ptgs2*^{tm1Jed}/J males to females (Jackson Lab., Bar Harbor, ME) generated COX-2 +/+, +/− and −/− mice, with a ratio of 23:57:21. These offspring were differentiated by genotyping of tail DNA using a PCR-based method. To increase the

number of wild-type controls, we purchased 50 wild-type mice of the same genetic background (B6129S7F2/J).

Four-week old mice were fed a ZD or ZS diet, forming 6 groups, ZD:COX-2−/−, ZD:COX-2+/−, ZD:COX-2+/+, ZS:COX-2−/−, ZS:COX-2+/−, ZS:COX-2+/+. The experiment was conducted in batches, as the mice became available. After 4 weeks of ZD diet, 36 ZD:COX-2+/+, 41 ZD:COX-2+/− and 14 ZD:COX-2−/− mice were administered 3 intragastric doses of NMBA at 2 mg/kg body weight, twice weekly. Based on our results with ZD wild-type mice (Fig. 5), we gave ZS mice, comprising 42 ZS:COX-2+/+, 60 ZS:COX-2+/− and 17 ZS:COX-2−/− mice, 6 NMBA doses in order to obtain higher tumor count in ZS:COX-2+/+ mice so that a possible protective effect of *COX-2* deficiency could be shown in forestomach tumorigenesis. The animals were killed 11 weeks after the first NMBA dose for tumor incidence analysis.

To study the effect of long-term dietary zinc deficiency on cell proliferation and LTA_4H expression in COX-2−/− mouse forestomach, weaning mice were maintained on ZD and ZS diet for 15 weeks, forming ZS:COX-2+/+, ZS:COX-2−/−, ZD:COX-2+/+ and ZD:COX-2−/− groups, with 7 mice per group.

Tumor analysis

At autopsy, tongues (rat) and stomachs (mouse) were excised. Tumors greater than 0.5 mm in diameter were mapped and counted. Tissues were fixed in buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin for histopathology or left unstained for immunohistochemical studies.

Biomarker assessment by immunohistochemistry

Immunohistochemistry on tissue sections was preformed as described.[13] Sections were incubated with respective primary antibodies, rabbit anti-COX-2 polyclonal antiserum (1:50 dilution, Caymen Chemical, Ann Harbor, MI), rabbit anti-NF-κB p65 polyclonal antiserum (1:100 dilution, Abcam, Cambridge, MA), rabbit anti-NF-κB phospho-p65 (serine 276) polyclonal antiserum (1:100 dilution, Cell Signaling, Danvers, MA), mouse anti-KRT 14 monoclonal antiserum (1:100 dilution, Novocastra Lab., Newcastle-upon-Tyne, UK), polyclonal rabbit anti-LTA_4H (1:500 dilution, Cayman Chemical), followed by incubation with appropriate biotinylated secondary antibodies and streptavidin horseradish peroxidase. Expression of proteins was localized by incubation with 3,3′-diaminobenzidine tetrahydrochloride (Santa Cruz Biotech., Santa Cruz, CA).

Cell proliferation by PCNA immunohistochemistry

Tissue sections were incubated with mouse anti-PCNA monoclonal antibodies (1:300 dilution, Santa Cruz Biotech) as previously described.[13] PCNA localization was visualized by incubation with 3-amino-9-ethylcarbazole substrate-chromogen (Dako, Carpinteria, CA).

Rat serum zinc and plasma celecoxib measurement

At sacrifice, blood was collected from the retro-orbital venous plexus of each rat after anesthesia with isoflurane (Ohmeda, NJ). Serum zinc analysis was by atomic absorption spectrometry.[11] Plasma samples for celecoxib analysis were isolated from 3 rats per experimental group and were obtained using a plasma separator tube with lithium heparin (Becton Dickinson, Franklin Lakes, NJ). Each rat was chosen randomly from a cage with 6 rats. Thus, the mean plasma level of celecoxib of 3 rats would represent that of 18 rats. Celecoxib was extracted from plasma by solid phase extraction.[37] Methanol extracts were then sent to LKT Laboratories (St. Paul) for celecoxib measurement by liquid chromatography–mass spectrometry.

Murine testis and hair zinc determination

At autopsy, testis from male and hair from female mice were obtained for zinc content determination.[16] Consistent with our previous data,[16,24] ZD mice (regardless of genotype) had significantly lower hair and testis zinc levels (μg/g) than ZS mice (hair, 111 *vs*. 157, difference = 46, 95% confidence interval (CI) = 61–31, $p < 0.001$; testis, 105 *vs*. 130, difference = 26, 95% CI = 34–16, $p < 0.001$).

Statistical analyses

Data on tumor multiplicity were analyzed by 2-way analysis of variance (ANOVA). Differences among the groups were assessed using *post hoc t*-tests with the Tukey-Kramer multiple comparisons (SAS statistical program, SAS Cary, NC). Confidence intervals were calculated using standard *t*-statistics and an alpha-level of 0.05. Individual differences in carcinoma incidence were assessed by Fisher's exact test. Confidence intervals for proportions were calculated using Wilson's method.[38] Statistical tests were 2-sided and were considered significant at $p < 0.05$.

Results

Serum zinc and plasma celecoxib levels

The chemoprevention experiment entailed administering 6 different diets to 7 different animal groups (Fig. 1*a*). To confirm that animals received their assigned diet, we measured serum zinc and plasma celecoxib levels after 15 weeks of intervention. Regardless of celecoxib content in the diet, rats fed a ZD diet had significantly lower serum zinc levels than ZS rats (Fig. 2*e*). Based on the plasma celecoxib levels presented in Figure 2*f*, the mean celecoxib concentration was 249 ng/mL (95% CI 5 161–337) in the 100 ppm group (ZD/Cxb100 and ZR/Cxb100) and 1,191 ng/mL (95% CI = 829–1553) in the 500-ppm group (ZD/Cxb500 and ZR+Cxb500). The 4.8-fold difference in plasma levels thus correctly reflects its intake from the diets containing 100 and 500 ppm celecoxib.

Lingual tumor incidence in control ZD rats

We first determined lingual tumor incidence and multiplicity in ZD rats before the start of intervention. At 9 weeks of NQO exposure, ZD rats had significantly greater tumor incidence and multiplicity than ZS rats (tumor incidence: 100% *vs*. 25%, difference = 75%, 95% CI = 42–91%, $p < 0.0001$; multiplicity: 8.3 *vs*. 0.67, difference 7.6, 95% CI = 6.7–8.5, $p < 0.001$). ZS-tongue typically showed a thickened epithelium, with PCNA-positive nuclei restricted to the basal/suprabasal cell layers (Fig. 1*b*, a and c). In contrast, ZD tongues often harbored SCC, with PCNA-positive nuclei in dysplastic epithelium and tumor tissue (Fig. 1*b*, b and d). Furthermore, 6 of 19 of ZD rats showed progression to malignancy *versus* none of the ZS animals ($p = 0.06$).

At 15 weeks after the cessation of NQO, lingual tumor incidence was 100% in ZD and ZS groups. Large tumor (size ≥ 1.5 mm) incidence and tumor multiplicity, however, were significantly higher in ZD than ZS animals (tumor incidence: 77% *vs*. 29%, difference = 48%, 95% CI = 26–65%, $p < 0.0001$; multiplicity: 16.1 *vs*. 7.0, difference = 9.1, 95% CI = 7.6–10.8, $p < 0.0001$; Figs. 2*b* and *c*). Furthermore, progression to malignancy was significantly more frequent in ZD (50%) than ZS (18%) rats (difference = 32%, 95% CI = 9.8–49%, $p = 0.007$; Fig. 2*d*).

Lingual cancer chemoprevention in ZD rats

We next compared the efficacy of celecoxib without ZR, with ZR, or ZR alone to prevent lingual carcinogenesis in ZD rats. At week 15, control ZD tongues regularly displayed multiple

tumors in the dorsum (Fig. 2*a*). Macroscopically, animals that received celecoxib alone (at both levels) had larger and more lingual tumors than rats given ZR only (Fig. 2*a*) or celecoxib plus ZR (data not shown).

Regardless of the intervention, the incidence of lingual tumors was 100% in all treatment groups. Celecoxib without ZR moderately reduced lingual tumor multiplicity of ZD group by 13% from 16.1 to 13.4 (ZD/Cxb100 vs. ZD, difference 2.7, 95% CI = 0.9–4.4, $p = 0.012$) and by 25% to 12.2 (ZD/Cxb500 *vs*. ZD, difference 4.0, 95% CI = 2.4–5.6, $p < 0.001$), however, it had no effect in decreasing the incidence of large tumors or SCC (Figs. 2*b*–2*d*). ZR alone effectively reduced the incidence of large tumors from the ZD level of 77–37%, the incidence of SCC from 50 to 18% and tumor multiplicity from 16.1 to 7.0 (large tumor incidence, difference = 40%, 95% CI = 21–56%, $p < 0.001$; SCC, difference = 32%, 95% CI = 13–48%, $p = 0.001$; multiplicity, difference = 9.2, 95% CI = 7.6–10.8, $p < 0.001$). Examples of pathology of tongue from representative groups ZD and ZD/Cxb100, showing SCC that covered the entire lingual section, ZR with SCC and ZR + Cxb100 with a large papilloma are shown in Figure 1*c*.

As expected, celecoxib plus ZR significantly reduced: (*i*) large tumor incidence from 77 to 32% and 30% (ZR+Cxb100 versus ZD, difference = 45%, 95% CI = 20–63%, $p < 0.001$; ZR +Cxb500 versus ZD, difference = 47%, 95% CI = 22–64%, $p < 0.001$), (*ii*) SCC incidence from 50 to 14% and 9% (ZR+Cxb100 *vs*. ZD, difference = 36%, 95% CI = 12–53%, $p = 0.004$; ZR+Cxb500 *vs*. ZD, difference = 41%, 95% CI = 19–56%, $p = 0.0006$) and (*iii*) tumor multiplicity from 16.1 to 6.2 and 5.0 (ZR+Cxb100 *vs*. ZD, difference = 10.0, 95% CI = 8.3–11.7, $p < 0.001$; ZR1Cxb500 *vs*. ZD, difference = 11.2, 95% CI = 9.6–12.8, $p < 0.001$). Although combining celecoxib and ZR produced a slight additive effect over ZR, this result is not significant.

The benefit of including ZR with celecoxib treatment is further affirmed when intervention with ZR + Cxb was compared with ZD/Cxb. There was a ~50% reduction in tumor multiplicity and a ~30% decline in large tumor incidence of (multiplicity: ZR + Cxb100 versus ZD/Cxb100, 6.2 *vs*. 13.5, difference = 7.3, CI = 5.8–8.8, $p < 0.001$; ZR + Cxb500 *vs*. ZD/Cxb500, 5.0 *vs*. 12.2, difference = 7.2, CI= 6 2–8.3, $p < 0.001$; large tumor incidence: ZR + Cxb100 *vs*. ZD/Cxb100, 32% *vs*. 63%, difference = 31%, 95% CI = 2–53%, $p = 0.045$; and ZR + Cxb500 *vs*. ZD/Cxb500, 30% *vs*. 74%, difference = 44%, 95% CI = 15–64%, $p = 0.007$; Fig. 2*b*). Together, these data show that nutritional deficit of zinc needs to be corrected in ZD animals for intervention to be successful.

Modulation of NF-κB p65 and COX-2 by dietary zinc

We first determined whether zinc modulates the transcriptional activator NF-κB, a transcription factor that controls the expression of several proinflammatory cytokines, as well as the inducible enzyme COX-2.[39] We probed the p65 subunit of NF-κB in archival serial esophageal sections from zinc-modulated rats of a previous study, which showed COX-2 overexpression under ZD conditions that was reduced upon ZR.[13] Similar to COX-2 expression, [13] NF-κB p65 expression in ZS esophagus was weak and diffuse (Fig. 3*a*, a). Importantly, NF-κB p65 was overexpressed in ZD esophagus (Fig. 3*a*, b), in a pattern similar to that of COX-2, from near serial sections.[13] Upon ZR, NF-κB p65 overexpression was reduced within hours (Fig. 3*a*, c) in a manner similar to that of COX-2 following ZR.[13] These results in serial esophageal sections show a high correlation of temporal and spatial relationship between NF-κB p65 and COX-2 cooverexpression under ZD conditions, and coreduction upon ZR. In addition, phospho-p65 serine 276 antibody detected strong nuclear expression in ZD esophageal epithelium, as compared with ZS esophagus (Fig. 3*a*, e *vs*. d), indicating stimulation and nuclear translocation of NF-κB p65 under ZD conditions. Upon ZR, phospho-p65 staining was similarly reduced (Fig. 3*a*, f). These data demonstrated in an *in vivo* cancer model that COX-2

and NF-κB cooverexpression and core-pression were modulated by dietary zinc. Our findings are consistent with a recent report, which showed in the lung from rats on a ZD diet that COX-2 overexpression was associated with NF-κB activation.[40]

Effect of chemopreventive agents on the expression of 3 predictive biomarkers COX-2, NF-κB p65 and LTA_4H

We next used immunohistochemistry to examine whether the effective inhibition of lingual cancer progression by celecoxib with ZR or ZR alone, as compared with celecoxib alone, was due to differential effects of these agents on expression of the 3 predictive biomarkers: COX-2, a target of celecoxib; NF-κB, a regulator of COX-2 expression and LTA_4H, a rate-limiting enzyme in the biosynthesis of LTB_4. To document the presence and the spatial localization of the 3 biomarkers in tumor and stroma tissue, immunohistchemistry was performed was performed on 19 ZD, 8 ZD/ Cxb500, 5 ZR and 8 ZR+Cxb500 tongues at 52 hr of intervention; and on every lingual SCC section at 15 weeks of intervention (24 control ZD, 8 ZD/Cxb100; 8 ZD/Cxb500; 9 ZR, 3 ZR + Cxb100 and 2 ZR1Cxb500).

Before intervention, ZD tongue typically showed strong and abundant expression of all 3 markers in dysplastic epithelia and tumor tissue (COX-2, Fig. 3*b*, a; NF-κB, Fig. 3*b*, e; and LTA_4H Fig. 3*b*, i). At 52 hr of intervention, ZD/Cxb500 tongue still displayed moderately strong and frequent expression of all 3 markers in hyperplastic epithelia and in tumor tissue (Fig. 3*b*, b, f and j). By contrast, all 5 ZR tongues had reduced, diffuse and infrequent cytoplasmic immunostaining of COX-2 in the proliferative epithelia; diffuse and moderate staining of NF-κB p65 in epithelium and tumor tissue and moderately strong but sparse staining of LTA_4H in stroma with infiltrating inflammatory cells (Fig. 3*b*, c, g and k). All 8 ZR + Cxb500 tongues showed sporadic and moderate staining of all 3 markers (Fig. 3*b*, d, h and l). Together, these data demonstrate that as early as 52 hr after intervention, celecoxib with ZR or ZR alone had a greater ability to reduce the overexpression of these predictive biomarkers than celecoxib without ZR.

At endpoint, lingual SCC from control ZD rats demonstrated persistent, strong and abundant expression of all 3 markers in tumor tissue (Figs. 4, a–c). Celecoxib without ZR reduced slightly the expression of these biomarkers (Fig. 4, d–f), as compared with ZD. As an example, ZD/Cxb100 carcinoma 61 still displayed moderately strong immunostaining of COX-2 and NF-κB p65 and strong and frequent staining of LTA_4H in carcinoma/stroma tissue (Fig. 4, d–f). ZR alone (Fig. 4, g–i) or celecoxib plus ZR treatment (Fig. 4, j–l) produced a general reduction in staining intensity and the number of cells stained of all 3 markers. These data affirm that celecoxib with ZR, or ZR alone was more effective than celecoxib without ZR in curbing the overexpression of these biomarkers. Persistent overexpression of these biomarkers was associated with a more aggressive tumor phenotype, as shown by the gross anatomy of ZD/Cxb100 tongue 61 (Fig. 2*a*).

NMBA-induced forestomach tumorigenesis in zinc-deficient COX-2−/− mice

We next examined the ability of genetic *COX-2* deletion to protect against NMBA-induced forestomach tumorigenesis in mice on zinc-deficient *versus* zinc-sufficient diet. Zinc-sufficient *COX-2* null mice typically displayed fewer forestomach tumors than wild-type controls (Fig. 5*a*, a–c). Forestomach tumor multiplicity was significantly lower in ZS:COX-2+/− mice than ZS:COX-2+/+ mice ([2.3 *vs*. 4.7], difference = 2.2, 95% CI = 1.7–3.5, $p < 0.001$) and in ZS:COX-2−/− than ZS:COX-2+/+ ([2.2 *vs*. 4.7], difference = 2.4, 95% CI = 1.7–3.5, $p < 0.001$). Additionally, none of ZS:COX-2−/− (0 of 17) or ZS:COX-2+/− (0 of 60) developed forestomach SCC versus 2.4% of ZS:COX-2+/+ (1 of 42) mice. Consistent with intestinal and skin tumor studies,[30,31] these data demonstrate that in nutritionally-complete mice *COX-2* deficiency protects against NMBA-induced forestomach carcinogenesis.

Under ZD conditions, *COX-2* deletion, however, did not provide any protection against forestomach tumorigenesis to heterozygous mice and led to an increased tumor multiplicity and tumor incidence in null mice (Fig. 5*a*, d–f). ZD:COX-2−/− mice had significantly greater tumor multiplicity and incidence than ZD:COX-2+/+ mice (multiplicity:4.4 *vs*. 1.5, difference = 2.8, 95% CI = 1.9–3.7, $p < 0.001$; tumor incidence: ZD:COX-2−/− [93%, 13 of 14] *vs*. ZD:COX-2+/+ [61%, 22 of 36], difference = 32%, 95% CI = 4–49%, $p = 0.04$, Figs. 5*b* and 5c). Importantly, 29% (4 of 14) of ZD:COX-2−/− and 4.9% (2 of 41) of ZD:COX-2+/− had forestomach SCC *versus* 2.7% (1 of 36) of ZD:COX-2+/+ mice. The difference in carcinoma incidence was statistically significant higher in ZD:COX-2−/− than ZD:COX-2+/− mice (difference=,26%, 95% CI = 5–52%, $p = 0.018$) and ZD:COX-2+/− mice (difference= 24%, 95% CI = 3–50% at $p = 0.031$). Despite a lower NMBA dosage, ZD:COX-2−/− mice had significantly higher SCC incidence than ZS:COX-2−/− mice (difference= 29%, 95%CI= 4–55%, $p = 0.032$) (Fig. 5*d*).

Histopathological examination revealed that in contrast to ZS:COX-2−/− forestomach that typically displayed a thickened epithelium with moderate basal cell proliferation (Fig. 6*a*, a), ZD:COX-2−/− forestomach showed an array of deep down-growths, papillomas, glandular metaplasia (data not shown) and SCC (mouse 38 and 74: Fig. 6*a*, b and c). Expression of KRT14, a squamous cell tumor marker in esophageal/forestomach tumorigenesis, [15,16] was found only in the basal cell layers of ZS:COX-2−/− forestomach epithelium (Fig. 6*a*, d) but in many cell layers and invasive tumor tissue of ZD:COX-2−/− mouse 38 and 74 (Fig. 6*a*, e and f). In summary, our data document that under conditions of zinc deficiency *COX-2* deletion enhances NMBA-induced forestomach tumorigenesis rather than inhibits carcinogenesis.

LTA_4H overexpression in ZD:COX-2−/− mouse forestomach tumors

We next determined by immunohistochemistry whether the lack of protection against NMBA-induced forestomach tumorigenesis in ZD:COX-2−/− versus ZS:COX-2−/− could be due, at least in part, to a shift of arachidonic acid to the 5-LOX/LTA_4H pathway. LTA_4H expression was assessed in 14 ZD:COX-2−/− forestomach, 17 ZS:COX-2−/− forestomach and 15 ZD:COX-2+/+ or ZS:COX-2+/+ forestomach with the highest tumor count. In wild-type ZD and ZS forestomach, weak to moderate staining of LTA_4H was detected in isolated areas of dysplasia and in stroma with infiltrating inflammatory cells (data not shown). In addition, strong COX-2 immunostaining was detected in dysplastic and tumor tissue of wild-type mice but not in COX-2−/− mice forestomach (data not shown).

Eighty-six percent of ZD:COX-2−/− (12 of 14) forestomach tumors showed strong nuclear and cytoplasmic LTA_4H expression in dysplastic epithelia and tumor tissue (Fig. 6*a*, h and i), in glandular metaplasia (Fig. 6*a*, h, inset) and in stroma with infiltrating inflammatory cells (Fig. 6*a*, h, inset). By contrast, only 29% of ZS:COX-2−/− (5 of 17) forestomach exhibited LTA_4H expression, which were sparse and infrequent in epithelia showing hyperplasia/dysplasia (Fig. 6*a*, g). Despite a lower NMBA dosage, the percentage of mice with LTA_4H overexpression in forestomach epithelia or stroma was significantly greater in ZD:COX-2−/− than ZS:COX-2−/− animals (forestomach, 86% *vs*. 29 %, difference = 56%, 95% CI = 21–75%, $p = 0.003$; stroma with inflammatory cells: 100% *vs*. 41%, difference = 59%, 95% CI = 27–78%, $p < 0.001$). Thus, these data lend support to the concept that under ZD conditions when the COX-2 pathway is blocked by genetic deletion, arachidonic acid metabolism could be shifted to the LTA_4H pathway.

Effect of long-term ZD on cell proliferation and LTA_4H expression in COX-2−/− mouse forestomach

We next determined if in *COX-2* null mice ZD alone might induce LTA_4H overexpression and cellular proliferation, thereby predisposing to carcinogenesis. Macroscopically, 29% (2 of 7)

of ZD:COX-2−/− mice versus none of the ZD:COX+/+ mice showed large forestomach lesions after 15 weeks of ZD diet (Fig. 5*e*). By contrast, *COX-2* null mice on ZS diet for 15 weeks showed a thin and normal forestomach mucosa without any lesions (data not shown). Additionally, PCNA immunohistochemistry revealed a highly hyperplastic ZD:COX-2−/− forestomach with abundant PCNA-positive nuclei in many cell layers and in focal hyperplastic lesions. By contrast, ZS:COX-2−/− forestomach mucosa was thin, with PCNA-positive nuclei mostly in basal cell layers (Fig. 6*b*, b and c *vs*. a). Importantly, hyperplastic ZD:COX-2−/− forestomach exhibited strong and abundant LTA_4H immunostaining in serial sections, as compared with ZS:COX-2−/− forestomach (Fig. 6*b*, e and f *vs*. d). Together, these data show that in COX-2−/− forestomach ZD alone causes unbridled cell proliferation and a shunting of arachidonic acid toward 5-LOX/LTA_4H pathway.

Discussion

We have shown in 2 different zinc-deficient rodent cancer models that targeting the COX-2 pathway alone without correcting nutritional zinc deficiency does not prevent UADT tumor progression. First, we demonstrated in ZD rat NQO-induced tongue cancer model that treatment by celecoxib alone is less effective than celecoxib with zinc replenishment or by zinc replenishment alone in preventing UADT cancer progression. In zinc-deficient rats, celecoxib at 100 and 500 ppm levels significantly decreased lingual tumor multiplicity by 13% ($p = 0.012$) and 25% ($p < 0.001$) but had no effect on the incidence of large tumors or carcinomas. Zinc replenishment alone, however, brought about a significant reduction of all 3 parameters: tumor multiplicity by 58%, incidence of large tumors by 40% and squamous cell carcinoma by 32%. Combination of celecoxib plus zinc replenishment had an additive over zinc replenishment alone, but the result is not statistically significant (Figs. 2*b*–2*d*). Our previous work showed that in ZD rats, zinc replenishment prevents NMBA-induced esophageal carcinogenesis.[22] The present data document the similar ability of zinc replenishment to inhibit lingual carcinogenesis in these animals. The lack of efficacy of celecoxib in curbing lingual cancer progression indicates its inability to block other cancer pathways that may be stimulated under nutritional ZD conditions. In this regard, in animals with complete nutrition, the COX-2 specific inhibitors nimesulide, etodolac and celecoxib inhibited NQO-induced lingual SCC development in rats.[33,34]

Second, we showed that in nutritionally complete ZS mice, genetic disruption of *COX-2* resulted in protection against NMBA-induced forestomach tumorigenesis, a result consistent with those of intestinal and skin tumor studies.[30,31] By contrast, *COX-2* null mice on a ZD diet developed significantly increased tumor multiplicity, incidences of forestomach tumors and carcinoma (Fig. 5). Additionally, zinc-deficient *COX-2* null mice chronically exposed to NQO had more lingual tumors than wild-type control mice, whereas similarly treated zinc-sufficient *COX-2* null mice had less lingual tumors than wild-type controls (unpublished data). The finding that overexpression of LTA_4H was induced by a zinc-deficient diet alone in the hyperplastic forestomach epithelium of *COX-2*−/− mice, and by zinc deficiency plus NMBA treatment in *COX-2*−/− forestomach carcinoma (Fig. 6) demonstrates that under ZD conditions alternative cancer pathways could be stimulated, thereby promoting forestomach carcinogenesis in ZD:COX-2−/− mice. One such pathway could be the shunting of arachidonic acid to the 5-LOX/LTA_4H pathway.

The precise role of the 5-LOX/LTA_4H pathway in cancer is not as clearly defined as the COX-2 pathway. About 2 decades ago, LTB_4 levels were shown to be >10-fold higher in human and hamster oral SCC than in control tissues, an observation that suggests a possible role for LTB_4 in the pathogenesis of head and neck cancer.[41] More recently, overexpression of LTA_4H and 5-LOX protein was demonstrated in rat and human esophageal adenocarcinoma [42,43] and hamster and human oral cancer.[44,45] Our data showing overexpression of LTA_4H

protein in lingual lesions/SCC from ZD rats, as well as in forestomach lesions/SCC from ZD:COX−/− mice, are consistent with the idea that 5-LOX/ LTA_4H pathway is of importance in oral carcinogenesis.[45]

It is now recognized that NF-κB provides a link between inflammation/immunity and cancer development/progression; and the IκB kinase/NF-κB activation pathway is a target for cancer prevention.[46] Uzzo *et al*.[47] reported that zinc supplementation induced NF-κB inhibition by blocking IκB kinase, thereby suppressing the tumorigenic potential of human prostate cancer cells. These authors concluded that zinc supplementation might have important implications in cancer prevention, predominantly through suppression of NF-κB signaling. Here, we demonstrated in archived esophageal serial sections from a previous study[13] that NF-κB (Fig. 3*a*) and COX-2 are cooverexpressed in ZD esophagus and corepressed upon ZR. Our data provide a link between COX-2 and NF-κB in the ZD-driven esophageal carcinogenesis. Furthermore, we showed that ZR alone or celecoxib with ZR was more effective than celecoxib alone in reducing the overexpression of the biomarkers COX-2, NF-κB and LTA_4H in ZD lingual lesions and SCC (Fig. 3 and Fig 4). These data are in agreement with the report that zinc in micromolar concentrations inhibit LTA_4H and LTB_4 biosynthesis in human polymorphonuclear leukocytes.[48]

Given that zinc deficiency *per se* causes extensive alterations in gene expression associated with preneoplasia in rat esophagus, including upregulation of *COX-2, MT-1* and *KRT-14*,[14–19] NF-kB (Fig. 3*a*), *S100A8/A9, SERPINB3* (unpublished data, presented at the 98th American Association of Cancer Research meeting, 2007), it may not be surprising that genetic deletion or pharmacological inhibition of *COX-2* alone does not lead to cancer prevention in zinc-deficient rodents. Zinc is an essential nutrient. Zinc plays an important role in the immune system due to its many roles in basic cellular function.[49] There are more than 300 Zn-containing enzymes. Zinc ions are key structural components of more than 1,000 proteins that are mainly nuclear transcription factors, which contribute to control of cell proliferation, differentiation and apoptosis through regulation of gene expression.[50] Based on the myriad of functions of zinc, the role of zinc deficiency as a cause of disease and as a determinant factor in the progression of disease is now gaining attention.[51]

Although data are scarce on the efficacy of multivitamin and mineral supplement use in the primary prevention of cancer, evidence accumulated to date suggests their potential benefits in cancer prevention in persons with poor nutritional status.[52] In a randomized, controlled trial of selenomethionine (a synthetic form of organic selenium) and/or celecoxib among residents in a poorly nourished Chinese population (Linxian), Limburg *et al*.[53] reported that neither selenomethionine nor celecoxib inhibited esophageal squamous carcinogenesis for high-risk subjects, but selenomethionine showed a protective effect among subjects with mild esophageal squamous dysplasia, thus providing the first report of a possible beneficial effect for any candidate esophageal SCC chemopreventive agent in a randomized controlled trial. In the earlier Linxian nutrition intervention trials of esophageal SCC, no significant reductions in the prevalence of esophageal or gastric dysplasia or cancer were seen with any of the 4 vitamin and mineral supplement groups that included zinc. The prevalence of gastric cancer among participants receiving retinol and zinc was 62% lower than those not receiving those supplements.[54] In a French trial, combined supplementation that included zinc reduced the rate of total cancer incidence and all-cause mortality by 31% in men but not in women who had higher serum levels of antioxidants.[55]

In summary, zinc deficiency is implicated in the etiologies of esophageal and oral cancers. Our data from 2 rodent cancer models demonstrate that in ZD animals while genetic or pharmacological disruption of *COX-2* does not prevent cancer progression, correcting nutritional zinc deficiency is beneficial in preventing UADT cancer development and

progression. Our findings support pursuit of zinc as a potential chemopreventive agent for UADT cancer prevention, particularly among subjects with preexisting nutritional zinc deficit. More broadly, this requirement for zinc replenishment in chemoprevention suggests that the nutritional status of patients should be considered in cancer treatment protocol.

Abbreviations

COX-2, cyclooxygenase-2
cxb, celecoxib
KRT14, cytokeratin
LTA_4H, leukotriene A_4 hydrolase
NF-κB, nuclear factor-κB
NMBA, *N*-nitrosomethylbenzylamine
NQO, 4-nitroquinoline 1-oxide
PCNA, proliferating cell nuclear antigen
SCC, squamous cell carcinoma
UADT, upper aerodigestive tract cancer
ZD, zinc deficiency
ZR, zinc replenishment
ZS, zinc sufficiency
5-LOX, 5-lipoxygenase

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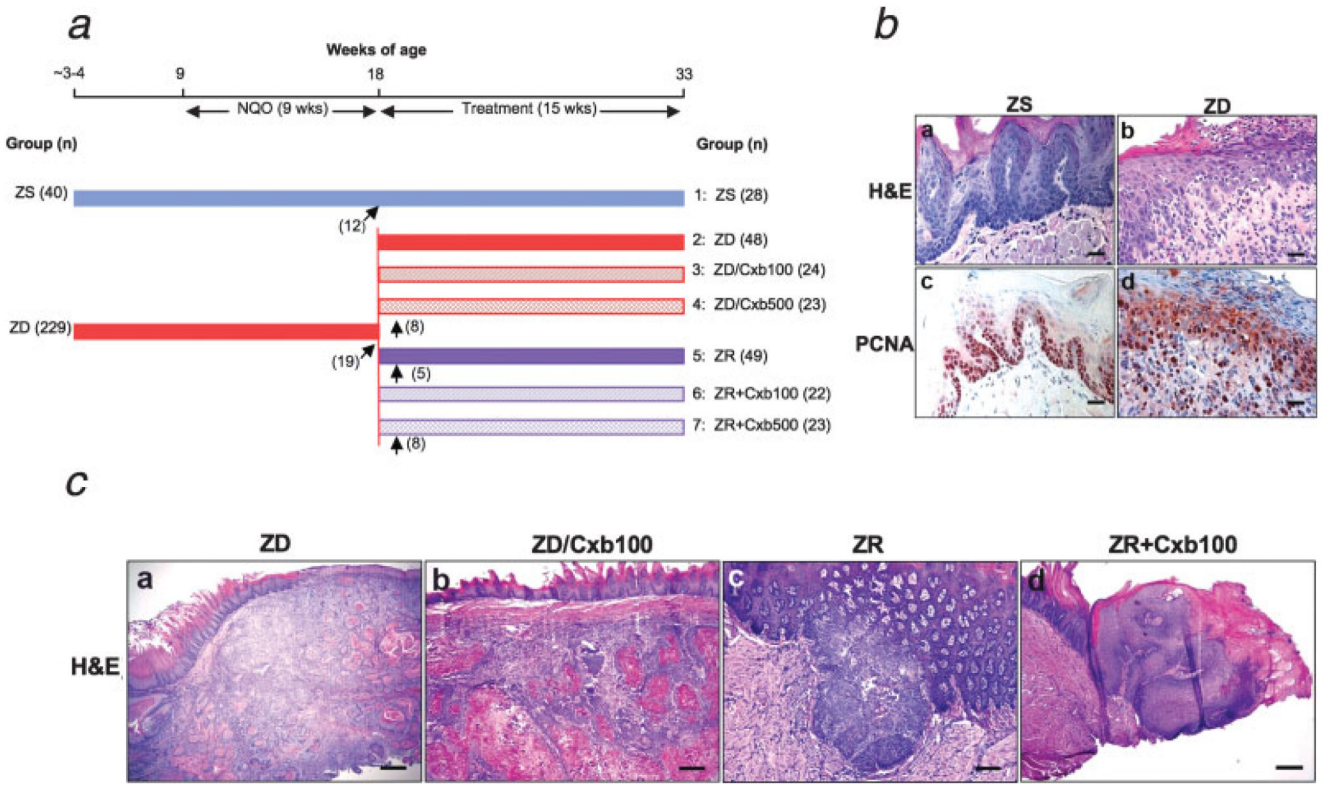

FIGURE 1.

(*a*) Experimental design. Weaning male rats were fed a ZS diet (40 rats) or ZD diet (229 rats) for 5 weeks and then exposed to 10 ppm NQO in deionized water for 9 weeks. NQO administration was then stopped and immediately replaced by deionized water. To determine the number of lingual lesions before the start of chemo-dietary intervention, nineteen ZD and twelve ZS rats were killed at 9 weeks. The remaining animals were taken off NQO administration. Twenty-eight ZS rats were untreated and continued on ZS diet. Two-hundred ten ZD rats were earmarked and randomly divided into 6 groups. Three groups remained on ZD diets that contained 0, 100 or 500 ppm celecoxib (Cxb), forming control ZD (48 rats), ZD/Cxb100 (24 rats) and ZD/Cxb500 (23 rats) groups. Three other groups were zinc-replenished (ZR) by switching to ZS diet, which had 0, 100 or 500 ppm Cxb, thus forming ZR (49 rats), ZR + Cxb100 (22 rats) and ZR + Cxb500 (23 rats) groups. Arrows indicate the times when animals were killed just before intervention and at 52 hr after intervention. (*b*) Histopathology and cell proliferation of tongues from ZS and ZD rats after exposure to NQO for 9 weeks and before intervention. Immunohistochemistry for PCNA was used to identify proliferating cells. (a, b) Hematoxylin and eosin-stained sections; (c, d) PCNA staining. ZS tongues typically showed a thickened epithelium (a) with PCNA-positive nuclei in basal and suprabasal layers (c). Six out of 19 ZD tongues showed microinvasion (b) with PCNA-positive nuclei in tumor tissue (d). (*c*) Histopathology of tongues at 15 weeks of intervention: (a) Invasive lingual SCC from an untreated ZD rat. (b) Invasive lingual SCC from a ZD/Cxb100 rat, (c) Invasive lingual SCC from a ZR rat and (d) Large papilloma from ZR + Cxb100 rat. Scale bars: 400 μm (*c*, a) 200 μm (*c*, b–d) and 25 μm (*b*, a–d).

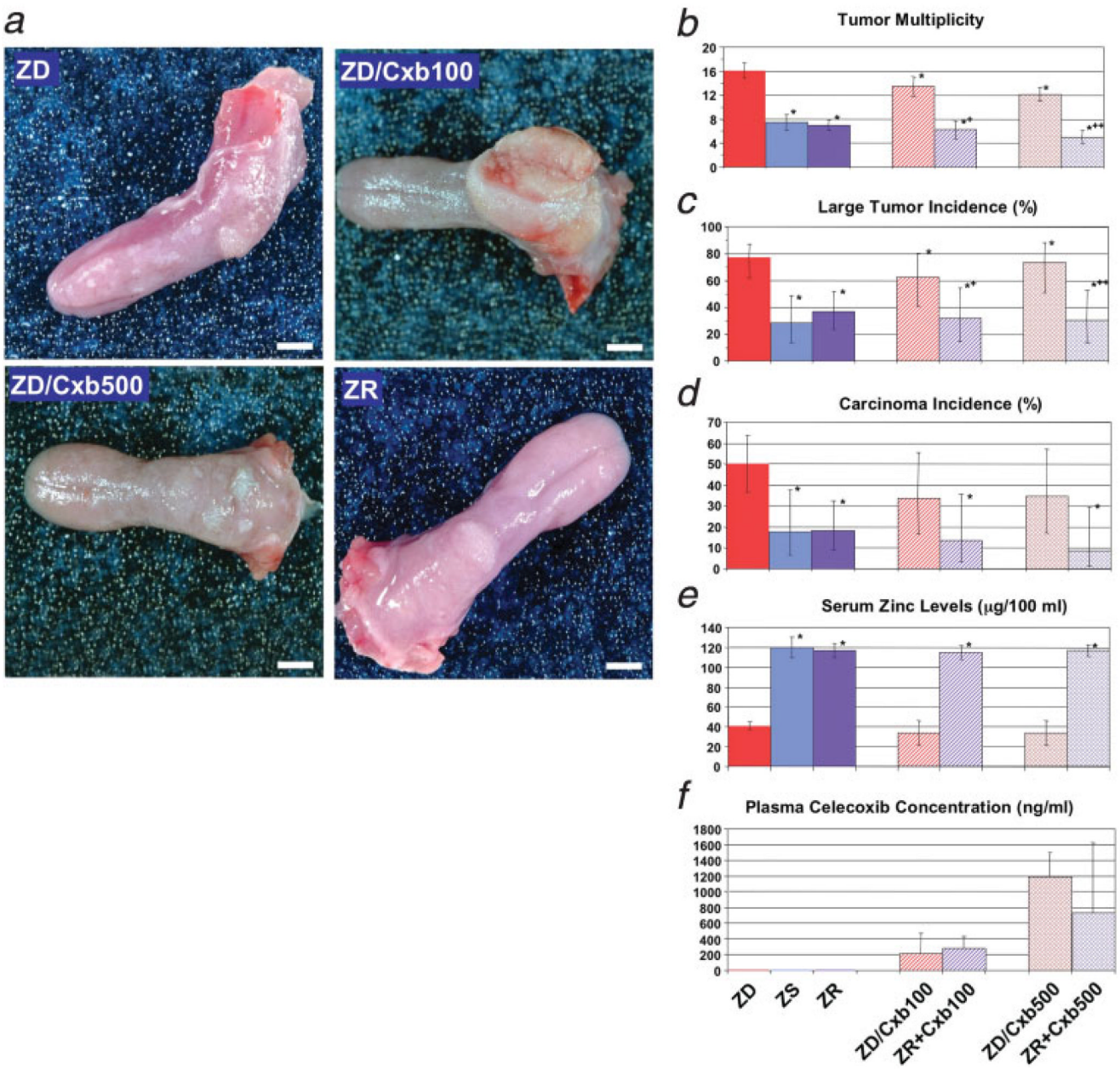

FIGURE 2.

Lingual tumor prevention by celecoxib with or without ZR in ZD rats that were formerly exposed to NQO. (*a*) Macroscopic appearance of tongues at 15 weeks of chemo-dietary intervention. ZD rat 53 showed multiple tumors on dorsal and lateral tongue. ZD/Cxb100 rat 61 showed a huge ulcerated tumor covering the entire posterior dorsum of the tongue. ZD/Cxb500 rat 85 had multiple large lesions on posterior dorsum and small lesions on anterior dorsum. ZR rat 111 showed 3 small lesions on the posterior dorsum of the tongue. (*b*–*f*) Tumor multiplicity, incidence of large tumors (tumors ≥ 1.5 mm), incidence of carcinoma, serum zinc levels and plasma celecoxib concentration were assessed at 15 weeks of intervention. (*b*) Tumor multiplicity (number of tumors per tongue). *All groups are statistically different from ZD group: ZS, ZR, ZR + Cxb100, ZR + Cxb500 and ZD/Cxb500 *versus* ZD, $p < 0.001$, ZD/Cxb100 *versus* ZD, $p = 0.012$. $^{+}$Statistically different from ZD/Cxb100 group, $p < 0.001$. $^{++}$Statistically

different from ZD/Cxb500 group, $p < 0.001$. (*c*) Large tumor incidence (%). *ZR (18 of 49), ZR+Cxb100 (7 of 22) and ZR+Cxb500 (7 of 23) *versus* ZD (37 of 48): $p = 0.00008$, $p = 0.0005$, $p = 0.0002$. ZS (8 of 28) *versus* ZD (24 of 48): $p = 0.00007$. $^{+}$ZR+Cxb100 (7 of 22) *versus* ZD/Cxb100 (15 of 24), $p = 0.045$. $^{++}$ZR+Cxb500 (7 of 23) *versus* ZD/Cxb500 (17 of 23), $p = 0.007$. (*d*) Carcinoma incidence (%). *ZR (9 of 49), ZR+Cxb100 (3 of 22) and ZR+Cxb500 (2 of 23) *versus* ZD (24 of 48): $p = 0.001$, $p = 0.004$, $p = 0.0006$. ZS (5 of 28) *versus* ZD (24 of 48), $p = 0.04$. (*e*) Serum zinc levels (μg/100 mL). Sample size for each group was 10–25 rats. *Serum zinc levels in ZS, ZR, ZR+Cxb100 and ZR+Cxb500 groups are significantly greater than ZD group, $p < 0.001$. (*f*) Plasma celecoxib concentration (ng/mL). Sample size for each group was 3 rats (each from a cage with 6 animals), thus representing the celecoxib plasma content of 18 animals. All statistical tests were 2-sided. Error bars = 95% confidence intervals. Scale bars: 5 mm (*a*).

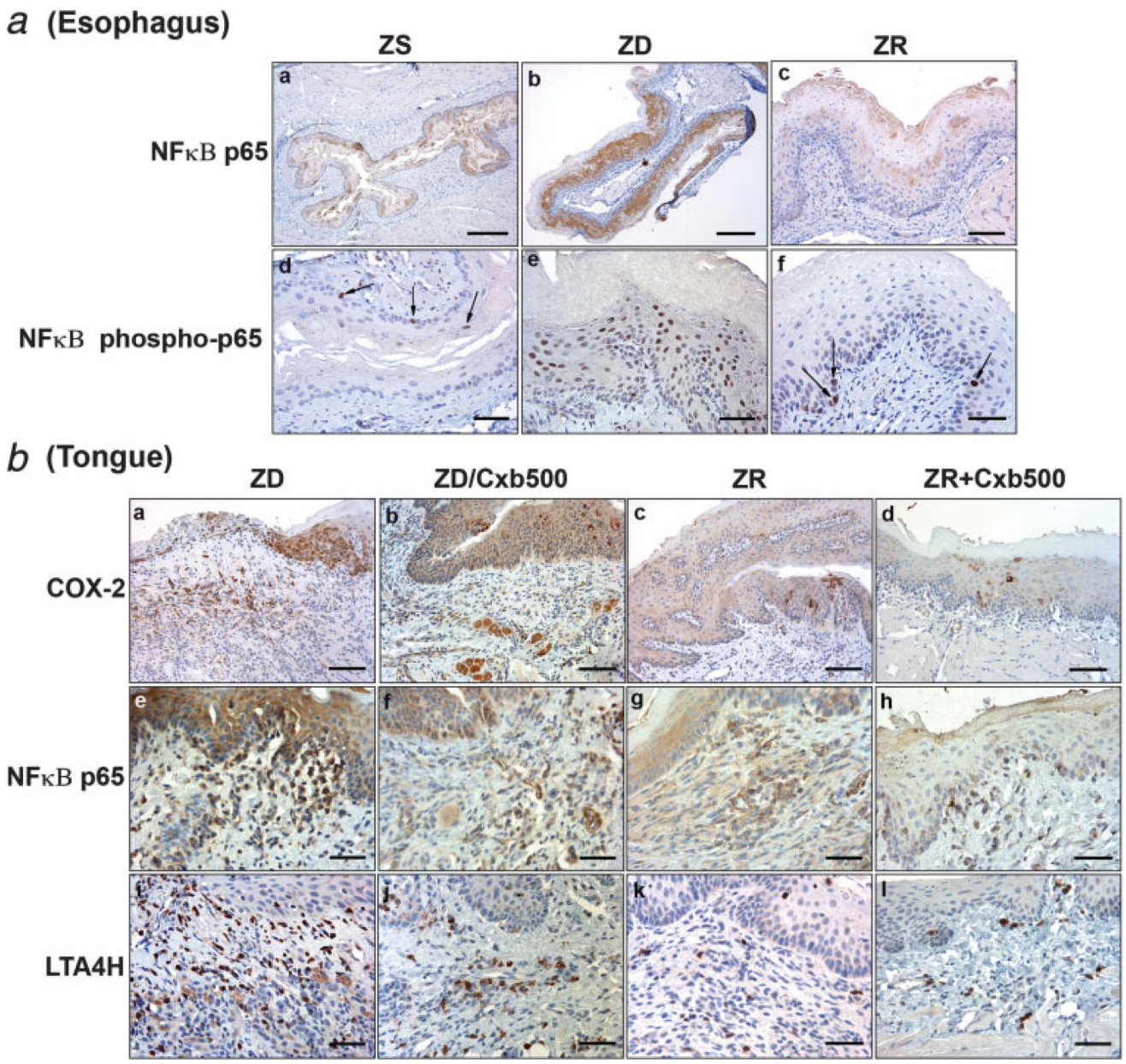

FIGURE 3.

(*a*) Esophagus. Localization of NF-κB p65 and NF-κB phospho-p65 protein in archival esophageal near-serial sections from zinc-modulated rats showing COX-2 modulation by zinc (Ref. 13). (*b*) Tongue. COX-2, NF-κB p65 and LTA_4H protein expression in tongue from NQO-treated ZD rats at 52 hr after switching to diets containing 500-ppm Cxb with ZR or without ZR. Representative sections were presented. (*a*) Esophagus. ZS esophagus typically showed moderately weak and sparse cytoplasmic staining of NF-κB p65 (a). NF-κB was intensely and abundantly expressed in ZD esophagi, with typical cytoplasmic and nuclear staining (b). ZR esophagus at 8 hr after zinc administration showed reduced staining (c) compared with ZD esophagus (b). Pattern of NF-κB p65 expression mirrored that of COX-2 in ZD esophagus (Ref. 13). Phospo-p65 nuclear staining was scarce and restricted to a few nuclei basal cell layer in ZS esophagus (d) but was abundant and in many cell layer in ZD

esophagus (e). At 8 hr after ZR, phospho-p65 expression was sparse and occurred mostly in the nuclei of basal cells (f). (*b*) Tongue. At 0 hr before intervention, ZD lingual SCC showed intense COX-2 (a), NF-κB p65 (e) and LTA_4H (i) protein expression in dysplastic epithelial cell layers and carcinoma. At 52 hr of intervention, ZD/Cxb500 lingual SCC showed moderately strong and frequent staining of COX-2 (b), NF-κB p65 (f) and LTA_4H (j) in dysplastic epithelial cell layers and carcinoma. ZR tongues displayed diffuse cytoplasmic and isolated perinuclear staining of COX-2 (c), diffuse and moderate staining NF-κB p65 (g) and sparse occurrence of LTA_4H expression in stroma (k). ZR+Cxb500 tongue had reduced and sparse occurrence of all 3 markers (COX-2, d; NF-κB p65, h and LTA_4H, l). Scale bars: 100 μm (*a*: a and b; *b*: a–d); 50 μm (*a*: c); 25 μm (*a*: d–f and *b*: e–l).

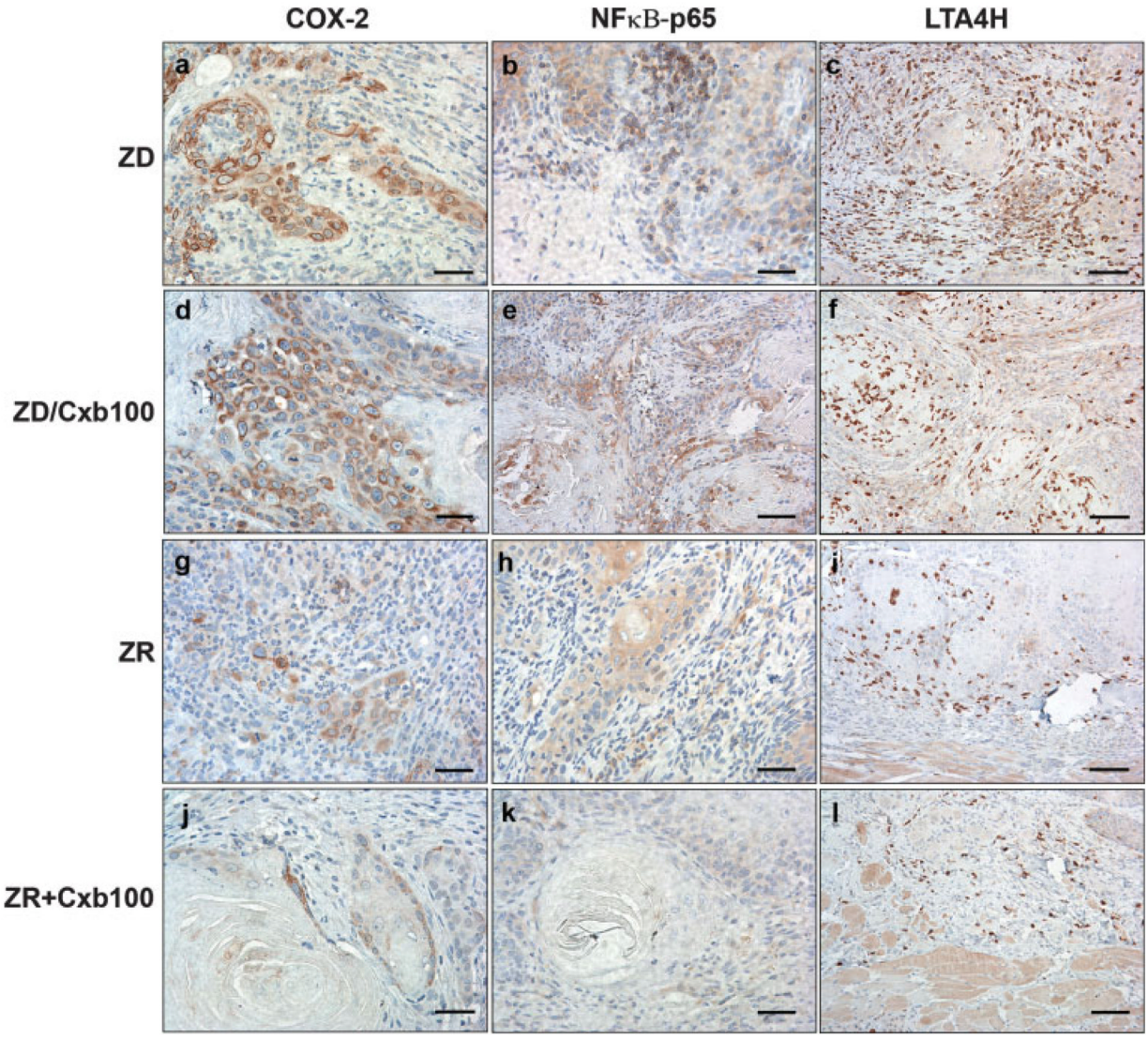

FIGURE 4.

COX-2, NF-κB p65 and LTA_4H protein expression in lingual SCC at 15 weeks after switching to diets containing 100-ppm Cxb with or without ZR, or ZR alone. Representative sections were presented. Control ZD lingual SCC 47 showed strong expression of COX-2, NFκB p65 and LTA_4H in carcinoma tissue and stroma (a–c). ZD/Cxb100 lingual SCC 61 had moderately strong expression of COX-2, NF-κB p65 and LTA_4H in carcinoma tissue (d–f). ZR lingual SCC 123 (g–i) and ZR+Cxb100 lingual SCC 143 (j–l) showed greatly reduced COX-2 staining, absent staining of NF-κB p65 and sparse expression of LTA_4H in stroma. Scale bars: 25 μm (a, b, d, e, g, h, j and k); 50 μm (c, f, i and l).

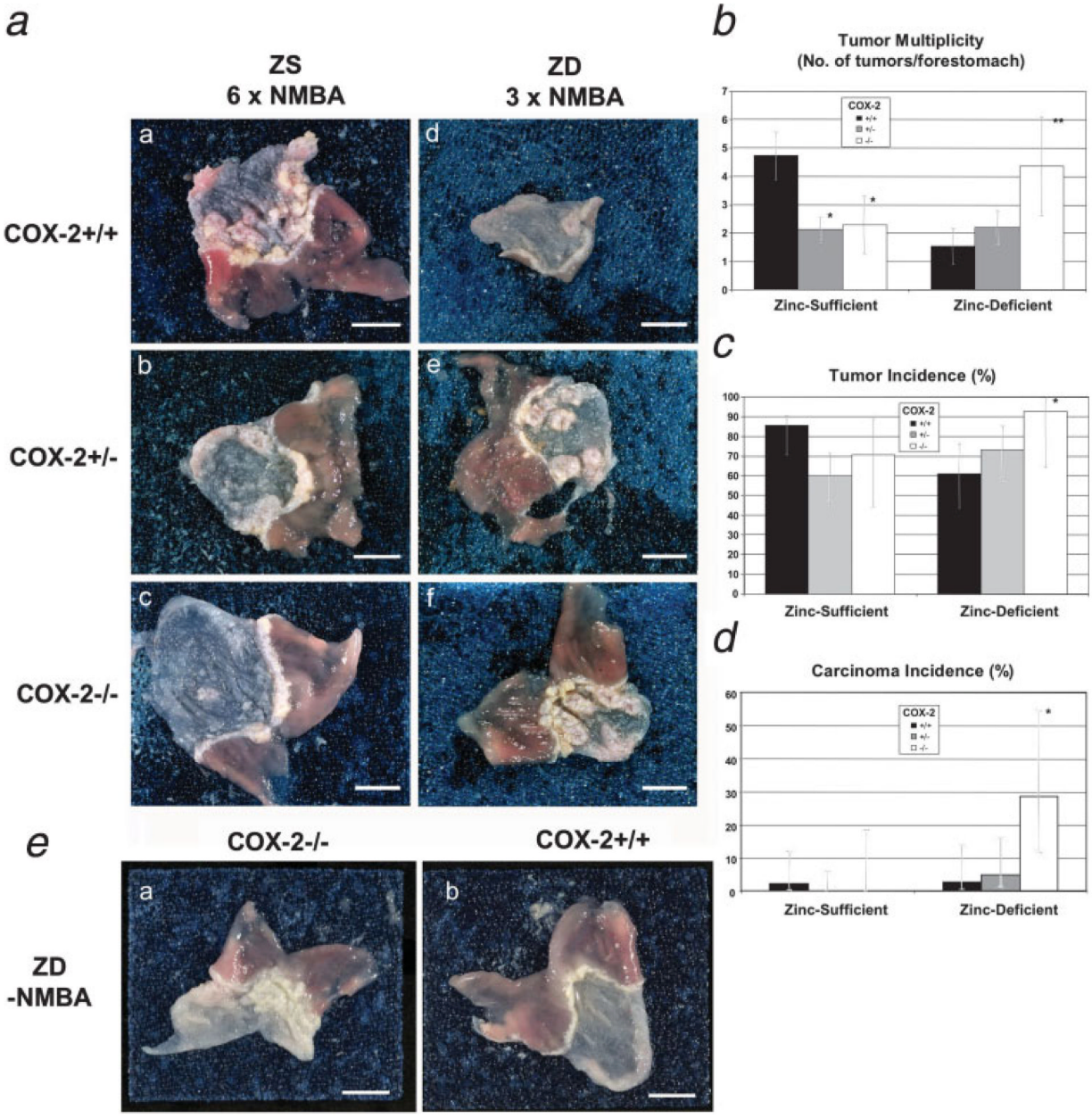

FIGURE 5.

Effect of dietary zinc deficiency on NMBA-induced forestomach carcinogenesis in COX-2 deficient mice. (*a*) Macroscopic appearance of forestomach. ZS and ZD mice were given 6 and 3 intragastric doses of NMBA, respectively, and killed 11 weeks later. Representative sections were presented. (a–c) ZS diet. ZS:COX-2+/+ forestomach 200 had 10 tumors (a), ZS:COX-2 +/− forestomach 173 showed 4 tumors (b) and ZS:COX-2−/− forestomach 199 showed 2 tumors (c). (d–f). ZD diet. ZD:COX-2+/+ forestomach 69 had 2 tumors (d), ZD:COX-2+/− forestomach 76 showed 5 tumors (e); and ZD:COX-2−/− forestomach 74 showed multiple fused tumors (f). (*b*) Tumor multiplicity (number of tumors per forestomach). *ZS:COX-2+/ − *versus* ZS:COX-2+/+, $p = < 0.001$; ZS:COX-2−/− *versus* ZS:COX-2+/+, $p = 0.001$. **ZD:COX-2−/− *versus* ZD:COX-2+/+, $p < 0.001$. (*c*) Tumor incidence (%) ZD:COX-2−/−

(13 of 14) *versus* ZD:COX-2+/+ (22 of 36), $p = 0.04$. (*d*) Carcinoma incidence (%) ZD:COX-2 −/− (4 of 14) versus ZD:COX-2+/+ (1 of 36), difference = 16.3%, 95% confidence interval [CI] = 2.5–55%, $p = 0.018$. All statistical tests were 2-sided. (*e*) Macroscopic appearance of forestomach after 15 weeks of ZD diet without NMBA treatment. (a) COX−/− mouse 108 showed a thickened forestomach mucosa with lesions. (b) COX+/+ mouse 109 showed a thickened mucosa. Error bars = 95% CI. Scale bars: 5 mm (*a*, *e*).

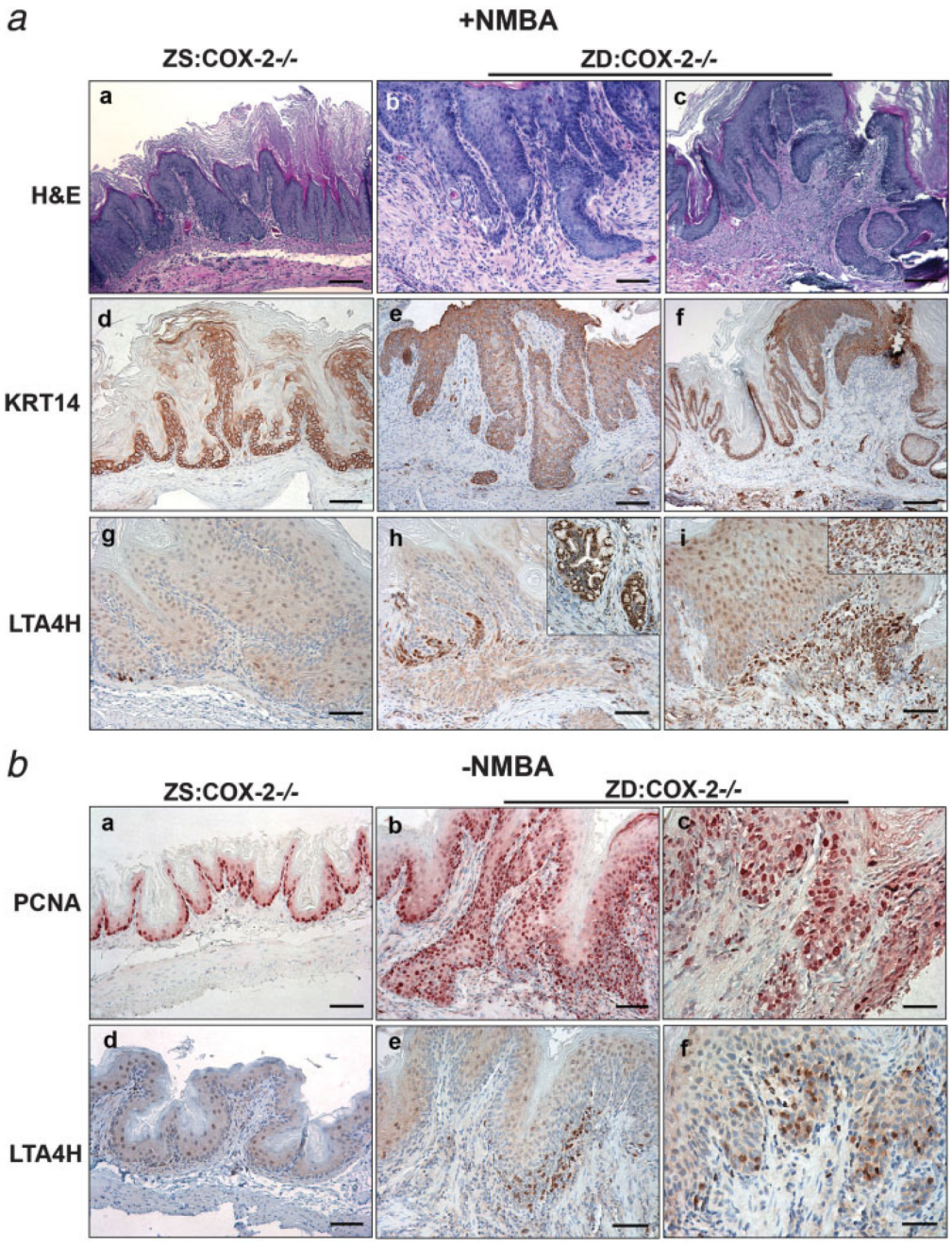

FIGURE 6.

(*a*) Histopathology, KRT 14 and LTA_4H protein expression in zinc-sufficient ZS:COX-2−/− and zinc-deficient ZD:COX-2−/− mice forestomach at 11 weeks after NMBA treatment. (*b*) Effect of 15 weeks of dietary zinc deficiency in the absence of the carcinogen NMBA on cellular proliferation (assessed by PCNA immunohistochemistry) and LTA_4H protein expression in COX-2−/− mice. Representative sections were presented. (*a*) At week 11 of NMBA treatment, ZS:COX-2−/− mouse 174 showed a thickened forestomach epithelium (a), with KRT14 expression restricted to basal and suprabasal cell layers (b) and sparse occurrence of LTA_4H protein expression (c). ZD:COX-2−/− mouse 38 and 78 showed invasive forestomach SCC (b, c), with KRT14 expression in carcinoma tissue (e, f), and LTA_4H

expression in dysplastic and carcinoma (h, i), glandular metaplasia (inset in h) and in stroma (inset in i). (*b*) At week 15 of ZS or ZD diet without NMBA treatment, ZS:COX-2−/− mouse forestomach was typically thin with PCNA-positive nuclei restricted to basal cells, with weak and diffuse LTA_4H protein expression in epithelium. ZD:COX-2−/− forestomach 104 and 108 showed abundant PCNA-positive cells in highly proliferative and dysplastic epithelia (b, c) and intense LTA_4H protein expression in near serial forestomach sections (e, f). Scale bars: 100 μm (*a*: a, c, f); 50 μm (*a*: b, d, e, g–i; *b*: a–c) and 25 μm (*b*: d, c, f).