Antizyme Overexpression in Transgenic Mice Reduces Cell Proliferation, Increases Apoptosis, and Reduces N-Nitrosomethylbenzylamine-induced Forestomach Carcinogenesis

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ABSTRACT

Antizyme (AZ) is known to be a regulator of polyamine metabolism that inhibits ornithine decarboxylase activity and polyamine transport, thus restricting polyamine levels. Transgenic mice with AZ expression targeted to the basal cell layer of the forestomach epithelium by the keratin 5 promoter were used to investigate whether AZ overexpression inhibited uncontrolled cell proliferation in zinc-deficient (ZD) mice and reduced their susceptibility to forestomach carcinogenesis by N-nitrosomethylbenzylamine (NMBA). Four-week-old keratin 5/AZ and wild-type (Wt) littermates were placed on ZD or zinc-sufficient (ZS) diets to form four groups: ZD:AZ; ZD:Wt; ZS:AZ; and ZS:Wt. After 5 weeks, 27–45 mice in each group were treated twice with NMBA and sacrificed 14 weeks later. Independent of zinc intake, AZ mice had significantly lower forestomach tumor incidence and tumor multiplicity than respective Wt littermates (P < 0.001): 21% of ZD:AZ versus 76% of ZD:Wt mice and 3% of ZS:AZ versus 35% of ZS:Wt mice developed tumors. Spermidine content was reduced in NMBA-treated ZD:AZ forestomachs. Zinc deficiency increased the forestomach cell proliferation in Wt mice, but this effect was blocked by AZ. Conversely, apoptosis was substantially higher in control and NMBA-treated AZ:ZD than respective ZD:Wt forestomachs. The restored ZD:AZ forestomach epithelium displayed strong expression of Bax, a proapoptotic protein, and weak staining of cyclin D1 and its catalytic partner Cdk4, key regulatory proteins controlling G1 to S progression. In contrast, proliferative ZD:Wt forestomach showed strong expression of Bcl-2, an antiapoptotic protein, and overexpression of cyclin D1/Cdk4. Treatment of ZD:Wt mice with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase, had similar results to AZ in reducing tumor incidence, spermidine content, decreasing cell proliferation, and increasing apoptosis. These results demonstrate that AZ may act as a tumor suppressor gene stimulating apoptosis and restraining cell proliferation, thereby inhibiting forestomach tumor development. Although effects of AZ on functions other than polyamine metabolism are possible, alterations in polyamines are the most likely explanation for the reduction in tumors, supporting the use of strategies to modulate polyamine levels for cancer chemoprevention in individuals at high risk of developing malignancies of the gastrointestinal tract.

INTRODUCTION

Polyamines are ubiquitous small basic molecules that play roles in many aspects of cellular physiology. Polyamines are essential for mammalian cell growth and development (1, 2), and gene disruptions of ODC (3) or S-adenosylmethionine decarboxylase, two key enzymes in polyamine biosynthesis are lethal at early stages of embryonic development (3, 4). Many studies have indicated a correlation between polyamine content and neoplastic growth (5), and the polyamine biosynthetic pathway may be a useful target for the design of cancer chemopreventive and chemotherapeutic agents (6–8). The irreversible ODC inhibitor DFMO is currently in clinical trials for chemoprevention of numerous types of cancers (9, 10).

ODC (EC 4.1.1.17), which catalyzes the formation of putrescine from ornithine, the first step of cellular polyamine biosynthesis, has been associated with neoplastic growth in many experimental studies. ODC overexpression and increased levels of polyamines alone or in cooperation with other oncogenes can transform cells in vitro (11–14). Increased ODC activity is associated with transformation caused by several oncogenes (15–18), and ODC transcription is negatively regulated by the Wilms’ tumor suppressor (19). Transgenic mice overexpressing ODC in the skin are more sensitive to tumor development in response to carcinogens (20) or UV radiation (21) and develop skin tumors without need for tumor promoters (22).

ODC activity is very highly regulated at multiple levels including transcription, translation and protein degradation (reviewed in Refs. 23–25). A major factor in the regulation of ODC and polyamine homeostasis is AZ (26, 27). AZ mRNA contains an internal stop codon preventing the synthesis of the active protein, but a polyamine-dependent +1 frameshifting event allows the translation of AZ mRNA into full-length AZ protein. AZ then binds to the ODC monomer, which prevents formation of the enzymatically active homodimer and, therefore, reduces ODC activity. More critically, the binding of AZ stimulates the degradation of ODC by the 26S proteasome in an ATP-dependent but ubiquitin-independent manner. Mammalian cells also possess a highly inducible polyamine transport system, which is activated when internal polyamine content falls. This uptake system is also inhibited by AZ, and there is some evidence that AZ may also stimulate polyamine excretion (27). Thus, AZ reduces polyamine content and contributes to polyamine homeostasis through multiple mechanisms. Recent studies have indicated that there may be a family of AZ molecules in addition to that first discovered, which is now termed AZ-1 (27, 28). The function of these multiple forms of AZ is not yet known.

Transgenic mice in which AZ-1 is expressed from the bovine K5 and K6 promoter elements have been derived (29). The AZ-1 cDNA construct used had a single nucleotide deletion (T205) to remove the requirement for polyamine-stimulated frameshifting in the translation of the mRNA. There are two potential start codons in the AZ-1 cDNA, and both are present in the transgene, but Western blots of epidermal extracts indicated that the second site was used preferentially in vivo (29). Both K5/AZ and K6/AZ transgenic mice developed normally and were phenotypically indistinguishable from Wt littermates. However, the transgenic AZ expression blocked the increase in skin ODC induced by tumor promoters, and reduced epidermal and dermal polyamine content, particularly spermidine. Carcinogenesis studies using a two stage protocol with initiation with 7,12-dimethylbenz(a)anthracene and treatment with the tumor promoter 12-O-tetradecanoylphorbol-13-acetate showed that two founder lines of K6/AZ were significantly lower forestomach tumor incidence and tumor multiplicity than respective Wt littermates (P < 0.001): 21% of ZD:AZ versus 76% of ZD:Wt mice and 3% of ZS:AZ versus 35% of ZS:Wt mice developed tumors. Spermidine content was reduced in NMBA-treated ZD:AZ forestomachs. Zinc deficiency increased the forestomach cell proliferation in Wt mice, but this effect was blocked by AZ. Conversely, apoptosis was substantially higher in control and NMBA-treated AZ:ZD than respective ZD:Wt forestomachs. The restored ZD:AZ forestomach epithelium displayed strong expression of Bax, a proapoptotic protein, and weak staining of cyclin D1 and its catalytic partner Cdk4, key regulatory proteins controlling G1 to S progression. In contrast, proliferative ZD:Wt forestomach showed strong expression of Bcl-2, an antiapoptotic protein, and overexpression of cyclin D1/Cdk4. Treatment of ZD:Wt mice with α-difluoromethylornithine, an inhibitor of ornithine decarboxylase, had similar results to AZ in reducing tumor incidence, spermidine content, decreasing cell proliferation, and increasing apoptosis. These results demonstrate that AZ may act as a tumor suppressor gene stimulating apoptosis and restraining cell proliferation, thereby inhibiting forestomach tumor development. Although effects of AZ on functions other than polyamine metabolism are possible, alterations in polyamines are the most likely explanation for the reduction in tumors, supporting the use of strategies to modulate polyamine levels for cancer chemoprevention in individuals at high risk of developing malignancies of the gastrointestinal tract.
mice had a delay in tumor onset and a substantial reduction in tumor multiplicity compared with normal littermates. K5/AZ mice also developed fewer papillomas than littermate controls, and combination of these lines to produce K5/AZ-K6/AZ double transgenic animals yielded an additive decrease in tumor multiplicity (29). These studies were carried out on a mixed B6D2 genetic background, but the results were confirmed in a defined genetic background after the transgenic lines were backcrossed onto the carcinogenesis-resistant C57BL/6J inbred strain as well as the sensitive DBA/2J strain (30). There was a greater effect with the K6/AZ lines, which is consistent with the more complete inhibition of ODC activity in K6/AZ compared with K5/AZ mice in response to 12-O-tetradecanoylphorbol-13-acetate application.

These results suggest that AZ acts as a tumor suppressor and that the transgenic mice expressing AZ can be used to investigate which pathways of carcinogenesis have an essential increase in polyamine metabolism as a contributory factor. AZ expression from the K5 promoter is not limited to the skin but may occur in other epithelial cells. Numerous studies have demonstrated that the K5 promoter can drive transgene expression in other stratified epithelia in addition to skin, such as the esophagus and forestomach (31–34), thymus (31, 32), and prostate (35).

Therefore, we have studied the incidence of tumors in K5/AZ mice after treatment with NMBA. This carcinogen is known to produce esophageal cancer in rats, and tumor development is accelerated by exposure to a ZD diet that induces proliferation in target cells (31, 37). Treatment of rats with NMBA and a ZD diet provides an important model system, which reproduces many aspects of the development of esophageal squamous cell carcinoma in humans in high risk areas such as northern China and Iran (38, 39). Mice also develop tumors readily in response to NMBA and a ZD diet (40, 41). However, in mice there is a higher incidence of tumors in the forestomach, which is considered to be a dilation of the lower esophagus (42), than in esophagus. A loss of p53 increased the sensitivity of ZD mice to NMBA-induced esophageal/forestomach carcinogenesis (41). The rapid rate of tumor induction/progression in ZD:p53−/− mice was accompanied by an increase in the rate of cell proliferation and a decrease in apoptosis. The studies reported here show that AZ expression has the opposite effect to zinc deficiency and lack of p53 in that it stimulates apoptosis, restrains cell proliferation, and inhibits forestomach tumor development. These results are supported by studies in which DFMO was tested and found to also reduce tumor development after NMBA treatment. These findings emphasize the potential value of using drugs targeting the polyamine metabolic pathway as cancer chemopreventive agents.

### MATERIALS AND METHODS

**Transgenic Mice.** This study was approved by the Thomas Jefferson University Institutional Animal Care and Use Committee and conducted under NIH guidelines. The generation of K5-AZ transgenic mice was described previously (29). All of the animals used for the experiments were from the fifth or sixth backcross generation to C57BL/6J mice; therefore, >98% of their genes are derived from this inbred strain. Breeding of transgenic males from a single founder line to C57BL/6J females (The Jackson Laboratory, Bar Harbor, ME) generated a total of 130 AZ transgenic mice and 136 Wt littermates (with about equal number of males and females) for the forestomach tumorigenesis study. The AZ and Wt offspring were differentiated by genotyping of tail DNA using a PCR-based method (29).

**Chemicals and Diets.** NMBA was purchased from Ash Stevens, Inc. (Detroit, MI). Custom-Formulated, egg white-based ZD and ZS diets containing 1.5 and 75 ppm zinc, respectively, were prepared by Teklad (Madison, WI). The ZD diet is nutritionally complete and is identical to ZS diet except for the concentration of elemental zinc (36).

**Zinc Determination.** Tissues were removed from male and hair from female animals at necropsy. Samples of testis or hair were dried to constant weight at 90°C and ashed in a furnace. Ashed samples were dissolved in 0.1 N HCl and the zinc content determined by atomic spectrometry as described (36). Zinc content was expressed as μg/g dry weight of testis or hair. Overt signs of zinc deficiency that are well-described for ZD rats (36), including foci of alopecia, skin lesions, and retarded growth, were not evident in ZD-Wt or ZD:AZ mice, which had similar body weights at end point (Table 1). However, zinc content in the testis (male) and hair (female) was significantly lower in ZD than ZS mice, regardless of genotype and other treatment (P < 0.001; Table 1).

**Forestomach Tumorigenesis Experiment in AZ Transgenic Mice.** The experiment was conducted in batches when the mice became available from our breeding colonies. Four-week-old mice (AZ male, 13.1 ± 1.5 g and female, 12.3 ± 1.4 g; Wt male, 13.0 ± 1.9 g and female, 12.6 ± 1.7 g) were housed 3–5 to a polycarbonate cage with a wire stainless steel floor. They were given free access to deionized drinking water. The mice were randomized into two dietary groups and were fed ad libitum a ZD or control ZS diet, forming four experimental groups: ZD-Wt, ZD-AZ, ZS-Wt, and ZS-AZ. After 5 weeks, 10 mice from each group (control mice) were sacrificed to determine the extent of cell proliferation and apoptosis in the forestomach. The remaining animals (27–45 mice/group and 15–29 mice/group in 2× NMBA and 4× NMBA studies, respectively) were treated with two or four intragastric doses of NMBA at 2 mg/kg body weight, twice weekly. NMBA-treated animals continued on their respective diet and were sacrificed 14 weeks after the first carcinogen treatment for end point tumor incidence analysis.

**Effect of DFMO on Forestomach Tumorigenesis in C57BL/6 Mice.** To evaluate the effect of DFMO in ZD mice, 75 weaning male C57BL/6 mice (11.7 ± 1.3 g) were purchased from Taconic Laboratory (Germantown, NY). The animals were randomly divided into two dietary groups as described above. To determine the extent of cell proliferation before NMBA dosing (0 h), 5 and 5 ZD and ZS mice were killed after 5 weeks of experimental diet. The remaining mice received two intragastric doses of NMBA at 2 mg/kg body weight per week. After the first dose, the animals were divided into four groups (14–20 mice/group): ZD/DFMO−; ZD/DFMO+; ZS/DFMO−; and ZS/DFMO+. DFMO− mice remained on deionized water, whereas DFMO+ animals were given deionized water containing 1% DFMO. All of the mice continued on their respective diet. After 2 weeks, 5 mice from each group were killed to determine the effect of DFMO on forestomach cell proliferation. The remaining 45 mice were sacrificed 12 weeks after the first NMBA dose for tumor incidence analysis.

**Tumor Analysis.** After anesthetization with isolurane (Ohmeda Inc., Madison, WI), the mice were sacrificed and subjected to complete necropsies. Whole stomachs were excised and opened longitudinally. Tumors in the forestomach were mapped and counted. Whole forestomachs were fixed in buffered formalin and embedded in paraffin. The forestomach was cut into 4–6 strips, across the SCJ. Then, 4-μm thick cross-sections were cut. Typically, there were 4 to 6 sections per slide, representing the entire forestomach/SCJ. Sections were either stained with H&E for histopathology or left unstained for immunohistochemical studies.

<table>
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<tr>
<th>Parameter</th>
<th>ZD-Wt</th>
<th>ZD-AZ</th>
<th>ZS-Wt</th>
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<td>133 ± 5</td>
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<td>27 ± 2</td>
<td>28 ± 2</td>
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<td>4× NMBA: Zinc content (μg/g, testis)</td>
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<td>128 ± 6</td>
<td>165 ± 13</td>
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<td>4× NMBA: Zinc content (μg/g, hair)</td>
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<td>131 ± 9</td>
<td>187 ± 122</td>
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<td>21 ± 2</td>
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Table 1 Effect of a zinc-deficient diet on zinc content and body weight

Four-week-old mice were fed ZD or ZS diet for 5 weeks, and were then given intragastrically, two or four NMBA doses, twice per week. The animals were sacrificed 14 weeks later. Values are shown as the mean ± SD. Zinc content (2× NMBA, 4× NMBA): testis (n = 5–16), hair (n = 5–18), ZD-Wt versus ZS-Wt, ZD-AZ versus ZS-AZ. P < 0.001. All statistical tests were two-sided.
Polyamine Analysis. At necropsy, a small strip of forestomach epithelium was cut and snap-frozen in liquid nitrogen. The samples were stored at −80°C until polyamine analysis. Each sample represented three pooled strips from mice of the same treatment group for the 2×NMBA experiment or a single tissue sample for the DFMO experiment. Polyamine content in forestomach was determined after separation by ion-pair reversed-phase high-pressure liquid chromatography using fluorescence detection after postcolumn derivatization with α-phthalaldehyde as described previously (43) and normalized to tissue wet weight (in grams). Normalization to mg protein/sample yielded similar results.

Cell Proliferation Determination by PCNA Immunohistochemistry. Monoclonal mouse anti-PCNA (Santa Cruz Biotech., Santa Cruz, CA) was used at a final incubation with 3-amin-9-ethylcarbazole-substrate-chromogen system (Dako Corp., Carpinteria, CA), and a light hematoxylin counterstain. Cells with red reaction product in the nucleus were considered positive for the presence of PCNA.

Apoptosis Analysis. Apoptosis was assessed by the TUNEL method and by morphological characterization of cells in H&E stained sections.

TUNEL Assay. The 3′-OH end labeling of DNA in tissue sections was performed with an ApopTag in situ peroxidase detection kit (Serologicals Corp., Norcross, GA). Sections were deparaffinized, rehydrated in a graded alcohol series, and incubated with proteinase K (20 μg/ml, 37°C for 10 min). Endogenous peroxidase in the sections was inhibited with 3% hydrogen peroxide, and slides were incubated (37°C for 1 h) with terminal deoxynucleotidyl transferase to catalyze the addition of digoxigenin-labeled nucleotides to the 3′-OH ends of fragmented DNA. Next, slides were incubated with horseradish peroxidase-conjugated antidigoxigenin antibodies, and DNA fragmentation was detected by staining with DAB. Finally, sections were counterstained with methyl green. Sections from rat mammary gland (Serologicals Corp.), in which extensive apoptosis occurs, served as a positive control. Negative controls omitted terminal deoxynucleotidyl transferase.

Morphological Criteria. The incidence of apoptosis was measured on good-quality sections stained with H&E. The morphology of apoptotic cells depends on their stage in the process. Apoptotic morphologies include diffuse cytoplasmic staining with only minimal nuclear condensation, distinct apoptotic bodies resulting from nuclear disintegration, or dense staining nuclei with normal nuclear structure (44). All three of the forms were considered to be equivalent, and the AI, expressed as a percentage, was calculated by dividing the number of apoptotic cells by the total number of cells in the tissue section.

K5 and AZ Immunohistochemistry. Antiserum to AZ was produced in rabbits immunized with a purified recombinant polyhistidine-tagged AZ fusion protein and then purified using an AZ-affinity column as described previously (29). After deparaffinization and rehydration in graded alcohols, forestomach sections were heated in citrate buffer [0.01 M (pH 6.0)] in a microwave oven (88–95°C; 3 × 5 min) before nonspecific binding sites were blocked with goat serum. Sections were incubated overnight at 37°C in a humidified chamber with rabbit anti-AZ antiserum at 1:200 dilution followed by incubation with biotinylated goat antirabbit antibody serum (1:500 dilution). Slides were then incubated with streptavidin horseradish peroxidase (1:1000 dilution). Cytoplasmic expression of AZ was localized by a final incubation with DAB and a light hematoxylin counterstain. K5 was detected using a polyclonal antibody against mouse K5 (Covance Research Products, Berkeley, CA) at a 1:500 dilution and visualized with the Vectastain Elite ABC kit with DAB chromagen (Vector Laboratories, Burlingame, CA).

Bax and Bcl-2 Immunohistochemistry. To detect Bax and Bcl-2, forestomach sections were incubated overnight at 37°C in a humidified chamber with a rabbit anti-Bcl-2 polyclonal antiserum (Santa Cruz) at 1:400 dilution, or with a rabbit anti-Bax polyclonal antiserum (Santa Cruz) at 1:200 dilution, followed by incubation with a biotinylated goat antirabbit antibody serum. Cytoplasmic Bcl-2 and Bax expression was visualized with DAB.

Cyclin D1 and Cdk4 Immunohistochemistry. To detect cyclin D1 and Cdk4, forestomach sections were incubated as described above with a rabbit anticyclin D1 polyclonal antibody (Lab Vision Corp., Fremont, CA) at a 1:100 dilution or with a rabbit anti-Cdk4 polyclonal antiserum (Santa Cruz) at 1:100 dilution, followed by incubation with a biotinylated goat antirabbit antibody serum. Nuclear cyclin D1 and Cdk4 expression was visualized with DAB.

Fig. 1. Reduction of NMBA-induced forestomach carcinogenesis in AZ transgenic mice. Four-week-old mice were fed ZD or ZS diet for 5 weeks, and were then given two (A and C) or four (B and D) NMBA doses, intragastrically twice per week. The animals were sacrificed 14 weeks later. A and B show the percentage of tumor incidence in the forestomach and SCI with the glandular stomach. The number of animals with tumors and the total number of mice is shown above each bar. C and D show the tumor multiplicity of forestomach tumors and the SE. Statistical analysis of significance of percentage of tumor incidence: 2× NMBA, ZD: AZ versus ZD: Wt, forestomach and SCI, P < 0.001; ZD: AZ versus ZD: Wt, forestomach, P = 0.003 and SCI, P < 0.001; ZD: Wt versus ZS: Wt, forestomach, P = 0.05; ZD: AZ versus ZS: AZ, forestomach, P = 0.032 and SCI, P = 0.013; 4× NMBA, ZD: AZ versus ZD: Wt, forestomach, P < 0.001 and SCI, P = 0.004; ZS: AZ versus ZS: Wt, forestomach, P = 0.048; ZD: Wt versus ZS: Wt, forestomach, P = 0.025 and SCI, P = 0.009. Statistical analysis of significance of tumors/forestomach: 2× NMBA, ZD: AZ versus ZD: Wt and ZD: Wt versus ZS: Wt, P < 0.001; 4× NMBA, ZD: AZ versus ZD: Wt and ZD: Wt versus ZS: Wt, P < 0.001. All statistical tests were two-sided.
Statistical Analysis. Tumor incidence differences were analyzed by two-tailed Fisher’s exact test, and data on cell proliferation and testis zinc level were analyzed by one-way ANOVA with the SAS statistical computer program as described (41). All of the statistical tests were two-sided and were considered statistically significant at $P < 0.05$.

RESULTS

Effect of AZ Expression on Forestomach Carcinogenesis. Fourteen weeks after two NMBA doses, ZD:Wt mice invariably showed a thickened and shrunken forestomach, with a 76% and 56% tumor incidence in the forestomach and SCJ, respectively, and a forestomach tumor multiplicity of 2.6 (Fig. 1, A and C). There was a large and highly significant reduction in tumor incidence in ZD:AZ mice (21% forestomach and 18% SCJ) and in tumor multiplicity (0.4). Although the overall tumor incidence was lower in ZS:Wt mice (which is consistent with previous results in C57BL/6 mice; Refs. 40, 41) ZS:AZ mice, relative to their ZS:Wt counterparts, also had significantly lower tumor incidence in the forestomach (3% versus 33%) and SCJ (0% versus 30%; Fig. 1A). Similar results were obtained in a second experiment in which the mice were given four doses of NMBA and sacrificed 14 weeks later for tumor incidence analysis (Fig. 1, B and D). Notably, only 15% of ZD:AZ mice had forestomach tumors versus 80% of their ZD:Wt counterparts, and tumor multiplicity was 0.5 versus 3.8. In the ZS mice, AZ expression caused a drop in forestomach tumors from 41% to 12%. Tumors at the SCJ were also reduced by AZ in both ZD and ZS mice. Esophageal tumors were not detected in ZD:Wt mice probably because the animals in the present study were exposed to fewer NMBA doses than in the previous study (40).

Tumor-bearing ZD:Wt mice regularly displayed large, fused forestomach tumors (Fig. 2A) or large, solitary tumors (Fig. 2B). On the contrary, ~80% of ZD:AZ mice showed a tumor-free forestomach that was mostly large and thin (Fig. 2C) or slightly thickened at the SCJ (Fig. 2D). The results shown in Fig. 2 were obtained with mice given two doses of NMBA but similar results (data not shown) were obtained after four doses of the carcinogen.

The expression pattern of AZ in the forestomach epithelium of K5/AZ transgenic mice was determined both indirectly by K5 immunostaining and directly by AZ immunostaining. Endogenous K5 was readily visualized in the forestomach of both AZ and control mice by immunohistochemistry. K5 staining was strong and uniform through-
out the basal cell layer of the forestomach and within areas of hyperproliferation. The staining was weak and patchy within the suprabasal layer. These patterns of K5 staining were consistent independent of diet, genotype, or carcinogen treatment (data not shown). These results are consistent with previous studies showing that the K5 promoter is active in the forestomach (31–34). AZ expression was visualized using immunohistochemical staining with an antiserum to purified recombinant rat AZ. This revealed higher levels of AZ expression in the cytoplasm of forestomach epithelial cells from K5/AZ mice relative to control animals (Fig. 3).

**Reduced Cell Proliferation in Control ZD:AZ Mice.** Visual inspection of control mice after 5 weeks of the ZD diet typically showed a thickened forestomach and SCJ in ZD:Wt mice but a large and thin forestomach in ZD:AZ animals, suggesting nutritional zinc deprivation did not have an effect on forestomach cell proliferation in the latter. Histopathologic examination of control forestomach sections showed that the level of cell proliferation was highest in ZD:Wt mice, followed by ZS:Wt, and then ZD:AZ ≥ ZS:AZ. Although AZ overexpression reduced cell proliferation in both ZD and ZS forestomach, its effect was more dramatic in ZD mice, and photomicrographs are presented of ZD tissue sections (Fig. 4). Relative to ZS:Wt forestomach that exhibited occasional and mild proliferation (data not shown), ZD:Wt forestomach regularly displayed a proliferative epithelium with small upward and downward focal hyperplastic lesions (Fig. 4A), showing many PCNA-positive nuclei (Fig. 4E), and overexpression of Cdk4 (Fig. 4J) and cyclin D1 (Fig. 4M) in these lesions. PCNA is an endogenous cell proliferation marker; cyclin D1 and its catalytic partner, Cdk4, are major G1-S regulatory proteins (45). These results are consistent with previous data that demonstrated that proliferative esophagi from untreated ZD rats had altered expression profiles for genes that control G1-S progression (43). In contrast, AZ mice on a ZD diet typically showed a thin forestomach epithelium of 2–5 cells thick (Fig. 4B), with PCNA-positive nuclei mainly in the basal cell layer (Fig. 4F), and weak and sporadic expression of Cdk4 (Fig. 4G) and cyclin D1 (Fig. 4N). Thus, AZ overexpression in ZD mice restrains Cdk4/cyclin D1 expression and inhibits cell proliferation in the forestomach.

Quantitative PCNA-immunohistochemistry showed that dietary zinc deficiency induced a high rate of cell proliferation in the forestomach of WT mice (Fig. 5A, Expt. 1), a result consistent with previous studies in rats (46) and mice (40). The PCNA-LI, a measure of cellular proliferation, was significantly higher in ZD:Wt than ZS:Wt forestomach. Overexpression of AZ in ZD mice counteracted the effect of zinc deficiency on cell proliferation and brought about a significantly reduced LI compared with ZD:Wt forestomach. Likewise, LI was significantly lower in ZS:AZ than ZS:Wt forestomach (Fig. 5A, Expt. 1).

Fig. 4. Cell proliferation in control and NMBA-treated ZD:AZ and ZD:Wt forestomachs. H&E staining (A–D), immunohistochemistry for PCNA (E–H), Cdk4 (I–L), and cyclin D1 (M–P) were used. Representative pictures of individual mice are shown. A, B, E, F, I, J, M, and N show results from mice fed ZD diet for 5 weeks with no NMBA treatment. ZD:Wt mice showed: a hyperplastic epithelium with focal hyperplastic lesions (A) displaying numerous PCNA-positive nuclei (3-amino-9-ethylcarbazole, *red*) in S phase, and G1/S/G2 phase (*red*); strong nuclear expression of Cdk4 (*red*); and strong nuclear staining of cyclin D1 (*red*) in basal cells and focal hyperplastic lesions. Conversely, ZD:AZ showed: a thin epithelium (*red*) with PCNA-positive cells mainly in the basal cell layer (F); moderate expression of Cdk4 in basal cells (J); and sporadic expression of cyclin D1 in basal cells (N). C, D, G, H, K, L, O, and P show results from mice fed ZD diet, treated with two NMBA doses, and sacrificed 14 weeks later. Sections from ZD:Wt mice showed: a dysplastic epithelium with deep focal hyperplastic lesions (*red*), abundant PCNA-positive nuclei (*red*); overexpression of cyclin D1 (*red*); and overexpression of Cdk4 in a dysplastic epithelium (K). Forestomach sections from ZD:AZ mice showed: a thin epithelium displaying many apoptotic cells and mitoses (*red*); infrequent PCNA-positive nuclei mainly in the basal cell layer (H); a few strongly stained Cdk4-positive nuclei (*red*); and cyclin D1-positive nuclei (*red*). Bars: 100 μm for A–C and G; 50 μm for D–F and H–P; 25 μm for the inset.
Increased Apoptosis in Control ZD:AZ Mice. Alfas were determined in high quality H&E-stained forestomach sections of control ZD and ZS mice of both AZ and Wt genotypes (Fig. 5B, Expt. 1). Zinc deficiency produced a significant decrease in the AI of Wt mice. Expression of AZ increased the AI, which was significantly higher in ZD:AZ compared with ZD:Wt forestomach and in ZS:AZ compared with ZS:Wt forestomach. In addition, TUNEL analysis (Fig. 6) showed frequent occurrence of darkly stained TUNEL-positive nuclei in the restored and sometimes still proliferative forestomach epithelium of ZD:AZ mice compared with ZD:Wt forestomach, which had only occasional incidences of apoptotic cells (Fig. 6, B versus A). Fig. 6 also shows immunohistochemical staining for Bax, a pro-apoptotic protein, and Bcl-2, an anti-apoptotic protein in forestomach sections. This revealed diffuse and weak staining of Bax in ZD:Wt sections (Fig. 6E) but strong staining in ZD:AZ epithelium (Fig. 6F). On the other hand, Bcl-2 expression was typically strong in the basal and proliferative areas of ZD:Wt (Fig. 6I) forestomach but weak and infrequent in ZD:AZ epithelium (Fig. 6J).

Cell Proliferation and Apoptosis in NMBA-treated ZD:AZ Mice. Detailed cell proliferation and apoptosis results are presented in Figs. 4–6 for treated ZD:AZ and ZD:Wt mice of the two NMBA dose experiment. At 14 weeks, the ZD:AZ forestomach demonstrated significantly lower LI than their ZD:Wt counterparts (Fig. 5A, Expt. 2) a result consistent with those from respective control groups (Fig. 5A, Expt. 1). Typically, ZD:Wt forestomach displayed numerous PCNA-positive nuclei in areas of hyperplasia, dysplasia, and papilloma, whereas ZD:AZ forestomach showed a few PCNA-positive nuclei mainly in the basal cell layer (Fig. 4, G versus H). In addition, ZD:Wt forestomach lesions exhibited overexpression of Cdk4 (Fig. 4K) and cyclin D1 (Fig. 4O) in these lesions, whereas ZD:AZ forestomach showed moderate expression of these two G1 to S regulatory proteins, mostly in basal cells (Fig. 4, L and P).

On the other hand, the AI was significantly higher in ZD:AZ than ZD:Wt forestomachs (Fig. 5B, Expt. 2). These data are in line with those from respective control groups (Fig. 5B, Expt. 1). H&E-stained and TUNEL-stained forestomach sections from ZD:AZ mice displayed frequent occurrence of apoptotic cells (Fig. 4D; Fig. 6D), whereas those from ZD:Wt animals showed only isolated occurrences (Fig. 4C; Fig. 6C). Bax expression was intense and abundant in ZD:AZ forestomach (Fig. 6H) but moderate and sparse in ZD:Wt forestomach (Fig. 6G). Conversely, Bcl-2 expression was moderate and mostly in the basal cells of ZD:AZ forestomach (Fig. 6L) but strong and abundant in ZD:Wt forestomach lesions (Fig. 6K).

Effect of AZ Expression on Polyamine Levels in the Mouse Foremostach. The most probable explanation for the striking effect of transgenic AZ expression on tumor incidence, cell proliferation, and apoptosis described above is that polyamine levels influence these processes, and that the inhibitory effects of AZ on ODC and polyamine transport alter polyamine levels. Direct assessment of alterations in ODC levels were not possible, because ODC activity measurements could not be made reliably on tissue samples because of the small amount of tissue available and the limited sensitivity of these assays. Except for putrescine, which was at the limit of detection, polyamine levels could be measured accurately in the forestomach tissue. In samples collected at 14 weeks after treatment with two doses of NMBA, there was a statistically significant reduction in the spermidine content of the ZD:AZ group compared with ZD:Wt (Fig. 7). However, the differences were small, and spermidine values were not statistically significantly decreased in the comparison of the ZS:AZ and ZS:Wt groups. A probable explanation for this is that the relevant epithelial cell population makes up only a small proportion of the tissue used for this analysis because the underlying connective tissue and muscle layers are also retained in this analysis. Therefore, additional study of the possible role of polyamines in cell proliferation and forestomach carcinogenesis in ZD mice was carried out using the ODC inhibitor DFMO.

Effect of DFMO on Polyamine Levels, Tumor Incidence, and Cell Proliferation in Forestomach of ZD Mice. ZD C57BL/6 mice were treated with the two-dose NMBA protocol, and half of the animals in each group received 1% DFMO in the drinking water starting after the first NMBA dose. DFMO treatment did lead to a small reduction in body weight but did not affect the zinc deficiency...
(Compare Table 2 with Table 1). Table 2 shows that 87% of male C57BL/6 mice developed forestomach tumors at week 12 after two doses of NMBA, with a tumor multiplicity of 3.7. ZD mice that were switched to drinking water containing 1% DFMO after 5 weeks of a deficient diet exhibited a greatly reduced tumor incidence of 9%, with a lower tumor multiplicity of 0.2 (Table 2). The spermidine content of the forestomach was reduced significantly by DFMO to about the same extent as that by AZ (compare Table 2 and Fig. 7). DFMO treatment also reduced the tumor incidence and the spermidine content in ZS mice treated with the two-dose NMBA protocol, although the tumor incidence in the ZS mice not receiving DFMO was considerably lower than in ZD mice (results not shown).

DFMO, administered after the establishment of increased cell proliferation by nutritional zinc deficiency, reversed the increased cell proliferation in the mouse forestomach (Fig. 8). After 5 weeks of the ZD diet, the forestomach typically displayed a proliferative epithelium with a thickness of 2–6 cells, with PCNA-positive nuclei mainly in the basal cell layer (Fig. 8A). At week 2 after NMBA treatment, there was an expansion in focal hyperplastic lesions that was accompanied by an increase in PCNA-positive S phase cells (Fig. 8B). At week 12, PCNA-positive cells were found in areas of hyperplasia, dysplasia, focal hyperplastic lesions, and papilloma (Fig. 8C). In contrast, when DFMO was given starting at the time of the first NMBA treatment, within 2 weeks after NMBA and DFMO administration, a restored epithelium with a thickness of 2–5 cells thick was in place, with PCNA-positive nuclei mainly in the basal cell layer (Fig. 8D). At week 12, the forestomach in the DFMO-treated group was mostly 2–5 cells thick with a few PCNA-positive nuclei, mainly in basal cells (Fig. 8E). Quantitative PCNA immunohistochemistry demonstrated a significantly lower PCNA-LI in ZD/DFMO than ZD/DFMO− forestomachs at week 12 (Fig. 8A, Expt. 3). In addition, these ZD/DFMO+ forestomachs showed a significantly higher AI (%) than the corresponding ZD/DFMO− forestomachs (Fig. 8B, Expt. 3).

**DISCUSSION**

The moderate expression of AZ from the K5 promoter has no obvious deleterious effects in these transgenic mice. Detailed exam-
There have been suggestions that AZ may cause a reduction in the expression of other proteins including cyclin D1, cdk4, Smad1, and SNIP1 (27, 47). These reports are highly preliminary and have not yet been confirmed. However, we cannot at present rule out the possibility that the tumor-suppressive effects of AZ are mediated or enhanced via effects on these pathways or others.

It is most probable that the cancer preventative effect is attributable to a reduction in cellular polyamine content. Although there was only a small decline in spermidine in the treated AZ mice compared with treated controls (Fig. 7), this reduction was similar to that produced by the administration of DFMO in doses that had a similar effect on tumor incidence. The close similarity between the effects on cell proliferation and apoptosis of DFMO treatment or transgenic AZ expression is strong evidence that AZ is modulating carcinogenesis through effects on polyamines. The importance of polyamines and ODC in esophageal and stomach carcinogenesis is also supported by studies showing that ODC increases in these tissues of rats responding to regimes that cause tumors (48, 49). Similarly, transgenic rats with human c-Ha-ras are highly susceptible to NMBA induction of esophageal tumors (50), and ODC is a downstream target of ras (17).

Two factors contribute to the cancer protective effect of AZ. Our findings clearly demonstrate that overexpression of AZ both stimulates apoptosis and inhibits ZD-induced cell proliferation, a condition known to promote forestomach carcinogenesis in ZD mice (40, 41). The antiproliferative effects of blocking increases in polyamine content have been documented extensively in cell culture and in vivo (2, 51). The ability of cancer chemopreventive agents including DFMO to cause reductions in the proliferative index is a key factor in the current evaluations of these agents in Phase II/III trials (7, 52–54). The stimulation of apoptosis by AZ or by DFMO, which is shown clearly in the results of Figs. 5 and 6, has been less widely recognized.

**Table 2. Reduction of NMBA-induced forestomach carcinogenesis by DFMO in C57BL/6 mice on a ZD diet**

Male weanling C57BL/6 mice were fed ZD diet for 5 weeks. The mice were then given two doses of NMBA. After the first dose, DFMO− groups received 1% DFMO in the drinking water. All mice were sacrificed at week 12. Statistical analysis of significance of % tumor incidence for both forestomach and squamocolumnar junction and tumor multiplicity, DFMO− versus DFMO+, P < 0.001. Statistical analysis of significance of polyamine content, spermidine: DFMO− versus DFMO+, P = 0.003. Statistical analysis of significance of body weight: DFMO− versus DFMO+, P < 0.01. All statistical tests were two-sided.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ZD:DFMO−</th>
<th>ZD:DFMO+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forestomach tumor incidencea (%)</td>
<td>13/15 (87)</td>
<td>1/11 (9)</td>
</tr>
<tr>
<td>Squamocolumnar junction tumor incidence (%)</td>
<td>14/15 (93)</td>
<td>1/11 (9)</td>
</tr>
<tr>
<td>Tumor multiplicityb</td>
<td>3.7 ± 3.0</td>
<td>0.2 ± 0.6</td>
</tr>
<tr>
<td>Polyamine content (nmol/g tissue)</td>
<td>7 ± 6</td>
<td>6 ± 7</td>
</tr>
<tr>
<td>Putrescine</td>
<td>328 ± 142</td>
<td>249 ± 104</td>
</tr>
<tr>
<td>Spermine</td>
<td>216 ± 112</td>
<td>217 ± 58</td>
</tr>
<tr>
<td>Zinc content (μg/g), testis</td>
<td>135 ± 4</td>
<td>133 ± 5</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>31 ± 2</td>
<td>26 ± 1</td>
</tr>
</tbody>
</table>

a Number of mice with tumors/total number of mice.

b Number of tumors per forestomach.

Fig. 8. Reversal of pre-established cell proliferation in forestomach of NMBA-treated ZD mice by DFMO. Mice were treated for 5 weeks with the ZD diet and then with two doses of NMBA as in the legend to Fig. 1. Half of the mice received DFMO in the drinking water (DFMO−) and the others did not (DFMO+). PCNA immunohistochemistry with counterstaining with hematoxylin was then carried out on forestomach samples. A–C show representative results for ZD, DFMO− mice: at 0 h (5 weeks after ZD diet), at 2 weeks (B), and 12 weeks (C) after two NMBA doses. Numerous PCNA-positive regions in small upward and downward focal hyperplastic lesions are seen at 0 h (A) with abundant PCNA-positive nuclei in expanded focal hyperplastic lesions at 2 weeks (B) and papillomas at 12 weeks (C). D and E show results for DFMO+ mice at 2 weeks (D) and 12 weeks (E). These show a restored epithelium of 2–3 cells thick with PCNA-positive nuclei, mainly in the basal cell layer (D and E). Bars, 100 μm.
than the effects on proliferation, because polyamine levels can have biphatic effects both inhibiting and increasing apoptosis (see Refs. 55, 56). However, it is consistent with results of DFMO treatment in the ZD rat esophagus (43) and of human gastric tumors growing in nude mice (57). Recent studies have shown that polyamine depletion triggers the mitochondrial-mediated cell death pathway (58).

More than 50% of human cancers including many esophageal squamous cell carcinomas exhibit alterations that cause a loss of activity of the p53 tumor suppressor protein. Mutations in p53 also occur frequently in rodent esophageal tumors induced by NMB 59. Recent studies using p53-deficient mice have shown that the combination of ZD with loss of p53 leads to a large increase in the rate of cell proliferation, a decrease in apoptosis, and the very rapid development of forestomach and esophageal tumors (41). Our studies on forestomach reported here and in the skin (29, 30) show that AZ has opposite effects to the loss of p53 and suggest that AZ may be a tumor suppressor gene. Several other findings support this possibility. The content of AZ mRNA is reduced in malignant oral keratinocytes, and AZ expression reversed their malignant phenotype (60). Expression of AZ blocked tumor formation by H-ras-transfected 3T3 cells in nude mice (61). AZ-mediated effects on cell proliferation have been documented by several groups (60–63). Therefore, additional study of the potential role of AZ as a tumor suppressor gene is warranted. Because of the unique regulation of AZ synthesis in which translational frameshifting is needed for production of the protein, the levels of AZ may not correlate with the mRNA content, and measurement of the active protein content will be required for such studies.

DFMO is currently in clinical trials to prevent cancer in high-risk groups (9, 64, 65). The success of the transgenic AZ approach may be because of the ability of AZ to augment a modest reduction of ODC along with a blockage of the uptake of exogenous polyamines. Therefore, an improved approach might be to combine low doses of DFMO, with a blockage of the uptake of exogenous polyamines. There-


Antizyme Overexpression in Transgenic Mice Reduces Cell Proliferation, Increases Apoptosis, and Reduces \( N \)-Nitrosomethylbenzylamine-induced Forestomach Carcinogenesis

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