Esophageal Cancer Prevention in Zinc-Deficient Rats: Rapid Induction of Apoptosis by Replenishing Zinc

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Background: Nutritional zinc deficiency in rats increases esophageal cell proliferation and the incidence of N-nitrosomethylbenzylamine (NMBA)-induced esophageal tumors. Replenishing zinc with a zinc-sufficient diet reduces these effects in zinc-deficient (ZD) rats. We investigated whether apoptosis was involved in the reduction of NMBA-induced esophageal tumors when ZD rats consumed a zinc-sufficient diet. Methods: Weanling rats were fed a ZD diet (zinc at 3–4 ppm) for 5 weeks to establish esophageal cell proliferation, then treated once with NMBA (2 mg/kg body weight), and divided into the following five groups (47–100 per group). One ZD group was fed the ZD diet, and four zinc-replenished (ZR) groups, ZR1, ZR24, ZR72, and ZR432, were fed a zinc-sufficient diet (zinc at 74–75 ppm) beginning 1, 24, 72, and 432 hours, respectively, after NMBA treatment. From 24 hours to 2 weeks after beginning a zinc-sufficient diet, esophagi from all ZR groups were analyzed for apoptosis and cell proliferation; ZD esophagi were the controls. Tumor incidence was determined 15 weeks after zinc replenishment. All statistical tests were two-sided. Results: Zinc replenishment initiated shortly after NMBA treatment effectively reduced esophageal tumorigenesis; 8% (three of 37) of ZR1, 14% (five of 37) of ZR24, 19% (five of 26) of ZR72, and 48% (19 of 40) of ZR432 rats developed esophageal tumors compared with 93% (14 of 15) of ZD animals (all P < 0.001). Importantly, 24 and 30 hours after zinc replenishment, esophagi had numerous apoptotic cells (% apoptotic cells: 0 hour = 2.9%, 95% confidence interval [CI] = 2.5% to 3.3%; 24 hours = 9.4%, 95% CI = 8.2% to 10.6%), and the expression of the proapoptotic Bax protein doubled. Within 48 hours, the ZR432 epithelium was three to five cell layers thick compared with 10–20 layers before zinc replenishment. Conclusions: Zinc replenishment of NMBA-treated ZD rats rapidly induces apoptosis in esophageal epithelial cells and thereby substantially reduces the development of esophageal cancer. [J Natl Cancer Inst 2001;93:1525–33]

The zinc-deficient (ZD) rat esophageal cancer model (1–6) is well suited for the analysis of mechanisms involved in the development and prevention of esophageal cancer and is relevant to human esophageal cancer. Sustained, uncontrolled cell proliferation occurs in the ZD esophageal epithelium, and ZD rats are much more sensitive to N-nitrosomethylbenzylamine (NMBA), which specifically induces esophageal tumors that are morphologically similar to human esophageal tumors (7). Epidemiologic data provide evidence that nutritional zinc deficiency (8–10) and exposure to carcinogenic N-nitrosamines, including NMBA (9,11–13), are associated with an increased risk of human esophageal squamous cell carcinoma. Increased esophageal cell proliferation has been noted in persons at high risk for esophageal cancer in China (14,15). In general, cell proliferation is associated with chemical carcinogenesis (16) and is used as a risk biomarker in cancer chemoprevention studies (17).

NMBA is used extensively to induce esophageal cancer in rats (13). Esophageal cytochrome P450 enzymes specifically bioactivate NMBA (18). Although the N-7 position of guanine in DNA is the major site of NMBA-mediated alkylation, methylation at the O-6 position is more biologically relevant because the O6-methylguanine adduct is associated with base mispairing and mutagenesis (19,20). NMBA methylates rat esophageal DNA with a concomitant accumulation of O6-methylguanine (21), and elevated levels of O6-methyldeoxyguanosine have been found in esophageal mucosal DNA from people living in areas that are associated with a high risk for esophageal cancer (22).

Using the ZD rat model, Fong et al. (3) have shown that, after a single, otherwise nontumorigenic dose of NMBA (23), sustained, increased cell proliferation in the ZD esophageal epithelium was associated with a highly tumorigenic response and accompanying genetic events. In addition, we demonstrated (24) that, if a zinc-sufficient diet was administered to ZD rats after the second of six NMBA doses, esophageal cell proliferation was effectively reversed and tumor incidence was reduced from 100% in ZD rats to 14% in pair-fed zinc-replenished (ZR) rats (whose food consumption matched that of ZD rats) and to 26% in ZR rats fed a zinc-sufficient diet ad libitum. Our results support the nutritional intervention studies (25) that were conducted on human populations in areas associated with a high risk for esophageal cancer. In these studies, human subjects who received supplemental multiple vitamins and minerals, including zinc, appeared to have reduced esophageal cell proliferation.

We have reported (5) that the ZD rat esophagus contained many proliferating cells in pre-existing focal hyperplastic lesions and that these cells had altered expression profiles for genes controlling the G1- to S-phase transition. Twenty-four hours after treatment with a single dose of NMBA, the size of the focal hyperplastic lesions increased; cyclin D1, cyclin-dependent kinase-4 (cdk4), and the retinoblastoma protein (pRb) were overexpressed in the lesions; however, p16ink4a was not
expressed. One week later, we found (5) esophageal tumors in ZD rats. Thus, tumor initiation by NMBA is extremely rapid in the ZD esophagus.

For decades, studies on oncogenesis have focused on the regulation of cell proliferation (26); in the last decade, studies have also focused on apoptosis [reviewed in (27–30)]. In a pathologic condition, apoptosis is a protective mechanism because damaged or abnormal cells are eliminated. Members of the Bcl-2 family of proteins are among the most important regulators of apoptosis. Some members, including Bcl-2, inhibit apoptotic cell death, whereas others, including Bax, promote apoptotic cell death.

In this study, we investigate whether zinc replenishment rapidly induces apoptosis in esophageal epithelial cells of NMBA-treated ZD rats, thus inhibiting tumorigenesis.

**Materials and Methods**

**Chemicals and Animal Diets**

NMBA was from Ash Stevens, Inc. (Detroit, MI). Custom-formulated, egg white-based, ZD and zinc-sufficient diets were prepared by Teklad (Madison, WI). The two diets were identical except for the amount of zinc carbonate, which was 3–4 ppm for the ZD diet and 74–75 ppm for the zinc-sufficient diet (2). Zinc levels in these diets were monitored regularly by atomic absorption spectroscopy.

**Experimental Design**

This study was approved by the Thomas Jefferson University Institutional Animal Care and Use Committee, Pittsburgh, PA, and was conducted under National Institutes of Health guidelines. The experimental design is depicted in Fig. 1. Three hundred thirty-five weanling male Sprague-Dawley rats (Taconic, Germantown, NY) were given a ZD diet and deionized water for 5 weeks to establish increased cell proliferation in the esophagus and are referred to as ZD rats. For the determination of the extent of esophageal cell proliferation before consuming a zinc-sufficient diet, seven ZD rats were killed (0 hour). The remaining 328 rats were given a single intragastric dose of NMBA (2 mg/kg body weight) and divided into five groups (62 rats in the ZD group, 100 in ZR1, 58 in ZR24, 47 in ZR72, and 61 in ZR432). Rats in the ZD group continued on the ZD diet. Rats in the other groups were replenished with zinc by being switched to a zinc-sufficient diet (and pair-fed to match the food consumption of ZD animals) beginning at 1, 24, 72, and 432 hours (18 days) after the NMBA treatment, thus forming ZR groups ZR1, ZR24, ZR72, and ZR432, respectively. ZD rats showed regrowth of hair, a sign of zinc replenishment, about 1 week after beginning the zinc-sufficient diet (24). To investigate cell proliferation and apoptosis in the esophageal epithelium, we killed rats in the ZR1 group 24 hours (n = 21 rats), 30 hours (n = 21 rats), 48 hours (n = 7 rats), 1 week (n = 7 rats), and 2 weeks (n = 7 rats) after zinc replenishment and in the control ZD group (n = 7–15 rats) at similar times after NMBA treatment. Because the ZR1 rats were fed the zinc-sufficient diet 1 hour after the single NMBA treatment, we will assume that 24 hours after zinc replenishment is comparable to 24 hours after NMBA treatment, etc. For the ZR24, ZR72, and ZR432 groups, seven rats from each group were also killed at 48 hours, 1 week, and 2 weeks after zinc replenishment. Detailed cell proliferation and apoptosis results, however, are not reported for these groups. The remaining rats (15 in the ZD group, 37 in ZR1, 37 in ZR24, 26 in ZR72, and 40 in ZR432) were killed 15 weeks after zinc replenishment to determine the incidence of tumors. In ZD rats, a single NMBA treatment produces esophageal tumors at an incidence of 80%–95% after 15 weeks (3,5,6).

**Tumor Analysis, Serum Zinc Analysis, and Isolation of Esophageal Tissues**

To minimize the effects of diurnal variation on the measurement of cell proliferation and cell death, we killed all rats between 9 AM and 12 noon. At that time, blood was collected from the retro-orbital venous plexus of each animal after being anesthetized with isoflurane (Datex-Ohmeda Inc., Andover, MA), and serum was prepared for zinc analysis. The level of serum zinc was determined by inductively coupled plasma-mass spectrometry (Perkin-Elmer Elan 6000; Perkin-Elmer, Inc., Wellesley, MA) as described previously (31). Whole esophagi were excised and opened longitudinally. Esophageal tumors greater than 1 mm in diameter were mapped and counted. A small portion of the upper one third of the esophagus was cut, fixed in buffered formalin, and embedded in paraffin. Serial cross-sections (4 μm) were stained with hematoxylin–eosin or left unstained for immunohistochemical studies.

**Determination of Cell Proliferation by Proliferating Cell Nuclear Antigen Immunohistochemistry**

Proliferating cell nuclear antigen (PCNA) localization is used to identify cell cycle subpopulations in G1, S, G2, and M phases: dark-staining nuclei = S-phase cells; light-staining nuclei = G1–S- and G2-phase cells; cells with cytoplasmic staining = usually mitoses; and nonstaining nuclei = quiescent (G0 phase) cells (32). We localized PCNA in esophageal sections as follows: The sections were deparaffinized, rehydrated in a graded alcohol series, and heated in 0.01 M sodium citrate (pH 6.0) in a microwave oven (90°C, for three 5-minute periods). Nonspecific binding sites then were blocked with goat serum (at room temperature for 1 hour), and the sections were incubated with mouse anti-PCNA monoclonal antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:250 dilution, then with biotinylated goat anti-mouse antibodies, and finally with streptavidin horseradish peroxidase. The location of PCNA was then visualized by incubation with the 3-amino-9-ethylcarbazole substrate–chromogen system (Dako Corp., Carpinteria, CA), and the sections were lightly counterstained with hematoxylin. Cells with a red reaction product in the nucleus were defined as positive for PCNA. Preliminary analysis (data not shown) found a good association between the number of S-phase cells measured with PCNA and the number measured with bromodeoxyuridine (2). The PCNA labeling index, expressed as a percent, was calculated by dividing the number of PCNA-labeled nuclei by the total number of cells counted per cross-section taken from the upper one third of the esophagus. Because ZR1 esophagi were mostly tumor free, for comparison, the proliferative and apoptotic data for ZD esophagi were thus assayed in tumor-free esophageal areas.

**Apoptosis Analysis**

Apoptosis was assessed by the terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate end labeling (TUNEL) method and by morphologic characterization of cells in sections stained with hematoxylin–eosin.
TUNEL assay. The 3′-OH end labeling of DNA in tissue sections was performed with an ApopTag in situ peroxidase detection kit (Intergen® Co., Purchase, NY). The sections were deparaffinized, rehydrated in a graded alcohol series, and incubated with proteinase K (20 μg/mL, 37°C for 10 minutes). Endogenous peroxidase in the sections was inhibited with 3% hydrogen peroxide, and the slides were incubated (37°C for 1 hour) with terminal deoxynucleotidyltransferase to catalyze the addition of digoxigenin-labeled nucleotides to the 3′-OH ends of fragmented DNA. Next, the slides were incubated with horse-radish peroxidase-conjugated anti-digoxigenin antibodies, and DNA fragmentation was detected by staining with 3,3′-diaminobenzidine tetrahydrochloride. Finally, the sections were counterstained with methyl green. Sections from rat mammary gland (Intergen® Co.), in which extensive apoptosis occurs, served as a positive control. Negative controls omitted terminal deoxynucleotidyltransferase.

Morphologic criteria. The morphology of apoptotic cells depends on their stage in the process. Apoptotic morphologies include 1) diffuse cytoplasmic staining with only minimal nuclear condensation, 2) distinct apoptotic bodies that were not evident in the esophageal sample.

Bax and Bcl-2 Immunohistochemistry

For the detection of Bax and Bcl-2, the sections were incubated overnight at 37°C in a humidified chamber with a rabbit anti-Bcl-2 polyclonal antiserum (Santa Cruz Biotechnology, Inc.) at a 1:3000 dilution or with a rabbit anti-Bax polyclonal antiserum (Santa Cruz Biotechnology, Inc.) at a 1:800 dilution, followed by incubation with a biotinylated goat anti-rabbit antibody serum. Bcl-2 and Bax expression was visualized with 3,3′-diaminobenzidine tetrahydrochloride. The percentage of cells positive for Bax or Bcl-2 was determined as described previously (33,34) and 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 (33).

Statistical Analysis

Data on cell proliferation and apoptosis were analyzed by one-way analysis of variance with the SAS statistical computer program (35). Tumor incidence differences were analyzed by a two-sided Fisher’s exact test (36). All statistical tests were two-sided and were considered to be statistically significant at P<.05.

RESULTS

General Observations and Serum Zinc Content After Zinc Replenishment

After 4 weeks on a deficient diet (zinc = 3–4 ppm), ZD rats showed retarded growth, loss of hair, immature hair coat, and definite foci of alopecia (2). After 1 week on a zinc-sufficient diet, the skin lesions regressed and hair regrowth was apparent in all ZR groups, and all of the animals had a normal hair coat in 2 weeks. Table 1 shows that the level of serum zinc in ZR1 rats rose from a ZD level of 44 μg/100 mL (95% confidence interval [CI] = 38 to 50 μg/100 mL) to 313 μg/100 mL (95% CI = 307 to 320 μg/100 mL) at 2 days and to 267 μg/100 mL (95% CI = 232 to 302 μg/100 mL) at 30 hours after zinc replenishment. Thereafter, the level of serum zinc declined to 178 μg/100 mL (95% CI = 156 to 200 μg/100 mL) at 48 hours, 150 μg/100 mL (95% CI = 130 to 170 μg/100 mL) after 2 weeks, and 98 μg/100 mL (95% CI = 88 to 108 μg/100 mL) after 15 weeks. The level of serum zinc at 15 weeks was lower than reported previously (24), but it was still within the accepted normal range for NMBA-treated and untreated zinc-sufficient rats of comparable age (37). After zinc replenishment, the levels of serum zinc in ZR24, ZR72, and ZR432 rats followed a pattern similar to that of ZR1 rats after replenishment (data not shown). The initial surge in the serum zinc level before the zinc was distributed to zinc-starved tissues, such as the esophagus, probably reflects the appearance of zinc after the first meal on the zinc-sufficient diet. Fong et al. (1) have observed that the level of esophageal zinc in ZD rats was substantially lower than that in control rats on a zinc-sufficient diet.

Table 1. Serum zinc levels in zinc-replenished rats

<table>
<thead>
<tr>
<th>Group*</th>
<th>0 h</th>
<th>24 h</th>
<th>30 h</th>
<th>48 h</th>
<th>1 wk</th>
<th>2 wk</th>
<th>15 wk</th>
</tr>
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<tbody>
<tr>
<td>ZD †</td>
<td>44</td>
<td>37</td>
<td>33</td>
<td>46</td>
<td>48</td>
<td>46</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>(38 to 50)</td>
<td>(21 to 53)</td>
<td>(25 to 41)</td>
<td>(39 to 53)</td>
<td>(38 to 58)</td>
<td>(37 to 55)</td>
<td>(34 to 52)</td>
</tr>
<tr>
<td>ZR ‡</td>
<td>—</td>
<td>313‡</td>
<td>267‡</td>
<td>178‡</td>
<td>170‡</td>
<td>150‡</td>
<td>98‡</td>
</tr>
<tr>
<td></td>
<td>(239 to 387)</td>
<td>(232 to 302)</td>
<td>(156 to 200)</td>
<td>(124 to 216)</td>
<td>(130 to 170)</td>
<td>(88 to 108)</td>
<td></td>
</tr>
</tbody>
</table>

*ZD = zinc-deficient; ZR = zinc-replenished (replenished 1 hour after N-nitrosomethylbenzylamine treatment).
†Results are mean values with 95% confidence interval (CI). Sample size for each time point was five to 11 rats. Serum zinc analysis was performed by inductively coupled plasma-mass spectrometry.
‡Statistically significantly different from respective ZD group, P<.001. All statistical tests are two-sided.
A surge in the expression of Bax, an apoptosis-promoting protein, was observed in the cytoplasm of the suprabasal cells undergoing apoptosis at 24 and 30 hours (Fig. 2, G; 30 hours: Fig. 3, O). In general, proliferative ZD esophagi weakly expressed cytoplasmic Bax at 24 and 30 hours, although stronger expression was detected in the cytoplasm of an occasional apoptotic cell (24 hours: Fig. 3, C; 30 hours: Fig. 3, K). Conversely, expression of Bcl-2, the apoptosis-inhibiting protein, was more intense in the proliferating cells of the ZD esophagi (24 hours: Fig. 3, D; 30 hours: Fig. 3, L) than in ZR1 esophagi (24 hours: Fig. 3, H; 30 hours: Fig. 3, P).

Within 48 hours of zinc replenishment, the epithelium of ZR1 esophagi had thinned to a thickness of three to five cells with mild folding and was covered by a thin keratin layer (48 hours: Fig. 2, H; 1 week: Fig. 2, I). In addition, esophageal cell proliferation was reduced substantially in ZR1 esophagi, as shown by PCNA immunohistochemistry, and PCNA-positive cells were found mostly in the basal cell layer (24 hours: Fig. 3, R; 48 hours: Fig. 3, T). In contrast, many PCNA-positive cells were observed in the basal and suprabasal layers of the proliferative ZD esophagi (24 hours: Fig. 3, Q; 48 hours: Fig. 3, S). At 15 weeks, ZR1 esophagi had a thin epithelium covered by a thin layer of keratin (Fig. 2, J), although papillomas were found in three of 37 ZR1 esophagi and focal hyperplastic lesions were found in two others (data not shown). In marked contrast, ZD esophagi consistently had parakeratosis, hyperkeratosis, hyperplasia, dysplasia, and focal hyperplastic lesions. Examples are shown in Fig. 2, C (48 hours) and D (1 week). At 15 weeks, 14 of 15 ZD rats had developed esophageal tumors (Fig. 2, E).

### Bax/Bcl-2 Immunoreactive Ratio

Under ZD conditions, Bax protein expression was mostly weak and scattered in the suprabasal cell layers of the highly proliferative ZD esophagi, whereas Bcl-2 protein expression was moderate to intense [data not shown, but see (6)]. A semiquantitative immunostaining analysis of ZD esophagus produced immunoreactive scores of 3.0 (95% CI = 2.0 to 4.0) at 0 hour and 3.3 (95% CI = 2.5 to 4.1) at 15 weeks for Bax expression and 6.7 (95% CI = 6.0 to 7.4) at 0 hour and 6.2 (95% CI = 5.8 to 6.6) at 15 weeks for Bcl-2 expression. These data result in a Bax/Bcl-2 ratio of 0.5 (95% CI = 0.3 to 0.7) at 0 hour and 0.5 (95% CI = 0.4 to 0.6) at 15 weeks (Table 3). The Bax/Bcl-2 ratio remained largely unchanged 24 and 30 hours after NMBA treatment. In contrast, zinc replenishment stimulated a considerable increase in Bax expression within 24 hours (Fig. 3, G), as reflected in an immunoreactive score of 6.0 (95% CI = 5.0 to 7.0) and a Bax/Bcl-2 ratio of 1.3 (95% CI = 1.1 to 1.5) (Table 3). This nearly threefold increase in the Bax/Bcl-2 ratio compared with that of the ZD esophagus reflects an increase in the expression of Bax protein and a decrease in the expression of Bcl-2 protein. Similarly, at 30 hours and at 15 weeks, ZR1 esophagi showed substantially higher Bax immunoreactive scores and lower Bcl-2 immunoreactive scores than ZD esophagi, again giving rise to a higher Bax/Bcl-2 immunoreactive ratio (Table 3).

### Rapid Induction of Apoptosis

Throughout the experimental period, the apoptotic index in ZD rats remained at a steady level of 2.9% (95% CI = 2.5% to 3.3%) at 0 hour to 3.2% (95% CI = 2.9% to 3.5%) at 15 weeks (Fig. 4; P<.001). Zinc replenishment elicited a rapid induction of

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**Table 2. Zinc replenishment initiated hours after N-nitrosomethylbenzylamine (NMBA) treatment: reduction of esophageal carcinogenesis**

<table>
<thead>
<tr>
<th>Animal group* (time between NMBA treatment and initiation of zinc replenishment)</th>
<th>Tumor incidence† (95% confidence interval)</th>
<th>No. of tumors per esophagus‡ (95% confidence interval)</th>
<th>Body weight, g (95% confidence interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZD (not replenished)</td>
<td>14/15 (93)</td>
<td>3.1 (1.9 to 4.3)</td>
<td>254 (236 to 272)</td>
</tr>
<tr>
<td>ZR1 (1 h)</td>
<td>3/37 (8)</td>
<td>0.08 (0 to 0.17)</td>
<td>243 (234 to 252)</td>
</tr>
<tr>
<td>ZR24 (24 h)</td>
<td>5/37 (14)</td>
<td>0.14 (0.03 to 0.25)</td>
<td>247 (238 to 256)</td>
</tr>
<tr>
<td>ZR72 (72 h)</td>
<td>5/26 (19)</td>
<td>0.27 (0 to 0.54)</td>
<td>247 (233 to 253)</td>
</tr>
<tr>
<td>ZR432 (432 h)</td>
<td>19/40 (48)</td>
<td>0.65 (0.40 to 0.90)</td>
<td>237 (229 to 245)</td>
</tr>
</tbody>
</table>

†Tumor incidence (number of rats with tumors/total number of rats) was analyzed by two-tailed Fisher’s exact test: ZR1 versus ZD, P<.001; ZR24 versus ZD, P<.001; ZR72 versus ZD, P<.001; ZR432 versus ZD, P<.001. Also, ZR1 versus ZR432, P<.001; ZR24 versus ZR432, P<.001; ZR72 versus ZR432, P<.001. Also, ZR1 versus ZR24, P<.001; ZR432, P<.001; ZR24 versus ZR432, P<.001; ZR72 versus ZR432, P<.001.

‡Tumor incidence (number of rats with tumors/total number of rats) was analyzed by two-tailed Fisher’s exact test: ZR1 versus ZD, P<.001; ZR24 versus ZD, P<.001; ZR72 versus ZD, P<.001; ZR432 versus ZD, P<.001. Also, ZR1 versus ZR432, P<.001; ZR24 versus ZR432, P<.001; ZR72 versus ZR432, P<.001.

*ZD = zinc-deficient; zinc-replenished (ZR1, ZR24, ZR72, and ZR432) animals were replenished, respectively, at 1, 24, 72, and 432 hours after NMBA dosing.

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Before NMBA treatment, hematoxylin–eosin-stained sections of ZD esophagi typically show a thickened epithelium with multiple deep foldings and occasional, small focal hyperplastic lesions that may be preinvasive lesions [data not shown, but see (6)]. Figs. 2 and 3 present the sequence of morphologic changes in esophageal sections from ZR1 rats that occur after zinc replenishment. Twenty-four and 30 hours after a single NMBA treatment, ZD esophageal epithelium was hyperplastic and 10–20 cells thick. Focal hyperplastic lesions and sporadic single apoptotic cells were noted. Representative esophageal sections from four ZR1 rats are shown (24 hours: Fig. 2, A, and Fig. 3, A; 30 hours: Fig. 2, B, and Fig. 3, I). ZR1 esophagi had substantially more apoptotic cells. At 24 hours, esophageal sections from 12 (57%) of 21 ZR1 rats showed clusters of apoptotic cells in the still proliferating esophageal epithelia (Fig. 2, F). Numerous suprabasal cells had condensed nuclear chromatin, an alteration that is indicative of an early stage of apoptosis (Fig. 3, E). At 30 hours, apoptosis was observed in esophageal sections from 18 (86%) of 21 ZR1 rats. As an example, Fig. 2, G, shows many apoptotic cells in the still hyperplastic cell layers, and Fig. 3, M, shows mature suprabasal cells undergoing apoptosis. The presence of apoptotic cells in ZR1 esophageal epithelia was confirmed by TUNEL analysis, which identified many dark-brown apoptotic cells with increasingly fragmented DNA in basal and mature suprabasal cells (24 hours: Fig. 3, F; 30 hours: Fig. 3, N). A few dark-brown apoptotic cells were identified in ZD esophagi (24 hours: Fig. 3, B; 30 hours: Fig. 3, J).
apoptosis in ZR₁ esophagi, and the apoptotic index rose rapidly from a ZD level of 2.9% (95% CI = 2.5% to 3.3%) to 9.4% (95% CI = 8.2% to 10.6%) at 24 hours and to 10.2% (95% CI = 9.1% to 11.3%) at 30 hours after zinc replenishment. The apoptotic index was statistically significantly higher in ZR₁ rats than in ZD rats at all times (Fig. 4). As a result, the total number of cells per cross-section of an esophagus in ZR₁ rats decreased from a ZD level of 2557 (95% CI = 2047 to 3067) to 1754 (95% CI = 1603 to 1905) at 24 hours and to 1784 (95% CI = 1677 to 1891) at 30 hours. At later time points, the total number of cells per cross-section ranged from 1305 (95% CI = 1241 to 1369) to 1422 (95% CI = 1341 to 1503). In contrast, no statistically significant difference in the total number of cells among ZD esophagi was evident at any time. Thus, zinc replenishment reduced esophageal cell layers through a rapid induction of apoptosis.

Rapid Reduction of Cell Proliferation

Consistent with previous reports (2,5), the high rate of esophageal cell proliferation induced by zinc deficiency was not increased further by treatment with NMBA. Throughout the experiment, the labeling index was 42%–47% for S-phase cells and 68%–77% for S- and G1–S/G2-phase cells (Fig. 5). Conversely, as early as 24–48 hours after zinc replenishment, cell proliferation in ZR₁ esophagi was reduced substantially, as determined by 1) the labeling index for S-phase cells, 2) the number of cells in S phase, and 3) the total number of cells (labeled plus unlabeled) per cross-section of an esophagus. As an example in ZR₁ esophagi, the labeling indexes for cells in S phase were 39.0% (95% CI = 36.2% to 41.8%), 32.4% (95% CI = 30.6% to 34.2%), and 29.1% (95% CI = 26.3% to 31.9%) at 24, 30, and 48 hours after zinc replenishment, respectively. These data were statistically significantly different from those obtained from ZD esophagi at similar times (Fig. 5). Thus, after zinc intervention, esophageal cell proliferation was reduced quickly (24).

DISCUSSION

In this study, we have shown that NMBA-induced esophageal carcinogenesis in a ZD rat is essentially stopped if a zinc-
Fig. 3. Apoptosis and cell proliferation in zinc-deficient (ZD) and zinc-replenished (ZR) esophagi at 24 (A–H, Q, and R), 30 (I–P), and 48 (S and T) hours after treatment with N-nitrosomethylbenzylamine (NMBA). Hematoxylin–eosin (H&E) staining, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate end labeling (TUNEL) analyses, and immunohistochemistry for Bax, Bcl-2, and proliferating cell nuclear antigen (PCNA) were used. Panels A–D, E–L, Q, and S: rats in the ZD group. Panels E–H, M–P, R, and T: rats in the ZR group (ZR1, replenished 1 hour after NMBA treatment). H&E-stained esophageal sections from ZD rats (A and D) show hyperplastic epithelium with mature differentiated suprabasal cells. ZR1 esophagi (E and M) had suprabasal cells undergoing apoptosis. TUNEL analysis shows sporadic apoptotic cells in the proliferative ZD esophageal epithelium (B and J) but numerous apoptotic cells in the basal and suprabasal layers in ZR1 esophagi (F and N). Apoptotic bodies are intensely stained dark brown by 3,3′-diaminobenzidine tetrahydrochloride and counterstained with methyl green. Bax in a ZD esophagus showing hyperplasia is weakly detected in the cytoplasm (C and K) but strongly detected (3,3′-diaminobenzidine tetrahydrochloride, dark brown) in suprabasal cells of ZR1 esophagus, showing apoptotic activities (G and O). Bcl-2 in the ZD esophagus with hyperplasia is weakly detected in the cytoplasm (D and L), but Bcl-2 in the ZR1 esophagi is weakly and diffusely detected in suprabasal cells (H and P).

Panels Q–T: PCNA immunohistochemistry detects many cells in S phase and G1–S/G2 phase in the basal and suprabasal layers of the proliferative esophagus from rats in the ZD group (24 hours; Q; 48 hours; S) but detects few of these cells, mainly in basal layer of the restored esophageal epithelium, in the ZR1 group (24 hours; R; 48 hours; T). PCNA-positive nuclei were stained red by 3-aminio-9-ethylcarbazole substrate chromogen and counterstained with hematoxylin. Panels A–P and T: scale bar = 25 μm; panels Q–S: scale bar = 50 μm.

sufficient diet is given to the rats soon after treatment with NMBA. The incidence of esophageal tumors was reduced from 93% to 8% in ZR1 rats (Table 2). Importantly, 24 hours after ZD rats were switched to a zinc-sufficient diet, apoptosis was soon triggered in the still proliferative ZR1 esophagus. By 48 hours, the ZR1 esophagus had an epithelium that was three to five cells thick, compared with 10–20 layers in the ZD esophagus. It is interesting that ZR432 rats that received a zinc-sufficient diet 432 hours after treatment with NMBA also had a thin epithelium within 48 hours (data not shown). These rats, however, had a statistically significantly higher incidence of tumors than rats replenished between 1 hour and 72 hours (Table 2). These findings agree with our previous conclusion that tumor initiation by NMBA is extremely rapid in the ZD esophagus (5) and, consequently, that apoptosis must be triggered shortly after zinc replenishment to curb tumor progression.

Apoptosis is a relatively fast process, taking 5–30 minutes for the cell to manifest morphologic changes and a few hours for clearance and digestion of the apoptotic bodies (30,38–40). The short duration of apoptosis and the fact that morphologic features characteristic of apoptotic cells can be detected only late in the process explain why apoptotic cells are found infrequently in histologic sections, even when many cells are lost (41). Intense apoptotic activity in a tissue is often associated with less than 5% of concurrently visible apoptotic cells (40). It is remarkable that our animal model with its extremely rapid tumor initiation allowed us to visualize two important biologic events in the earliest stages of tumor regression. First, 24 and 30 hours after NMBA treatment and zinc replenishment, about 10% of cells in the ZR1 esophagi (Fig. 4) were undergoing apoptosis (Fig. 2, F and G; Fig. 3, E, F, M, and N). Second, we concomitantly observed a surge in Bax expression (Fig. 3, G versus C and O versus K) and a reduction in Bcl-2 expression (Fig. 3, H versus D and P versus L) in the cells undergoing apoptosis, compared with ZD esophagi at similar times after NMBA treatment. These findings identify a cell death program that is dependent on members of the Bcl-2 gene family (42–44). Apoptotic sensitivity is determined by alterations in the ratio between proapoptotic and antiapoptotic members of the Bcl-2 protein family rather than by the level of expression of any single member (42,45). We found that, in the highly proliferative ZD esophagus, the Bax/Bcl-2 immunoreactive ratio was 0.5 (95% CI = 0.3 to 0.7) and remained at this level throughout the experiment (Table 3). By contrast, the Bax/Bcl-2 ratio increased to 1.3 (95% CI = 1.1 to 1.5) 24 hours after zinc replenishment in ZR1 esophagus (Table 3) and remained at an elevated level throughout the experiment. Thus, zinc replenishment increases Bax expression and reduces Bcl-2 expression, which alters the
Table 3. Bax and Bcl-2 protein expression in zinc-deficient (ZD) and zinc-replenished (ZR) esophagi*

<table>
<thead>
<tr>
<th>Time after zinc replenishment</th>
<th>Group† (n)</th>
<th>Immunoreactive score‡</th>
<th>Bax/Bcl-2 ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>(95% CI)</td>
<td>(95% CI)</td>
</tr>
<tr>
<td>0 h</td>
<td>ZD (7)</td>
<td>3.0 (2.0 to 4.0)</td>
<td>6.7 (6.0 to 7.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 (0.3 to 0.7)</td>
</tr>
<tr>
<td>24 h</td>
<td>ZD (7)</td>
<td>3.9 (3.2 to 4.6)</td>
<td>8.2 (7.6 to 8.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 (0.4 to 0.6)</td>
</tr>
<tr>
<td></td>
<td>ZR, (21)</td>
<td>6.0 (5.0 to 7.0)</td>
<td>4.6 (3.9 to 5.3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.3 (1.1 to 1.5)</td>
</tr>
<tr>
<td>30 h</td>
<td>ZD (10)</td>
<td>3.0 (2.3 to 3.7)</td>
<td>7.8 (6.7 to 8.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.4 (0.3 to 0.5)</td>
</tr>
<tr>
<td></td>
<td>ZR, (20)</td>
<td>5.8 (4.9 to 6.7)</td>
<td>4.8 (4.2 to 5.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.2 (1.0 to 1.4)</td>
</tr>
<tr>
<td>15 wk</td>
<td>ZD (10)</td>
<td>3.3 (2.5 to 4.1)</td>
<td>6.2 (5.8 to 6.6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0.5 (0.4 to 0.6)</td>
</tr>
<tr>
<td></td>
<td>ZR, (17)</td>
<td>4.5 (4.0 to 5.0)</td>
<td>2.9 (2.4 to 3.4)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1.5 (1.0 to 2.0)</td>
</tr>
</tbody>
</table>

*CI = confidence interval.
†ZR, replenished 1 hour after N-nitrosomethylbenzylamine treatment. n = number of animals.
‡Immunoreactive scores = sum of (grade of percentage of positive cells) x (grade of intensity of staining) for each component of the esophageal section. For Bax expression, ZR, versus ZD, P = .019 at 24 hours, P<.001 at 30 hours, and P = .012 at 15 weeks. For Bcl-2 expression, ZR, versus ZD, P<.001 at 24 hours, 30 hours, and 15 weeks. For the Bax/Bcl-2 ratio, ZR, versus ZD, P=.007 at 15 weeks. All statistical tests were two-sided.

Fig. 4. Apoptosis index in zinc-replenished (ZR) esophagi. The index is expressed as the percent of apoptotic cells in the total number of cells per cross-section of an esophagus. For statistical analysis, seven to 10 zinc-deficient (ZD) rats and seven to 20 ZR, rats (replenished 1 hour after treatment with N-nitrosomethylbenzylamine (N MBA)) were used. All statistical tests were two-sided, and error bars are 95% confidence intervals at representative time points. *ZR, versus ZD, P<.001, for each time.

Thus, by using the ZD rat esophageal cancer model (1–6), we have demonstrated, to our knowledge for the first time, that apoptosis is triggered in the esophageal epithelium shortly after N MBA-treated ZD rats are given a zinc-sufficient diet and, as a result, N MBA-induced esophageal tumor formation is stopped.
in the very early stages of the tumorigenesis pathway. We have also demonstrated that increased expression of the Bax protein accompanies the induction of apoptosis in the esophageal epithelium. Thus, our results indicate that chemopreventive or therapeutic regimens that simulate zinc replenishment, by very rapid induction of overexpression of Bax, could be tested in this animal model. More broadly, this study suggests that zinc supplementation may have a role in the prevention of esophageal cancer in persons who show a high risk for the disease and who do not have an adequate level of zinc intake.

REFERENCES


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NOTES

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